University of Malta

Faculty of Medicine and Surgery

Department of Physiology and Anatomy

Investigating the Effect of Differentiation Therapy using extracts from *Holothuria poli* (sea cucumber) on the haemopoietic malignancy.

A dissertation presented to the Faculty of Medicine and Surgery in partial fulfilment of the requirements for the award of Doctor of Philosophy at the University of Malta.

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List of Abbreviations

Abbreviation	Term
ALL	Acute lymphoblastic leukaemia
AML	Scute myeloid leukaemia
APL	Acute promocyeolocytic luekaemia
ATRA	All-*trans* retinoic acid
CLL	Chronic lymphatic leukaemia
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
TF	Transcription factors
ROS	Reactive oxygen species
ETS	Erythroblast Transformation Specific
KLF	Krüppel-like factor
MNP	Marine Natural Products
BSA	brine shrimp (*Artemia salina*) lethality assay
EPA-PLs and	Eicosapentaenoic acid-enriched phospholipids
SCS	Sea cucumber saponins
CR	Complete Regeneration

List of Definitions

Gonophoric species	These are species whereby each individual is either male or female and hence exhibit two distinct sexes (diecious).
Stenohaline	Aquatic organisms that are able to only tolerate a narrow range of salinity.
Anlage	Term used in embryology to describe the initial clustering of the embryonic cells that will eventually serve as the foundation from which various organs or body parts develop.
Holostane	Toxic triterpene glycosides present in Holothurians.
Benthic	Organisms that live at the bottom of the sea.

Abstract

Leukaemia is a cancer of the blood and is known to affect both males and females all around the world, including Malta. The main research question of this investigation is to study and investigate the potential of an extract from holothurians (sea cucumbers) to be used as a differentiating agent in the treatment of leukaemia. Leukaemia is a cancer which involves a block to the normal differentiation of a white blood cell and cause it to proliferate at a very fast rate in immature blast cells. The aim of differentiation is to remove the block allowing the cell to continue its normal maturation and death.

A number *of H. poli* were allowed to eviscerate their intestines and were then allowed to regenerate them in a suitable condition which did not harm the animals. Coelomic fluid was obtained from organisms using a specific bleeding method at various stages of regeneration. MTT and NBT assays were carried out and it was noted whether there were signs of reduced growth with an increase in oxidative burst which show that there is a possibility of differentiation taking place.

It was concluded that at day 9 from the evisceration, there was a clear indication that this was happening. It was also confirmed statistically. The fluid was then separated into two fractions; that of < 5 kDa and > 5 kDa and it is has been shown that the effect is a combination of both fractions. Flow cytometry using antibodies has confirmed a change in the surface marker expression of the HL60 cell line cells.

Marine organisms are known to be a reservoir of biochemicals which are still being discovered and isolated and this investigation will aid research in the treatment of cancer. Hence the results of this dissertation are expected to have an impact on the study and extraction of such differentiation compounds.

PREFACE

Investigating the Effect of Differentiation Therapy using extracts from Holothuria poli (sea cucumber) on the haemopoietic malignancy.

There are six hallmarks which are associated with cancer, and these include: immortality, ongoing production of growth factors from oncogenes, anti-growth signals from tumour suppressor genes, resistance to apoptosis, angiogenesis, and metastasis (Hanahan & Weinberg, 2000). Cancerous cells are transformed cells that have undergone extensive genetic changes. They show self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, sustained angiogenesis, tissue invasion and limitless replicative potential (Cree, 2011).

Leukaemia is a group of haemal disorders that arise due to the blockage of white blood cells during their development. Numerous scientific studies have been conducted on leukaemia in order to determine the risk factors and causes of these cancers, however the information is still fragmented. Although treatment can suffice in many cases, its effectiveness can increase and prevention strategies can be established given that these knowledge gaps are filled (Udensi & Tchounwou, 2014).

Many papers have been published related to the cytotoxic effects and apoptotic effects of various sea cucumber extracts (Wijesinghe, Jeon, Ramasamy, Wahid, & Vairappan, 2013); Salimi, *et al*., 2017), the number of papers associated with extracts from sea cucumber, specifically the coelomic fluid and differentiation is much more limited. Over the last decade, the University of Malta has been active with research efforts aimed at the identification of extracts which could have a potential use on the differentiation leukaemia cell lines. Most of these extracts were from either of plant origin or else of synthetic chemicals (Agius Anastasi, Cassar and Schembri-Wismayer, 2012). This research study will serve as the first research on differentiation using an extract from a marine organism.

In this research study it was decided to focus on the species *Holothuria poli*, which is an echinoderm belonging to the class Holothuria. These species are known to be able to eviscerate their intestines when under stress and then be able to regenerate

them completely in a limited time span without affecting their survival. These species are not known to contract cancer and during the development of their digestive tract, they undergo differentiation and cell division at fast rates, in the presence of compounds which are analogous to compounds present in humans when they are terminally sick. Such compounds include proteins such as mortalin and survivin (Mashanov, Zueva, Rojas-Catagena, & Garcia-Arraras, 2010).

Leukaemia cancers are treated with cytotoxic drugs; however, a new approach is to cause these cells to differentiate into non-malignant cells that will eventually undergo apoptosis. Leukaemia occurs due to a block in myeloid differentiation. By using biological agents, differentiation can be forced to continue and hence cause the cell to enter the apoptotic pathway. This approach is based on the assumption that Leukaemia cells can, thought specific treatment, be reprogrammed to reduce the rate of proliferation, and continue their path to form normal cells which can undergo apoptosis (Leszczyniecka, Roberts, Dent, Grant, and Fisher, 2001). This offers an alternative to curing cancers other than the conventional cytotoxic therapies and in the process reducing or completely removing the side effects of these drugs and ensure complete cure of these conditions (Nowak, Stewart & Koeffler, 2009). Very good results have been obtained both *in vitro* and *in vivo* to cure promyelocytic leukaemia by this form of therapy (Fang, *et al*., 2010; Huang, *et al*., 1988). In these experiments All-trans retinoic acid (ATRA) was used as it was proven to restore the natural progression of differentiation to these leukaemia cells (Bruserud, Gjertsen, & Huang, 2000). In a study carried out by Castalgne, *et al*., (1990), all trans retinoic acid was used to treat twenty-two patients with acute promyelocytic leukaemia, 14 of which showed complete remission. This has been only one of the chemicals tried for differentiation and studies are being carried out to date.

The sea cucumbers have two defence mechanisms. These animals can either discharge sticky threads or else exhibit autotomy (Wilkie, 2001). In this process the animal adaptively detaches parts of its body, and releases them out of its anus, being then able to regenerate the missing parts very quickly (Mashanov *et al*., 2010) in a period of about 27 days in the holothurian *Eupentacta fraudatrix* (Mashanov *et al*., 2005). When organisms belonging to Aspidochirota undergo evisceration, the intestine is detached from the oesophagus and the cloaca, and also from the

supporting mesenteria. These autotomized parts of the digestive tract, as well as the associated visceral organs such as gonads, and one or both respiratory trees, are then expelled though the rupture in the cloacal wall (Emson and Wilkie, 1980; García-Arraras *et al*., 1998; Wilkie, 2001).

Holothuria species are known to possess high amounts high value-added compounds, many of which have therapeutic properties and also for nutraceutical, pharmaceutical and cosmeceutical products (Pangestuti & Arifin, 2017; Siahaan, Pangestuti, Munandar, & Kim, 2017). So far cancer formation has never been reported in studies of visceral regeneration in holothurians even though they constantly renew cells in their adult tissues and quickly regrow most of their tissues after traumatic injury; all instances which give opportunity for carcinogenic changes to occur. Tumour formation has never been documented in animals captured in the wild. This implies that sea cucumbers, must have, through evolution, developed a particularly strong set of anti-tumour mechanisms.

Introduction

PART 1 – Literature Review

1.1 Echinoderms

Echinoderms are a group of around 7000 known marine species, that are invertebrates, characterised by a body plan which is secondary radially symmetrical and possess tube feet. Such organisms have a calcareous endoskeleton (Moore, 2006). Echinoderms are classified as deuterostomes. In deuterostomes, the blastopore develops into the anal opening during the embryonic development and are characterised by radial cleavage. Chordates and hemichordates are also classified in the same clade, and therefore, from an evolutionary perspective, echinoderms and chordates show similarities and closely related developmental features, biochemistry, and physiology (Kamyab, Kellermann, Kunzmann & Schupp, 2019). Therefore, it is expected that analogous compounds will be found in these two phyla. The phylum Echinodermata is divided into 5 main classes: Asteroidea (sea stars), Ophiuroidea (brittle stars), Echinoidea (sea urchins), Holothuroidea (sea cucumbers) and Crinoidea (sea lilies) (Kondo & Akasaka, 2012).

1.1.1 Biology of the genus *Holothuria*

Holothurians or as they are commonly known, sea cucumbers, are exclusively marine organisms, which live in a wide range of depths, from shallow areas to 5000 m in all regions of the world's oceans and are mostly benthic (Dolmatov, 2021). Members of the class Holothuroidea are very distinctive from the members belonging to the other four classes of phylum Echinodermata. This variation can be seen in terms of shape, skeleton, and other characteristics including their great regenerative capabilities which are superior to those of other classes in the phylum (Sun *et al.*, 2017). Sea cucumbers are very important organisms in the Mediterranean and in the marine ecosystems in general, as they are involved in nutrient recycling, sediment mixing and bioturbation (Barnes & Ruppert, 1994). They are also known to stimulate growth of microalgae in their surrounding environment. In countries such as China and Japan, sea cucumbers are also food items and have been used for pharmaceutical

and cosmetic products (Valente, Serrão, & González-Wangüemert, 2015; Sun *et al.*, 2017).

The most diverse family in the class Holothuroidea is the sea cucumber family Holothuriidae which includes around 1250 species from all around the world (Esmat, Said, Soliman, El-Masry & Badiea, 2013; Guo *et al.*, 2015; Luparello *et al.*, 2019). Within this family, the genus Holothuria includes 18 subgenera with seven of these subgenera occurring in the Mediterranean Sea. There are 5 Holothuria species which are most frequent in the Mediterranean Sea, and these are *Holothuria mammata, Holothuria tubulosa, Holothuria forskali, Holothuria sanctori and Holothuria poli* (Borrero-Pérez, Pérez-Ruzafa, Marcos & González-Wangüemert, 2009).

Sea cucumbers can make up 80% of the whole biomass of benthic invertebrates in some areas around the world, however they are often targeted by fisheries. Poor management practices and overfishing of holothurian stocks in the Indo-Pacific region are becoming of great concern for the protection of this species in the Mediterranean Region, as an increased catch of holothurian species found in the Mediterranean region to keep up with the demand has been noted (Sun *et al.*, 2017; Moussa & Wirawati, 2018). *Holothuria poli* are the most sought-after sea cucumber due to the highest dry weight richest in protein (Moussa & Wirawati, 2018).

Sea cucumbers are characterised by being flexible, elongated, worm-like organisms, with a leathery skin and gelatinous body (Senadheera, Dave, & Shahidi, 2020). These organisms are bottom dwellers and deposit or suspension feeders (Mashanov & Garcia-Arraras, 2011). Bottom feeders feed by taking up sand, absorbing what they can from it and then egesting the unwanted material (Moore, 2006). These organisms show selectivity towards what is ingested from the detritus they take in, which in turn is formed from different sources. *Holothuria poli* are able to assimilate food sources which are very difficult to digest by most other consumers, such as lignocellulose from sea grass, reducing competition. This is possible due to the occurrence of microbes in their gut which produce protease, amylase and cellulase (Boncagni, Rakaj, Fianchini & Vizzini, 2019), an occurrence which highlights the existence and the importance of these microorganisms in the life of the sea cucumbers.

These organisms do not have a well-developed calcareous skeleton as seen in other echinoderms like the sea urchin and the star fish and has been reduced to microscopic spicules found on the surface of the organism. These calcareous ossicles are of mesodermal origin, where each ossicle is a single crystal and has no connections with others. They are, in fact, all separated from each other by living tissues, a thick layer of dermal connective tissue. Due to this, these animals can attain large sizes without moulting and their body can be rigid or soft, enabling them to change size and hardness according to the need. The taxonomy of sea cucumbers is based on the shapes (such as buttons, anchors, wheels, and plates amongst others) and combinations of such spicules (figure 1) (Toral-Granda, 2005).

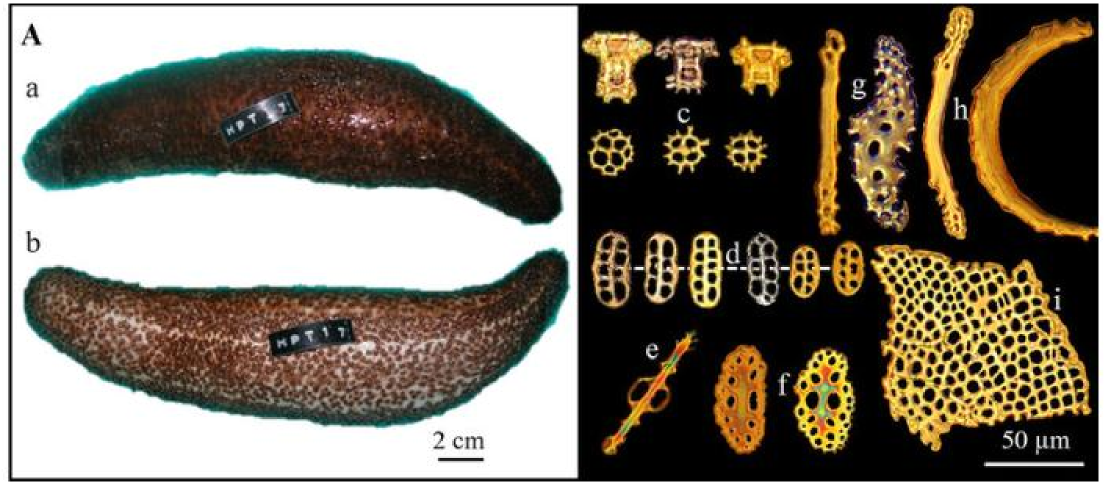


Figure 1: Holothuria (Roweothuria) poli ossicles. Aa. Dorsal surface. Ab. Ventral surface. Ac. Tables of dorsal and ventral surfaces. Ad. Buttons of dorsal and ventral surfaces. Ae. Elongated plates of pedicels. Af. Enlarged plates of pedicels. Ag. Straight rods. Reference: Mezali, et al,(2021).

The organism used for this study is an organism belonging to the genus *Holothuria*, the species *Holothuria poli*.

1.1.2 Taxonomy and Biology of Holothuria poli

Holothuria (Roweothuria) poli Delle Chiaje, 1824 is a species that is exclusive to the Mediterranean (Valente *et al.*, 2015). *H. poli* are usually found in places where there is a rock to sand interface, occupying areas from the intertidal zones to deep trenches (Mendes, Marenzi & Di Domenico, 2006). This gives them the sand that is required by these organisms for feeding purposes, as well as an enhanced possibility of camouflage. The sea cucumbers are usually covered by other marine biota, thin layer of sand and fragments of calcareous shells mimicking their natural habitat,

which makes them very difficult to spot by predators (Mendes *et al.*, 2006; personal observation). Despite its high ecological and economic importance, there is a lack of information related to the biology of this species. There is a knowledge gap in the genetic diversity, structure, connectivity, and stocks of *H. poli* along the Mediterranean (Valente *et al.*, 2015) as well as other anatomical and physiological characteristics such as their reproductive cycles. It is known that they are a gonochoric species without sexual dimorphism (this is common in aspidochirotid holothurians); spawning and fertilization are external during the summer, with a distinct annual reproductive cycle (Aydin and Erkan, 2015). Despite the limited information about their reproductive cycle, in a study carried out between 2000 and 2004 by Tanti and Schembri (2006) which involved a large collection of organisms collected from the Maltese waters, *Holothuria poli* was identified as being the most abundant sea cucumber in the Maltese Islands. Hence, using these organisms in this study is not ecologically disruptive as the species is not on the verge of extinction. In addition, collection of coelomic fluid (the extract) for the tests required to provide pharmacological knowledge will not lead to the killing of many organisms, which is important for biological, ecological, and ethical purposes. This is because the bleeding procedure will be used, described in detail is section 2.4.1 and 2.4.2, which does not kill the specimen from which it is extracted, and the fluid is easily regenerated by the animal. An ERA (formerly known as MEPA) permit has been obtained, in order to collect specimens for this study as shown in Appendix 1. The pyramid drawn below (Figure 2) illustrates the taxonomy of the *Holothuria poli:*

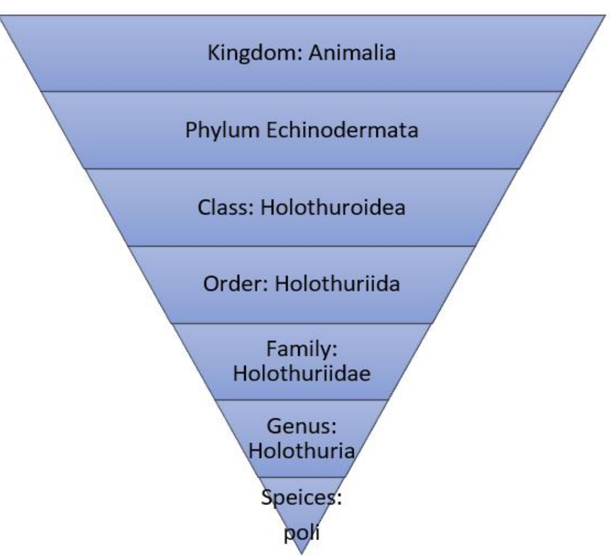


Figure 2: Taxonomical classification of Holothuria poli. Classification obtained from the World Register of Marine Organisms. Reference: Drawn by Author.

Organisms belonging to the species *Holothuria poli* have a mouth at one end surrounded by about twenty short retractable tentacles and a cloacal opening on the other end. *Holothuria poli* are secondary bilaterally symmetrical and lie on their sides with three rows of tube feet on their ventral side, referred to as the sole and the other two are found on the dorsal side. They vary in colour; however, they are usually dark and are characterised by white tips on their podia which are used for locomotion and feeding. The colour of the top and lower surface of the organisms is different, with the lower surface being lighter than the upper surface. Their length also varies, depending on the depth from which they were caught, most probably this being related to the fact that the deeper they are, the less likely it is that they were affected by human interference, allowing to attain larger sizes (Moussa & Wirawati, 2018). The cross section of a specimen belonging to *H. poli* can be seen in figure 3 below:

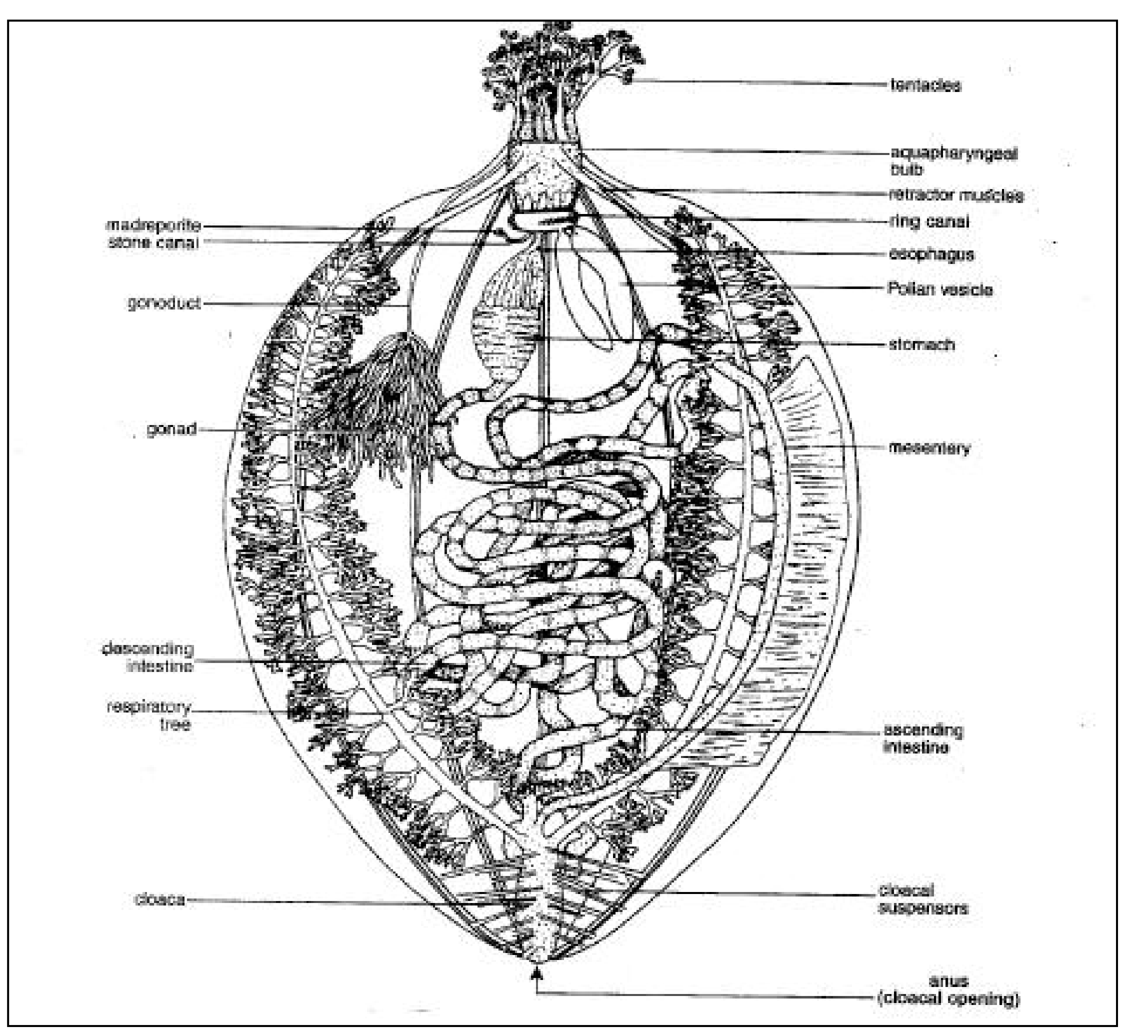


Figure 3: Cross section through H. poli (Barnes & Ruppert,1994)

Another characteristic feature of these organisms are their respiratory trees which are responsible for the uptake of oxygenated water. Sea cucumbers have a water vascular system, and the coelom of such organisms is very conspicuous. The coelomic epithelial cells in holothurians has been shown to be involved in the formation of luminal epithelium of the gut, in muscle development and regeneration (Dolmatov, 2021). The coelomic epithelium is a derivative of mesoderm and causes the formation of a large body cavity filled with coelomic fluid. The coelomic fluid contains a lot of coelomocytes (Canicattí, D'ancona, & Farina-Lipari, 1989, Barnes & Ruppert, 1994, Moore, 2006). Besides being free in the coelomic fluid these cells are also found in tissues, in the water vascular system, and the haemal system. Such cells perform a variety of functions, including, amongst others, cellular and humoral immune responses, excretion, gas exchange and nutrient storage (Canicattí *et al*., 1989). A generally accepted classification involves six types of coelomocytes in echinoderms including: phagocytes (phagocytic amoebocytes - which originate in the stone canal and show an intense phagocytic activity), morula (or spherule) cells which are responsible for the melanin pigment, vibratile cells, progenitor (lymphocyte like) cells which are associated with the defence mechanism of these organisms, haemocytes and crystal cells (Barnes & Ruppert, 1994; Eliseikina & Magarlamov, 2002; Moore, 2006).

Clotting in *H. poli* is a complex process in which three stages are involved. A clot forms when cells progressively aggregate into a large mass. Complex scavenger structures consisting of a variable number of nodules surrounding an internodular mass, conventionally referred to as brown bodies, are encapsulating bodies (Canicatti, Rizzo, & Montinari, 1992). Brown bodies are made up of possibly amoebocytes and spherulocytes, and their function is to retain unwanted material such as parasitic organisms. These brown bodies are eventually removed from the coelom through the organisms cloacal opening.

The coelomic fluid may vary in composition from sea water (Moore, 2006). However, these organisms are stenohaline and find it difficult to maintain a great osmotic gradient from the surrounding water (Vidolin, Santos-Gouvea & Freirei, 2002).

1.1.3 Microbial associations

H. poli specimens host a number of microorganisms within them (Nerva *et al*., 2019). The composition of these microorganism varies depending on the habitat and general health of the sea cucumber; however, Holothurians are generally infested by parasites such as bacteria, protozoa, gastropods, and others (Eeckhaut, Parmentier, Becker, Gomez da Silva & Jangoux, 2004) with some of these organisms having a communalistic or symbiotic relationship with the holothurians (Marchese *et al*., 2020). A microbiological investigation was carried out on the *Holothuria poli* species collected from Abu-kir shore of Alexandria, Egypt in the Mediterranean in 2012. It was found out that the sea cucumbers contained five human Gram-negative pathogenic bacteria which were identified to species level. These were *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella sp*. and *Shigella sp*. In addition, *Candida albicans*, a yeast was also isolated (Omran & Allam, 2012). Farouk, Ghouse and Ridzwan (2007) isolated 30 bacterial strains from various parts of the sea cucumber, *Holothuria atra*, without finding any signs of external or internal infection. This indicates, that whilst these microorganisms are pathogenic on their own, they show a relationship with the sea cucumbers whereby it allows them to survive, possibly by producing metabolites that are useful to the host or help protect them against predation. The associations between sea cucumbers and these microorganisms are not always clear.

H. poli are known to suffer from various diseases including rotting edges, stomach ulcerations, gas bubble disease, off-plate syndrome, and skin ulcer disease (Spiteri, 2010). The first sign of the latter is a white spot that appears on the integument of individuals that extends quickly onto the whole integument leading to the death of the specimen within a very short period of time. Usually, such a disease is caused by *Vibrio*. This can be seen in figure 4 below.

Figure 4: Holothurian showing skin-ulcer disease. Image, taken by author, enlarged x1.75 for a better view of the symptoms.

1.1.4 Regeneration

Regeneration is not common to all organisms; however, Echinoderms are known to have a high regenerative power and in fact, these organisms are able to regrow any parts that would have been amputated. Organisms belonging to the class Asteroidea can regrow their arms if these are severed and organisms belonging to *Holothuria* can regrow their intestine if this is eviscerated. This regeneration involves rapid multiplication of cells which can be compared to the rapid multiplication of cancer cells (Mashanov & Garcia-Arraras, 2011). One important characteristic feature of regeneration in echinoderms is that there is no consistent evidence for the presence of stem cells in any of these animals except for primordial germ cells and possibly coelomocytes (Dolmatov, 2021). Dolmatov (2021) states that regeneration in echinoderms is therefore thought to occur through the differentiation of cells from remnants of organs left after evisceration through a system of dedifferentiation and trans differentiation of specialised cells.

1.1.5 Evisceration

Evisceration is the expulsion of the intestine from the organism. Sea cucumbers including *H. poli* have this ability to eviscerate their intestine completely and then regenerate all the missing components completely in a specific time span, the latter being species dependent. Species of *Holothuria*, use evisceration of the intestines as one of two possible species-specific defence mechanisms that they employ. These animals can either possess Cuvierian Tubules and discharge sticky threads or else exhibit autotomy (Wilkie, 2001; García-Arrarás, Lázaro-Peña & Díaz-Balzac, 2018). In the latter process, the animal adaptively detaches parts of its body, and releases them out of its anus or mouth, being then able to regenerate the missing parts very quickly (García-Arrarás *et al*., 2018; Mashanov *et al*., 2010) in a period of around 25 to 30 days depending on the species. In fact, the holothurian *Eupentacta fraudatrix* is able to undergo regeneration in a period of about 27 days (Mashanov, Dolmatov & Heinzeller, 2005), whilst *Holothuria glaberrima* takes about 1 month (García-Arrarás *et al*., 2018).

Autotomy is an intrinsic mechanism, mediated by the nervous system (Wilkie, 2001) and involves the separation of the anatomical components along predetermined breakage zones hence excluding any variations between animals and is not affected by the severity of the trauma (Mashanov & Garcia-Arraras, 2011). When organisms belonging to Aspidochirota (including *H. poli*) undergo evisceration, the intestine is detached from the oesophagus and the cloaca, and also from the supporting mesentera. These autotomized parts of the digestive tract, as well as the associated visceral organs such as haemal vessels, gonads, and one or both respiratory trees, are then expelled though the rupture in the cloacal wall (Emson & Wilkie, 1980; Wilkie, 2001; Mashanov & Garcia-Arraras, 2011; García-Arrarás *et al*., 2018). There are some differences in the organisation of the digestive tract of different holothurians (Mashanov & Garcia-Arraras, 2011), however the same authors state that sites, within each particular species which are known to undergo autotomy can be described as permanent sites of weakness. Mashanov *et al*., (2010) state that the process of the regeneration of the digestive tract involves a number of steps usually following a precise order of de-differentiation of the specialised cells that would have remained following evisceration, their migration, together with cell death, cell division, and re-differentiation.

The purpose and triggers that cause evisceration still remain poorly understood and not much literature is found in this regard. Whilst in some sea cucumbers, the gut is eviscerated when in the presence of a predators acting a predator diversion device, in other sea cucumbers, it is eviscerated seasonally. One theory is that such expulsion is essential to remove enteric pathogens from the system which, if left indefinitely, harm the host (Shinn, 1985). Other causes of evisceration are as a means of reducing metabolic costs in adverse conditions such as those occurring as a result of accumulation of waste products in tissues or the occurrence of adverse conditions in the environment such as a reduction in oxygen concentration, water pollution, and temperature rise (Emson & Wilkie, 1980; Dolmatov, Nguyen & Kamenev, 2012).

In a study carried out by Zhang *et al*., (2019) to investigate the relationship between the genome of the Holothurian *A. japonicus* and visceral regeneration, it was determined that there is a genetic component to this process. Transcriptome and proteome analyses of organ regeneration was carried out on the regenerating gut that occurred after evisceration was artificially induced in the organism. The occurrence of a specific tandem-duplicated prostatic secretory protein of 94 amino acids (PSP94)-like gene family and a significantly expanded fibrinogen-related protein (FREP) gene family was noted to be activated during this process. This sequencing was done using a combination of Illumina shot gun and PacBio Single Molecule Real-Time (SMRT) sequencing.

To date, regeneration has only been described in a reasonable level of detail from organisms belonging to three different orders of sea cucumbers – Synallactida, Holothuriida and Dendrochirotida. In total four holothurian species have been discussed, these being *Apostichopus japonicus*, *Holothuria glaberrima*, *Eupentacta fraudatrix* and *Cladolabes schmeltzii* (Dalmatov, 2021). The molecular data of these organisms can be interpreted relatively well as many factors such as morphological events of regeneration, morphogenesis at cellular level, and sources of regeneration of various organs were studied in great details and subsequently well identified. Each of the species above has its own characteristics of regeneration, highlighting the complexity of this process. *C. schmeltzii* regenerates body fragments through transverse fission, A. *japonicus* and *H. glaberrima* eviscerate the internal organs through the anal orifice, following which they form two gut anlages, and then

regenerate the gut from the epithelia of cloaca (posterior anlage) and oesophagus remnant (anterior anlage). These two organisms show a great similarity even at a cellular level of regeneration. The holothurian *E. fraudatrix* is capable of anterior evisceration and shows a major distinguishing feature in the regeneration of the digestive system in that enterocytes are formed through the trans differentiation of coelomic epithelial cells (Dolmatov, 2021).

Knowledge on the regeneration of *H. poli* is limited. However, it is known that regeneration in *H. poli* involves the anterior part of the regenerating alimentary developing into the descending and the ascending limb whilst the posterior part gives rise to the large intestine (García-Arrarás *et al*., 2018). The digestive tract of these organisms consists of five tissue layers which involve the mucosa (closest to the lumen), muscular layer, outer layer of connective tissue and ciliated peritoneum which leads to the coelom. Regeneration of the intestine is in continuous communication with the coelom (due to anatomy explained in section 1.1.2) where most biomolecules would be exchanged and transported. Progress in the understanding of both cellular and molecular events required for the visceral regeneration is sea cucumbers has occurred over the last 20-25 years (Mashanov & Garcia-Arraras, 2011).

In the study carried out by Garcı´a-Arrara´s *et al*., (2018), six fundamental stages of regeneration have been identified in *H glabberina*. Stage 1 involves wound healing of the areas of rupture, stage 2 is identified by the appearance of a thickening in the edges of the free mesenteries which corresponds blastema like structure. Stage 3 corresponds to the initial appearance of luminal epithelium outgrowth from the oesophagus and the cloaca, whilst stage 4 involves active lumen formation. Stage 5 is characterised by the presence of a continuous lumen which is formed via a thin tube, and it includes all the different layers and the final stage, stage 6, occurs when the intestine has grown to its original form.

The time it takes for the intestine to become fully functional again is not necessarily the same as the time it takes to become completely regenerated. *H. glaberrima* becomes functional in the third week of regeneration, whilst that of *H. scarba* within 8 days of regeneration (García-Arrarás *et al*., 2018). Although these cellular processes have been clearly studied extensively, the molecular machinery

remains greatly unknown to date (Quispe-Parra, Medina-Feliciano, Cruz-Gonzále, Ortiz-Zuazaga, & García-Arrarás, 2021). Functional and signal pathway analysis indicate that there is an overall upregulation of transcription processes, translation, and protein transport. This is expected as reconstruction of organs requires proteins. Proteasome related transcripts are also upregulated, and this is indispensable for stem cell maintenance and cell signalling (Quispe-Parra *et al.*, 2021).

The great power of regeneration causes a problem related to an increased risk of mutations. Due to the constant renewing of the cells in their adult tissues and due to fast regeneration of tissues after traumatic injury, the occurrence of carcinogenic changes is expected to be high (Mashanov & Garcia-Arraras, 2011). However, according to the same authors, to date, cancer formation has never been reported in studies of visceral regeneration in holothurians. This implies that sea cucumbers, must have, through evolution, developed a particularly strong set of anti-tumour mechanisms. This is also seen in other regenerating organisms. Apart from great regenerative power, and a lessened tendency to get any form of cancer, Guo *et al.*, (2015) explain that organisms belonging to the species *H. poli* have been shown to possess a wide range of biological compounds with great therapeutic effects and unique biological and pharmacological effects. A more detailed introduction to the various biological compounds that are obtained from Holothurians will be given in section 1.2.2.2.

The loss of coelomic fluid from the animals upon evisceration, through the same opening through which the internal organs are expelled, causes the loss of a large number of coelomocytes which in turn causes a great stress to the animal (Dolmatov, 2021). This will in turn result in the blocking of many biological functions as well as the activation of protective functions. It has been shown that many genes related to signalling systems, metabolic processes and those associated with the immune system are downregulated after evisceration (Quispe-Parra *et al.*, 2021). Upon evisceration, the aim of the organism is to restore the cellular and protein composition of the coelomic fluid, which would allow it to activate the immune system and heal the wounds associated with his process. Humoral immunity causes the synthesis and release of a large number of factors including lectins which are known to interact with extracellular sugar residues forming part of bacterial walls, in turn protecting the organism. In *A. japonicus*, production of such compounds starts

after day 3 following evisceration, as first endogenous reserves are used up. However, expression of some putative immune related genes such as serum amyloid and melanotranspherins keep on being expressed and synthesised even in the late stages of regeneration, indicating their possible dual role of immunity as well as morphogenesis (Dolmatov, 2021). In a study by Ding (2021), the components of ejected and non-ejected coelomic fluid of *A. japonicus* was studied and compared using ultraperformance liquid chromatography combined with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). It was determined that a total of nine metabolites were found to have increased in the ejected coelomic fluid and eleven metabolites to have decreased in the ejected coelomic fluid of *A. japonicus* undergoing evisceration when compared to the coelomic fluid in organisms that have not undergone evisceration. Such metabolites include phosphatidylethanolamine, glucosylceramide, L-tryptophan, carbamic acid, and cyclohexylamine. In addition, the ejection of the coelomic fluid revealed significant changes in specific signalling pathways, including but not limited to Glycosyl-phosphatidyl-inositol-anchor biosynthesis, regulation of autophagy and nitrogen metabolism. These results contribute valuable data on the potential physiological mechanisms underlying evisceration behaviour of *A. japonicus*.

In a study by Vazzana, Siragusa, Arizza, Buscaino & Celi (2015), heat shock proteins specifically HSP90, HSP70 were found to increase 5-fold within 1 hour of evisceration of *H. tubolosa* and remained relatively high in the first two days of regeneration when compared to intact organisms. HSP90 is an extracellular tissue-repairing factor in humans whilst HSP70 is known to trigger cellular immune responses, has a role in wound epithelialisation, performs a role as an immune system activator and increases resistance to oxidative stress (Wallin, Lundqvist, Moré, von Bonin, Kiessling & Ljunggren, 2002). In the same study by Vazzana *et al*., (2015), HSP27 where also found to be present right after evisceration and this protein is associated with the regulation of cell migration and differentiation and also with protection from the stressful effects afflicted on the animal by the formation of the wound. Another immediate response to evisceration is the restoration of the number of coelomocytes. One hour post evisceration, the coelomocytes are composed of a large number of juvenile cells which are comparable to stem cells within the fluid, some of which express the pluripotency marker piwi. In *H tubulosa,* 24 hours post

evisceration, this number decreases as the juvenile cells differentiate into amoebocytes and morula cells (Dolmatov, 2021).

Oxidative stress is one of the effects caused by evisceration. This can be seen by an increase in the production of reactive oxygen species (ROS). The antioxidant enzyme system of cells, comprised of various components including glutathione S-transferase, superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase, is activated. This provides protection against ROS (Birben, Sahiner, Sackesen, Erzurum & Kalayci, 2012). The genes of the main enzymes involved in this antioxidant system were identified in holothurians. In a study by Boyko, Girich, Tkacheva, & Dolmatov, 2020, 11 factors, namely Ef-EGR1, Ef-ELF, Ef-GATA3, Ef-ID2, Ef-KLF1/2/4, Ef-MSC, Ef-PCGF2, Ef-PRDM9, Ef-SNAI2, Ef-TBX20, and Ef-TCF24 were identified in the holothurian *Eupentacta fraudatrix.* With the exception of TCF24, all these factors are known to be involved in the regeneration, development, epithelial-mesenchymal transition, and immune response in other animals. The authors of this study suggest that these transcription factors may also be involved in the trans differentiation of coelomic epithelial cells into enterocytes in holothurians. It was shown that the total antioxidant capacity in *A. japonicus* increases in the first 10 days post-evisceration and doesn't decrease until the completion of regeneration (Zang, Tian, Dong & Dong, 2012). However, the dynamics of the activity of antioxidant enzymes varies between different tissues. The same study suggests that the stressful effect on evisceration occurs on the entire animal's body and in all its tissues. They determined that the total antioxidant capacity as well as the superoxide dismutase activity also increases in muscles that were not damaged by evisceration.

A total of 11 genes of Transcription factors (TF), belonging to 6 TF classes, that showed higher transcriptional activity during trans differentiation were identified and characterized in *E. fraudatrix* (Boyko, Girich, Tkacheva, & Dolmatov, 2020). This occurs between 3-10 days post evisceration with the maximum being seen in days 5–7 post evisceration. The classes determined were those of the Tryptophan cluster (Ef-*elf*), C2H2 zinc finger (Ef-*prdm9*, Ef-*egr1*, Ef-*klf1/2/4* (*klf2*), Ef-*snai2*), bHLH (Ef-*tcf24*, Ef-*msc*, Ef-*id2*), C4 zinc finger (Ef-*gata3*), polycomb group ring finger (Ef-*pcgf2*), and T-box (Ef-*tbx20*). The genes whose expression increased during this period were associated with trans differentiation of a part of coelomic epithelial cells, the dedifferentiation and proliferation of undifferentiated coelomic

epithelial cells (Boyko *et al.*, 2020; Dolmatov, 2021). Ef-ELF belongs to the Ets family, components of which act as powerful regulators of cell proliferation, angiogenesis, haematopoiesis, tumour transformation, and differentiation. Proteins of the PRDM family are important epigenetic regulators of development, and pluripotency in mice whilst the KLF proteins play diverse roles in cell proliferation and development. Both proteins of the PRDM family as well as KLF proteins play an important role in the cell differentiation. *Ef-egr1* is also involved in the processes of dedifferentiation and proliferation and it is known that EGR1 is involved in the cell cycle progression of various tumour types, and also in hepatic regeneration in mammals (Dolmatov, 2021). Various other genes have been expressed in *E. fraudatrix (*Boyko *et al.*, 2020), however further discussion is beyond the scope of this study.

The proliferative activity in the regenerating digestive tract is probably triggered and modulated by the Wnt signalling pathway. It has been shown that during the regeneration of the anterior organs of *E. fraudatrix*, the Wnt signalling pathway is activated (Bello *et al.*, 2020; Dolmatov, 2021). Gene transcripts of four ligands (*wntA*, *wnt4*, *wnt6*, and *wnt16*) and three receptors (*frizzled1/2/7*, *frizzled4*, and *frizzled5/8*) have been identified and it has been shown that the transcripts of these genes localized in coelomic epithelium and organs of the water–vascular system. The expression of *wnt16* in vertebrates is associated with the development or regeneration of connective-tissue structures, the transformation of the extracellular matrix, and the activation of metalloproteinases After 5-7 days post evisceration, the expression of *wntA*, *wnt4*, *wnt6*, *frizzled1/2/7*, and *frizzled4* increases abruptly and reaches its maximum and then is either reduced or insignificantly changes expression at day 10 post evisceration. After 14- and 20-days post evisceration, changes in the expression of all Wnt and frizzled genes are minimal (Dolmatov, 2021). In *A. japonicus*, transcripts of the *Wnt4*, *Wnt6*, *Wnt7*, *Wnt8*, and *WntA* genes are encountered with *Wnt7* and *Wnt8* becoming maximally expressed after 1-3 days post evisceration. Their expression decreases drastically in the days that follow. *Wnt6* transcripts are recorded at their maximum expression at day 7 post evisceration. Their expression is then maintained at a high level through the period of regeneration. Similar results of studies on localization of expression were obtained for *H.*

glaberrima, however *Wnt9*, was observed in this species and not in *A. japonicus* (Dolmatov, 2021).

Apoptosis is observed during regeneration in holothurians, and this is known to make a significant contribution to various physiological processes that are occurring during tissue remodelling, regeneration, and morphogenesis (Dolmatov, 2021). The greatest number of apoptotic cells is observed in the coelomic epithelium of *H. glaberrima* after 3–14 days following evisceration (Mashanov *et al*., 2010). This greater than normal apoptotic activity, together with coelomocytes producing ROS, could be two of the mechanisms which activate cell proliferation. The *survivin* gene, known as BIRC5 also plays a role in holothurian gut regeneration. This gene is conserved throughout eukaryotes and is important for cell division and also inhibiting cell death. It is expressed in coelomic and luminal epithelia during the gut regeneration in *H. glaberrima* (Mashanov *et al*., 2010).

1.1.6 Distinguishing between *H. poli* and other Holothurians

Great care needs to be taken when identifying *H. poli* from other holothurians found in the Maltese waters. *Holothuria forskali* is also characterised by white tips and a black spot at the apex however organisms belonging to this species rarely contract and possess Cuvier tubules, hence do not eviscerate but release a white sticky strand when threatened or manipulated. *Holothuria tubulosa* is another similar species, but the colour difference between the upper and lower side of the organism is more marked than *H. poli.* In addition, contrary to *H. poli*, this species is never covered with sediment or sand. *Holothuria sanctori* is dark brown in colour and may have yellow circles at the base of the papillae. This species has a different surface texture due to the numerous sclerites present on the surface of the integument.

1.1.7 Conclusion

Holothurians, apart from being important from an ecological point of view, they are also important from a socio-economic perspective. They are delicacies in various parts of the world, especially in Asia (Nerva *et al*., 2019), play an important role in the ecosystem, have a high regenerative power, and have an ability to never show any form of cancer (Mashanov & Garcia-Arraras, 2011). Very limited information can be found about the biology of *H. poli* in the Mediterranean Sea and to date very little or

no information can be found on their application to medicine. This makes this study of great interest in the fields of biology and medicine as it will allow new information to come to light about the possible relationship of these organisms to differentiation therapy of leukaemia.

1.2 The marine environment

The marine environment is affected by both natural and anthropogenic stressors and these cause major changes in species distribution. Climate change, opening of artificial pathways and maritime traffic are some of the factors which affect changes in the Mediterranean biodiversity (Stern *et al*., 2019). These may lead to the introduction of alien species and together with pollution, affect the intimate relationship between water quality and biodiversity. Given that the marine environment is a vast resource that is being studied and exploited for various reasons by many countries, including for the search of natural products, marine scientific research and technological innovation, water quality and biodiversity are essential aspects that need to be studied and discussed. These will allow for better investigation of ocean life, whilst at the same time conserving it and allowing for the sharing of marine genetic resources beyond national jurisdictions (Harden-Davies & Gjerde, 2019).

1.2.1 The Blue growth strategy and the Blue Mediterranean Project.

Sustainable development is based on three pillars which are interdependent and mutually reinforcing, these being economy, socio-cultural and the environment. It aims towards a holistic approach between these three areas, integrating environmental protection and fulfilling human needs for the benefit of current and future generations (Klarin, 2018). The European 2020 strategy aims at achieving goals that are smart, sustainable and involve inclusive growth. Five ambitious targets have been set by the European Union (EU), covering employment, research and development, climate change and energy sustainability, education, and the fight against poverty and social exclusion (Stec & Grzebyk, 2018).

The Blue Growth strategy is a long-term approach aimed at supporting sustainable growth in the marine and maritime sectors as a whole. Seas and oceans are drivers for the European economy and have great potential for innovation and growth.

In fact, Blue-Action opens matchmaking dialogue between the users of the project, data modelling and their analyses and the core scientific groups. The goal is of strengthening the competitiveness and growth of businesses which need climate and weather data or analysis for enhancing their existing core business activities; those that rely on improved forecasting capacity. A focus of Blue-Action is on providing knowledge and security in the blue economy, focussing on marine knowledge, to improve access to information about the sea and maritime spatial planning, to ensure efficient and sustainable management of activities at sea. The Mediterranean Sea accounts for 28% of all endemic species in the world and is therefore considered a biodiversity hotspot and for 30% of the global sea-borne trade by volume indicating the importance of the Mediterranean basin as an area of biological as well as economic importance.

The Blue Mediterranean Project (BlueMed) is a medium-term plan launched in 2014 during the Italian Presidency of the European Union for promoting the blue economy in the Mediterranean Basin through cooperation between all Mediterranean countries for a healthy, safe, and productive Mediterranean Sea. BlueMed is contributing to reach several Sustainable Development Goals of the UN 2030 Agenda. Specifically, they are targeted to focus on Sustainable Development Goals (SDG)14-Life below Water, SDG12- Sustainable Consumption and Production and SDG17-Partnerships for Sustainable Development (赵建文, 2016).

1.2.2 Marine Natural products

The concept of marine natural products was put forward in the 1950's. The marine habitat is a complex environment that is characterised by great biodiversity both in terms of organisms inhabiting it as well as the active biomolecules that they produce, with an estimated number of marine organisms ranging into 100 million (Fattorusso, Gerwick, & Taglialatela-Scafati, 2012). Whilst seawater is known to contain 10^6 microorganisms per mL of water, the sediment contains 10^9 microorganisms per g. Although these numbers vary with various authors, a vast array of biologically active compounds have been extracted from various marine organisms in a process known as bioprospecting and more than 22,000 marine natural products have been extracted by 2012 (Fattorusso *et al.*, 2012) and 28,609 until 2016 (Kamyab *et al.*, 2019) indicating that the interest in these compounds is increasing at a

very fast rate. This opens the door to a range of biomolecules potentially left to be found in the future and hence the marine environment is undoubtedly an enormous resource (Kamyab *et al*., 2019).

Natural products are compounds that are obtained from living organisms and may give their effect in pure form. However more often than not, given that the organism coexists in an ecosystem, interacting with other organisms whether externally or internally, natural products are found to act as a combination of two or more biomolecules interacting together. These molecules, in combination, may either enhance each other's effect, work antagonistically to each other or else give a different effect altogether than either one would on its own (Singh & Geetanjali, 2018). The chemistry of these compounds plays a major role in the determination of the interaction of these molecules. In comparison to synthetic compounds, natural products are now being more sought after for use in medicine and in the pharmaceutical industry on an international level. This has made biodiversity to become the most important natural resource for medicinal purposes (Singh & Geetanjali, 2018). Natural products are, more often than not, as efficient and less harmful sources of drug molecules when compared to synthesized organic compounds (Molinski *et al*., 2009).

Marine organisms that are being studied for natural products can be divided into three main groups: marine microorganisms (including phytoplankton), marine algae and marine invertebrate (Hu *et al*., 2011) which include but are not limited to tunicates, sponges, molluscs, and echinoderms (Victor & Sharma, 2015). To date, marine invertebrates have been the main contributor to natural products and even though a large number have been extracted and isolated in the past decades with many exhibiting potential and promising use as therapeutic agents displaying remarkable bioactivity, only a few have successfully reached been developed as active drugs (Hu *et al*., 2011). Out of the 28,609 natural products extracted from marine organisms until 2016, 35% were isolated from echinoderms, however the diversity was not high (Kamyab *et al*., 2019). To date, 2000 studies related to marine natural products (MNP) have been published from which 52 marine invertebrate-derived compounds reached clinical trials with only seven compounds, isolated from sponges, molluscs, tunicates, and their associated bacteria, having so far been approved (Kamyab *et al*., 2019). Unfortunately, 45 of the total 52 MNPs have been discontinued from clinical trials

due to low production yields and/or high costs (Kamyab *et al*., 2019). Based on the past 75 year of research, some conclusions regarding the various marine phyla as reservoirs of marine natural products can be drawn. Data on sessile marine organisms such as sponges, ascidians, and corals in relation to marine natural products is very diverse, however information from echinoderms is much more limited (Kamyab *et al*., 2019). Organisms belonging to the phylum porifera have given a lot of novel molecules and have lent themselves to research in that they are easily obtained from the sea with a good mass (Fattorusso *et al*., 2012). Crinoids and ophiuroids possess pigment and lipid derivatives these being aromatic sulfonated compounds as their major groups of bioactive compounds (Kamyab *et al*., 2019). Marine invertebrate taxa involving slow moving organisms display unique features such as the secretion of powerful chemicals to defend themselves against predation, competition, and other ecological pressures (Liu, Zheng, Shao & Wang, 2019). This is as a result of the fact that these organisms cannot run away from predators as they slow moving (Kamyab *et al*., 2019). In addition, many marine organisms show symbiotic relationships with microorganisms (Liu *et al*., 2019) which produce chemicals to facilitate chemical defence against detrimental microorganism colonisation.

Marine natural products have been classified into different categories but one way they can be divided is into seven classes based on their chemical structures. These are terpenoids, steroids (including steroidal saponins), alkaloids, ethers (including ketals), phenols (including quinones), strigolactones, and peptides (Hu *et al*., 2011). Both primary and secondary metabolites from marine invertebrates are screened for their potential effect as natural products. Primary metabolites include amino acids, simple sugars, nucleic acids, and lipids, and are essential for cellular processes and hence the organism's survival. Secondary metabolites, such as alkaloids and terpenoids, are not directly involved in critical physiological processes, and often play a role in interspecific and other ecological interactions. (Singh & Geetanjali, 2018). Li, Himaya and Kim (2013) state that triterpenoids are the most abundant secondary metabolites present in sea cucumbers which show remarkable bioactivity. Hence the occurrence of such bioactive compounds increase the chances for successful biomolecule discoveries from such species and consequent patenting and commercialization.

Extraction, separation, isolation, and purification of novel natural products from marine organisms remains a challenging task for various reasons. The fact that these molecules are found in a salt water as a medium can prove difficult to extract without the loss of the active ingredient. The fact that molecules, whether being primary or secondary metabolites, interact together, can prove difficult to determine their direct function, especially when this is coined with the fact that there is a lot of missing data yet gathered. Such processes can be made easier by using hybrid methods such as LC-MS or LC-NMR, amongst others. In addition, in order to obtain complete chemical structures which includes the appropriate stereochemistry, there needs to be highly purified compounds and reference compounds which might be difficult to obtain (Bucar, Wube & Schmid, 2013). The chemical synthesis of triterpenoids is often costly and inefficient (Rascón *et al*., 2016) and may not yield enantiomerically pure terpenes. Large scale microbial production in order to have enough secondary metabolites to be able to test adequately requires expensive feedstocks (Wu *et al*., 2013).

1.2.2.1 Natural products originating from Echinoderms.

Organisms belonging to the phylum Echinodermata have been shown to possess various useful chemicals which show bioactive properties (Kamyab *et al*., 2019) related to treating bacterial, fungal, and viral infections, chemicals which can help in the blood clotting process, and others which cause cytotoxic effects (Layson, Rodil, Mojica & Deocaris, 2014). In a study by Cuong *et al*., (2017), eight triterpene diglycosides, of which four were novel compounds were isolated ant they showed toxicity on five human cancer cell lines, these being Hep-G2 (hepatoma cancer), KB (epidermoid carcinoma), LNCaP (prostate cancer), MCF7 (breast cancer), and SK-Mel2 (melanoma). Natural products from echinoderms are mostly aliphatic (about 46%) in nature, followed by carbohydrates, steroids, terpinoids, alkanoids and a very small percentage of amino acids and peptides. In 2017, 36 new metabolites were reported from echinoderms, and these included sulfated alkanes 2,6-dimethylheptyl sulfate and octyl sulfate and decyl sulfate (Kamyab *et al*., 2019). Bhakuni & Rawat (2005) indicate that echinoderms usually contain polyhydroxysteroids and saponins, the latter having haemolytic activity.

1.2.2.2 Natural products originating from Holothurians.

Extracts from sea cucumbers have a range of functions, such as anti-constipation, against hypertension and asthma and also for nutraceutical, pharmaceutical and cosmeceutical products (Esmat *et al*., 2013; Pangestuti, & Arifin, 2017; Siahaan *et al*., 2017).

Thus far, approximately 300 diverse compounds, such as saponins (Caulier *et al*., 2011), chondroitin sulfates, glycosaminoglycan (GAGs), phenolics, cerberosides glycoprotein, glycosphingolipids, terpenoids, essential fatty acids (Hassan and Hassan, 2012), polysaccharides (Wu *et al*., 2013) and peptides (Pérez-Vega, Olivera-Castillo, Gómez-Ruiz & Hernández-Ledesma, 2013), have been isolated from sea cucumber; these compounds elicit anti-angiogenic (Tian *et al*., 2005), anticoagulant (Du, Long & Chen, 2014), antimicrobial (Wang *et al*., 2018), antioxidant (Liu *et al*., 2019), antithrombotic, and antitumor (Al Marzouqi *et al*., 2011) effects. Cytotoxic glucoerebrosides and long-chain bases have been extracted from the sea cucumber *Cucumaria frondosa* (Xu *et al*., 2013). The sulfated alkenes (5Z)-dec-5-en-1-yl sulfate and (3E)-dec-3-en-1-yl sulfate were isolated from the sea cucumber *Apostichopus japonicus* (Fattorusso *et al*., 2012). A high molecular weight form of fucosylated chondroitin sulfate, known as Holothurian glycosaminoglycan, has been extracted from sea cucumbers and it has been found to have both an antithrombotic effect as well the ability to inhibit tumour metastasis (Zhao *et al*., 2013). Attoub *et al.*, (2013) have identified an extract known as Frondoside which has shown to have a suppressive effect on lung tumour growth, angiogenesis, invasion, and metastasis. Sea cucumber extracts also contain saponins (triterpene glycosides) which have anti-inflammatory and antitumour compounds. Other extracts of sea cucumbers contain methyltetradecanoic acid which is associated with the treatment of asthma and ulcerative colitis. In an article by Bahrami, Zhang & Franco (2014), it was stated that novel saponins, such as triterpene glycosides which have a lot of industrial, agricultural, and pharmacological functions, have been extracted from the viscera of the Australian Sea Cucumber *Holothuria lessoni*.

The various metabolites isolated can be divided into the following sections: Triterpene glycosides saponins which account for more than 300 different molecules, as well as other molecules including but not limited to steroids, terpenoids,

isoprenoids, nonisoprenoids, quinones, brominated compounds, nitrogen heterocyclics and nitrogen sulphur heterocyclics and glycosaminoglycans (Bhakuni & Rawat, 2005; Soltani *et al*, 2014; Luparello *et al.*, 2019). Other compounds include carotenoids, bioactive peptides, vitamins, minerals, fatty acids, collagens, gelatines, chondroitin sulfates (polysaccharides) and amino acids, glycosaminoglycans and phenolics (Luparello *et al.*, 2019). Vitamins include vitamins A, C, B1, B2 and B3. Sea cucumbers also contain trace elements such as calcium, iron, magnesium, and zinc.

Triterpenoids are units made up of 30C atom which form 6 isoprene units' synthesis by cyclisation of squalene. Triterpenoids can be found free a linear or cyclic molecules or else in combinations with sugars forming glycoside and ester. Triterpenoid saponins are glycosides consisting of glycone (sugar moiety) and aclycone (triterpenoid component). They are generally water soluble and characterised strong foam-forming in aqueous solutions. Saponins are toxic and have significant chemotherapeutic effects such as bladder, breast cervix and colon. Cancer progression involves a number of underlying intrinsic cellular and extrinsic micro environmental factors. Saponins are known to inhibit proliferation, induce apoptosis as well as attenuation of invasion and metastasis. Saponins also inhibit angiogenesis, has anti-inflammatory effects, antioxidative effects amongst others (Du *et al*., 2014).

An extract from the sea cucumber *Holothuria nobilis* was obtained and marketed as Echinoside A (Li *et a*l., 2010). It has shown features in the induction of DNA double strand break in a Top2-dependent manner which is characteristic to this extract and is different in action from any other known Top2α inhibitors. This extract inhibits the noncovalent binding of Top2α to DNA by competing with DNA for the DNA-binding domain of the enzyme. This causes an interference in the Top2α-mediated prestrand passage cleavage/religation equilibrium over the poststrand passage one. As a result, echinoside A induced DNA double-strand breaks (Li *et a*l., 2010).

The following table (Table 1) gives an overview of the range of compounds extracted from Holothuroids and these include triterpene glycosides, peptides, polysaccharides, and lipids.

Table 1: Table showing major classes of secondary metabolites, biological activity, and example of sea cucumber from which it was extracted. Reference: Kamyab et al., 2019).

Major classes of secondary metabolites	**Biological activity**	**Species**	**References**
Triterpene glycoside	Antifungal, anticancer, ichthyotoxic	*Holothuria atra, Holothuria fuscocinerea*	Yamanouchi (1955), Kobayashi *et al*. (1991), Popov *et al*. (1994), and Zhang *et al*. (2006d)
Triterpene glycoside	Anticancer, antifungal, antiprotozoal	*Apostichopus japonicus*	Kitagawa *et al*. (1976), Wang *et al*. (2012)
Triterpene glycoside	Antifungal	*S. japonicus*	Yano *et al*. (2013)
Polysaccharides	Antihyperlipidemic, antioxidant	*A. japonicus*	Liu *et al*. (2012)
Sulfated polysaccharides	Anticoagulant, antithrombin, antiparasitic	*Ludwigothurea grisea*	Borsig *et al*. (2007), and Marques *et al*. (2016)
Lipid	Anticancer, antihyperlipidemic	**Stichopus variegatus, Acaudina molpadioides, Bohadschia argus*	Sugawara *et al*. (2006), Ikeda *et al*. (2009), Zhang *et al*. (2012), and Du *et al*. (2015)
Peptide	Antihypertension	*Acaudina molpadioides*	Zhao *et al*. (2009)
Peptide	Antibacterial	*Holothuria scabra*	Gowda *et al*. (2008)
Phenolic	Anti-inflammatory	*S. japonicus*	Song *et al*. (2016)

compounds			
Phenolic compounds	Anticancer	*Holothuria parva*	Amidi *et al.* (2017)
Pigments	Antioxidant	*Holothuria atra*	Esmat *et al.* (2013)
Pigments	Antioxidant	*Plesiocolochirus minaeus*	Maoka *et al.* (2015)
Mucopolysaccharide	Antitumor, immunomodulatory effect	*S. japonicus*	Song *et al*. (2013)
Saponin	Anticancer	*C. frondosa*	Janakiram *et al.* (2010) and Jia *et al*. (2016)

Many of these metabolites exhibited cytotoxic effects to some degree. Other cytotoxic compounds such as glucoerebrosides and long-chain bases have been extracted from the sea cucumber *Cucumaria frondosa* (Xu *et al*., 2013). Literature about such cytotoxic compounds in *H. poli* is limited.

Even though a large number of different biomolecules have been isolated and identified, the mechanisms by which these components affect various diseases and synergistically or antagonistically interact with one another at molecular and system levels are not entirely clear (Yun *et al*., 2012; Guo *et al*., 2015). Great research is being done in this area. In a study conduced by Yun *et al*., (2012) the mechanisms through which a compound called stichoposide C, extraceted from *Thelenota anax,* induces apoptosis in leukaemia were investigated. It was determined that stichoposide C causes apoptosis of leukaemia cells in a a dose-dependent manner. It also causes the activation of Fas and caspase-8, cleavage of Bid, mitochondrial damage, and activation of caspase-3 reducing tumor growth of HL-60 *in vivo*.

1.2.3 Biosynthesis

Biosynthesis involves the study of the biochemical processes which are utilised by living organisms in order to convert simple molecules into more complex molecules (Blunt, Copp, Keyzers, Munro & Prinsep, 2014). The first comprehensive review of this topic occurred in 1983 and since then much research has been done.

The main distinguishing feature of the holothurians is the occurrence of the triterpenoid glycosides of the holostane type which is exclusive to these organisms (Blunt *et al*., 2014). Glycosides are compounds with one or more sugars which are combined via a glycosidic linkage to a non sugar molecule (Singh & Geetanjali, 2018). Several factors contribute to the triterpenoid composition of *Holothuria* as it is affected by the sources of the sterols. These can be from the diet of the animal, the absorption of sterols from their symbiotic microorganisms, the ability of the holothurian to modify the absorbed dietary sterol, and any de novo biosynthesis. In *Holothuria* there are two main biosynthetic *de novo* routes that operate, one via lanosterol and the other via parkeol (Blunt *et al*., 2014; Claereboudt *et al*., 2019,). However, experiments still need to confirm this assumption. Such experiment will increase the chances for successful drug discovery and consequent patenting and commercialization.

Being deposit feeders, they are involved in recycling organic matter, mixing the sediment layer, and stimulating the growth of many microalgae (Nerva *et al*., 2019, Boncagni *et al*., 2019). The origin and mode of formation of secondary metabolites in marine organisms is believed to be similar to the well documented biosynthetic pathways of the secondary metabolites of terrestrial plants and animals.

There is a knowledge gap in the origin and mode of formation of secondary metabolites in marine organisms, but it is believed to be similar, in part to the well documented biosynthetic pathways of the secondary metabolites of terrestrial plants and animals. Although a lot of research has been done in this area, as shown by Petersen, Kellermann, & Schupp, (2019), a lot of research still needs to be done in this area for a more detailed and complete picture.

1.2.4 Major area of concern when using organisms from the marine environment

Since this study offers pioneer research in the area, the nature of the active ingredient with a potential positive effect is not known. There were some major areas of concern that needed to be taken note of in this investigation as they might potentially affect the study, and these were:

1.2.4.1 The origin of primary and secondary metabolites:

The metabolic activities and hence the different types of chemicals produced by the different species of sea cucumbers depends on their habitat, their mode of nutrition, and their interactions with other organisms. Sea cucumbers are benthic and act as a biotic substrate whereby organisms such as bacteria, viruses and fungi constantly live on or within the organism whereby they use it as a source of food, or a place where they can complete their life cycle (Sun *et al*., 1989). In a study by Sun *et al*., (1989) eleven genera of bacteria and four genera of yeast were isolated from various parts of the sea cucumber *Stichopus japonicus*, whilst in a study by Farouk *et al*. (2007) 30 bacterial strains were extracted from *Holothuria sp*. The degree and exact nature of the association with microorganisms varies from species to species. The pathways of transfer of nutrients between symbiotic partners is of great importance, particularly when sessile animals are involved and raise questions about the real origin of the metabolites produced by the association (Bhakuni & Rawat, 2005). For example, highly branched fatty acids from sea cucumbers are known to have originated from their bacteria inhabiting these organisms (Fattorusso *et al*., 2012). In a microbiological investigation carried out by Omran & Allam, (2012) on the *Holothuria poli* species collected from Abu-kir shore of Alexandria, Egypt in the Mediterranean, remarkable antifungal activities on *Aspergillus niger, Scloretium* sp, *C. albicans, Aspergillus flavus* and *Malassezia furfur*, were found as well as limited antibacterial activity against Gram-negative bacteria (*Salmonella choleraesuis* and *Aeromonas hydrophila*). On the contrary, Omran & Eissa., (2006) isolated nothing from *H. poli* teguments collected from the same area in July 2005 suggesting that the relationship is based on the microorganisms present at the same time and the quality of the water. In a study by (Nerva *et al*., 2019), 10 mycoviral genomes were

identified, with a total of 24 genome segments (Nerva *et al.*, 2019). In addition, coelomic fluid contains coelomocytes which secrete proteins inside the fluid, essential to give a degree of immunity, hence affecting the microbial community.

1.2.4.2 The osmotic nature of the coelomic fluid:

The organisms under study are marine organisms and hence their coelomic fluid contains a large amount of salt as they are stenohaline and the active ingredients are water soluble. Water soluble extracts allow for bacterial and fungal growth over a short period of time, and these may degrade the active compound. False results due to endotoxins produced by microorganisms would also give false positives. Literature shows that many of these endotoxins exhibit antitumour activities and include many lipopolysaccharides. Although brief heating could be used in order to eliminate this problem, it could also degrade the active ingredient. Evaporation of water in order to from a concentrated aqueous extract is also problematic due to the high heat of vaporisation of water. However, freeze-drying in order to lyophilise the solid is a good way of overcoming this problem, still not solving the problem associated with high salinity.

Working with molecules with a high mass such as some proteins rather than smaller masses which is usually the case with terpenes would have made separation much easier. For example, in the study involving *Holothuria* carried out by Luparello *et al.*, (2019), the coelomic fluid is fractioned into two fractions, one <10KDa and one of >10 KDa. The salt remained in the lower fraction and the upper fraction, which consisted mainly of protein, was used for testing.

Being water soluble, ethanolic/methanolic extracts would be needed and the active ingredient would be soluble in such solvent. This mixture of compounds which is to date very difficult to separate into its constituent chemicals. The ethanolic/methanolic mixture extract can be successively extracted with hexane, chloroform, ethyl ethanoate and then divided into water soluble and water insoluble fractions. Hexane and chloroform, being nonpolar would possess lipophilic components such as esters, ethers, hydrocarbons of terpenoids, sterols, fatty acids and others and extraction of the compounds from these solvents is much easier than from

water due to salt not being able to dissolve in such solvents. It usually involves standard chromatographic techniques. However, there would be great losses in every separation step and if the amount of active ingredient is very small, this will make extraction nearly impossible.

The high concentration of salt in the coelomic fluid of these organisms would negatively affect the Leukaemia cells being tested, as it would offer a great osmotic gradient, causing the cells to shrink and die. Desalination processes exist and these involve Ion exchange chromatography, Reverse-Phase columns, High/Medium pressure chromatography, a combination of ion-exchange and size-exclusion chromatography and possibly other methods. These all require a knowledge of the size of the molecules being looked for and are not adequate for the isolation of low molecular weight compounds from aqueous extracts of marine organisms.

In conclusion, biological activity, if at all present, can, in fact, be in one or more fractions as has been reported in studies (Luparello *et al.*, 2019). The chemical nature of the mixture of these bioactive compounds is possibly very complex and is not yet clearly elucidated, hence it would be difficult to single out a specific technique of extraction.

In a study by Agustina, Bella, Karina, Irwan, & Ulfah, (2021), with the aim to identify the sea cucumber *Holothuria atra* and its secondary metabolite content, maceration was used to extract the secondary metabolites whilst isolation of secondary metabolite was carried out by column chromatography. Maceration is a multistep extraction where the original solid is ground to power from in order to increase the surface area and allow it to mix more with the solvents. Solvent is then added and the pressed-out liquid as well as the strained solvent are separated from any solid material by filtration. Throughout these steps agitation is done to increase extraction yield (Srivastava, *et al.*, 2021). For chromatography, the mobile phase employed was a mixture of n-hexane : ethyl acetate (8:4), whilst the stationary phase was silica gel GF254. Phytochemical screening was performed to indicate the presence of secondary metabolites. For example, Wagner's reagent gives a reddish-brown precipitate in the presence of alkaloids, whilst Lieberman-Burchard's reagent gives a greenish blue ring in the presence of a steroid.

In a study by Hassan and Hassan (2012), the body wall and the coelomic fluid of three sea cucumbers *Holothuria scabra*, *Holothuria leucospilota* and *Holothuria atra* were used for the extraction of particular molecules. Extract were shaken well at 120 rpm for 1 week with ethanol (70%), methanol, ethyl acetate and chloroform/methanol (2:1). The supernatant residue of each sample was collected and stored at 4°C before testing the sample on the various bacteria. Detection and separation of the various carotenoids was done by HPLC (Agilent Technologies 1200 Series, German) with UV/ VIS detector and column: Eclpise XDB C18, 5 micrometer (4.6\150 mm) at 480 nm as shown by figure 5:

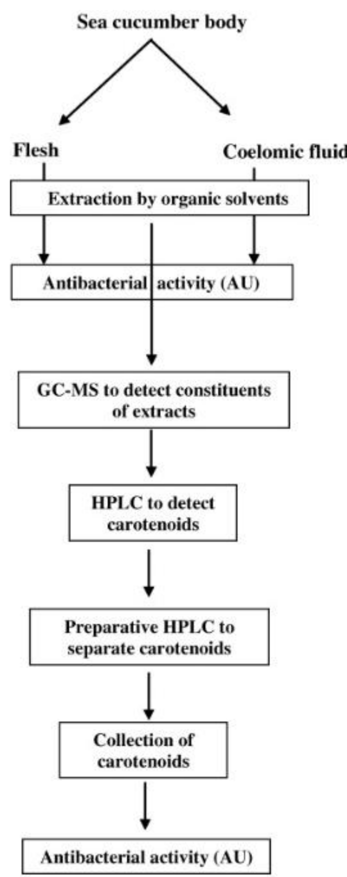


Figure 5: Diagrammatic representation of the extraction methods used by Hassan and Hassan (2012)

Chromatographic separation of the coelomic fluid from *Holothuria poli* has been carried out by Canicatti & Parrinello (1983), by passing the coelomic fluid through a Bio-gel A5m column (1.5 x 90 era) equilibrated with PBS. The samples obtained were then concentrated using ultrafiltration in a Diaflo equipped with a UM 2 membrane (Amicon Corp., Lexington, Mass). The final extract was then dialyzed

overnight against the starting buffer. UV absorbance at 280 nm was used for analysis. The aim in this particular study was to separate proteins that show hemagglutinating or haemolytic activity against rabbit erythrocytes.

Notwithstanding these problems cytarabine (from *Cryptotethia crypta* – sponge) (Adrian, 2007), trabectedin (from *Ecteinascidia turbinata* - sea squirt and *Candidatus Endoecteinascidia frumentensis*, a microorganism which lives in a symbiotic relationship with tunicates), eribulin mesylate (synthetic analogue halichondrin B from *Halichondria* - sponges and monomethyl auristatin E (MMAE) (derived from *Dolabella auricularia* – a mollusc) (Correia-da-Silva, Sousa, Pinto, Kijjoa, 2017), are four compound that see their origin in marine organisms and are used in the treatment of various cancers (Adrian and Collin, 2018). Cytarabine, was synthesized after a spongonucleosides biomolecule containing arabinose was extracted from the sponge, *Cryptotethia crypta*. This molecule has been used in the treatment of various forms of leukaemia. Its mode of action is to replace a cytosine residue in DNA, stopping DNA synthesis during the cell cycle (Adrian, 2007).

1.3 Haematopoiesis and Leukaemia

1.3.1 Normal Haematopoiesis

Haematopoiesis is a process which predominantly takes place in the bone marrow of certain bones including but not limited to ribs, sternum, and pelvic bones, through which blood cells are made. Pluripotent hematopoietic stem cells (HSC) are responsible for the formation of all the different blood and lymph elements and the regulation of its growth and differentiation processes are essential for normal haematopoiesis to take place (Sawyers, Denny, and Witte, 1991; Schmaier and Lazarus, 2012; Chatterjee *et al*., 2016). HSCs are characterised by a capacity to self-renew maintaining a stem cell pool essential for haematopoiesis to occur. Haematopoiesis involves the interaction of multiple genes, cytokine activity together with the activity of other protein factors (Munker, Hiller, Glass, & Paquette, 2007), and is therefore a multi-step development which needs to be carefully controlled. If not strongly regulated, diseases such as cancers may occur. This control is greatly controlled by the bone marrow microenvironment, which is also known as the as the bone marrow niche. Regulation is also brought about by cell-to-cell contact, which is mediated by integrins which are cell adhesion molecules (Munker *et al*., 2007).

The highly specialised bone marrow niche can be broadly separated into two sections, the outer edge of the bone marrow (endosteal niche) which is composed of quiescent HSC's and the inner core of the bone marrow (vascular niche) which is composed of actively dividing cells and mesenchymal stem cell (MSC) derivatives (Boulais & Frenette, 2015). These derivatives include stromal fibroblast cells, endothelial cells, and osteoblasts amongst others (Weiss & Sakai, 1984; Dorshkind 1990). The various chemicals and growth factors being produced in a particular niche will determine and effect the functions of the HSCs (Arai, 2006) making the bone marrow a complete factory of blood cell production. Osteoclasts are important not only in promoting the formation of HSC niches but also in maintaining them. They are important in the release of calcium from bone which is essential for the maintenance of HSC's and also for the release of growth factors such as basic fibroblast growth factor and transforming growth factor ß which regulate the fate of HSCs through various mechanisms such as those involved in regulating quiescence and self-renewal of these cells (Itkin, Kaufmann, Gur-Cohen, Ludin and Lapidot, 2013). Stromal cells are essential for the migration and differentiation of HSC's as they are essential for cytokine and extracellular matrix molecule production allowing for contacts between different cells (Boulais & Frenette, 2015). Examples of cytokines involved in haematopoiesis are thrombopoietin (TPO) involved in the regulation of the development of megakaryocytes and platelets, Stem cell factor (SCF) which binds to its tyrosine receptor c-Kit and Notch ligands which include Jagged and Delta ligands both being crucial for lymphopoiesis, HSC production and differentiation (Duncan *et al*, 2005). HSC's express both TPO and its receptor c-mpl which are essential for self-renewal of HSC's. HSC's also express c-Kit receptor which is essential for the survival of HSC by averting apoptosis. Pathophysiological consequences such as the various forms of Leukaemia occur as a result of changes in this chemical niche during the production of the blood cells (Ogawa, 2014, Chatterjee *et al*., 2016).

During normal haematopoiesis, HSCs exist in two subsets, these being long term hematopoietic stem cells (LT-HSC) and short-term haematopoietic stem cells (ST-HSC). LT-HSC are found in bone marrow niches whilst ST-HSC may be mobilised. Self-renewal is indefinite for LT-HSCs whilst definite for ST-HSCs. This definite period is of about 8-12 weeks long. The pluripotent stem cell commits itself

to certain cell lineages at an early stage. One stem cell can self-renew and differentiate into progenitor cells. Once a cell differentiates and becomes specialised, it will lose its plasticity and with it its ability to proliferate (King, 2006).

The self-renewal ability of HSC's is regulated through two main pathways, these being telomerase activity (an enzymes which is needed to repair the chromosomes so that they do not become shorter after replication) and apoptosis (programmed cell death) (Blackburn, 2001).

Human haematopoiesis involves a number of colony stimulating factors (CSF) (such as macrophage colony-stimulating factors (M-CSF), granulocyte colony-stimulating factors (G-CSF), and granulocyte-macrophage colony-stimulating factors (GM-CSF)), as well as interleukins which are glycoproteins and play a key role in the regulation of the processes required for the differentiation in both macrophage and granulocytic lineages (King 2006, Litwack, 2017). These proteins alter cell kinetics by reacting with specific receptors on the cell surface membrane (King, 2006). By binding to their cytokine receptor, activation occurs, leading to the activation of the Janus kinases (JAKs) through phosphorylation. This in turn creates signal transduction and activates the STAT transcription pathway. Translocation and binding of the activated STATs to target genes occurs and this is essential for transcription of many important genes that are needed for the survival, proliferation, and differentiation of cells. In Myeloid cell differentiation, interleukin-3 causes cell proliferation of the pluripotent stem cell. IL-3 will then cause more specific CSF's to be switched on and initiate the processes of differentiation and proliferation. Granulocyte CSF will promote granulocyte development whilst macrophage CSF causes development of the macrophage lineage (King, 2006).

Human haematopoiesis (figure 6) occurs via two lineages, the myeloid (or myelopoiesis) and lymphoid lineage (or lymphopoiesis). The common myeloid progenitor will keep on differentiating through a series of steps to form various cells including megakaryocytes, red blood cells, granulocytic (the neutrophils, eosinophils, and basophils) and monocytic lineages. Through the lymphoid origin, natural killer cells and B and T lymphocytes are produced (Miyamoto *et al*., 2002).

Leucocytes can be divided into 5 main groups; namely neutrophils, lymphocytes, monocytes, eosinophils, and basophils based on their function but also on their morphology. Normal blood would contain 40-75% neutrophils and 2-10% monocytes. Both neutrophils and monocytes are associated with immunity, and both are associated with phagocytosis of unicellular organisms such as bacteria and yeast, involved in inflammation and remove debris from the blood. Monocytes acts as antigen presenting cells and are therefore involved with lymphocytes in order to generate antibodies, release cytokines and are important for the expression of tissue factors. It is believed that once it starts differentiating it will commit to that lineage (Miyamoto *et al*., 2002). At each stage there occurs the expression of surface markers denoted as cluster of differentiation at it defines the stage of differentiation.

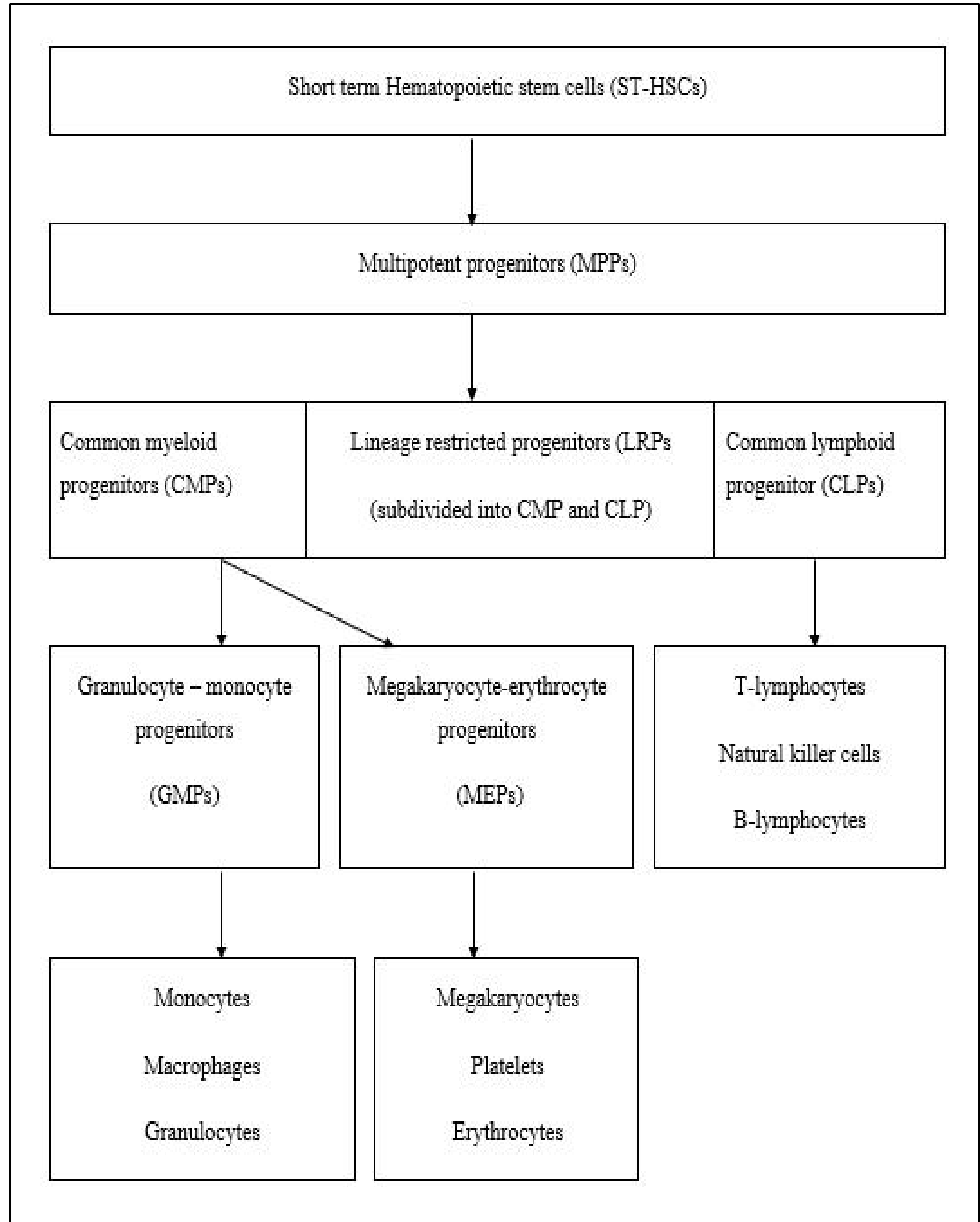


Figure 6: Flow chart showing normal haematopoiesis. Drawn by Author. Adapted from: Miyamoto et al., 2002.

White blood cells can also be distinguished based on the size and shape of their nucleus as well as the presence or absence of granules in their cytoplasm. Neutrophils are polymorphonuclear leukocytes as they have an irregular nucleus which has 3-5 lobes. Neutrophils are also granulocytes. These granules contain enzymes and chemicals that are used in the process of phagocytosis and are found at a neutral pH. On the other hand, monocytes are mononuclear leucocytes which have a round or kidney shaped and regular nucleus and are agranulocytes. The nucleus in monocytes

occupies 70-80% of the cells and they may have vacuoles in the cytoplasm (Munker *et al*., 2007). Figure 7 shows the occurrence of haematopoiesis in humans, giving special attention to the size and shapes of nuclei.

The hematopoietic system produces the required number of different types of blood cells during an individual's lifetime. There is a carefully balanced mechanism of differentiation, proliferation and self-renewal and any changes to these mechanisms will result in irregular generation of abnormal cells (Mashanov, *et al*, 2010). These mechanisms can be affected by cellular conditions and external pressures. They would lead to an irregular differentiation of cell and premature generation of immature/abnormal cells and presence of populations which have distinct sub-clones that are genetically heterogeneous when compared to the other cells in circulation causing the adverse effects associated with various cancers (Udensi & Tchounwou, 2014). It has been shown that the balance between cell replication and apoptosis can be shifted in order to meet the needs during growth and regeneration (Mashanov & Garcia-Arraras, 2011). Apoptosis is very important in several stages of carcinogenesis, and even in established cancer. For example, Bcl2 overexpression indicates a poor prognosis because it is an indication of the occurrence of proliferation with the absence of apoptosis and high resistance to chemotherapy. Genetic defects such as loss of the p53 break also predisposes an organism towards high cancer risk as the p53 suppressor protein is a key protein in integrating responses to DNA damaged cells. It will cause the cell cycle to stop and initiate apoptosis. There is therefore an inverse relationship between differentiation and proliferation, but this is only valid if the two features are compared at the beginning and the end of a developmental sequence (King 2006). It's important to note that these two processes might also occur simultaneously at other stages. Cells at one stage of differentiation are sensitive to one growth factor, so different growth factors are needed for different differentiation stages. In established tumours, there is faster growth and poorer differentiation (King, 2006) highlighting the inverse relationship between the development of cancerous properties and differentiation.

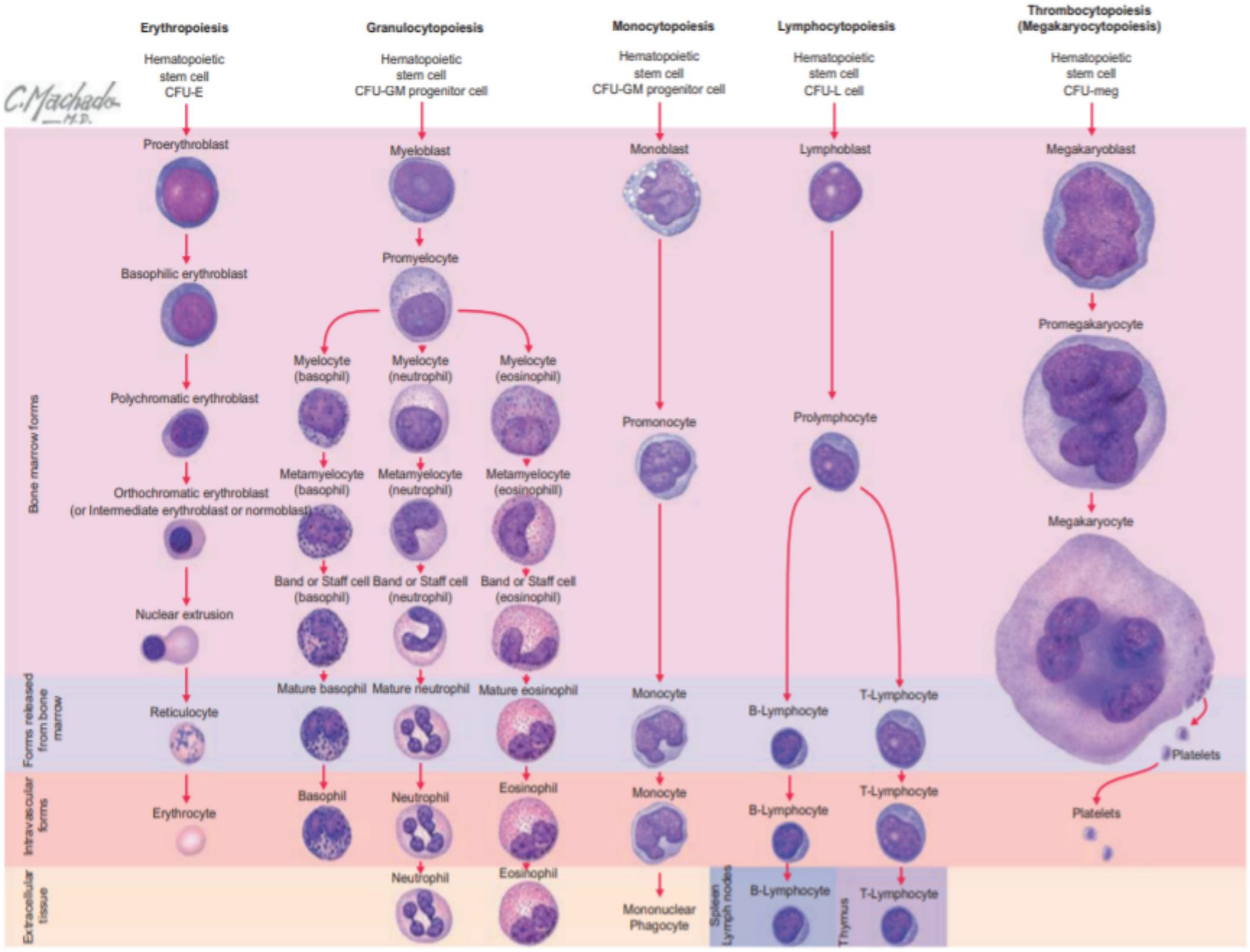


Figure 7: Figure showing Haematopoiesis with special reference to the nuclei of each cell. Reference: Ward, Cherian & Linden. 2018

1.3.2 Leukaemia

The pathophysiology behind the development of leukaemia lies in the loss of the HSC's function and behaviour as a result of malignant transformation events. Marrow hypercellularity also occurs due to overshoot of functionally inactive undifferentiated cell proliferation. Hence in Leukaemia there is dysregulation and dysfunction of both HSCs and their niche (Chatterjee *et al*., 2016). Leukaemia's are haem proliferative disorders and occur as a result of a blockage in the differentiation process occurring in these progenitor cells (King, 2006, Udensi & Tchounwou, 2014). Blocked maturation at different stages of differentiation of the white blood cells give rise to the several subtypes of leukaemia (King, 2006). For example, a blockage at the myeloid stem cell gives rise to AML-M0 and AML-M1, whilst blockage at the promyelocyte stage causes AML-M3 (refer to table 2). It could also be that blockage at various stages down the differentiation line causes a particular subtype. For example, CML is known to occur either due to a differentiation at the myelocyte stage or further down the differentiation line. Blockage can happen in the common granulocyte - macrophage progenitor or less often in the megakaryocyte – erythrocyte progenitor. Studies show that leukaemia can be initiated through anomalous HSCs which become leukaemia stem cells (LSCs). These LSC's exploit the marrow niche for self-renewal (Riether, Schürch, & Ochsenbein, 2015). In addition to having a blockage in differentiation, LSC's are also accompanied by specific chromosomal abnormalities. This alters the gene expression, giving the additional mutations required to convert the cell into a leukemic state and hence giving them the malignancy a proliferative advantage (Nowak, Stewart, & Koeffler, 2009). Such cells are less likely to undergo apoptosis and show an increased tendency to proliferate. As cells differentiate, because of signals obtained by the cells, they divide more slowly and consequently by blocking differentiation in cancers, such as leukaemia, growth rate increases, which is one of the hallmarks of cancer (King, 2006). To date the relationship between the stage at which a cell is blocked from differentiating and the genetic change is not clear for most Leukaemia's.

A number of mutations are known to give rise to Leukaemia stem cells and these mutations can be classified as class 1 or class 2. Class one mutations are those which effect signalling pathways, whilst class 2 are those that involve transcription

preventing the cell from maturing. Class 1 mutations include mutations in the tyrosine protein kinase (KIT), tyrosine kinase 3 (FLT3) and Retroviruses associated DNA sequences (RAS). The KIT gene codes for a receptor tyrosine protein c-KIT. Binding of cytokines to the c-KIT results in a number of protein phosphorylation leading to the activation of signal transduction pathways such as MAP kinase and phosphatidylinositol-3 kinase (P13K). Mutations in the mentioned gene result in the receptor being activated in the absence of a ligand and therefore constant phosphorylation happen leading to cellular proliferation (Takahashi, 2011). Mutations in the FLT3 gene also allow for constant phosphorylation, leading to cell proliferation and the pathways affected are the MAP kinase and STAT. Uncontrolled proliferation is also brought about by mutations in the RAS genes (Chatterjee *et al*., 2016).

Mutations in nucleophosmin 1 (NPM1), CCAAT/enhancer-binding protein α (CEBPα), and Runt-related transcription factor 1 (RUNX1) are class 2 mutations. It has been reported that class 1 mutations vary between the initial and relapse stages of the diseases, whilst class 2 mutations remain conserved. This suggests that class 2 mutations are more likely to happen in the early stages of LSC formation. The gene NPM1 codes for a protein which acts as a chaperone protein for nucleic acids and proteins. A change in the conformation of this protein leads to knocking down of onco-suppressors as well as activation of the c-myc oncogene. LSCs cannot terminally differentiate and have defects in apoptotic mechanisms (Heath *et al*., 2017). Detailed, comparative studies of the pathophysiological conditions in different haematological disorders will be helpful in elucidating the possible mechanisms behind the transformation of normal marrow physiology to various pathophysiologies (Chatterjee *et al*., 2016).

Leukaemia is classified according to the type of white blood cell affected (Figure 8). If a lymphoid cell is affected it is termed as lymphoblastic leukaemia, whereas if a myeloid cell is affected, it is known as myelogenous leukaemia. Each type of Leukaemia has its own characteristics and gene changes vary with types of Leukaemia; however, translocations are very common in all types of leukaemia, none of which involve cytokine genes (King, 2006). In fact, according to King (2006) overexpression of cytokines causes hyperplasia not cancers. Leukaemia can be acute

or chronic with chronic leukaemia being the most common type of leukaemia with its occurrence in men being double than that in females and Acute myeloid leukaemia is five time more common than acute lymphocytic leukaemia (King, 2006).

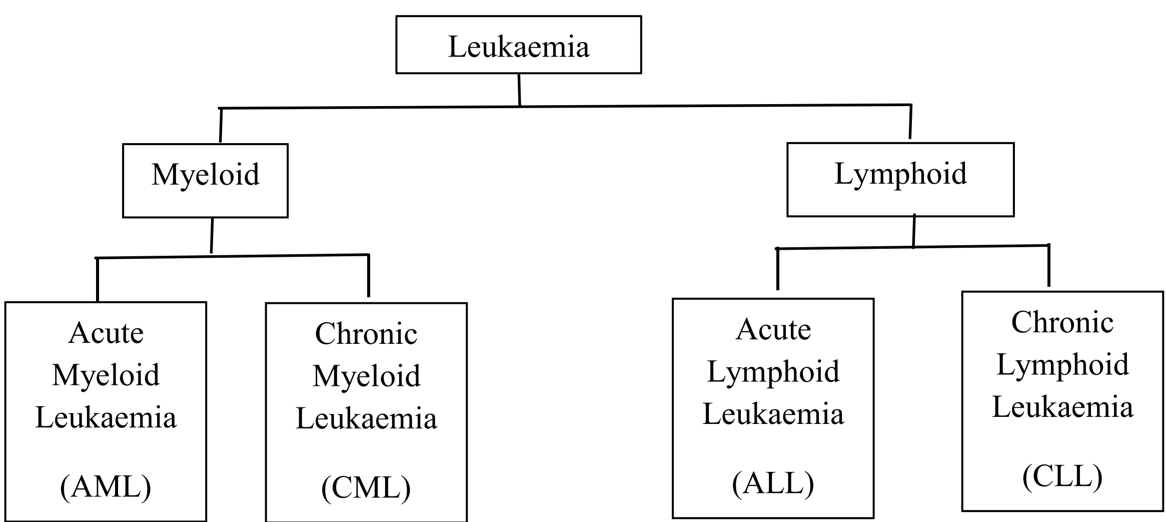


Figure 8: Figure showing classification of Leukaemia. Drawn by author.

Acute leukaemia is characterised by blast cells, which are immature cells that multiply rapidly allowing for the disease to progress very quickly, whilst chronic leukaemia is composed of more mature cells than acute leukaemia, however they still do not develop normally. In both cases the immature cells permeate the bone marrow and prevent haematopoiesis from occurring normally. These blasts enter the blood stream and are transported to other organs. In acute myelogenous leukaemia, a myeloid stem cell is transformed, causing it to expand and proliferate supressing normal haematopoiesis. These cells proliferate under the influence of myeloid growth factors. Segments from two different genes fuse together and produce a chimeric gene consisting of the 5' end of one gene and the 3' end of the other gene. From these genes, a new leukaemia-associated messenger RNA is transcribed, and a new protein is synthesized (Munker *et al.,* 2007).

In chronic myeloid leukaemia, there might be no early symptoms and might go undiagnosed for years. It is characterised by an abnormality of the Philadelphia chromosome (with one or more additions) which results from the reciprocal

translocation between chromosomes 9 and 22. This translocation fuses the BCR gene to the ABL gene on chromosome 9. The product of this is a chimeric gene that encodes a fusion protein with enhances tyrosine kinase activity (Vetrie, Helgason, & Copland, 2020).

The molecular defects causing acute myeloid leukaemia are considerably more heterogeneous (giving rise to at least 24 different genetically defined subtypes) than those seen chronic myeloid leukaemia (Vetrie, Helgason, & Copland, 2020). Figure 9 shows scientific and clinical milestones for CML and AML leukaemia stem cell research.

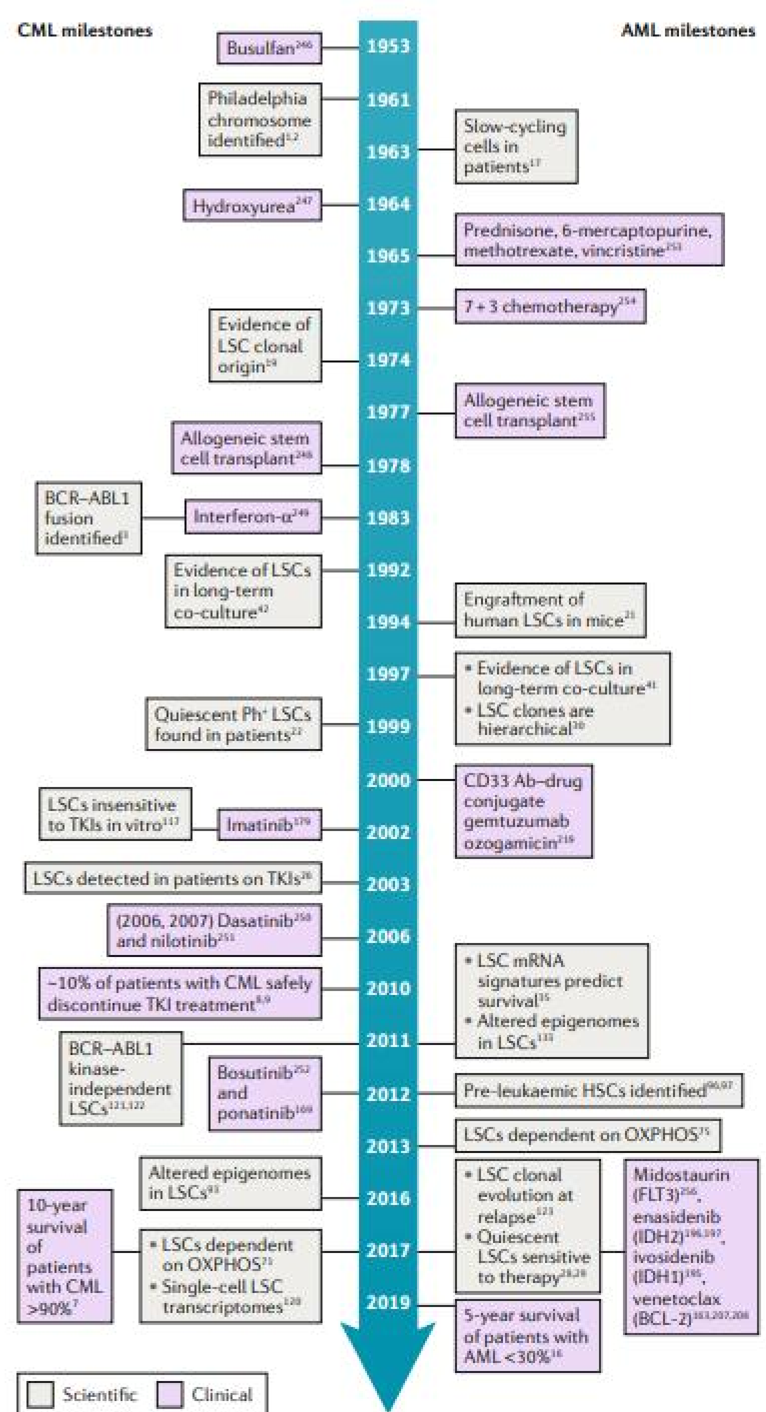


Figure 9: Scientific and clinical milestones relevant to CML and AML Leukaemia stem cell research. (Vetrie, Helgason, & Copland, 2020)

Acute leukaemia can be classified into 3 main ways: The French-American-British (FAB) classification, the World Health Organisation (WHO) classification, and the classification by the European Group for immunological Classification of Leukaemia. The following (table 2) is the French-American-British (FAB) system of classification of AML and their recognised subtypes. It is based on the morphology and other cytochemical studies.

Table 2: Table showing FAB system of classification of AML. Reference: Reference: (Udensi & Tchounwou, 2014)

Classification	Name
M0	Undifferentiated AML
M1	Acute Myeloblastic leukaemia with minimal maturation
M2	Acute Myeloblastic leukaemia with maturation
M3	Hyper granular promyelocytic leukaemia
M4	Acute myelomonocytic leukaemia
M5	Acute monocytic leukaemia
M6	Acute erythroid leukaemia
M7	Acute megakaryoblast leukaemia
M8	Acute basophilic/eosinophilic leukaemia

1.3.3 Chromosomal abnormalities in AML

AML is characterized by a high degree of heterogeneity with respect to chromosome abnormalities, gene mutations, and changes in expression of multiple genes and microRNAs (Madan & Koeffler, 2020). Non-random chromosomal translocations which result in gene arrangements are associated with most of the cases of AML. Chromosomal abnormalities include monosomies or deletions of part or all of chromosomes 5 or 7 and trisomy 8. In addition, chromosomal changes also occur on the long arm of chromosome 11; balanced translocations between chromosomes 15 and 17; chromosomes 8 and 21 and other chromosomes such as 31 and 22. Inversion on chromosome 16 can also occur in AML. The translocation between chromosomes 15 and 17 is always associated with a subtype of AML, referred to as APL and leads to the expression of PML-RARα onco-fusion gene in hematopoietic myeloid cells. 749 chromosomal aberrations have been associated to AML; however, their prevalence is different, with some being very common occurrence whilst other being very rare (Kumar, 2011).

1.4 Treatment options

1.4.1 Chemotherapy

The common treatment for leukaemia is chemotherapy which is a systemic treatment. According to the American Society of Cancer, more than 100 drugs exist that are in chemotherapy; some of these act on their own whilst others are used in combination with other chemicals. These drugs usually attack specific stages in the cell cycle and attack fast reproducing cells. Therefore, timing and dosage depends on many factors, including the stage at which the cell cycle is stopped. Since healthy cells also reproduce, they would also be attacked by the drug being used leading to a number of side effects on the organism receiving the treatment. Chemotherapy can either cure, control, or act as a palliative treatment.

Chemotherapy drugs are divided into groups having usually similar mode of action, chemical nature, side effects, and also interaction with other drugs. Such groups are alkylating agents which are not phase specific, antimetabolites which attack the cell during S phase of the cell cycle, Antitumor antibiotics which interfere with enzymes involved in DNA replication, Topoisomerase which affect the enzyme topoisomerase that separates the DNA strands during replication, Mitotic inhibitors which affect the cell in the M stage of the cycle, Corticosteroids, and other miscellaneous chemotherapy drugs. Most of these treatments are used in the treatment of the various Leukaemia types (Lee & Huang 2013).

Even though there is a vast range of chemicals that can be used in the curing of leukaemia, such treatments can also give a lot of side-effects which will negatively affect the organism, sometimes leading to death. For instance, since alkylating agents affect the DNA, they might affect the bone marrow cells leading to an acute form of leukaemia, 5 to 10 years after completion of the original treatment. Some antitumour antibiotics can negatively affect the heart. With topoisomerase inhibitors, a secondary leukaemia can develop as early as 2-3years after completion of the original treatment (Lee & Huang 2013).

Improved technology is allowing for targeted therapy. Certain chemicals such as imagine, bortezomib, and gefitinib attack cells which show mutated versions of

certain genes or cells that express too many copies of a particular gene. These are all key signs that the cell is replicating uncontrollably.

1.4.2 Immuno reactions and other methods

Cancer immunotherapies seek to establish a pool of long-term living T cells that have antitumour activity (Emens & Middleton., 2015). There are various types of immunotherapies, and these include immune checkpoint inhibitors, which block checkpoints that would suppress the immune system under normal circumstances to respond better to cancer cells, monoclonal antibodies, T-cell transfer therapy and vaccines (Stanculeanu, Daniela, Lazescu, Bunghez, & Anghel, 2016). The same authors highlight that vaccines contain mixtures of short peptides, delivering both cytotoxic T cell epitopes as well as helper T cell epitopes or long peptides that include both activate a durable T cell response. As with other mechanisms to treat cancer, resistance to immunotherapy may lead to failure of this method.

Other options also exist such including drugs such as imatinib and autologous bone marrow transplantation, however this is beyond the scope of this project. All these measures have greatly improved survival rates for patients (King, 2006). A new approach is through differentiation treatment whereby the leukemic cells are forced to differentiate into non-malignant cells that will eventually undergo apoptosis.

1.4.3 Differentiation therapy

Leukaemia occurs due to a block in myeloid differentiation as a result of maturation arrest at an early development stage (Nowak *et al.*, 2009) and uncontrolled clonal expansion of myeloid precursors (Madan & Koeffler, 2020). For example, M1 type of Leukaemia occurs when the cells stop at myeloid stem cell level whilst M3 type of Leukaemia happens when cells stop at the promyelocyte stage (refer to Table 2). Through the inability to undergo apoptosis, leukaemia cells increase in numbers in the bone marrow, outcompeting normal blood cells. These leukaemia cells will eventually move into the peripheral blood where they will be different from normal blood cells based on the expression of the surface antigens as well as their chromosomal abnormalities. By using biological agents, differentiation can be forced to continue and hence cause the cell to enter the apoptotic pathway. One assumption made in this form of therapy is that the neoplastic cell types exhibit

reversible defects which can therefore be reversed using appropriate treatment. This treatment is expected to reprogramme the cells and induce apoptosis.

Differentiation therapy offers an alternative to curing cancers other than the conventional cytotoxic therapies, and in the process reducing or completely removing the side effects of these drugs and ensures complete cure of these conditions (Nowak, *et al*., 2009). Very good results have also been obtained both *in vitro* and *in vivo* to cure promyelocytic leukaemia by this form of therapy (Breitman, *et al*., 1981; Huang, *et al*., 1988; Fang *et al*., 2010; Stubbins & Karsan, 2021). In such experiments All-*trans*-retinoic acid (ATRA) was used as it was proven to restore the natural progression of differentiation to these leukaemia cells (Bruserud, Gjertsen, & Huang, 2000). In a study carried out by Castaigne *et al*., (1990), all *trans*-retinoic acid was used to treat twenty-two patients with acute promyelocytic leukaemia, 14 of which showed complete remission. Other agents bringing about differentiation are Cyclic AMP analogues, HMBA, Polar-aplanar compounds, Aza-cytidine, Demethylating agents, TPA and bryostatin, PKC agonists and antagonists and Sodium butyrate. Although a wide array of chemicals have been used to bring about differentiation, the drawback is that many of these chemicals are too toxic to be used in vivo in the right concentrations and hence research is focusing on finding other chemicals that can cause differentiation, whilst at the same time be used in good concentrations that are not toxic to humans (Madan & Koeffler, 2020). In fact, although differentiation therapy has given some very good results with certain subtypes of Leukaemia, differentiation didn't give clinical benefits for other subtypes and hence chemotherapeutic regimes still remain the standard treatment option (Madan & Koeffler, 2020).

Differentiation involves the coordinated switching on and off of genes specific to the cell type concerned, which is usually caused by an altered regulation of transcription involving DNA-protein interactions (King, 2006). Since differentiation also required proliferation, any transcriptional alterations have to be passed on to the daughter cells and these require DNA methylations. For example, the cytosine-guanine dinucleotide sequence in DNA can be methylated at the 5-position of cytosine through the function of enzymes and once this occurs, transcription factors will not bind to it, diminishing transcription (King, 2006).

1.5 Assessing Differentiation

1.5.1 Cell lines used and their morphology.

In vitro testing allows for the testing of various extracts and other biological material in laboratory equipment using immortal cell lines which can be easily obtained, and which grown under specifics conditions are likely to give genetically homogenous cells and hence more reliable results. The availability of such cell lines allows for pathophysiological studies to be done. In this study, four different Leukaemia cell lines were initially selected. These were HL60, KG1A, K562 and NB4R2. These cells differentiate into diverse phenotypes; monocytes or granulocytes.

HL-60 cell line involves cells showing acute myeloid leukaemia (AML) that was established in 1977. This cell line is commonly used as a model system for studying myeloid cell development because these cells can be induced to develop into macrophages and granulocytes in vitro by using various chemicals such as dimethyl sulfoxide (DMSO) as well as ATRA (Callens *et al*., 2010). Once formed, they will exhibit useful functional and morphological markers. The signal transduction pathways which lead to maturation in both processes are very similar to each other (Carter, *et al*., 2020). The HL-60 suspension is predominantly made up of promyelocytes, having an occurrence of greater than 90-95% (Breitman *et al*., 1980). The cells in suspension appear large, blast-like with characteristic large, rounded nuclei containing 2-4 distinct nucleoli (more visible in cells with finer chromatin), and a basophilic cytoplasm with azurophilic granules, whilst the remaining cells exhibit morphologies which are typical to more mature myeloid cells such as myelocytes, neutrophils and monocytes). The HL-60 can multiply and hence proliferate rapidly, however, once differentiation occurs, cell proliferation slows down.

KG1A was obtained from a male patient who was suffering from AML and was isolated from the KG1 cell line in 1980. The KG1 cell line was isolated from a patient who was diagnosed with acute myeloblastic leukaemia following erythroleukemia. KG1a cells are at an earlier developmental stage when compared to HL-60. These KG1 cells are more difficult to differentiate than HL60 and are predominantly myeloblasts. The KG1a has lost myeloid features and has three new characteristics giving it a new karyotype. These changes involve a low-level expression of the T cell receptor beta mRNA; high-level expression of T3 delta

mRNA which is intracellular and expression of the CD7/gp40 T cell-associated membrane antigen (Furley, 1986).

The K562 cell line was originally obtained in 1975 from a patient with chronic myeloid leukaemia (CML) in terminal blast crisis by Lozzio & Lozzio(1975). The cells in this cell line usually carry the Philadelphia chromosome marker (Lozzio & Lozzio., 1975 and Yılmazer, 2018) which results in the formation of a bcr:abl fusion protein. .

NB4R2 is a derivative of the NB4 cell line which is resistant to differentiation by ATRA. The NB4 cell line was isolated from long-term cultures of APL blast cells on bone-marrow stromal fibroblasts, as reported by Lanotte *et al*. (1991). NB4 cells possess the characteristic translocation t(15;17) resulting in the fusion between the PML gene and the RARα. The resistance by the NB4R2 cell line is due to a mutation present in the PML-RARα fusion protein (Idres, Benoît, Flexor, Lanotte & Chabot, 2001).

Cytokines such as G-CSF influence the formation of myeloid progenitor cells (CFU-G). This cell can then differentiate into myeloid precursors such as myeloblasts, promyelocytes, myelocytes and metamyelocytes which are morphologically recognisable. Myeloblasts are cells that are relatively large, with a large nucleus possessing fine chromatin and several nucleoli. Cytoplasmic granules are not present and then divide into promyelocytes. The latter are slighter larger neutrophilic precursor with granules now present in their cytoplasm. Once these cells divide, they will form myelocytes with smaller granules and which can be further divided into metamyelocytes. These cells can no longer divide and have an indented nucleus and numerous granules in their cytoplasm. The band is a stage in differentiation where the nucleus is not very much yet indented. Figure 10 shows the stages in granulocyte morphology. The mature neutrophil is characterised by a dense nucleus which has 2 to 5 lobes and a pale cytoplasm. Granules are present and these can be one of two types, azurophilic (primary) granules and secondary granules. These granules are lysosomal in origin, however the azurophilic granules contain myeloperoxidase, acid phosphatase and acid hydrolases, whilst the secondary granules have lactoferrin, collagenase and lysozymes (Munker *et al.,*2007). Blockage at the promyelocyte is characteristic of HL60 and NB4 (Blanter *et al*., 2021).

Mitotic pool			Post mitotic pool		
Myeloblast	Promyelocyte	Myelocyte	Metamyelocyte	Band	Mature neutrophil
Nucleoli	granules				

Figure 10: Granulocytic development of HL60 cells Reference: Drawn by Author.

Growth factors can drive the myeloid progenitor cell to differentiate into a monocyte and later into a macrophage. Figure 11 shows the stages in monocyte development which can be seen as cells that are larger than most other blood cells, with an abundant cytoplams, with mnay fine vacuoles and fine granules. The nucleus is large and usually indented with clumped chromatin.

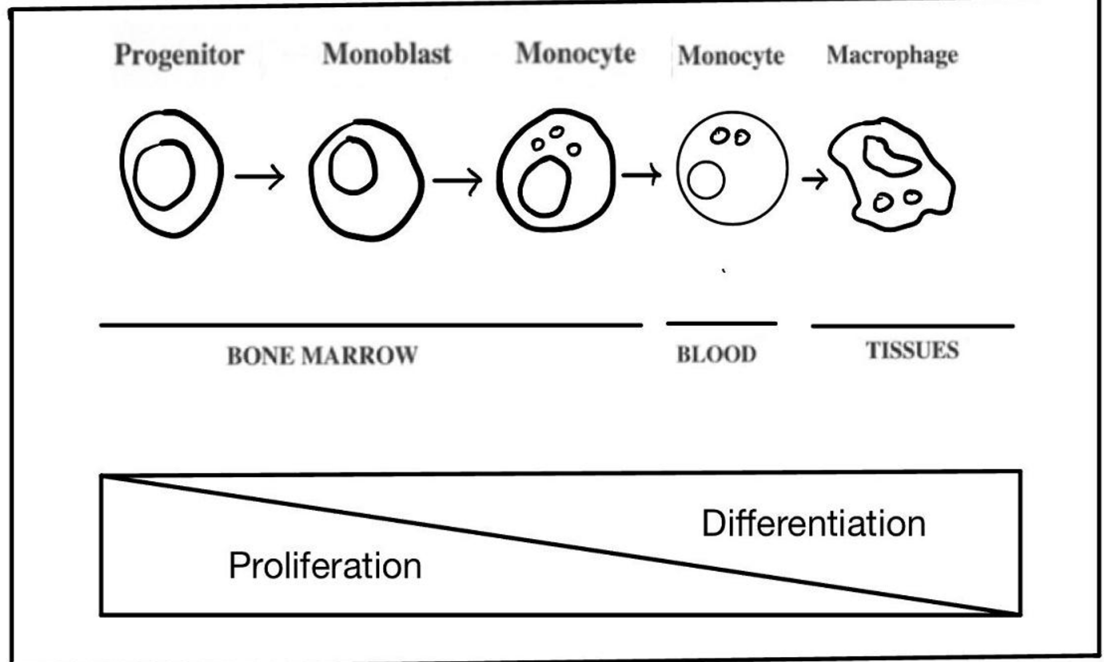


Figure 11: Schematic representation of monopoeisis. There is an increase in differentiation and a decrease in proliferation as the progenitor matures to a macrophage. References: Moss and Hilton, 2000.

1.5.2 Other characteristics of differentiated cells

The differentiation of cells into macrophages or granulocytes can be initiated either extrinsically by the reaction of different chemicals on receptors on the cell surface or intrinsically by stochastic processes (Miller *et al.*, 2002). HL-60 cells can be made to differentiate into monocyte/macrophage by compounds such as 1,25-dihydroxyvitamin D3, PMA, and sodium butyrate, and into granulocytes with compounds such as dimethyl sulfoxide (DMSO), and other compounds as diverse as retinoic acid and actinomycin D. In conjunction, histochemical changes also occur. Incubation with DMSO over a period of 5 days will lead to differentiation of HL-60 into granulocytes. Upon differentiation, cells become more irregular with a progressive decrease in size, have a greater number of vacuoles, the nucleus/cytoplasmic ratio decreases, and the nucleus becomes first kidney shaped and later lobed which is a characteristic of neutrophils. The cytoplasm becomes more diffuse. In addition, changes in histochemistry appear, including decreased myelperoxidase activity and the appearance of cells capable of reducing nutroblue tetrazolium (a granulocyte marker). Sometimes, there is a lack of uniformity in the response of HL-60 to different inducers that have the same overall effect, and this created confusion about the interpretation of data obtained. Qualitative and quantitative differences between inducers have been summarised by Collins (1987). However, whether the extent to which these differences are due to differences between inducers or to differences between sublines of HL-60 cells is unclear. In addition, the mechanisms controlling the differentiation of HL-60 into monocytes and granulocytes remain unclear, despite the amount of recent literature available. KG1a are found at an earlier stage of differentiation when compared to HL60, however it's expected to follow the same patterns of differentiation. The human erythroleukemic cell line (K562) has been shown to differentiate into embryonic and foetal haemoglobin without the expression of adult haemoglobin by DMSO and PMA (Baliga *et al.*, 1993)

The process of differentiation is also associated with a change in cell surface proteins. Cell surface proteins are important for a variety of cellular functions such as signal transduction, receptors for certain growth factors, adhesion, and extracellular enzymes, amongst others (Attar, 2014). The same author has shown that various cell

surface markers can be used to identify mature monocytes and granulocytes whilst markers such as CD34 are used to show the presence of stem cells. CD11b is expressed during the development of a promyelocyte into a neutrophil. Whilst the surface marker is not expressed on the promyelocyte, this marker is then expressed on all the other differentiating cells. CD11b cells are also expressed on monoblasts, promonocytes and monocytes. CD14 is expressed on monocytes (Young & Al-Saleem, 2008).

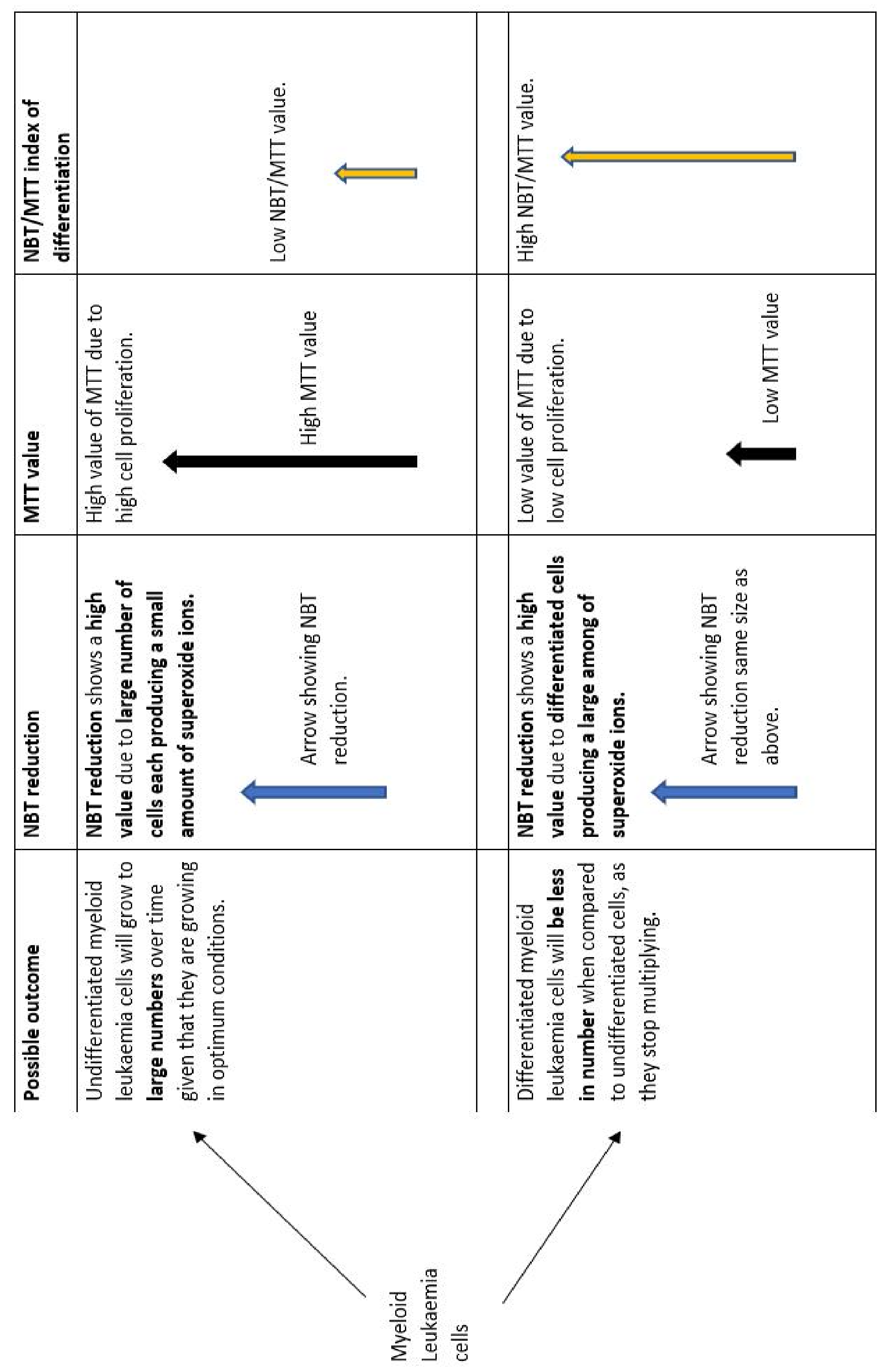
1.5.3 MTT and NBT

Tetrazolium salts are some of the most widely used tool in cell biology which are used to measure metabolic activity of mammalian cells *in vitro* (Berridge *et al*., 2005). The MTT *in vitro* assay is a method by which cell viability, proliferation and toxicity is assessed using a colorimetric assay (Abe & Matsuki, 2000). The basis of this test is to reduce yellow tetrazolium into its insoluble purple formazan**.** Tetrazolium dye reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes, largely in the cytosolic compartment of the cell. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The degree of light absorption depends on the solvent. The MTT Reagent will still show a low background absorbance value in the absence of cells. Therefore, an accurate quantification for any changes in cell proliferation would be obtained by determining the linear relationship between cell number and signal produced.

Tetrazolium dye assays are used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. Rapidly dividing cells exhibit high rates of MTT reduction, while cells with low metabolic activity reduce very little MTT. Consequently, a great reduction in MTT value compared to the control shows that the compound is cytotoxic. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

NBT is a water-soluble dye that is reduced to insoluble intracellular blue-black formazan by healthy immune cells, such as differentiated granulocytes. These release reactive oxygen species abundantly that will reduce NBT to give a dark blue-black colour. This respiratory burst does not occur readily in the less differentiated leukaemia cells and is therefore a good measure of degree of differentiation.

The higher the ratio, the greater the degree of possible differentiation caused by the extract. This ratio is of great significance as it gives a measure of degree of differentiation (Schembri-Wismayer & Cassar, 2017). Taking this ratio, rather than the individual NBT and MTT values is very important as the ratio gives a relationship between oxidative burst and the number of viable cells. Differentiated cells lose their ability to proliferate resulting in a reduced number of cells. On the contrary undifferentiated cells keep on proliferating indefinitely. A high NBT/MTT ratio signifies a low cell number whilst a low ratio would signify a large number of cells. This can be seen in figure 12:



Possible outcome	NBT reduction	MTT value	NBT/MTT index of differentiation
Undifferentiated myeloid leukaemia cells will grow to **large numbers** over time given that they are growing in optimum conditions.	**NBT reduction** shows a **high value** due to **large number of cells each producing a small amount of superoxide ions.** Arrow showing NBT reduction.	High value of MTT due to high cell proliferation. High MTT value	Low NBT/MTT value.
Differentiated myeloid leukaemia cells will **be less in number** when compared to undifferentiated cells, as they stop multiplying.	**NBT reduction** shows a **high value** due to **differentiated cells producing a large among of superoxide ions.** Arrow showing NBT reduction same size as above.	Low value of MTT due to low cell proliferation. Low MTT value	High NBT/MTT value.

Figure 12: Figure showing NBT/MTT ratio highlighting the effects of proliferation and differentiation on this value. Reference: Drawn by author

1.5.4 Flow cytometry

Flow cytometry is a very powerful tool with a diverse range of applications in multiple disciplines and these include but are not limited to immunology, virology infectious disease monitoring and cancer biology. For flow cytometry analyses, individual cells flow through single or multiple lasers whilst being suspended in a buffered salt-based solution with one of the aims being to recognise any cells that are undergoing differentiation (McKinnon, 2018). This will be explained in greater detail.

The cell cycle is a process a cell goes through which will ultimately result in its division into two daughter cells. The cell cycle is split into 3 main stages, these being interphase, nuclear division and finally cytokinesis. Interphase itself is subdivided into 3 stages, these being G1, S and G2 phases. In G1, the cell grows physically, and the amounts of proteins and organelles increases. During S phase, which is synthesis phase, DNA replication occurs, and in G2, there is further cell growth and organisation of the cell's contents in preparation of nuclear division.

The DNA is constantly under the threat of being damaged through various ways such as mutations in the number and sequence of bases or the breakdown of phosphodiester bonds. Epidemiological studies and observations using animal models show that syndromes which make humans more susceptible to cancer arise from mutations. Various agents can bring about such changes, and these can be produced by the cell itself or can be external. Free oxygen radicals can cause a lot of damage and arise from normal cellular metabolic reactions or when the organism is exposed to an external source of ionisation radiation. However, cells have developed a number of mechanisms to combat these effects and they are collectively termed cell-cycle checkpoints. A variety of mechanisms have evolved which are capable of detecting the various changes that can happen to the DNA. DNA may be repaired; the cell cycle progression may be haltered, and the cell might be caused to undergo apoptosis (Kastan, & Bartek, 2004).

There are four main cell cycle check points during the cells cycle that can be deregulated in cancer cells, these being G0/G1 checkpoint, G1 checkpoint, the G2 checkpoint, and the mitosis-associated spindle assembly checkpoint (SAC). The G0/G1 checkpoint controls the cells commitment to division through the presence or

absence of growth factors such as cyclin D1, CDK 4 and 6, where upstream regulation of such factors, or inactivation of the RB1 gene will lead to dysregulation. This checkpoint in largely controlled by the Rb/E2F signalling pathway, whereby the release of the transcription factor E2F from Rb allows the activation of genes that are required to activate genes required to promote entry into the S phase. The G1 checkpoint is mainly controlled by the p53/p21 and MDM2 pathway and is essential in delaying DNA replication should there be any DNA damage. Loss of this checkpoint can be caused by mutations or inactivation of the gene p53 which leads to mutated DNA to be synthesised as the time allowed for DNA repair will be reduced. The intra-S checkpoint is regulated by the ATM and ATR genes. Following cell damage, the ATM kinase will be activated and inhibits cell cycle progression whilst when the ATM kinase is activated it will arrest the formation of DNA replication forks, essentially inhibiting DNA replication (Kastan, M., Bartek, J, 2004). G2 checkpoint involves a DNA damage checkpoint where initiation of mitosis is delayed upon DNA damage. This is achieved through the sequestration of cyclin B1/cdk1 in the cytoplasm. Within the same checkpoint, decatenation may also occur and is activated in response to the presence of the catalytic inhibitor topoisomerase IIα (topo IIα). The spindle assembly check (SAC) points is important to delay the onset of anaphase until all chromosomes exhibit bipolar attachment to the mitotic spindle through the APC/C and RubR1. Impairment of this checkpoint often leads to unequal partitioning of the chromatids (Bower, Vance, & Psioda, 2017). This is summarised in figure 13 below:

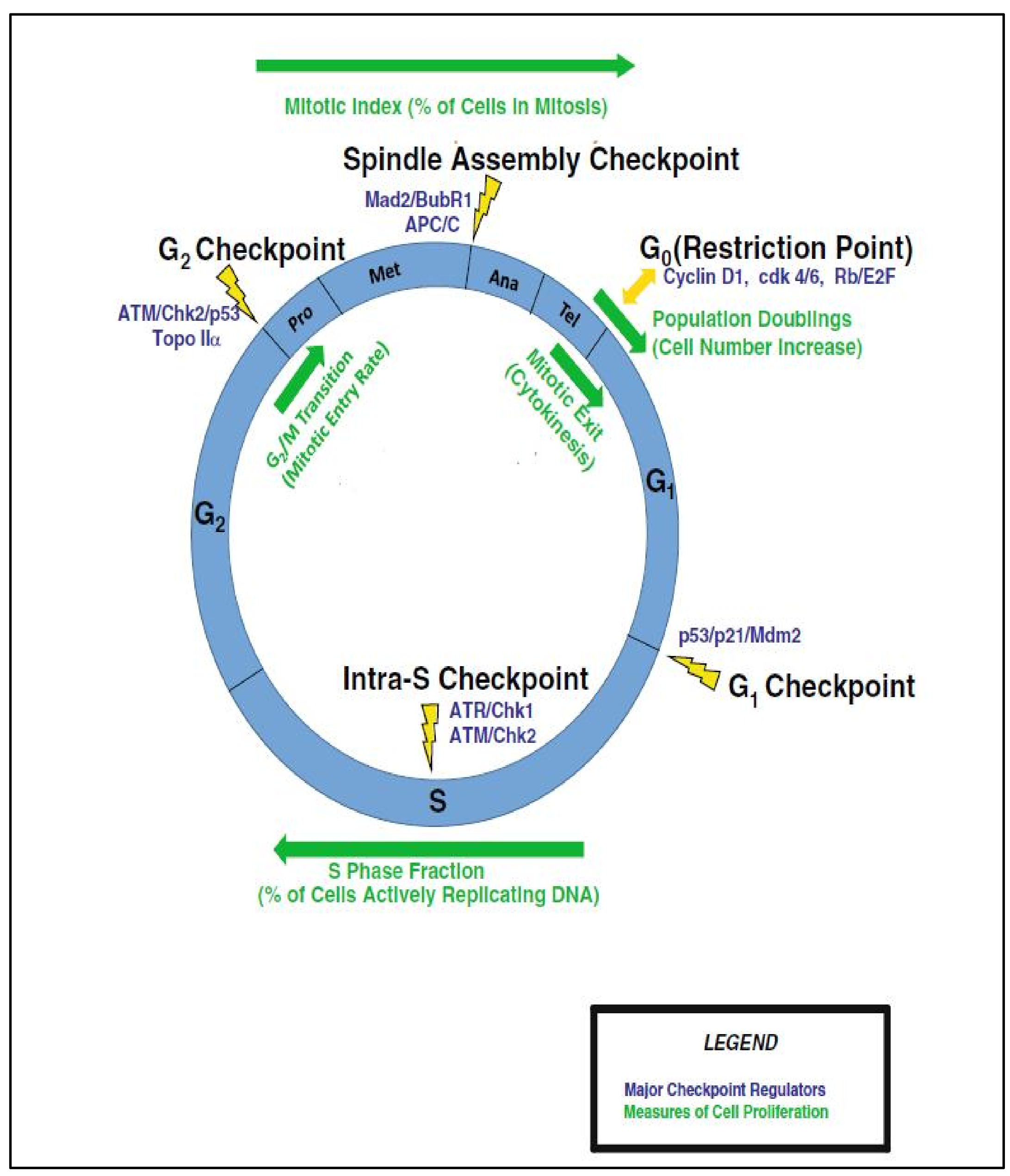


Figure 13: Figure showing cell cycle regulation. G0, G1, S, G phases of the cell cycle are shown inside the blue circle in the centre of the figure. Mitosis is split into prophase (Pro), metaphase (Met), anaphase (Ana), and telophase (Tel). The G0 Restriction Point is designated with a yellow dual headed arrow to illustrate the reversible nature of cell cycle entry and exit. Checkpoints are shown by the yellow lightning bolts. Components of major regulatory pathways triggering each checkpoint are listed in dark blue font near the checkpoint in which they play a role. Adapted from: Bower et al., 2017.

The cell cycle analysis quantifies, through the use of the flow cytometer, the DNA content in cells. S phase is related to the replication of the DNA and hence the among of DNA doubles in that section, where it will remain double until it is separated and divided into two cells during Mitosis. Figure 14 shows the relative DNA content at different stages in the cell cycle.

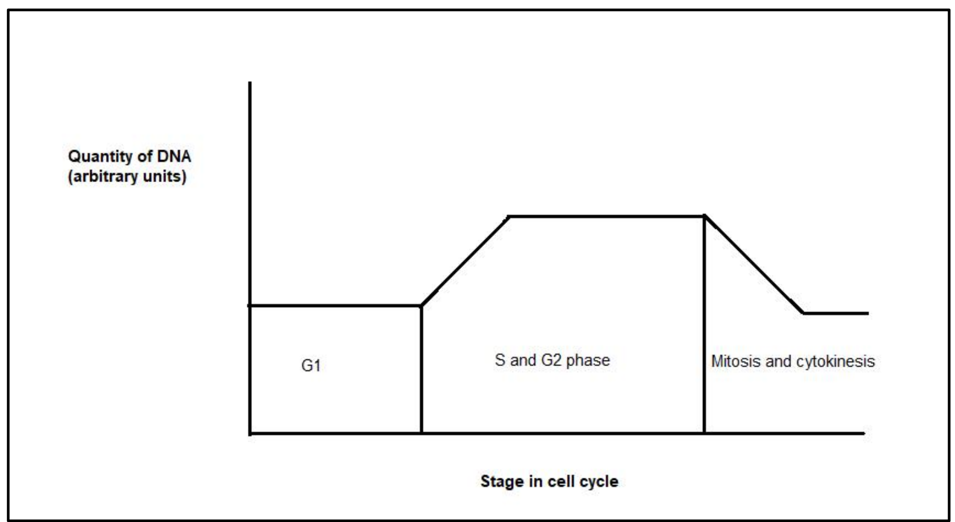


Figure 14: Figure showing the quantity of DNA in arbitrary units at different stages in the cell cycle. Reference: Figure drawn by author

The Cell cycle analysis assays requires the DNA to be stained with a saturating amount of DNA binding dye. Cells are then fixed with a 70% ethanol solution which is required to allow the cells to become fixed and more permeable to the dye, as otherwise the cells will expel the dye out and finally stained with a suitable dye such as propidium iodide (McKinnon, 2018). 70% ethanol should not be made with PBS as this would cause protein precipitation on fixation. Cells that are in the S phase and G2 will have more DNA than those cells in G1 and hence they will take more dye than those in G1. These will be detected by a more brilliant florescence. When performing this method, cell loss will inevitably happen due to electrostatic attraction to tubes. Another drawback of this technique is that cells in early S phase cannot be distinguished from cells in G1 and those in late S phase cannot be clearly distinguished form those of the G2 phase.

Figure 15 shows the plot that is obtained through the flow cytometer, showing the relative number of cells present in each of the different stages in the cell cycle. As can be seen most of the cells will be expected to be found in G1 of the phase.

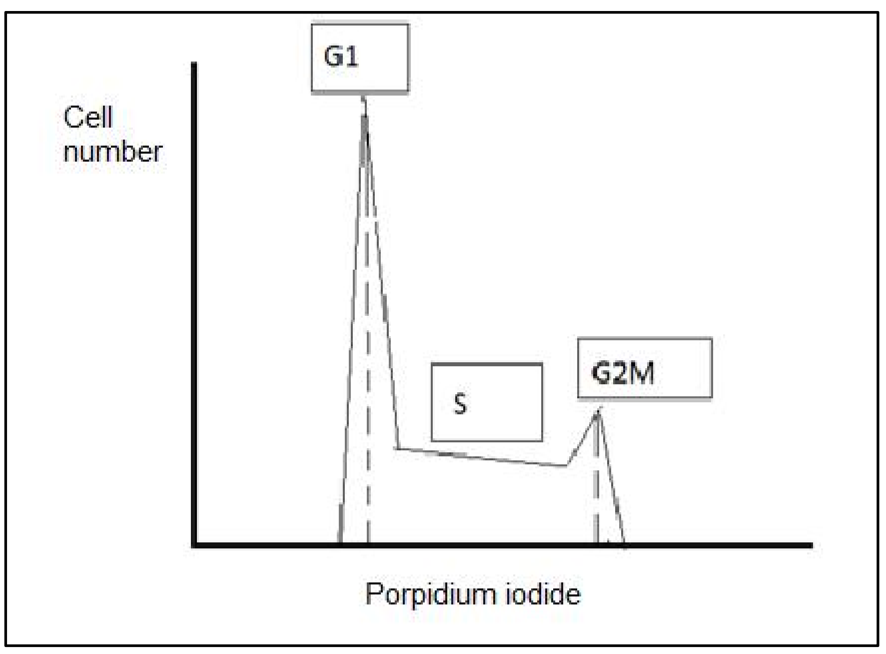


Figure 15: Figure showing the cell number vs PI used in a plot. The plot is an example of what is usually obtained through a cell cycle analysis.

The ability of the immune system to function properly works through different leukocytes interacting with each other and with various other components, including other cells, pathogens, and other antigens. Markers for cluster of differentiation (CD) are used to identify cell surface molecules present on leukocytes which mediate this interaction. The Human Leucocyte Differentiation Antigens Workshops (HLDA) have provided for the proper classification of such molecules since 1984 (Zola *et al*., 2005). Through flow cytometric analysis, combinations of antibodies, allow for the identification of specific cell types, determination of the degree of cell differentiation and recognition of abnormal cells. In addition, these antibodies are not only associated with a cell type but vary with maturity in their relevant expression.

When a sample enters the flow cytometer, the cells must be ordered into a stream of single particles that be seen individually by the machines detection system. This process is done by the outer sheath fluid, which is under high pressure and therefore moves faster than the cells. As a result, it creates a drag effect that causes the sample in the central core to narrow, creating a single file, a process known as hydrodynamic focusing. These cells will then flow in a single file through the illumination source, allowing them to be analysed one a time. Lasers produce a single wavelength of light which is collected by a photomultiplier tube. Light scattering of

beam of laser will provide information about the cell's properties (McKinnon, 2018). During analysis, each particle is analysed for visible light scatter and one or multiple fluorescence parameters, these two parameters being independent of each other. When laser beams pass through cells, it is scattered. The visible light scatter is measured in two different directions, namely the forward scatter (FSC) and the side scatter (SSC). The FSC gives an indication of the relative size of the cell and the SSC indicates the granularity of the cell. Samples are prepared for fluorescence measurement by staining with fluorescently conjugated antibodies when using antibodies (McKinnon, 2018). A combination of the forward and side scatters is used to roughly differentiate cell types in a heterogeneous sample. Dead cells have a lower forward scatter and a higher side scatter than living cells. All this is illustrated in figure 16 below.

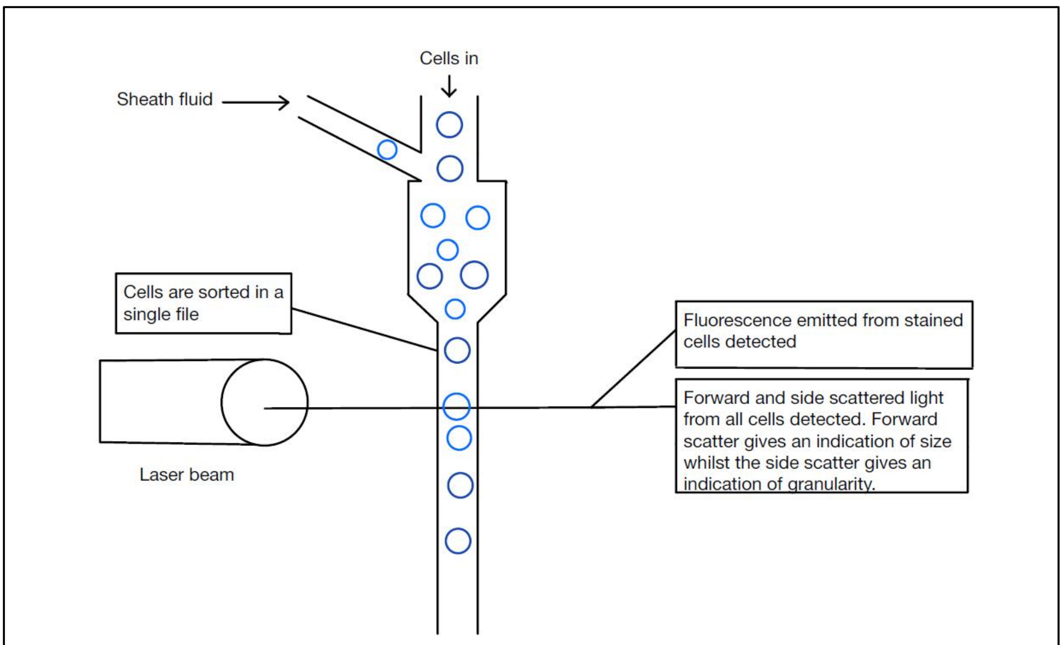


Figure 16: Figure showing cell sorting and effect of laser beam in flow cytometry.

1.6 Background to the study

The International Agency for the Research of Cancer is a specialised cancer research agency of the WHO which was established in 1965. Statistics from this agency show that by the end of 2020, the total number of Leukaemia cases in both sexes at all ages was of 474519 (out of which 100020 were from Europe) with 311594 individuals (out of which 62262 were from Europe) dying from it (Key Cancer Data and Key Figures on IARC: 2020–2021, WHO accessed on 7 August 2020).

When looking at the local scenario, around 1700 people were diagnosed with cancer in Malta in 2010 (the National Cancer plan 2010-2015). On average, between 2010 and 2014, cancer was the cause of 29.5% of deaths from illness or disease (excluding violent or accidental deaths) (The National Cancer Plan for the Maltese Islands, 2017-2021, accessed on 10 June 2017). There is a projected increase in the proportion of deaths attributable to cancer over the next 20 to 30 years where it is estimated that by 2040, cancer mortality will reach approximately 35% of all deaths in females and 40% of all deaths in males. The total number of deaths due to leukaemia in Malta in 2010 were of 34 patients according to the National mortality centre (2013). More recent statistics were not found.

There are various types of treatments that can be given to patients suffering from various types of cancer. Chemotherapy, immunotherapy, and other methods are on the market, with each treatment having its own rate of success, together with advantages and disadvantages for the patient. However, one main disadvantage is the numerous side effects that are felt by the patient upon treatment. A new form of therapy, differentiation therapy, aims at unblocking the cancer cells forcing them to continue to develop into normal cells, with the aim to be used on their own or in conjunction with other forms of therapy, reducing the side effect and increasing the chance of a good outcome of complete remission for the patient. This form of therapy seeks the use of active biomolecules form various organisms, terrestrial or aquatic.

The marine environment is a habitat with great biodiversity and organisms which haven't yet been studied. Such marine organisms are known to contain a lot of bioactive components that could potentially be used for the treatment of cancer by differentiation. Although many biomolecules can be shown to have therapeutic

effects, many of these molecules don't make it to clinical trials due to reasons discussed in section 1.2.2. Hence, the search for novel compounds is ongoing and a lot of organisms are being researched. Holothurians are one such group of marine organisms which have shown to have to be rich in bioactive compounds with many biological effects including antibacterial, antifungal, and anticancer, amongst others (Erwin *et al*., 2010; Fattorusso *et al*., 2012; Guo *et al*., 2015; Petersen *et al*., 2019). Their regenerative properties, evolutionary relationship to vertebrate chordates and the wide range of molecules that they possess makes them ideal candidates for research of biomolecules with the aim of differentiation therapy.

PART 2: Aims and Objectives

1.7 Aims and objectives.

This project aims to improve the therapeutic possibilities of leukaemia and consequently is of great significance in the research and medical field. Leukaemia cancer types are characterised by undifferentiated cells, and therefore success in causing any level of unblocking of leukaemia cells would be more data that can be used in the development of new possible agents that can be tested for the treatment of such cancers. This would offer another realistic opportunity for effective medication.

The main research question in this project is to determine whether an extract from *Holothuria poli* can be used to cause any level of differentiation in Leukaemia. The concept of this project is based on the fact that at different points of regeneration, after evisceration, the chemical composition of the coelomic fluid changes in line with the processes taking place within the organism, specifically for the differentiation of the newly regenerated gut and the proliferation of cells for the completion of its growth. Hence the coelomic fluid is considered as being a dynamic fluid and it was important to determine at which point during regeneration a positive effect could be obtained.

The objectives are:

1. Taking day 0 to be the day of evisceration, determine which is the day of regeneration that gives the best results in terms of possible differentiation.
2. To determine the range of concentration of extract expressed as a % that can be used in order to possibly cause differentiation on leukaemia.
3. To determine which cell line from NB4R2, KG1A, HL60 and K562 is most susceptible to give the best possible differentiation results.
4. To determine, as much as possible, the nature of the active ingredient.

Since the search for bioactive compounds from the marine environment is new in the Department of Anatomy at the University of Malta, many new techniques related to growth of the specimens in artificial conditions as well as extraction of the biomolecules had to be developed from scratch and therefore a lot of preparatory work went into this research project, prior to the collection of data, which would enable the objectives to be reached. This groundwork, although very laborious and

time consuming had to be carried out in order to be able to have a solid foundation for the rest of the project and any future projects which can take off using this research as the base. This will be discussed in detail in Chapter 2 – Materials and Methods.

The following is an outline of the scheme to be followed in order to reach the objectives of this study (figure 17).

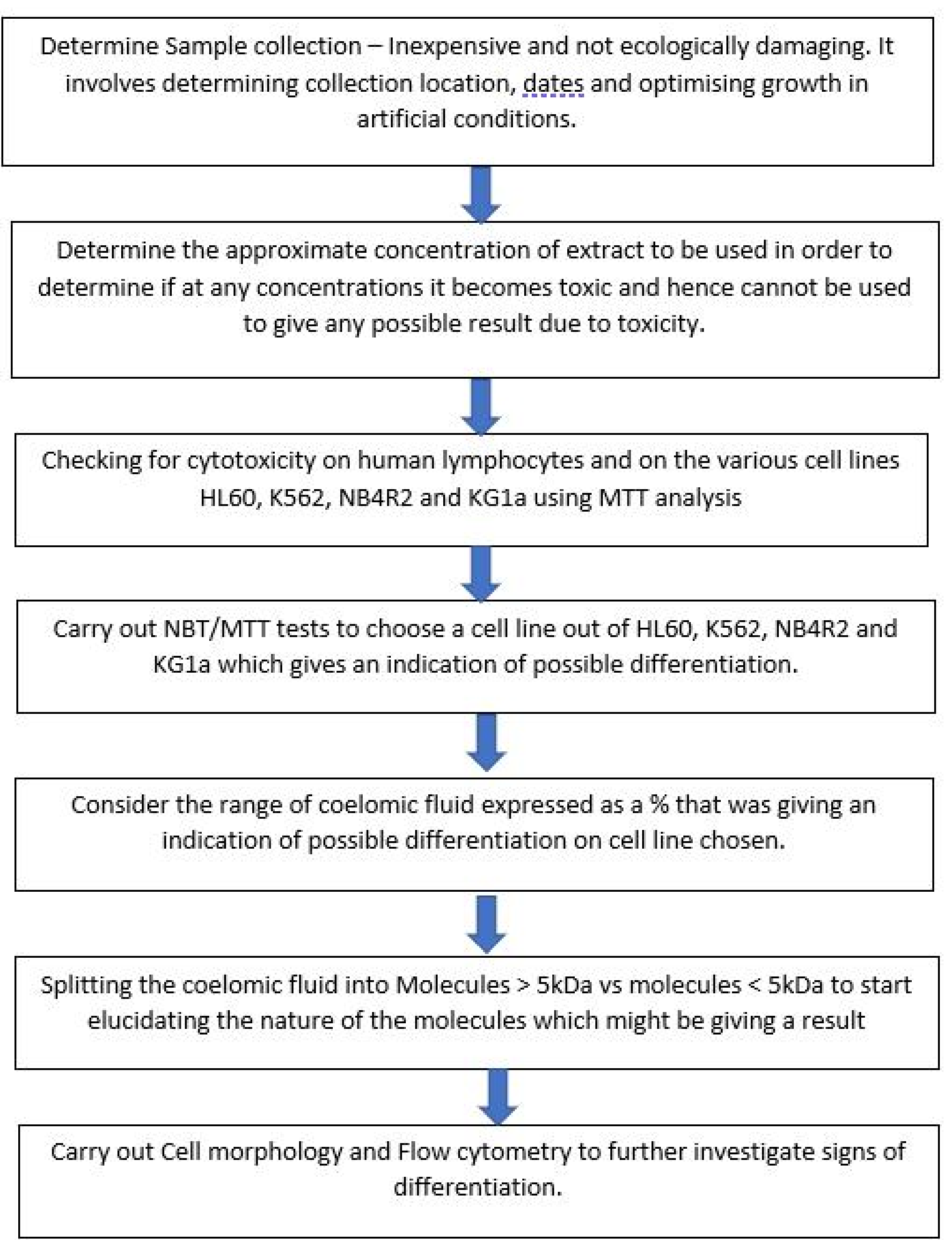


Figure 17: Figure showing schematic diagram of steps outlining objectives of this study.

2 Materials and Methods

2.1 List of Materials

The following three tables illustrate the chemicals used (table 3), the equipment used (table 4), and the cell lines employed (table 5) throughout the different experiments with the aim for them to serve as an easy reference when they are mentioned in the different methods outlined in this section. Table 3 gives the list of chemicals, including abbreviations and codes, as well as the company name from where they were bought. Table 4 illustrates the equipment used together with some important notes related to supplier, whilst table 5 gives a list of cell lines used together with some notes related to their origin.

2.1.1 List of chemicals used.

Table 3: Table showing list of reagents used, together with abbreviations and codes used and the company from where they were purchased.

Reagent	Abbreviation and codes	Company name
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	MTT M5655	Sigma-Aldrich® Germany
Bovine Serum Albumin	BSA A2153	Sigma-Aldrich® Germany
Dimethyl sulfoxide	DMSO – reagent grade D5879	Sigma-Aldrich® Germany
Foetal bovine serum	FBS F5724	Sigma-Aldrich® Germany
Histopaque	- 10770	Sigma-Aldrich® Germany
Iscove ’ s Modified Dulbecco	IMDM 12440053	Sigma-Aldrich®

Medium		Germany
Leishman's stain	- L6254	Sigma-Aldrich® Germany
Nitroblue tetrazolium chloride	NBT N6639	Sigma-Aldrich® Germany
Penicillin-Streptomycin*	Penstrep P0781	Sigma-Aldrich® Germany
Phorbol 12-myristate 13-acetate	PMA P8139	Sigma-Aldrich® Germany
Phosphate buffered saline	PBS D5652	Sigma-Aldrich® Germany
Propidium Iodide	PI P4170	Sigma-Aldrich® Germany
Roswell Park Memorial Institute Media	RPMI-1640 R6504	Sigma-Aldrich® Germany
Sodium hydrogen carbonate	$NaHCO_3$ 11810-025	Gibco USA
Sodium hydroxide	NaOH 795429	Sigma-Aldrich® Germany
Tryptan Blue	- T8154	Sigma-Aldrich® Germany

* At a concentration of 10,000 U/ml Penicillin and 10 mg Streptomycin per ml in 0.9% NaCl

2.1.2 List of equipment used.

Table 4: Table showing list of equipment used throughout the research projects together with names of companies from where they were purchased and any additional notes.

Equipment	Notes
Autoclave	Tuttnauer 1730M, USA
Centrifuge	5810-R Eppendorf, UK
Compounds microscope	Olympus, BX60, Blegium
Class II laminar flow hood	SafeFAST Elite, FASTER, Italy
Cytospin 2	Shandon, UK
Digital balance	Lebman Digital Analytical, USA
Graduated centrifuge tube	Labbox™, France
33 mm Millex® filters	Merck Millipore 20µm, USA
T25 flask	CytoOne, UK
T75 flasks	VWR International, UK
1.5 ml microcentrifuge tubes	Labbox™ , France
Incubators	LEEC, Australia
Inverted Microscope	Motic, AE2000, Spain
pH meter	Jenway 3310, UK
Plate shaker	Thermomixer comfort, Eppendorf, Germany
Salinity Refractometer	Salinity Refractometer for Seawater and Marine Fishkeeping Aquarium 0-100 PPT with Automatic Temperature Compensation, Spain
Portable Kitchen Balance	Sencor, China
Sterile filter unit with filter bell	Millipore, Sterivex™ ,Germany

Concentrator	Eppendorf Vacufuge Plus Concentrator, Germany

2.1.3 List of Cell lines employed in the research project.

Table 5: Table showing cell lines tested in the research project together with some important notes attributed to the cell line.

Cell line	Notes
HL60	French-American-British association classification: M2 Purchased from Cell Line Service (CLS) GmbH, Germany Acute myeloid leukaemia Catalogue nos.: 300209 Passage number = 86 Media: RPMI-1640 Minimum cell concentration: $5x10^5$ cells/ml. Maximum cell concentration: $1x10^6$ cells/ml
K562	Purchased from Cell Line Service (CLS) GmbH, Germany Chronic myeloid leukaemia Catalogue nos.: 300224 Passage number = 10 Media: RPMI-1640 Purchased from Cell Line Service (CLS) GmbH, Germany Minimum cell concentration: $2x10^5$ cells/ml. Maximum cell concentration: $1x10^6$ cells/ml
KG1a	French-American-British association classification: M1

	Purchased from Cell Line Service (CLS) GmbH, Germany Acute myeloid leukaemia Catalogue nos.: 300234 Passage number = 38 Media: IMDM Minimum cell concentration: $1x10^5$ cells/ml. Maximum cell concentration: $1x10^6$ cells/ml
NB4-R2	French-American-British association classification: M3 Provided by Professor Mario Tschan from the Institute of Pathology at the University of Bern, Switzerland. Acute myeloid leukaemia Catalogue nos.: NA Passage number = 17 Media: RPMI-1640 Minimum cell concentration: $5x10^5$ cells/ml. Maximum cell concentration: $1x10^6$ cells/ml

2.2 Cell culture

2.2.1 Aseptic techniques.

All experiments outlined in this section were carried out using aseptic techniques as they prevented fungal and bacterial infections which in turn would have given false positives.

An overall laboratory cleanliness was always maintained, to minimise any possibility of infection. Equipment such as spatulas, measuring cylinders and other glass equipment were washed with twice with tap water and twice with distilled water prior to being used in an experiment in order to make sure that all traces of any chemicals or media were completely removed hence not affecting in any way the experiments. In addition, apparatus used for toxic material such as MTT were not use for non-toxic ones. All biological waste was mixed with bleach and disposed of in an adequate manner.

All cells were grown in incubators that were kept clean by periodically washing with water and then occasionally employing an inbuilt sterilising cycle. The incubators were always kept at a temperature of 37°C and a carbon dioxide concentration of 5%. All the tests carried out using the different cell lines were done in a Laminar flow hood BSL, class II. This type of hood protects both the outside and inside environment, i.e., it protects the researcher as well as the samples from contamination. The flow hood was subjected to UV for 30 minutes prior to each experiment and was then cleaned with 70% ethanol whilst the filtered laminar air flow was running. This air flow supplying the flow hood was being handled with HEPA (High Efficiency Particulate Air filter) filtered air ensuring that no contaminants were present, protecting the samples being handled. Any materials used inside the flow hood where always sprayed with ethanol to avoid contamination. Gloves and any exposed skin that would go into the laminar flow were sprayed thoroughly with 70% ethanol and any jewellery and sleeves were removed.

Flasks are kept closed (sterile packs) and always opened in the Laminar Flow hood. Two main flasks were used during this investigation: the T75 flasks were filled with cells suspension up to a maximum 20 ml and the T25 flasks were filled with cells suspension up to a maximum of 8 ml. The media used, RPMI-1640 (Roswell Park

Memorial Institute Media) and IMDM (Iscove's Modified Dulbecco Medium) were always sterile filtered and any liquids such as distilled water or PBS and solids such as tips and centrifuge tubes were always autoclaved prior to being used in experiments. The FBS used to supplement the RPMI and IMDM was always aliquoted and used under sterile conditions and the complete RMPI and IMDM media always included penstrep (to control bacterial growth) and when in need amphotericin (to protect against fungal growth). FBS and penstrep were both stored at -20°C, where thawed once in their original container, aliquoted into sterile tubes into smaller quantities which could be refrozen. Before use, the contents of a sterile tube was thawed. This was imperative for the long-term storage of these products. Once thawed, the lifespan of these compounds was of a month, whereas in the frozen state they could be kept up for 6 months. Once thawed, the aliquots of FBS and Penstrep were added to the media in order to complete it, thus the life span of the complete cell culture media was also of one month.

Prior to starting an experiment, at least 24 hours before being used in the actual experiment, some media and some of the cell suspension to be used were placed in a T25 flask in a 37°C incubator. This was done to be able to detect any infection present. Any infection would have made the solutions look turbid accompanied by a change in colour of the indicator of the media and hence would need to be discarded, source of contamination identified and a fresh solution to be used.

2.2.2 Media used in the research project.

Leukaemia cells are not adherent and therefore float in the media. They form a cell suspension the media in which they are growing. These cell cultures need to be grown in optimal conditions in terms of nutrients available, pH, number of cells growing within the flask and other factors.

The Media used, primarily RPMI-1640 (used to grow HL-60, K562 and NB4R2 cell lines as recommended by the cell line's supplier product sheet) and IMDM (used to grow KG1A cell line as recommended by the cell line's supplier product sheet) were purchased in the powdered form and a solution was prepared when required. The vials containing the powdered media were only opened prior to

immediate use. Given that dry media is hygroscopic, leaving the powdered media partly unused would have caused it to become unsuitable for future use as its mass and composition would vary. Therefore, all the powered contents were used to make up media solutions at one go. Both media contain phenol red which is a pH indicator and provides a measure of cell growth which is easily visible. A decrease in pH causes phenol red to change from red (pH 8.2) to yellow (pH 6.4) hence indicating increased carbonic acid due to cell proliferation or possibly an infection (Morgan *et al.*, 2019). $NaHCO_3$ provides the buffering system in both these media.

2.2.2.1 Preparation of RPMI.

10.90 g of the powered medium containing L-glutamine was weighed using a clean weighing boat. This mass was then quantitatively transferred to a 1L measuring cylinder and the weighing boat was washed with distilled water, with the washing being placed in the measuring cycling. 800 ml of distilled water was added, and the solution was stirred continuously using a magnetic stirrer. 2.0 g of sodium hydrogen carbonate was added, and the pH was maintained between 6.9 and 7.2 by the dropwise addition of a 6 M solution of hydrochloric acid or sodium hydroxide. The pH was checked using a pH meter and when the required pH was reached, the solution was made up to a 1000 ml with distilled water.

The solution prepared was not sterile and hence the media was sterile filtered before use, using a high-pressure dispensing system. A pressure vessel for filtration (Millipore XX6700P05) was fitted with tubing and washed twice with tap water and twice with distilled water. This step was necessary not only to clean the equipment but also to make sure that water flowed unobstructed. The reservoir inlet tube was attached to a diaphragm pump (KNF Neuberger N022AN.18) at the pressurised exit. The set up was closed and switched on to make sure that it was airtight. The media was then poured into the reservoir. The set up was placed in a class II Laminar Flow hood, which had previously been aseptically cleaned. A sterile filter bell fitted with a 0.20 μm pore sized filter (Millipore Sterivex™ SVGPB1010) was fitted to end of the tube and placed over TC-treated cell culture T75 flasks. The pump was switched on and the pressure was kept at around 1 bar throughout the process. Once the flask was filled with 200 mL of medium, a new flask was used. This process was repeated until the whole litre of media was split into 5 T75 flasks, which were closed, properly

labelled, and stored at 4°C for up to 6 months. Care was taken to avoid a build-up of pressure or leaking of the solution.

2.2.2.2 Preparation of IMDM.

17.70 g of IMDM powder was weighed using a clean weighing boat. This mass was then quantitatively transferred to a 1L measuring cylinder and the weighing boat was washed with distilled water, with the washing being placed in the measuring cycling. 800 ml of distilled water was added, and the solution was stirred continuously using a magnetic stirrer. 3.7 g of sodium hydrogen carbonate was then added. The pH was maintained between 6.9 and 7.2 by the dropwise addition of a 6 M solution of hydrochloric acid or sodium hydroxide. The pH was checked by using a pH meter and when the required pH was reached, the solution was made up to a 1000 ml with distilled water. The solution prepared is not sterile and hence the media was sterile filtered before use as described above, using a high-pressure dispensing system, and passing the medium through a sterile filter bell fitted with a 0.20 µm pore sized filter. Care was taken to avoid a build-up of pressure or leaking of the solution. Once filtered it was then stored at 4°C. In this incomplete state, without the addition of serum, it can last for up to 6 months.

2.2.2.3 Completion of media.

Prior to being used on cells, the complete media were prepared as follows: RPMI-1640 was completed by the addition of 10 % FBS and 1% penstrep whilst IMDM was completed by the addition of 20 % FBS and 1 % penstrep. The FBS supplied the cells with vitamins, minerals, proteins, hormones, growth factors and carbohydrates (Gstraunthaler, Lindl, & van der Valk, 2013). Penstrep controlled bacterial growth of both Gram-positive and Gram-negative bacteria. After these additions the medium is described as complete, and in this state, it can last for up to 4 weeks, refrigerated; therefore, it was carefully labelled and dated whenever prepared.

In order to maintain the functionality of both FBS and Penstrep, repeated freeze-thaw cycles are avoided as this would have resulted in denaturation of the proteins present in the liquid. Both these substances are aliquoted once thawed from the -20°C freezer into volumes that are suitable for the cell culture techniques being carried out and then frozen once again. The second time they are thawed, they would

be used. FBS was aliquoted into 20 ml aliquots in sterile 50 ml tubes whilst Penstrep was kept as aliquots of 200 μL in sterile 1.5ml microcentrifuge tubes.

2.2.3 Cell lines

In vitro tests provide reliable information that is accurate in a relatively short time. This reduces the number of animal tests that would be required in order to screen biological materials, reducing the negative effect on living organisms and hence any ethical issues which might arise (Pearson, 1986). In addition, cell culture allows for more human specific testing avoiding the use of animals whose reactions might be different from those of a human.

Four cell lines were tested, and these were HL60, K562, NB4R2 and KG1a, however this was narrowed down to HL60 and K562 for various reasons described at a later stage and finally tests focused on HL60. They were stored indefinitely in liquid nitrogen allowing aliquots to be used as required without repeated freezing and thawing.

2.2.3.1 Counting cells using a haemocytometer

Cell counting was done prior to every experiment, before freezing and before subculturing. A viable cell count gave an indication of the health of the Leukaemia cells suspension which allowed for proper use of suspensions to be made. Whenever cells were counted, a haemocytometer with a Neubauer Improved chamber was used. Counting of cells prior to using the cell suspension for an experiment was an imperative step as it was extremely important to start with a similar concentration of Leukaemia cells, in order to make the results obtained from the various treatments comparable.

A haemocytometer is a glass chamber that can take a precise volume of solution and gives an accurate measure of number of cells in a specific volume. When viewed under the light microscope it can be seen as two large grids etched on the glass as seen in figure 15 (on the left) and measures 3 mm by 3 mm. One of these grids is found on the upper part of the chamber and one on the lower part of the chamber, separated by a groove.

The Trypan blue dye exclusion assay was used when counting cells from a Leukaemia cells suspension as this stain is taken up by dead cells but not so by live cells (Strober, 2015). Hence counting using this stain gave a more precise value of the size of leukaemia cells at hand as the dead ones would be excluded and hence only those cells that are viable would be counted. 200 µl trypan blue were diluted by 1400 µl PBS in a microcentrifuge tube. 100 µl of a homogenous Leukaemia cells suspension were placed in a microcentrifuge tube and 100 µl of diluted trypan blue solution were added to the same microcentrifuge tube. The microcentrifuge tube was closed, shaken well and the cells were left to incubate for 10 minutes. Exposure to trypan blue of longer than 15 minutes would have resulted in all the cells being stained.

When preparing the haemocytometer, both the glass cover slip and the haemocytometer where properly cleaned. This was done by washing with distilled water, sprayed with 70 % ethanol, and then left to dry on a paper towel inside the laminar flow hood. The glass cover slip was firmly attached to the rails of the haemocytometer. The Leukaemia trypan blue cell suspension was then thoroughly shaken again to form a homogenous solution and a 2 ml sterile glass pasteur pipette was used to take up some cells and place them into the haemocytometer chamber by capillarity. Whilst the pipette was placed inside the suspension inside the microcentrifuge tube, the top part of the pipette was closed using the thumb causing some of the cell suspension to be contained. The sterile tip was then placed into the haemocytometer chamber and the thumb was removed from the top part of the pipette allowing for the suspension to seep into the chamber, once again by capillarity. This was repeated for both sides of the haemocytometer. This method of filling was used over the use of a micropipette as the latter might lead to underfilling or overfilling possibly leading to the detachment of the glass slide slip. When assembled appropriately, capillarity will allow the chambers to fill into a fixed volume only. The haemocytometer was then placed under the microscope and viewed under high power. If upon inspection any air bubbles were noticed, the assembled haemocytometer would have to be disassembled, cleaned and the procedure would have to be started all over again.

4 smaller grids (a smaller grid being shown in blue in figure 18) measuring 1 mm x 1 mm) were used in total, two from the upper chamber and two from the lower

chamber. Cells falling on the left and top of each smaller grid were counted but those falling on the right and bottom of each smaller grid were not counted. This method ensured that a cell is not counted twice. An average was then taken and that gave the number of cells x 10^4 per ml of cell suspension. The unstained cells were counted, and a total viability cell count was done. The values obtained from 4 small grids (where each small grid represent 1×10^4 of a ml) were averaged and the value obtained when counting was then multiplied by 2 so as to account for the cell culture: trypan blue dilution. This value was then converted into number of cells per ml. This is all shown in the equation 1.

Equation 1: Equation used to calculate the total number of viable cells with dye exclusion assay and a haemocytometer with Neubauer improved Chamber.

$$\text{Cells per ml of suspension} = \text{averaged viable cell count} \times \text{dilution factor} \times 10^4$$

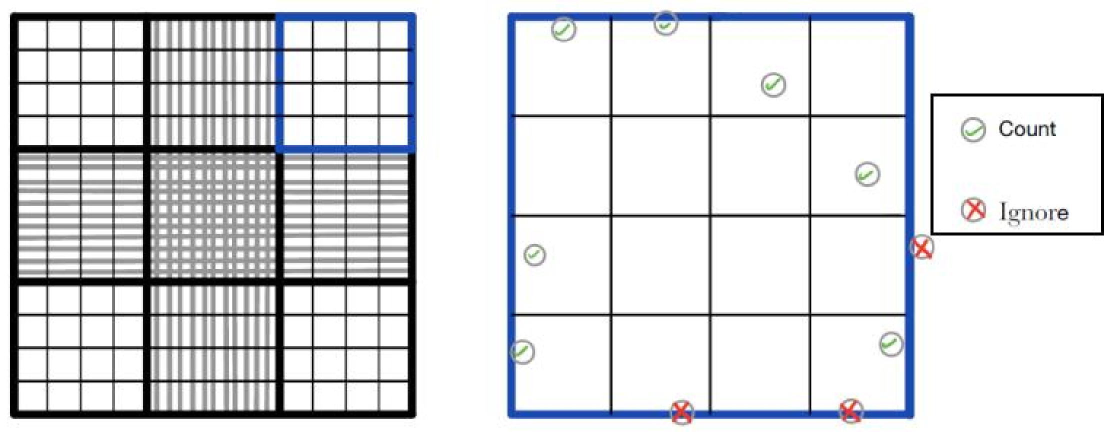


Figure18: Haemocytometer as viewed under x400 magnification using a light microscope. Figure drawn by author.

When counting was completed, the haemocytometer and glass cover slip were once again washed using distilled water, followed by ethanol, and allowed to dry. Extreme care had to be taken when cleaning the haemocytometer as any scratches would result in a change in total volume kept within the assembled chamber, leading to inaccurate viable cell concentrations. This was imperative to prevent cells and debris from being deposited in the grooves of the haemocytometer which would impair further use and readings obtained from it. The cell suspension inside the chambers was not mixed with the original cell suspension as the former is not sterile anymore.

Despite this technique of dye exclusion being a relatively simple test, there are some drawbacks associated with it. This method is based on the viability of the cell membrane integrity and even though a cell might be compromised, the integrity of the cell membrane might still be maintained for a short period of time, which would be coinciding with the time of counting. The opposite may also be the case, whereby the cell membrane integrity is compromised and yet the cell would be viable. In addition, given that the uptake of the dye is being assessed subjectively, small amounts of dye being taken up by an injured cell might go unnoticed and hence giving false viability result (Strober, 2015). A better approach would be to use flow cytometry; however, this was deemed to not be important as the trypan blue essay was enough to allow successful tests to be carried out.

2.2.3.2 General Maintenance of cell lines

The cell media give an indication, based on its colour, on the number of cells present in culture and on the possible contamination of the cell suspension.

2.2.3.3 Freezing and thawing techniques.

- **Freezing**

On the day prior to freezing, the health of the cells was checked, by visualising under the microscope and the number of cells was counted using a haemocytometer and kept to around $5 \times 10^6 - 1 \times 10^7$ cells per ml. The cells were sub-cultured with new medium in each flask. The following day, the cells were checked to make sure that they were healthy and strong. This was done by making sure that the number did not decrease and that most cells appeared round. The fluid in each flask was centrifuged at 1500 rpm for 5 min and the pellet was then re-suspended in 1 ml FBS were T25 flasks were used and 1.5 ml FBS when T75 flasks were used. 10 % by volume DMSO was added as an anti-freezing agent to the re-suspended pellet and placed into cryovials. The cryovials were immediately frozen to -80°C in a 'slow-freezing box' and -196°C (liquid nitrogen) subsequently.

- **Thawing**

In order to thaw, 15 ml centrifuge tubes were prepared for each cell vial and were labelled appropriately. 5 ml of Media were placed into each of two test tubes. A beaker was filled with tap water at ~37 °C and the samples were collected from the liquid nitrogen freezer or the -80 °C freezer and placed in the beaker. All the details of each vial were listed down, and the vials were then subsequently wiped with ethanol. If different cell lines were thawed at the same time, or cells of the same cell line but with different passage number, different beakers were used, and these were marked appropriately. Once thawed, the samples were transferred into the ready-labelled tube with media using sterile pipettes. Transfer to the media was done as soon as the cell suspension had thawed as DMSO is a differentiating agent and can therefore alter the results. The tubes were mixed well and centrifuged at 1500 rpm for 5 mins. The supernatant was decanted, and the pellet was placed in a fresh 5 ml media. The solution was spun at 1500 rpm for 5 mins. The supernatant was removed, and the pellet was re-suspended in a fresh 5 ml complete media. The cells were transferred into a new T25 / T75 flask and incubated at 37°C and 5% CO_2.The tap of the culture flask was opened a quarter of a turn to allow gaseous exchange to take place.

2.2.3.4 Sub culturing

Sub culturing was an essential process in order to keep the cells at their optimal cell density and hence ensure healthy cell proliferation. Each time a cell suspension is sub cultured, its passage number (P) increases and is recorded diligently on the flask.

The time between sub-culturing depended on the time it took for the cells to grow. HL60 and KG1a have an optimal density of $1x10^5$-$1x10^6$ viable cells / ml, K562 the optimal density is $1x10^6$ viable cells / ml and NB4R2 its $2x10^5$ – $5x10^5$ viable cells / ml (CLS product information sheet). HL-60 cells, NB4R2, and K562 cells were maintained in RPMI with 10µL/ml Penstrep and 10% foetal bovine serum. KG1a were maintained in IMDM with 20% FBS and 10µL/ml Penstrep. Cell cultures were incubated at 37°C with 5% CO_2.

For cell lines K562 and HL60, sub culturing was done about every 3-5 days as the doubling time was that of about 34 hours for HL60 (Foa *et al*., 1982) and about 20.4 hours for k562 (Rutherford *et al*., 1981). KG1A had a doubling time of 55-60 hours (Koeffler *et al*., 1980) and so were sub-cultured on a weekly basis. NB4R2 were proving very difficult to grow in culture and were differentiating without the addition of any extracts a few days after thawing. In the rare occurrence were NB4R2 were grown in culture, these were tested normally.

During their time in culture, the cells were examined periodically using an inverted microscope and their density and general conditions, such as roundness of cells, were studied. When the cells were still healthy, and the medium capacity was not yet reached, new medium, incubated at 37°C, was added. When the cell density was higher than that which is acceptable, subculturing was performed. This involved spinning down the cell suspension by centrifuging in for 5 minutes. All the medium was removed, and the remaining pellet resuspended in fresh medium, incubated at 37°C, was split equally between three flasks. Every time the cells were split, the passage number increased by 1. The flasks were placed in an incubator and stored at a temperature of 37°C and a concentration of CO_2 of 5%.

2.2.3.5 Plating out experiments

When plating, healthy Leukaemia cell cultures were selected, pooled, and counted using a haemocytometer. By centrifugation and re-suspension in the appropriate complete medium, a cell concentration of $2x10^5$ cells per ml was prepared and 100 µLwere poured into 96-well plates and subsequently subjected to different extracts. When preparing the cell suspension or solutions of the extracts to be tested, double the concentration of that required was prepared as this was then diluted by half upon plating. In addition, the wells on the perimeter of any plate placed in the incubator were always filled with sterile distilled water so as to minimise differences in the results, resulting from evaporation.

2.3 Biological samples

Holothurians are a group of echinoderms which have a very important biological role in that they are key organisms in the marine environment. Being deposit feeders, they are involved in recycling organic matter, mixing the sediment layer, and stimulating the growth of many microalgae (Nerva *et al*., 2019; Boncagni *et al*., 2019).

The first practical step was to learn how to identify locations in which to find these organisms. A preliminary search for these organisms was performed by snorkelling monthly in different locations around Malta which involved habitats described in literature as their preferred natural habitat, i.e., the interphase between rocks and sands, especially in areas where there was sand with *Posidonia oceanica*. Besides being very capable of camouflaging by covering themselves with algae and other organisms, *H poli* also have the ability to burrow. They also have the tendency to live in aggregates and their faecal matter is very specific consisting of cylinders of sand. It was determined that once faecal matter is found, it could be expected to find a good number of specimens making it easier to collect the required number of organisms required for each trial.

H. poli were identified by their white tips and when there was uncertainty by using their spicules (as explained in the section 1.1.1) (figure 19). This was done by scraping the surface of the animal and placing the cells on the surface of a slide. A few drops of water were added and then investigated using a microscope (Motic, AE2000) (Personal Communication with Prof. P.J. Schembri, Department of Biology, UOM).

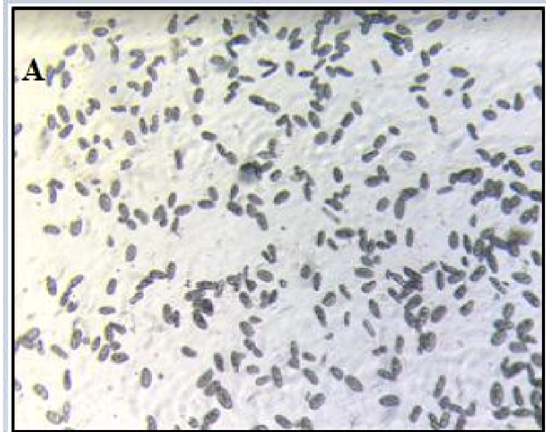


Figure 19: Holothuria (Roweothuria) poli ossicles taken by author. Figure A is at a x100 magnification whilst figure B is at a x400 magnification.

Those specimens not belonging to this species were placed back into their original habitat to reduce as much as possible the negative effect on the marine environment. The measure used when catching these organisms was taken to be the mass and not their length as the latter varies depending on handling (Moussa & Wirawati, 2018). When caught in hand, they tend to distend their body and when threatened they tend to compress their body. Calculating their mass as soon as they are caught, gave the closest indication of their similarity. The animals could not be allowed to become stressed as they would start the process of evisceration. The organisms above and below a predetermined mass range were thrown back into the sea. The collected sea cucumbers were patted dry using a cloth and weighed on a portable digital balance (Sencor, China). An increase in size, might possibly be correlated with age, which can possibly introduce another variable in the study. The organisms collected were kept in plastic boxes at a density of about 3 animals per litre of water for as little as possible and were immediately transported to where they were going to be kept. This took place in October-December 2013.

2.3.1 Studying the internal anatomy of *H. poli*.

Three *H. poli* were collected from St Thomas Bay, Marsascala, in November 2013. They were then placed in a container on ice and in the fridge in order to cause a 'stupor' with reduced sensation slowing down the metabolism for 2 hours and then in a container containing 70% ethanol, in order to be preserved for dissection so that their internal biology could be studied and compared to literature. These organisms were preserved before dissection to stop their body contractions when cut open which makes it very difficult to study them. These dissections helped in the understanding of their internal anatomy. Dissection was initiated by a ventral incision through the body wall and extended through the anterior, buccal end. Detailed notes of their intestines were made, and diagrams were drawn. This was done for three organisms to note repeatability of structures given variability in sex and age of the organisms collected.

2.3.2 Understanding evisceration of *H. poli*.

Three *H. poli* were collected from St Thomas Bay in November 2013. They were placed in plastic containers in a very small amount of water and allowed to eviscerate (Figure 20). In order to induce evisceration, the animals would have to be left in a very small amount of fresh sea water, to induce stress (verbal communication with Prof. P.J. Schembri, 2013, Jenzri, 2022). Organisms undergoing evisceration would form an indent, nearly twisting onto itself, and then expulsion of the intestine would happen over a period of time. This amount of time was not the same for every organism, but care was taken to move the organisms as soon as it eviscerated. Once this was achieved, they were placed in a container on ice and in the fridge in order to cause a 'stupor' with reduced sensation slowing down the metabolism for 2 hours and then in a container containing 70% ethanol, in order to be preserved for dissection so that their internal biology could be studied and compared to literature. Dissections were done as described in 2.3.1. This step was essential in the understanding of how evisceration occurred and hence the structures left behind after autotomy could be identified. This information, together with a study of the intestine eviscerated gave a complete picture that confirmed what had been pre learnt from literature. This was done for three organisms to note repeatability of structures given variability in sex and age of the organisms collected.

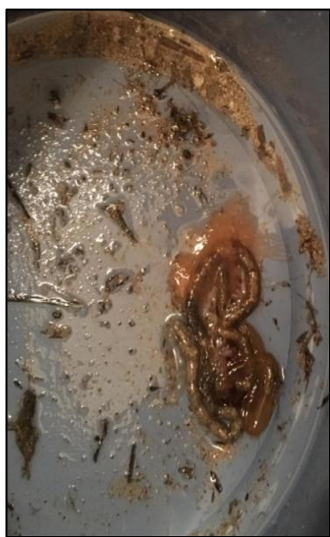


Figure 20: From left to right –in the first photo, the black arrow shows how the first step of evisceration can be seen a constriction indicating that evisceration will happen. In the second photo, evisceration is taking place from the oral opening. In the final photo there is the eviscerated intestine. Photos taken by author. Scale: x0.5

2.3.3 Determination of location for the collection of specimens

The next step was to identify the right place from where these organisms could be obtained. Slugs bay was the first location to be investigated starting in December 2013, however due to difficulty associated with coming up and down, to and from the bay, even though the number of organisms there were abundant, the location was not further investigated. When taking into consideration number of organisms present (assessed by amount of faeces present), ease by which these organisms could be collected and the ease by which they, together with the water they would be in could be carried and transported, two locations were identified. These were Mistra Bay and St Paul's Bay. However, after further consideration, it was determined that St Paul's Bay was the best location as it was deemed to have better circulation of water, less boats are moored in the areas, although there is a kiosk, it not open all year round, whilst in Mistra bay there are many caravans and a number of boat houses, permanent houses and a restaurant which is open all year round negatively affecting the area. The water in St Pauls Bay was constantly being changed due to partial exposure to wind and yet since the area is quite closed, it less susceptible to sudden changes in water characteristics with day-to-day changes in weather than Mistra Bay. Number of boats travelling to the area at the time of collection was also much less in St Paul's Bay when compared to Mistra Bay. In St Pauls Bay there was very easy access by car and the water could be collected from the shore itself. In addition, the depth of the water was not more than 3m which meant that organisms could be collected without the need of diving.

Sea cucumbers were collected from the bay called il-Bajja ta' l-Ghazzenin in Saint Paul's Bay (figure 21) from a depth varying between 3m and 4m depending on weather conditions, currents, and the location of the specimens. Once collected, these organisms were blotted dry using a cloth and weighed using a digital balance. Those in the range of 65-85g were placed in a large plastic container measuring 1.2m by 75cm by 30cm which contained a layer of sand and water collected from the area. Permits for MEPA were obtained for this transfer of sand and animals prior to the start of the collection part of the research and this can be seen in Appendix 1. Figure 21 shows is a map of Malta, showing the location from where the sea cucumbers were collected for this research project.

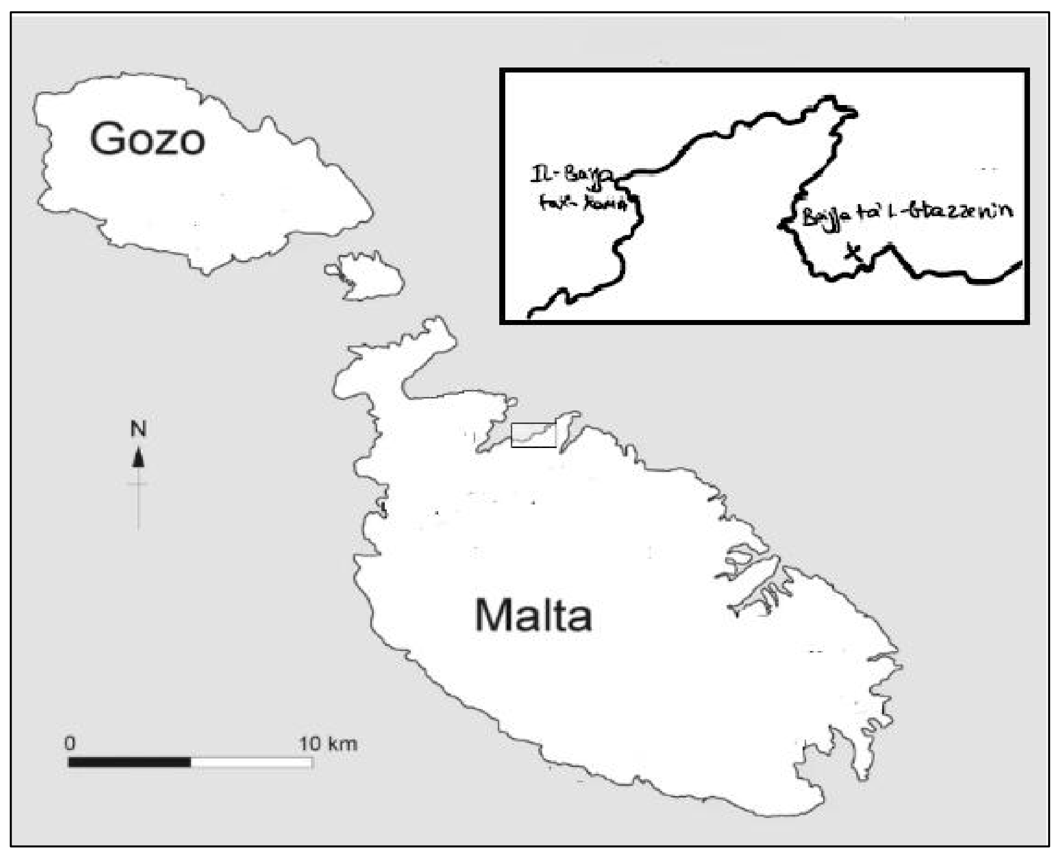


Figure 21: Map of Malta and Gozo. Location of collection of sea cucumbers marked with an X on the insert. Adapted from Cassar et al., 2018.

2.3.4 Determination of best time period for harvesting.

The next step involved determining the period during which the specimens would be collected. The distribution of these organisms is known to be affected by the seasons (Mendes, *et al*, 2006). When considering information about population density and seasonal distribution of *H. poli* from the previous exercise, periods December to March and June to August were automatically not taken as possible months for harvesting. In summer (June, July, August) these animals would be found burrowing deep down to avoid the summer heat whilst in winter (December, January, February, March). they would be easily carried away by strong currents. In addition, due to the low temperature and low salinity associated with the winter season, these animals undergo aestivation (Tolon, 2017) making it difficult to collect them. Hence possible time frames were April – May and October – November. The last remaining factor to determine the time of collection was the growth in artificial conditions as well as biological factors involving life cycle.

2.3.5 Growth in Artificial Conditions

When collecting organisms, it was noted that the easiest way was to find their faeces first, which is very distinctive. Finding the faeces would have meant that the holothurians are in the area. Through personal observation it was determined that once one organism was found, many others would be found in the same area, at very close proximity. The same behaviour was observed in artificial conditions, whereby the specimens tended to clump together during some periods in the aquaria but then be separated during other periods. It was essential, however, to keep a constant watch on these animals, as its very easy for them to be blocked by others or by structures (like the pump) found in the aquaria and die, affecting the water quality and the survival of all the others living in the same aquarium.

It was also noted that acclimatisation was essential. Prior to allowing them to eviscerate, the organisms were allowed to grow for a week in the aquaria. This allowed them to adapt to the new environment without compromising their survival. Allowing them to eviscerate as soon as they are caught resulted in many organisms dying (personal observation).

Holothuria are known to grow well in artificial conditions given that the conditions in which they are allowed to grow is suitable for their needs in terms of pH, salinity, temperature, aeration, and other factors. Their dietary needs have not yet been completely clearly elucidated and more research needs to be conducted in this area. However, a study conducted by Boncagni *et al*., 2019, it was determined that *H. poli* showed high yields under varying feeding regimes showing their robustness in growing in artificial conditions.

H poli are susceptible to changes in salinity. They are known to be much less negatively impacted with higher salinity than with lower salinity (Tolon, 2017). In addition, they are also known to be affected by temperature, where at high temperatures they go into a period of no growth (Tolon, 2017) and are more susceptible to diseases (personal observation). In addition, they are known to form microbial associations with microorganisms found in their environment, especially the sediment (Gao *et al*, 2014; Liu *et al*, 2019). Hence control of the conditions when

being grown in artificial environments is not only important for the growth of the organisms but also for possible repeatability of results.

Growth in artificial conditions (i.e., in aquaria) was done as a trial for the months of April, May, October and November 2014. This was done by collected 12 individuals every mentioned month, allowing them to eviscerate by stressing them in a little amount of water and then placing 3 specimens in each of four different aquaria. One aquarium was prepared three days before with sea water collected from the same area, aerated continuously by means of a pump for the three days and treated with antibacterial and antifungal treatments. The other aquarium was filled with water on the same day as the organisms were collected and aerated on the same day. In the other two aquaria the same concept was used, however water used was Artificial in nature. Some sand form the site of collection was placed at the bottom of each aquarium.

Collecting organisms in April and May and growing them in aquaria would have caused overharvesting to make sure that enough survived in artificial condition for the duration of the experiment as during these periods organisms were dying at a high rate of white spot disease (as described in section 1.1.3). This is because contact with a warmer air temperature (whilst eviscerating) was noted to cause them to become more susceptible to this disease creating a number of drawbacks as even though the room was air conditioned, this was not enough for the disease to be avoided. Even on the coldest setting of the air conditioner, it was noted that once organisms were exposed to high temperatures, cooling them would not be enough to restore them back to normality. First of all, an unnecessary strain on the wild population of these organisms would have occurred making it non ethical. Once an organism becomes sick, the whole tank would need to be cleared out as it was noted that the white spot disease was easily transmitted to the rest of the individuals found in the same aquarium. Clearing out the tanks every time and start all over again would have resulted in a lot of animals dying for no reason and a lot of difficult manual work. Finally, presence of white spot disease, even if somehow controlled would have meant that the organisms starting off the experiment were in their suboptimal conditions and probably a change in the composition of their coelomic fluid. This would have further increased the number of variables that would have had to be taken

note of. During the months of October and November, it was noted that most efficient and successful growth of such organisms took place in aquaria.

It was also noted that at this time of year, individuals are non-gonadal as no gonads were noticed when their intestines eviscerated, as opposed to other months. In November, individuals of *H. poli* would be in a resting phase, whereby the gonads which would have already matured until fertility and would have now retracted after spawning (Slimane-Tamacha *et al.*, 2019). Hence, upon evisceration, less strain will be felt by the animal as it has less organs to regenerate making the process more likely to happen successfully.

It was essential to find a balance between being able to collect organisms in a period which would make this collection possible in terms of weather and animal availability as well growth of the actual organisms in artificial conditions. Keeping the date of collection to a two-month period was deemed to be the best way forward as this gave a greater certainty that these organisms would be in a very similar stage of their life cycle, given that organisms of similar size were collected.

After the attempts to grow these organisms in aquaria it was determined that the best way was to keep the specimens healthy was to employ aquaria that were prepared three days prior to the actual experiment and then employ continuously circulating water on top of a layer of sediment obtained from the same area of collection. Once animals were placed in the aquaria, the air pump was switched on for 12 hours every alternate day, as otherwise continuous exposure to aeration caused an increase in mortality. The water used was changed every three days to be kept as fresh as possible by using water from the same site, again to maintain as much as possible constant conditions. Fresh seawater is known to have a pH stabilising effect (Baensch, 1983). Artificial sea water was tested; however, growth was not as successful as when using the same sea water from the area. It was deemed more useful for these organisms to have a continuous supply of water from their originating location, as it allowed for the same microenvironment to be maintained.

2.3.6 Optimisation of set up.

The final setup involved 6 aquaria being set up, each one independent of the other but all kept under the same conditions of temperature (using AC), aeration (using pumps) and other factors such as salinity and pH. This was done to allow for the animals to be checked continuously as when any of the organisms' showed signs of infection, they had to be removed immediately, as otherwise infection would have increased drastically affecting all the animals in the aquarium. Although the labs at the biology department were considered as an option for this function, they were deemed not as appropriate as at the time they were all connected, and hence if one group became contaminated it would have affected the rest of the organisms leading to a great loss of organisms and time. The location chosen to keep the aquaria in the set up described above was very close to UOM and hence transport of organisms to the lab for testing was easy and did not pose any threat to the organisms or any negative effect on the result. The organisms grew well the aquaria and rate of mortality was reduced to practically 0%. These results were obtained when the period of collection was October/November. When tests needed to be carried out on the specimens, the animals would be carried to UOM on top of ice, to cause them to go into stupor hence reducing stress which would trigger the release of different biomolecules in the coelomic fluid.

The preparation of the aquaria involved a number of steps. The aquaria were thoroughly washed with fresh water cold water and soap and then hot water. They were then dried using clean cloths as thoroughly as possible so as not to leave puddles of water in the aquaria. All the aquaria were then allowed to air dry for about 3-4 days, after which the process was repeated one more time. Once the aquaria were clean and dry, they were washed 3 times with ethanol to make sure that no microorganisms from any previous growing specimens were present, as these could potentially interfere with the new batch of specimens. Once they were air dried, water from the location of collection of the organism was collected in jerry cans that were washed three times with the same water. 6 jerry cans of 25L each were filled in each time as they were very heavy. They were then taken to the location where the aquaria were held, and the water was placed equally in the aquaria. This process of filling up water was carried out twice, in order to have enough water for all the aquaria. The

room was then set up at a temperature of 20 °C. Pumps where then attached, and the water was left to aerate for three days prior to placing sand collected from the area of collection and any sea cucumbers. Once sea cucumbers were placed in the aquaria, half the water was exchanged with freshly collected sea water. Prior attempts to grow the sea cucumbers, not following this procedure had failed and at least one organism would become infected with white spot disease, infecting the others.

Prior to optimising this method, another 3 methods had been employed, each proving itself to be ineffective. The first method that was tried out involved cleaning the aquaria with ethanol only. Instead of changing half the water with fresh sea water every three days, addition of salt ice cubes and cold ice water which was placed in the fridge was used. This proved to be not only ineffective but very difficult to keep up with, so this method was abandoned.

Through a discussion with the owner of pet shop who displays marine aquaria, empty clean clay pots were added to the water circulation, placed at a higher level than the aquarium itself. They were filled with beads and water was made to pass through them whilst circulating. This would have induced bacteria to grow on them, potentially increasing the nitrates in the water (verbal communication with pet shop owner) as well as increase the air content, as air was taken up as water flowed down. Although this process seemed to work initially, the aquaria, together with the filters were more prone to clogging and there where a number of occasions were water overflowed out of the pots, making it not a viable option.

The other option that was tried out was to use artificial sea water, to which antifungal and antibacterial liquids were added. However, since it was noted that the surrounding water affected with growth of microorganisms inside the sea cucumber, this method was abandoned in order to make the environment more similar to the natural environment.

2.3.7 Understanding regeneration in *H. poli*.

In Holothurians, regeneration following evisceration takes an average of a month to be completed, however this varies between species and is also affected by the environment they are growing in (García-Arrarás & Greenberg, 2001). Artificial conditions as those present in an aquarium, are known to alter the behaviour as well as

the regeneration time when compared to that found in the natural environment (García-Arrarás & Greenberg, 2001). Hence it was essential to determine the regeneration time for *H. poli* growing under these conditions.

Thirty *H. poli* were collected at one go from the chosen site in November 2014 and caused to eviscerate. Once evisceration was complete, they were then kept alive in aquaria at a density of one specimen per litre as described above. The pH was tested regularly so as to make sure it was in the range of 7.8 and 8.3 using a pH metre. A refractometer (Salinity Refractometer for Seawater and Marine Fishkeeping Aquarium 0-100 PPT with Automatic Temperature Compensation, Spain) was used to make sure that salinity was under control at 35ppm, and an air pump was used to keep the water well aerated.

The animals were allowed to regenerate in the aquaria, but 3 specimens were taken every three days in order to carry out tests. The same method of preparation and dissection was carried out as described above is section 2.3.2. The process of regrowth could be elucidated in detail, each time part of the re-growing intestine, together with the coelomic fluid, were collected in order to be tested. All the coelomic fluid except for 1 ml was centrifuged in a 15 ml conical bottomed tube at 1500 rpm to remove the coelomocytes, properly labelled and then fast frozen at -80°C. Literature showed that in general holothurians take around a month to regenerate their intestine (García-Arrarás & Greenberg, 2001). In this research study it was determined that *H. poli* take 24 days for complete regeneration. The three-day sampling method was chosen on the fact that it was deemed to give a sample size large enough and taken at regular intervals, for a good comparison of any potential effects of extracts from the sea cucumbers to take place and be noticeable.

Once regeneration was studied in more detail in these organisms, the part of the *Holothuria poli* to be used for the tests had to be determined. There were two main components that could have been used, namely the regenerating part of the intestine and the coelomic fluid.

Originally, the intestine was going to be used for this study and it has been suggested by Dolmatov (2021) that the regenerating parts of the organism during regeneration following evisceration synthesise a large number of factors such as

lectins which are important for humoral immunity. The method followed for the culturing of the intestinal cells obtained from the specimens was that from Odintsova *et al.,* (1994). However, after a few days in culture, the cells were being continuously infected by bacterial and fungal growth. Therefore, the method was modified as follows:

The regenerating part of the intestine was dissected and placed in sterile sea water in a flow hood, containing 1ml penstrep and 1 ml amphotericin for about 30 minutes. This was done so as to start the process of removing bacteria and fungi from the surface of the dissected animal which seemed to have a symbiotic relationship with these organisms. Sea cucumbers live symbiotically with a number of bacteria and fungi that are resistant to many antibiotics. In a particular study carried out by Jiang *et al*., (2014), the resistance mechanisms of 87 *Vibrio parahaemolyticus* isolates isolated from *Apostichopus japonicas* were investigated. The results showed that all isolates were resistant to ampicillin and cephazolin, with fewer of them being resistant to streptomycin and cefuroxime sodium. Therefore, antibiotics not in these categories were identified using the BNF and after some trials with various antibiotics, the antibiotics Ciprofloxacine (quinoline) and Doxycycline (tetracycline) were the ones that gave the best result, with no infection being observed when used in culture with the cells from the regrowing intestine described below. The dissected intestine was then placed in 5ml Ciprofloxacine of concentration (2 mg/ml of seawater) and 5ml Doxycycline of concentration (2 mg/ml of seawater) for 30 minutes and the process was repeated for 3 times, each time using a fresh sample of antibiotic.

When this was complete, the dissected part was placed in pure ethanol for 2 minutes to ensure eradication of these microorganisms as much as possible and then washed immediately in sterile sea water for a period of 10 minutes to remove the ethanol. Longer exposure to alcohol would have led to complete cell death.

The dissected part was then cut into 1 mm^3 sections to increase the surface area for enzymes to break down the material holding the cells together and centrifuged for 5 minutes at 1500 rpm at 15 ^{o}C so as to obtain the cell pellet. The cell pellet was re-suspended in 2 ml collagenase (2.5 mg/ml sea water) and placed in a petri dish for 2 hours in order to cause cells to detach from each other as much as possible. The cell suspension was then centrifuged for 10 minutes at 1500 rpm at 15 ^{o}C. The pellet was

re-suspended in sterile sea water and centrifuged for 10 minutes at 1500 rpm at 15 ^{o}C. This step was carried out 3 times so as to wash off any collagenase from the cells. The cell pellet was re-suspended in 600 µl gentamycin (40 mg/L sea water), 600µl Ciprofloxacine (2 mg/ml sea water) and 600 µl Doxycycline (2 mg/ml seawater) at a concentration of 2 mg/ml and left standing for 30 minutes. The cell suspension was once more centrifuged for 10 minutes at 1500rpm at 15 ^{o}C. Finally, the cell pellet was re-suspended in sterile sea water to remove the antibiotics, centrifuged for 10 minutes at 1500rpm at 15 ^{o}C , with this step being carried out three times in order to ensure complete washing. The pellet was then resuspended in 6ml of complete lebnovitz medium supplemented with 1 ml Ciprofloxacine (2 mg/ml), 1 ml Doxycycline (2 mg/ml), 1 ml amphotericin (1.25 mg/ml, and 1 ml sterile filtered coelomic fluid in a T75 flask and placed in the dark. Diluted, cell free coelomic fluid was added to the cells to ensure that the cells were still growing in some of the growth factors that they are usually found in, as the digestive tract is bathed in coelomic fluid. Dilution involved addition of enough water to the coelomic fluid to obtained the same % osmolality as the lebnovitz medium. This was achieved using a refractometer (Salinity Refractometer for Seawater and Marine Fishkeeping Aquarium 0-100 PPT with Automatic Temperature Compensation, Spain). To remove the coelomytes the fluid was centrifuged at 1000 g for 10 min at 4°C. The coelomocytes produce a pellet at the bottom of the conical bottomed tube.

Intestinal cells from most of the days post evisceration were not giving any viable growth. However, those intestinal cells obtained from organisms being at their 9th day of regeneration, were surviving for a few days. Despite the cells surviving for a few days, the cells were not dividing *in vitro* for long enough allow them to interact with the surrounding environment and produce biomolecules for testing. So, any chemical interactions that occur *in vivo*, were being lost *in vitro*. This path of growing intestinal cells had to be halted and other parts of the organism had to be chosen for the research project.

The coelomic fluid was hence chosen for the research, based on the fact that the intestine is bathed in the coelomic fluid and hence any interactions between the differentiating cells at the two ends of the re-growing intestine were facilitated by the coelomic fluid, probably containing secretions from the growing cells and hence it was very likely to give biological activity. In addition, as shown in the introduction,

the coelomic fluid plays a very important role in antioxidation. The coelomic fluid collected from the organisms sampled was used to check for cytotoxicity on NB4R2, KG1A, K562 and HL60 as well as lymphocytes described in section 2.4.5.

2.3.8 Specimen collection for trials

By January 2015, the best months for collection had been identified and during the following months, the method to be followed was outlined. Collection of specimens and tests related to possible differentiation on the coelomic fluid of the collected specimen were initiated in October 2015 and were collected over 4 specific times: 15th October 2015, 15th October 2016, 15th October 2017, 17th November 2019. In October 2019, the water was continuously being very murky, with low visibility, not allowing for collection. Table 6 gives a more detailed explanation about what was done with the coelomic fluid collected from the various sampling sets.

Table 6: Summary of the 4 different sampling sets.

15th October 2015	30 animals collected in order to have 3 specimens of coelomic fluid to be pooled for tests on day 0, 3, 6, 9 ,12, 15, 18, 21 and 24. 3 extra animals were collected in case any organisms got infected along the period of regeneration.
15th October 2016	30 animals collected in order to have 3 specimens of coelomic fluid to be pooled for tests on day 0, 3, 6, 9 ,12, 15, 18, 21 and 24. 3 extra animals were collected in case any organisms got infected along the period of regeneration.
15th October 2017	30 animals collected in order to have 3 specimens of coelomic fluid to be pooled for tests on day 0, 3, 6, 9 ,12, 15, 18, 21 and 24. 3 extra animals were collected in case any organisms got infected along the period of regeneration.
The coelomic fluid of 3 organisms was pooled for each sampling date and tested as a triplicate (technical triplicate). The same was done for each of the other sampling dates, giving a total of 3 biological triplicates where n=9 overall. Pooling was deemed an essential step to reduce variability.	
17th November 2019	12 animals collected in order to have 3 specimens of coelomic fluid for tests on the 9th day of regeneration. 2 extra animals were collected in case any organisms got infected along the period of regeneration.
These were then used for Flow cytometry and morphology.	

Once collected these organisms were allowed to grow for a week in aquaria in order to acclimatise to their new artificial environment. Every three days, half the water was replaced with fresh sea water obtained from the same area where the organisms were collected from. The aquaria were kept oxygenated as the water needs to be kept circulating all the time (Canicatti & Ancona, 1989, Luparello *et al*., 2019). The pH and salinity were measured every time sample of water were taken from the area. Any variations were noted as these would have affected the growth of the organisms. The Air conditioner of the room was maintained at 20°C, to avoid the atmospheric heat from affecting these organisms. During this time, the animals were fed Spirulina power and shrimp feed which was kindly donated by Mr. Angus Sharma from the Malta Aquaculture Research Centre, San Lucjan, Birzebbugia. When regenerating their intestine, the organisms were not fed as this would have negatively affected the cleanliness of the water for no reason, as the sea cucumbers would not be feeding until complete regeneration took place. However, it was imperative that prior to the experiment the animals were not starved so that their physiology would remain as close to natural as possible.

When the experiment was about to begin, the specimens were placed individually in plastic boxes with water covering only the bottom of the plastic. This increased the stress of the organisms and caused them to eviscerate (personal communication with Prof. P.J. Schembri, Department of Biology, UOM). Once evisceration occurred, the organisms were checked for visible signs of infection and any organisms possessing these infections had to be removed as the infection is easily transmitted to all the others present in the same tank as previously explained. The others were immediately returned to the aquaria as otherwise exposure to air for a prolonged time causes them to develop the white spot disease immediately. Every three days, 3 organisms were collected, placed in a container on ice and in the fridge in order to cause a 'stupor' with reduced sensation slowing down the metabolism for 2 hours. The specimens were taken to the lab. A small incision was done on their dorsal side around 1-2 cm in length. The coelomic fluid was collected using a syringe, placed in a tube, labelled, and then treated as explained in section 2.4.1 and 2.4.2. The organisms were then quickly taken to an aquarium, allowed to heal, and returned back to the sea if there was no sign of infection.

Throughout their time in the aquaria, the animal's general health was constantly kept in check and any signs of infection were immediately taken note of and the animal was immediately removed from the tank and transferred to another tank. Those animals that healed were then transferred to the sea again whilst those that didn't make it were disposed of. No infected organisms were ever transferred to the sea so as not to damage the wild community of *H. poli* in the area. An infection was preceded with a change in smell emanating from the water and a change in the consistence of the outer wall of the animals. If they caught an infection, the body of these organisms decomposed within a few days and the water was then discarded and the aquarium would be thoroughly washed with soap and water, followed by ethanol. It was then left to air dry completely and allowed to stand for a few days before being used again. If these precautionary measures where not taken, reoccurrence of infection within that aquarium would be more likely. Addition of antibiotics and antifungal was avoided unless absolutely necessary so as to reduce the impact these chemicals would have on the composition of the coelomic fluid. The health of these organisms was essential as any changes would cause an immune response and hence changes in the coelomic fluid would take place.

Even though organisms were collected during the same period, some variables included the sex of the organism, age, and general health which was not visible to the naked eye and could not be controlled. Organisms were chosen based on the size and where always checked for any visible features which might have indicated an infection, mitigating as much as possible these variables. In the coelomic fluid there are coelomocytes suspended which are related to immunity. The coelomocytes produce chemicals to function, and this is secreted into in the coelomic fluid. Hence organisms that are perfectly healthy would have produced different proteins as an immune response changing the composition of the coelomic fluid. It was difficult to choose the organism based on sex. These organisms expel their sex organs during evisceration, which are different in male and female. However, in October, the organs would not have always formed and so it wasn't a variable that could be taken note of. It was assumed that sex in these organisms should not offer a great variable in terms of coelomic fluid.

Collecting organisms from the same location was extremely important. It is a given fact that changes in the environment stresses would have an effect on the health

of organisms inhabiting that area. In *H poli* this is even more important as they are bottom feeders and hence any changes occurring in the external environment of the organisms would have a marked affect the composition of the coelomic fluid. The microclimate in which the organisms lived which could lead to different genera and species of microorganisms to grow in association with these organisms altering the secondary metabolites that are produced and exchanged through the coelomic fluid. Therefore, even though the same species was being collected, collecting organisms from various locations might have caused different results because of the different environmental factors.

Great care was taken to avoid misidentification as much as possible and comparing coelomic fluid from three organisms helped reduced the effect of having possibly a coelomic fluid different from that of *H. poli*.

2.4 Laboratory methods

2.4.1 Coelomic fluid (extract) extraction

Three methods to obtain coelomic fluid were tried prior to choosing the bleeding method as the main method. These methods were a) using a hypodermic syringe to withdraw the coelomic fluid, b) a small incision between 1-2cm (bleeding procedure), which would have allowed the organisms to heal itself and c) complete dissection. The method employing a syringe was not practical at all, as the moment the organisms felt the prick of the needle, they would harden their body to the extent that the needle would not be able to penetrate into the cavity and if it did, no fluid would come out of the cavity. Complete dissection would have resulted in the killing of many organisms, making it unethical. Therefore, it was decided that the bleeding method was the best option as it allowed for the coelomic fluid to be collected whilst at the same time the organisms could be returned to an aquarium where they would easily regenerate the cells needed to close the incision and hence be returned to their natural environment. This had to be done really quickly whilst using force to keep the incised part open for long enough for the coelomic fluid to be retrieved.

2.4.2 Preparation of extract.

The bleeding procedure involved making an incision measuring 1-2 cm on the anterior-dorsal side (Luparello *et al*., 2019) using a scalpel. Care was taken to prevent

injuries to the internal organs of the sea cucumbers. Since the contained coelomocytes might affect the chemical composition of the coelomic fluid (Canicattí, 1990) over time by giving false positives interfering with the results obtained, the fluid was centrifuged at 1000 g for 10 min at 4°C. The coelomocytes produce a pellet at the bottom of the conical bottomed tube. This was done, keeping in mind that some compounds might be lost at this stage, however it was an essential step which could not be eliminated. The coelomic fluid was then filtered using 33 mm Millex® filters with a pore size of 0.20µm to remove any microorganisms present in it. This was done to remove any microorganisms, sterilising the fluid, avoiding infections and false positives. The final step was to make the coelomic fluid of a concentration that could be used in trials when using Leukaemia cells. Dialysis or dilution where two possible options.

For dialysis: The coelomic fluid was placed into the dialysis tubing and this was subsequently placed is a solution of PBS for 3 hours. The tubing was then removed and placed in a fresh PBS solution for another 3 hours. This was repeated for 4 times in order to maximise the efficiency, however, this method resulted in the fluid being exposed to the normal ambient temperatures for long hours, prior to the experiment increasing the chance of small molecular weight compounds which tend to be volatile to diffuse away and for the extract to potentially start degrading, which is very common with marine extracts. In addition, since the identity of the active ingredient was unknown, the pore size was difficult to choose. The lowest pore size could have been chosen and dialysed against distilled water (instead of PBS) so as to have a bigger gradient for removal of salt, with the process being carried out in a cold room reducing losses of volatile compounds. However, in order to avoid, as much as possible, losses of molecules, dilution was chosen.

Diluting with distilled water was therefore determined to be a more efficient system and was employed throughout. The concentration of the fluid was measured using a refractometer (Salinity Refractometer for Seawater and Marine Fishkeeping Aquarium 0-100 PPT with Automatic Temperature Compensation, Spain) and compared to that of RPMI measured using the same reader. Enough autoclaved distilled water was then added to get the coelomic fluid of the same concentration of

RPMI making it suitable for use with Leukaemia cell suspensions without affecting their osmotic gradient. This resulted in the dilution of the coelomic fluid, a factor which had to be accounted for throughout the experiments. This allowed for all the components of the coelomic fluid to remain untouched. In addition, the effect of salt ions associated with sea water salinity were offset by the fact the minute amounts were added. Addition of more fluid to compensate for this dilution, meant that the less RPMI would be available for the cells to grow in during the incubation period of 120 hours following seeding and had to be accounted for during the interpretation of the results.

Dilution was carried out by first determining the salinity of the pooled coelomic fluid available (as this was not always the same). This was compared to RPMI as it is known that cells grow very well in that medium. Enough autoclaved distilled water was added to the coelomic fluid, each time rechecking the salinity, until the salinity was the same as that of the RMPI. The new volume was noted. The dilution factor was then calculated by the following formula:

Equation 2: Equation used to determine the dilution factor.

$$Dilution\ factor = \frac{Final\ volume\ of\ diluted\ solution}{Initial\ volume\ of\ undiluted\ solution}$$

The calculated factor was considered when calculating the amount added to give the % concentrations. The following table (table 7) illustrates a numerical example of how the dilution factor was calculated and how the % concentrations were calculated.

Table 7: Table showing preparation of various concentrations of coelomic fluid and the dilution factor calculations.

Working for the preparation of the extract				
Original Concentration of fluid (% osmolality)	**Volume of fluid (mL)**	**Concentration of RMPI (% osmolality)**	**New volume following dilution (mL)**	**Working: Dilution factor**
36	10	30	15	$\frac{15}{10} = 1.5$

Working out different % concentrations of coelomic fluid					
%	**2**	**4**	**6**	**8**	**10**
Volume of Coelomic fluid without dilution (μL)	4	8	12	16	20
Volume of RPMI without dilution (μL)	96	92	88	84	80
Volume of Coelomic fluid with dilution (μL)	6	12	18	24	30
Volume of RPMI with dilution (μL)	94	88	82	76	70

Pooled coelomic fluid from three different organisms was used in order to decrease the effect of variations in concentration of any chemicals due to different organisms being used. The animals for the technical triplicates were collected at the same time of year, however other variables which couldn't be controlled were present. Such variables included the sex, age, and health conditions of the animal. The coelomic fluid was centrifuged at 2000 rpm to sediment the cells and then stored at -80 °C (Canicatti *et al*., 1989).

2.4.3 Storage of extract: Freeze-drying.

10 ml of sample was placed in 50 ml conical bottomed tube and the samples were subjected to a vacuum by the use of a pump (lyophilisation). The samples were left there until all the liquid had evaporated leaving behind dry solid.

2.4.4 Nitroblue Tetrazolium (NBT) and Thiazolyl blue Tetrazolium Bromide (MTT assays for differentiation and cell viability.

NBT and MTT essays were used for two main functions. The NBT and MTT assay can be used to determine the potential differentiation taking place when using the extract on different Leukaemia cells, whilst the MTT assay on its own gives an indication of toxicity. Phagocytes perform oxidative respiratory burst in response to various stimuli using NADPH oxidase as a catalyst. The products of these reactions are the superoxide anion (O_2^-) together with hydrogen peroxide (H_2O_2), both of which serve as inflammatory mediators aiding in the killing of pathogens.

Many techniques are available for the detection of intracellular superoxide production that can be used for research and clinical diagnostic purposes, and these include the NBT assay, fluorometric assays, the peroxidase-dependent luminol-amplified chemiluminescence assay, or the oxidation of Fc OxyBurst. Although chemiluminescent assays allow for kinetic studies to be easily followed, they are relatively insensitive when compared to the NBT assay. The NBT assay is easily performed, however the results are only semi-quantitative and therefore not conclusive, and the result depends on the amount of superoxide produced, the cells releasing it, the stimulus given and the location where the superoxide is produced (Sim *et al.*, 2006). PMA is one such chemical which causes oxidative burst and the peroxide producing capacity of human phagocytes in response to this chemical are peripheral eosinophils > neutrophils > monocytes (Sim Choi, Woo Kim, Cha, & Kim, 2006).

NBT can be reduced to formazan and this reduction is then determined spectrophotometrically by determining absorbance values at 630 nm with 405 nm as a reference wavelength. This avoids technical disadvantages such as observer bias, difficulty in standardisation and difficult evaluation of results when using

conventional microscopic NBT (Sim *et al*., 2006). The reduction varies depending on degree of differentiation of each cell. To avoid a large number of cells with minimal differentiation giving an NBT spectrophotometric reading similar to fully, but lower in number, differentiated cells, normalization of the NBT absorbance per cell was required.

The number of viable cells was calculated by carrying out MTT cytotoxicity assay. Although non-direct cell counting was performed, MTT assay gave a spectrophotometric reading relative to number of viable cells. The absorbance is measured at 570 nm with 690 nm as the reference wavelength. Living cells carry out reactions catalysed by mitochondrial dehydrogenases affecting the mitochondrial metabolic rate, and the MTT assay is based on the fact that the tetrazolium salt is reduced to insoluble purple formazan crystals based on the mitochondrial metabolic rate. The total amount of formazan crystals produced are directly proportional to the number of viable cells in culture (Rai *et al*., 2018). Although the MTT assay is relatively easy to use, precise and can be applied to various cells lines, it is based on enzymatic reactions and therefore inhibitors might affect the readings, leading to false results (Ganot, Meker, Reytman, Tzubery, & Tshuva, 2013).

In order to confirm that the extract was not in any way inhibiting the MTT results, the following procedure was carried out: Two suspensions of cells and extracts were prepared and incubated for 3 days. MTT was added to both cell suspensions. However, MTT was added directly to the first suspension, whilst it was added only to the second suspension once the extract was washed off with PBS. The two results were obtained and since the results were not significantly different it was determined that MTT could be directly added to cell suspensions with extract.

Hence, NBT and MTT assays were used to determine the potential differentiation by working the ratio of NBT/MTT ratio. Toxicity was determined by measuring MTT expression. However, results obtained here would then need to be further confirmed, as results are not quantitative and give no indication of any molecular mechanisms taking place (Ganot *et al*., 2013).

2.4.4.1 MTT

MTT was obtained in powdered form and kept at 2°C. Prior to the experiment, the amount of MTT needed for that particular experiment was calculated and prepared accordingly. The MTT solution was prepared by dissolving the required amount of MTT in autoclaved PBS. The concentration employed was that of 5 mg/ml. The mixture was prepared under sterile conditions and was kept in a conical bottomed tube covered in foil in the fridge at a temperature of 8°C for use on both days of testing, i.e., 72 hours and 120 hours after seeding. The mixture was mixed well using the vortex until no clumps were visible prior to use.

Seeded plate of incubated Leukaemia cells was shaken at 750 rpm for 5 minutes. 20 µL MTT:PBS solution was added to each well and incubated for 4 hours and 37°C. After 4 hours, the MTT plate was centrifuged at 2000 rpm for 10 minutes and the medium was then removed completely by turning the plate on a tissue. 120 µL of DMSO (99.0% minimum purity) were added to each well and the plate was shaken at 750 rpm for 5 minutes to ensure homogeneity and absorbance was read at 562 nm with 570 nm as the reference wavelength using a UV/Vis microplate reader.

2.4.4.2 NBT

NBT was obtained in powdered form and kept at 2°C. Prior to the experiment, the amount of NBT needed for that particular experiment was calculated and prepared accordingly. The concentration of NBT used was of 2 mg/ml. The mixture was prepared under sterile conditions and was kept in a conical bottomed tube covered in foil in the fridge at a temperature of 8°C for use on both days of testing, i.e., 72 hours and 120 hours after seeding. The mixture was mixed well using the cortex until no clumps were visible prior to use.

Seeded plate of incubated Leukaemia cells was centrifuged at 2000 rpm for 10 minutes and the supernatant was discarded. Whilst centrifugation was taking place, 0.01% of PMA (concentration) was added to NBT in order to activate it. 50µL activated NBT was placed in each well and incubated at 37°C. After 20mins, 70 µL 1M HCl was added to stop the reaction. The plate was centrifuged at 2000 rpm for 10 minutes and the supernatant was removed. 50µL 2M KOH and 150 µL DMSO (99.0% minimum purity) were subsequently added. Readings were taken on a

spectrophotometer with a wavelength of 630 nm and a reference wavelength of 405 nm in order to obtain a relative reading of NBT crystals within each well.

2.4.4.3 Calculating $^{NBT}/_{MTT}$

All values for NBT and MTT and hence the NBT/MTT ratio were normalised in relation to the negative control for differentiation (that is for cells grown in RPMI-1640 and IMDM) for the reading obtained at 72 hours after seeding. Using the reading obtained above, an NBT/MTT ratio could be worked out as follows:

Equation 3: Equation showing NBT/MTT ratio.

$$\text{NBT/MTT ratio}=\frac{Value\ of\ NBT}{Value\ of\ MTT}$$

A low value for this NBT/MTT ratio implies that the cells are proliferation whilst a high value for this NBT/MTT ratio implies that cell differentiation might be taking (refer to figure 12) .

MTT values equal to or lower than 15% of the MTT value for cells in RPMI-1640 or IMDM were considered as showing toxicity. Results for NBT/MTT ratios showing 1.5 times to 2 times the value obtained for the negative control for differentiation was taken as the cut off point for assessment for differentiation and hence taken as a positive result.

2.4.5 Preliminary testing to determine the range of % by volume of extract which possibly causes differentiation.

To determine the range of fluid in ratio to the whole cell suspension that might possibly cause differentiation coelomic fluid from organisms in their day 9 of regeneration was chosen for this particular test based on the results obtained in section 2.3.7 whereby it was seen that the cells from the intestine in day 9 had shown a greater aptitude towards growing *in vitro* and section 2.4.5 when cytotoxicity was investigate.

Different % of coelomic fluid were used, these being 5%, 10%, 15%, 20% and 25% by volume of the original extract. A % of coelomic fluid higher than 25% could not be included as when accounting for the dilution factor, the extract would contain a

small amount of RPMI, whereby the cells would not have a lot quantity of nutrients available for growth. The different % solutions were prepared by dissolving the appropriate amount of coelomic fluid in RPMI as explained previously in table 7.

50 μL of each of these solutions containing different % of extract, together with 100% RPMI growth medium acting as a negative control for differentiation and 1.6% DMSO acting as the positive control for differentiation, were then plated in triplicates in two - 60 well plates. 50 μL of leukaemia cell suspension at a concentration of $1x10^5$ cells per mL HL-60 mixture was added to each well and the two plates were placed in the incubator at 37°C and 5% carbon dioxide concentration.

After 72 hour and 120 hours from seeing, the MTT test was carried out as explained in section 1.4.3 and the results were tabulated. A graph of NBT/MTT ratio vs % extract was plotted. This was then used to determine at which values the coelomic fluid is more likely to give differentiation.

2.4.6 Checking for toxicity of the coelomic fluid.

The extract being looked into needs to possess a potential for differentiation. Cytotoxicity, though an efficient way of killing leukaemia cells could also results in the destruction of healthy human cells, a characteristic which would reduce the efficiency of the extract is use in the medical field.

a. The cytotoxicity effect of the fluid on the various cell lines namely HL60, K562, NB4R2 and KG1A based on the ranges found in a. above.
b. The cytotoxicity effect of the extract on human lymphocytes expressed as %'s determined in a.

2.4.6.1 Preliminary testing of extracts on leukaemia cell lines to determine presence or absence of cytotoxic component.

Taking into consideration that previous results showed that it took about 24 days for complete regeneration, coelomic fluid from days 3, 6, 9, 12, 15, 18, 21 and Complete regeneration (CR) which is equivalent to day 24 following evisceration were used for testing. Coelomic fluid from days 3, 6, 9, 12, 15, 21 and (CR) from three different organisms were pooled together and centrifuged as described before.

MTT test described above, were carried out 72 hours and 120 hours after seeding were carried out for the various tests. The following table summarises the variables tested:

Table 8: Table showing cell line, % coelomic fluid used per extract.

Cell line	% coelomic fluid	Extracts tested
HL60	2%, 4%, 6%, 8% and 10%	Extract from 3, 6, 9, 12, 15, 18, 21 and CR following evisceration. Positive and negative control for toxicity.
K562	2%, 4%, 6%, 8% and 10%	Extract from 3, 6, 9, 12, 15, 18, 21 and CR following evisceration. Positive and negative control for toxicity.
KG1a	2%, 4%, 6%, 8% and 10%	Extract from 3, 6, 9, 12, 15, 18, 21 and CR following evisceration. Positive and negative control for toxicity.
NB4R2	2%, 4%, 6%, 8% and 10%	Extract from 3, 6, 9, 12, 15, 18, 21 and CR following evisceration. Positive and negative control for toxicity.

Graphs of MTT vs % extract for each cell line for all the different regeneration days were plotted. These were then analysed for toxicity, proliferation, or any signs of possible differentiation.

2.4.6.2 Testing of the extract (coelomic fluid) to check for Cytotoxicity on human lymphocytes.

Cytotoxicity on human lymphocytes was also checked to ensure that the extract at the % chosen was not toxic on human lymphocytes, the latter being an example of healthy proliferating normal cells. If the coelomic fluid proved to be toxic on human

lymphocytes, any positive results obtained in this study would have been of limited use.

Pooled coelomic fluid extracted from 3 organisms in their day 9 of regeneration was used. The coelomic fluid was prepared as described in section 2.4.2. Buffy coats were collected from the National Blood Transfusion Services in Malta and the following procedure was carried out within 12 hours of collection of blood to avoid the white cells from clumping.

10 ml of the buffy coat was transferred in a 50 ml graduated centrifuge tube, under sterile conditions. The blood was then diluted using sterile PBS in order to reduce the density of the blood allowing for a better separation of the red blood cells from the white blood cells and further inhibiting clotting. The ratio of blood:PBS was 1:3. Histopaque was allowed to get to room temperature. 10 ml of histopaque was placed in a graduated centrifuge tube and the diluted blood was added to the histopaque dropwise to avoid clumping. The samples formed were then centrifuged for 2500 rpm for 30 minutes with the breaks of the centrifuge switched off to ensure that the layers formed were not disturbed. Without shaking, the plasma was removed, and the white hazy was collected, i.e., the white cells and placed in a 50 ml graduated centrifuge tube. As much as possible no red blood cells were aspirated when collecting the white blood cells. ELB:PBS solution in the ratio of 3:1 was added to the sample containing the white blood cells and mixed well. This was incubated for 10 minutes, shaking the cells at intervals. The cells were then centrifuge at 1500 rpm for 5 mins. This step was repeated until a completely white pellet was collected. The supernatant was removed by decantation, and 10 ml complete RMPI-1640 was added to the pellet obtained. The sample was transferred to a T75 flask and was left overnight, incubated at 37°C and 5% CO_2, for the monocytes to adhere to the bottom of the plastic T75 flask.

The ELB was used to lyse erythrocytes and was prepared at least 24 hours before as it required autoclaving. 0.04 g EDTA, 7.49g ammonium chloride, 0.745 g potassium chloride ad 0.79 g ammonium carbonate were added to 600 ml deionised water. The solution was stirred using a magnetic stirrer until all the different solids dissolved and was then adjusted to a volume of 1 L using deionised water. The solution was autoclaved and allowed to get back to room temperature before use.

The overnight incubation allowed the monocytes to adhere to the plastic. Following the overnight incubation, the floating lymphocytes were collected in a new T75 flask and 2 % of the final volume of Phytohemagglutinin was added, in order to stimulate proliferation. The lymphocytes were then incubated at 37°C and 5% CO_2 for 2 days.

In order to test whether the extract to be investigated in this research project was toxic to lymphocytes, the MTT assay was used. 50 µl of the extract at 10% was then plated in triplicate in two, 60 well plates. 50 µL of lymphocyte suspension at $1x10^5$ cells per ml was added to each well and the two plates were placed in the incubator at 37°C and 5% carbon dioxide concentration. After 24 hours, 48 hours and 72 hours from seeding, the MTT test was carried out and the results were tabulated. A graph of NBT/MTT ratio vs concentration was plotted and analysed.

2.4.6.3 Concluding remarks

Even though through these various tests it could be concluded that the fluid was not toxic to lymphocytes not completely toxic to any cell lines, HL60 and K562 were chosen as the two cell lines on which all experiments would be based. Both these cell lines were more easily obtainable and easily grown *in vitro* by the author. HL60 has been shown to undergo complete myeloid differentiation, differentiating into granulocyte-like cell or monocyte-like cells *in situ* depending on the chemicals employed and is therefore a good cell line to work with in this project (Birnie, 1988). Birnie (1988) states that the HL60 genome contains an amplified c-myc proto-oncogene whereby the mRNA levels from this gene are correspondingly high in undifferentiated cells but reduce quickly when they start to differentiate. K562 are known to differentiation into foetal and embryonic erythrocytes, giving an alternative route to differentiation that can be possibly caused by the extract used (Baliga *et al.*, 1993). Upon visual inspection of the cytotoxicity tests NB4R2 and KG1a didn't show as reliable results as those of HL6O and K562, as will be shown is the results chapter.

Once it was determined that the extract was not cytotoxic to human lymphocytes or to the cell lines chosen, and the dilution factor was enough to sustain growth, tests were carried out in order to determine, the limits of concentration of coelomic fluid expressed as a % by volume, which would potentially have the ability to cause differentiation.

2.4.7 Investigating the effect of the extract on HL60 and K562 cell line for any possible effects of differentiation.

Counting from day 0 as being the first day at which the animals eviscerated their intestine through day 3, 6, 9, 12, 15, 18, 21 and CR, the latter being defined as complete regeneration, various concentrations up to 10% of total medium volume of coelomic fluid were tested on HL60 and K562 cell lines using normalized NBT/MTT ratio relative to the value of untreated cells 72 hours and 12 hours after the beginning of the test. For each condition there were 3 separate biological experiments, each having 3 technical replicates, therefore n = 9. The NBT/MTT assays were conducted 72 hours and 120 hours after treatment as these correspond to the time points for control-induced monocytic and granulocytic differentiation, respectively. This was used to assess the ratio between the oxidative burst given off by differentiating cells and the proliferation of such cell. This index could be then used as an indication of possible differentiation taking place.

2.4.7.1 Testing the extract using the index of differentiation.

The coelomic fluid for day 0 (day of evisceration), 3, 6, 9, 12, 15, 18, 21 and organisms that had completely regenerated their intestines was centrifuged for 10 minutes at 4000 rpm in order to remove the coelomocytes. The supernatant was then sterile filtered using 33 mm Millex® filters with a pore size of 20µm to remove any microorganisms present in it. It was then mixed with RPMI to obtain concentrations of 2%, 4%, 6% and 8%, 10% by volume for each day after evisceration and tested on the various cell lines.

For each cell line: 50 µL of each of these solutions, together with 100% RPMI growth medium acting as a negative control for toxicity and 10% DMSO acting as the positive control for toxicity, were then plated in triplicates in two, 60 well plates. 50 µL of leukaemia cell suspension at a concentration of 1×10^5 cells per mL was added to each well and the two plates were placed in the incubator at 37°C and 5% carbon dioxide concentration. After 72 hour and 120 hours from seeding, the MTT and NBT tests were carried out and the results were tabulated. A graph of NBT/MTT ratio

vs % extract was plotted. Results were compared to the NBT/MTT ratio when leukaemia was treated with DMSO.

2.4.8 Determining the nature of the active ingredient

2.4.8.1 Testing two fractions of the extract.

8ml of sterile pooled coelomic fluid obtained from organisms at days 9 following regeneration was prepared as explained in section 2.4.2. 1 mL was put aside in order to test for protein concentration. The coelomic fluid was the spun using Spin-X® UF6 concentrator using centrifuge 5810-R Eppendorf for 10 minutes at 4000 rpm. This resulted into two fractions, with the unfiltered one containing components with a size greater than 5 KDa and the filtered one containing component having a size of less than 5 KDa.

1mL from each of the two layers obtained was used to determine the protein concentration using the Bradford assay as follows: 20 µL of reagent S was added to each mL of reagent A (alkaline copper tartrate solution) and this was labelled reagent A'. 7 dilutions of BSA (Bovine Serum Albumin) protein standard were prepared containing 0.125, 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mg/mL. 5µL of standard and samples were pipetted into a clean dry microtiter plate. 25µL of reagent A' was added to each well. 200 µL if reagent B (Folin Reagent) was added to each well and the plate was gently agitated for 5 seconds to mix the reagents. Any bubbles that formed were popped using a dry clean pipette tip. Cross-contamination was avoided, and absorbance was read at 750 nm. A standard curve was prepared, and the protein concentration of the samples was determined.

The following table (table 9) illustrates the absorbance of each of the BSA concentrations prepared in order to allow the plotting of a graph which acts as reference graph for the data obtained from the samples.

Table 9: Table showing different replicates for absorbance at specific protein concentrations.

Protein Concentration mg/mL	**Abs 1 (750nm)**	**Abs 2 (750nm)**	**Abs 3 (750nm)**	**Average (750nm)**
1.5	0.18836	0.31312	0.31718	0.31515
1.25	0.22157	0.32528	0.2849	0.27725
1	0.24688	0.23197	0.23141	0.236753
0.75	0.23099	0.22689	0.22685	0.228243
0.5	0.17747	0.21463	0.20836	0.200153
0.25	0.1886	0.18293	0.17976	0.183763
0.125	0.16834	0.16347	0.15552	0.162443

These values where then plotted on a graph for better visualisation.

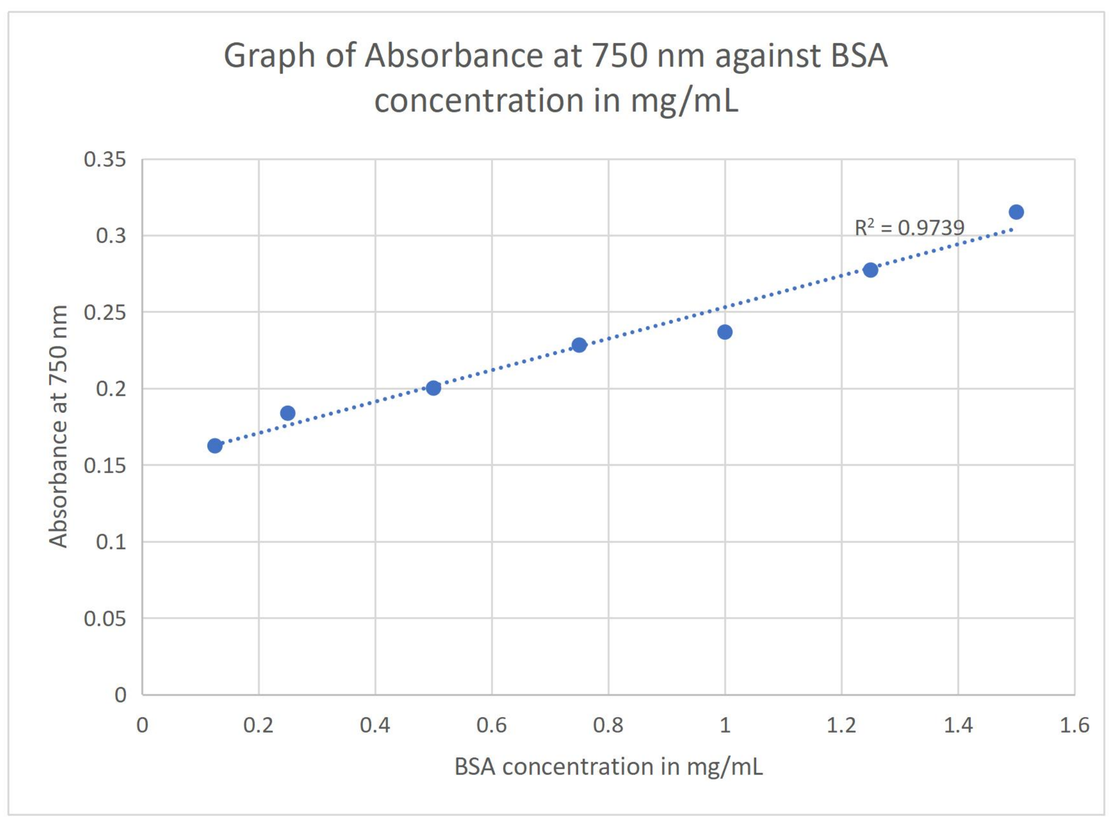


Figure 22: Graph of Absorbance at 750 nm vs BSA concentration in mg/mL used as a reference.

The remainder of the coelomic fluid was then mixed with RPMI to obtain concentrations of 2%, 4%, 6% and 8%, 10% by volume for each day after evisceration and tested on HL-60, and K562. NBT/MTT tests were carried out to determine which solution gave an indication of a positive result.

For each cell line and for coelomic fluid from each day following regeneration: 50 µL of each extract, together with untreated cells acting as a negative control for differentiation and 1.6% DMSO acting as the positive control for differentiation, were then plated in triplicates in two - 60 well plates. 50 µL of leukaemia cell suspension at a concentration of $1x10^5$ cells per mL was added to each well and the two plates were placed in the incubator at 37°C and 5% carbon dioxide concentration. After 72 hour and 120 hours from seeding, MTT and NBT tests were carried out and the results were tabulated. A graph of NBT/MTT ratio vs % extract was plotted. Results were compared to the NBT/MTT ratio when leukaemia was treated with DMSO.

2.4.8.2 Extraction in a solvent.

In the next step, the <5KDa fraction of the extract from organisms from day 9 of regeneration was treated with two specific solvents, ether (ethoxyethane) and ester (ethyl ethanoate). For this section, the coelomic fluid used had been stored and so was previously freeze dried as described in section 2.4.3.

Freeze dried coelomic fluid that had been previously extracted from organisms from day 9 and day 12 following regeneration, was resuspended into 10 ml of distilled water and to this mixture, 10 ml of ethoxyethane, an ether, was added. The mixture was shaken for 30 minutes and was then allowed to stand for an hour until separation occurred. The ether layer was collected, and the ether was then evaporated off using a vacuum pump. The solute left behind when all the ether was removed was then resuspended in 10 ml RPMI and tested as follows: The fraction of the coelomic fluid of each respective day was made into the following concentrations: 2%, 4%, 6% and 8% and 10% by volume for each extract, and tested on HL-60, and K562 cell lines. The ether layer did not require any sterile filtration as ether does not support growth of any living organisms. The residual water fraction left behind was not tested as all the salt was left in this fraction. The purpose of this extraction was to remove if not all, most of the salt from the coelomic fluid and therefore have a more definite result using the extracted biomolecules from the sample. This procedure was then repeated for another solvent, ethyl ethanoate, which is an ester. Two solvents used have different polarities, with the ether being less polar than the ester. This would have allowed most of the biomolecules found in the <5KDa fraction to be separated into two parts, further increasing the chance of determining the nature of the biomolecule have an effect of initiating differentiation.

For each cell line and each extract being tested: 50 µL of each of these solutions, together with 100% RPMI growth medium acting as a negative control for differentiation and 1.6% DMSO acting as the positive control for differentiation, were then plated in triplicates in two, 60 well plates. 50 µL of leukaemia cell suspension at a concentration of $1x10^5$ cells per mL was added to each well and the two plates were placed in the incubator at 37°C and 5% carbon dioxide concentration. After 72 hour and 120 hours from seeding, the MTT and NBT tests were carried out and the results

were tabulated. A graph of NBT/MTT ratio vs % extract was plotted. Results were compared to the NBT/MTT ratio when leukaemia was treated with DMSO.

2.5 Statistical analysis

There were many variables in the data obtained, namely the cell line that was more susceptible to showing signs of differentiation, the day from regeneration where the coelomic fluid has to be taken to have the maximum possible effect on differentiation, the range in concentration expressed as a % by volume and the nature of the molecule exerting a possible effect. In each case the independent variable (i.e., under the control of the researcher) was percentage of coelomic fluid whereas the dependent variables (i.e., observed variable) were the effects occurring in the different days for NBT and MTT. Statistical analysis were carried out using R statistical package Version 4.0.0.

A histogram was plotted, and the pattern of distribution was noted. If the histogram showed skewness or exponential distribution, a log transformation, which is a special case of the Box-Cox transformation were lambda = 0, will be applied in an attempt to make the date more closely resemble a normal distribution (Feng *et al.*, 2014). Log transformation will also make it easier to visualise the various data sets.

The first statistical analysis involved testing for normality. The Shapiro Wilk Test was used in order to determine if the variables under study were normally distributed or otherwise. This test was chosen based on the fact that due to standardisation there were ties, readings with the same values in the data and it was more robust, and therefore appropriate with smaller sample sizes (namely those with less than 50 readings). The Shapiro Wilks test works by quantifying as a percentage the similarity between observed distribution and normal distribution curves. The null hypothesis is that a variable is normally distributed and is rejected if $p < 0.05$.

If the data was found to be normally distributed, Anova or Ancova tests would have been used to determine if there were any significant differences in the data. If testing for the effect of a categorical independent variable on a continuous dependent variable, then Anova would be employed, whilst if testing for the effect of a

categorical independent variable on a continuous dependent variable whilst at the same time controlling for the effects of one or more continuous covariates, Ancova would be used. The assumption underlying these two statistical tools is homogeneity of variance which would be tested using Levene's test. In the event that statistical significance was determined, a post hoc test would be carried out to determine between which pairs of data such significant difference was noted. Tukey's HSD test for pairwise comparison was conducted.

If the data is found to not be normal, the Kruskal Wallis test will be used as it is a nonparametric test. It assumes that the dependent variable is measured at the ordinal or continuous level, consists of two or more categorical and independent groups and the distribution in each group is similar. This test determines whether the medians of two or more groups are different. The null hypothesis is that the population medians are equal, and the null hypothesis is rejected if $p < 0.05$. The Dunn's post hoc test was then conducted to find which group pairings are significantly different in the case that the Kruskal Wallis test results in a difference across groups. The null hypothesis is that there is no difference between groups and the null hypothesis is rejected if $p < 0.05$. This procedure is outline is figure 23:

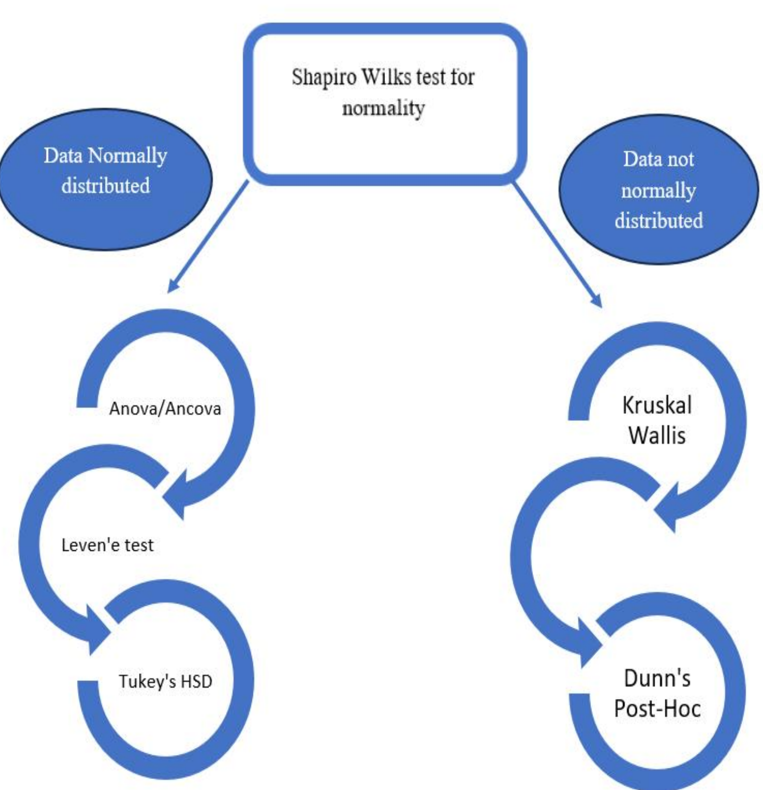


Figure 23: Parametric and non-parametric tests designed to be used for statistical analysis.

The statistical analysis carried out was the following:

1. Statistical analysis on all the data collected (cell lines, time during which leukaemia cell were exposed to coelomic fluid, % by volume of extract, day from which coelomic fluid was extracted). This analysis helped to highlight any statistically significant differences between the data collected. This would help determine which days from evisceration gave an extract which is statistically significantly different from the other days post evisceration.

2. Statistical analysis to determine the range of % by volume of coelomic fluid needed to bring about possible differentiation for HL60 and K562 cell lines.

3. Statistical analysis to compare the two fractions < 5 KDa and >5 KDa and hence determine whether they behave similarly or differently from each other and the complete fluid.

4. Statistical analysis to compare the extract obtained in an ether solvent and in an ester solvent.

Graphs were plotted for visualisation of trends in all the scenarios described above.

The following figure, figure 24, outlines the rationale behind the sequence of methods outlined above.

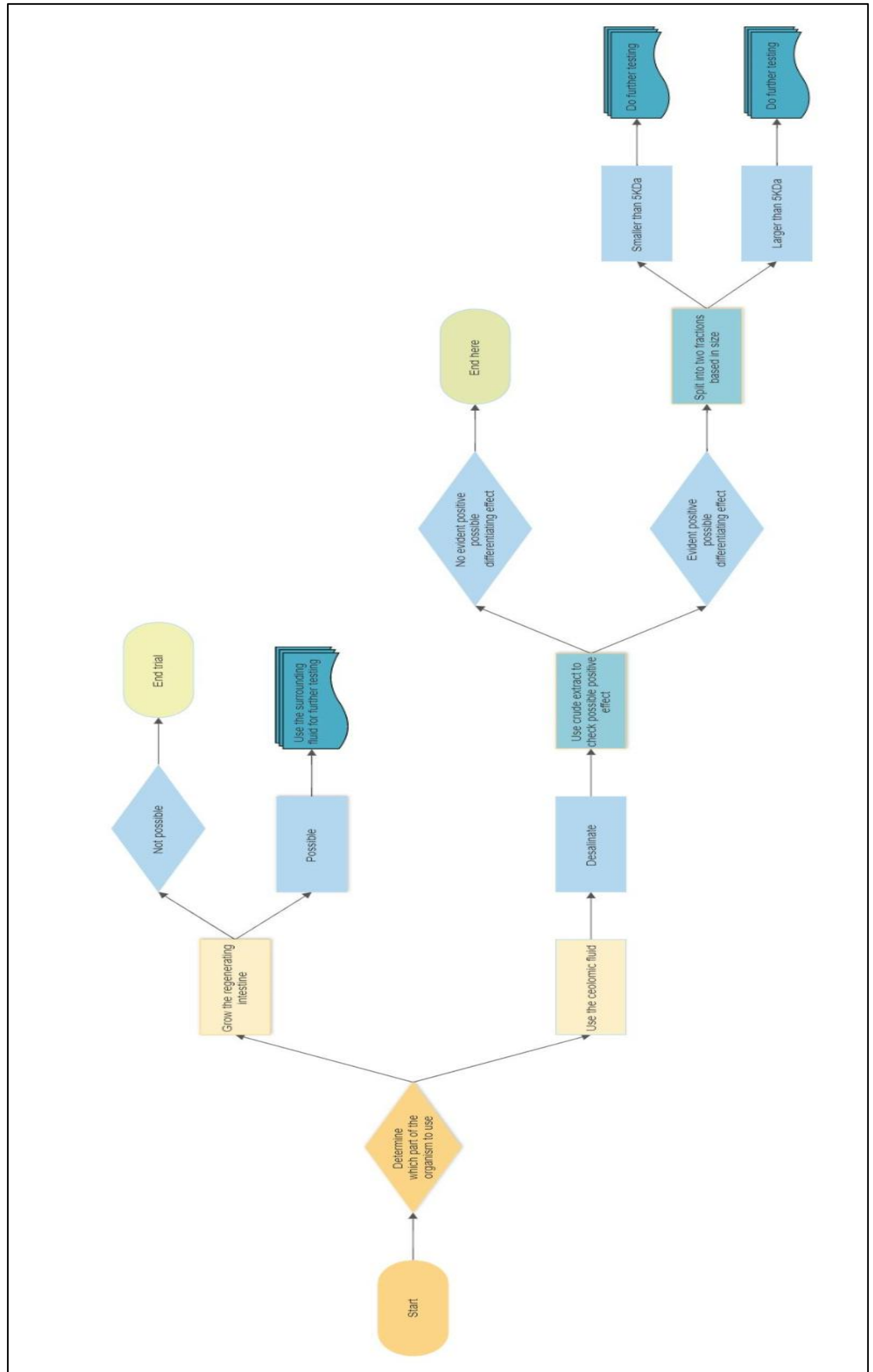


Figure 24: Figure showing steps taken to arrive separating the extract into two portions depending on the size of the molecules. Photo by author

2.6 Further tests

2.6.1 Cell morphology

The first confirmatory test used to test for differentiation following the tests described above, was to check cell morphology.

0.15 g of Eosin-Methylene Blue also known as Leishman stain (Sigma-Aldrich®) were weighed and dissolved in 100 ml of methanol (Sigma-Aldrich®). The solution formed was mixed thoroughly using a magnetic stirrer and was then stored in brown glass bottles. It was incubated for 24 hours prior to use in an incubator at 37°C. Prior to being used, the solution was filtered using Whatman no.3 filter paper to remove undissolved particles which would have given errors in the results. Healthy Leukaemia cell cultures were selected, pooled, and counted using a haemocytometer. By centrifugation and re-suspension in RPMI, a cell concentration of $1x10^5$ cells per well was prepared and poured into 96-well plates and were subjected to different extract test conditions.

The following was carried out 72 and 120 hours after the start of the experiment. Cytospin were performed to spin the contents of different wells onto different slides. The slides and filter paper were labelled using pencil, and the cytospin racks (figure 25) were washed well with PBS and drained. The slide and filter paper were connected (labelled side to the right-hand side) and the loaded wells were placed in the cytospin. 200 µL of the corresponding cell suspension from each well was pipetted into each cytospin well. The cells were spun for 5 minutes at 1000 RPM using Cytospin 2 (Shandon, UK).

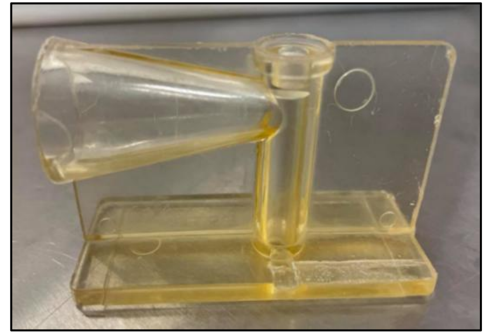


Figure 25 The well-used for the cytospin and the metal rack needed to keep the wells. (Photo: by author)

The slides were dismantled from the cytospin racks (together with the filter paper) and were left to air dry for 20 minutes. Once dry, 1ml Leishman stain was pipetted over each slide and kept for 5 minutes to fix. 1ml Leishman buffer was added and mixed gently by blowing (figure 26). They were left to stain for 20 minutes and it was ensured that a shimmering gold sheen on the surface was formed. The slides were rinsed with water and then left to dry. The slides were studied visually for differentiation, using an upright compound microscope (Olympus, BX60).

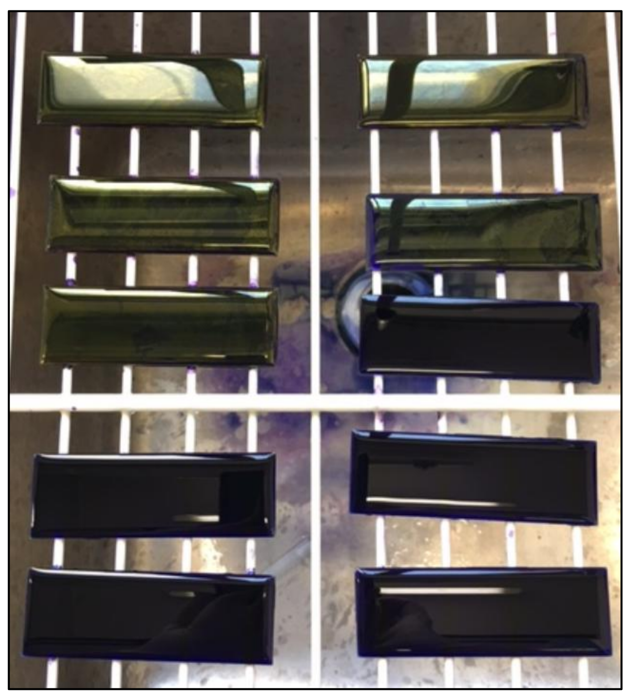


Figure 26: Leishman stain. Photo taken by author.

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2.6.2 Flow cytometry – test with antibodies:

Flow cytometry was carried out on the coelomic fluid obtained from three organisms found in their 9th day of regeneration. Their coelomic fluid was pooled and two concentrations were used, these being 4% and 8% coelomic fluid. The controls were untreated cells growing in RPMI acting as a negative control for differentiation and 1.6% DMSO acting as the positive control for differentiation. The tests were carried out on the HL60 cell line.

Cells were counted using trypan blue and a haemocytometer and 500 µL of Leukaemia cells were seeded at 1×10^5 cells ml^{-1} per well. To each well, 500 µL of extract were added together with 500 µL of RPMI and 500 µL of 1.6% DMSO of purity 99.9% that acted as the negative and positive control for differentiation, respectively. The surrounding wells were filled with sterile distilled water and incubated in a humidified box at 37 °C and 5% CO_2.

On the day of seeding, and 72 and 120 hours after seeding extracellular staining was performed. The contents of each well from the plate were emptied into a separate 5ml FACS tube, centrifuged at 2000 rpm and the media decanted. The cell pellet in each tube was washed with PBS so as to remove residual media. After removal of the PBS solution, 500 µL blocking agent (90% PBS, 10% FBS) was added to each tube to reduce non-specific binding and the cell suspension was incubated at room temperature for 10 minutes. After 10 minutes elapsed, the cell suspension was vortexed and split into two samples, where a tube containing 250 µL would be treated with antibodies, whilst the other tube containing the other 250 µL cell suspension would be treated with isotype. The blocking agent was then removed and the cell pellet in each tube was washed with PBS for three times.

2 master mixes were prepared in the dark, one containing antibodies and one their isotypes. 100 µL of each master mix was placed into a separate tube and incubated in the dark, on ice, on a rotating platform for 30 minutes. After the time had elapsed, the tube was centrifuged at 300 rpm and the antibodies/isotype solution was decanted. The cell pellet was washed with PBS three times and finally resuspended in 500 µL staining buffer. Flow cytometric analysis was conducted using a FACS Calibur flow cytometer (BD FACS Calibur™).

Side scatter plot and forward scatter plots were obtained, and these results where then analysed. Forward scatter plots give an estimation of the size of the cells whilst side scatter plots give an estimation of the granularity of the cells. An example of such plots can be seen in figure 27. CD34, CD11b and CD14 were the antibodies used.

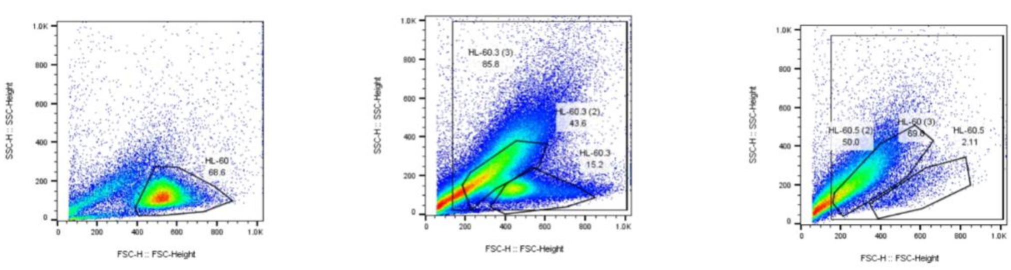


Figure 27: Forward and Side Scatter plot for at the start of the trial used for flow cytometry. Black structures indicate gates drawn.

The data obtained in these plots is then gated for better analysis. Through gating, populations of cells with common characteristics are analysed and can then be further analysed through histograms that show percentage change from controls.

2.7 Conclusions to the methods section

At this point in the investigation, it was not deemed important to look into the chemical nature of the compound which might be possibly giving a good effect in terms of differentiation. This is because the separation and purification is so complex, as shown in the literature review, that this study merits an investigation of its own. In addition, the interaction of biomolecules is also very complex and would need more analysis. The origin of the biomolecules is difficult to establish at this point.

3 Results

3.1 Essential groundwork

The first part of this research project required a lot of snorkelling in various areas around the Maltese Islands in order to be able to identify the best location from where to obtain the specimens in terms of number of animals available and ease of collecting them and the required sand and water. In addition, the determination of the right time of the year when harvesting of the specimens was going to be done was also essential. This was very time consuming and laborious. Prior to collection of organisms, preparation of the aquaria had to take place and this in itself was also very laborious.

Growth in artificial conditions, i.e., in aquaria was also a very laborious process and took quite some time to optimise the growth. One of the main issues that was occurring was that the sea cucumbers were collecting as a group in corners of the sea aquaria and special attention had to be taken to make sure that none of them died as that would have caused the process to be restarted all over again. This was the case in October 2015 where sampling had to take place twice. Once all the organisms had acclimatised and eviscerated and everything was ready so that the experiment could begin, in one of the aquaria, the sea cucumbers occluded the pump causing them to die and disintegrate, affecting the water quality of the aquarium and the well-being of the others. This meant that the whole process had to be restarted all over again, including the deep cleaning of the aquaria and the re collection of new organisms. Following this incident, more precautions were taken, and the aquaria were checked very often during the day and night to make sure that this didn't happen.

Every October / November, the weather was analysed for the whole period and winds were noted. During 2019, collection had to be postponed by a whole month as the weather was so windy that it made it impossible to collect the sea cucumbers as visibility was very bad, so the sandy bottom was not stable, and it was not safe to be done.

3.2 Growth and regeneration of the *H. poli* in an artificial environment.

The first step in this research project was to successfully grow the *H. poli* in an artificial environment. This was achieved using suitably sized aquaria, containing natural sea water obtained from the same site from which the organisms were obtained. This water was kept at a salinity of 38 parts per thousand, pH of 8.3 ± 0.2 and constantly well aerated. In a study carried out by Luparello *et al*., (2019), constant aeration was a requirement for the proper rearing of *Holothuria*.

The organisms occupied all the parts of the tank and moved around freely. Growth inside the aquarium under the described conditions, did not inhibit their normal growth pattern and in fact, complete regeneration of their intestine following evisceration occurred. A number of dissections were carried out on the regenerating organisms in order to determine three things. First it was essential to determine whether complete regeneration occurred, to determine whether the animals would survive in artificial conditions until regeneration did occur and to determine the amount of time it would take for the organisms to regenerate all their intestines back. The period it takes for regeneration to be completed is usually of about a month (Mashanov & Garcia-Arraras, 2011), however it had to be confirmed as artificial conditions could affect the organisms and potentially their regeneration time. The organisms did survive successfully, and it was determined that in order for *H poli* to completely regenerate their intestine under the artificial conditions described, it took approximately 24 days. Figures 28 shows the whole eviscerated intestine.

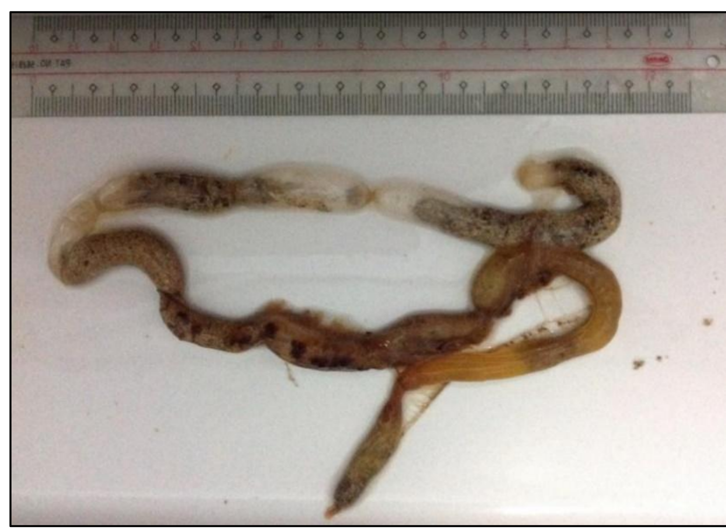


Figure 28: Figure showing eviscerated gut of H.poli.

Figure 29 shows a dissection of a regenerating organism at day 9 from evisceration. Following evisceration, the gut of the *H. poli* is left with the mesentery attached to the body wall and both of the terminal fragments of the digestive tube are still present (Mashanov & Garcia-Arraras, 2011). When culturing the cells from the tips of these growing parts, the growing part always had a darker colour than the fully developed parts of the gut.

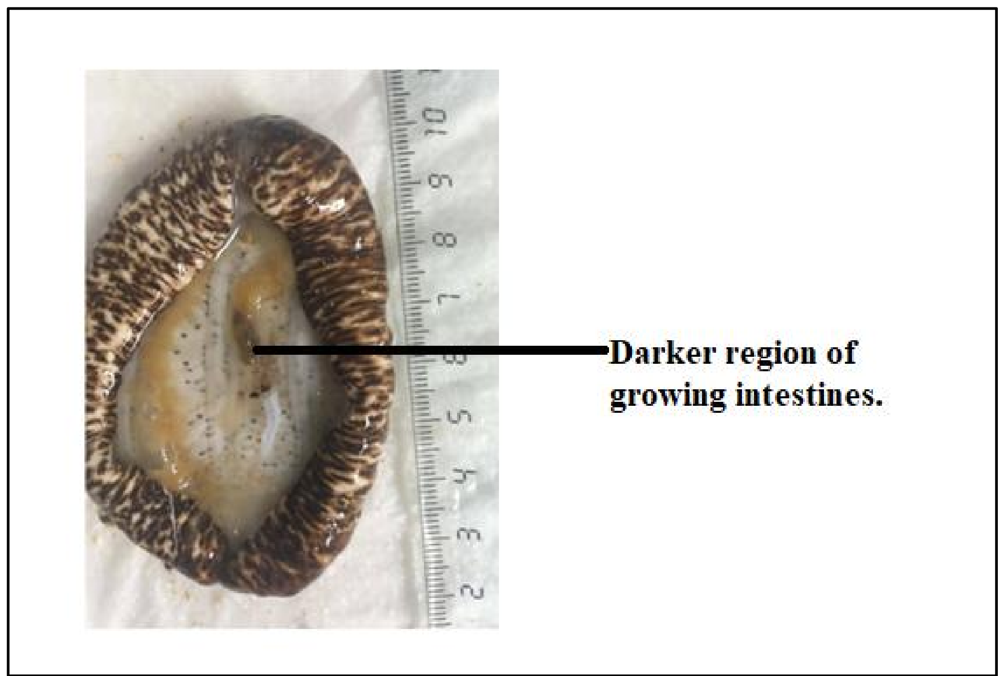


Figure 29: Dissected organism showing regenerating internal organs. Mesentery can be seen attached to the body wall. Photo: taken by author

It was also established that organisms harvested in October /November were the most likely to survive in the aquaria and during this period there was the least loss in organisms and no or almost no infection. When organisms were harvested in summer, as this would have given the best conditions for collection of the organisms, the sand and the sea water, the rate of mortality of these organisms in captivity was very high and was therefore not viable. Even though the room was air conditioned, the heat trauma experienced by the specimens whilst being collected and collected in the hotter months was enough to cause them to be more susceptible to pathogens and disease, specifically white spot disease.

3.3 Determining which component of the animal to use

The first part of this project involved an attempt to grow in culture cells from the regenerating parts of the regrowing intestine. Cells taken from organisms that were on day 9 of their regeneration, were surviving in the medium for a few days with minimal replication taking place. In this case, it was noted that over a period of 72 hours, the cells were increasing in numbers and then dying suddenly, even though different attempts to the change the protocol were done. Cells taken at different intervals of the regeneration process, namely day 3, 6, 12, 15, 18, 21 and Cr, didn't show any particular growth in media. Hence the extracts that could potentially be obtained using such a method were not enough for the design of this experiment, both in terms of cell growth at different days from the start of regeneration as well as the amount of active ingredient that could potentially be obtained. The method suggest by Odintsova *et al.,* (1994) was initially tested without any modifications. However the cultures were becoming constantly infected by microorganisms and intestinal cell growth was not achieved. The method was then optimised by the addition of antibiotics. Even though the problem with microorganisms was solved, growth of the intestinal cells *in vivo* was still not accomplished. Addition of diluted, cell free coelomic acid was another optimisation, which improved the results for coelomic fluid obtained from day 9 of regeneration. Therefore, it was deemed better to try an alternative pathway and the attention was focused on the coelomic fluid.

The coelomic fluid was hence chosen for the research, based on the fact that the regenerating gut was bathed by the coelomic fluid and any interactions between the differentiating cells at the two ends of the re-growing intestine were facilitated by the coelomic fluid and hence it was very like to give biological activity.

3.4 Preliminary testing to determine the range of % by volume of extract which does not show toxicity on Leukaemia cells.

The MTT assay was carried out on HL60 cells exposed to different % concentrations, to give an indication of potential biological activity with increasing concentration of extract. This was done by comparing the absorbance with cells in RPMI medium alone which serves as a negative control for toxicity as it allows for

proliferation to happen and 1.6% DMSO which acts as a positive control for differentiation. This assay was carried out 72 hours after exposure (figure 29) as well as at 120 hours after exposure (figure 30). 72 hours and 120 hours are the time points for monocytic and granulocytic differentiation induced by positive controls (refer to section 1.5.2). The percentages of coelomic fluid used were 5%, 10%, 15%, 20% and 25% (as dilutions in RPMI medium). These values were chosen on the basis that a value of less than 5% is potentially too small to give any form of result. Values higher than 25% were probably going to be either toxic or the dilution required to reach such percentage concentration to result in a significant lowering of the amount of growth factors due to less medium available, hence reducing the ability to sustain proliferation for 120 hours after seeding. Statistical analysis was carried out on this data to check for any statistical significance difference between the different extract concentrations and the two controls, RPMI (negative control for differentiation) and 1.6% DMSO (positive control for differentiation).

Figures 29 and 30 show the relationship between MTT assay absorbance and % concentration of coelomic fluid after 72 hours and 120 hours of being exposed to treatment respectively. The extract was obtained from day 9 following regeneration of the animal based on the fact that intestinal cells from day 9 showed the most promising results in growing in vitro. The fact that day 9 showed a possible positive effect for differentiation and is being used in this test was not assumed throughout the rest of the project. Various coelomic fluid from various days of regeneration will be tested and it's through these tests that the best day following regeneration to be used as an extract for possible differentiation will be identified.

Figures 30 and 31 show DMSO at 1.6% decreases MTT reduction to approximately half when compared to RPMI showing that number of cells are decreasing due to a decrease in proliferation. At this concentration, DMSO is known to act as a differentiating agent of HL60 through the granulocyte pathway (Chatterjee *et al.*, 1997). Through a first visual analysis, it was noted that when taking into consideration the margin of error as shown by the error bars, the data for 5% and 10% coelomic fluid, a similar absorbance value to that of DMSO was observed.

Visual inspection of the data showed that at % extract higher than 10% extract, there was a decrease in height of the bar graph when compared to 1.6% DMSO which

is the control for differentiation. Values which are less than that of 1.6% DMSO is associated with a probable cytotoxicity, whilst values which are higher than those associated with 100% RMPI are associated with proliferation. There was an attempt to carry out a statistical test to determine differences and similarities. Since the data was determined to be not normally distributed for either 72 hours after seeding or 120 hours after seeding when checked with the Shapiro Wilks test, non-parametric statistical tests were carried out as described in section 2.5. Kruskal Wallis test for therefore carried out and it indicated a significant difference between pairs with p value of <0.05. A post hoc Dunn test was carried out to find which pairs were causing significant differences.

For both time points (72 hours and 120 hours exposure to treatment), at a p value of <0.05 only the extract at a 25% concentration showed difference from 1.6% DMSO, whilst at p value of 0.1 differences to 1.6% DMSO caused by the extract with a 20 and 25%. Since there are only 3 technical triplicates for pooled coelomic fluid from three organisms, there was not enough data for the Kruskal Wallis to be reliable and so visual effects were also taken into consideration (figures 30 and 31). p values can be found in Appendix 5 - Part A.

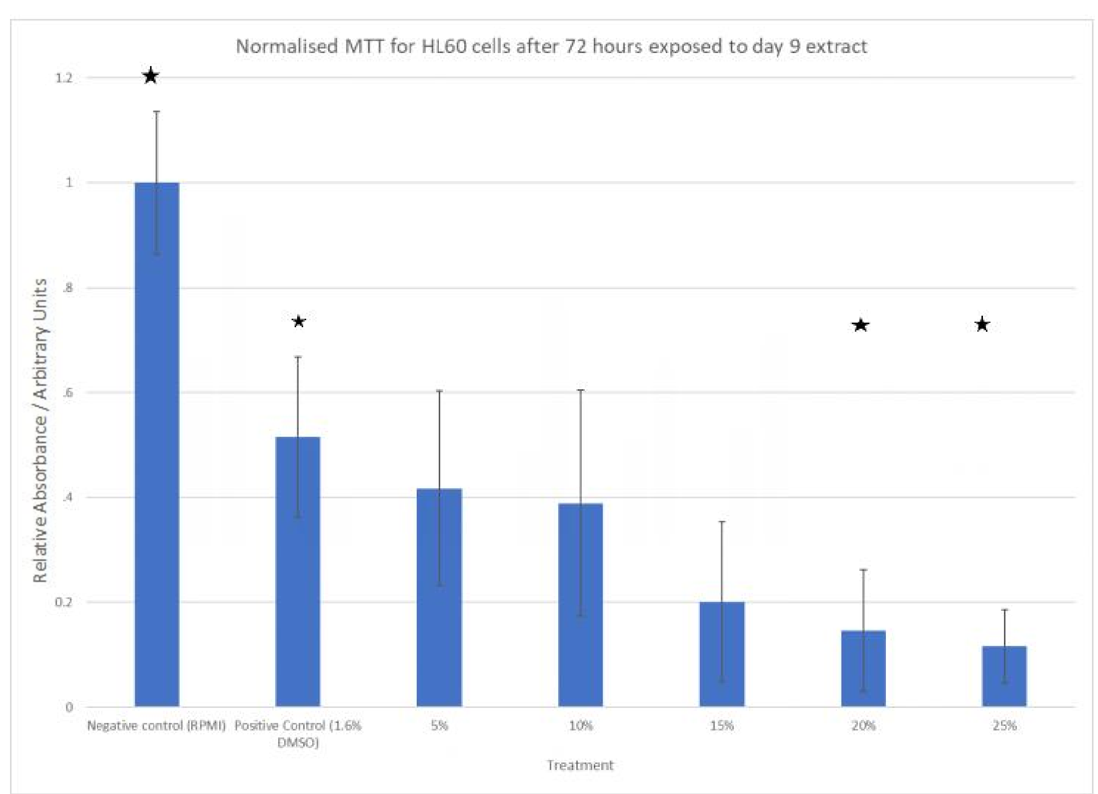


*Figure 30: Graph showing Relative absorbance vs negative control for differentiation, Positive 1.6% DMSO control for differentiation and various concentrations of extracts expressed as % of coelomic fluid on HL60 cells exposed to 72 hours of treatment obtained from organisms in their 9th day post evisceration . The readings are for technical triplicates f for the pooled coelomic fluid of three organisms for each reading hence n=3. Error bars show standard error. Raw data is found in appendix 5 Part A.Passage number = 84 Data is normalised against RMPI. * shows significant difference at p <0.01*

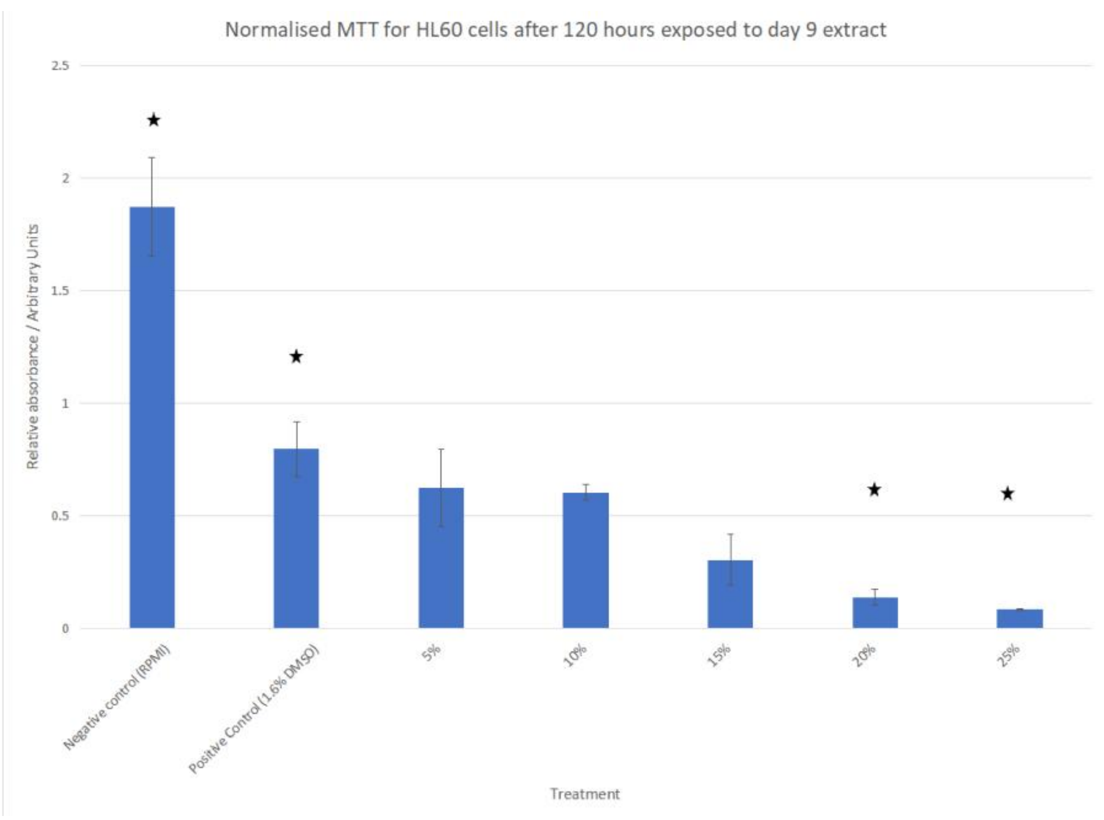


*Figure 31: Graph showing Relative absorbance vs negative control for differentiation, Positive 1.6% DMSO control for differentiation and various concentrations of extracts expressed as % of coelomic fluid on HL60 cells exposed to 120 hours of treatment obtained from organisms in their 9th day post evisceration . The readings are for technical triplicates for the pooled coelomic fluid of three organisms for each reading hence n=3. Error bars show standard error. Raw data is found in appendix 5 Part A. Passage number = 84 Data is normalised against RMPI. * shows significant difference at p <0.01*

Through the statistical analysis and visual inspection of the data, it was therefore concluded that the range of % coelomic fluid that should be tested during further tests was that of 2% up to 10% for each test done.

3.5 Cytotoxicity.

3.5.1 Preliminary testing of extracts on Leukaemia cell line to determine presence or absence of cytotoxic component.

There is a great deal of data associated with this section as graphs for each cell line were drawn and examined. Each cell line had 16 graphs associated with, two for each extract obtained from the various days of evisceration, one graph representing the cells exposed to 72 hours after treatment and one representing cells exposed to 120 hours after treatment. This information is summarised in the following table 10:

Table 10: Table showing data collected for Cytotoxicity tests on four cell lines.

MTT for NB4R2 cell line	Graphs for leukaemia cells exposed to holothurian extracts at 3, 6, 9, 12, 15, 18, 21 and CR days after evisceration for 72 hours and 120 hours after exposure to treatment
MTT for KG1a cell line	Graphs for leukaemia cells exposed to holothurian extracts at 3, 6, 9, 12, 15, 18, 21 and CR days after evisceration for 72 hours and 120 hours after exposure to treatment
MTT for K562 cell line	Graphs for leukaemia cells exposed to holothurian extracts at 3, 6, 9, 12, 15, 18, 21 and CR days after evisceration for 72 hours and 120 hours after exposure to treatment
MTT for HL60 cell line	Graphs for leukaemia cells exposed to holothurian extracts at 3, 6, 9, 12, 15, 18, 21 and CR days after evisceration for 72 hours and 120 hours after exposure to treatment

Visual inspection of the preliminary data for NB4R2 and KG1a showed that data was not consistent. The following is a representative example of the data obtained for these two cell lines.

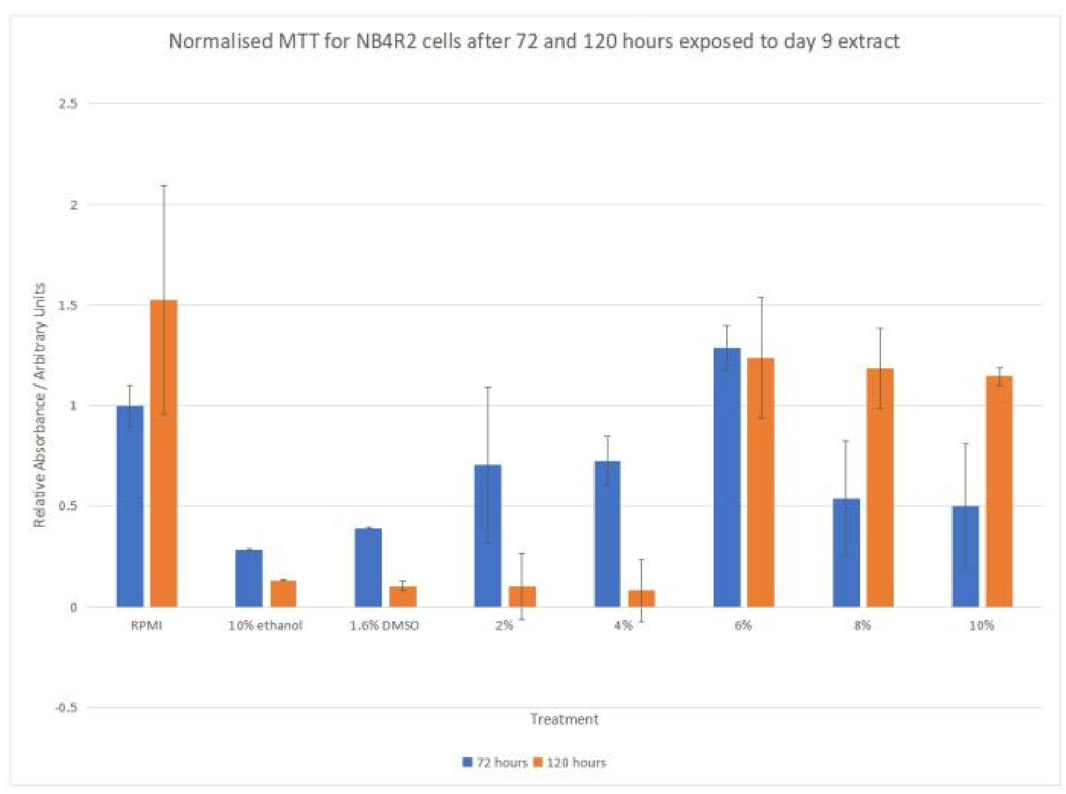


Figure 32: Relative Absorbance (arbitrary units) vs Treatment for Normalised MTT for NB4R2 cells after 72 hours and 120 hours exposed to extract obtained from organisms at the 9th day post evisceration. Bars represent mean MTT readings for pooled coelomic fluid obtained from 3 individual organisms. Error bars represent standard error. N=3. Passage number is 15. Data is normalised against RPMI at 72 hours

Figure 32 is a representative example, and it shows that there does not appear to be any toxicity of the extract on NB4R2 cells when using an extract from organisms in their 9th day post evisceration. In some cases, like 6% concentration by volume of extract even showed a degree of proliferation as the values is even higher than that of RPMI which allows for proliferation of cells. The fact that concentration which are so close to each other give such different results in the context of this research project was not something that was suitable.

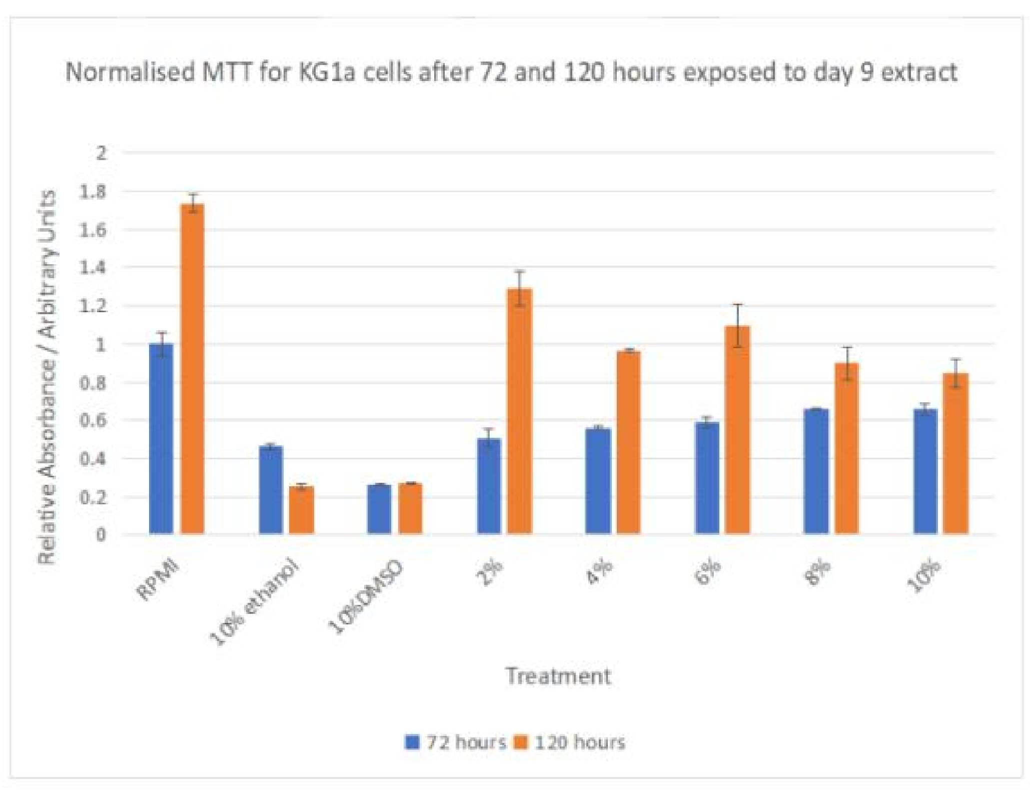


Figure 33 Relative Absorbance (arbitrary units) vs Treatment for Normalised MTT for KG1a cells after 72 hours and 120 hours exposed to extract obtained from organisms at the 9th day post evisceration. . Bars represent mean MTT readings for pooled coelomic fluid obtained from 3 individual organisms. Error bars represent standard error. N=3. Passage number is 8. Data is normalised against RPMI. At 72 hours.

Figure 33 shows a very similar relationship between treatments and controls as that seen in figure 31. Following visual inspection of results, and taking into consideration all the factors mentioned previously, it was decided to move forward in this research project using cell lines which gave the best results.

Hence it was decided that the cell lines chosen to further the investigation would be HL60 and K562, which are representative of an AML and CML cell line respectively. Graphs for relative absorbance for the MTT test for HL60 and K562 will not be shown in this section as they will be displayed further on in this chapter.

3.5.2 Cytotoxicity on human lymphocytes

After determining a range of activity and the fact that some extracts are not toxic to the various cell lines, the MTT assay was carried out by testing the extract of human lymphocytes. In the case that the extract gives a positive result in terms of possible differentiation activity, it was imperative to ascertain that it was not toxic to normal human cells as otherwise its possible use in the human treatment would be very limited. The absorbance values obtained are given in the graph below where RPMI medium only acting as a negative control for cytotoxicity is compared to 10% DMSO which is acting as a positive control for cytotoxicity. At this concentration, DMSO brings about a cytotoxic affect. The cells were also treated with 10 % coelomic fluid. Results were taken at 24, 48 and 72 hours after exposure. Results of this test were obtained 24 hours, 48 hours and 72 hours following incubation as it was deemed essential to determine whether the coelomic fluid was killing the lymphocytes and if yes, at which rate. Since the cells were proliferating, there was no need to continue the experiment any further. This can be seen in figure 34 below:

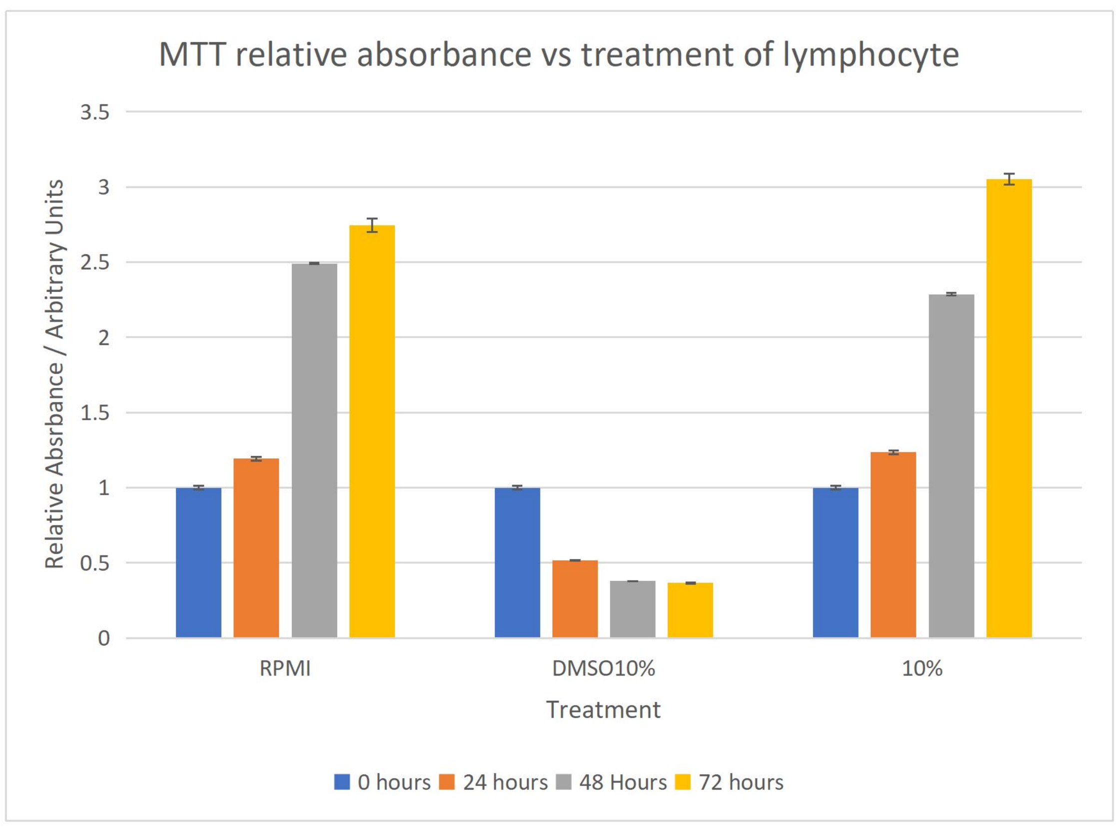


Figure 34: Graph showing mean effect of extracts obtained from organisms in their 9th day post evisceration on lymphocyte proliferation after 24 hours, 48 hours and 72 hours of treatment compared to day 0. Coelomic fluid from 3 specimens was pooled and was used, each being tested as a technical triplicate and hence 3x3 with n=9. Error bars represent standard error. Average values for MTT used to plot this graph are in Appendix 5 Part c.

Results show an increase in the MTT reading for RPMI negative medium control, as well as the 10% treatment, but not for DMSO 10% cytotoxic control in which there was a decrease. The 10% extract shows a similar behaviour to RPMI with a slow increase after 24 hours, a fast increase after 48 hours and another increase after 72 hours exposure to treatment. This implies that extract is not toxic to human lymphocytes. Data can be found in Appendix 5 part C.

3.6 Differentiation Effect of the *Holothuria poli* extract on HL60 and K562 cell line.

When testing all the various extracts on the two cell lines, the data collected was vast and would not lend itself for direct statistical analysis and comparison of all the data as a whole. The independent variables were the cell lines (HL60 or K562), the day post evisceration at which the coelomic fluid was obtained (namely day 3, 6, 9, 12, 15, 18, 20, 21 and CR). Each test involved data for three different biological samples each with a triplicate at the different percentage concentration, the various concentrations of coelomic fluid tested as % by volume (2%, 4%, 6%, 8% and 10%) at 72 hours and 120 hours from exposure to treatment. With the independent variable being absorbance of NBT/MTT ratio. Pooling of coelomic fluid was done as a compromise to reduce as much as possible the effect of variations in the composition of the coelomic fluid between organisms. There are various sources of variability in this research project, including sex, age, general condition of the organism, all of which effect the composition of the coelomic fluid and pooling reduced such effects. All this data had to be combined and narrowed down in order to be able to better visualise any changes. The Data was normalised against RPMI for 72 hours exposure to treatment.

Normal distribution is a pre-requisite condition for a class of statistical analysis called parametric statistics. In normally distributed data, mean and mode are the same and therefore data can be expressed with two parameters mean and variance. The original raw data did not follow normal distribution and hence a log transformation was performed to reduce skewness of data making parametric statistical techniques possible for analysis. The data needs to have a log-normal distribution in order to be treated in this way, otherwise the transformation won't

work. This was the case with the data of this research study. Hence non-parametric statistical analysis techniques had to be used.

Log transformations is a very popular method used in biomedical research (Feng *et al*., 2014) which allows the comprehensive data that was obtained from this set of experiments in this study to be compressed in such a way that it could be easily visualised, aiding in interpretation as the data is shifted to fit in the range whilst at the same time still remaining comparable as the data was normalised to RPMI at 72 hours for each cell line prior to being logged. This can be seen in figure 35.

Preliminary visual inspection of this log transformation shows that the biggest difference is when using coelomic fluid from day 9 following regeneration for both HL60 and K562. There is also a spike at day 12, however not as big as that of day 9. The values appear to be going back to normal in the successive days and then shoot up and down for days 21 and CR. One of the limitations of log transformations is that they do not necessarily mirror the original data (Feng *et al*., 2014). Hence statistical analysis was used to determine whether what was being visualised in the log transformation above is statistically significant or otherwise using the original data. Statistical analysis confirmed that at $p<0.05$, the readings obtained from extracts of day 9, 21 and CR were significantly different from the others. Figure 34 shows the results obtained through the log transformation.

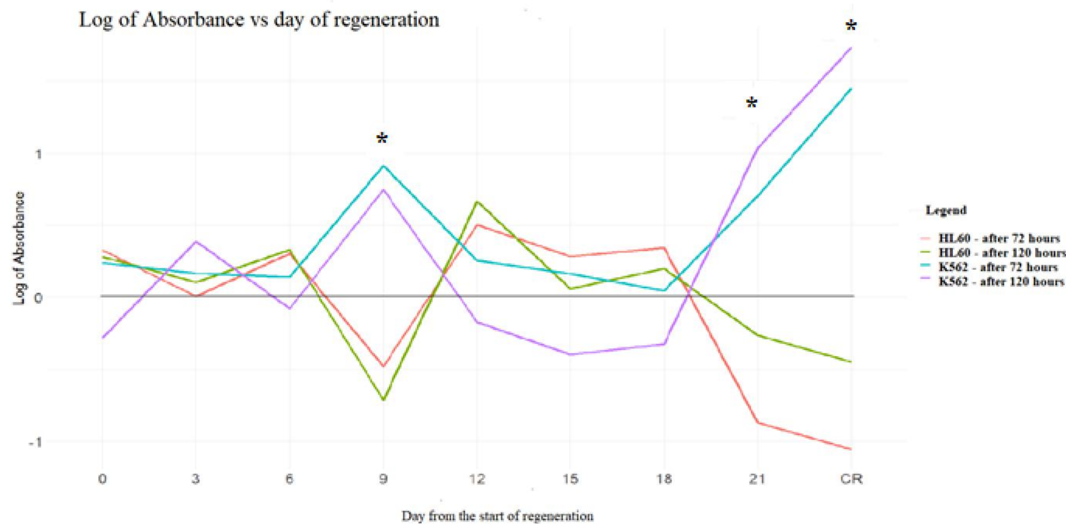


*Figure 35: Log representation of the normalised (against RPMI 72 of treatment) NBT/MTT data obtained for extracts from all days post evisceration being tested. Each day studied at 72 hours and 120 hours following incubation of treatment for two cell lines HL60 and K562. The points marked in * have indicated statistical significance.of $p < 0.05$. Part of the Data is found in Appendix 5 - Part F.*

Whether the value in the log transformation is above or below the 0 line, does not imply anything on the significance of the results however it gives an indication that a change is occurring at that particular point. A value higher than 0 doesn't mean that there is an increase and a value lower than 0 doesn't mean a decrease.

Although the log transformation was partially successful at reducing the skewness of the data, the data was still not normally distributable and hence non-parametric statistical analysis was carried out. The Kruskal Wallis test followed by Dunn's post hoc test was carried out on the data. Statistical significance showed that in the case of the cell line HL60, at 72 hours after treatment, it was determined that the NBT/MTT readings for day 9 were significantly different from all other percentages but not from day 3, 21 and CR. Days 21 and CR were significantly different from all other extracts except for day 9 and each other. All p values that show statistical significance are found in Appendix 5-part D, marked in red. All p values related to exposure of cells to 72 hours of treatment were <0.001.

In the case of the cell line HL60, 120 hours exposed to treatment, it was determined that the NBT/MTT readings for day 9 were significantly different from all other percentages but not from day 21 and CR. Day 21 showed a significant difference from all days except day 3, 9, 15 and CR and those of CR were

significantly different except for those of day 9 and day 21. All p values related to exposure of cells to 120 hours of treatment were mostly $p<0.001$ or $p<0.05$.

MTT results for extracts obtained from organisms at day 21 and CR post evisceration on HL60 cell line, 72 hours after treatment showed that the extract was toxic. This can be visualised in figures 36 and 37.

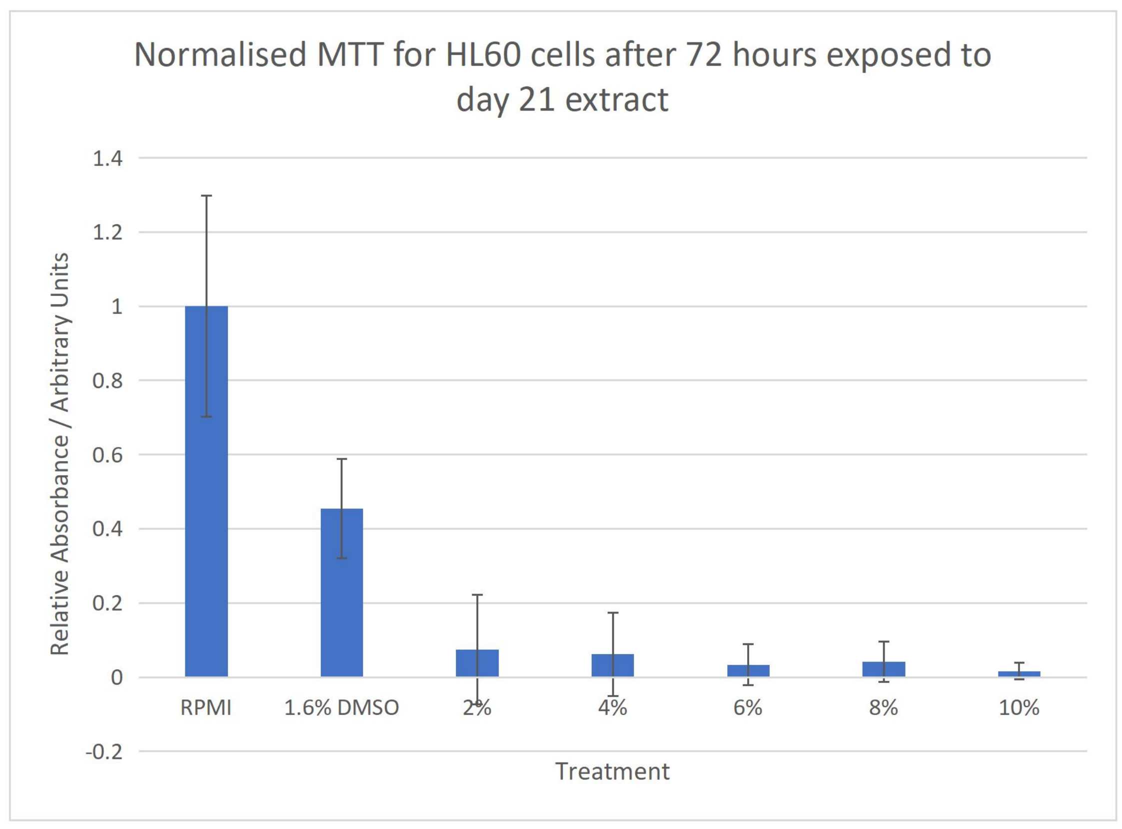


Figure 36: Graph showing Normalised MTT reading for HL60 cells exposed to 72 hours treatment mean effect of extracts obtained from organisms in their 21st day post evisceration. Coelomic fluid from 3 specimens was pooled and was used, each being tested as a technical triplicate and hence 3x3 with n=9. Error bars represent standard error. Average values for MTT used to plot this graph are in Appendix 5 Part G

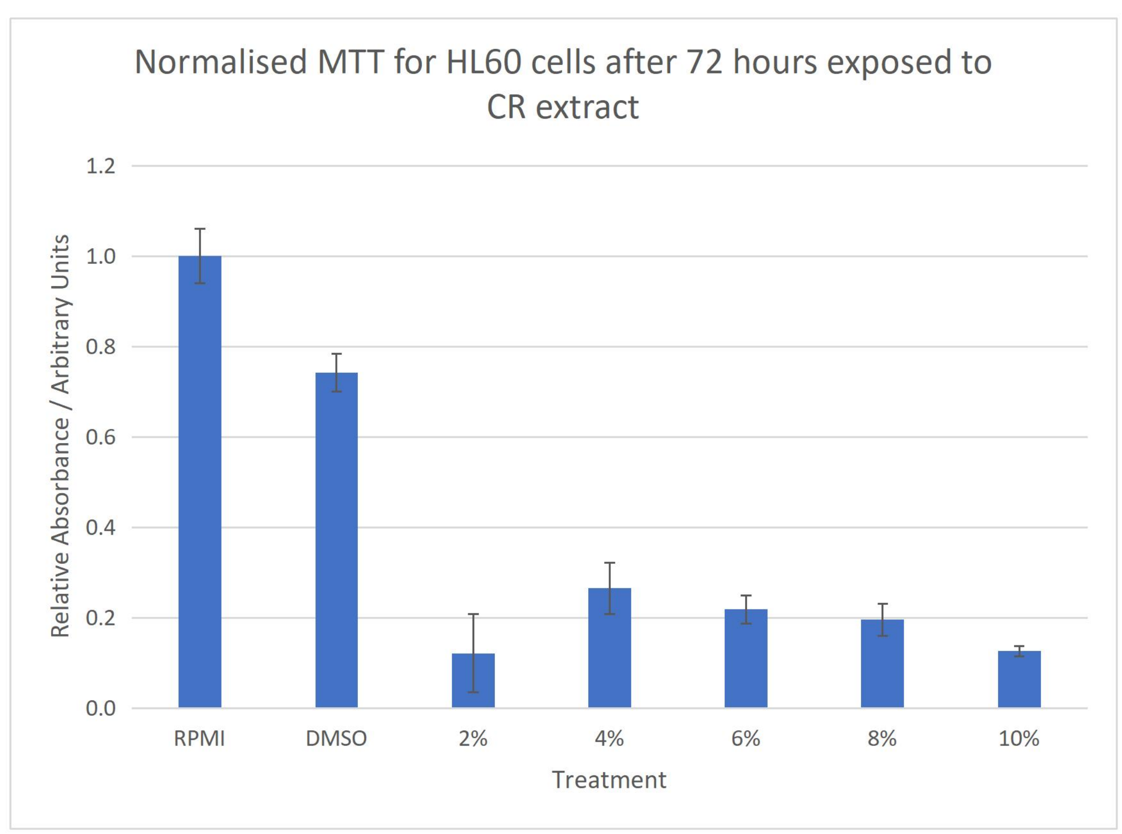


Figure 37: Graph showing Normalised MTT reading for HL60 cells exposed to 72 hours treatment mean effect of extracts obtained from organisms in their CR day post evisceration. Coelomic fluid from 3 specimens was pooled and was used, each being tested as a technical triplicate and hence 3x3 with n=9. Error bars represent standard error. Average values for MTT used to plot this graph are in Appendix 5 Part G

The significant difference obtained in the log transformation for day coelomic fluid obtained from day organisms in their 21st and CR days post evisceration can therefore be attributed to toxicity. Day 9 which also showed statistical significant difference in the results, was then studied.

In the case of the cell line K562 at 72 hours from treatment, it was determined that the NBT/MTT readings for day 9 were significantly different from all other percentages but not from day 21 and CR. CR was significantly different from all the days except day 9 and 21. In the case of the cell line K562 at 120 hours from treatment, it was determined that the NBT/MTT readings for day 3 were significantly different from day 0, 12, 15 and 18 but not from the others. Those of day 9 were significantly different from all other days except day 3 and 21. Day 21 showed a

significant difference from all days except day 3 and 9 and CR and those of CR were significantly different from all the others but not those of days 3 and day 21. All the p values that show statistical significance are found in Appendix 5-part D, marked in red. P values for cells exposed to both 72 hours of treatment and 120 hours of treatment were very low, all below $p < 0.05$, showing great statistical difference.

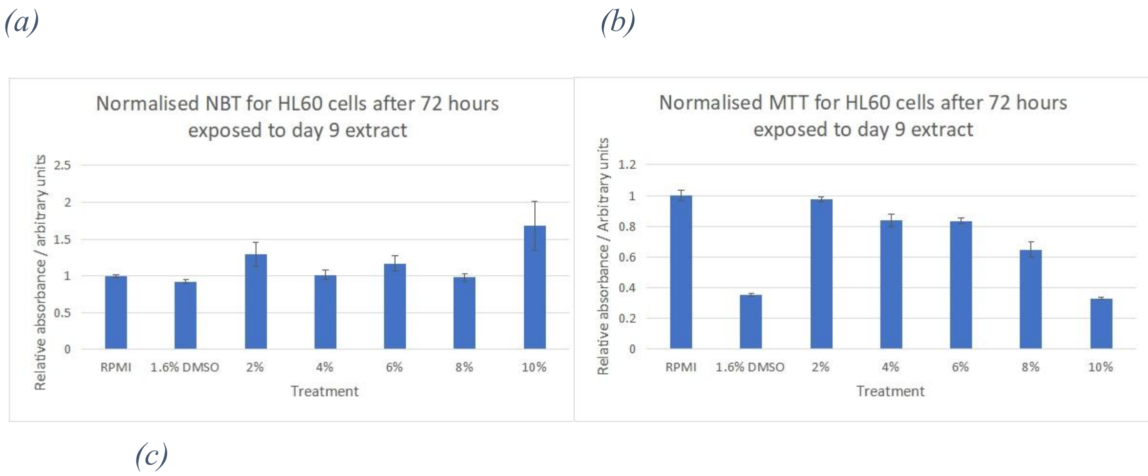
Hence it was concluded that the best approach was to focus on coelomic fluid from day 9 of regeneration for further tests however, the graphs for day 12 will also be illustrated in the next section as visual inspection of the log transformation showed that there could possibly be some valuable results.

3.7 Effect of 2% to 10% range of coelomic fluid

3.7.1 HL60 cell line using coelomic fluid from day 9 of regeneration.

Figure 38 represents Normalised NBT, MTT and NBT/MTT for HL60 cells after 72 hours of being exposed to coelomic fluid obtained from organisms in their day 9 of regeneration. Data can be found in Appendix 5 Part E.

(a) *(b)*



(c)

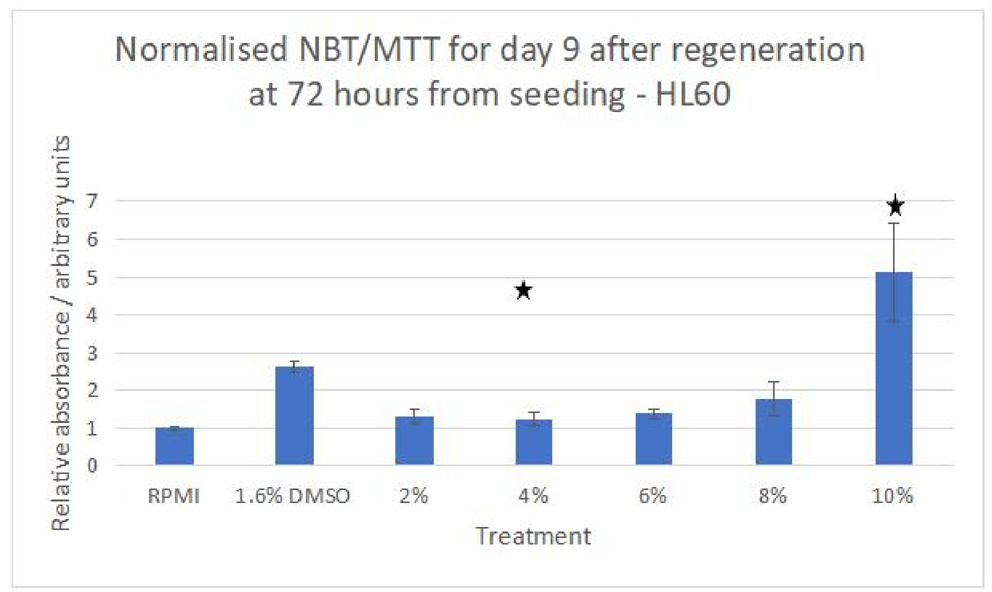


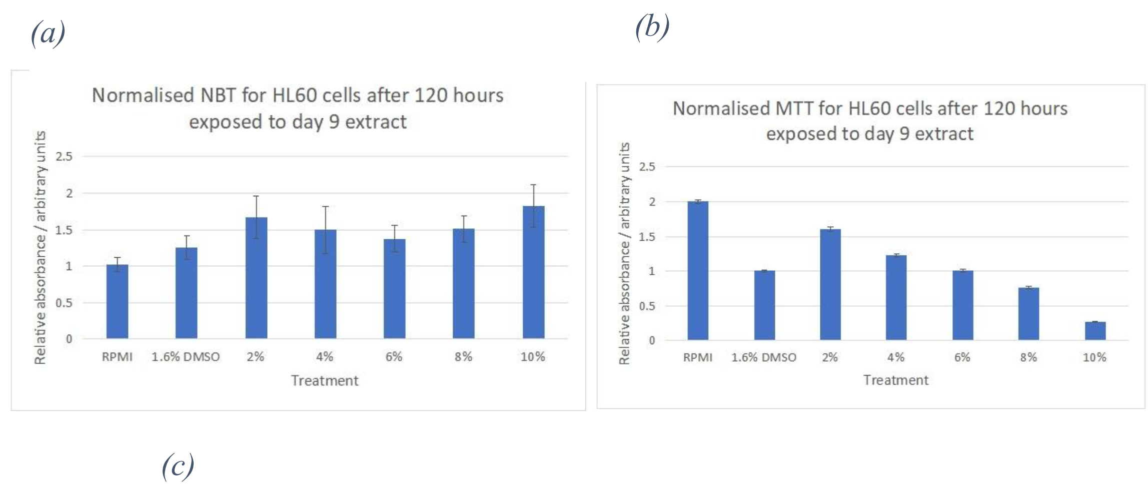
*Figure 38: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 72 hours of treatment. (c) NBT: MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours. * statistical significant difference at p< 0.05*

The readings at 10% show an NBT/MTT value which is higher that than of 1.6% DMSO. When looking at the separate graphs of NBT and MTT, at the same concentration, there is an increase in NBT which corresponds to increased oxidative burst capability, a sign of phagocytic differentiation and a decrease in MTT, suggesting that the cells are not dividing as much. The bar related to the extract at 10% is visually similar to that of 1.6% DMSO which acts as the control.

Statistical analysis was carried out on the data. Since the data was not normally distributed, non-parametric analysis was performed. The results for this statistical analysis are found in Appendix 5 – part H. Through statistical analysis it was determined that the NBT/MTT readings for RPMI were statistically significantly different for all other treatments performed with $p<0.001$. DMSO was seen to be not statistically different from any concentration of extract. This might imply that there is a possible effect in terms of differentiation. A statistical difference was noted between extracts at 4% and 10% at a p value of <0.05.

Figure 39 represents Normalised NBT, MTT and NBT/MTT for HL60 cells after 120 hours exposed to coelomic fluid obtained from organisms in their day 9 of regeneration. Data can be found in Appendix 5 Part F.

(a) *(b)*



(c)

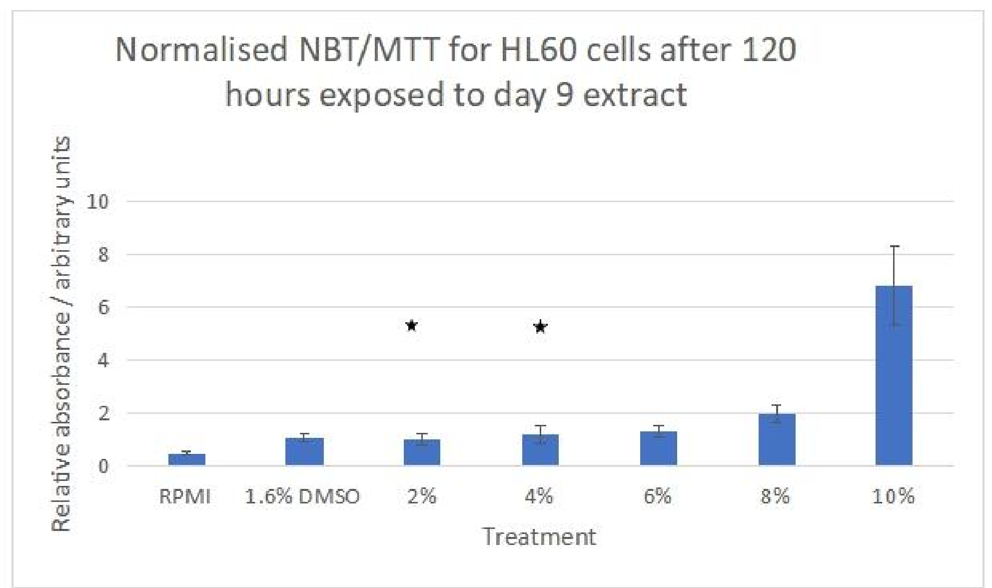


*Figure 39:Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 120 hours of treatment. (c) NBT: MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.. * statistical significant difference at p< 0.05*

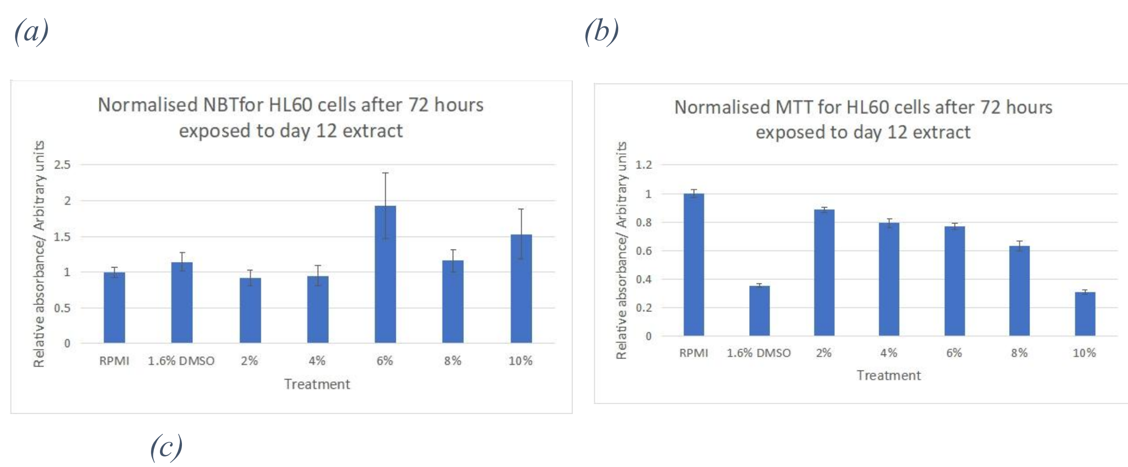
Graphs here show that for 10% coelomic fluid NBT reading increased and MTT reading decreased so much that it could also mean toxicity. Lower values of concentration are behaving more closely to the behaviour of 1.6% DMSO.

Statistical analysis was carried out on the data. Since the data was not normally distributed, non-parametric analysis was performed. The results for this

statistical analysis are found in Appendix 5 – part H. It was determined that the NBT/MTT readings for RPMI were statistically different for all other treatments performed with a $p<0.001$. DMSO was seen to be not statistically different from any extract except for the lower concentrations of 2% and 4% by volume with a $p<0.05$. 2% and \$% by volume of extract are also statistically different from 10% by volume of extract with a $p<0.05$. So, in this case, the range of action on differentiation, if at all, is between 6% and 10% by volume. This would need more tests, such as morphology to be confirmed.

3.7.2 HL60 cell line using coelomic fluid from day 12 of regeneration.

These graphs are being shown for visualisation of results trends. From previous statistical analysis (section 3.6), its data using coelomic fluid from day 9 of regeneration that is statistically significant and so detailed statistical analysis will be done with that data set. However, it was felt, that a visual comparison of the data when using coelomic fluid from day 12 would help look for variations since the log transformation showed a gradual change from day 9. Figure 40 represents normalised NBT, MTT and NBT/MTT for HL60 72 hours from coelomic fluid obtained from organisms in their day 12 of regeneration. Data can be found in Appendix 5 Part F.



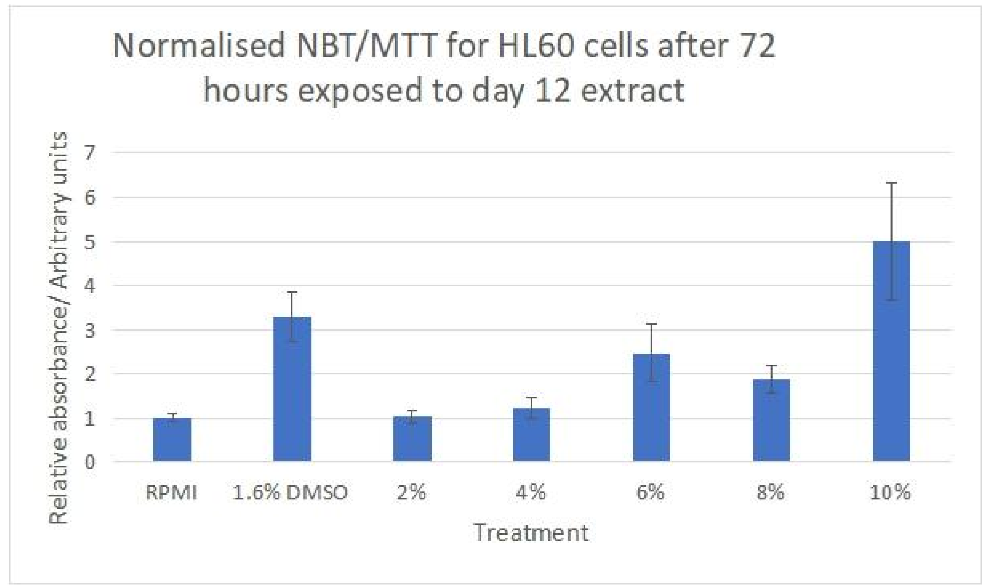
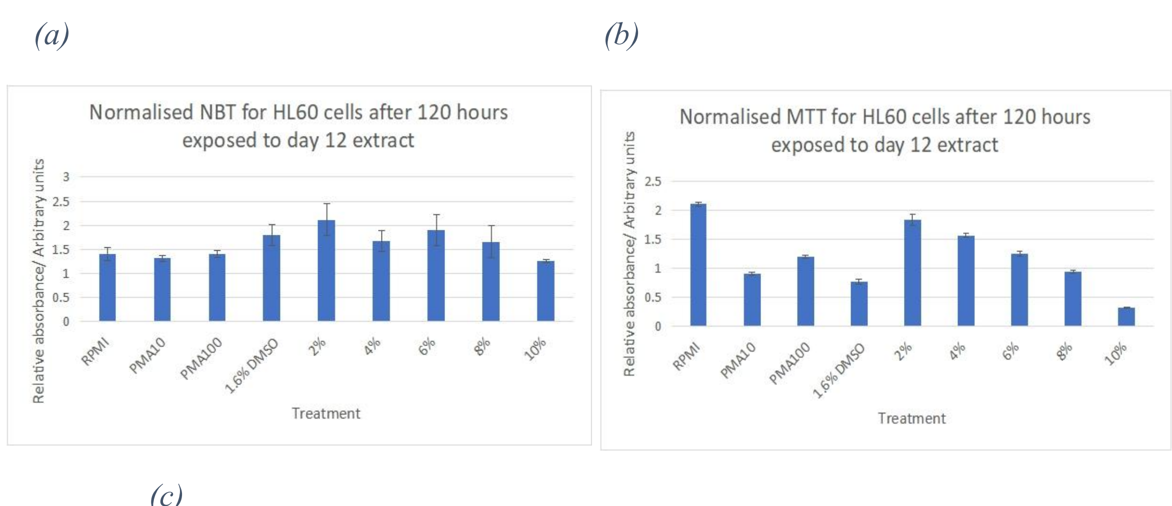


Figure 40 Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 72 hours of treatment. (c) NBT: MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.

The results noticed for the NBT and MTT tables as well as NBT/MTT are very similar to those seen in the results for extract obtained from day 9. Whilst there is a variation in NBT readings, MTT values decrease with an increase in % extract by volume, resulting in various NBT/MTT readings with that at 10% being greater than that of 1.6% DMSO.

Figure 41 represents normalised NBT, MTT and NBT/MTT for HL60 120 hours from coelomic fluid obtained from organisms in their day 12 of regeneration. Data can be found in Appendix 5 Part F.



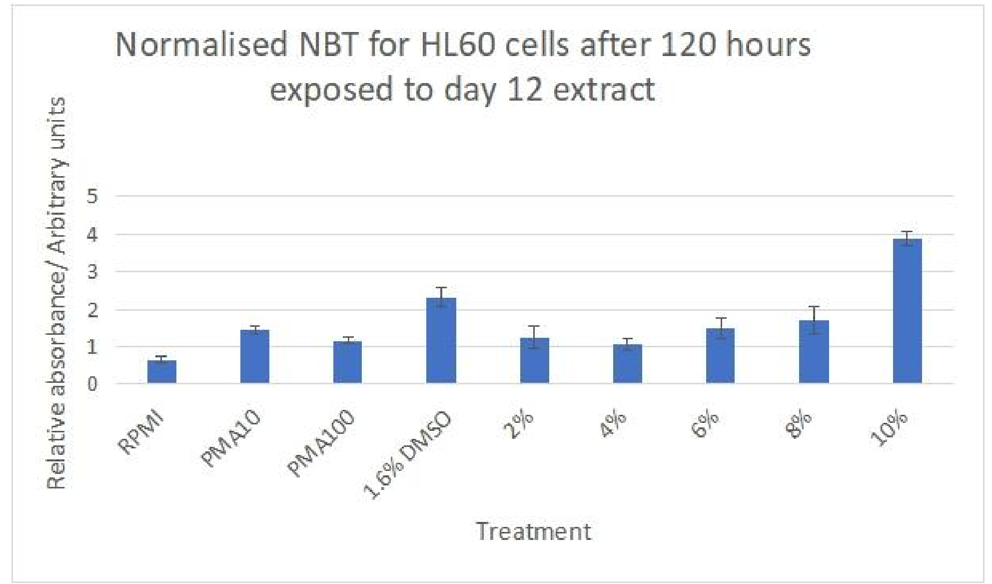


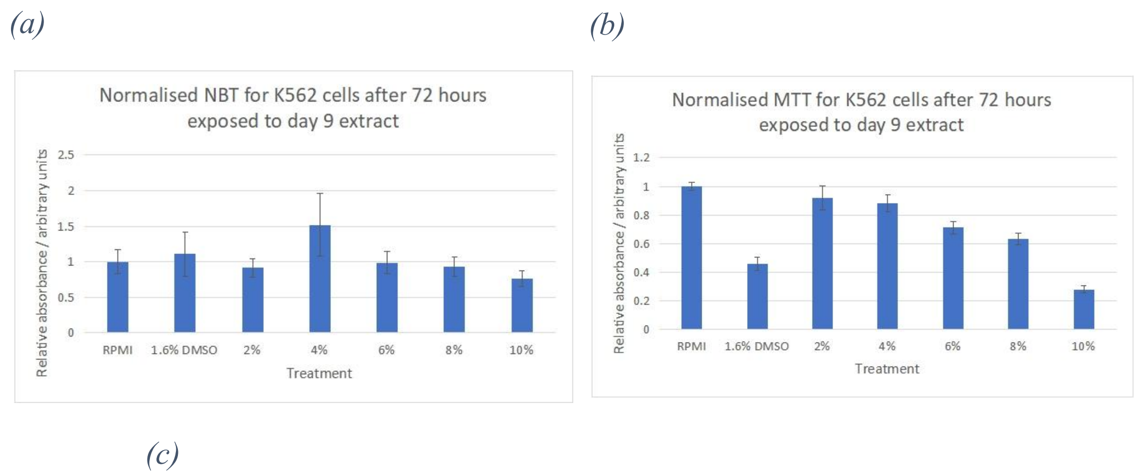
Figure 41 Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 120 hours of treatment. (c) NBT: MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.

The same patterns seen in figure 40 is seen in figure 41. It is highly possible that the coelomic fluid is toxic at 10% by volume.

3.7.3 K562 cell line using coelomic fluid from day 9 of regeneration.

Figure 42 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 72 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data can be found in Appendix 5 Part F.

(a) *(b)*



(c)

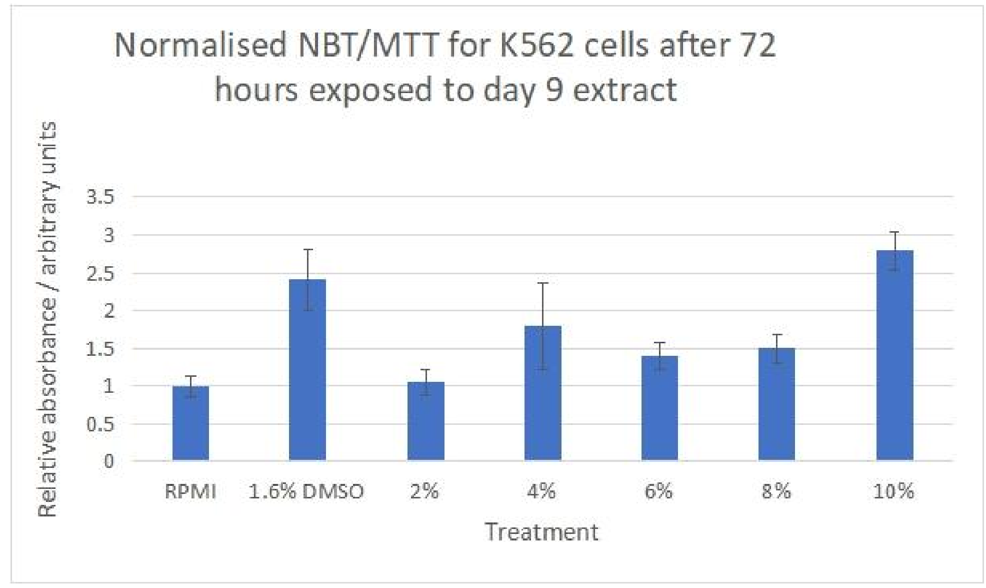


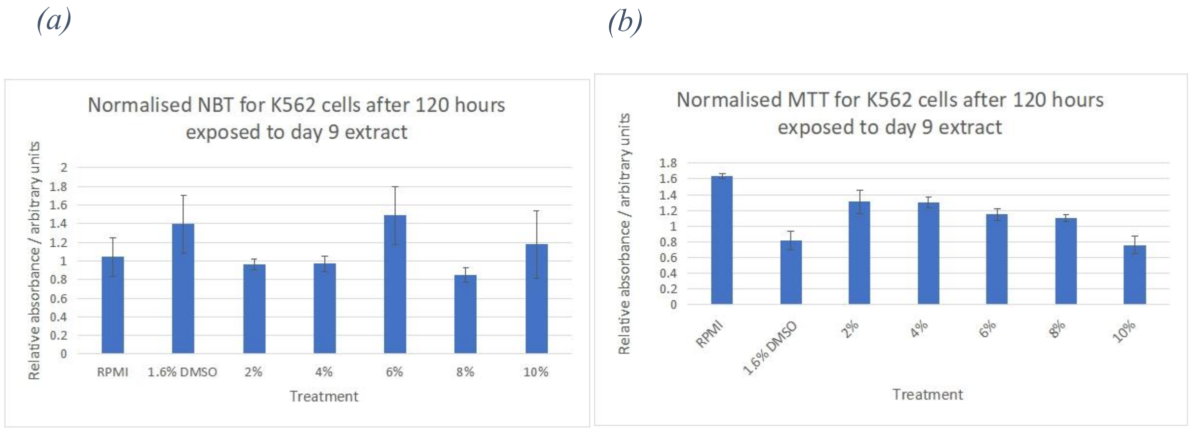
Figure 42: Effect of coelomic fluid from day 9 of regeneration on K562 cells after 72 hours of treatment. (c) NBT: MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.

In the case of the K562 cells lines, similar pattern to that previously described is seen for MTT, and hence it can be inferred that increasing concentration of extract, is increasing toxicity. NBT shows a spike for 4% extract and then is remaining relatively stable throughout the other values. The NBT spike at 4% is also accompanied by a corresponding high MTT value. This could be indicative of a proliferative effect.

Data was determined to be not normally distributed and hence non-parametric statistical analysis was carried out. Results for this statistical analysis were found in Appendix 5 – part H. It was determined that the NBT/MTT readings for RPMI were statistically significantly different ($p<0.001$) for all other treatments performed. Reading for 1.6% DMSO was seen to be not statistically different from any extract. This statistical analysis is not sustaining visual analysis related to the the idea that at higher values the coelomic fluid might be becoming cytotoxic due to a considerable decrease in MTT reading when compared to 1.6% DMSO which is the agent of differentiation. This will be analysed through morphological analysis (see figure 46).

Figure 43 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 120 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data can be found in Appendix 5 Part F.

(a) *(b)*



(c)

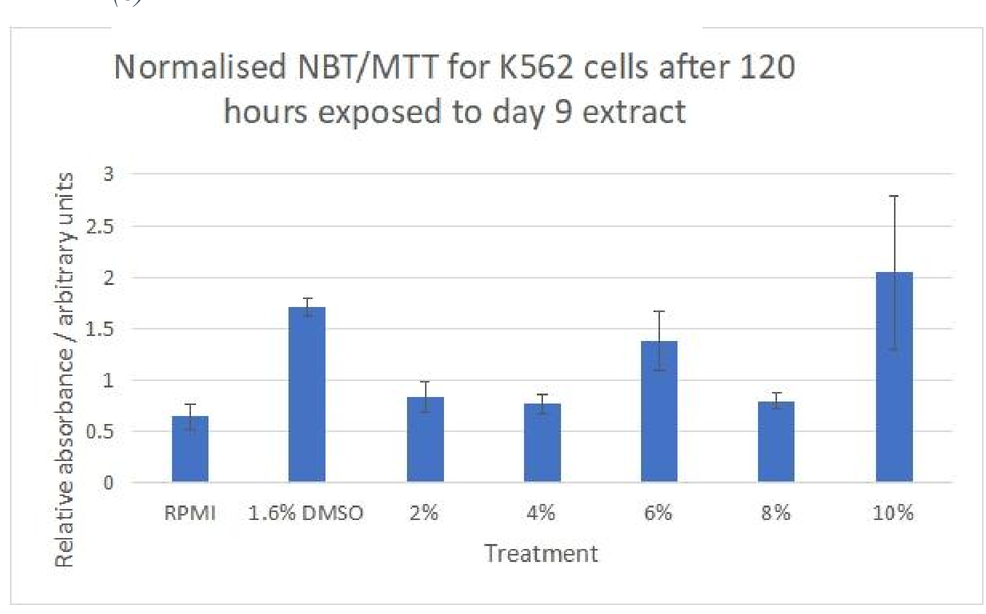


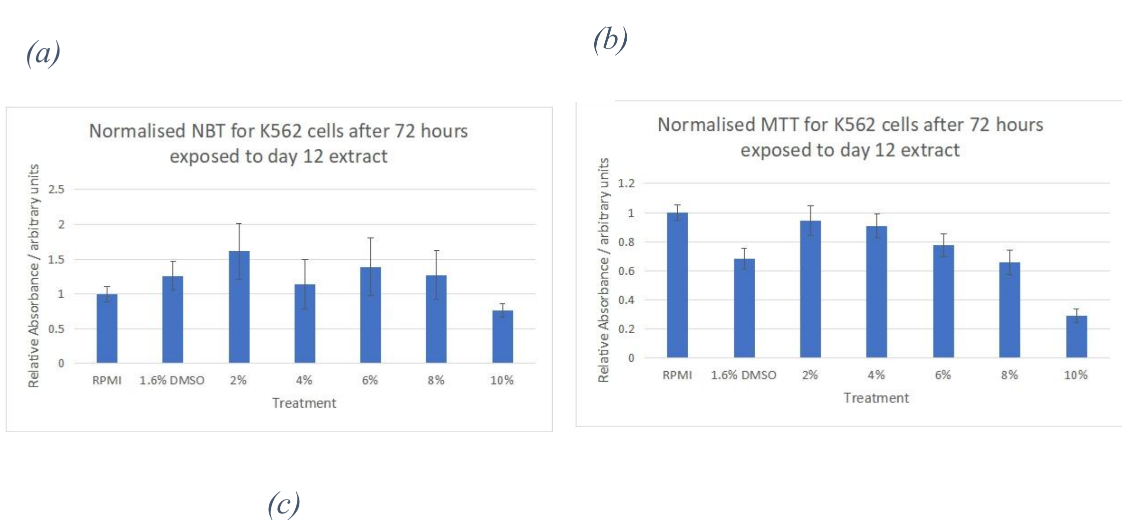
Figure 43: Effect of coelomic fluid from day 9 of regeneration on K562 cells after 120 hours of treatment. (c) NBT: MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.

After 120 hours exposed to the extract, it was noted that there was a peak at 6% concentration rather than at 4% concentration of extract (unlike what was seen after 72 hours). Here cytotoxicity was not noted visually at 10% concentration as the peak was not as low as in the other graphs presented. When analysed statistically it was shows that NBT/MTT readings for RPMI were statistically significantly different ($p<0.001$) for all other treatments performed. 1.6% DMSO was seen to be not statistically different from any coelomic extract.

3.7.4 K562 cell line using coelomic fluid from day 12 of regeneration.

Figure 44 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 72 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data can be found in Appendix 5 Part F.

(a) *(b)*



(c)

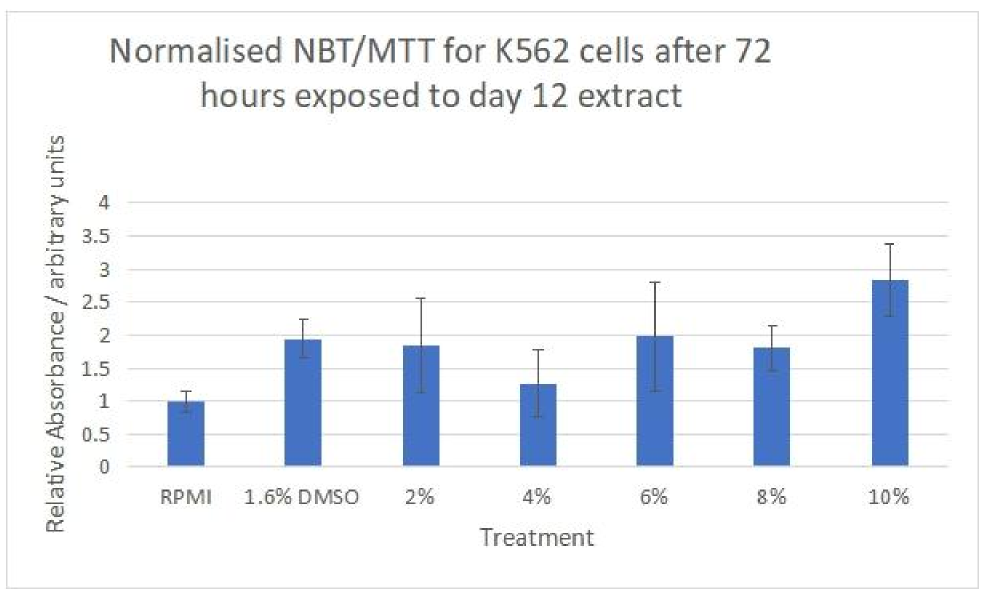
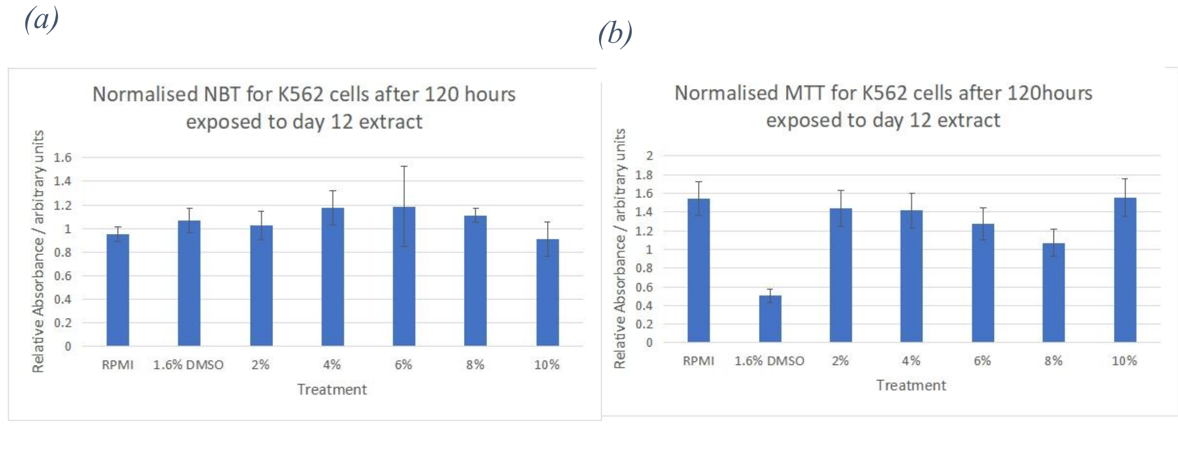


Figure 44:Effect of coelomic fluid from day 12 of regeneration on K562 cells after 72 hours of treatment. (c) NBT: MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.

The data here is not showing any specific pattern as seen in other graphs presented. There are peaks at random concentrations for NBT readings although an increase in toxicity as concentration of extract increases, is consistent and can be noted by the decrease in MTT values.

Figure 45 represents normalised NBT, MTT and NBT/MTT for K562, 120 hours from coelomic fluid obtained from organisms in their day 12 of regeneration. Data can be found in Appendix 5 Part F.

(a) *(b)*



(c)

Normalised NBT/MTT for K562 cells after 72 hours exposed to day 12 extract

Relative Absorbance / arbitrary units

2.5
2
1.5
1
0.5
0

RPMI 1.6% DMSO 2% 4% 6% 8% 10%

Treatment

Figure 45: Effect of coelomic fluid from day12 of regeneration on K562 cells after 120 hours of treatment. (c) NBT: MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.

Here there seems to be an increase in NBT reading up to 6% concentration followed by a decrease. However there seems to be proliferation as the values for MTT are consistently high.

3.7.5 Conclusion to this section.

Morphological analysis of HL60 cells treated for 120 hours with 10% by volume coelomic fluid obtained from organisms in their 9th day post evisceration, shows that there is considerable cell death. The following figure 46 is a representative photo of the general health and development of the cells at this extract concentration.

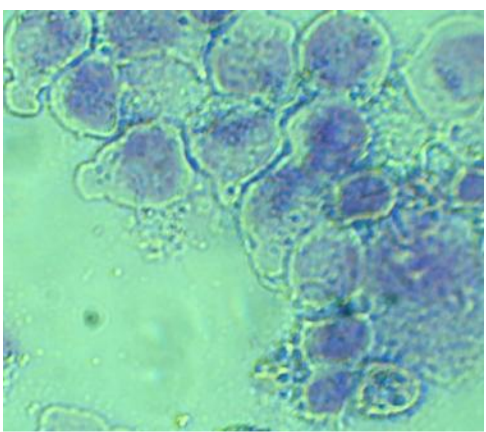


Figure 46: Representative image of morphological analysis of HL60 cells treated for 120 hours with 10% by volume coelomic fluid.. Coelomic fluid was obtained from organisms in their 9th day post evisceration.

Even though statistical analysis is not showing 10% by volume to be different from 1.6% DMSO in terms of NBT/MTT ratio, morphological analysis shows that cells at this concentration are undergoing cell death.

3.8 Determining the nature of the active ingredient

3.8.1 Protein concentration determination

The results for absorbance for protein concentration determination obtained for the samples were the following:

Table 11: Table showing absorbance readings for samples of the extract obtained from day 9 from regeneration.

	Reading 1	**Reading 2**	**Reading 3**	**Reading 4**	**Average**
Total	0.24007	0.16277	0.20751	0.200117	0.202617
> 5kDa	0.095682	0.091539	0.092044	0.093088	0.093088
< 5kDa	0.049656	0.048729	0.048582	0.048989	0.048989

NB. These values were taken prior to any dilution.

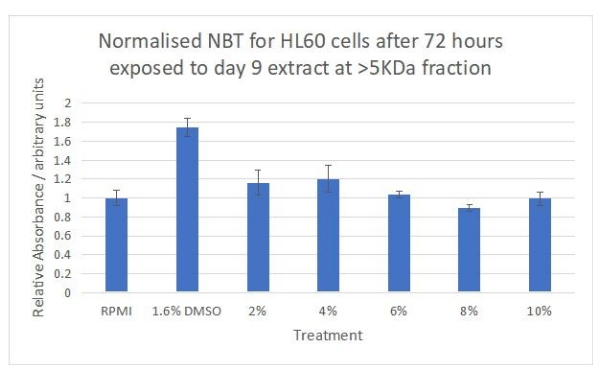
According to the graph, the average value of 0.202617 for absorbance corresponds to 0.5mg/mL of protein. The amount of protein in the two fractions upon spinning the coelomic fluid does not add up to the total value before spinning the coelomic fluid. This implies that some protein might have been held by the mesh in between the two fractions. This implies that some other molecules might also not be able to pass and are not collected in either section. Some biomolecules are therefore lost in the process. This will affect their effectiveness during the test.

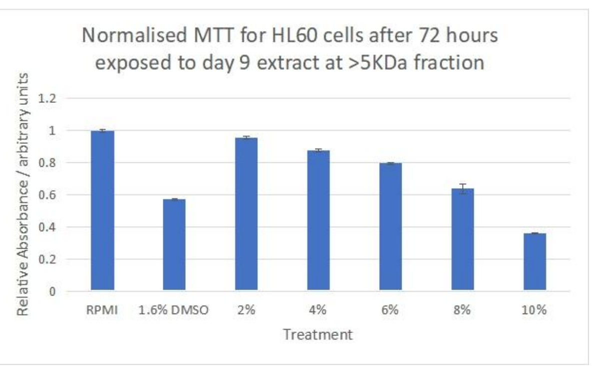
3.8.2 Effect of two fractions: >5KDa and <5KDa

3.8.2.1 HL60 cell line exposed to day 9 extract.

Figure 47 represents normalised NBT, MTT and NBT/MTT for HL60 cells, exposed to 72 hours of coelomic fluid obtained from organisms in their day 9 post evisceration. Data is related to >5 KDa fraction. Data can be found in Appendix 5 Part J.

(a) *(b)*





(c)

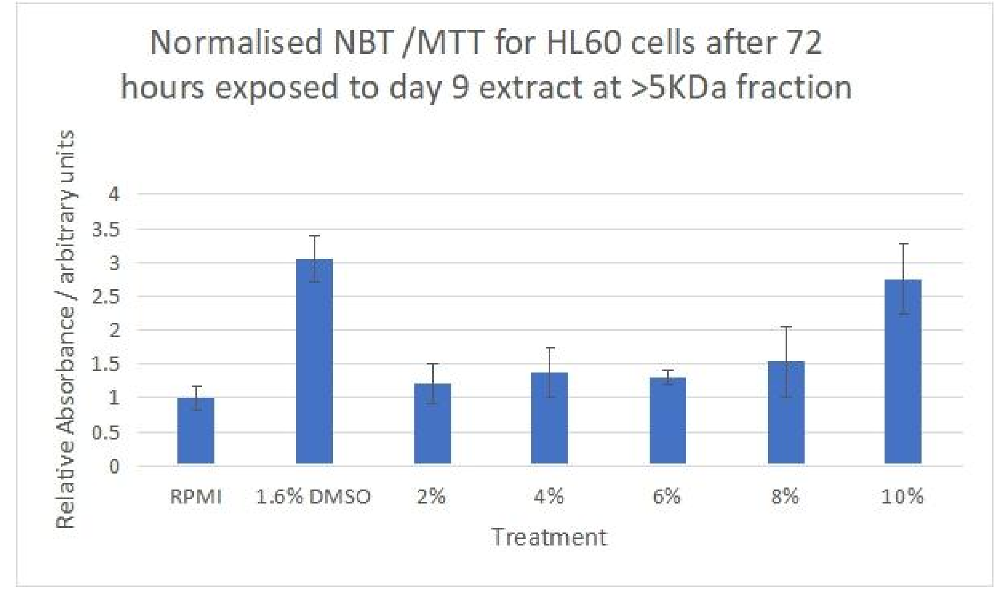
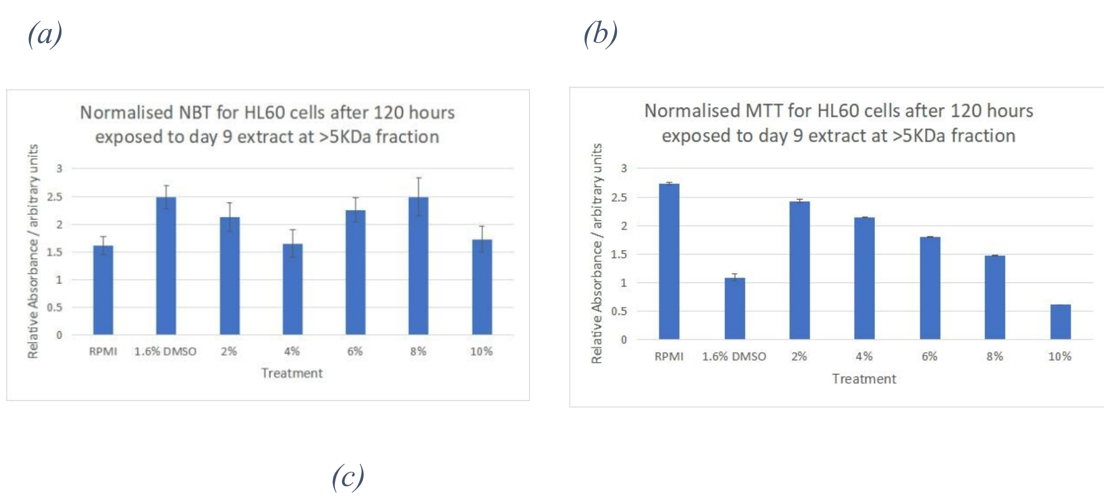


Figure 47: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 72 hours of treatment for the >5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.

In these graphs it can be noted that the value for NBT for any % concentration of extract is never as high as that of 1.6% DMSO but seem to be more in the range of RPMI. For MTT readings, there is a consistent decrease, once again possibly pointing towards toxicity at higher values.

Figure 48 represents normalised NBT, MTT and NBT/MTT for HL60 exposed for 120 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to >5 KDa fraction. Data can be found in Appendix 5 Part J

(a) *(b)*



(c)

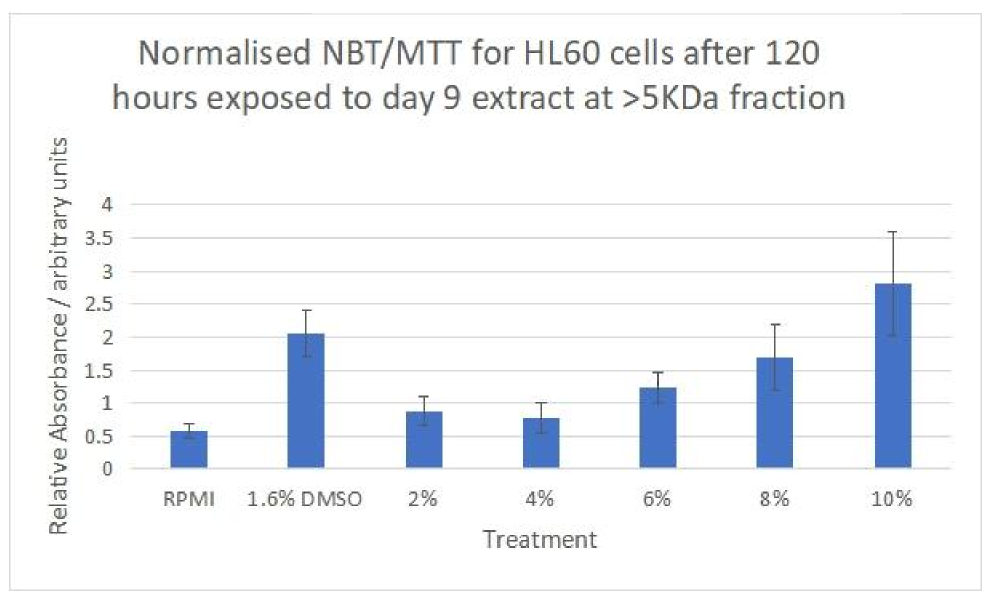
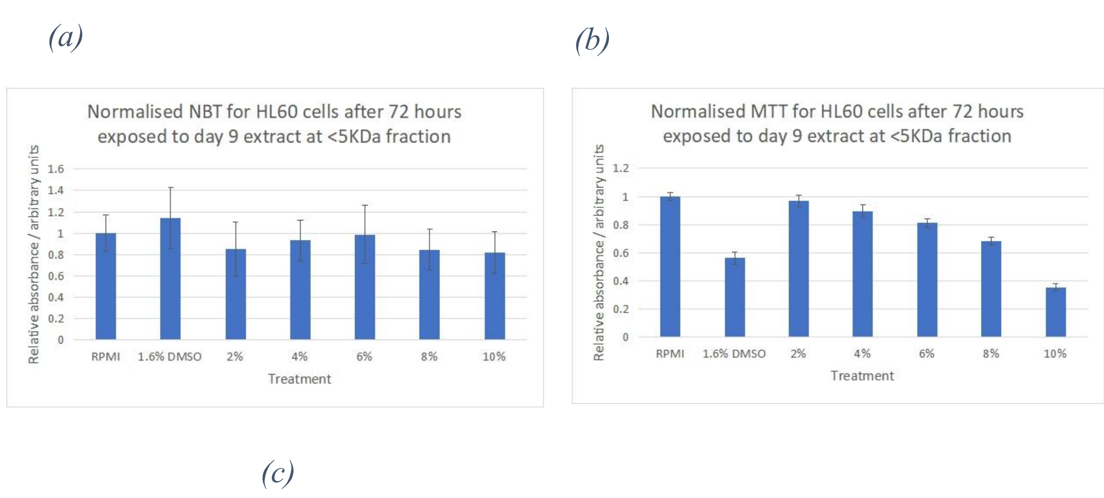


Figure 48: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 120 hours of treatment for the >5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours.

Graphs drawn there visually shows that the coelomic fluid at 8% and 10% could potentially be behaving like 1.6% DMSO. When looking at NBT values, it can be seen that coelomic fluid at 8% is behaving in a very similar fashion as 1.6% DMSO, whilst at 10% there seems to be toxicity as the value for MTT is very low.

Figure 49 represents normalised NBT, MTT and NBT/MTT for HL60 cell exposed for 72 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to <5 KDa fraction. Data can be found in Appendix 5 Part J

(a) *(b)*



(c)

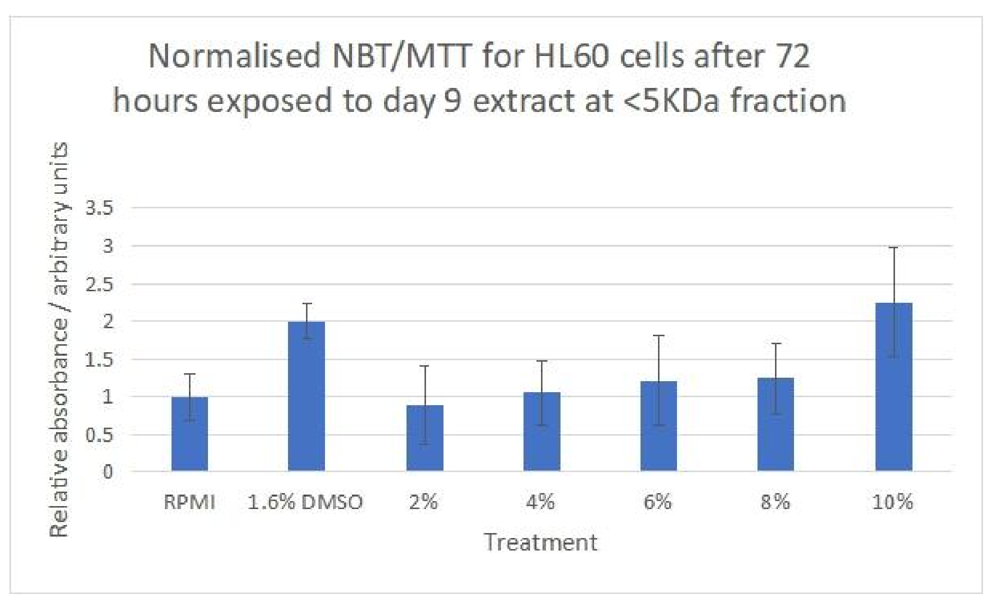
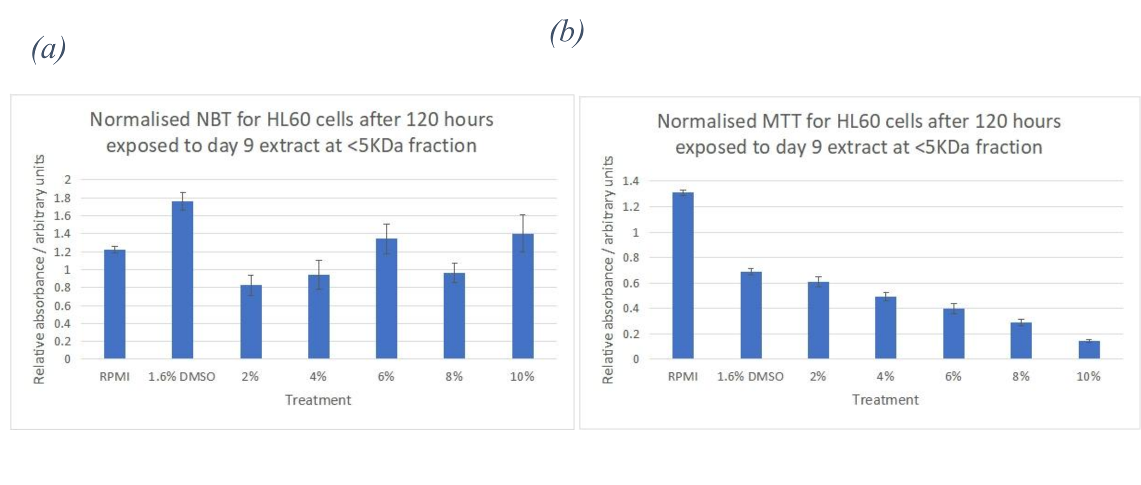


Figure 49: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 72 hours of treatment for the <5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours..

When looking at the data for NBT and MTT separately, one can notice that in the case of NBT, at all levels of concentrations, extracts seem to be working in a very similar fashion as both RPMI and 1.6% DMSO, however for MTT, there is a steady decrease, implying possible cytotoxicity on increasing in concentration of extract.

Figure 50 represents normalised NBT, MTT and NBT/MTT for HL60 cells exposed for 120 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to <5 KDa fraction. Data can be found in Appendix 5 Part J



(c)

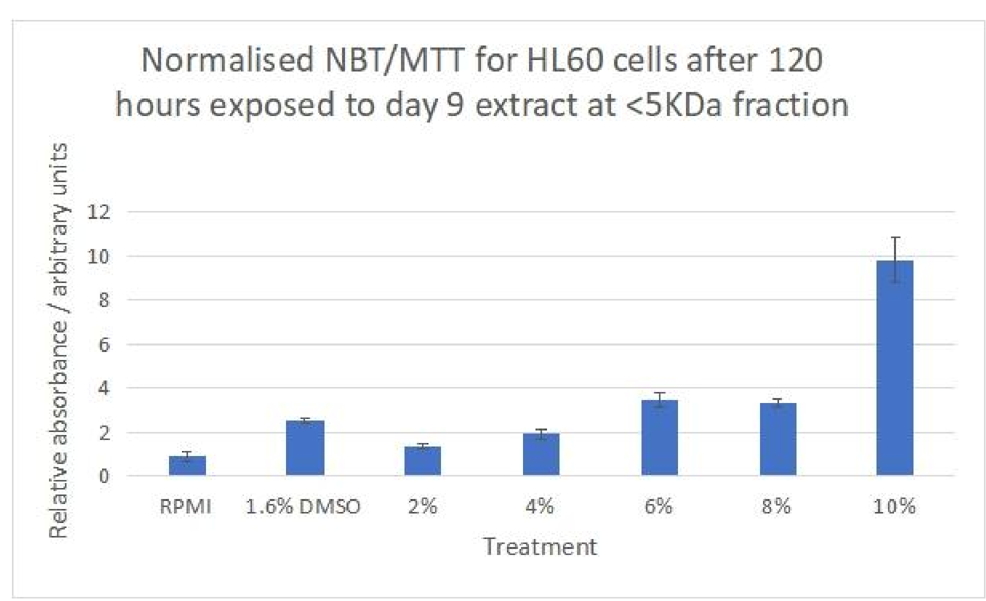


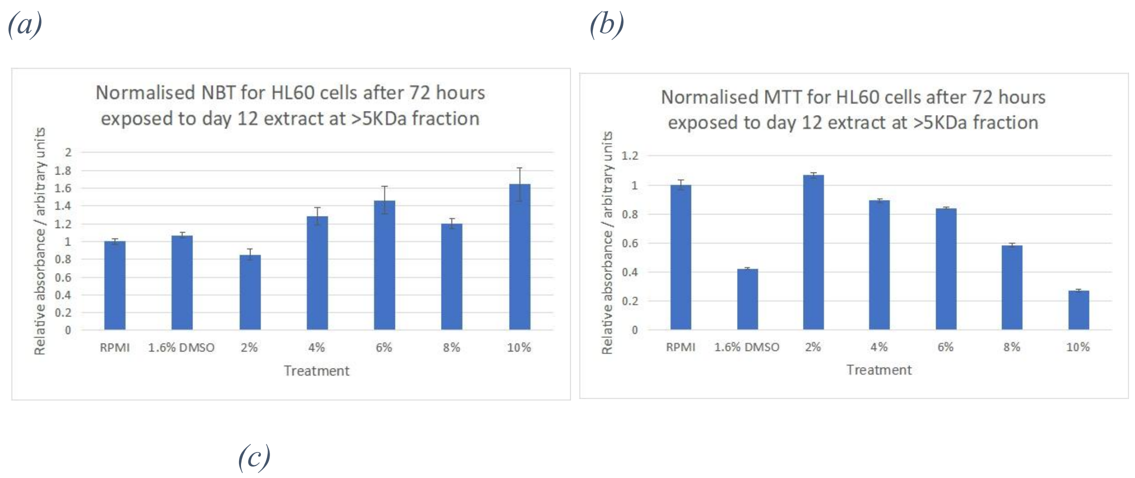
Figure 50: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 120 hours of treatment for the <5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control of day 3 of testing. .

Data was determined to be not normally distributed and hence non-parametric statistical analysis was carried out. Results for this statistical analysis were found in Appendix 5 – part I.Statistical analysis shows that the effect on HL60 cells of the two fractions, < 5KDa and > 5 KDa after 72 hours of exposure to extract, are statistically significantly different from each other with a $p < 0.05$. The > 5 KDa is not statistically significant from the complete fraction at $p < 0.05$. The < 5 KDa is statistically significant from the complete fraction at $p < 0.001$

Statistical analysis shows that the effect on HL60 cells of the two fractions, < 5KDa and > 5 KDa after 120 hours of exposure to extract, are not statistically significantly different from each other with a $p < 0.05$.Both fractions are statistically different from the complete fluid, but the < 5 KDa fraction is more statistically more significantly different with a $p < 0.001$ than the >5 KDa with a $p < 0.005$

3.8.2.2 HL60 cell line exposed to day 12 extract.

Figure 51 represents normalised NBT, MTT and NBT/MTT for HL60 cells after 72 hours exposure to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to >5 KDa fraction. Data can be found in Appendix 5 Part J



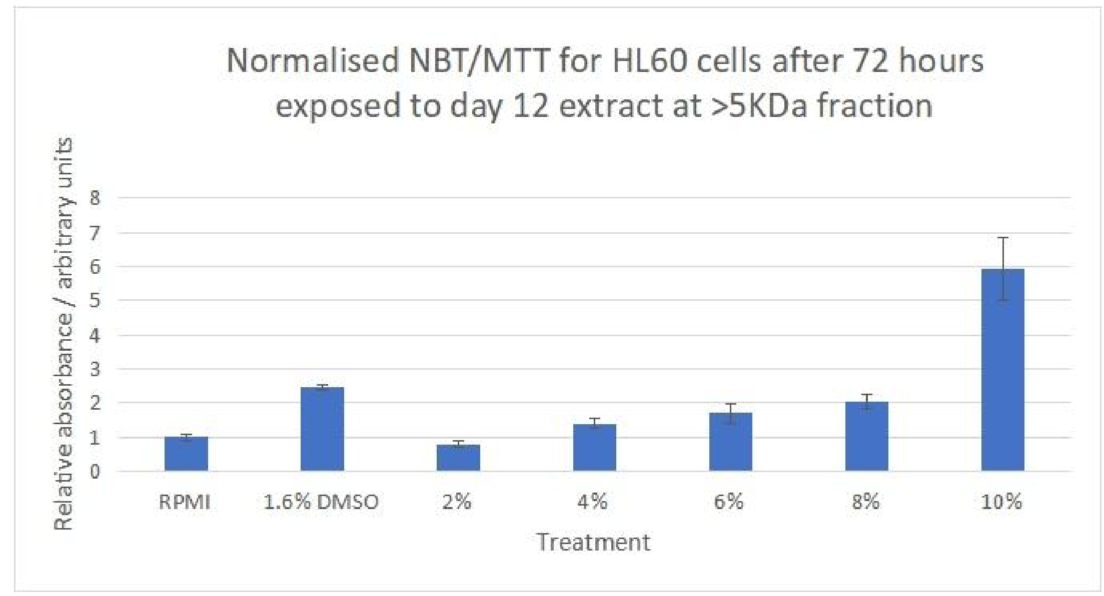
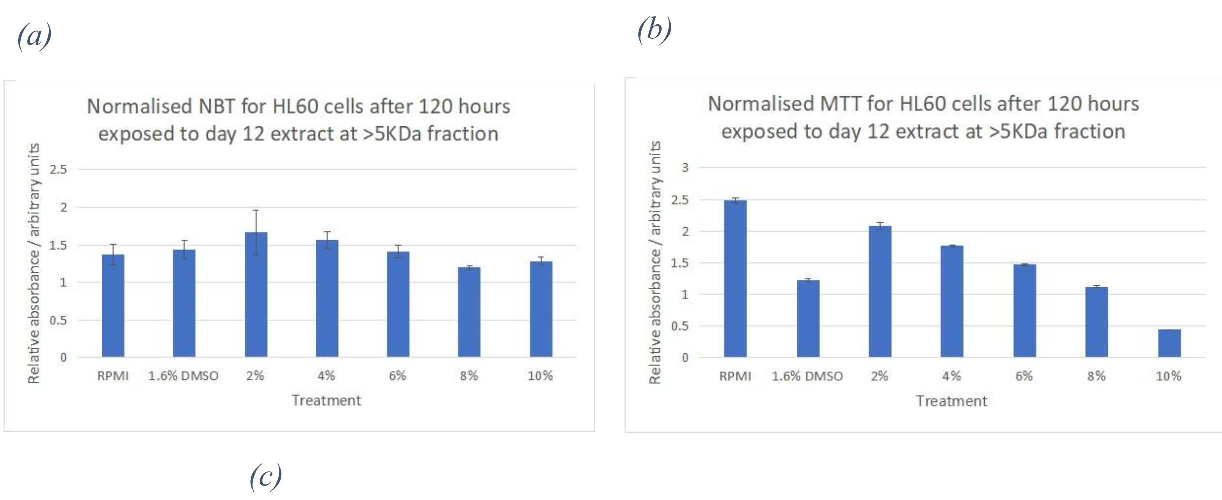


Figure 51: Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 72 hours of treatment for the >5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Data here is very consistent with that data observed for cells exposed to coelomic fluid from day 9.

Figure 52 represents normalised NBT, MTT and NBT/MTT for HL60 cells after 120 hours exposure to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to >5 KDa fraction. Data can be found in Appendix 5 Part J

(a) *(b)*



(c)

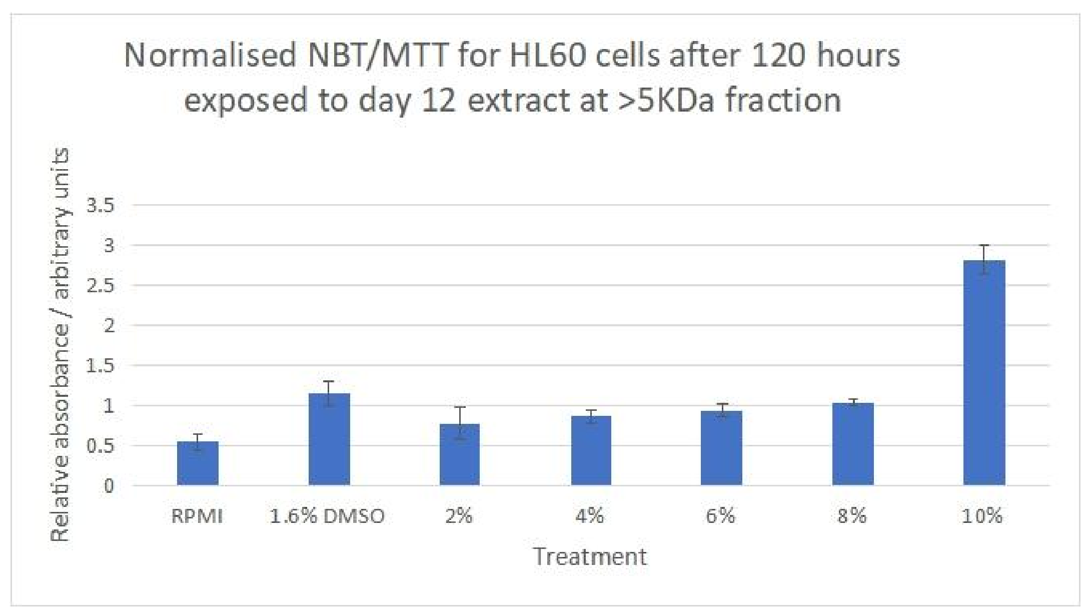
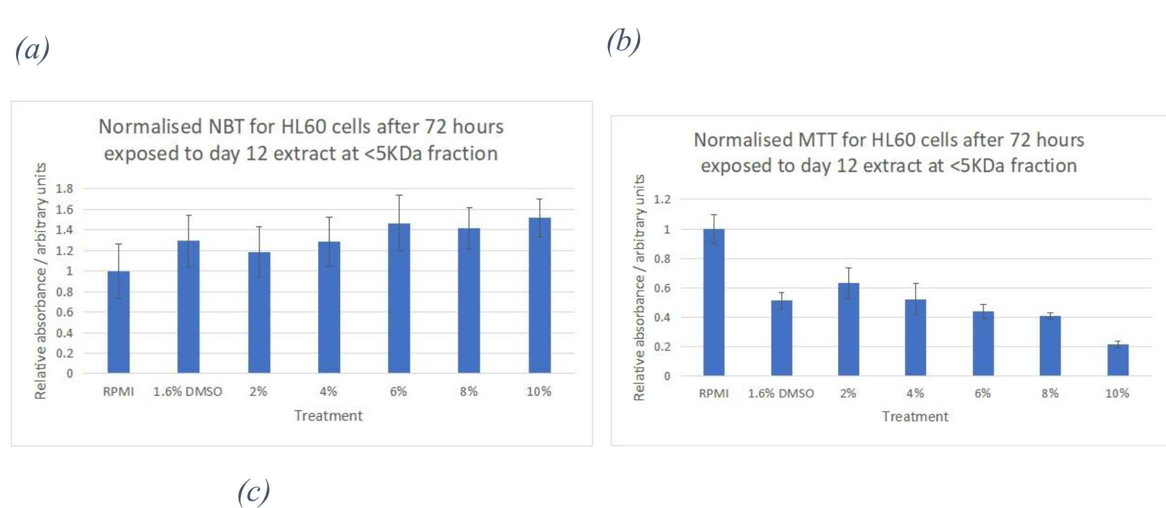


Figure 52: Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 120 hours of treatment for the >5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. VValues were normalised by the RPMI control at 72 hours

In the data shown above, there seems to be a visual similarity between 1.6% DMSO and 6 and 8% concentrations of extract, for NBT readings, MTT readings and NBT/MTT readings. Once again, 10% by volume of coelomic fluid seems to show toxicity.

Figure 53 represents normalised NBT, MTT and NBT/MTT for HL60 cells after 72 hours exposure to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to <5 KDa fraction. Data can be found in Appendix 5 Part J



(c)

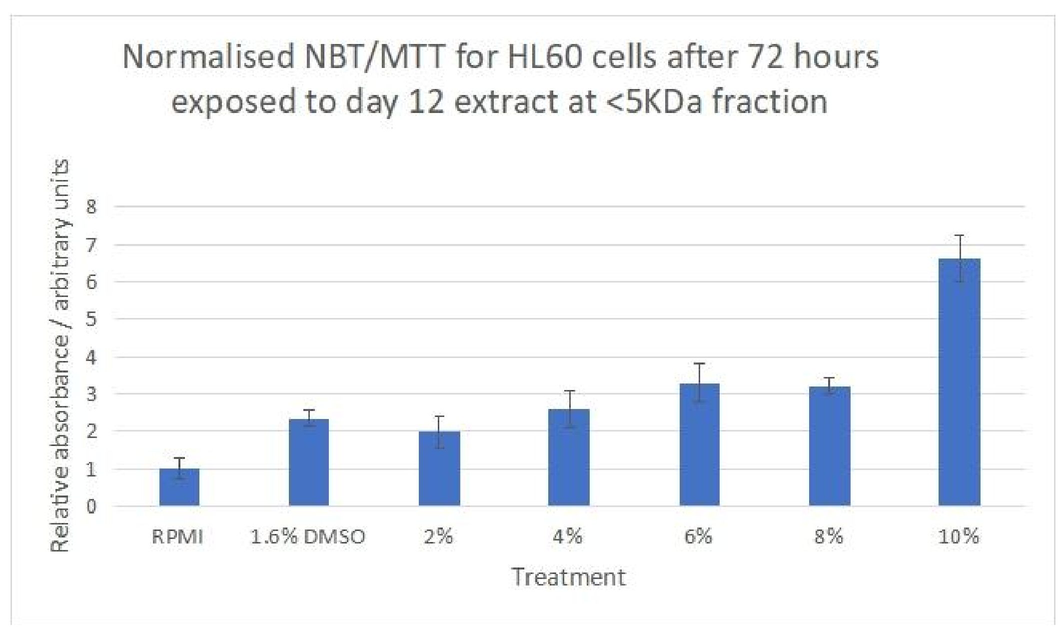
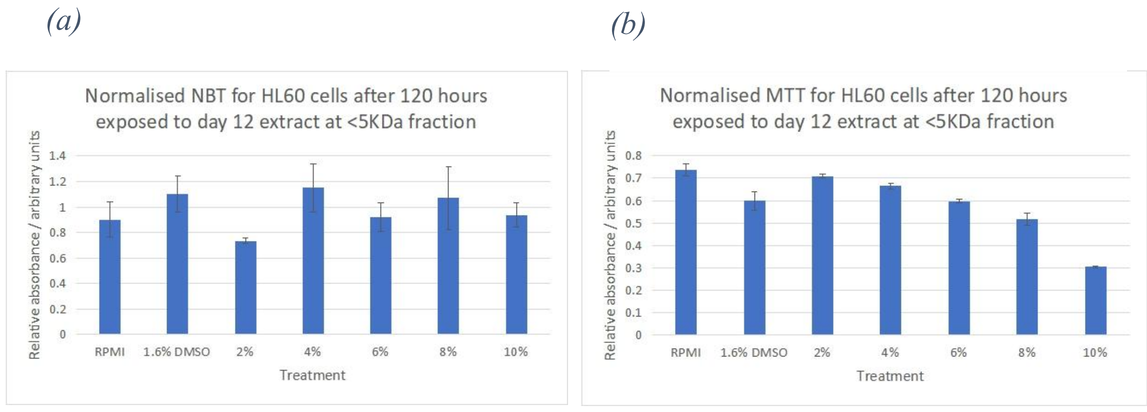


Figure 53: Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 72 hours of treatment for the <5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

This data shows an increase in the value for NBT and a decrease in the value for MTT which is very consistent throughout all the various concentrations of extracts.

Figure 54 represents normalised NBT, MTT and NBT/MTT for HL60 cells after 120 hours exposure to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to <5 KDa fraction. Data can be found in Appendix 5 Part J



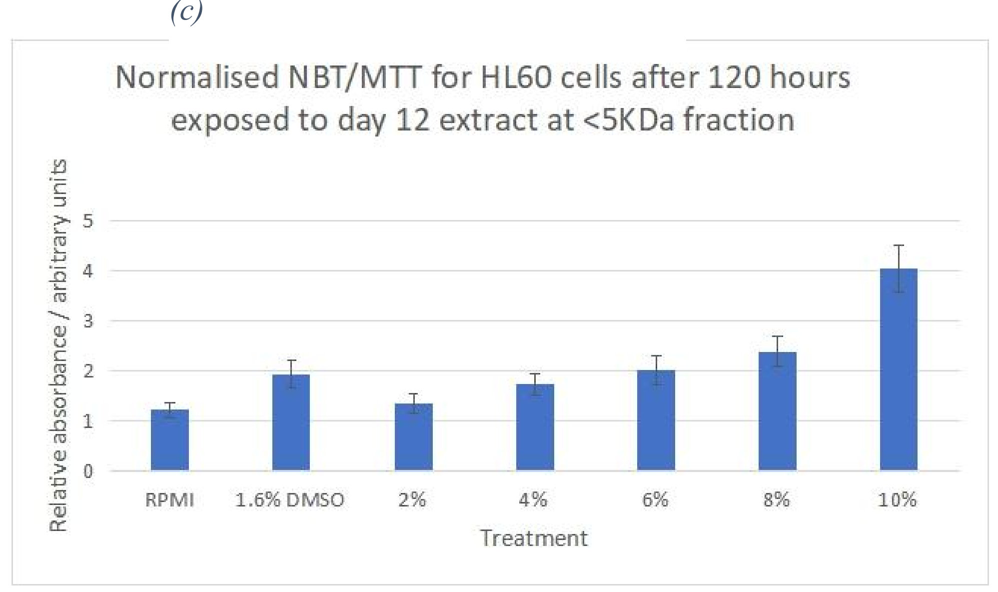


Figure 54: Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 120 hours of treatment for the <5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Here it can be noted that readings for NBT vary throughout the various concentrations of extract, whilst MTT is always decreasing with an increase in the % concentration of extract.

3.8.2.3 K562 cell line exposed to day 9 extract.

Figure 55 represents normalised NBT, MTT and NBT/MTT for K562 cells after 72 hours exposure to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to >5 KDa fraction. Data can be found in Appendix 5 Part J

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(a) *(b)*

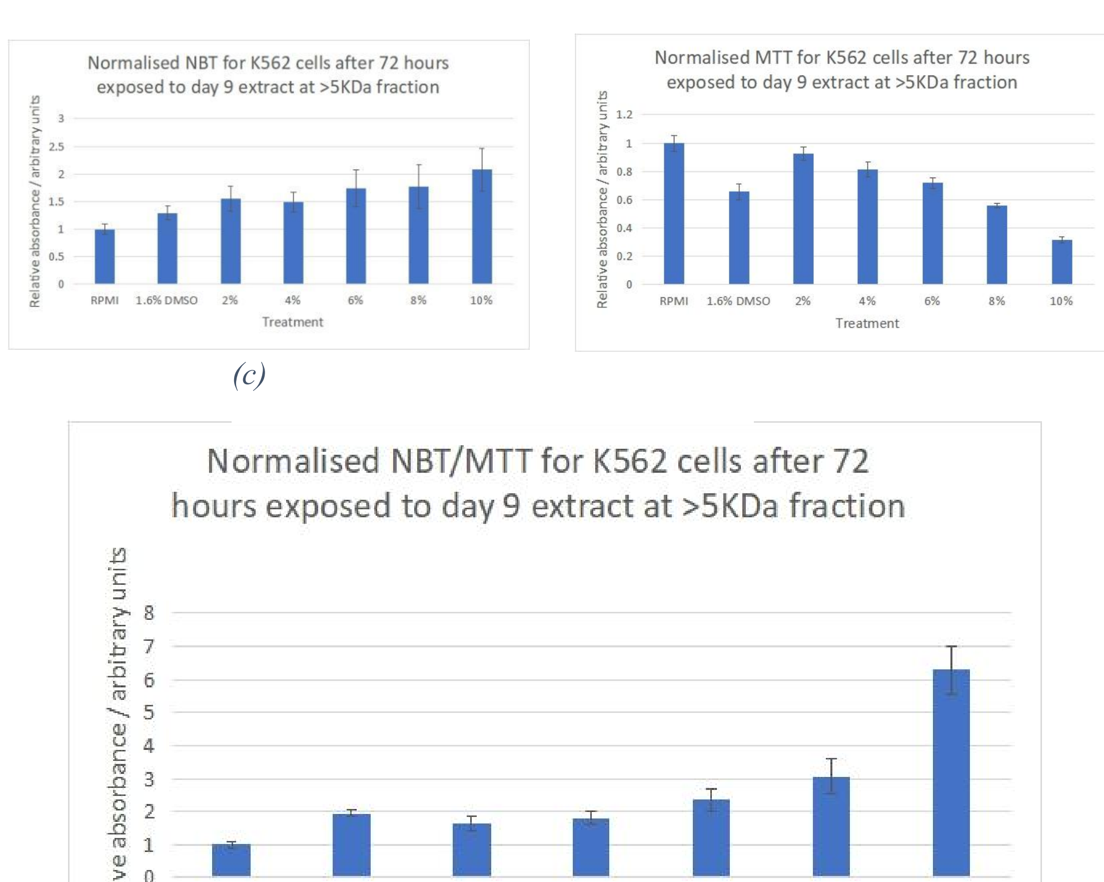
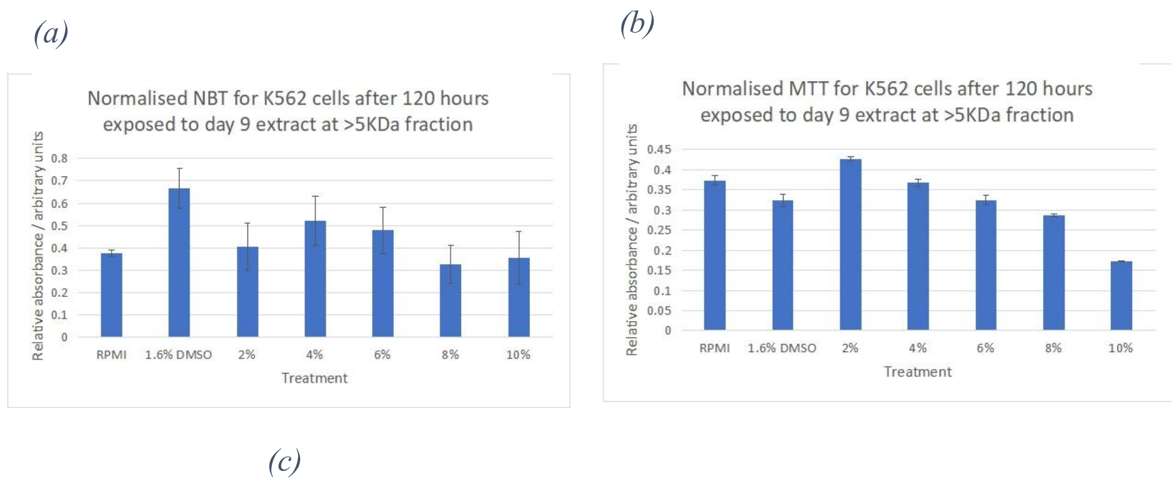


Figure 55: Effect of coelomic fluid from day 9 of regeneration on K562 cells after 72 hours of treatment for the >5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Here it can be noted that there is a consistent increase in NBT as the % concentration increases, whilst the value for MTT decreases with an increase in % concentration.

Figure 56 represents normalised NBT, MTT and NBT/MTT for K562, cells after 120 hours exposure to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to >5 KDa fraction. Data can be found in Appendix 5 Part J



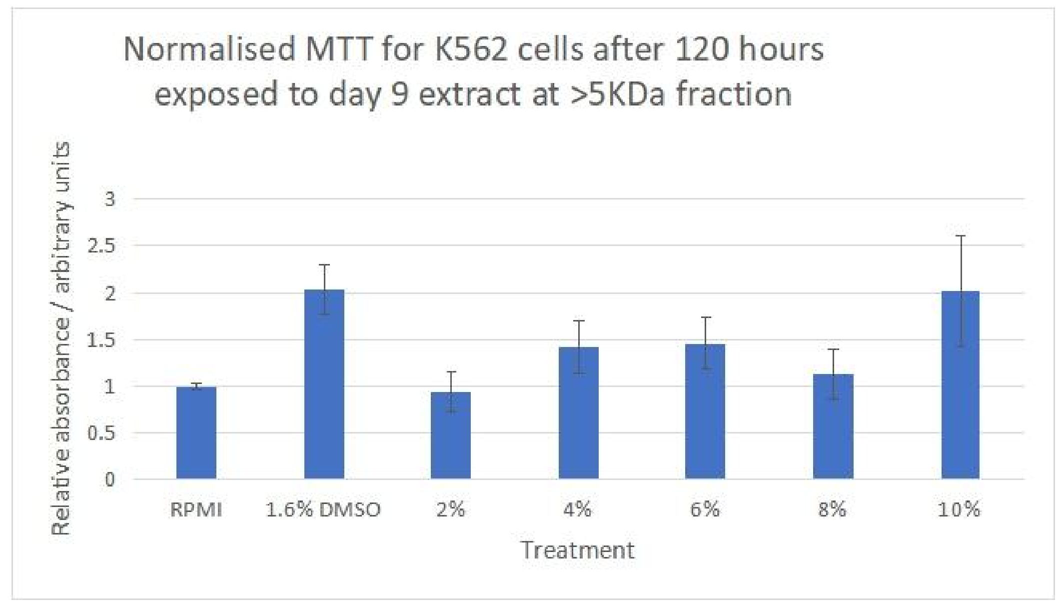
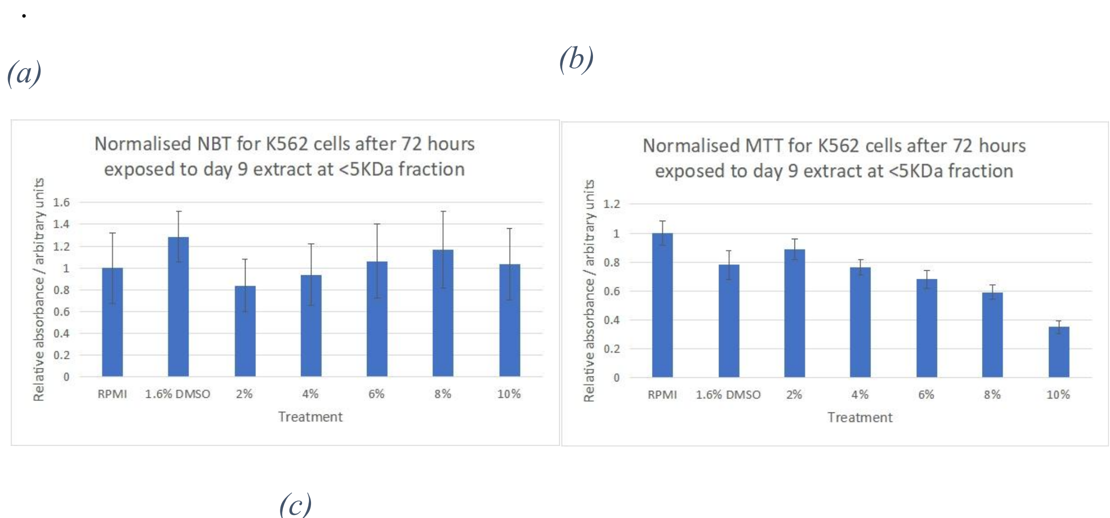


Figure 56: Effect of coelomic fluid from day 9 of regeneration on K562 cells after 120 hours of treatment for the >5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Here it can be noted that readings for NBT vary throughout the various concentrations of extract, whilst MTT is always decreasing with an increase in the % concentration of extract.

Figure 57 represents normalised NBT, MTT and NBT/MTT for K562 cells after 72 hours exposure to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to <5 KDa fraction. Data can be found in Appendix 5 Part J

.



(c)

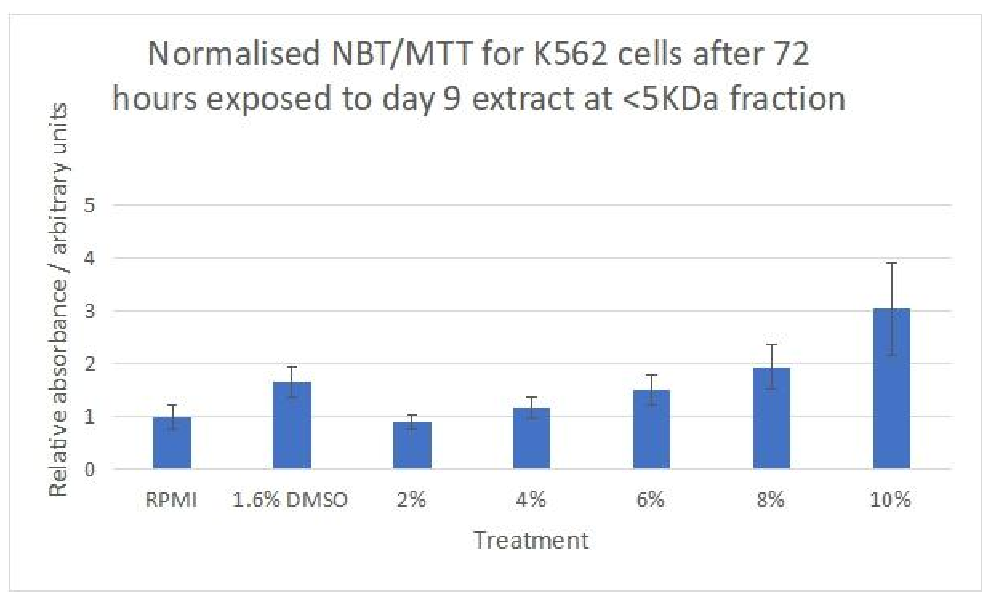
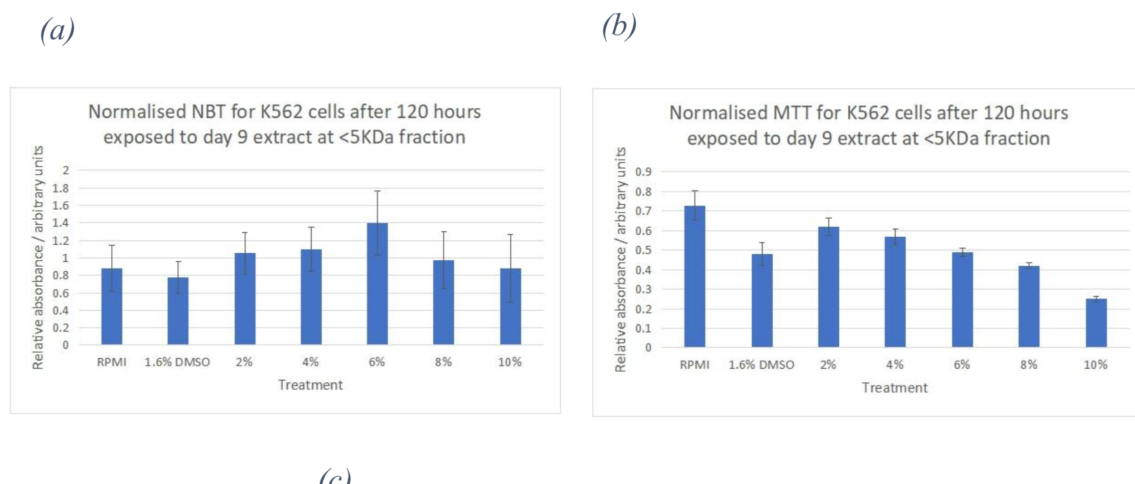


Figure 57: Effect of coelomic fluid from day 9 of regeneration on K562 cells after 72 hours of treatment for the <5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Here NBT values increase up to 8% concentration of extract, then decrease, whilst MTT readings decrease throughout the increase in % concentration of extract.

Figure 58 represents normalised NBT, MTT and NBT/MTT for K562 cells after 120 hours exposure to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to <5 KDa fraction. Data can be found in Appendix 5 Part J



(c)

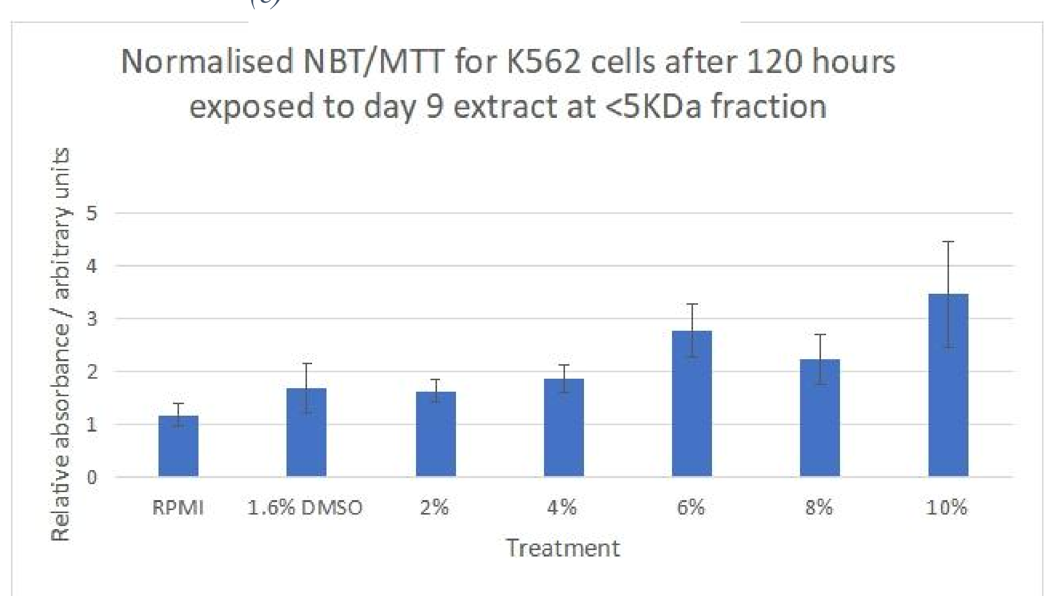


Figure 58: Effect of coelomic fluid from day 9 of regeneration on K562 cells after 120 hours of treatment for the <5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Here NBT values increase up to 6% concentration of extract, then decease, whilst MTT readings decrease throughout the increase in % concentration of extract.

Through statistical analysis (appendix 5 - Part I) it was determined that in the case of extracts on the K562 cell line, 72 hours after being exposed to treatment, both the >5 KDa and the <5 KDa fractions were significantly different ($p<0.005$)from the complete extract. However, the two fractions, those that are <5KDa and >5KDa were not statistically different from each other.

In the case of extracts on the K562 cell line, 120 hours after treatment, statistical analysis showed that there was not statistically significantly difference between any extracts and the values are all larger than 0.05. The p value when comparing difference between complete extract with <5KDa is the highest showing there is the least significant difference between these two.

.

3.8.2.4 K562 cell line exposed to day 12 extract.

Figure 59 represents normalised NBT, MTT and NBT/MTT for K562 cells after 72 hours exposure to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to >5 KDa fraction. Data can be found in Appendix 5 Part J

.

(a) *(b)*

Normalised NBT for K562 cells after 72 hours exposed to day 12 extract at >5KDa fraction

Relative absorbance / arbitrary units

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

RPMI 1.6% DMSO 2% 4% 6% 8% 10%

Treatment

Normalised MTT for K562 cells after 72 hours exposed to day 12 extract at >5KDa fraction

Relative absorbance / arbitrary units

1.4
1.2
1
0.8
0.6
0.4
0.2
0

RPMI 1.6% DMSO 2% 4% 6% 8% 10%

Treatment

(c)

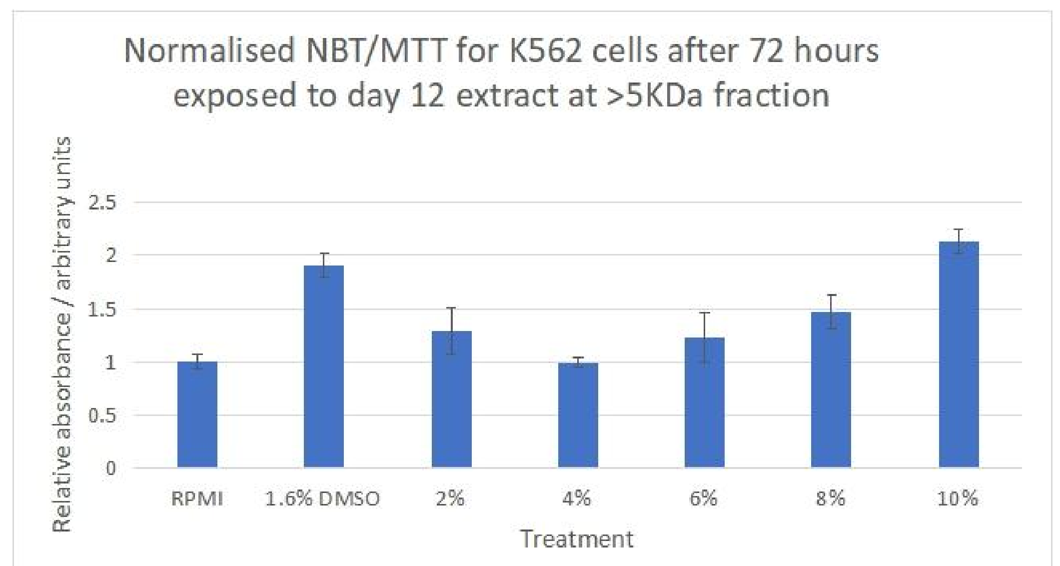


Figure 59 Effect of coelomic fluid from day 12 of regeneration on K562 cells after 72 hours of treatment for the >5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

There is no evident pattern in the data here. The consistent increase in possible cytotoxicity with an increase in concentration is still noted.

Figure 60 represents normalised NBT, MTT and NBT/MTT for K562 cells after 120 hours exposure to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to >5 KDa fraction. Data can be found in Appendix 5 Part J

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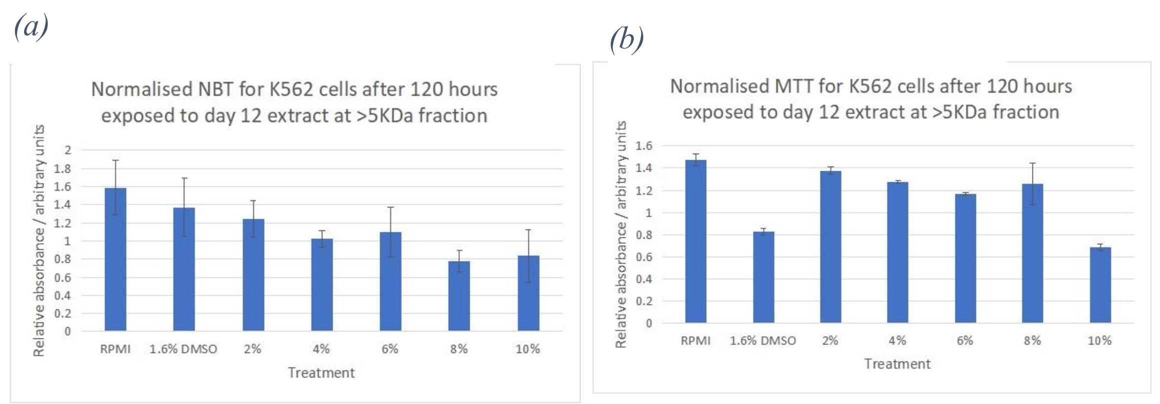


(c)

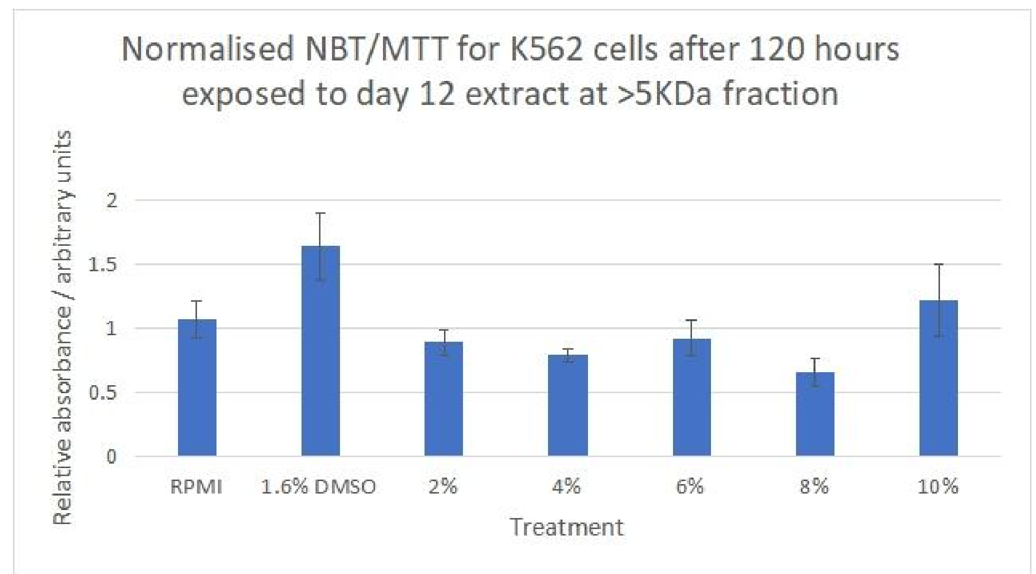


Figure 60: Effect of coelomic fluid from day 12 of regeneration on K562 cells after 120 hours of treatment for the >5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

There doesn’t seem to be a distinct pattern in the changes observed. However, the consistent increase in possible cytotoxicity with an increase in concentration is still noted.

Figure 61 represents normalised NBT, MTT and NBT/MTT for K562 cells after 72 hours exposure to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to <5 KDa fraction. Data can be found in Appendix 5 Part J

.

(a)

Normalised NBT for K562 cells after 72 hours exposed to day 12 extract at <5KDa fraction

(b)

Normalised MTT for K562 cells after 72 hours exposed to day 12 extract at <5KDa fraction

(c)

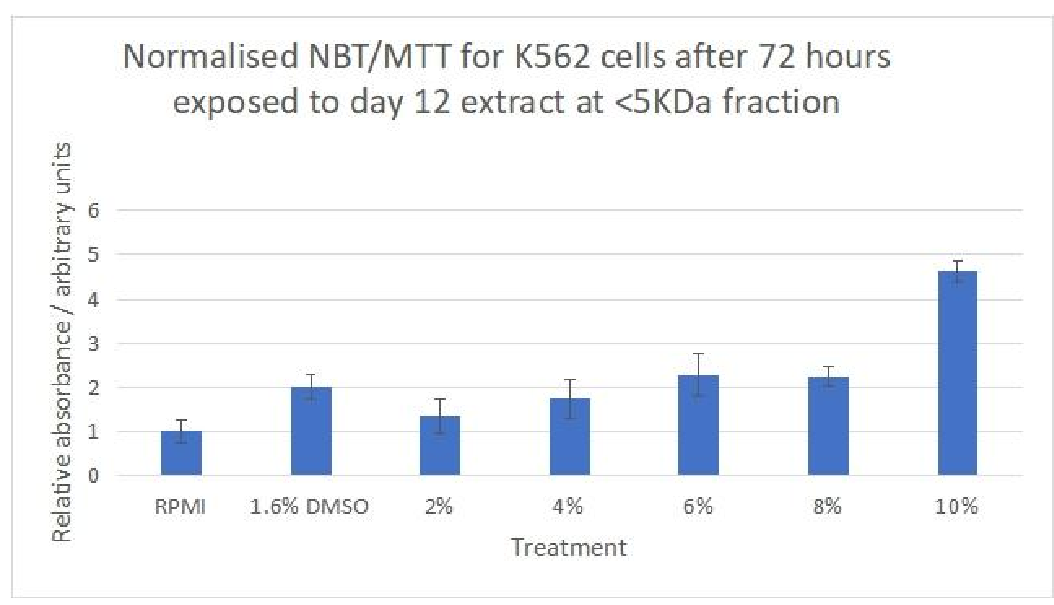
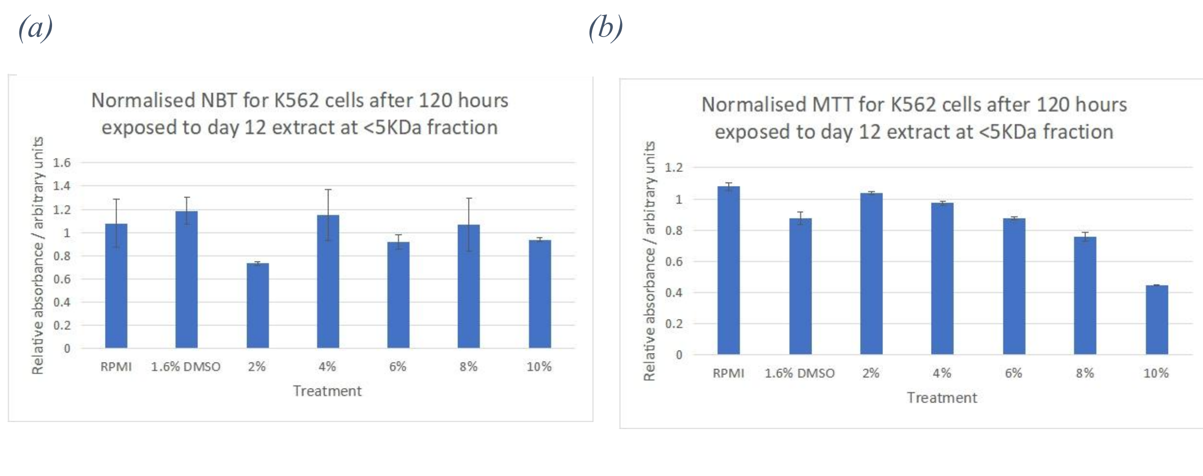


Figure 61: Effect of coelomic fluid from day 12 of regeneration on K562 cells after 72 hours of treatment for the <5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

The data here seems to be visually very similar to that seen in figure 56. Here it can be noted that there is a consistent increase in NBT as the % concentration increases, whilst the value for MTT decreases with an increase in % concentration.

Figure 62 represents normalised NBT, MTT and NBT/MTT for K562 cells after 120 hours exposure to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to <5 KDa fraction. Data can be found in Appendix 5 Part J

(a) *(b)*



(c)

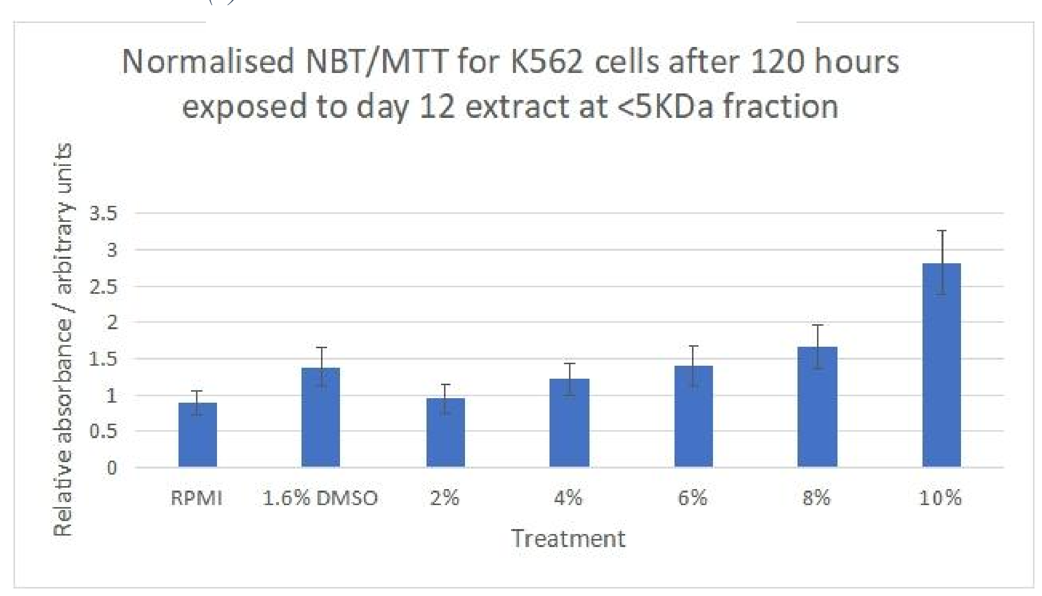


Figure 62: Effect of coelomic fluid from day 12 of regeneration on K562 cells after 120 hours of treatment for the <5KDa fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

The results obtained here seem to be in line with most of the results associated with the K562 cell line. There isn't a particular pattern associated with NBT reading as % concentration increases, and there is the consistent decrease in MTT readings as % concentration increases. Therefore the most reliable results are those associated with HL60.

3.8.3 Extraction in solvents

When taking readings for absorbance, absorbance for DMEM was taken as the baseline for comparison of all other readings. DMEM comes in a container with a label showing a list of the components and their concentration. The total concentration of solute was of 10 µg/ µL and this was equivalent to an absorbance of 13 = 10 µg/ µL. The absorbance given by the complete fluid as well as the two fractions obtained in the ether and ester solvents was compared to this baseline in order to get an indication of the concentration of the solutes present in µg/ µL. Salt doesn't dissolve in these solvents, so it was automatically removed during the extraction. The following readings were taken for the sample dissolved in 5 mL DMEM without indicator, so that its colour doesn't interfere with the absorbance reading.

Table 12: Table showing Mass of solid residue from individual organisms used in this part of this study. Three different specimens were taken for this part, and the coelomic fluid was separately analysed and its content quantified.

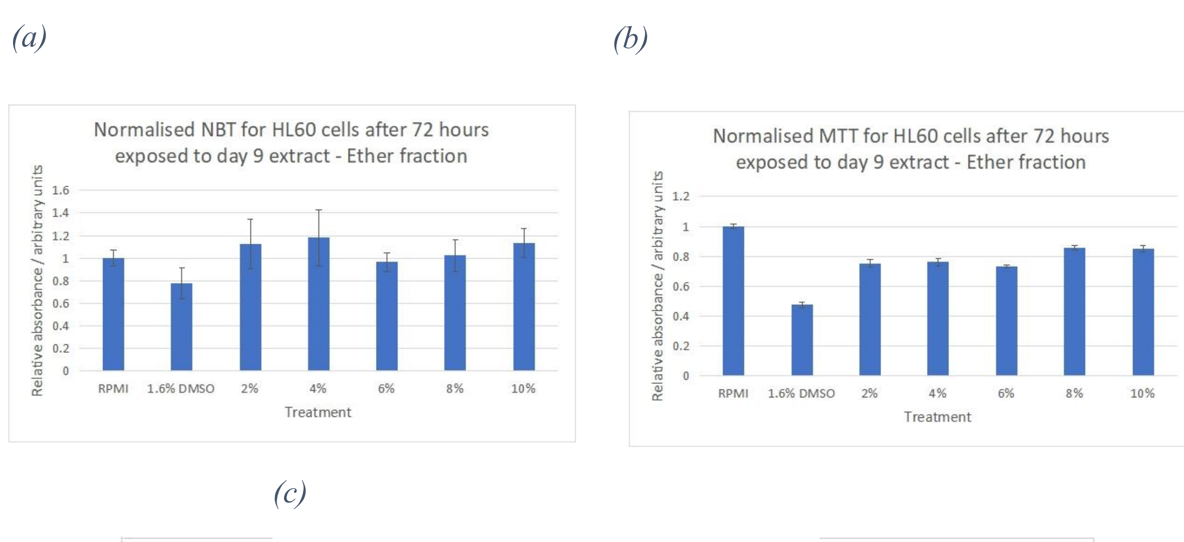
Sample identity	**Day**	**Mass of specimen (g)**	**Solvent used in extraction**	**Mass of conical bottomed tube + sample (g)**	**Mass of empty conical bottomed tube (g)**	**Mass of solid residue from animal (g)**
A1	9	46.04	Ether	13.02	12.53	0.49
A2	9	52.67	Ether	13.07	12.53	0.48
A3	9	48.19	Ether	13.18	12.53	0.65
B1	12	58.41	Ether	13.23	12.53	0.7
B2	12	49.87	Ether	13.15	12.53	0.62
B3	12	47.36	Ether	13.21	12.53	0.68
C1	9	42.41	Ester	12.94	12.53	0.41
C2	9	42.46	Ester	12.99	12.53	0.46
C3	9	45.73	Ester	13.11	12.53	0.58
D1	12	54.64	Ester	13.17	12.53	1.05
D2	12	43.01	Ester	13.49	12.53	0.96
D3	12	46.08	Ester	13.26	12.53	0.73

Table 13: Calculation of mass of solute (excluding salt) in µg/ µL found in the individual organisms. Three different specimens were taken for this part, and the coelomic fluid was separately analysed and its content quantified.

Sample identity	**Absorbance given by freeze-dried extract before extraction in the ether or ester solvent.**	**Initial concentration µg/ µL**	**absorbance given by the extract after it was shaken with ether or ester solvent.**	**Final concentration µg/ µL**
A1	3.25	2.50	0.4004	0.308
A2	3.01	2.32	0.4225	0.325
A3	2.45	1.88	0.4043	0.311
B1	2	1.53	0.35	0.269
B2	3.35	2.58	0.3211	0.247
B3	3.16	2.43	0.39	0.300
C1	1.5	1.15	1	0.769
C2	1.65	1.27	1.01	0.777
C3	1.8	1.38	1.09	0.841
D1	3.5	2.69	0.75	0.577
D2	2.85	2.19	0.9906	0.762
D3	3.11	2.39	0.880	0.677

3.8.3.1 HL60 cell line exposed to day 9 extract.

Figure 63 represents normalised NBT, MTT and NBT/MTT for HL60 exposed for 72 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to Ether fraction. Data can be found in Appendix 5 Part L



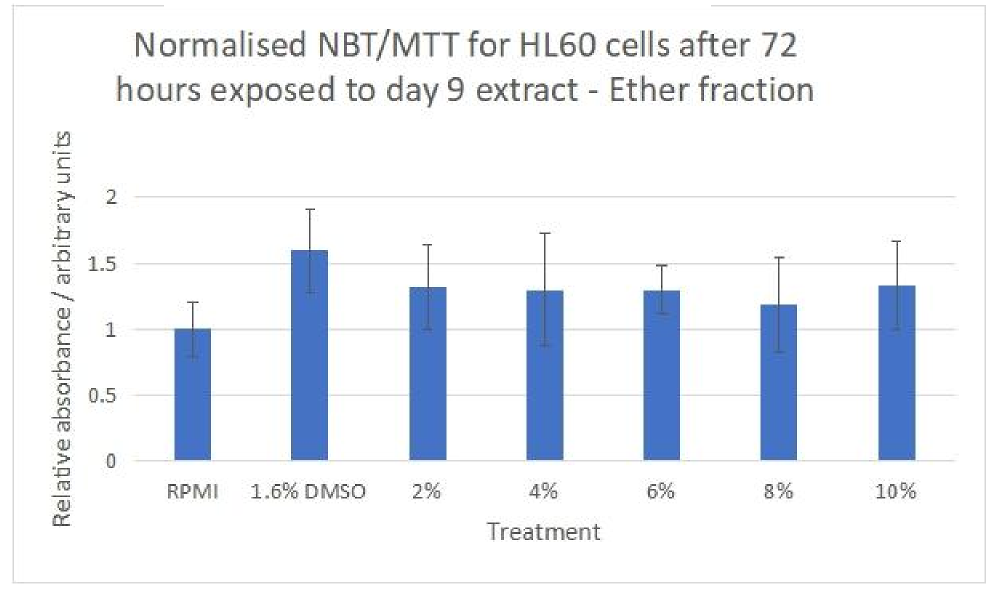
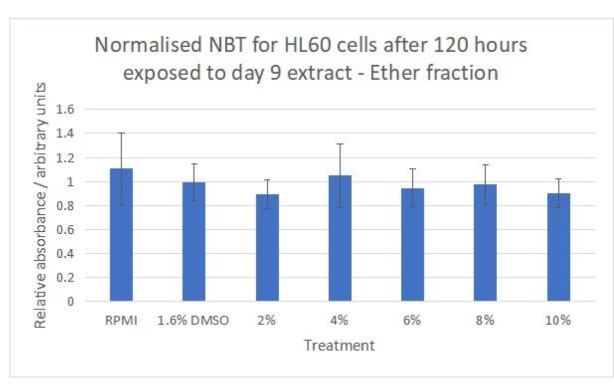


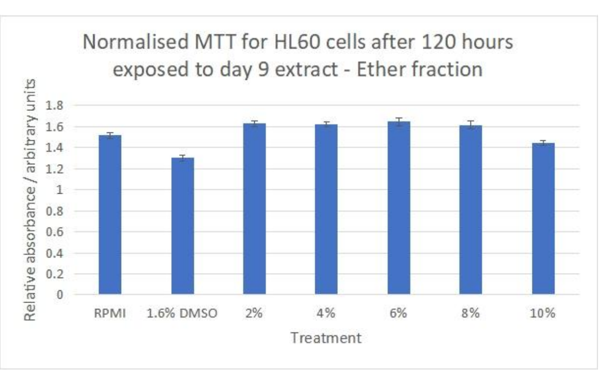
Figure 63: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 72 hours of treatment for the ether fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Here it could be noted that data for both MTT and NBT was consistently high across all concentrations. Through visual inspection only, it is very difficult to notice any similarity between extracts, RPMI and 1.6% DMSO.

Figure 64 represents normalised NBT, MTT and NBT/MTT for HL60, 120 hours from coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to Ether fraction. Data can be found in Appendix 5 Part L

(a) *(b)*





(c)

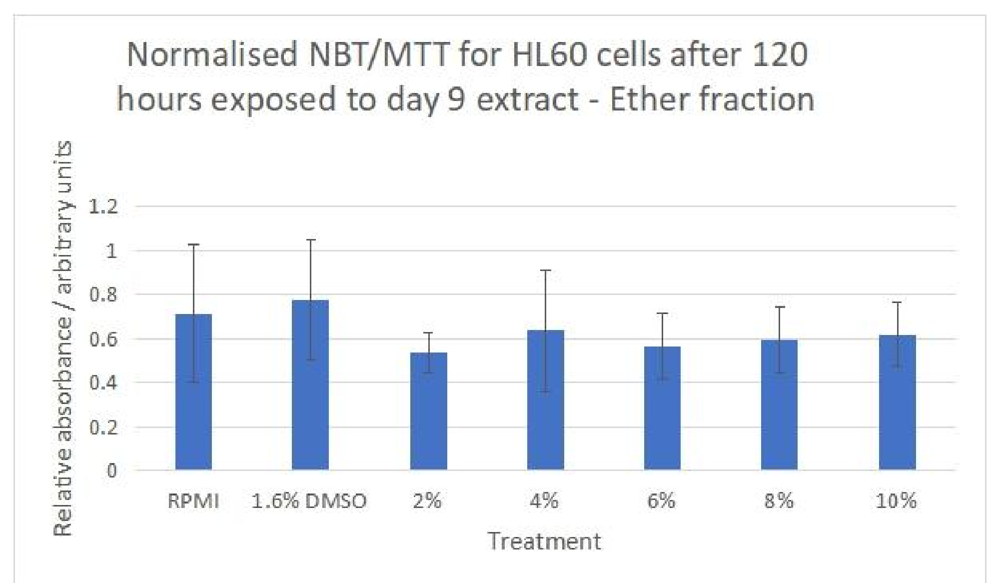


Figure 64: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 120 hours of treatment for the ether fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Here it could be noted that data for both MTT and NBT was consistently high across all concentrations. Through visual inspection only, it is very difficult to notice any similarity between extracts, RPMI and 1.6% DMSO.

Figure 65 represents normalised NBT, MTT and NBT/MTT for HL60 exposed for 72 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to Ester fraction. Data can be found in Appendix 5 Part L

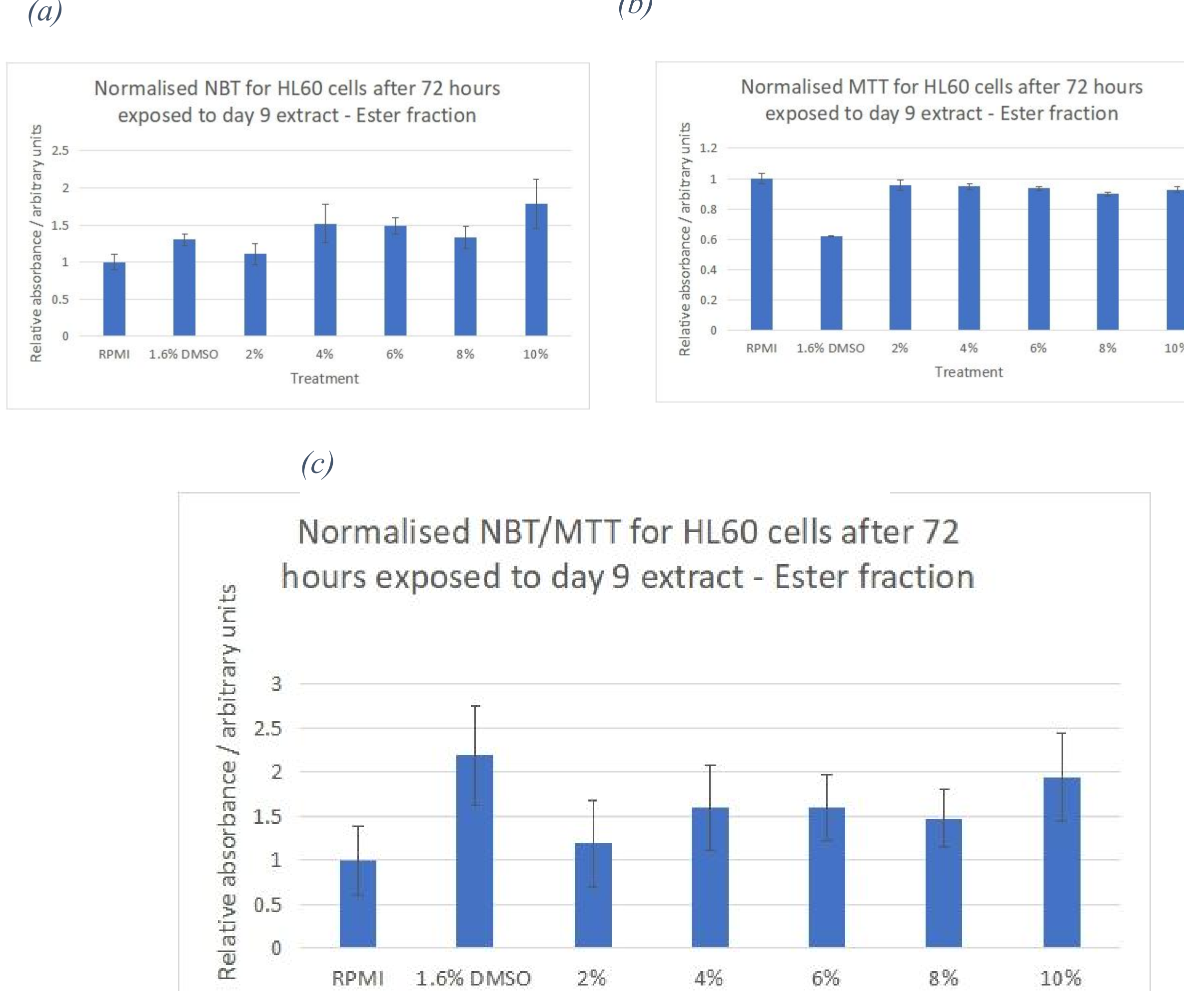
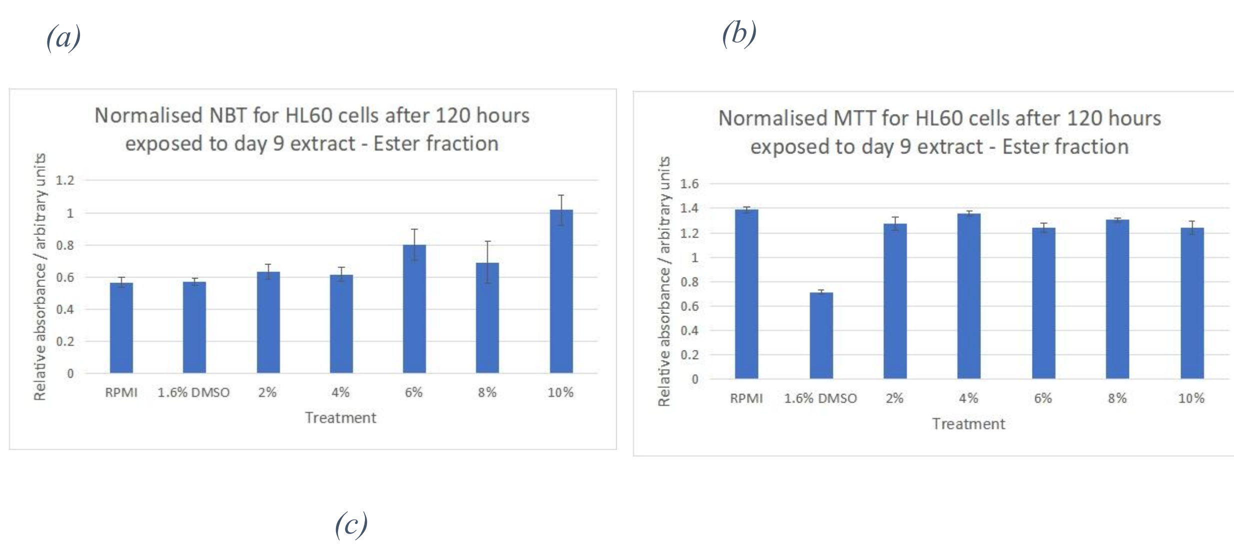


Figure 65: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 72hours of treatment for the ester fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Here it could be noted that data for both MTT and NBT was consistently high across all concentrations. Through visual inspection only, it is very difficult to notice any similarity between extracts, RPMI and 1.6% DMSO.

Figure 66 represents normalised NBT, MTT and NBT/MTT for HL60 exposed for 120 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to Ester fraction. Data can be found in Appendix 5 Part L

(a) *(b)*



(c)

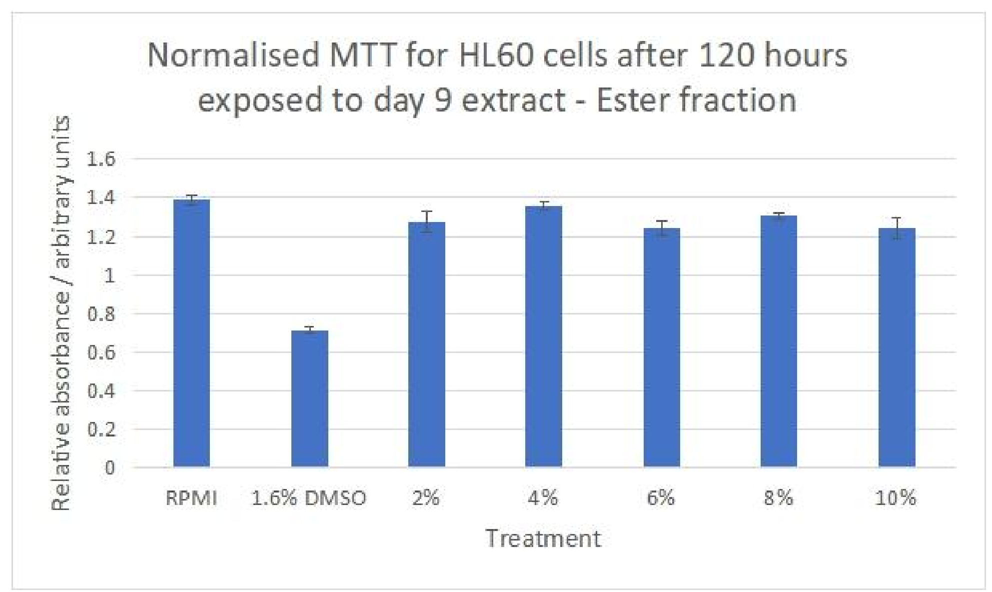


Figure 66: Effect of coelomic fluid from day 9 of regeneration on HL60 cells after 120 hours of treatment for the ester fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

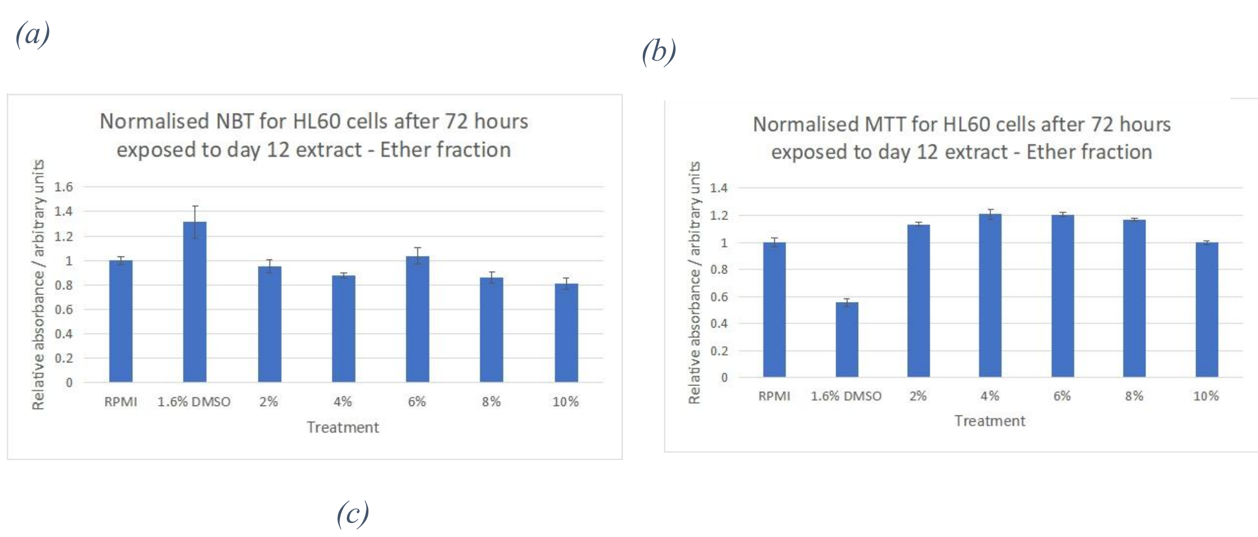
Here it could be noted that data for both MTT and NBT was consistently high across all concentrations. Through visual inspection only, it is very difficult to notice any similarity between extracts, RPMI and/or 1.6% DMSO.

Statistical analysis was carried out and it was noted that from the results obtained when the cells were left exposed to treatment for 72 hours, all fractions were significantly different with a $p<0.05$. The significant difference between <5KDa fraction and the ester and ether fractions was in both cases very high with a $p < 0.001$. The statistical significant difference between the ester fraction and the ether fraction were still very strong with a $p<0.05$ but not as strong as the difference between the <5KDa and both ester and ether fractions.

In the case when the cells were left exposed to treatment for 120 hours, all fractions were statistically significant different except for the ether fraction when compared to <5KDa fraction.

3.8.3.2 HL60 cell line exposed to day 12 extract.

Figure 67 represents normalised NBT, MTT and NBT/MTT for HL60 exposed for 72 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to Ether fraction. Data can be found in Appendix 5 Part L



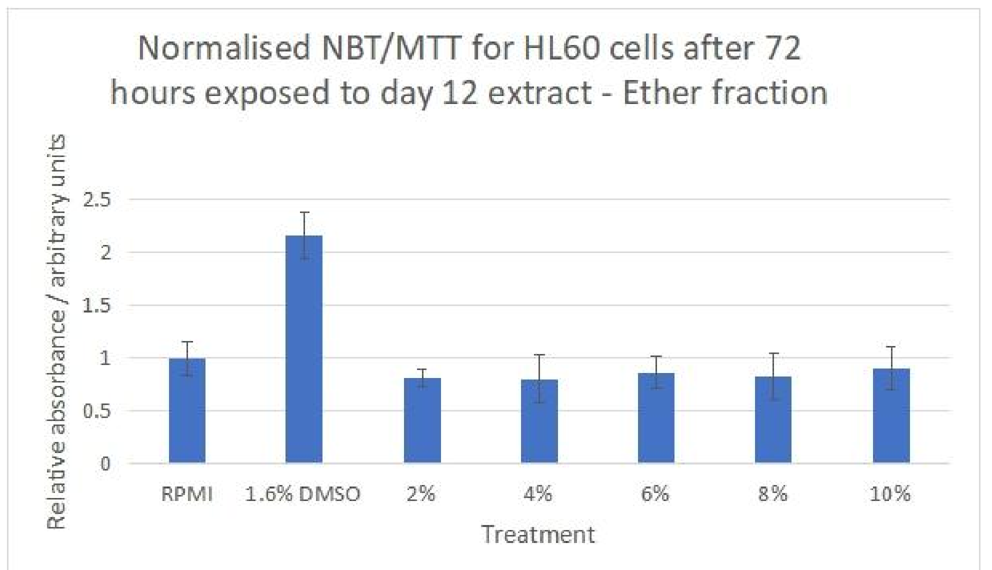
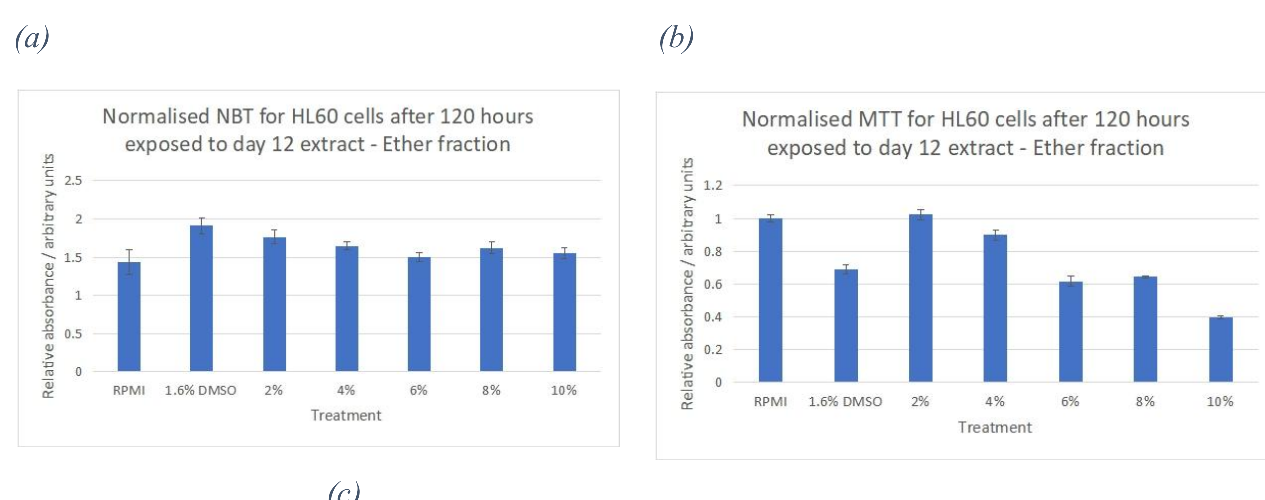


Figure 67: Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 72 hours of treatment for the ether fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Figure 68 represents normalised NBT, MTT and NBT/MTT for HL60 exposed for 120 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to Ether fraction. Data can be found in Appendix 5 Part L

(a) *(b)*



(c)

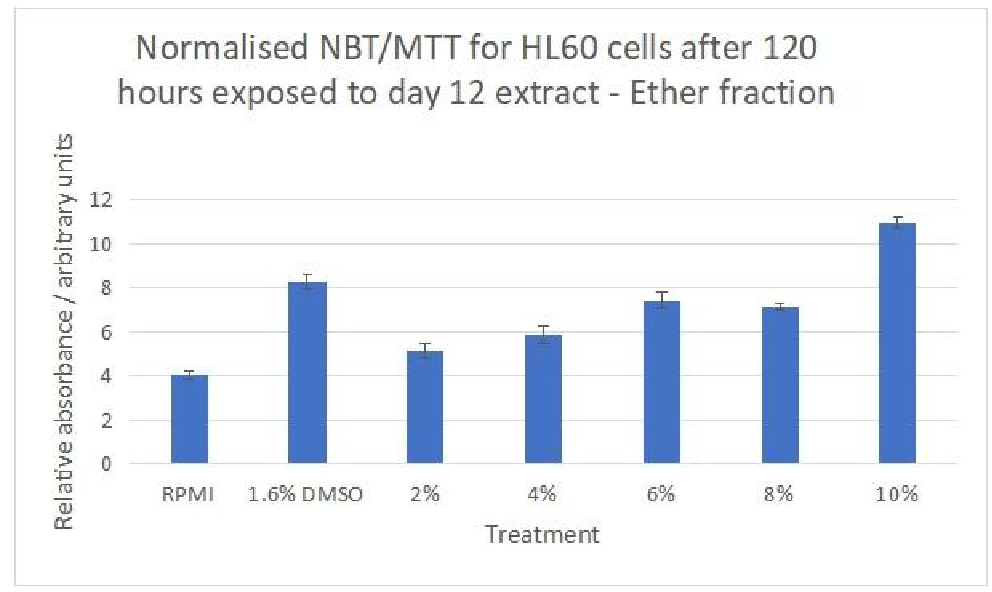
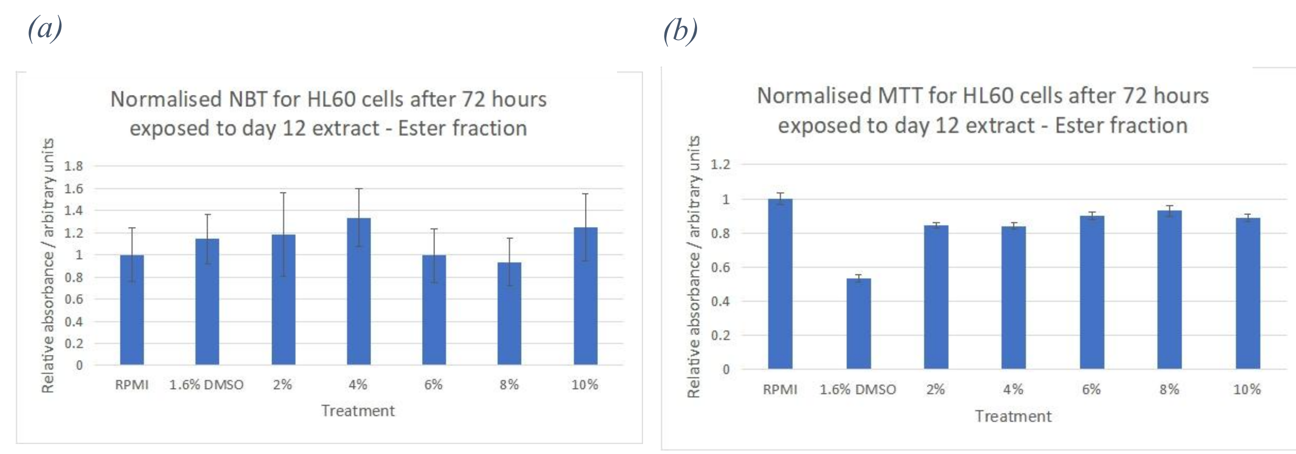


Figure 68: Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 120 hours of treatment for the ether fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. VValues were normalised by the RPMI control at 72 hours

Figure 69 represents normalised NBT, MTT and NBT/MTT for HL60 exposed for 72 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to Ester fraction. Data can be found in Appendix 5 Part L



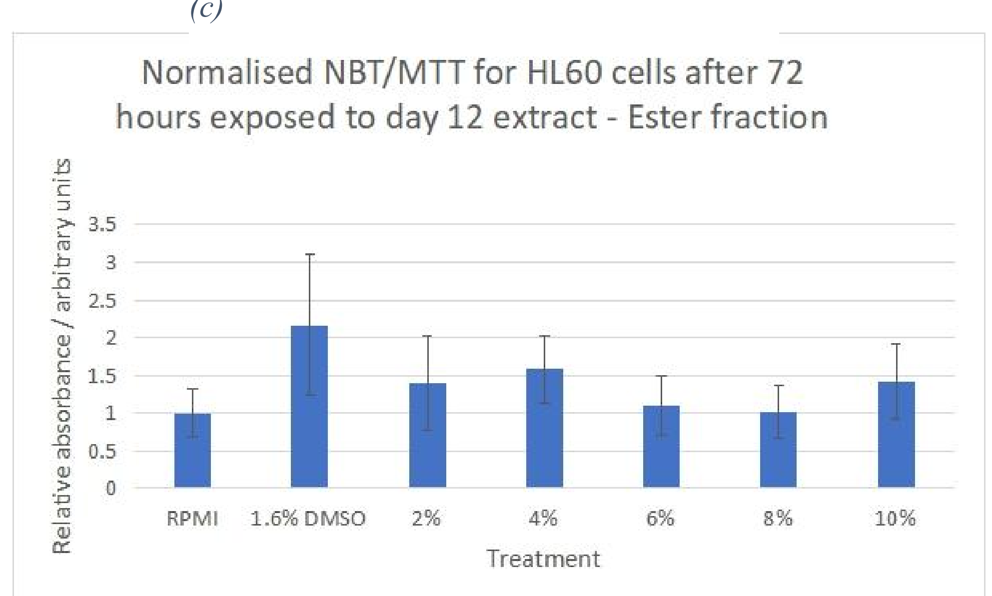
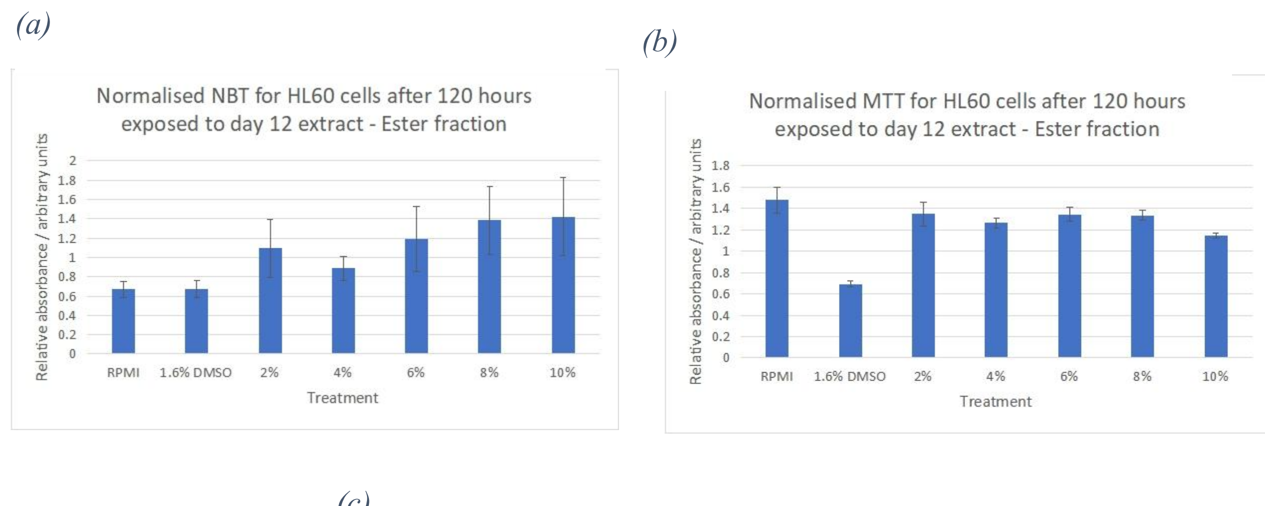


Figure 69: Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 72 hours of treatment for the ester fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Figure 70 represents normalised NBT, MTT and NBT/MTT for HL60 exposed for 120 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to Ester fraction. Data can be found in Appendix 5 Part L



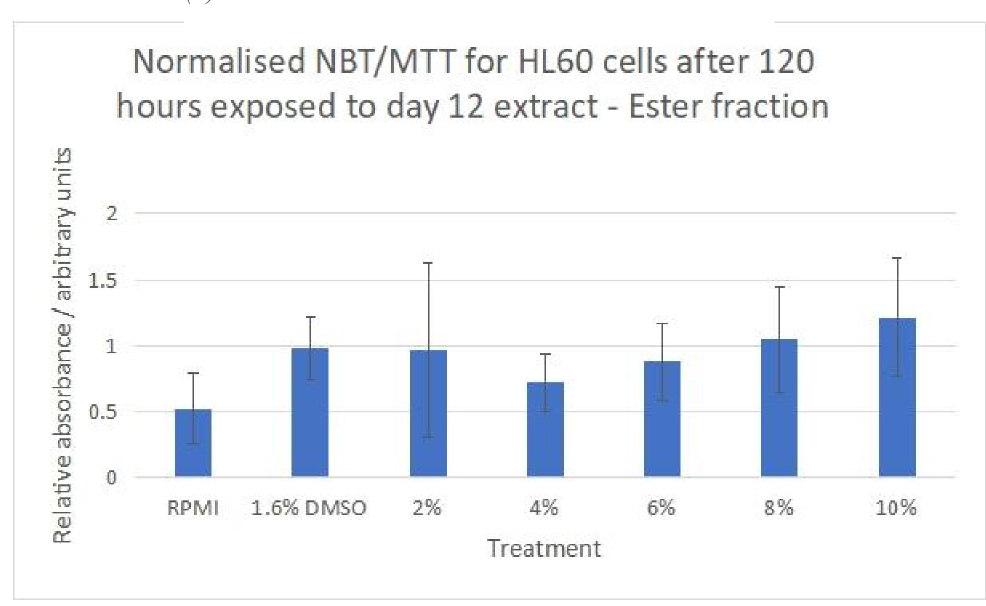
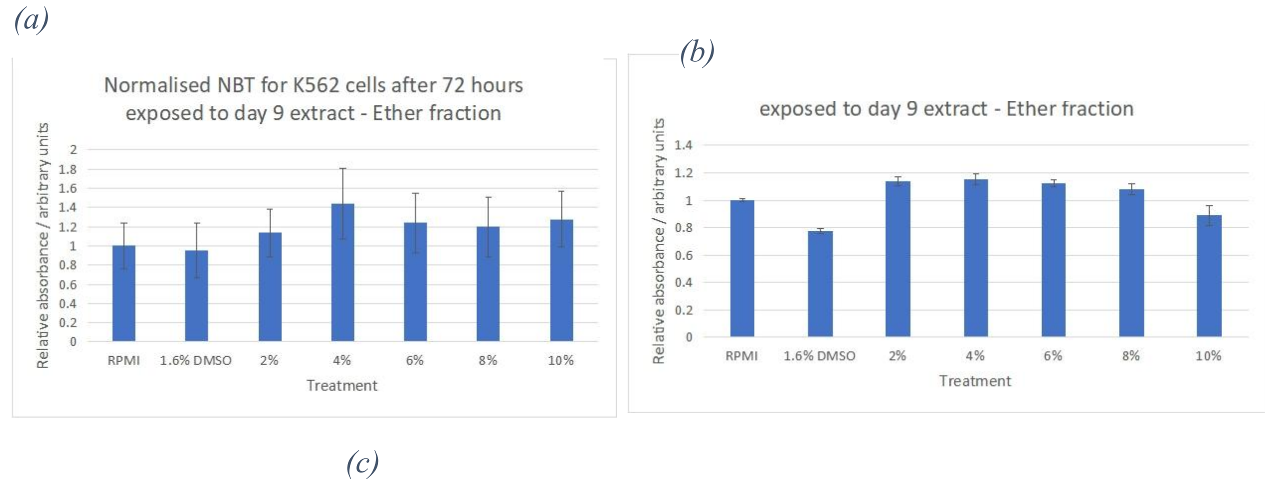


Figure 70: Effect of coelomic fluid from day 12 of regeneration on HL60 cells after 120 hours of treatment for the ester fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

3.8.3.3 K562 cell line exposed to day 9 extract.

Figure 71 represents normalised NBT, MTT and NBT/MTT for K562, 72 hours from coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to Ether fraction. Data can be found in Appendix 5 Part L



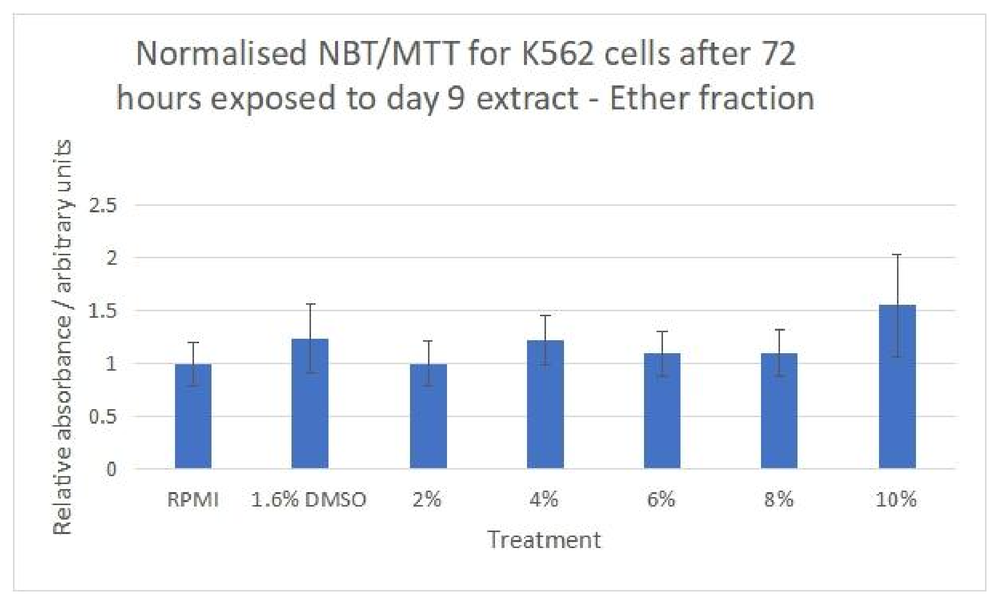
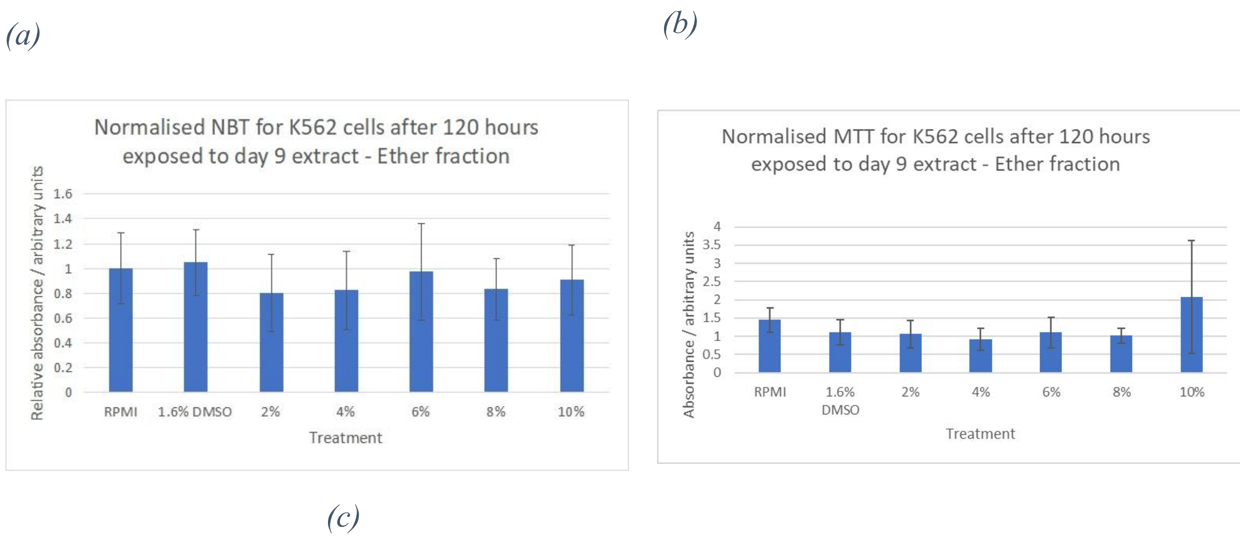


Figure 71 Effect of coelomic fluid from day 9 of regeneration on K562 cells after 72 hours of treatment for the ether fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Figure 72 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 120 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to Ether fraction. Data can be found in Appendix 5 Part L



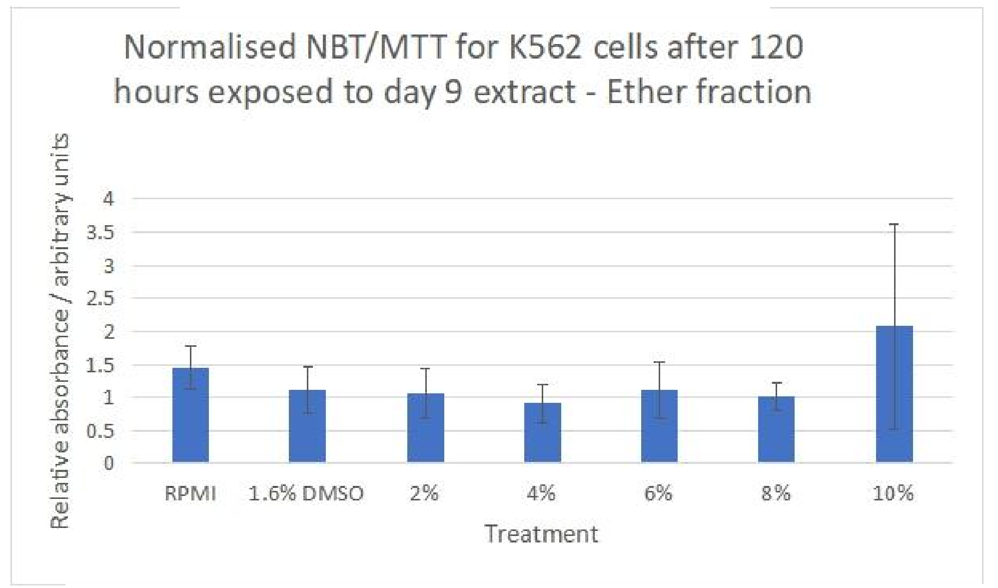


Figure 72 Effect of coelomic fluid from day 9 of regeneration on K562 cells after 120 hours of treatment for the ether fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Figure 73 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 72 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to Ester fraction. Data can be found in Appendix 5 Part L

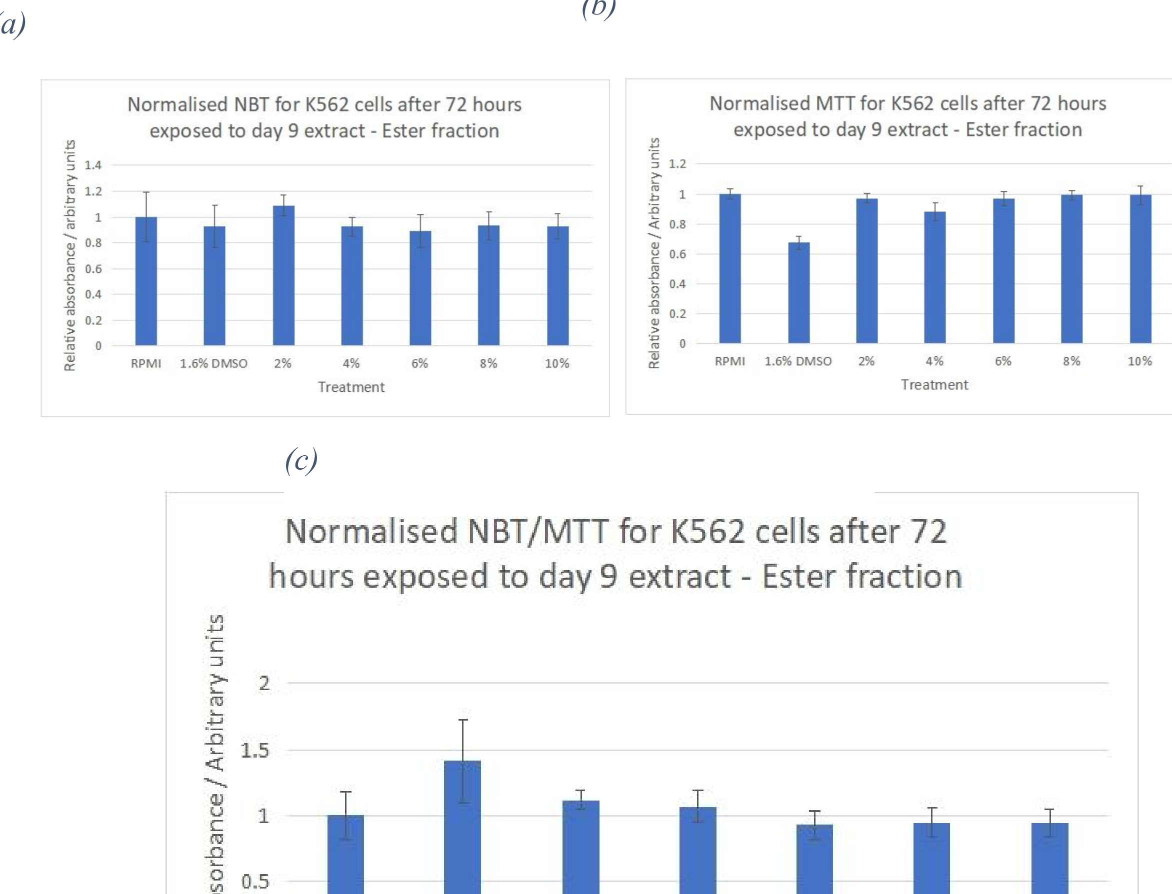


Figure 73 Effect of coelomic fluid from day 9 of regeneration on K562 cells after 72 hours of treatment for the ester fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Figure 74 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 120 hours to coelomic fluid obtained from organisms in their day 9 of regeneration. Data is related to Ester fraction. Data can be found in Appendix 5 Part L

(a)

Normalised NBT for K562 cells after 120 hours exposed to day 9 extract - Ester fraction

Relative absorbance / arbitrary units

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7

RPMI 1.6% DMSO 2% 4% 6% 8% 10%

Treatment

(b)

Normalised MTT for K562 cells after 120 hours exposed to day 9 extract - Ester fraction

Relative absorbance / arbitrary units

0 0.2 0.4 0.6 0.8 1 1.2

RPMI 1.6% DMSO 2% 4% 6% 8% 10%

Treatment

(c)

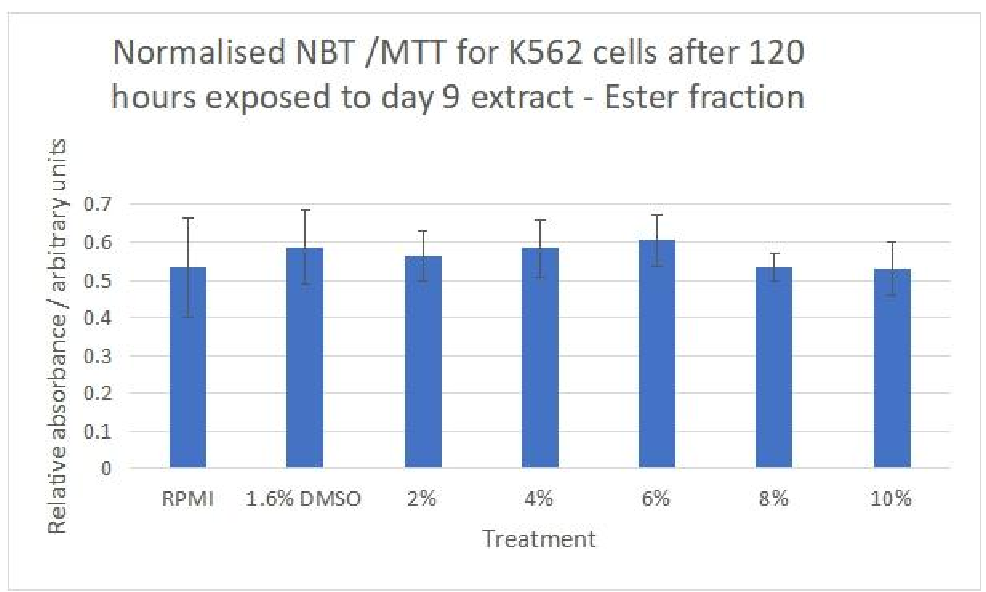
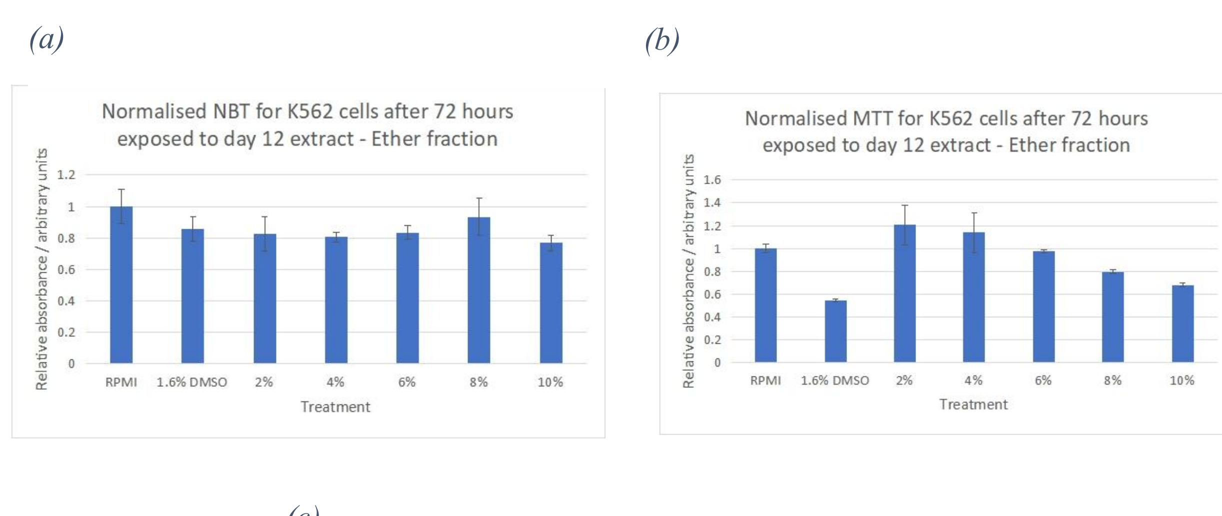


Figure 74 Effect of coelomic fluid from day 9 of regeneration on K562 cells after 120 hours of treatment for the ester fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Statistical analysis was carried out on this data. When looking at the results obtained from cells exposed to 72 hours of treatment, the ester fraction was statistically significantly different from the <5 KDa fractions ($p<0.001$). However, the two fractions – extract in ether and in ester- were not statistically different from each other. In the case of results obtained 120 hours after treatment, the ether fraction, ester fraction and the <5KDa fractions were all statistically significantly different from any other ($P<0.001$)

3.8.3.4 K562 cell line exposed to day12 extract.

Figure 75 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 72 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to Ether fraction. Data can be found in Appendix 5 Part L



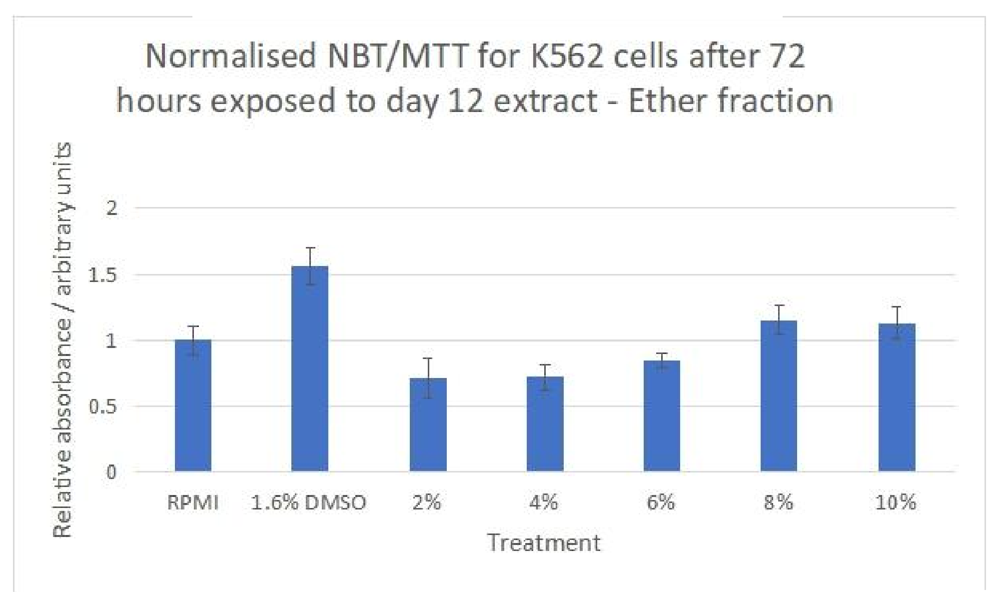
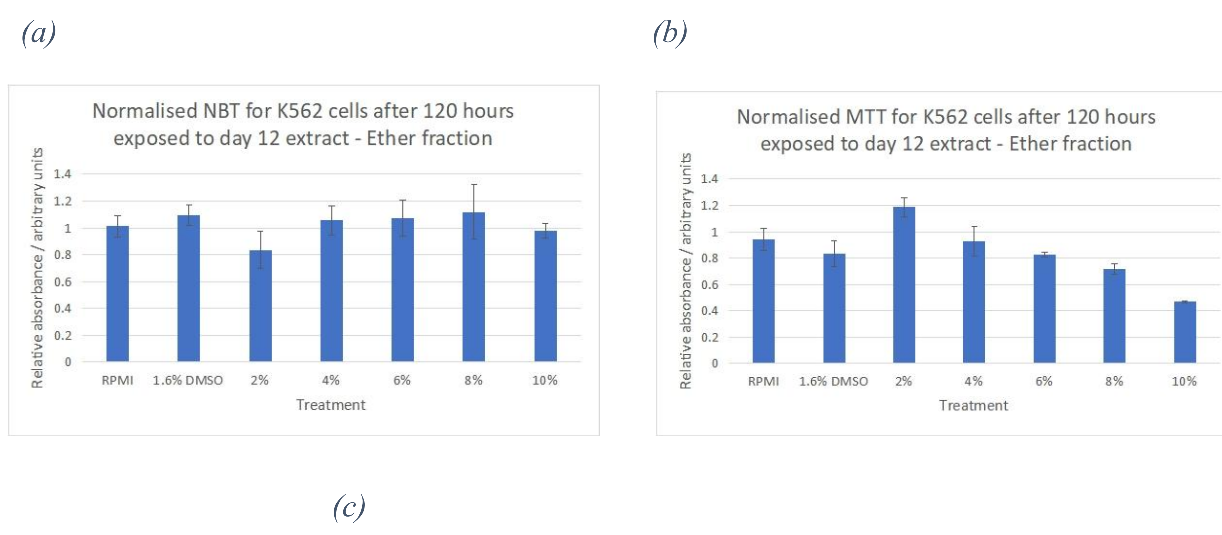


Figure 75 Effect of coelomic fluid from day 12 of regeneration on K562 cells after 72 hours of treatment for the Ether fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Figure 76 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 120 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to Ether fraction. Data can be found in Appendix 5 Part L



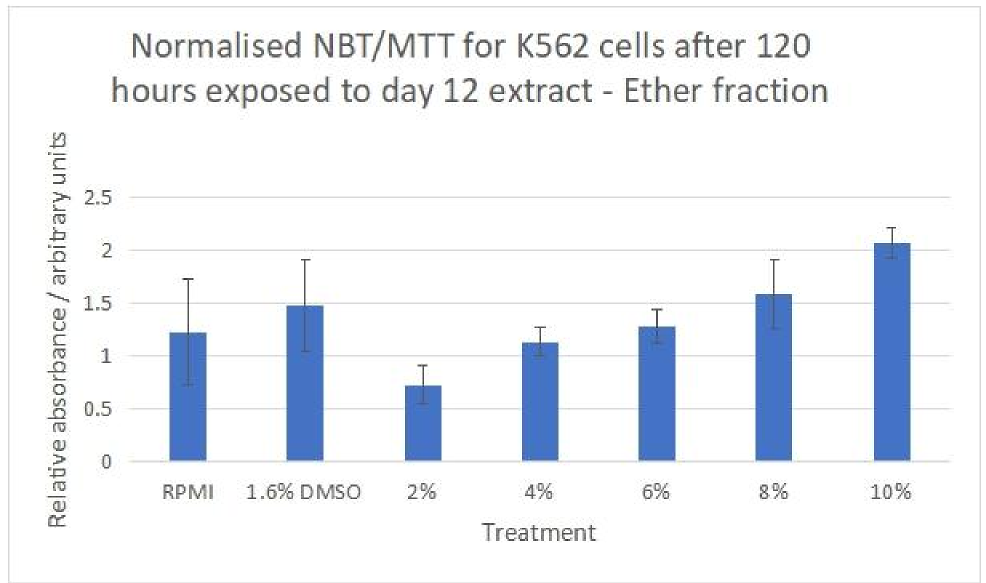
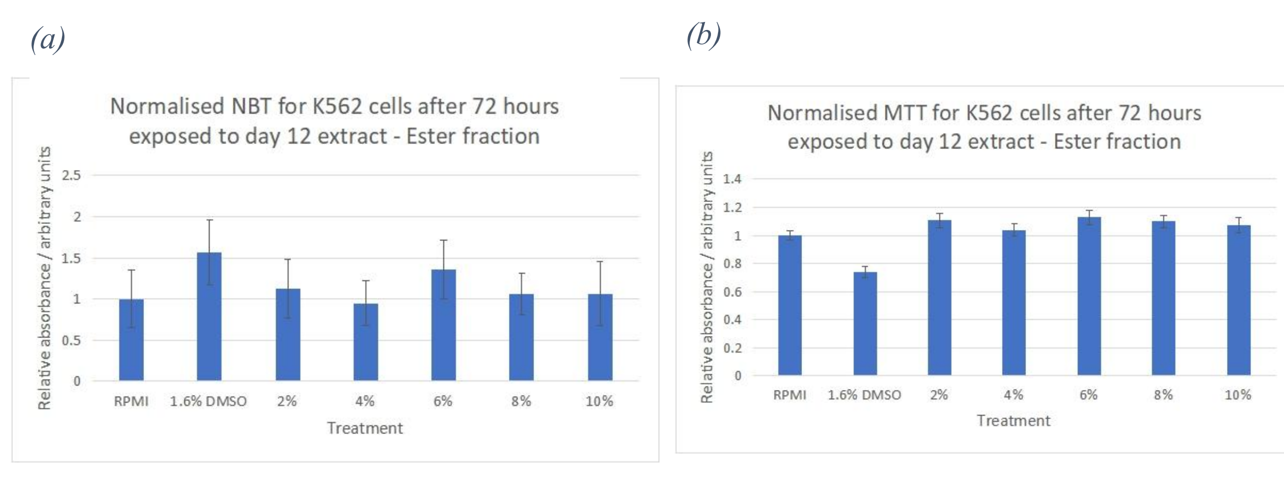


Figure 76 Effect of coelomic fluid from day 12 of regeneration on K562 cells after 120 hours of treatment for the Ether fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Figure 77 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 72 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to Ester fraction. Data can be found in Appendix 5 Part L



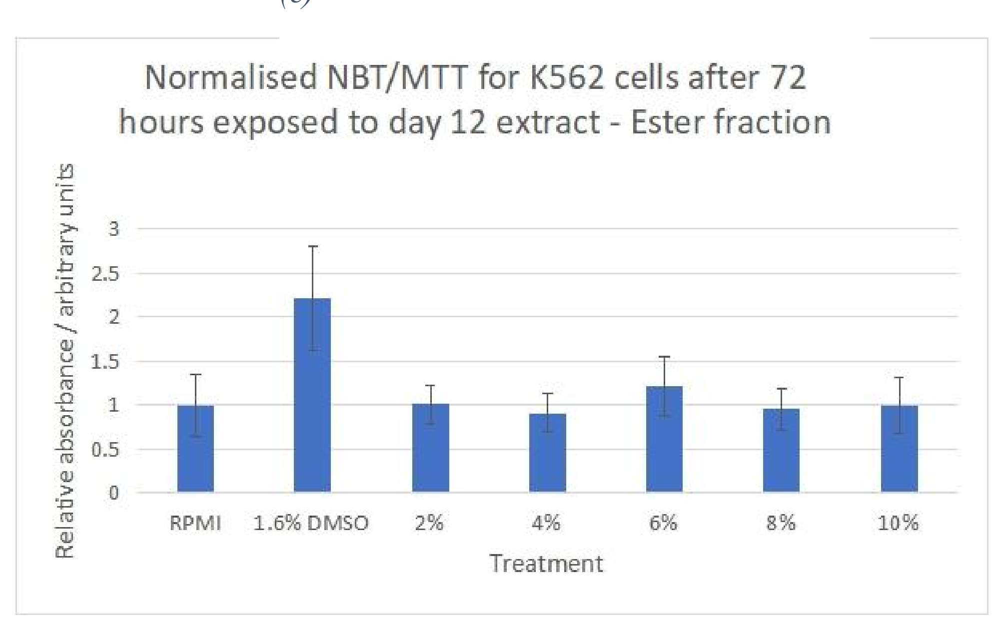
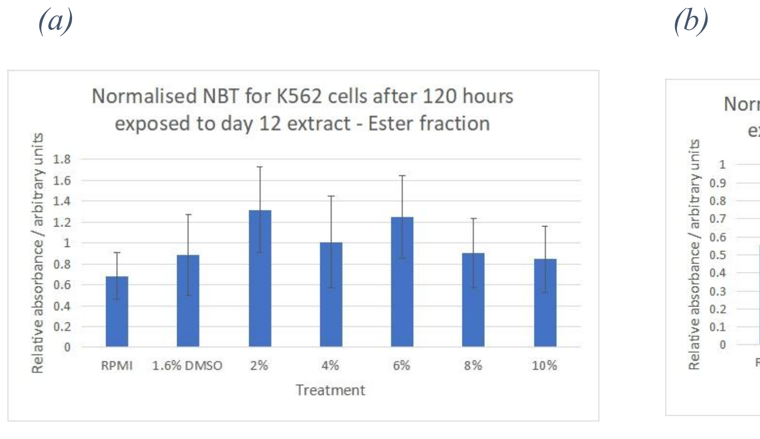


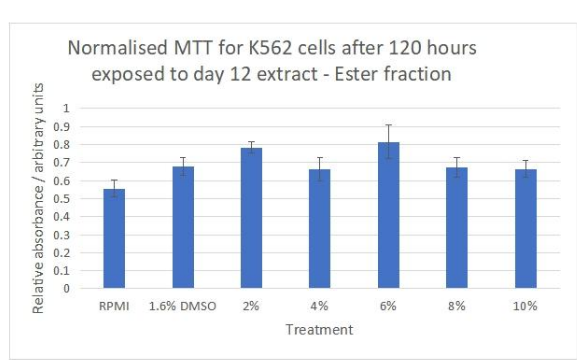
Figure 77 Effect of coelomic fluid from day 12 of regeneration on K562 cells after 72 hours of treatment for the Ester fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

Figure 78 represents normalised NBT, MTT and NBT/MTT for K562 exposed for 120 hours to coelomic fluid obtained from organisms in their day 12 of regeneration. Data is related to Ester fraction. Data can be found in Appendix 5 Part L

(a)



(b)



(c)

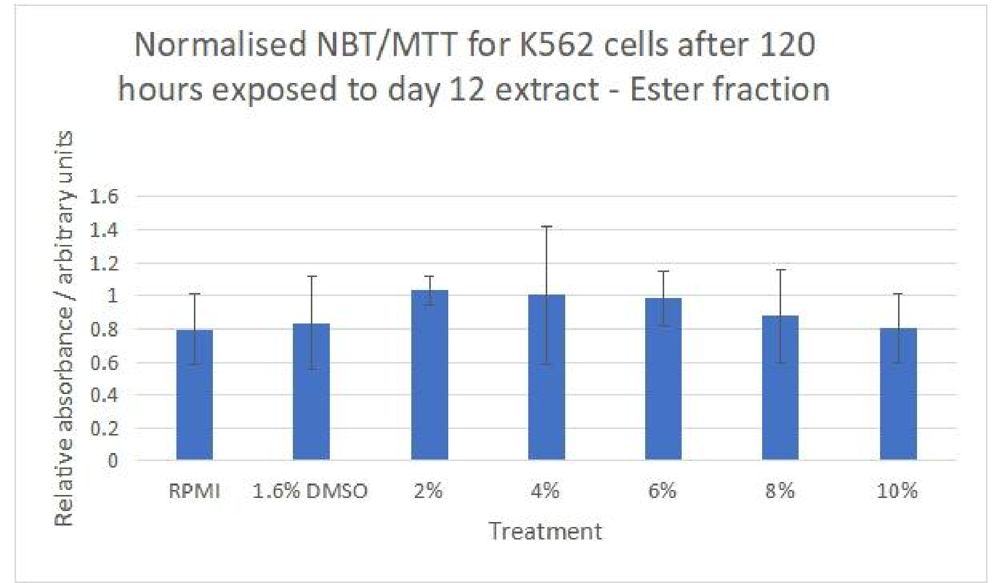


Figure 78 Effect of coelomic fluid from day 12 of regeneration on K562 cells after 120 hours of treatment for the Ester fraction (c) NBT:MTT ratio calculated by (a) Absorbance of reduced NBT divided by (b) Absorbance caused by MTT. Values are an average of 3 individual experiments have 3 individual experiments have 3 individual experiments have 3 replicated each, therefore (n=9). Error bars are constructed using the Standard error. Values were normalised by the RPMI control at 72 hours

3.9 Morphological analysis and Flow cytometry

3.10 Morphological analysis

The following is a representative photographic images of the untreated cell morphology for HL60 cells.

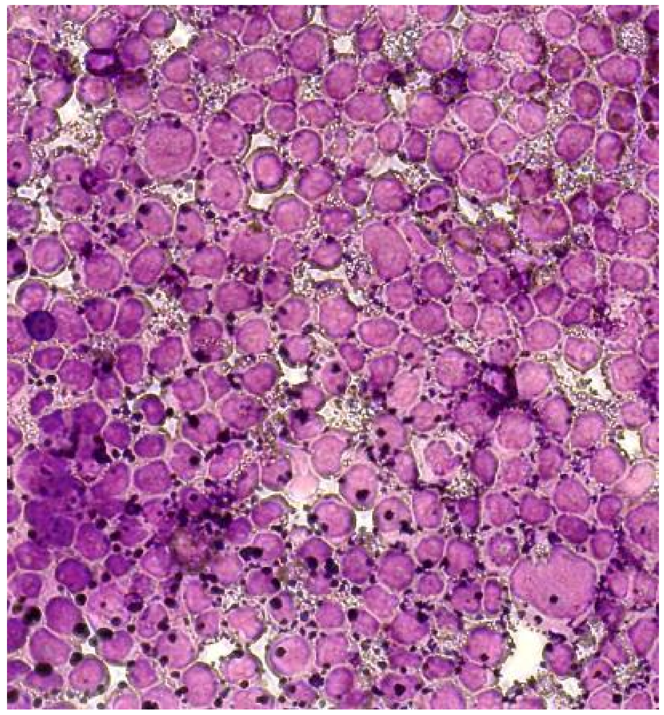
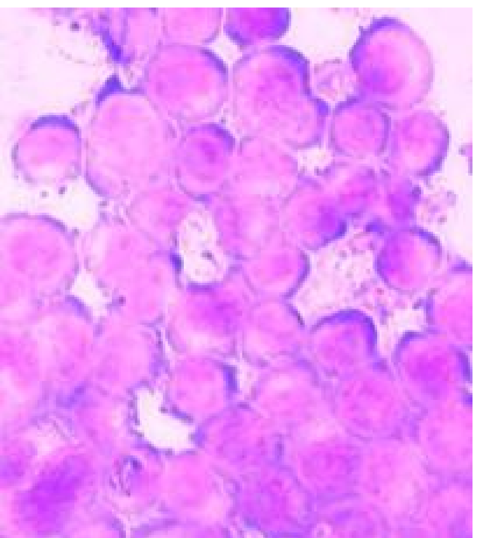


Figure 79: Representative figure showing untreated HL60 Leukaemia cells. Magnification x400

The cells exhibit the typical characteristics associated with the HL60 cell line described in Sections 1.3 and 1.5.

The following figures figures show morphological changes that were observed for the HL60 cell line 72 hours after being exposed to 8% coelomic fluid obtained from organisms in their 9th day post evisceration.



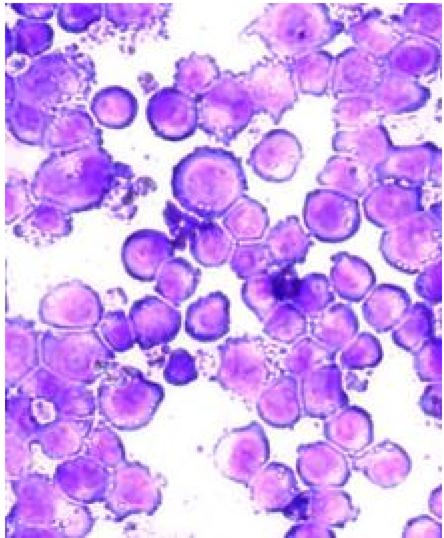
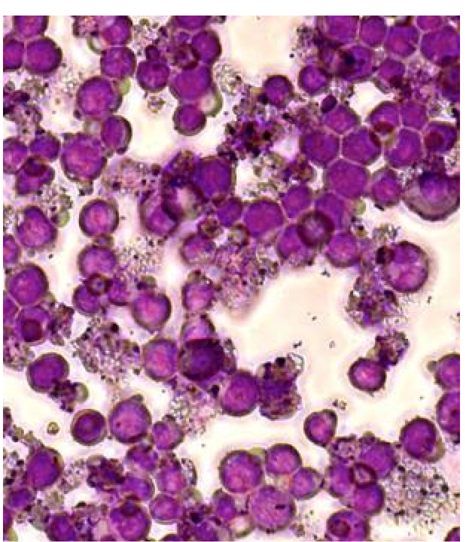


Figure 80: Representative photos showing morphology of HL60 cells after 72 hours exposed to 8% by volume of coelomic fluid obtained from organisms in their 9th day post evisceration. Images are x400 magnification.

The following figures figures show morphological changes that were observed for the HL60 cell line 120 hours after being exposed to 8% coelomic fluid obtained from organisms in their 9th day post evisceration.



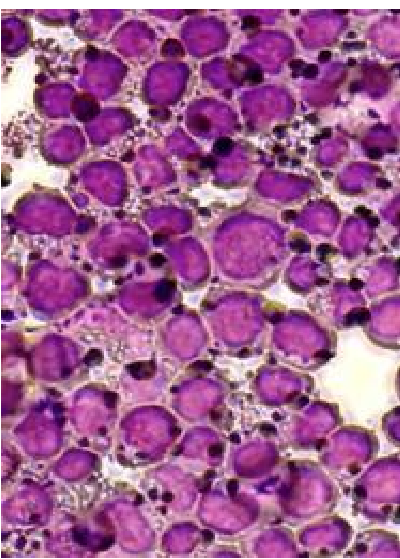


Figure 81: Representative photos showing morphology of HL60 cells after 120 hours exposed to 8% by volume of coelomic fluid obtained from organisms in their 9th day post evisceration. Images are x400 magnification.

From the images obtained, an increase in the amount of heterochromatin can be noted to occur from 72 hours following treatment to 120 hours following treatment. This

can be noted as the nuclear region is stained darker. In addition, there seem to be a decrease in the amount of cell division taking place as frequency of dividing cells (observed through dividing nuclei) is visually less. There is some cell death which is an indication that extreme proliferation is not taking place.

3.11 Flow cytometry

During differentiation, cells become initially smaller and contain more granules. These changes increase side scatter. Those cells were gated for analysis. The following figures are the histogram plots obtained when carrying out flow cytometry.

The first set corresponds to HL60 cells on day 0 of the experiment. Therefore, these cells are untreated and are forming a suspension in RPMI.

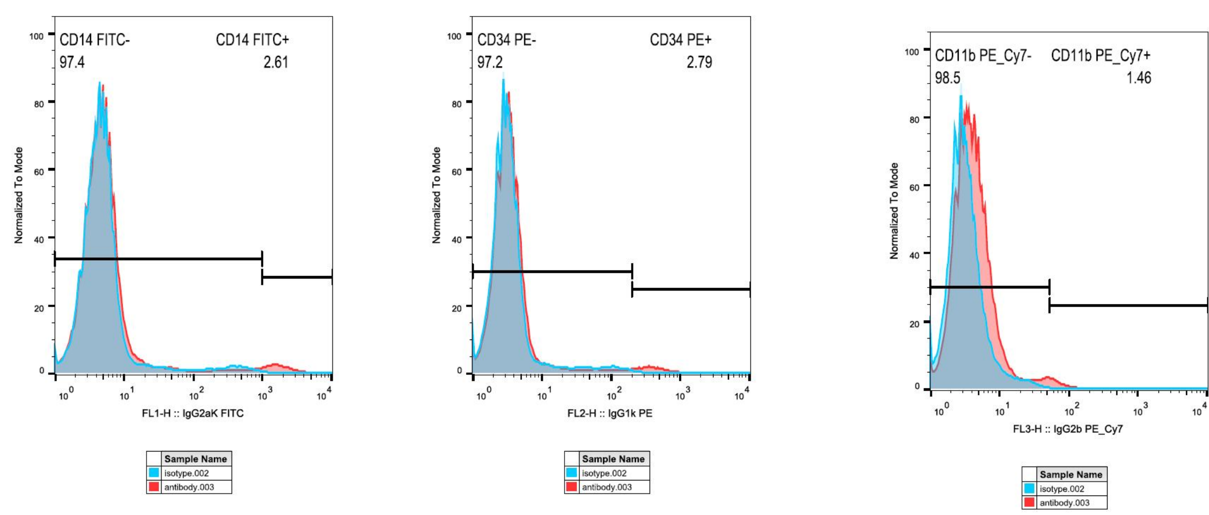


Figure 82: Figure showing isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on untreated cells at day 0.

It can be noted that at the beginning of the experiment no shifts could be noted as the cells are mostly undifferentiated and hence the antibodies markers are not expressed, with no increased binding by antibody over isotype control.

Flow cytomtery was then performed on HL60 cells after 72 hours exposed to treatment. The following results were obtained:

a. Untreated cells – No exposure to any treatment acting as a negative control for differentiation. These cells had been incubated in a suspension with RPMI. Although they were proliferating, they were not differentiating and so there was no increased binding by antibody over isotype control

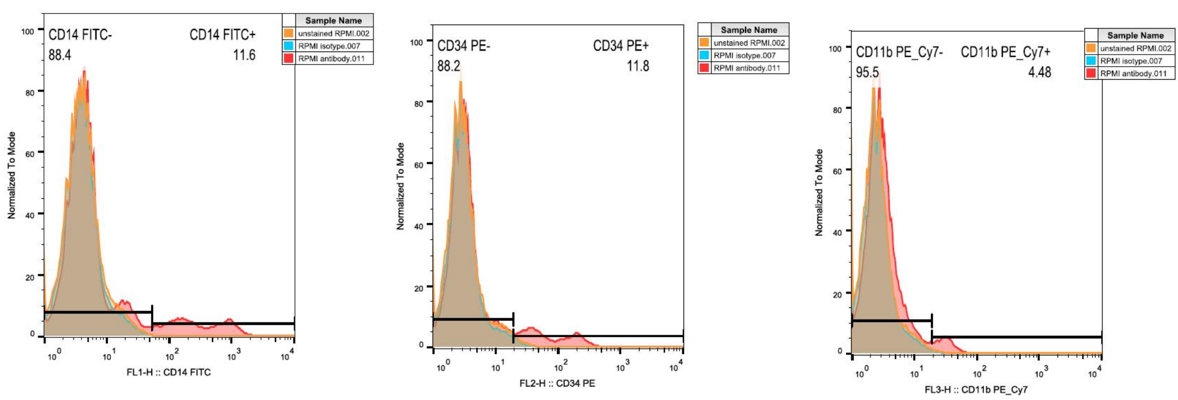


Figure 83: Figure showing unstained (orange), isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on HL60 cells treated with RPMI for 72 hours.

b. Positive control for granulocytic differentiation, cells exposed to 1.6% DMSO.

Antibodies CD11b gave the highest shift, that of 60.4%. Less shift was observed with CD14 (4.63%) and CD34 (4.62%)

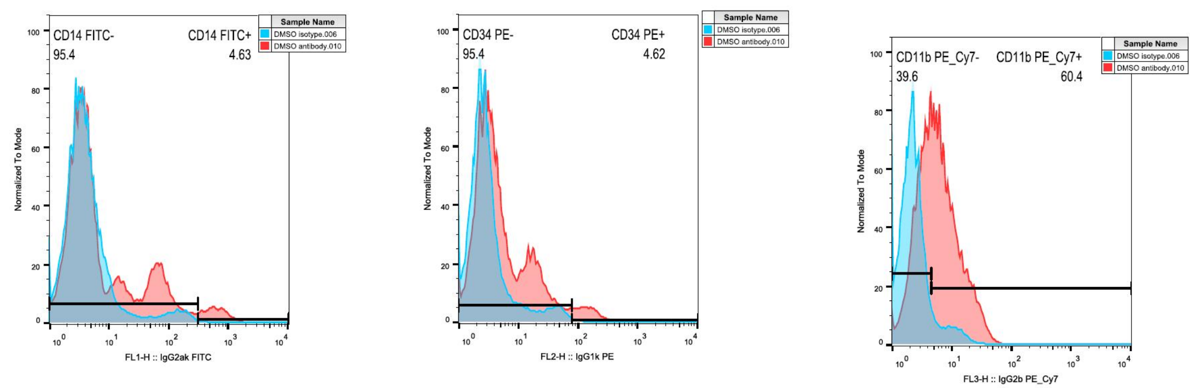


Figure 84: Figure showing, isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on HL60 cells treated with DMSO for 72 hours.

c. HL 60 cells exposed to 4% by volume as concentration of coelomic fluid obtained from organisms in their 9th day post evisceration. Here there were very minor changes when compared to untreated cells.

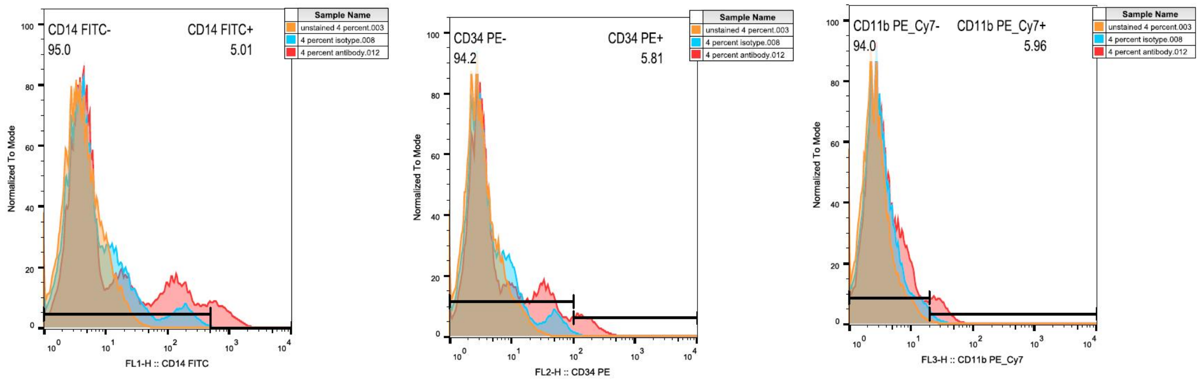


Figure 85: Figure showing unstained (orange), isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on HL60 cells treated with 4% coelomic fluid obtained from organisms at day 9 post evisceration for 72 hours.

d. HL 60 cells exposed to 8% by volume as concentration of coelomic fluid. obtained from organisms in their 9th day post evisceration All three markers gave a considerable change. CD14 gave a shift of 45.1%, CD34 gave a shift of 45.5% and CD11b gave a shift of 43.6%.

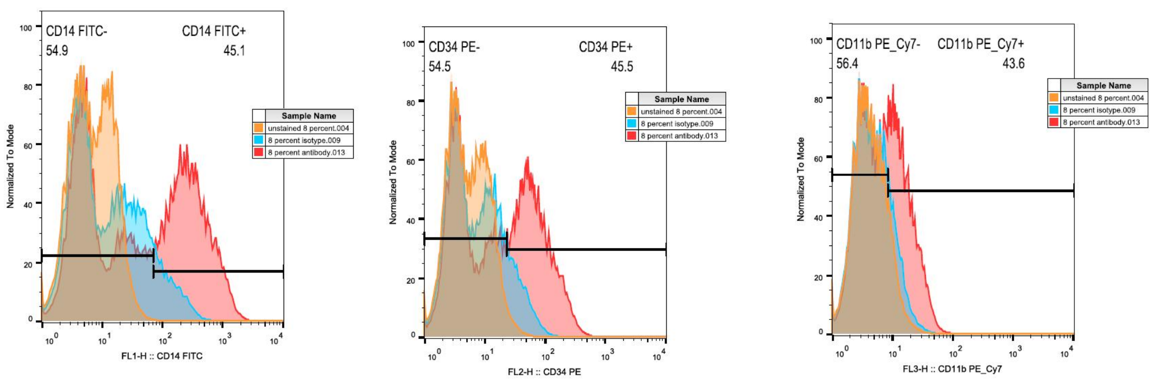


Figure 86: Figure showing unstained (orange), isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on HL60 cells treated with 8% coelomic fluid obtained from organisms at day 9 post evisceration for 72 hours.

After 120 hours exposed to treatment, the following results were obtained:

a. Untreated cells – These cells were still forming a suspension in RPMI. Although they were proliferating, they were not differentiating and so there was not expression of any cell surface markers. There was a slight increase in expression from the reading obtained after 72 exposed to RPMI.

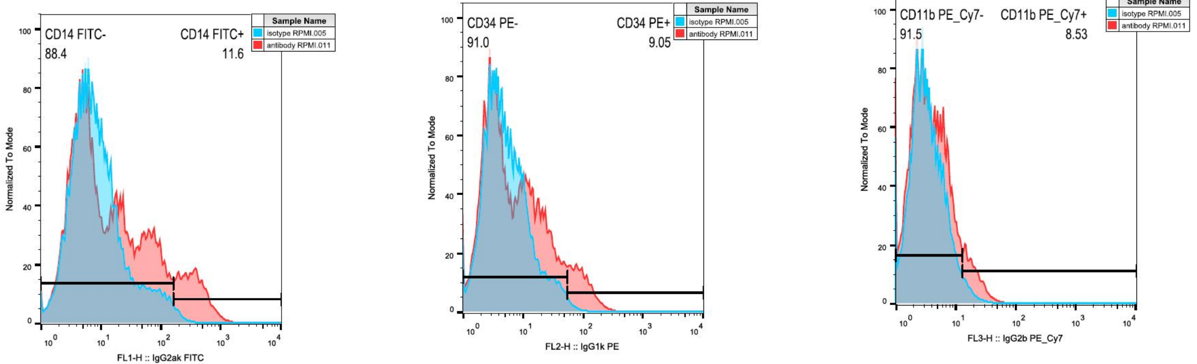


Figure 87: Figure showing unstained (orange), isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on HL60 cells treated with RPMI for 120 hours.

b. Positive control for granulocytic differentiation, cells exposed to 1.6% DMSO.

Antibodies CD11b gave the highest shift, that of 42.6% (as expected by granulocytic differentiation), less that what was expressed after 72 hours of treatment. A slightly more expression was observed with CD14 (5.55%) and CD34 (4.84%).

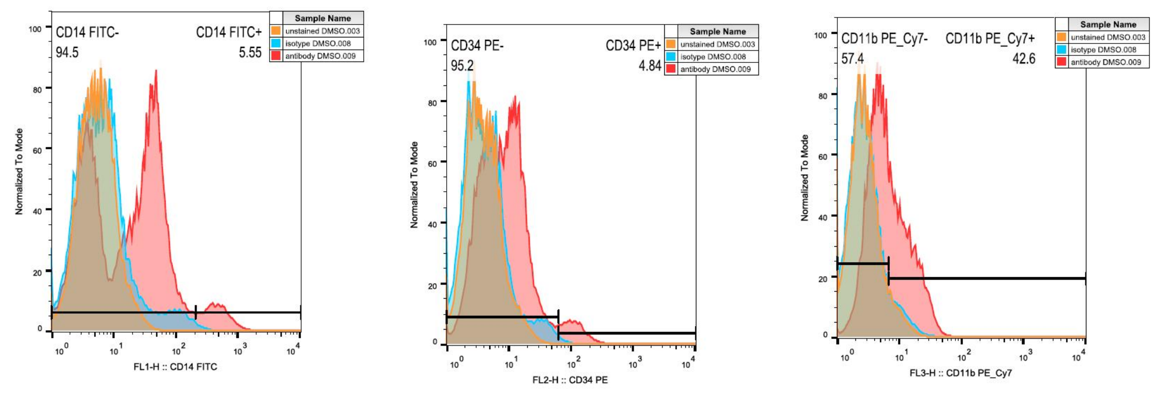


Figure 88: Figure showing unstained (orange), isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on HL60 cells treated with DMSO for 120 hours

c. HL 60 cells exposed to 4% by volume as concentration of coelomic fluid.

There was a slightly more expression for the three markers than was observed for cells left for 72 hours exposed to treatment.

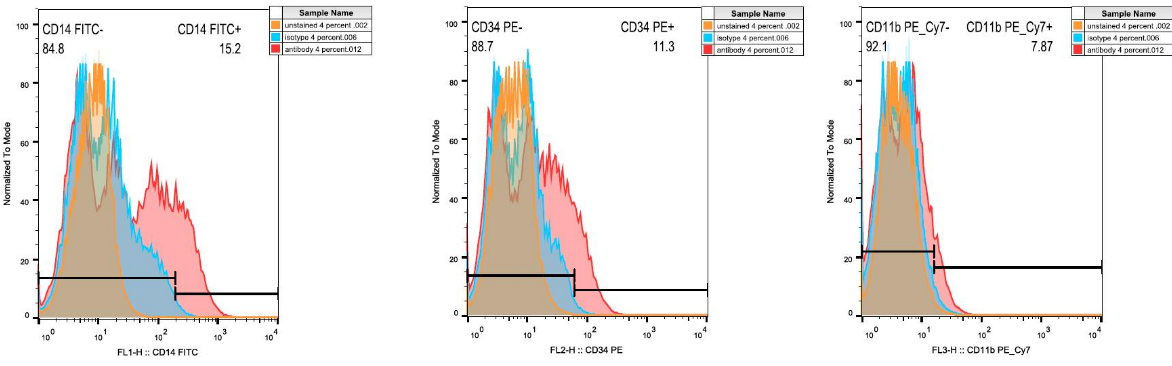


Figure 89: Figure showing unstained (orange), isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on HL60 cells treated with 4% coelomic fluid obtained from organisms at day 9 post evisceration for 120 hours.

d. HL 60 cells exposed to 8% by volume as concentration of coelomic fluid.

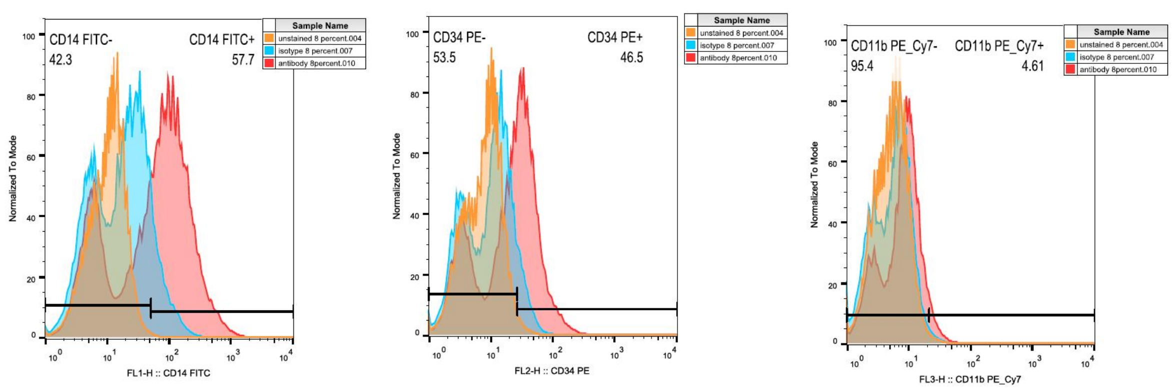


Figure 90: Figure showing unstained (orange), isotype (blue) and antibody (red) expression for CD14, CD34 and CD11b on HL60 cells treated with 8% coelomic fluid obtained from organisms at day 9 post evisceration for 120 hours.

All three markers gave a considerable change in expression. CD14 increased from 45.1% to 57.7%, CD34 increased from 45.5% to 46.5% and expression of CD11b decreased drastically from 43.6% to 4.61%.

Through flow cytometry it was seen that a percentage as low as 4% by volume didn't give a big morphological change, however a more noticeable change was obtained at 8%. It can be safely concluded therefore that around 8% by volume of coelomic fluid is the right concentration to unblock, even slightly the HL60 leukaemia cells.

4 Discussion

The main research question in this project was to determine whether an extract from *Holothuria poli* can be used to cause any level of differentiation in Leukaemia. Differentiation therapy is one of the ways that is being researched to fight myeloid malignancies (Stubbins & Karsan, 2021) with the aim of possibly replacing or reducing the more commonly and more toxic used methods such as chemotherapy. Adrian & Collin (2018) have reported that the triterpenoid glycoside named Frondoside A extracted from the sea cucumber *Cucumaria frondosa,* has shown to work synergistically with chemotherapeutic agents, including gemcitabine and cisplatin, without giving any side effect related to body weight, blood cell count or renal functions of patients. Frondoside A is cytotoxic and not an agent of differentiation.

A lot of literature is available related to the use of extracts from holothurians which cause cytotoxicity of various cancer cell lines including HL60 cell lines (Wijesinghe, Jeon, Ramasamy, Wahid, & Vairappan, 2013; Xu *et al*., 2013; Fedorov, 2016). Certain studies show that cytotoxicity can be targeted to other cancerous cells. In studies such as those by Salimi, *et al*., (2017), *Holothuria parva* extracts were used to test for cytotoxicity to both cancerous and non-cancerous B lymphocytes and their mitochondria and they determined that the extract was cytotoxic only to the cancerous B lymphocyte and not the non-cancerous B lymphocytes. This was not concentration dependent, and an increase in concentration still showed the same effect. The fact that the biomolecules from holothurians, exhibit different biochemical functions, opens the door to the possibility that biomolecules from Holothurians can also cause differentiation.

Literature related to the use of extracts from sea cucumbers to cause differentiation, in line with the main aim of this research project is very limited. Hence there were many variables to look into and through an elimination process try and reach a conclusion to answer the research questions. Results of this research project are very promising, and although they are not conclusive, they have shown that some changes to the HL60 cell line is happening, possibly unblocking it slightly from its blast state. Any level of differentiation that occurred was essentially brought

about by an extract composed of coelomic fluid from *Holothuria poli*. The extract was obtained from organisms at their 9th day of gut regeneration, following evisceration. Whilst a lot of studies investigating for biomolecules that could be used for the treatment of various forms of cancer use extracts from holothurians obtained by macerating different part of the sea cucumber being investigated (Zhang & Zhu, 2017; Salimi *et al*., 2017), some studies have also used the coelomic fluid as the extract for their tests (Luparello *et al*., 2019; Luparello *et al*., 2022).

4.1 Growth of Holothuria in artificial conditions.

The first step of this research project involved identifying a location around Malta that could be used to collect the specimens. Proper identification of the holothurians being collecting and the artificial conditions in which these organisms where grown were optimised. Sea cucumbers are known to be affected greatly by stressful situations, where changes in their behaviour, physiology and biochemical reactions will occur when exposed to stress (Ji, Dong & Dong, 2008). Huo *et al*., (2018) described the negative effects of hypoxia exposure to the sea cucumber *Apostichopus japonicus*, which include but are not limited to degradation of respiratory trees, increase in the expression of heat shock protein 70 and glutathione peroxidase and a change in the digestive enzyme activities. It has also been noticed that when these animals are exposed for prolonged periods of exposure to air, or periods of high temperature, they tend to eviscerate (Dolmatov, Nguyen & Kamenev, 2012) and / or develop diseases such as white spot disease (personal observation) (refer to figure 4) and / or undergo aestivation (Ji, Dong & Dong, 2008). In fact, when attempting to collect organisms of *Holothuria poli* in the summer months, these animals where found burrowed down in the sand, probably to reduce exposure to fluctuations in temperature, making them very difficult to catch. When caught in hot weather conditions, the exposure to the hot temperatures during collection time made the animals very susceptible to white spot disease. In a study by Ji, Dong & Dong (2008) on the sea cucumber *Apostichopus japonicus*, it was noted that a temperature change of just 4°C caused the release of heat shock proteins such as HSP 70 and antioxidant molecules.

Therefore, the way these organisms were caught, transported, and handled had to be optimised. With this in mind, it was determined that the best time of collection and rearing of such organisms was in the October – November period. It not only made it easier to collect, but also increased the chance of survival in artificial conditions. The disadvantage of collecting organisms in this time period was sometimes due to wind and waves which caused turbidity making it difficult to collect the organisms. However, the optimised methods used to collect and rear these organisms allowed the specimens to acclimatise to their new environment, eviscerate when required and regenerate completely their intestine without or very little occurrence of white spot disease. This made the collection process effective, allowing for tests to be carried out in a timely manner and avoiding stress on the wild populations as the need to recollect more animals was greatly reduced.

Collecting organisms from the same location was extremely important to reduce variability. In future studies, one of the aspects that could be investigated is to compare the effect of coelomic fluid obtained from organisms in their 9th day of regeneration collected from different locations. It is known that changes occurring in the external environment of the organisms would have a marked effect on the composition of the coelomic fluid and the microorganisms inhabiting it (Nerva *et al*., 2019) and therefore possibly on the outcome of the experiments.

In a study by Ji, Dong & Dong (2008) on the sea cucumber *Apostichopus japonicus,* the animals were grown in aquaria, where the sea water was filtered using a sand filter and half or more of the water was changed for fresh sea water on a daily basis. In addition, aeration was provided in a continuous fashion and the animals were allowed to be in the light for 12 hours and in the dark for another 12 hours. In this research project it was noted, through several trials of rearing these organisms in aquaria, that by changing half the water every three days with fresh sea water collected from the same location that the organisms were collected, was sufficient to ensure continuous growth. Aeration was also provided in a continuous fashion. The most effective pumps were immersible pumps with grooves at the bottom. Although these pumps allowed for the best aeration, one flaw was the fact that some specimens tended to be attracted to these grooves, possibly due to circulating air. So, the pumps had to be placed in such a way that the animals could not get stuck in any grooves. This was not always successful and when animals did get stuck in these grooves,

exposure to that high quantity of air would have caused their body to disintegrate, thus fouling the water.

Even though the behaviour of *H poli* could change when grown in artificial environments as a result of the difficulty encountered in precisely mimicking their natural environment, Bhakuni & Rawat (2005) state that in general, for most organisms, the metabolites produced in artificial environments are identical to those produced in their natural environment. This is not true for all marine organisms and there are several organisms in which metabolite production is highly affected by temporal and environmental changes (Karthikeyan, Joseph & Nair, 2022).

4.2 Holothuria regeneration.

Holothurians are deuterostomes and therefore they are evolutionary closer to human beings than most other invertebrates and are more likely to possess chemicals which are similar or analogous to those found in humans (Sun *et al*., 2017). Hence, it was very interesting trying to determine if there are any bioactive molecules within these organisms with differentiation properties and test them on human cancer cells. This was done in an effort to try and unblock the stage in the development of the cell lineage causing the particular cancer type and induce the cell to move on in its path to differentiating in a more normal manner.

Like most echinoderms, *H. poli* specimens (used in this study) have a great capability of regeneration. In the case of *H. poli*, they are capable of regenerating their intestine following evisceration (Ding *et al*., 2021). Regeneration follows a period of great cell differentiation followed by cell proliferation. Rapid cell proliferation is also one of the Hallmarks of cancer in human beings, however unlike humans, Holothurians are not known to ever suffer from cancer (Mashanov & Garcia-Arraras, 2011). This formed the rationale behind this project. Such organisms offer a metabolic pool of compounds with the ability to control cell differentiation and proliferation and maintaining the balance between the two. This is extremely important in cancer treatment as the balance between cell death and cell proliferation would have been compromised.

In *H poli*, once regeneration occurs, the most anterior and most posterior segments which are the oesophagus, and the cloaca respectively are retained. Upon evisceration, the wounds present in the anterior and posterior ends are immediately closed and reorganisation of the mesentery occurs with an overall extension of the width, strengthening and shortening of the free margin of the structure in order to allow more efficient regeneration. In nature this is extremely important as it allows the animal to start feeding earlier ensuring its better survival (Mashanov & Garcia-Arraras, 2011).

Regeneration represents a secondary post embryonic development. Even though the outcome of both regeneration and embryogenesis involve the formation of the same structure, regeneration cannot be deemed as an exact reproduction of embryogenesis. In regeneration there are unique processes such as wound healing and dedifferentiation which do not occur during embryogenesis (Mashanov & Garcia-Arraras, 2011).

Cell proliferation and apoptosis have been studied extensively in the aspidochirotid *Holothuria glaberrima* in studies by García-Arrarás, *et al*, (2018); Mashanov, *et al*, 2010; Mashanov & Garcia-Arraras, 2011). García-Arrarás, *et al*, (2018) state that even though Holothuria regeneration has been described and documented since Aristotle's time, the information was always a description of macroscopic morphological events. Neither the cellular events nor the mechanisms used by Holothurians in their regeneration process have been clearly established. Morphallactic mechanisms have been proposed, which indicates a process of transformation, reorganisation and redifferentiation with little or no cell division and epimorphic mechanisms which involve the formation of a blastema mass at the wound site which consists of undifferentiated dividing cells giving rise to new tissues have been postulated (Quispe-Parra, *et al*, 2021). García-Arrarás, *et al*, 2018 have also suggested that evisceration causes a burst of cell division which reaches its maximum stage whilst the anterior and posterior rudiments grow towards each other and will then return to normal values.

The artificial growth of cells from the regenerating parts was tested. These cells are not immortal and therefore could not be grown for a long period of time. This path had to be abandoned as there would have been difficulty in obtaining enough possible

active ingredients for the tests that were required to carry out this investigation. However, from the various attempts that were done, a protocol was finalised which allowed some degree of growth, and an interesting result was obtained. Those cells coming from day 9 following regeneration were the ones to give the most promising result, with these cells being the ones that remained alive for the longest period of time. This could imply that the regenerative and / or proliferative ability of these cells was the strongest. Garcı´a-Arrara´s *et al*., (1998) have shown that the largest percent of cells dividing in the mucosa was first noted during day 9 of evisceration, together with the first peak of cell division in the serosa and the least in submucosa (figure 91).

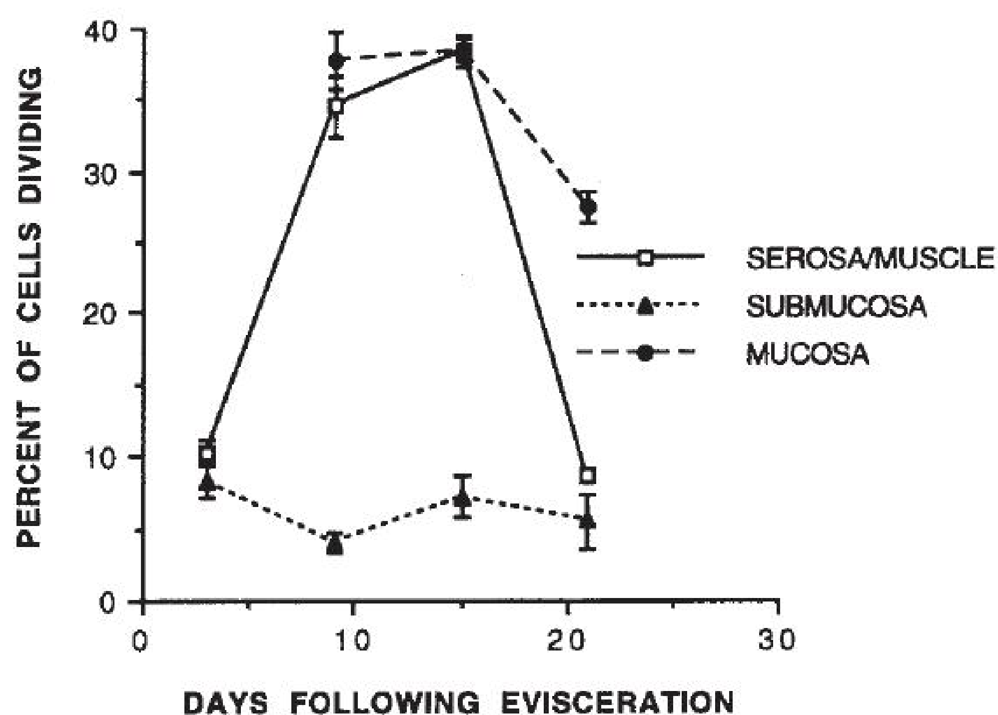


Figure 91: Figure showing percentage of cells dividing vs days following evisceration in Holothuria glaberrima Garcı´a-Arrara´s et al., 1998).

Figure 91 shows a lot of change going on during day 9, the percentage of cells dividing in the mucosa were highest at day 9. Those of the serosa, which is on the outside of the digestive tract and hence closest to the coelomic fluid were showing an increase too. This shows that since there is a large number of cell divisions, there is also great differentiation taking place, otherwise, there would only be a blastema (stem cell) mass. This is in line with the dates that seem to give a positive result in

this research. Day 9 following regeneration gave results which showed a significant difference when compared to other coelomic fluid.

Although this information by itself is not conclusive, it can be taken as a first possible observation, i.e., at around day 9 there are changes happening in the developing intestine, which allow stimulation for better growth *in vitro* and which is probably secreting a lot of biomolecules needed for regeneration. Since the procedure of growing the cells and extracting any chemicals from them was not followed, the coelomic fluid was then chosen for further tests. The coelomic fluid bathes the internal organs and should be very rich in the metabolic compounds produced and secreted by the developing intestines at each point in time. Even though a note was taken about day 9 being promising, when choosing the coelomic fluid as the extract, several days following regeneration were considered and it was through visual and statistical analysis that further decisions were made.

4.3 Marine Natural products

Given that 70% of the earth is covered by the sea with a biodiverse ecological system inhabiting it, the potential number of biomolecules that can be obtained from the marine environment is extremely high. In fact, compounds derived from marine invertebrates have also already contributed a great deal to contemporary medicine (Ghosh, 2022). Karthikeyan *et al*., (2022) gave a review through which they have identified the derivatives of structurally unique marine natural products which exhibit different potent bioactivities. These include pharmaceutical activities such as antibacterial, cytotoxic, and antiviral amongst others, as they contain reactive groups such as hydroxyl, amine and -SH bonds in their chemical structures which help them act as antioxidants. Such compounds include but are not limited to Xanthones, peptides, terpenoids, lipopeptides, polysaccharides, proteins, *etc*. Terpenoids are a modified class of terpenes with different functional groups and methyl group that can be oxidised, moved, or removed at various positions and can be subdivided into monoterpenes, sesquiterpenes, diterpenes, sesterpenes and triterpenes based on the number of carbon units (Perveen, 2018). They are found in various sea cucumbers. To date, around 100 terpenoids at least half of which have novel structures have been

isolated and they are known to exhibit antibacterial, cytotoxic, and antifouling activities (Liu *et al*., 2019). Many terpenoids have been used to develop anticancer agents (Perveen, 2018). Triterpene glycosides are predominant in sea cucumbers. For example, the coral reef sea cucumber *Actinopyga lecanora* possess tetra glycosylated triterpenes and diglycoslyated holoturin both showing antileishmanial activity (Kumar, Chaturvedi, Shukla, Lakshmi, 2007). Branched chain acids reported from sea cucumbers are probably of bacterial origin. Phospholipids and glycosphingolipids appear to be universally present in holothurians (Kamyab *et al*., 2020)

Karthikeyan *et al*., (2022) highlighted that some marine natural products could tackle the possible side effects of the pharmacological treatment but obtaining these natural products from marine organisms adds another hurdle as its quite difficult due to low production yields and/or high costs (Bhakuni & Rawat, 2005).

There is a lot of research being done to help in the discovery and synthesis of organic compounds from marine organisms (Freitas, Rodrigues, Rocha-Santos, Gomes & Duarte, 2012, Barbosa, Valentão, & Andrade, 2014, Gallego, Bueno, & Herrero, 2019). However, there are various problems associated with the use of marine extracts as molecules of biological importance. The three main problems are the high salt content of the fluid in marine organisms, the fact that the marine environments, together with the biodiversity that inhabits them are still not well studied and understood and the fact that many microorganisms inhabit the organism under study making it difficult to know what the origin of any biomolecules of interest is. Furthermore, native forms of marine natural products are not always patentable and so need to be modified. It is also very difficult for marine natural products to get to the clinical trial phase, due to the very complex structure of the actual biomolecules, the pathway and target receptor of the biomolecules, and the technology available for sampling and extraction. This opens a plethora of new research opportunities including the techniques used for characterisation of the actual structure of the biomolecule as well as the possible chemical and biochemical synthesis.

As already mentioned, one of the main problems associated with this research project is the high salt content of the fluid in marine organisms. The coelomic fluid has a salt concentration similar to that of sea water, hence it is not physiologically adequate for human cells. The high salinity would cause the cells to shrink and lose

their function. Consequently in the research of active biomolecules from such extracts, usually various methods of extraction are employed resulting in an inevitable great loss of active material. The most common traditional methods of extraction are three: maceration, percolation and Soxhlet extraction using solvents such as ethyl ethanoate, ethanol, acetone, water, and methanol (Ghosh, 2022). These solvents are chosen based on the polarity of the molecules. Some studies show that in order to enhance the extraction of a substance, certain acids including tartaric acid are added to the solvents. Thermolabile substances are degraded using the Soxhlet method of extraction and greater volumes of solvents are used, together with longer processing times are employed to extract hydrophilic substances, resulting in great losses. In addition, separation is always accompanied by losses and if the amount of active biomolecule present in the coelomic fluid of the organisms in this research project is already low, then further losses would have resulted in the loss of functionality. Separation of specific molecules such as triterpene glycosides from the sea cucumber *Holothuria nobilis* has been separated using chromatographic techniques. Through chemical and spectral analysis, the structure of the 12 triterpene glycosides present in the extract have been identified (Zhang, Dai, Chen, Ding, & Wu, 2008).

There are some emerging techniques in the area of extraction from marine sources such as fermentative extraction, pulse electric field assisted method, microwave-assisted method, ultrasound assisted method, supercritical fluid, membrane separation technologies, extrusion-assisted extraction, amongst others (Srivastava *et al.*, 2021). Usually, as happens in the traditional techniques, combined extraction techniques are used. In the case of this research, the nature of the active ingredient being sought after was not known and hence determining a method of extraction was very difficult. The ingredient might be lost due to it being in low quantities, being volatile or its structure being negatively altered due to any desalination processes taking place. Low molecular weight compounds would be negatively impacted by desalting methods and their size is not very different from that of the salt and so they would be easily lost on the desalinating gels and membranes used in such processes. Prolonged evaporation of water will not only leave a large amount of salt behind but may also lead to the destruction of any biologically active molecules. Bacterial and fungal growth may occur in water which would affect the active ingredient giving false positive results. Alcohol can be added in order to

inhibit the growth of such microorganisms, adding however, another variable during testing on living cells (Bhakuni & Rawat, 2005). Following extraction, filtration would have also been needed again causing a problem of pore size. The variety of natural organic substance is very vast and includes, amongst others, peptides, phenolic compounds, fatty acids, polysaccharides, and proteins, some being primary metabolites whilst others being secondary metabolites (Agustina *et al*., 2021).

Another limitation of this study is the fact that it was very difficult to determine the active ingredients in the coelomic fluid. This was also the case in other studies (Luparello *et al.*, 2019) and various studies show synergic activities of various biomolecules at different, specific concentrations (Luparello *et al.*, 2019, Hopkins *et al*., 2020). In the study carried out by Guo *et al*. (2018), the authors used extracts from sea cucumbers to try and treat non-alcoholic fatty liver disease which is currently treated by bariatric surgery and drugs that leave the patient with a lot of side effect. Eicosapentaenoic acid-enriched phospholipids (EPA-PLs) and sea cucumber saponin (SCS) can theoretically be used separately to treat non-alcoholic fatty liver disease as they both effect the mechanism of action. However, the raw material to make Eicosapentaenoic acid-enriched phospholipids (EPA-PLs) is scarce, EPA itself is easily oxidised and sea cucumber saponins are mostly toxic. The authors, during their study extracted the saponins from the sea cucumber *Pearsonothuria graeffei* and Eicosapentaenoic acid-enriched phospholipids (EPA-PLs) from the sea cucumber *Cucumaria frondosa*. After proper treatment, they performed experiments using various combinations of EPA-PLs and SCS using mice affected with non-alcoholic fatty liver disease. Through this study it was determined that when EPA-PL and SCS are used in combination each at half dose, the resulting effect was more effective at reducing orotic acid induced symptoms than when using either EPA-PL or SCS alone or a combination of EPA-PL and SCS at full dose. The study by Guo *et al*. (2018) therefore highlights that the biomolecules used, not only show a synergistic effect when used together in the right ratios, but also the fact that the biomolecules can be obtained from different species of sea cucumbers. Different ratios of such biomolecules not only can have no effect at all, but also a completely different effect. This supports the results obtained in this study. In this research project, when separating the extract into both two fractions based on molecular size (<5KDa and >5KDa) or the <5KDa into fractions in the two solvents ether and ester, a clear-

cut effect on which particular fraction was showing a similar result in giving an effect on the HL60 cell line as the complete fluid was not achieved. This is because, the biomolecules that are involved, most probably do not work independently of each other in isolation but work in conjunction with one another.

In this present study, in the case of extracts on the K562 cell line exposed to 72 hours of treatment and HL60 cell line exposed to treatment with coelomic fluid from day 9 of regeneration, showed that the effect of both the >5 KDa and the <5KDa fractions were significantly different from the complete extract (refer to section 3.8.2). This implies, that the separation technique might have removed the actual molecules that lead to a positive result, and / or reduced their concentration so much due to sedimentation during the filtration process. However, the two fractions those that are <5KDa and >5KDa were not statistically different from each other in either cell line. This in line with what has been observed by Guo *et al*., (2018) in that any positive result can be given by the interaction of more than one type of molecule, which interact together and affect each other. In the case of extracts on the HL60 cell line exposed to 72 hours of treatment with coelomic fluid from day 9 of regeneration, showed that the >5KDa fraction was not significantly different from the complete fluid, but it was statistically significantly different from the <5KDa fraction. This could imply that within the >5KDa fraction there is an ingredient which is found in the complete fluid and affects differentiation.

Marine drugs face serious challenges because of a lack of scientific and technological approaches hindering therapeutic advances (Guo *et al*., 2015). This imposes problems when looking at the molecules in a holistic and dynamic manner which allows to determine its interactions among various biological components. Natural products can be described as being compounds that are structurally complex and they possess a well-defined spatial orientation. This implies that they have evolved over time to interact with their biological targets giving them a good starting point in terms of use in the pharmaceutical industry (Montaser, 2011). Furthermore, the complexity of marine ingredients, unknown targets in the human body, and the active mechanism of marine drug efficiencies increase the difficulties encountered in marine drug studies. Therefore, a scientific and comprehensive method used to discover drugs from marine sources should be established (Guo *et al*., 2015).

4.4 Active biomolecules

Table 1 in the introduction chapter, gives an outline of various types of biomolecules extracted from Holothurians and their effect. Health benefit effects of sea cucumbers have been validated through scientific research and have shown medicinal value such as wound healing, neuroprotective, antitumor, anticoagulant, antimicrobial and antioxidant (Pangestuti & Arifin, 2017). Others are known to be inflammatory and antifungal (Esmat *et al*., 2013). Khotimchenko (2018) has reviewed the pharmacology potential of sea cucumber extracts. Although no extracts from *Holothuria poli* were reviewed as having pharmacology potential, various other species of sea cucumbers possess biomolecules that effect cancer. The molecules, Triterpene glucosides and cerebrosides have been reported in this review as having cytotoxic effects on various types of cancers. Some of these molecules have unique structures highlighting the potential of these marine organisms as a reservoir of new biomolecules.

Jin *et al*., (2009) have compared the effect of two triterpene glycosides obtained from 2 different holothurian species in causing apoptosis in HL-60, NB4, and THP-1 cell lines. They have concluded that whist both triterpene glycosides cause apoptosis on leukaemia cell lines, different holothurians cause apoptosis to happen via caspase-dependent or caspase-independent mechanisms. The same effect by compounds belonging to the same class of molecules can be driven by a different mechanism. Zhang & Zhu (2017) have identified another triterpene glycoside, nobiliside D, from the sea cucumber *Holothuria nobilis*. Using xCELLigence Real-Time Cell Analysis the researchers have determined that this compound exhibits an inhibitory effect on K562 and U937 cell lines amongst other cancer cell lines, indicating that this compound has a potential to be an effect treatment for a range of cancers. At the beginning of this research project, not much literature was available related to extracts from *Holothuria* species inducing differentiation in leukaemia, making isolation and extraction of potential bioactive compounds more difficult.

Samples for testing need to be collected and through *in vitro* tests, an active sample/s is extracted. These *in vitro* tests are fast, inexpensive, and reliable (Lin *et al*.,

2008). In this present research project samples were collected and through *in vitro* tests their cytotoxicity on various cells lines was determined, together with their possible effect on differentiation of Leukaemia. The sample which shows activity was then analysed and its biomolecules separated, and their structure elucidated through various techniques (Lin *et al*., 2008). *In vivo* activity is tested through toxicity testing and preclinical and clinical studies as well as structural studies, leading to the development of the therapeutic drug. This process employed in research and development labs is outlined through the following scheme (figure 92):

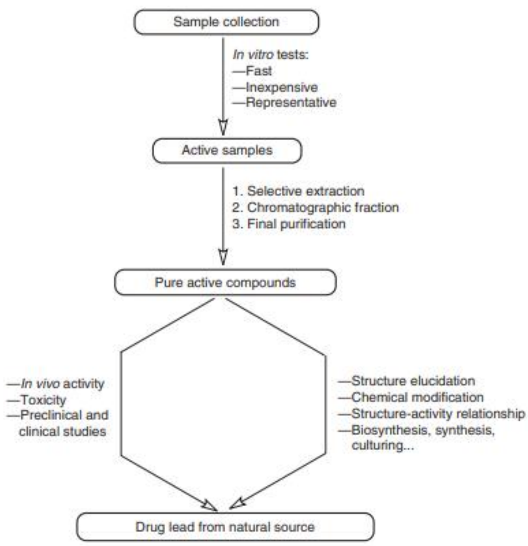


Figure 92: Figure showing an outline of the various steps involved in the extraction of biomolecules for clinical testing. Reference Lin et al., 2008.

A hurdle in the search of the active biomolecules lies in the fact that the actual active ingredient/s might be in such small quantities (Valliappan, *et al*., 2015) that a large number of specimens are needed, increasing stress on wild populations, or difficult to extract with the technology present today.

The marine environment is not a constant environment and its constitution changes with temperature changes, currents, and storms amongst other factors. As a result of these variable conditions and given that *Holothuria poli* are in constant communication with their external environment including when they feed on the sediment, the same organism may produce different metabolites at different times (Karthikeyan *et al*., 2022). This makes it more difficult to pinpoint which is the actual metabolite responsible for a particular pharmaceutical effect. In addition, due to the interaction between microorganisms and the invertebrate host, and the secondary

metabolites produced by these microorganisms, it's not only very difficult to pinpoint the origin of the possible useful metabolite (Peterson *et al*., 2019), but its presence or absence will also depend on the presence or absence of the particular microorganisms, which in turn depends on the environmental conditions at the time. *H. poli* host a number of microorganisms within them (Nerva *et al*., 2019) which vary depending on the habitat and general health of the sea cucumber. However, even healthy Holothurians are generally colonised by organisms such as bacteria, protozoa, gastropods, and others (Eeckhaut *et al*., 2004**)** with some of these organisms having either a parasitic or a communalistic or symbiotic relationship with the holothurians (Marchese *et al*., 2020). Marine microorganisms involved in relationships with animals, plants and fungi are difficult to successfully culture, making it difficult to determine their origin, and hence difficult to distinguish between commensalistic, saprophytic, symbiotic, or parasitic. These microorganisms also secrete compounds which interact with the animals and plants, and which are usually associated with the chemical defence mechanisms of the animal or plant at hand, making it very difficult to distinguish between these products and the products produced directly by the animals or plant (Fattorusso *et al*., 2012). In this research study, 3 organisms were used for every test, in order to always have a biological triplicate, reducing the effect of variation of biomolecules between organisms.

There are concerns that natural products being isolated in recent years are from unidentified microorganisms, making their sources not clearly defined and hence creating a situation where identification of the biological material is not unequivocal (Blunt *et al*., 2014).

The fact that concordant results were being obtained for NBT/MTT and morphology readings in the HL60 cell line when using extract from the 9th day of regeneration, but clearly different to the results at different time points after evisceration might imply that the nature of the biomolecule is one that is constantly found in the coelomic fluid of the organism, and so, rather than being produced by microorganisms within the holothurians, the active biomolecule is probably one that is being secreted by the animal itself in relation to the evisceration / regeneration. Several factors contribute to the triterpenoid composition of *Holothuria* as it is affected by the sources of the sterols.

4.5 The extract.

In this research project, it was essential to start form the premise that the coelomic fluid is not cytotoxic to the various cell lines or to human lymphocytes. There are various chemicals that are known to cause differentiation of leukaemia cells but are toxic to healthy cells, thus limiting their potential use in the treatment of cancers, including extracts from various sea cucumbers. Such extracts include molecules such as saponins (Guo *et al*., 2018) and sea cucumbers are known to contain saponins (Van Dyck, Gerbaux & Flammang, 2009; Caulier *et al*., 2011; Bahrami, Zhang & Franco, 2014; Kamyab *et al*., 2020). In a study by Luparello *et al*, (2019), coelomic fluid extracted from the sea cucumber *Holothuria tubulosa* was used as a cytotoxic agent against triple-negative MDA-MB231 breast cancer cells.

Even though results, in this present study, showed that specific coelomic fluids obtained from specific days of regeneration were not cytotoxic (such as the fluid obtained from day 9 and 12 on K562 and HL60 cell lines), it does not exclude that the effective biomolecule for differentiation, once isolated, would also not possibly be cytotoxic. It could also be that other molecules in the fluid at the time and its specific concentration reduce toxicity, as was reported to be the case outlined by Guo *et al*., (2018) relating to the effect of the different concentrations of eicosapentaenoic acid-enriched phospholipids and sea cucumber saponin on orotic acid-induced non-alcoholic fatty liver disease in rats.

In a study by Silchenko *et al*., (2021), six new triterpene tetra-, penta- and hexaosides, were isolated from the sea cucumber *Psolus chitonoides.* Three of these biomolecules were different from other glycosides found in holothurians that belong to the *Psolidae* family. They had a different structure in that they possess an unusual aglycone bond and lacking a lactone. Some of these novel molecules also had a 3-*O*-methylxylose residue as a terminal unit. This was also never found in holothurians that belong to the *Psolidae* family. All these novel molecules were found to be cytotoxic to various cell lines including HL60. This discovery highlights that specific sea cucumbers, although related can have a myriad of compounds which are different,

and which could have the potential of showing various activities including differentiation.

In this research project, the concentration of the coelomic fluid was defined as % by volume. This was done because isolation of the biomolecules present in the aqueous coelomic fluid into a solid state, without salt and without losing most of the original biomolecules was not possible at the beginning of the research project. In the beginning of this research project, one of the things that was carried out was to evaporate the extract and determine the mass so as to work with more conventional and possibly more accurate units like mass per volume or parts per million (ppm). Originally, samples were freeze dried at the Physiology and Biochemistry Department at UOM and the following (figure 93) was being obtained:

Figure 93 Figure showing white foam left behind in the round bottomed flask when freeze drying coelomic fluid from day 0 and day 9. Photo: Taken by Author.

The equipment available (Labconco freezer) had attachments for round bottomed flasks and so the only possible way to freeze dry the coelomic fluid was by placing it in such a flask. This was not ideal as the solid was then very difficult to remove, resulting in a lot of losses. The white foam is probably due to high protein and high salt content within the coelomic fluid. Eventually, later on during this research, a new equipment (Eppendorf Vacufuge Plus Concentrator) was available at the Department of Pharmacology at UOM where freeze drying of the coelomic fluid

was done directly from the 50 mL conical bottomed tubes, making it easier to work with the solid obtained.

One of the major sources of errors in this study is the supply problem and target identification, which were also outlined by (Karthikeyan *et al.*, 2022). Since the identity of the actual biomolecule/s which might have been responsible for inducing differentiation in HL60 were not known, and the solid was very difficult to obtain, it was very difficult to work with conventional units of ppm or µg/ml.

Two different methods, namely quantifying the amount of protein using BSA analysis and extraction in solvents, were attempted, allowing the the actual mass of solute present in coelomic fluid (excluding salt) to be determined. One was done by determining the protein concentration and use that value as a relative concentration of solute, meaning that the assumption that other molecules with the coelomic fluid are changing in the same proportion. The second method was to extract the coelomic fluid into two solvents, ether, and ester, since one is more polar than the other. Different molecules move into the different solvents at different rates, and the quantity can then be quantified once the solvent is evaporated off.

By employing the first method described, the results obtained are shown in table 11, an average mass of 0.5mg/mL of protein was determined. Whilst the protein concentration of the complete coelomic fluid could be quantified as the absorbances were within the range of the standards, those of the separate fractions were too low to be actually quantified. The quantity of proteins in the different extracts at various stages of regeneration could have been investigated, however, this was not performed as it was difficult to find data to back this concept. An important observation was however made from this part of the study. It was noted, in table 11, that when splitting the extract into two fractions, those bigger than 5KDa and those smaller than 5KDa, the amount of protein was always larger in the >5KDa fraction. This means that the number of larger proteins is bigger than the smaller ones. Since the total proteins bigger and smaller than 5KDa did not add to the total initial value, some proteins were remaining stuck in the filter of the separating tube, contributing to losses.

Extraction of the biomolecule/s in this research project was done through extraction in two solvents ether and ester. Although a mass of solid (without salt) was actually obtained, it was impossible to separate the components found in each

fraction, thus more detailed research about the nature of these biomolecules needs to be conducted to help in this study. The ether ethoxyethane is less polar than the ester ethyl ethanoate. However, in most cases, when analysing the data statistically, there were no clear-cut results or patterns of where there was, it was not statistically significant different. Once again showing that there might be more than one compound that is acting on the leukaemia cells, extracted in the different solvents. The usual method used to isolate a sample from an active crude extract can be summarised in the following diagram (figure 94):

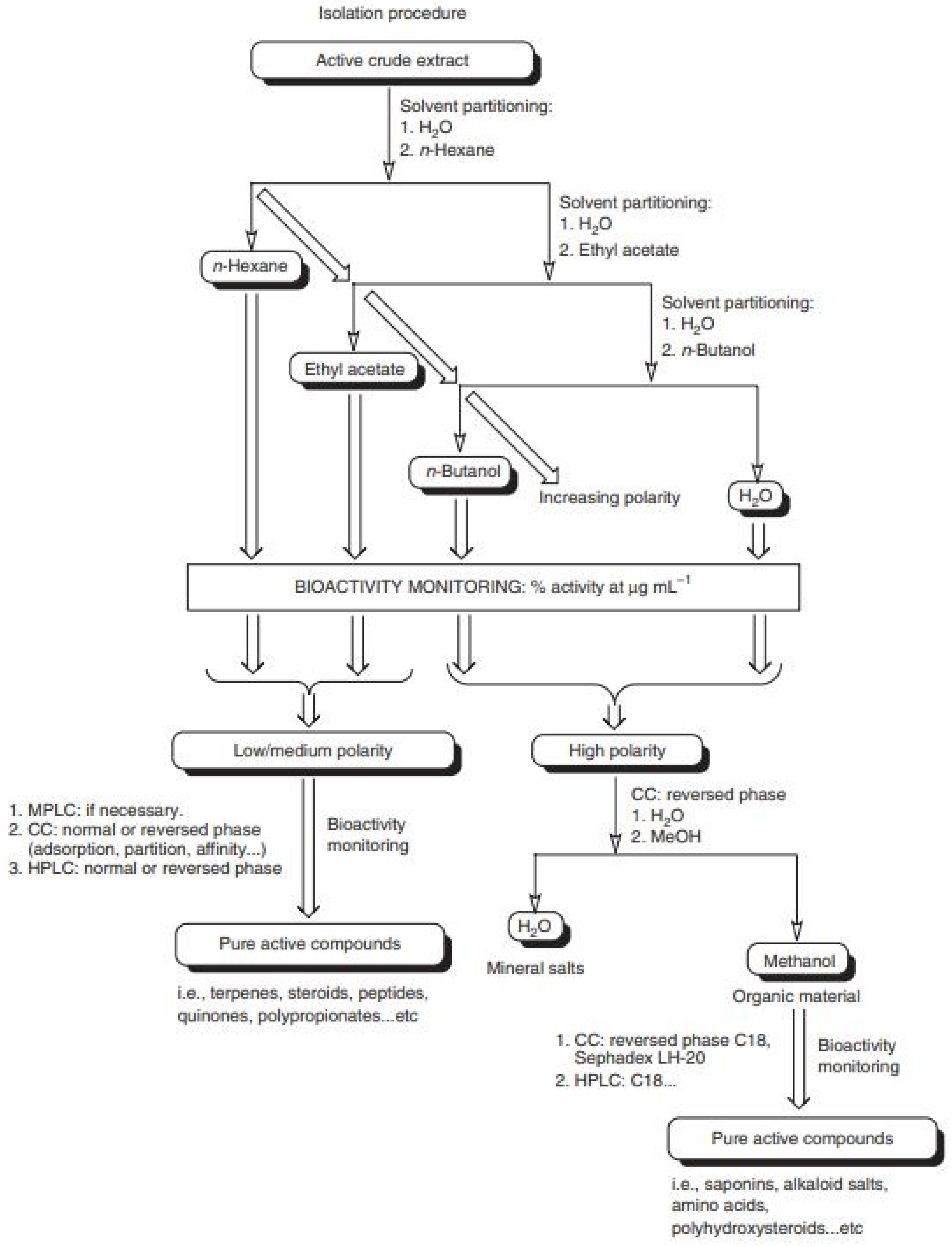


Figure 94: Figure showing the various extraction methods available to isolate pure compounds based on their nature. Reference: Lin et al., 2008.

However, in this present study, the aim was not to completely separate it into the various fractions but to show that potentially different molecules with different polarities, and thus solubilities, are interacting together. This methodology is backed by literature. Molecules from marine sources tend to enhance or reduce each other's

effect (Yun et al., 2012; Guo et al., 2015) and hence secondary bioactive molecules will need to be eventually mixed together in a fashion that has a synergistic effect. This will allow several diseases to be treated in a more efficient way with less adverse reactions when compared to certain medical treatment today.

When the extracts were spun and divided into 2 fractions, that with less than 5KDa weight and those with more than 5KDa weight, the various biomolecules were made to split according to their size. The lower molecular weight molecules correspond to molecules that are likely steroid and terpenes in nature, whilst those of higher molecular weights correspond to proteins (Prof Vincenzo Arizza, University of Palermo). One major source of error is the possible loss of the substances in the filter separating the two different sized fractions. This also implied that some molecules that could be potentially having a positive effect on the process of differentiation were getting stuck to separation place, resulting in loss functionality.

The coelomic fluid obtained from the organisms under study was expected to show different behaviour at different concentrations due to different amounts of biological molecules present. As noted, extracts from these organisms have the potential of being cytotoxic (Luparello *et al.,* 2019). The aim of this research project is to cause differentiation of the cells and based on literature (Mashjoor & Yousefzadi, 2019) it was expected that the higher the concentration of coelomic fluid used, the more cytotoxic it would become. In the study carried out by Mashjoor & Yousefzadi (2019), new marine natural products with anticancer potential from three different Holothuria species (*H. scabra*, *H. parva*, and *H. leucospilota)* obtained from the Persian Gulf were tested. This was achieved by testing three fractions: extracts in hexane, ethyl acetate and methanol and using two toxicity assays on each, brine shrimp (*Artemia salina*) lethality assay and MTT assays in human cancer cell lines (MCF-7) and HeLa. Their results showed that toxicity depended on concentrations, however they also postulate that there can be variations on how toxic a compound in a fraction is depending on what cell line is being tested. When performing tests using 2% -10% by volume as concentration, a recurrent result that 10% by volume extract was possibly toxic was obtained (refer to figures 39-46). Results in this study also showed that the effect of coelomic fluid at a range of 5%-10% by volume of coelomic fluid on the HL60 cell line was similar to the behaviour of 1.6% DMSO which acts as a positive differentiation control (refer to figures 39-42).

Days 21 and CR coincide with near / complete regeneration respectively. Preliminary cytotoxicity tests show that the coelomic fluid from those days of regeneration is in effect cytotoxic (refer to figures 36 and 37). The cells of the regenerating intestine, coinciding with day 9, would be undergoing a high rate of division (as described by figure 70).

4.6 Morphology and Flow cytometry

4.6.1 Morphology

The structure of DNA can be changed into various forms which differ in their ability to be transcribed (Fang 2020). Whilst Heterochromatin is compact and is not involved in protein synthesis, euchromatin is less condensed and involved in protein synthesis. In addition, heterochromatin gives specific genomic domains specific properties. It is responsible for many essential genomic changes such as reducing the activity of mobile elements all with the aim of contributing to the genetic stability (Allshire & Madhani, 2018). Heterochromatin also reduces that amount of repeating DNA sequences (Allshire & Madhani, 2018) which would otherwise cause hypomethylation, leading to malignancy (Roman-Gomez *et al*., 2008 Bergman & Cedar, 2013) Mutations in particular genes such as those related to the cohesion-complex (essential for mediating lopping as well as establish specific boundaries within the chromatin structure), as well as the insulator-binging protein CTCF have been shown to cause cancers such as acute myeloid leukaemia(Fang 2020). These mutations cause the chromatin structure to change abnormally causing different gene expression to occur from that expected (Fang 2020).

Although the figures presented are not conclusive to a morphological change, Kaplinsky, Estrov, Freedman, & Cohen (1986) argue that observations through their studies indicate that differentiation of HL-60 cells can still be taking place even if a change in morphology is not co-occurring as the cellular commitment to commit might not yet be seen as morphological change.

4.6.2 Flow Cytometry

NBT, MTT and NBT/MTT readings for all of the various tests showed more visual coherence in the HL60 cell line than the K562 cell line. In addition, Silchenko *et al*., 2022 have determined that cells from the HL60 cell line is more susceptible to the effect of extracts from Sea Cucumber *Psolus chitonoides*. Therefore, flow cytometry was carried out on cells from this particular cell line as there was a greater chance that a differentiating agent in the extract used in this research project would be more effective.

The results using 4% by volume of coelomic fluid at day 9 post evisceration, showed very little expression of the three markers used, CD14, 11b and CD34. However, more expression was seen when using 8% coelomic fluid as expected from the previous finding of this research project.

CD14 is a human monocyte differentiation antigen (Wu, Zhang, Lei, & Lei, 2019). It is a pattern recognition receptor which enhances the immune responses. The fact that coelomic fluid at 8% by volume obtained from organisms in their 9th day of regeneration, has induced HL60 cell lines to express this marker, is an indication that there was unblocking of HL60 from its blast state. This was also supported by an increase in expression of CD11b. CD11b is a marker for myeloid lineage cells including monocytes, granulocytes, and natural killer cells (Fagerholm, 2006). Whilst the expression of CD14 continued to increase from 72 hours exposed to treatment to 120 hours exposed to treatment, the expression of CD11b was seen to decrease from 72 hours exposed to treatment to 120 hours exposed to treatment. Decrease in CD11b could imply that the cells are going more into a monocyte lineage rather than a granulocyte lineage.

When HL60 are exposed to DMSO, they mature into granulocytic cells, whilst when exposed to PMA HL60 cells acquire a macrophage appearance. Fontana, Colbert, & Deisseroth, (1981) cloned a population of HL60 cells and exposed them to dimethyl formamide and PMA. It was noted that 120 hours after exposure to treatment with dimethyl formamide 95% of the incubated cells acquired granulocytic markers and lacked macrophage markers. However, 120 hours after exposure to treatment with dimethyl formamide 95% of the incubated cells acquired myeloid

macrophage markers and lacked myeloid markers. The same authors have described the HL60 cells as Bipotent stem cells. The expression of CD34 is associated with stem cells (Naeim, 2018). In this study, an increase in expression of CD34 was noted from 72 hours to 120 hours after exposure to treatment. Whilst it could mean that cells are still dividing as they are cancerous, it could also possibly mean that the HL60 cells would have unblocked forming stem cells, before choosing a lineage to mature to.

5 Conclusions and Future work.

The main conclusion from this study is that coelomic fluid from *Holothuria poli* has some effect on overcoming the differentiation block of some leukaemia cell lines, especially when using the HL60 cell line. Through this research project, most of the objectives set at the beginning of this study were successfully resolved, except for the identification of the active ingredient. It can be concluded that the coelomic fluid from organisms in their 9th day of regeneration has great potential towards differentiation of Leukaemia cell line HL60 into monocytes, like 1.6% DMSO. Changes in cell morphology confirm that changes were taking place in the HL60 cells. This by no means does it imply that any other coelomic fluid from any other organism in a different regenerating day cannot also possess the biomolecules needed to cause the same morphological changes. It shows however that the combination of biomolecules in the coelomic fluid of organisms of day of regeneration have a combination that is good enough to start causing changes, calling for more research in the area.

This research project served as an initial step in the research of novel products with the aim to attempt differentiation of leukaemia cells from marine organisms. However more in-depth research needs to be done in order to ensure that molecules that are causing a possible effect are identified and their concentration to give an actual proper effect of differentiation quantified. Hence future work should involve with following:

The coelomic fluid should be dialysed using dialysis tubing immersed in distilled water in order to remove the salts. Once this is achieved, the biomolecules should be separated into various solvents from non-polar to more polar. All the different fractions need to then be biotested.

Various analysis techniques including GC-MS and LC-MS should be used to distinguish the contents of the solvent fraction which was active for day 9. Results are then compared to the coelomic fluid of a non-active day in the same solvent to attempt to identify the active ingredient. Results should also be compared to coelomic fluid from days 21 and CR for possible cytotoxic compounds for cancer treatments.

The life cycle of the *Holothuria poli* needs to be studied more in depth, both in terms of the actual biological life cycle, as well as the regeneration process. Farming of these organisms should also be considered, optimised, and carried out, so that the effect on wild populations is reduced as much as possible.

The method of collection of the coelomic fluid can also be further optimised. One way by which this could be done is to cause stupor to the animals by placing on ice, and then in a cold room fit in a needle into the coelom and collect the coelomic fluid through a syringe. This will reduce further the negative impact on the organisms.

H. poli should be collected together with sand from the area and water, as was the case in this study, but they should be analysed for microorganisms present in each and a complete analysis of the various biomolecules present should be performed using various analytical techniques, including extraction in solvents and analysis using GC-MS, NMR, IR, and other techniques used for identification. The results from the three different locations – the organism, the sand and the water should then be analysed and compared. This should be immediately followed by evisceration (outside of the location in which they are grown) and the same process should be carried out again, but this time also including a screen of any microorganisms and biomolecules present in the eviscerated intestines, in order to look for any changes. This whole process would need to be repeated not only multiple times in the same season from the same location, but also from the same species collected from different location in the same season and the whole process repeated once again throughout the four seasons. This laborious but essential process would give a clearer picture of changes that are happening in the composition of biomolecules throughout the

seasons, resulting from evisceration as well as those occurring (if any) from a difference in location. This is important both from an ecological point of view as it will help shed light on the complex interactions of the sea cucumbers with the environment and with the internal and external microorganisms and also from a medical research point of view as it will shed light on presence, absence and changes in biomolecules present at any point in time.

An optimised method for the growth of cells from the regenerating intestines also needs to be addressed. Biomolecules produced and secreted from these cells into the coelomic fluid might be an alternative source of biomolecules that could be screened for potential effect on differentiation of leukaemia cells lines.

Although natural products obtained from living organisms may give their effect in pure form, more often than not, given that the organism coexists in an ecosystem, interacting with other organisms whether externally or internally, natural products are found to act as a combination of two or more biomolecules interacting together. These molecules, in combination, may either enhance each other's effect, work antagonistically to each other or else give a different effect altogether than either one would on its own. This study shows that there is a very high probability that there is an interaction between some biomolecules as results obtained showed that both the >5kDa and <5kDa fractions gave results which might possibly indicate that differentiation is taking place. So once the data described above is obtained and changes in composition are highlighted, various biomolecules can be investigated on various cell lines, in isolation and in conjunction with others, so that interaction of these biomolecules and their possible effects on differentiation can start to be investigated in a methodical manner.

Finally, other species of sea cucumbers should also be analysed and tested and the possibility of using various molecules from various species be evaluated.

It is essential for the scientific community to further the studies to obtain a more detailed, pharmacological activity and toxicity profile of biomolecules obtained from Holothurians. These organisms are a vast resource of useful biomolecules and so such studies would prove to be indispensable for the discovery of novel marine natural products with possible use in differentiation.

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Appendix 1- MEPA Permits

File Ref NP 134/13

Ms Deborah Vella
24 Triq il-Qalb Mqaddsa
Birkirkara

12th November 2013

Application to carry out a research on *Holothuria polii*

Reference is made to your request for a permit to carry out activities in connection with the capture and keeping of *Holothuria polii*. It is to be noted that the species *Holothuria polii* is not listed as a protected species, thus a MEPA permit to carry out this activity is not required. However precautions should be taken not to damage the natural environment of the sea bed by following the attached list of precautionary conditions. Furthermore if the specimens are to be collected from any one of the Marine Protected Areas then a permit would be required to carry out this research within these protected areas. Attached please find site map of Marine protected areas marked in blue for ease of reference.

1. It is the responsibility of the organiser, Ms Deborah Vella (ID 394483M) to ensure that all reasonable precautions are taken so that no harm is caused to the environment or any protected site or area and cultural heritage.

2. The activity organiser shall ensure that disturbance of any specimen of any species present at the sites should be kept to a minimum. It is crucial to avoid affecting any protected species. No protected species should be taken or transported away from the area.

3. Disturbance to ecological communities within the area in question, especially benthic ones, should be kept to a minimum. Disturbance of the sediment should also be kept to a minimum.

4. It would be appreciated if the organiser could provide MEPA with a report of the activities undertaken and results obtained

   Such report should:
   i. contain information about the locality and date where the authorised activities took place;
   ii. specify the aim of the activity being carried out;
   iii. describe the methodology employed;
   iv. provide results/outcome concerning the activities carried out in connection with the work mentioned in (ii) above;
   v. indicate the information that should be treated as confidential as outlined in Regulation 48 of the Flora, Fauna and Natural Habitats Protection Regulations, 2006.

   This report is to be submitted to nature.permitting@mepa.org.mt,

6. This letter is being forwarded saving third party rights. This letter does not exempt the permit holder from obtaining any other permits, licences, or clearances as may be required by law.

Darrin T Stevens
Unit Manager
Ecosystems Management Unit
f/Director of Environment

Appendix 2
Change in title.

Doctoral School
University of Malta
Msida MSD 2080, Malta

Tel: +356 2340 3608/3254
doctoralschool@um.edu.mt

www.um.edu.mt

18 September 2020

Ms Deborah Vella
24 Triq Il-Qalb Imqaddsa
Birkirkara BKR 4665

Student Code: 394483M

Dear Ms Vella

Change in Title

I refer to correspondence dated 19 June 2020.

I would like to inform you that Senate has approved the change in title of your Ph.D. thesis to 'Investigating the Effect of Differentiation Therapy Using Extracts from Holothuria Poli (Sea Cucumber) on the Haemopoietic Malignancy'.

With best wishes for your studies.

Yours sincerely

Colin Borg
Academic Registrar – Designate

c.c. Dean, Faculty of Medicine and Surgery
Professor Pierre Schembri Wismayer, Principal Supervisor
Director of Finance
Officer i/c Faculty of Medicine and Surgery
SIMS Office

Appendix 3
Frec appoval

FRECMDS_1920_177 - FOR RECORDS Inbox ×

FACULTY RESEARCH ETHICS COMMITTEE <research-ethics.ms@um.edu.mt> Thu, 9 Jul 2020, 13:19

to me, Pierre

Dear Ms Vella,

Since your self-assessment resulted in no issues being identified, FREC will file your application for record and audit purposes but will not review it.

Any ethical and legal issues including data protection issues are your responsibility and that of your supervisor.

Ruth Stivala | Secretary
B.A.(Hons)(Melit.),M.A.(Melit.)

Faculty Research Ethics Committee
Faculty of Medicine and Surgery
Medical School, Mater Dei Hospital
+356 2340 1214

https://www.um.edu.mt/ms/students/researchethics

Appendix 4
R Script

R script

```
library(readxl, quietly = TRUE)

library(dplyr, quietly = TRUE)

library(ggplot2, quietly = TRUE)

library(tidyverse, quietly = TRUE)

library(reshape2, quietly = TRUE)

library(nortest, quietly = TRUE)

library(multcompView, quietly = TRUE)

library(PMCMRplus, quietly = TRUE)

rm(list = ls())



#Choose Cell Line

######

cleaned_data_day_cl <- read_excel("C:/Users/catan/Downloads/Debbie Thesis/Cleaned Data - Testing.xlsx", sheet = "Data - Choose Day & Cell Line")

cleaned_data_day_cl =  cleaned_data_day_cl%>%

                    mutate(log_transform = log(normalised_value),

                          days_since_test = factor(days_since_test),

                          test_day_no = factor(test_day_no.),

                          solution_pt = factor(solution_pt))


normal_results = cleaned_data_day_cl %>%

  group_by(cell_line, days_since_test) %>%

  summarise(sw_p = shapiro.test(log_transform)$p.value,

          interpretation = ifelse(shapiro.test(log_transform)$p.value < 0.05, "Not Normal", "Normal"),
```

```
        kw_result = kruskal.test(log_transform~test_day_no)$p.value)



hl60_3_ph = kwAllPairsDunnTest(log_transform ~ test_day_no,
                    data = cleaned_data_day_cl %>% filter(cell_line == 'HL60',
days_since_test == 3),
                    p.adjust.method = 'bonferroni')$p.value
hl60_5_ph = kwAllPairsDunnTest(log_transform ~ test_day_no,
                    data = cleaned_data_day_cl %>% filter(cell_line == 'HL60',
days_since_test == 5),
                    p.adjust.method = 'bonferroni')$p.value
k562_3_ph = kwAllPairsDunnTest(log_transform ~ test_day_no,
                    data = cleaned_data_day_cl %>% filter(cell_line == 'K562',
days_since_test == 3),
                    p.adjust.method = 'bonferroni')$p.value
k562_5_ph = kwAllPairsDunnTest(log_transform ~ test_day_no,
                    data = cleaned_data_day_cl %>% filter(cell_line == 'K562',
days_since_test == 5),
                    p.adjust.method = 'bonferroni')$p.value
#####


#Choose Concentration
#####
rm(list = ls())
cleaned_data_conc <- read_excel("C:/Users/catan/Downloads/Debbie Thesis/Cleaned
Data - Testing.xlsx", sheet = "Data - Choose Concentration")
cleaned_data_conc =  cleaned_data_conc%>%
  mutate(log_transform = log(normalised_value),
         days_since_test = factor(days_since_test),
```

```
        test_day_no = factor(test_day_no.),

        solution_pt = factor(solution_pt))


normal_results = cleaned_data_conc %>%

  filter(test_day_no == 9) %>%

  group_by(days_since_test, cell_line)%>%

  summarise(sw_p = shapiro.test(log_transform)$p.value,

          interpretation = ifelse(shapiro.test(log_transform)$p.value < 0.05, "Not
Normal", "Normal"),

          kw_result = kruskal.test(log_transform~solution_pt)$p.value)


hl60_3_ph = kwAllPairsDunnTest(log_transform ~ solution_pt,

                              data = cleaned_data_conc %>% filter(cell_line == 'HL60',
days_since_test == 3),

                              p.adjust.method = 'bonferroni')$p.value

hl60_5_ph = kwAllPairsDunnTest(log_transform ~ solution_pt,

                              data = cleaned_data_conc %>% filter(cell_line == 'HL60',
days_since_test == 5),

                              p.adjust.method = 'bonferroni')$p.value

k562_3_ph = kwAllPairsDunnTest(log_transform ~ solution_pt,

                              data = cleaned_data_conc %>% filter(cell_line == 'K562',
days_since_test == 3),

                              p.adjust.method = 'bonferroni')$p.value

k562_5_ph = kwAllPairsDunnTest(log_transform ~ solution_pt,

                              data = cleaned_data_conc %>% filter(cell_line == 'K562',
days_since_test == 5),

                              p.adjust.method = 'bonferroni')$p.value


#####
```

```
#Choose Protein

#####

rm(list = ls())

cleaned_data_prt <- read_excel("C:/Users/catan/Downloads/Debbie Thesis/Cleaned Data - Testing.xlsx", sheet = "Data - Choose Protein")

cleaned_data_prt =  cleaned_data_prt%>%
  mutate(log_transform = log(normalised_value),
         days_since_test = factor(days_since_test),
         test_day_no = factor(test_day_no.),
         solution_pt = factor(solution_pt),
         protein = factor(protein))


normal_results = cleaned_data_prt %>%
  group_by(cell_line, days_since_test)%>%
  summarise(sw_p = shapiro.test(log_transform)$p.value,
            interpretation = ifelse(shapiro.test(log_transform)$p.value < 0.05, "Not Normal", "Normal"),
            kw_result = kruskal.test(log_transform~solution_pt)$p.value)


hl60_3_ph = kwAllPairsDunnTest(log_transform ~ protein,
                               data = cleaned_data_prt %>% filter(cell_line == 'HL60', days_since_test == 3),
                               p.adjust.method = 'bonferroni')$p.value
hl60_5_ph = kwAllPairsDunnTest(log_transform ~ protein,
                               data = cleaned_data_prt %>% filter(cell_line == 'HL60', days_since_test == 5),
                               p.adjust.method = 'bonferroni')$p.value
```

```
k562_3_ph = kwAllPairsDunnTest(log_transform ~ protein,
                         data = cleaned_data_prt %>% filter(cell_line == 'K562',
days_since_test == 3),
                         p.adjust.method = 'bonferroni')$p.value
k562_5_ph = kwAllPairsDunnTest(log_transform ~ protein,
                         data = cleaned_data_prt %>% filter(cell_line == 'K562',
days_since_test == 5),
                         p.adjust.method = 'bonferroni')$p.value



#####

#Choose Component
#####
rm(list = ls())
cleaned_data_cpt <- read_excel("C:/Users/catan/Downloads/Debbie Thesis/Cleaned
Data - Testing.xlsx", sheet = "Data - Choose Component")
cleaned_data_cpt =  cleaned_data_cpt%>%
  mutate(log_transform = log(normalised_value),
         days_since_test = factor(days_since_test),
         test_day_no = factor(test_day_no.),
         solution_pt = factor(solution_pt),
         component = factor(component))

normal_results = cleaned_data_cpt %>%
  group_by(cell_line, days_since_test)%>%
  summarise(sw_p = shapiro.test(log_transform)$p.value,
```

```
interpretation = ifelse(shapiro.test(log_transform)$p.value < 0.05, "Not Normal", "Normal"),

kw_result = kruskal.test(log_transform~component)$p.value)


hl60_3_ph = kwAllPairsDunnTest(log_transform ~ component,

                data = cleaned_data_cpt %>% filter(cell_line == 'HL60', days_since_test == 3),

                p.adjust.method = 'bonferroni')$p.value

hl60_5_ph = kwAllPairsDunnTest(log_transform ~ component,

                data = cleaned_data_cpt %>% filter(cell_line == 'HL60', days_since_test == 5),

                p.adjust.method = 'bonferroni')$p.value

k562_3_ph = kwAllPairsDunnTest(log_transform ~ component,

                data = cleaned_data_cpt %>% filter(cell_line == 'K562', days_since_test == 3),

                p.adjust.method = 'bonferroni')$p.value

k562_5_ph = kwAllPairsDunnTest(log_transform ~ component,

                data = cleaned_data_cpt %>% filter(cell_line == 'K562', days_since_test == 5),

                p.adjust.method = 'bonferroni')$p.value
```

Appendix 5
Data Analysis

Part A – Preliminary testing to determine the range of % by volume of extract which does not show toxicity on Leukaemia cells.

Table showing p values obtained when statistical analysis was carried out on data used for figures 30 and 31.

P Value 0.1

Day 3	5%	10%	15%	20%	25%	DMSO 1.6
10%	1.00	NA	NA	NA	NA	NA
15%	1.00	1.00	NA	NA	NA	NA
20%	1.00	1.00	1.00	NA	NA	NA
25%	0.87	1.00	1.00	1.00	NA	NA
DMSO 1.6	1.00	1.00	1.00	0.31	0.10	NA
RPMI	1.00	1.00	0.31	0.05	0.01	1.00

Day 5	5%	10%	15%	20%	25%	DMSO 1.6
10%	1.00	NA	NA	NA	NA	NA
15%	1.00	1.00	NA	NA	NA	NA
20%	1.00	1.00	1.00	NA	NA	NA
25%	0.63	1.00	1.00	1.00	NA	NA
DMSO 1.6	1.00	1.00	1.00	0.37	0.06	NA
RPMI	1.00	1.00	0.37	0.06	0.01	1.00

Data for figure 30:

	RPMI	**1.6% DMSO**	**5% extract**	**10% extract**	**15% extract**	**20% extract**	**25% Extract**
MTT reading 1	7.95	4.422	3.654	3.557	1.349	1.083	0.878
MTT reading 2	8.422	3.922	3.553	2.81	1.734	1.43	0.881
MTT reading 3	8.2	4.314	3.053	3.183	1.855	1.079	1.09

Treatment	Negative control (RPMI)	Positive Control (1.6% DMSO)	5% extract	10% extract	15% extract	20% extract	25% Extract
Normalised MTT	1	0.515139	0.417548	0.388654	0.294807	0.20096	0.146183
Standard error	0.136335	0.151901	0.185802	0.21564	0.152553	0.116339	0.070172

Data for figure 31

	RPMI	1.6% DMSO	5% extract	10% extract	15% extract	20% extract	25% Extract
MTT Reading 1	15.105	6.743	5.224	4.937	2.552	1.07	0.694
MTT Reading 2	15.782	6.502	5.366	5.017	2.652	1.189	0.678
MTT Reading 3	15.149	6.329	4.795	4.901	2.271	1.141	0.683

Suspension	Negative control (RPMI)	Positive Control (1.6% DMSO)	5% extract	10% extract	15% extract	20% extract	25% Extract
Normalised MTT value	1.8855	0.808542	0.646468	0.607643	0.317679	0.137901	0.083754
Standard error	0.3385	0.1205	0.071	0.04	0.05	0.0595	0.008

Part C: Average MTT values used for graph 34.

	RPMI	DMSO10%	10%
0 hours	1	1	1
24 hours	1.192484149	0.517810231	1.235301532
48 Hours	2.490711229	0.378453652	2.285427884
72 hours	2.744614313	0.366651242	3.0507386

Part D– Selection of regeneration date from which to extract coelomic fluid.

Cell Line	Day Since Test	Shapiro Wilk (p-value)	Normality Interpretation	Kruskall Wallis (p-value)	Pairwise Comparison Test
HL60	3	0.031	Not Normal	0.000	Dunn's Test
HL60	5	0.036	Not Normal	0.000	Dunn's Test
K562	3	0.000	Not Normal	0.000	Dunn's Test
K562	5	0.000	Not Normal	0.000	Dunn's Test

The following are p tables - red signifies statistical significance.

In the following table 3 signifies 72 hours exposed to treatment

Cell Line	K562
Day Since Test	3

	0	3	6	9	12	15	18	21	CR
0		1.000	1.000	0.006	1.000	1.000	1.000	1.000	0.000
3	1.000		1.000	0.004	1.000	1.000	1.000	1.000	0.000
6	1.000	1.000		0.000	1.000	1.000	1.000	0.260	0.000
9	0.006	0.004	0.000		0.035	0.000	0.000	1.000	1.000
12	1.000	1.000	1.000	0.035		1.000	1.000	1.000	0.001
15	1.000	1.000	1.000	0.000	1.000		1.000	0.265	0.000
18	1.000	1.000	1.000	0.000	1.000	1.000		0.013	0.000
21	1.000	1.000	0.260	1.000	1.000	0.265	0.013		0.234
CR	0.000	0.000	0.000	1.000	0.001	0.000	0.000	0.234	

In the case of the cell line K562 at day 3, it was determined that the NBT/MTT readings for day 9 were significantly different from all other percentages but not from day 21 and CR. CR was significantly different from all the days except day 9 and 21.

In the following table 5 signifies 120 hours exposed to treatment.

Cell Line	K562
Day Since Test	5

	0	3	6	9	12	15	18	21	CR
0		0.004	1.000	0.000	1.000	1.000	1.000	0.000	0.000
3	0.004		0.272	1.000	0.048	0.000	0.003	1.000	0.109
6	1.000	0.272		0.043	1.000	1.000	1.000	0.000	0.000
9	0.000	1.000	0.043		0.004	0.000	0.000	1.000	0.018
12	1.000	0.048	1.000	0.004		1.000	1.000	0.000	0.000
15	1.000	0.000	1.000	0.000	1.000		1.000	0.000	0.000
18	1.000	0.003	1.000	0.000	1.000	1.000		0.000	0.000
21	0.000	1.000	0.000	1.000	0.000	0.000	0.000		1.000
CR	0.000	0.109	0.000	0.018	0.000	0.000	0.000	1.000	

In the case of the cell line K562 at day 5, it was determined that the NBT/MTT readings for day 3 were significantly different from day 0, 12, 15 and 18 but not from the others. Those of day 9 were significantly different from all other days except day 3 and 21. Day 21 showed a significant difference from all days except day 3 and 9 and CR and those of CR were significantly different from all the others but not those of days 3 and day 21.

In the following table 3 signifies 72 hours exposed to treatment

Cell Line	HL60
Day Since Test	3

	0	3	6	9	12	15	18	21	CR
0		1.000	1.000	0.001	1.000	1.000	1.000	0.000	0.000
3	1.000		1.000	0.137	0.100	1.000	1.000	0.000	0.000
6	1.000	1.000		0.000	1.000	1.000	1.000	0.000	0.000
9	0.001	0.137	0.000		0.000	0.000	0.000	1.000	0.555
12	1.000	0.100	1.000	0.000		1.000	1.000	0.000	0.000
15	1.000	1.000	1.000	0.000	1.000		1.000	0.000	0.000
18	1.000	1.000	1.000	0.000	1.000	1.000		0.000	0.000
21	0.000	0.000	0.000	1.000	0.000	0.000	0.000		1.000
CR	0.000	0.000	0.000	0.555	0.000	0.000	0.000	1.000	

In the case of the cell line HL60 at day 3, it was determined that the NBT/MTT readings for day 9 were significantly different from all other percentages but not from day3, 21 and CR. Days 21 and CR were significantly different from all other extracts except for day 9 and each other.

In the following table 3 signifies 120 hours exposed to treatment

Cell Line	HL60
Day Since Test	5

	0	3	6	9	12	15	18	21	CR
0		1.000	1.000	0.000	0.127	1.000	1.000	0.035	0.001
3	1.000		1.000	0.000	0.040	1.000	1.000	0.181	0.008
6	1.000	1.000		0.000	0.435	1.000	1.000	0.008	0.000
9	0.000	0.000	0.000		0.000	0.000	0.000	1.000	1.000
12	0.127	0.040	0.435	0.000		0.000	1.000	0.000	0.000
15	1.000	1.000	1.000	0.000	0.000		1.000	0.200	0.005
18	1.000	1.000	1.000	0.000	1.000	1.000		0.002	0.000
21	0.035	0.181	0.008	1.000	0.000	0.200	0.002		1.000
CR	0.001	0.008	0.000	1.000	0.000	0.005	0.000	1.000	

In the case of the cell line HL60 at day 5 it was determined that the NBT/MTT readings for day 9 were significantly different from all other percentages but not from day 21 and CR. Day 21 showed a significant difference from all days except day 3, 9, 15 and CR and those of CR were significantly different except for those of day 9 and day 21.

Part E –Data used for the *log transformation and .

***this is only part of the data used for the log transformation as there is the equivalent data for both cells lines for all days investigated for 72 and 120 hours of exposure.**

Data for figure 38

NBT 72 hours - HL60 - day 9									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.975	0.601	2.958	0.878	2.473	0.839	0.891	1.084	2.117
Reading 2	0.845	1.118	0.642	0.992	0.816	0.991	0.905	0.704	1.671
Reading 3	0.951	1.016	1.037	0.844	1.054	1.195	1.217	1.08	3.09
Reading 4	0.865	1.152	1.131	0.961	1.019	0.84	0.867	0.806	0.867
Reading 5	0.837	0.742	0.993	0.743	1.199	0.737	1.787	0.87	0.753
Reading 6	0.96	0.902	0.966	0.847	0.975	0.684	0.788	0.784	0.695
Reading 7	0.993	1.37	0.813	0.73	1.054	1.028	0.967	0.794	0.842
Reading 8	0.918	0.742	0.921	0.834	1.056	0.917	1.037	0.918	0.882
Reading 9	0.951	1.016	1.037	0.844	1.054	1.195	1.217	1.08	3.09
Average	0.921667	0.962111	1.166444	0.852556	1.188889	0.936222	1.075111	0.902222	1.556333
Stdev	0.058517	0.24069	0.686923	0.086166	0.491801	0.182942	0.305935	0.146535	0.99116
st error	0.019506	0.08023	0.228974	0.028722	0.163934	0.060981	0.101978	0.048845	0.330387
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	1.043882	1.265582	0.925015	1.289934	1.015793	1.166486	0.978903	1.688608
Stdev	0.058517	0.24069	0.686923	0.086166	0.491801	0.182942	0.305935	0.146535	0.99116
st error	0.019506	0.08023	0.228974	0.028722	0.163934	0.060981	0.101978	0.048845	0.330387

MTT 72 hours - HL60 - day 9									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.993	0.882	0.797	0.335	0.829	0.709	0.697	0.606	0.276
Reading 2	0.781	0.838	0.797	0.293	0.784	0.426	0.697	0.582	0.275
Reading 3	0.876	0.801	0.719	0.251	0.846	0.707	0.72	0.591	0.265
Reading 4	0.901	0.817	0.521	0.28	0.885	0.858	0.718	0.658	0.284
Reading 5	0.91	0.823	0.718	0.19	0.838	0.775	0.776	0.532	0.305
Reading 6	0.802	0.799	0.735	0.085	0.784	0.677	0.681	0.607	0.275
Reading 7	0.713	0.851	0.803	0.341	0.816	0.735	0.673	0.578	0.269
Reading 8	0.75	0.766	0.66	0.247	0.748	0.71	0.663	0.166	0.257
Reading 9	0.719	0.776	0.754	0.278	0.725	0.637	0.582	0.5	0.253
Average	0.827222	0.817	0.722667	0.255556	0.806111	0.692667	0.689667	0.535556	0.273222
Stdev	0.097244	0.036606	0.088931	0.078737	0.050481	0.117863	0.052369	0.145748	0.015401
st error	0.032415	0.012202	0.029644	0.026246	0.016827	0.039288	0.017456	0.048583	0.005134
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	0.987643	0.873606	0.308932	0.97448	0.83734	0.833714	0.647414	0.330289
Stdev	0.097244	0.036606	0.088931	0.078737	0.050481	0.117863	0.052369	0.145748	0.015401
st error	0.032415	0.012202	0.029644	0.026246	0.016827	0.039288	0.017456	0.048583	0.005134

NBT/MTT 72 hours - HL60 - day 9									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.981873	0.681406	3.711418	2.620896	2.983112	1.183357	1.278336	1.788779	7.67029
Reading 2	1.081946	1.334129	0.805521	3.385666	1.040816	2.326291	1.298422	1.209622	6.076364
Reading 3	1.085616	1.268414	1.442281	3.36255	1.245863	1.69024	1.690278	1.827411	11.66038
Reading 4	0.960044	1.410037	2.170825	3.432143	1.151412	0.979021	1.207521	1.224924	3.052817
Reading 5	0.91978	0.90158	1.383008	3.910526	1.430788	0.950968	2.302835	1.635338	2.468852
Reading 6	1.197007	1.128911	1.314286	9.964706	1.243622	1.01034	1.157122	1.291598	2.527273
Reading 7	1.392707	1.609871	1.012453	2.140762	1.291667	1.398639	1.43685	1.373702	3.130112
Reading 8	1.224	0.968668	1.395455	3.376518	1.411765	1.291549	1.564103	5.53012	3.431907
Reading 9	1.32267	1.309278	1.375332	3.035971	1.453793	1.875981	2.091065	2.16	12.21344
Average	1.129516	1.179144	1.623398	3.914415	1.472538	1.411821	1.558503	2.004611	5.803492
Stdev	0.165363	0.287091	0.86612	2.326553	0.582412	0.468634	0.403156	1.360793	3.891352
st error	0.055121	0.095697	0.288707	0.775518	0.194137	0.156211	0.134385	0.453598	1.297117
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	1.043937	1.43725	3.465568	1.303689	1.249934	1.379797	1.774752	5.138034
Stdev	0.165363	0.287091	0.86612	2.326553	0.582412	0.468634	0.403156	1.360793	3.891352
st error	0.055121	0.095697	0.288707	0.775518	0.194137	0.156211	0.134385	0.453598	1.297117

Data for figure 39

NBT 120 hours - HL60 - day 9									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.919	1.761	1.191	0.699	0.965	1.145	1.361	1.233	0.98
Reading 2	0.608	2.434	0.76	2.208	2.266	1.124	2.426	1.768	0.994
Reading 3	0.644	0.774	1.294	0.725	0.785	0.879	0.663	0.946	0.737
Reading 4	0.935	1.88	1.083	1.281	3.171	1.192	1.305	1.358	2.467
Reading 5	0.787	1.561	0.929	1.149	2.218	1.247	0.954	0.972	3.356
Reading 6	1.107	1.069	1.524	1.357	1.614	3.943	1.621	2.411	2.233
Reading 7	1.562	0.776	0.686	1.254	0.738	0.962	0.919	1.816	1.595
Reading 8	0.836	0.728	0.655	0.638	0.757	0.867	0.718	0.646	1.043
Reading 9	1.075	1.992	1.733	1.068	1.335	1.057	1.453	1.386	1.742
Average	0.941444	1.441667	1.095	1.153222	1.538778	1.379556	1.268889	1.392889	1.683
Stdev	0.288425	0.625803	0.377609	0.479973	0.85523	0.970566	0.547578	0.538262	0.86608
st error	0.096142	0.208601	0.12587	0.159991	0.285077	0.323522	0.182526	0.179421	0.288693
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalised	1.021459	1.564195	1.188065	1.251236	1.66956	1.496805	1.376733	1.511272	1.82604
Stdev	0.288425	0.625803	0.377609	0.479973	0.85523	0.970566	0.547578	0.538262	0.86608
st error	0.096142	0.208601	0.12587	0.159991	0.285077	0.323522	0.182526	0.179421	0.288693

MTT 120 hours - HL60 - day 9									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.677	0.862	0.166	0.944	1.373	1.021	0.905	0.689	0.215
Reading 2	1.541	0.782	0.102	0.946	1.302	1.094	0.884	0.636	0.207
Reading 3	1.635	0.837	0.12	0.93	1.227	1.001	0.794	0.682	0.27
Reading 4	1.49	0.729	0.122	0.034	1.345	1.011	0.826	0.612	0.23
Reading 5	1.634	0.629	0.152	0.926	1.418	1.126	0.869	0.594	0.196
Reading 6	1.744	0.7	0.128	0.953	1.362	1.01	0.849	0.547	0.233
Reading 7	1.73	0.878	0.101	0.91	1.427	0.99	0.819	0.679	0.211
Reading 8	1.764	0.714	0.091	0.962	1.353	0.985	0.861	0.643	0.245
Reading 9	1.678	0.732	0.23	0.885	1.128	0.913	0.72	0.582	0.211
Average	1.654778	0.762556	0.134667	0.832222	1.326111	1.016778	0.836333	0.629333	0.224222
Stdev	0.091857	0.083145	0.043154	0.300254	0.095421	0.061983	0.055403	0.049497	0.022819
st error	0.030619	0.027715	0.014385	0.100085	0.031807	0.020661	0.018468	0.016499	0.007606
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	2.000403	0.921827	0.162794	1.006044	1.603089	1.229147	1.011014	0.760779	0.271054
Stdev	0.091857	0.083145	0.300254	0.043154	0.095421	0.061983	0.055403	0.049497	0.022819
st error	0.030619	0.027715	0.100085	0.014385	0.031807	0.020661	0.018468	0.016499	0.007606

NBT/MTT 120 hours - HL60 - day 9									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.548002	2.042923	7.174699	0.740466	0.70284	1.12145	1.503867	1.78955	4.55814
Reading 2	0.394549	3.112532	7.45098	2.334038	1.740399	1.027422	2.744344	2.779874	4.801932
Reading 3	0.393884	0.924731	10.78333	0.77957	0.639772	0.878122	0.835013	1.387097	2.72963
Reading 4	0.627517	2.578875	8.877049	1.284691	2.357621	1.179031	1.579903	2.218954	10.72609
Reading 5	0.48164	2.481717	6.111842	1.240821	1.564175	1.10746	1.097814	1.636364	17.12245
Reading 6	0.634748	1.527143	11.90625	1.423924	1.185022	3.90396	1.909305	4.407678	9.583691
Reading 7	0.90289	0.883827	6.792079	1.378022	0.517169	0.971717	1.1221	2.674521	7.559242
Reading 8	0.473923	1.019608	7.197802	0.663202	0.559497	0.880203	0.833914	1.004666	4.257143
Reading 9	0.640644	2.721311	7.534783	1.20678	1.183511	1.157722	2.018056	2.381443	8.255924
Average	0.566422	1.921408	8.203202	1.227946	1.161112	1.358565	1.516035	2.25335	7.732693
Stdev	0.159104	0.855733	1.945188	0.505695	0.631383	0.961109	0.63036	1.00117	4.424862
st error	0.053035	0.285244	0.648396	0.168565	0.210461	0.32037	0.21012	0.333723	1.474954
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.501473	1.701089	7.262581	1.087143	1.027973	1.202785	1.342199	1.994969	6.846023
Stdev	0.159104	0.855733	1.945188	0.505695	0.631383	0.961109	0.63036	1.00117	4.424862
st error	0.053035	0.285244	0.648396	0.168565	0.210461	0.32037	0.21012	0.333723	1.474954

Figure 40:

NBT 72 hours - HL60 - day 12									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.816	1.165	0.868	1.771	1.467	0.243	4.189	1.951	0.632
Reading 2	1.296	4.159	2.481	0.578	0.608	0.451	3.576	0.59	3.599
Reading 3	0.647	0.925	0.723	0.572	0.598	0.538	0.571	0.559	0.708
Reading 4	0.643	0.576	0.668	0.48	0.55	0.561	0.605	0.461	0.571
Reading 5	0.662	2.274	1.018	0.955	0.626	1.094	0.924	1.041	1.053
Reading 6	0.929	1.125	1.467	0.853	1.063	1.23	0.844	1.022	1.115
Reading 7	0.848	2.403	1.17	0.986	0.418	1.574	2.181	0.792	0.684
Reading 8	0.974	1.994	1.263	1.045	0.866	0.891	1.048	1.224	2.48
Reading 9	0.866	1.039	0.729	0.773	0.876	0.688	0.878	1.266	0.917
Average	0.853444	1.74	1.154111	0.890333	0.785778	0.807778	1.646222	0.989556	1.306556
Stdev	0.206532	1.11019	0.56722	0.386531	0.323793	0.425516	1.361488	0.464069	1.038444
st error	0.068844	0.370063	0.189073	0.128844	0.107931	0.141839	0.453829	0.15469	0.346148
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	2.038797	1.352298	1.043224	0.920713	0.946491	1.928916	1.159484	1.53092
Stdev	0.206532	1.11019	0.56722	0.386531	0.323793	0.425516	1.361488	0.464069	1.038444
st error	0.068844	0.370063	0.189073	0.128844	0.107931	0.141839	0.453829	0.15469	0.346148

MTT 72 hours - HL60 - day 12									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.814	0.913	0.711	0.252	0.755	0.741	0.707	0.641	0.324
Reading 2	0.904	0.909	0.8	0.308	0.816	0.844	0.679	0.581	0.288
Reading 3	0.81	0.909	0.721	0.34	0.671	0.743	0.722	0.621	0.263
Reading 4	0.905	0.897	0.717	0.336	0.815	0.572	0.706	0.628	0.272
Reading 5	0.875	0.908	0.785	0.34	0.8	0.703	0.637	0.594	0.303
Reading 6	0.939	0.965	0.778	0.316	0.686	0.654	0.688	0.545	0.292
Reading 7	0.915	0.776	0.699	0.251	0.659	0.584	0.584	0.356	0.242
Reading 8	0.714	0.835	0.676	0.281	0.762	0.585	0.551	0.425	0.221
Reading 9	0.71	0.781	0.709	0.273	0.758	0.592	0.567	0.426	0.17
Average	0.842889	0.877	0.732889	0.299667	0.746889	0.668667	0.649	0.535222	0.263889
Stdev	0.086059	0.064836	0.043421	0.036418	0.061079	0.095055	0.0662	0.105475	0.047057
st error	0.028686	0.021612	0.014474	0.012139	0.02036	0.031685	0.022067	0.035158	0.015686
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.040469	0.869496	0.355523	0.886106	0.793303	0.769971	0.634985	0.313077
Stdev	0.086059	0.064836	0.043421	0.036418	0.061079	0.095055	0.0662	0.105475	0.047057
st error	0.028686	0.021612	0.014474	0.012139	0.02036	0.031685	0.022067	0.035158	0.015686

NBT/MTT 72 hours - HL60 - day 12									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.002457	1.276013	1.220816	7.027778	1.943046	0.327935	5.925035	3.043682	1.950617
Reading 2	1.433628	4.575358	3.10125	1.876623	0.745098	0.53436	5.266568	1.015491	12.49653
Reading 3	0.798765	1.017602	1.002774	1.682353	0.891207	0.724092	0.790859	0.900161	2.692015
Reading 4	0.710497	0.64214	0.93166	1.428571	0.674847	0.980769	0.856941	0.734076	2.099265
Reading 5	0.756571	2.504405	1.296815	2.808824	0.7825	1.556188	1.450549	1.752525	3.475248
Reading 6	0.98935	1.165803	1.885604	2.699367	1.549563	1.880734	1.226744	1.875229	3.818493
Reading 7	0.926776	3.096649	1.67382	3.928287	0.634294	2.695205	3.734589	2.224719	2.826446
Reading 8	1.364146	2.388024	1.868343	3.718861	1.136483	1.523077	1.901996	2.88	11.22172
Reading 9	1.219718	1.330346	1.028209	2.831502	1.155673	1.162162	1.548501	2.971831	5.394118
Average	1.022434	1.999593	1.556588	3.111352	1.056968	1.264947	2.52242	1.933079	5.108272
Stdev	0.263018	1.260826	0.685732	1.699734	0.442802	0.739246	1.952906	0.913551	3.97559
st error	0.087673	0.420275	0.228577	0.566578	0.147601	0.246415	0.650969	0.304517	1.325197
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.955718	1.522433	3.043082	1.033776	1.237191	2.467073	1.890663	4.996186
Stdev	0.263018	1.260826	0.685732	1.699734	0.442802	0.739246	1.952906	0.913551	3.97559
st error	0.087673	0.420275	0.228577	0.566578	0.147601	0.246415	0.650969	0.304517	1.325197

Data for figures 41

NBT 120 hours - HL60 - day 12									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.041	1.325	1.248	1.363	1.122	0.964	0.977	1.276	1.02
Reading 2	0.94	1.271	1.144	2.299	2.771	1.057	1.894	0.892	0.984
Reading 3	1.208	1.351	1.075	1.004	2.518	1.123	1.654	4.063	0.996
Reading 4	0.934	0.925	0.8	2.743	1.147	2.85	3.97	1.276	1.236
Reading 5	1.175	0.789	1.685	2.037	1.315	1.322	1.07	1.081	1.216
Reading 6	1.14	1.203	1.199	1.193	1.392	1.235	1.078	1.109	1.102
Reading 7	2.265	1.085	1.257	1.202	3.823	1.103	1.144	1.065	1.091
Reading 8	0.959	1.103	1.254	1.163	1.067	2.08	1.044	1.116	1.099
Reading 9	1.15	1.103	1.157	0.817	1.071	1.13	1.757	0.885	0.949
Average	1.201333	1.128333	1.202111	1.535667	1.802889	1.429333	1.620889	1.418111	1.077
Stdev	0.412722	0.185014	0.230289	0.660328	0.994191	0.626104	0.947731	1.001373	0.100468
st error	0.137574	0.061671	0.076763	0.220109	0.331397	0.208701	0.31591	0.333791	0.033489
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.407629	1.322093	1.408541	1.799375	2.112485	1.674782	1.899232	1.661633	1.261945
Stdev	0.412722	0.185014	0.230289	0.660328	0.994191	0.626104	0.947731	1.001373	0.100468
st error	0.137574	0.061671	0.076763	0.220109	0.331397	0.208701	0.31591	0.333791	0.033489

MTT 120 hours - HL60 - day 12									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.714	0.853	1.027	0.682	1.819	1.449	1.175	0.81	0.268
Reading 2	1.917	0.921	0.791	0.825	0.876	1.336	1.108	0.891	0.244
Reading 3	1.954	0.727	1.069	0.741	1.666	1.43	1.166	0.873	0.267
Reading 4	1.695	0.862	1.094	0.713	1.806	1.435	1.101	0.866	0.26
Reading 5	1.653	0.723	1.069	0.687	1.702	1.295	1.162	0.796	0.25
Reading 6	1.851	0.668	1.035	0.647	1.566	1.307	0.906	0.709	0.263
Reading 7	1.777	0.629	1.013	0.755	1.506	1.208	0.909	0.718	0.313
Reading 8	1.774	0.774	1.042	0.544	1.476	1.182	0.95	0.698	0.316
Reading 9	1.714	0.771	1.021	0.285	1.53	1.268	1.045	0.817	0.293
Average	1.783222	0.769778	1.017889	0.653222	1.549667	1.323333	1.058	0.797556	0.274889
Stdev	0.103745	0.095206	0.089083	0.158378	0.282135	0.09824	0.110485	0.073931	0.02626
st error	0.034582	0.031735	0.029694	0.052793	0.094045	0.032747	0.036828	0.024644	0.008753
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	2.115608	0.913261	1.207619	0.77498	1.838518	1.569997	1.255207	0.946217	0.326127
Stdev	0.103745	0.095206	0.089083	0.145198	0.282135	0.09824	0.110485	0.073931	0.02626
st error	0.034582	0.031735	0.029694	0.048399	0.094045	0.032747	0.036828	0.024644	0.008753

NBT/MTT 120 hours - HL60 - day 12									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.607351	1.553341	1.21519	1.998534	0.616822	0.665286	0.831489	1.575309	3.80597
Reading 2	0.49035	1.380022	1.446271	2.786667	3.163242	0.791168	1.709386	1.001122	4.032787
Reading 3	0.618219	1.858322	1.005613	1.354926	1.511405	0.785315	1.418525	4.654066	3.730337
Reading 4	0.551032	1.073086	0.731261	3.847125	0.635105	1.986063	3.605813	1.473441	4.753846
Reading 5	0.710829	1.091286	1.576239	2.965066	0.77262	1.020849	0.920826	1.35804	4.864
Reading 6	0.615883	1.800898	1.158454	1.843895	0.888889	0.944912	1.189845	1.564175	4.190114
Reading 7	1.27462	1.72496	1.240869	1.592053	2.538513	0.913079	1.258526	1.483287	3.485623
Reading 8	0.540586	1.425065	1.203455	2.137868	0.7229	1.759729	1.098947	1.598854	3.477848
Reading 9	0.670945	1.43061	1.133203	2.866667	0.7	0.891167	1.68134	1.083231	3.238908
Average	0.675535	1.481954	1.190062	2.376978	1.283277	1.084174	1.523855	1.754614	3.95327
Stdev	0.234432	0.283633	0.241588	0.795306	0.941857	0.462377	0.837436	1.108375	0.565597
st error	0.078144	0.094544	0.080529	0.265102	0.313952	0.154126	0.279145	0.369458	0.188532
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.660712	1.449437	1.163949	2.324822	1.255119	1.060385	1.490419	1.716114	3.866527
Stdev	0.234432	0.283633	0.241588	0.795306	0.941857	0.462377	0.837436	1.108375	0.565597
st error	0.078144	0.094544	0.080529	0.265102	0.313952	0.154126	0.279145	0.369458	0.188532

Data for figures 42

NBT - 72 hours - K562 Day 9									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.89	0.947	1.11	0.803	1.913	4.07	1.35	1.754	0.849
Reading 2	0.905	0.953	0.74	0.758	0.924	1.032	0.91	1.147	0.916
Reading 3	1.221	0.776	1.008	0.887	0.864	0.837	0.976	0.771	0.757
Reading 4	0.954	1.198	1.056	0.921	0.837	0.827	1.648	0.873	0.705
Reading 5	0.934	0.841	1.547	1.162	1.048	0.914	0.881	0.93	0.955
Reading 6	1.157	1.347	1.128	3.718	0.909	4.061	0.811	0.947	0.747
Reading 7	1.755	1.455	1.403	1.697	1.306	1.489	1.213	1.405	1.163
Reading 8	1.929	1.549	1.421	1.628	1.625	2.164	1.966	1.507	1.503
Reading 9	2.227	1.324	2.23	1.705	1.506	2.807	2.088	1.882	1.581
Average	1.330222	1.154444	1.293667	1.475444	1.214667	2.022333	1.315889	1.246222	1.019556
Stdev	0.50723	0.28258	0.429372	0.926886	0.390601	1.33755	0.482851	0.406167	0.327044
st error	0.169077	0.094193	0.143124	0.308962	0.1302	0.44585	0.16095	0.135389	0.109015
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	0.867858	0.972519	1.109171	0.913131	1.520297	0.989225	0.936853	0.766455
Stdev	0.50723	0.28258	0.429372	0.926886	0.390601	1.33755	0.482851	0.406167	0.327044
st error	0.169077	0.094193	0.143124	0.308962	0.1302	0.44585	0.16095	0.135389	0.109015

MTT - 72 hours - K562 Day 9									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.953	0.418	0.365	0.455	0.79	0.699	0.618	0.547	0.199
Reading 2	0.999	0.473	0.155	0.546	0.796	0.828	0.671	0.576	0.219
Reading 3	1.049	0.182	0.13	0.319	0.619	0.703	0.597	0.626	0.262
Reading 4	0.937	0.167	0.149	0.571	1.017	0.923	0.713	0.527	0.261
Reading 5	1.076	0.19	0.209	0.311	0.666	0.93	0.8	0.608	0.236
Reading 6	1.056	0.543	0.348	0.766	1.188	0.935	0.681	0.662	0.348
Reading 7	1.161	0.747	0.273	0.509	1.202	0.935	0.881	0.75	0.345
Reading 8	1.017	0.669	0.221	0.462	1.19	1.168	0.959	0.813	0.367
Reading 9	1.201	1.109	0.301	0.411	1.242	1.224	0.804	0.882	0.424
Average	1.049889	0.499778	0.239	0.483333	0.967778	0.927222	0.747111	0.665667	0.295667
Stdev	0.087823	0.311822	0.087334	0.139358	0.251045	0.179723	0.122161	0.123431	0.077337
st error	0.029274	0.103941	0.029111	0.046453	0.083682	0.059908	0.04072	0.041144	0.025779
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.476029	0.227643	0.460366	0.921791	0.883162	0.71161	0.634035	0.281617
Stdev	0.087823	0.311822	0.087334	0.139358	0.251045	0.179723	0.122161	0.123431	0.077337
st error	0.029274	0.103941	0.029111	0.046453	0.083682	0.059908	0.04072	0.041144	0.025779

NBT/MTT - 72 hours - K562 Day 9									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.933893	2.26555	3.041096	1.764835	2.421519	5.822604	2.184466	3.206581	4.266332
Reading 2	0.905906	2.014799	4.774194	1.388278	1.160804	1.246377	1.356185	1.991319	4.182648
Reading 3	1.163966	4.263736	7.753846	2.780564	1.3958	1.190612	1.634841	1.231629	2.889313
Reading 4	1.018143	7.173653	7.087248	1.61296	0.823009	0.895991	2.31136	1.656546	2.701149
Reading 5	0.86803	4.426316	7.401914	3.736334	1.573574	0.982796	1.10125	1.529605	4.04661
Reading 6	1.095644	2.480663	3.241379	4.853786	0.765152	4.343316	1.190896	1.430514	2.146552
Reading 7	1.511628	1.947791	5.139194	3.333988	1.086522	1.592513	1.376844	1.873333	3.371014
Reading 8	1.896755	2.315396	6.429864	3.52381	1.365546	1.85274	2.050052	1.853629	4.095368
Reading 9	1.854288	1.193868	7.408638	4.148418	1.21256	2.293301	2.597015	2.133787	3.728774
Average	1.249806	3.120197	5.808597	3.015886	1.31161	2.246694	1.755879	1.878549	3.491973
Stdev	0.403286	1.855345	1.825906	1.213288	0.49131	1.706827	0.542231	0.573636	0.759756
st error	0.134429	0.618448	0.608635	0.404429	0.16377	0.568942	0.180744	0.191212	0.253252
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	2.496545	4.6476	2.413084	1.049451	1.797635	1.404921	1.503073	2.794013
Stdev	0.403286	1.855345	1.825906	1.213288	0.49131	1.706827	0.542231	0.573636	0.759756
st error	0.134429	0.618448	0.608635	0.404429	0.16377	0.568942	0.180744	0.191212	0.253252

Figure 43

NBT - 120 hours - K562 Day 9									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.95	1.545	2.15	1.238	1.284	1.443	1.529	1.138	0.944
Reading 2	1.268	2.113	0.748	1.304	1.231	1.483	1.716	1.147	0.987
Reading 3	1.091	2.215	0.899	1.349	1.215	1.265	0.936	0.99	1.33
Reading 4	2.859	1.12	0.971	1.361	1.513	1.088	1.504	1.627	1.15
Reading 5	1.032	4.11	0.776	4.004	1.422	0.894	4.044	0.778	0.843
Reading 6	1.135	1.09	0.931	1.882	0.986	1.564	2.132	1.104	4.128
Reading 7	1.0205	1.88	1.5245	1.2935	1.2495	1.354	1.2325	1.064	1.137
Reading 8	1.2015	1.6015	0.8395	1.593	1.1085	1.5235	1.924	1.1255	2.5575
Reading 9	1.9455	2.615	0.8735	2.6825	1.4675	0.991	2.774	1.2025	0.9965
Average	1.389167	2.032167	1.079167	1.856333	1.275167	1.2895	1.976833	1.130667	1.563667
Stdev	0.625467	0.926197	0.462661	0.92682	0.170594	0.245458	0.93975	0.223934	1.091378
st error	0.208489	0.308732	0.15422	0.30894	0.056865	0.081819	0.31325	0.074645	0.363793
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.044312	1.52769	0.811268	1.395506	0.958612	0.969387	1.486093	0.849983	1.175493
Stdev	0.625467	0.926197	0.462661	0.92682	0.170594	0.245458	0.93975	0.223934	1.091378
st error	0.208489	0.308732	0.15422	0.30894	0.056865	0.081819	0.31325	0.074645	0.363793

MTT - 120 hours - K562 Day 9									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.733	1.34	0.278	0.597	0.692	1.522	1.391	1.266	1.095
Reading 2	1.865	0.7395	0.627	0.658	1.762	1.517	1.299	1.256	1.1
Reading 3	1.766	0.553	0.206	0.758	1.741	1.203	1.442	1.218	1.097
Reading 4	1.768	0.716	0.341	0.614	1.622	1.535	1.301	1.179	0.993
Reading 5	1.843	0.418	0.233	1.64	1.465	1.58	1.39	1.306	1.025
Reading 6	1.674	2.057	0.293	0.799	0.577	1.456	0.69	1.154	0.6
Reading 7	1.533	0.856	0.362	0.715	1.46	1.064	1.084	0.925	0.261
Reading 8	1.675	0.771	0.363	0.624	1.492	1.068	1.065	1.104	0.53
Reading 9	1.628	0.623	0.347	1.325	1.559	1.413	1.178	1.005	0.502
Average	1.720556	0.897056	0.338889	0.858889	1.374444	1.373111	1.204444	1.157	0.800333
Stdev	0.105068	0.504954	0.121901	0.368434	0.434582	0.205525	0.235381	0.126409	0.325064
st error	0.035023	0.168318	0.040634	0.122811	0.144861	0.068508	0.07846	0.042136	0.108355
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.638798	0.854429	0.322785	0.818076	1.309133	1.307863	1.147211	1.102021	0.762303
Stdev	0.105068	0.504954	0.121901	0.368434	0.434582	0.205525	0.235381	0.126409	0.325064
st error	0.035023	0.168318	0.040634	0.122811	0.144861	0.068508	0.07846	0.042136	0.108355

NBT/MTT - 120 hours - K562 Day 9									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.548182	1.152985	7.733813	2.073702	1.855491	0.948095	1.099209	0.898894	0.8621
Reading 2	0.679893	2.857336	1.192982	1.981763	0.698638	0.977587	1.321016	0.913217	0.897273
Reading 3	0.61778	4.005425	4.364078	1.779683	0.697875	1.051538	0.649098	0.812808	1.212397
Reading 4	1.617081	1.564246	2.847507	2.216612	0.932799	0.708795	1.156034	1.379983	1.158107
Reading 5	0.559957	9.832536	3.330472	2.441463	0.970648	0.565823	2.909353	0.595712	0.822439
Reading 6	0.678017	0.529898	3.177474	2.355444	1.708839	1.074176	3.089855	0.956672	6.88
Reading 7	0.665688	2.196262	4.211326	1.809091	0.855822	1.272556	1.136993	1.15027	4.356322
Reading 8	0.717313	2.077173	2.312672	2.552885	0.742962	1.426498	1.806573	1.019475	4.825472
Reading 9	1.195025	4.197432	2.517291	2.024528	0.941309	0.701345	2.354839	1.196517	1.98506
Average	0.808771	3.157032	3.520846	2.137241	1.044931	0.969601	1.724774	0.991505	2.555463
Stdev	0.35906	2.78385	1.851694	0.272871	0.432135	0.278777	0.869679	0.230065	2.230468
st error	0.119687	0.92795	0.617231	0.090957	0.144045	0.092926	0.289893	0.076688	0.743489
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.647117	2.526018	2.817115	1.710059	0.836075	0.775802	1.380034	0.793328	2.044688
Stdev	0.35906	2.78385	1.851694	0.272871	0.432135	0.278777	0.869679	0.230065	2.230468
st error	0.119687	0.92795	0.617231	0.090957	0.144045	0.092926	0.289893	0.076688	0.743489

Figure 44

NBT - 72 hours - K560 day 12									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.718	0.764	0.712	1.871	1.867	0.903	0.97	0.827	0.866
Reading 2	0.885	0.78	0.763	1.804	2.064	0.886	1.063	0.987	0.851
Reading 3	1.266	0.757	0.84	1.794	2.237	0.985	1.03	0.806	0.724
Reading 4	1.098	0.737	0.742	1.498	0.888	0.806	4.061	1.772	0.753
Reading 5	0.941	0.788	2.948	0.969	2.61	0.836	3.761	0.96	0.709
Reading 6	0.85	0.702	0.805	0.899	2.99	4.185	0.882	0.744	0.711
Reading 7	1.39	1.122	1.89	1.284	1.295	1.353	1.382	3.817	1.232
Reading 8	1.551	1.604	1.322	2.686	1.497	1.544	1.199	1.729	1.218
Reading 9	1.565	2.321	1.259	1.361	2.711	1.311	1.31	2.658	1.523
Average	1.251556	1.063889	1.253444	1.574	2.017667	1.423222	1.739778	1.588889	0.954111
Stdev	0.324132	0.553858	0.745718	0.545773	0.69995	1.068679	1.243419	1.050335	0.296114
st error	0.108044	0.184619	0.248573	0.181924	0.233317	0.356226	0.414473	0.350112	0.098705
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalised	1	0.850053	1.001509	1.257635	1.612127	1.137163	1.390092	1.269531	0.76234
Stdev	0.324132	0.553858	0.745718	0.61629	1.21868	1.068679	1.243419	1.050335	0.296114
st error	0.108044	0.184619	0.248573	0.20543	0.406227	0.356226	0.414473	0.350112	0.098705

MTT - 72 hours - K560 day 12									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.697	0.105	0.089	0.473	0.634	0.638	0.441	0.392	0.135
Reading 2	0.877	0.122	0.151	0.446	0.535	0.695	0.562	0.419	0.212
Reading 3	0.653	0.165	0.111	0.376	0.734	0.582	0.591	0.411	0.181
Reading 4	0.785	0.121	0.095	0.47	0.591	0.595	0.534	0.409	0.157
Reading 5	0.875	0.173	0.138	0.49	0.711	0.689	0.551	0.511	0.16
Reading 6	0.837	0.147	0.15	0.572	0.639	0.714	0.561	0.379	0.161
Reading 7	1.079	0.238	0.441	0.848	1.229	1.122	0.981	0.905	0.429
Reading 8	1.099	0.401	0.369	0.958	1.18	1.19	1.011	0.923	0.438
Reading 9	1.065	0.436	0.34	0.832	1.276	1.022	0.955	0.905	0.45
Average	0.885222	0.212	0.209333	0.607222	0.836556	0.805222	0.687444	0.583778	0.258111
Stdev	0.164874	0.123672	0.134847	0.212963	0.300552	0.237528	0.225381	0.248244	0.137338
st error	0.054958	0.041224	0.044949	0.070988	0.100184	0.079176	0.075127	0.082748	0.045779
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	0.239488	0.236475	0.685955	0.945023	0.909627	0.776578	0.65947	0.291578
Stdev	0.164874	0.123672	0.134847	0.212963	0.300552	0.237528	0.225381	0.248244	0.137338
st error	0.054958	0.041224	0.044949	0.070988	0.100184	0.079176	0.075127	0.082748	0.045779

NBT/MTT - 72 hours - K560 day 12									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	2.464849	7.27619	8	3.955603	2.944795	1.415361	2.199546	2.109694	6.414815
Reading 2	1.009122	6.393443	5.05298	4.044843	3.857944	1.27482	1.891459	2.355609	4.014151
Reading 3	1.938744	4.587879	7.567568	4.771277	3.047684	1.69244	1.742809	1.961071	4
Reading 4	1.398726	6.090909	7.810526	3.187234	1.502538	1.354622	7.604869	4.332518	4.796178
Reading 5	1.075429	4.554913	21.36232	1.977551	3.670886	1.213353	6.825771	1.878669	4.43125
Reading 6	1.015532	4.77551	5.366667	1.571678	4.679186	5.861345	1.572193	1.963061	4.416149
Reading 7	1.28823	4.714286	4.285714	1.514151	1.053702	1.205882	1.408767	4.21768	2.871795
Reading 8	1.411283	4	3.582656	2.803758	1.268644	1.297479	1.185955	1.873239	2.780822
Reading 9	1.469484	5.323394	3.702941	1.635817	2.124608	1.282779	1.371728	2.937017	3.384444
Average	1.452378	5.301836	7.414597	2.829101	2.683332	1.844231	2.867011	2.625395	4.123289
Stdev	0.477344	1.066385	5.512294	1.230206	1.269991	1.513596	2.491109	0.993028	1.108261
st error	0.159115	0.355462	1.837431	0.410069	0.42333	0.504532	0.83037	0.331009	0.36942
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	3.650453	5.105144	1.94791	1.847544	1.269801	1.974012	1.807653	2.838993
Stdev	0.477344	1.066385	5.512294	0.848211	2.132319	1.513596	2.491109	0.993028	1.63839
st error	0.159115	0.355462	1.837431	0.282737	0.710773	0.504532	0.83037	0.331009	0.54613

Figure 45

NBT - 120 hours - K560 day 12									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.162	1.093	1.001	0.923	1.549	1.133	0.967	1.758	1.028
Reading 2	1.096	1.293	1.194	1.454	1.61	1.176	1.12	1.393	0.993
Reading 3	1.113	0.951	1.112	1.148	0.972	0.886	1.055	1.342	0.945
Reading 4	0.948	1.305	0.861	1.721	0.983	2.041	0.856	1.278	1.088
Reading 5	1.01	0.917	0.887	1.663	0.881	1.707	1.222	1.508	1.261
Reading 6	1.481	0.86	0.846	0.805	0.839	1.213	0.923	1.318	0.793
Reading 7	1.319	1.281	1.991	1.433	1.497	1.671	1.563	1.154	2.245
Reading 8	1.452	1.581	1.734	1.49	1.837	2.13	4.107	1.39	1.029
Reading 9	1.148	1.186	1.053	1.383	1.411	1.295	1.568	1.408	0.862
Average	1.192111	1.163	1.186556	1.335556	1.286556	1.472444	1.486778	1.394333	1.138222
Stdev	0.186482	0.231411	0.405649	0.314699	0.369481	0.432419	1.015808	0.167795	0.435966
st error	0.062161	0.077137	0.135216	0.1049	0.12316	0.14414	0.338603	0.055932	0.145322
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalised	0.952504	0.929244	0.948065	1.067116	1.027965	1.176491	1.187944	1.11408	0.909446
Stdev	0.186482	0.231411	0.405649	0.314699	0.369481	0.432419	1.015808	0.167795	0.435966
st error	0.062161	0.077137	0.135216	0.1049	0.12316	0.14414	0.338603	0.055932	0.145322

MTT - 120 hours - K560 day 12									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.528	0.211	0.169	0.368	1.155	1.325	1.066	0.845	1.453
Reading 2	1.41	0.215	0.202	0.379	1.203	1.275	1.114	0.917	1.263
Reading 3	1.583	0.15	0.196	0.4	1.246	1.295	1.122	0.746	1.422
Reading 4	1.467	0.143	0.204	0.389	1.335	1.24	1.172	0.752	1.323
Reading 5	1.001	0.159	0.188	0.387	1.238	1.102	1.015	1.03	1.4
Reading 6	1.214	0.153	0.173	0.432	1.279	1.153	1.163	1.069	1.542
Reading 7	1.381	0.195	0.227	0.549	1.361	1.294	1.156	1.058	0.955
Reading 8	1.305	0.22	0.266	0.574	1.382	1.298	1.117	1.036	1.522
Reading 9	1.398	0.271	0.258	0.543	1.277	1.28	1.225	1.074	1.484
Average	1.365222	0.190778	0.209222	0.446778	1.275111	1.251333	1.127778	0.947444	1.373778
Stdev	0.175629	0.042845	0.034557	0.083675	0.07438	0.074843	0.061563	0.135956	0.180946
st error	0.058543	0.014282	0.011519	0.027892	0.024793	0.024948	0.020521	0.045319	0.060315
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1.542237	0.215514	0.23635	0.504707	1.440442	1.413581	1.274005	1.07029	1.551902
Stdev	0.552968	0.086979	0.096758	0.21104	0.581864	0.550742	0.509433	0.43994	0.612087
st error	0.184323	0.028993	0.032253	0.070347	0.193955	0.183581	0.169811	0.146647	0.204029

NBT/MTT - 120 hours - K560 day 12									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.760471	5.180095	5.923077	0.635237	1.341126	0.855094	0.907129	2.080473	2.793478
Reading 2	0.777305	6.013953	5.910891	1.151227	1.338321	0.922353	1.005386	1.519084	2.620053
Reading 3	0.703095	6.34	5.673469	0.807314	0.780096	0.68417	0.940285	1.798928	2.3625
Reading 4	0.646217	9.125874	4.220588	1.300831	0.73633	1.645968	0.730375	1.699468	2.796915
Reading 5	1.008991	5.767296	4.718085	1.187857	0.711632	1.549002	1.203941	1.464078	3.258398
Reading 6	1.219934	5.620915	4.890173	0.522049	0.655981	1.052038	0.793637	1.232928	1.835648
Reading 7	0.955105	6.569231	8.770925	1.500524	1.099927	1.291345	1.352076	1.090737	4.089253
Reading 8	1.112644	7.186364	6.518797	0.978975	1.329233	1.640986	3.676813	1.341699	1.792683
Reading 9	0.821173	4.376384	4.081395	0.931941	1.104933	1.011719	1.28	1.310987	1.587477
Average	0.889437	6.242235	5.634156	1.001773	1.010842	1.18363	1.321071	1.504265	2.570712
Stdev	0.195384	1.350764	1.439855	0.31679	0.291236	0.360652	0.908914	0.309676	0.793557
st error	0.065128	0.450255	0.479952	0.105597	0.097079	0.120217	0.302971	0.103225	0.264519
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	0.612401	4.297942	3.879264	0.689747	0.695991	0.814961	0.909592	1.035726	1.770002
Stdev	0.195384	1.350764	1.439855	0.31679	0.291236	0.360652	0.908914	0.309676	0.793557
st error	0.065128	0.450255	0.479952	0.105597	0.097079	0.120217	0.302971	0.103225	0.264519

Part G: Normalised data for figures 36 and 37

Normalised MTT for HL60 cells after 72 hours exposed to day 21 extract for figure 36.

	RPMI	1.6% DMSO	2%	4%	6%	8%	10%
Normalised	1	0.454	0.073	0.061	0.0337	0.0414	0.0161
Standard deviation	0.516	0.232	0.256	0.195	0.0964	0.0940	0.0395
Standard error	0.298	0.1348	0.1479	0.112	0.0556	0.0543	0.0228

Normalised MTT for HL60 cells after 72 hours exposed to day CR extract for figure 37.

	RPMI	1.6% DMSO	2%	4%	6%	8%	10%
Normalised	1.000	0.742	0.122	0.265	0.218	0.196	0.126
Standard Deviation	0.105	0.072	0.150	0.098	0.054	0.060	0.019
Standard error	0.060	0.042	0.087	0.057	0.031	0.035	0.011

Part H: Statistics for selection of % coelomic fluid.

Cell Line	Day Since Test	Shapiro Wilk (p-value)	Normality Interpretation	Kruskall Wallis (p-value)	Pairwise Comparison Test
HL60	3	0.000	Not Normal	0.000	Dunn's Test
HL60	5	0.001	Not Normal	0.000	Dunn's Test
K562	3	0.001	Not Normal	0.000	Dunn's Test
K562	5	0.000	Not Normal	0.000	Dunn's Test

P values for K562 cell line exposed to coelomic fluid from day 9 post evisceration. 3 signifies 72 hours after treatment whilst 5 signifies 120 hours after treatment. Values in red are those which show statistical significance at $p < 0.05$. 0.02 represents coelomic fluid at 2%, 0.04 represents coelomic fluid at 4%, 0.06 represents coelomic fluid at 6%, 0.08 represents coelomic fluid at 8%, 0.1 represents coelomic fluid at 10%.

The following are p tables - red signifies statistical significance.

3 represents 72 hours exposed to treatment

Cell Line	K562
Day Since Test	3

	RPMI	DMSO	PMA10	PMA10(	0.02	0.04	0.06	0.08	0.1
RPMI		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DMSO	0.000		1.000	0.389	1.000	1.000	1.000	1.000	1.000
PMA10	0.000	1.000		1.000	0.043	1.000	0.332	1.000	1.000
PMA100	0.000	0.389	1.000		0.001	0.081	0.011	0.106	1.000
0.02	0.000	1.000	0.043	0.001		1.000	1.000	1.000	0.120
0.04	0.000	1.000	1.000	0.081	1.000		1.000	1.000	1.000
0.06	0.000	1.000	0.332	0.011	1.000	1.000		1.000	0.775
0.08	0.000	1.000	1.000	0.106	1.000	1.000	1.000		1.000
0.1	0.000	1.000	1.000	1.000	0.120	1.000	0.775	1.000	

5 represents 120 hours exposed to treatment.

Cell Line	K562
Day Since Test	5

	RPMI	DMSO	PMA10	PMA10(	0.02	0.04	0.06	0.08	0.1
RPMI		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DMSO	0.000		0.020	0.298	1.000	1.000	1.000	1.000	1.000
PMA10	0.000	0.020		1.000	0.000	0.002	0.369	0.010	1.000
PMA100	0.000	0.298	1.000		0.006	0.038	1.000	0.175	1.000
0.02	0.000	1.000	0.000	0.006		1.000	1.000	1.000	0.072
0.04	0.000	1.000	0.002	0.038	1.000		1.000	1.000	0.332
0.06	0.000	1.000	0.369	1.000	1.000	1.000		1.000	1.000
0.08	0.000	1.000	0.010	0.175	1.000	1.000	1.000		1.000
0.1	0.000	1.000	1.000	1.000	0.072	0.332	1.000	1.000	

P values for HL60 cell line exposed to coelomic fluid from day 9 post evisceration. 3 signifies 72 hours after treatment whilst 5 signifies 120 hours after treatment. Values in red are those which show statistical significance at $p < 0.05$. 0.02 represents coelomic fluid at 2%, 0.04 represents coelomic fluid at 4%, 0.06 represents coelomic fluid at 6%, 0.08 represents coelomic fluid at 8%, 0.1 represents coelomic fluid at 10%.

Cell Line	HL60
Day Since Test	3

	RPMI	DMSO	PMA10	PMA10(	0.02	0.04	0.06	0.08	0.1
RPMI		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DMSO	0.000		0.007	0.421	0.165	0.069	0.636	1.000	1.000
PMA10	0.000	0.007		1.000	1.000	1.000	1.000	1.000	0.003
PMA100	0.000	0.421	1.000		1.000	1.000	1.000	1.000	0.196
0.02	0.000	0.165	1.000	1.000		1.000	1.000	1.000	0.072
0.04	0.000	0.069	1.000	1.000	1.000		1.000	1.000	0.028
0.06	0.000	0.636	1.000	1.000	1.000	1.000		1.000	0.306
0.08	0.000	1.000	1.000	1.000	1.000	1.000	1.000		0.941
0.1	0.000	1.000	0.003	0.196	0.072	0.028	0.306	0.941	

Cell Line	HL60
Day Since Test	5

	RPMI	DMSO	PMA10	PMA10(	0.02	0.04	0.06	0.08	0.1
RPMI		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
DMSO	0.000		0.532	0.086	0.006	0.019	0.110	1.000	1.000
PMA10	0.000	0.532		1.000	1.000	1.000	1.000	1.000	0.918
PMA100	0.000	0.086	1.000		1.000	1.000	1.000	1.000	0.165
0.02	0.000	0.006	1.000	1.000		1.000	1.000	1.000	0.014
0.04	0.000	0.019	1.000	1.000	1.000		1.000	1.000	0.039
0.06	0.000	0.110	1.000	1.000	1.000	1.000		1.000	0.208
0.08	0.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000
0.1	0.000	1.000	0.918	0.165	0.014	0.039	0.208	1.000	

Part I: Statistics for > 5 KDa or < 5 KDa.

Cell Line	Day Since Test	Shapiro Wilk (p-value)	Normality Interpretation	Kruskall Wallis (p-value)	Pairwise Comparison Test
HL60	3	0.000	Not Normal	0.000	Dunn's Test
HL60	5	0.000	Not Normal	0.000	Dunn's Test
K562	3	0.000	Not Normal	0.000	Dunn's Test
K562	5	0.000	Not Normal	0.000	Dunn's Test

The following represent p values. Red signifies statistical significance. In the following table 3 signifies 72 hours exposed to treatment, P represents the >5KDa fraction and T represents the <5KDa fraction.

Cell Line	K562
Day Since Test	3

	Complete	P	T
Complete		0.005	0.000
P	0.005		0.211
T	0.000	0.211	

In the following table 5 signifies 120 hours exposed to treatment, P represents the >5KDa fraction and T represents the <5KDa fraction.

Cell Line	K562
Day Since Test	5

	Complete	P	T
Complete		0.251	1.000
P	0.251		0.327
T	1.000	0.327	

In the following table 3 signifies 72 hours exposed to treatment, P represents the >5KDa fraction and T represents the <5KDa fraction.

Cell Line	HL60
Day Since Test	3

	Complete	P	T
Complete		0.436	0.000
P	0.436		0.004
T	0.000	0.004	

In the following table 5 signifies 120 hours exposed to treatment, P represents the >5KDa fraction and T represents the <5KDa fraction.

Cell Line	HL60
Day Since Test	5

	Complete	P	T
Complete		0.037	0.001
P	0.037		0.576
T	0.001	0.576	

Part J: Data for > 5 KDa or < 5 KDa.

Data for figure 63

NBT 72 hours - HL60 Day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	0.02	0.04	0.06	0.08	0.1
Reading 1	1.291	1.358	1.228	2.462	2.053	1.564	1.452	1.191	1.228
Reading 2	1.828	1.557	1.902	2.04	1.638	1.654	1.425	1.249	1.405
Reading 3	1.258	1.554	1.435	2.556	1.449	1.79	1.36	1.136	1.814
Reading 4	1.476	1.374	1.955	2.67	1.554	1.447	1.375	1.256	1.21
Reading 5	1.292	1.045	1.47	1.956	1.47	1.332	1.587	1.261	1.192
Reading 6	1.249	1.363	1.177	2.422	1.279	2.206	1.372	1.105	1.112
Reading 7	1.347	1.377	1.829	2.686	2.275	2.228	1.389	1.311	1.47
Reading 8	1.355	1.172	1.163	2.027	1.161	1.305	1.289	1.303	1.186
Reading 9	0.957	1.08	1.178	2.212	1.108	0.975	1.244	0.975	1.328
Average	1.339222	1.32	1.481889	2.336778	1.554111	1.611222	1.388111	1.198556	1.327222
Stdev	0.230029	0.185577	0.330645	0.284736	0.390987	0.414379	0.097988	0.109542	0.2155
st error	0.076676	0.061859	0.110215	0.094912	0.130329	0.138126	0.032663	0.036514	0.071833
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	0.985647	1.106529	1.744877	1.160458	1.203103	1.036505	0.894964	0.99104
Stdev	0.230029	0.185577	0.330645	0.284736	0.390987	0.414379	0.097988	0.109542	0.2155
st error	0.076676	0.061859	0.110215	0.094912	0.130329	0.138126	0.032663	0.036514	0.071833

MTT 72 hours - HL60 Day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	0.02	0.04	0.06	0.08	0.1
Reading 1	0.475	0.514	0.432	0.264	0.484	0.469	0.421	0.368	0.195
Reading 2	0.492	0.477	0.446	0.277	0.494	0.399	0.357	0.381	0.185
Reading 3	0.497	0.462	0.431	0.297	0.42	0.446	0.393	0.36	0.159
Reading 4	0.487	0.464	0.432	0.287	0.468	0.431	0.376	0.375	0.166
Reading 5	0.434	0.449	0.431	0.269	0.472	0.445	0.389	0.382	0.183
Reading 6	0.491	0.46	0.439	0.287	0.511	0.45	0.399	0.371	0.173
Reading 7	0.491	0.42	0.173	0.2705	0.441	0.386	0.391	0.187	0.19
Reading 8	0.529	0.483	0.183	0.292	0.494	0.418	0.411	0.201	0.1625
Reading 9	0.521	0.456	0.229	0.278	0.436	0.431	0.387	0.196	0.1815
Average	0.490778	0.465	0.355111	0.280167	0.468889	0.430556	0.391556	0.313444	0.177222
Stdev	0.027161	0.025656	0.121105	0.011242	0.030665	0.026082	0.018595	0.089397	0.012669
st error	0.009054	0.008552	0.040368	0.003747	0.010222	0.008694	0.006198	0.029799	0.004223
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.947476	0.723568	0.570863	0.9554	0.877292	0.797827	0.638669	0.361105
Stdev	0.027161	0.025656	0.121105	0.012278	0.030665	0.026082	0.018595	0.089397	0.012669
st error	0.009054	0.008552	0.040368	0.004093	0.010222	0.008694	0.006198	0.029799	0.004223

NBT/MTT 72 hours - HL60 Day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS	0.02	0.04	0.06	0.08	0.1
Reading 1	2.717895	2.642023	2.842593	9.325758	4.241736	3.334755	3.448931	3.236413	6.297436
Reading 2	3.715447	3.264151	4.264574	7.364621	3.315789	4.145363	3.991597	3.278215	7.594595
Reading 3	2.531187	3.363636	3.329466	8.606061	3.45	4.013453	3.46056	3.155556	11.40881
Reading 4	3.030801	2.961207	4.525463	9.303136	3.320513	3.357309	3.656915	3.349333	7.289157
Reading 5	2.976959	2.327394	3.410673	7.271375	3.114407	2.993258	4.079692	3.301047	6.513661
Reading 6	2.543788	2.963043	2.681093	8.439024	2.502935	4.902222	3.438596	2.978437	6.427746
Reading 7	2.743381	3.278571	10.57225	9.92976	5.15873	5.772021	3.55243	7.010695	7.736842
Reading 8	2.561437	2.426501	6.355191	6.941781	2.350202	3.12201	3.136253	6.482587	7.298462
Reading 9	1.836852	2.368421	5.144105	7.956835	2.541284	2.262181	3.21447	4.97449	7.316804
Average	2.73975	2.843883	4.791713	8.348706	3.332844	3.766952	3.553271	4.196308	7.542612
Stdev	0.502789	0.414045	2.465545	1.042938	0.901577	1.069263	0.316487	1.565365	1.541705
st error	0.167596	0.138015	0.821848	0.347646	0.300526	0.356421	0.105496	0.521788	0.513902
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	1.038008	1.74896	3.047251	1.216478	1.374926	1.296933	1.531639	2.75303
Stdev	0.502789	0.414045	2.465545	1.042938	0.901577	1.069263	0.316487	1.565365	1.541705
st error	0.167596	0.138015	0.821848	0.347646	0.300526	0.356421	0.105496	0.521788	0.513902

Data for figure 64

NBT 120 hours - HL60 Day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	2.827	2.228	2.721	3.943	2.378	3.759	3.952	4.14	1.921
Reading 2	2.432	2.015	2.38	3.769	2.732	2.815	3.065	1.848	2.871
Reading 3	2.927	2.424	2.33	3.883	2.959	2.033	3.23	2.212	2.09
Reading 4	2.289	1.662	2.947	4.005	3.829	1.559	3.244	4.039	2.257
Reading 5	2.015	2.113	2.452	2.766	3.021	1.423	2.01	4.048	2.395
Reading 6	2.04	2.471	1.724	2.601	3.073	2.036	2.491	3.804	3.94
Reading 7	1.515	1.75	1.654	2.877	1.735	1.659	2.227	3.926	1.686
Reading 8	1.708	1.535	1.902	3.68	1.901	2.754	3.738	4.077	1.893
Reading 9	1.693	1.615	1.548	2.482	4.003	1.894	3.219	1.912	1.817
Average	2.160667	1.979222	2.184222	3.334	2.847889	2.214667	3.019556	3.334	2.318889
Stdev	0.499705	0.354243	0.49732	0.635032	0.771297	0.755761	0.656586	1.016676	0.705955
st error	0.166568	0.118081	0.165773	0.211677	0.257099	0.25192	0.218862	0.338892	0.235318
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalised	1.613374	1.477889	1.630963	2.489505	2.126525	1.653696	2.254708	2.489505	1.731519
Stdev	0.499705	0.354243	0.49732	0.635032	0.771297	0.755761	0.656586	1.016676	0.705955
st error	0.166568	0.118081	0.165773	0.211677	0.257099	0.25192	0.218862	0.338892	0.235318

MTT 120 hours - HL60 Day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.437	0.678	0.5	0.595	1.145	1.079	0.866	0.658	0.312
Reading 2	1.416	0.62	0.582	0.555	1.245	1.045	0.908	0.757	0.319
Reading 3	1.367	0.611	0.626	0.684	1.1	1.074	0.893	0.735	0.287
Reading 4	1.348	0.7	0.432	0.583	1.27	1.047	0.891	0.753	0.285
Reading 5	1.308	0.761	0.429	0.565	1.082	1.001	0.856	0.726	0.313
Reading 6	1.243	0.783	0.615	0.592	1.1	1.04	0.88	0.711	0.297
Reading 7	1.319	0.892	0.722	0.672	1.282	1.072	0.847	0.739	0.312
Reading 8	1.259	0.85	0.817	0.588	1.266	1.016	0.956	0.716	0.284
Reading 9	1.393	0.901	0.81	0.517	1.247	1.07	0.855	0.733	0.304
				0					
Average	1.343333	0.755111	0.614778	0.5351	1.193	1.049333	0.883556	0.725333	0.301444
Stdev	0.067196	0.110592	0.146968	0.194588	0.084204	0.027359	0.033953	0.029441	0.013575
st error	0.022399	0.036864	0.048989	0.064863	0.028068	0.00912	0.011318	0.009814	0.004525
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	2.737152	1.538601	1.25266	1.09031	2.430835	2.138103	1.800317	1.477926	0.614218
Stdev	0.067196	0.110592	0.146968	0.194588	0.084204	0.027359	0.033953	0.029441	0.013575
st error	0.022399	0.036864	0.048989	0.064863	0.028068	0.00912	0.011318	0.009814	0.004525

NBT/MTT 120 hours - HL60 Day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.967293	3.286136	5.442	6.626891	2.076856	3.483781	4.56351	6.291793	6.157051
Reading 2	1.717514	3.25	4.089347	6.790991	2.194378	2.69378	3.375551	2.441215	9
Reading 3	2.141185	3.967267	3.722045	5.676901	2.69	1.892924	3.617021	3.009524	7.28223
Reading 4	1.698071	2.374286	6.821759	6.86964	3.014961	1.489016	3.640853	5.363878	7.919298
Reading 5	1.54052	2.77661	5.715618	4.895575	2.792052	1.421578	2.348131	5.575758	7.651757
Reading 6	1.641191	3.155811	2.803252	4.393581	2.793636	1.957692	2.830682	5.350211	13.26599
Reading 7	1.148597	1.961883	2.290859	4.28125	1.353354	1.547575	2.62928	5.312585	5.403846
Reading 8	1.356632	1.805882	2.328029	6.258503	1.50158	2.71063	3.910042	5.694134	6.665493
Reading 9	1.215363	1.792453	1.911111	4.800774	3.210104	1.770093	3.764912	2.608458	5.976974
Average	1.60293	2.707814	3.902669	5.621567	2.402991	2.107452	3.408887	4.627506	7.702516
Stdev	0.329262	0.769667	1.749905	1.051774	0.659209	0.702718	0.696116	1.492267	2.360805
st error	0.109754	0.256556	0.583302	0.350591	0.219736	0.234239	0.232039	0.497422	0.786935
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.585064	0.988344	1.424462	2.051854	0.877084	0.769213	1.244233	1.689025	2.811394
Stdev	0.329262	0.769667	1.749905	1.051774	0.659209	0.702718	0.696116	1.492267	2.360805
st error	0.109754	0.256556	0.583302	0.350591	0.219736	0.234239	0.232039	0.497422	0.786935

Data for figure 65

NBT 72 hours HL60 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.85	1.434	1.169	2.391	1.442	2	1.199	1.41	1.79
Reading 2	2.162	1.332	1.737	1.9095	1.602	1.25	1.909	1.402	1.094
Reading 3	1.827	2.213	1.153	2.184	1.194	2.451	2.157	1.491	1.656
Reading 4	1.684	4.337	1.392	2.6145	1.748	1.607	2.155	2.056	1.55
Reading 5	1.914	1.459	1.117	2.5485	1.126	1.882	1.014	2.063	1.553
Reading 6	2.195	1.868	1.097	1.3365	2.51	2.123	1.653	1.482	1.397
Reading 7	2.091	2.272	3.107	4.305	2.954	1.442	3.229	1.305	1.462
Reading 8	2.498	2.732	2.792	1.8915	1.179	2.493	3.103	2.919	2.831
Reading 9	3.367	3.185	2.888	3.213	2.943	3.044	2.966	2.464	2.692
Average	2.176444	2.314667	1.828	2.488167	1.855333	2.032444	2.153889	1.843556	1.780556
Stdev	0.508519	0.981486	0.852709	0.863498	0.749328	0.568361	0.809184	0.565997	0.588924
st error	0.169506	0.327162	0.284236	0.287833	0.249776	0.189454	0.269728	0.188666	0.196308
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalised	1	1.063508	0.839902	1.143225	0.852461	0.933837	0.989637	0.847049	0.818103
Stdev	0.508519	0.981486	0.852709	0.863498	0.749328	0.568361	0.809184	0.565997	0.588924
st error	0.169506	0.327162	0.284236	0.287833	0.249776	0.189454	0.269728	0.188666	0.196308

MTT 72 hours HL60 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.749	1.677	1.698	0.975	1.38	1.482	1.215	1.119	0.558
Reading 2	1.644	1.578	1.524	0.792	1.653	1.521	1.188	1.065	0.519
Reading 3	1.629	1.887	1.656	0.885	1.632	1.464	1.26	1.179	0.639
Reading 4	1.563	1.812	1.707	0.822	1.491	1.449	1.416	1.107	0.489
Reading 5	1.518	1.707	1.617	0.906	1.653	1.734	1.389	0.99	0.525
Reading 6	1.695	1.881	1.656	0.846	1.518	1.227	1.314	0.975	0.555
Reading 7	1.638	1.77	1.467	1.053	1.674	1.353	1.311	1.125	0.591
Reading 8	1.482	1.77	1.62	0.795	1.704	1.413	1.491	1.158	0.672
Reading 9	1.686	1.812	1.593	1.167	1.41	1.443	1.269	1.224	0.666
Average	1.622667	1.766	1.615333	0.915667	1.568333	1.454	1.317	1.104667	0.579333
Stdev	0.086773	0.099295	0.078591	0.127507	0.120941	0.135706	0.098681	0.082742	0.066703
st error	0.028924	0.033098	0.026197	0.042502	0.040314	0.045235	0.032894	0.027581	0.022234
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	1.088332	0.995481	0.564297	0.966516	0.896056	0.811627	0.680772	0.357025
Stdev	0.086773	0.099295	0.078591	0.127507	0.120941	0.135706	0.098681	0.082742	0.066703
st error	0.028924	0.033098	0.026197	0.042502	0.040314	0.045235	0.032894	0.027581	0.022234

NBT/MTT 72 hours HL60 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.057747	0.855098	0.688457	2.452308	1.044928	1.349528	0.986831	1.260054	3.207885
Reading 2	1.315085	0.844106	1.139764	2.410985	0.969147	0.821828	1.606902	1.316432	2.1079
Reading 3	1.121547	1.172761	0.696256	2.467797	0.731618	1.67418	1.711905	1.264631	2.591549
Reading 4	1.077415	2.393488	0.815466	3.180657	1.172368	1.109041	1.521893	1.857272	3.169734
Reading 5	1.26087	0.854716	0.690785	2.812914	0.681186	1.085352	0.730022	2.083838	2.958095
Reading 6	1.294985	0.993089	0.66244	1.579787	1.653491	1.730236	1.257991	1.52	2.517117
Reading 7	1.276557	1.283616	2.117928	4.088319	1.764636	1.06578	2.463005	1.16	2.473773
Reading 8	1.68556	1.543503	1.723457	2.379245	0.691901	1.764331	2.081154	2.520725	4.212798
Reading 9	1.997034	1.757726	1.812932	2.753213	2.087234	2.109494	2.337273	2.013072	4.042042
Average	1.342978	1.299789	1.14972	2.680581	1.199612	1.412197	1.632997	1.666225	3.03121
Stdev	0.308281	0.521923	0.579302	0.682569	0.516777	0.425912	0.589336	0.472709	0.715412
st error	0.10276	0.173974	0.193101	0.227523	0.172259	0.141971	0.196445	0.15757	0.238471
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.967841	0.856098	1.995998	0.893248	1.051541	1.215953	1.240694	2.257081
Stdev	0.924844	1.565769	1.737907	0.691453	1.550332	1.277735	1.768009	1.418126	2.146235
st error	0.308281	0.521923	0.579302	0.230484	0.516777	0.425912	0.589336	0.472709	0.715412

Data for figure 66

NBT 120 hours HL60 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.79375	1.8235	1.635	3.95	2.001	1.78	2.076	2.525	2.699
Reading 2	2.658	1.833	1.705	4.065	2.041	1.982	3.87	2.493	3.877
Reading 3	2.5695	1.8235	2.457	3.982	2.28	1.648	2.852	2.137	2.124
Reading 4	2.7465	1.835	1.44	4.069	2.093	1.975	3.062	2.173	3.363
Reading 5	2.658	1.8085	1.58	3.929	1.204	2.849	3.384	2.069	2.897
Reading 6	2.59125	1.781	1.513	3.987	1.5514	2.856	2.554	2.157	3.949
Reading 7	2.2995	1.866	1.757	3.663	1.608	1.876	2.869	2.109	3.1
Reading 8	2.883	1.751	1.403	3.188	1.662	1.813	2.877	1.712	3.162
Reading 9	2.7045	1.915	1.477	3.611	1.73	1.681	2.789	1.473	2.313
Average	2.656	1.826278	1.663	3.827111	1.796711	2.051111	2.925889	2.094222	3.053778
Stdev	0.165764	0.04693	0.320767	0.289879	0.334002	0.468426	0.50127	0.333436	0.627721
st error	0.055255	0.015643	0.106922	0.096626	0.111334	0.156142	0.16709	0.111145	0.20924
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.220339	0.839111	0.76409	1.758424	0.825526	0.942414	1.344343	0.962222	1.403104
Stdev	0.110509	0.04693	0.320767	0.289879	0.334002	0.468426	0.50127	0.333436	0.627721
st error	0.036836	0.015643	0.106922	0.096626	0.111334	0.156142	0.16709	0.111145	0.20924

MTT 120 hours HL60 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.256	0.806	0.683	1.166	0.938	0.932	0.766	0.513	0.226
Reading 2	2.21	0.834	0.798	1.2	1.109	0.865	0.768	0.537	0.246
Reading 3	2.114	0.813	0.722	1.186	1.027	0.785	0.757	0.557	0.266
Reading 4	2.108	0.921	0.735	1.038	1.169	0.888	0.724	0.525	0.268
Reading 5	2.135	0.845	0.76	1.006	1.078	0.86	0.73	0.469	0.27
Reading 6	1.109	0.721	0.758	1.182	0.986	0.757	0.583	0.517	0.243
Reading 7	1.047	0.855	0.896	1.052	0.829	0.73	0.485	0.384	0.192
Reading 8	1.068	0.932	0.949	1.102	0.892	0.706	0.52	0.37	0.191
Reading 9	1.086	0.898	0.972	1.122	0.843	0.654	0.487	0.374	0.212
Average	1.681444	0.847222	0.808111	1.117111	0.985667	0.797444	0.646667	0.471778	0.234889
Stdev	0.575036	0.065433	0.104747	0.071862	0.119896	0.093582	0.125393	0.075602	0.031335
st error	0.191679	0.021811	0.034916	0.023954	0.039965	0.031194	0.041798	0.025201	0.010445
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.036223	0.522117	0.498014	0.688442	0.607436	0.491441	0.398521	0.290742	0.144755
Stdev	0.067081	0.065433	0.104747	0.071862	0.119896	0.093582	0.125393	0.075602	0.031335
st error	0.02236	0.021811	0.034916	0.023954	0.039965	0.031194	0.041798	0.025201	0.010445

NBT/MTT 120 hours HL60 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.238364	2.262407	2.393851	3.38765	2.133262	1.909871	2.710183	4.922027	11.94248
Reading 2	1.202715	2.197842	2.136591	3.3875	1.840397	2.291329	5.039063	4.642458	15.76016
Reading 3	1.215468	2.242927	3.403047	3.357504	2.220058	2.099363	3.767503	3.836625	7.984962
Reading 4	1.302894	1.9924	1.959184	3.920039	1.790419	2.224099	4.229282	4.139048	12.54851
Reading 5	1.244965	2.140237	2.078947	3.905567	1.116883	3.312791	4.635616	4.411514	10.72963
Reading 6	2.336564	2.47018	1.996042	3.373096	1.573428	3.772787	4.380789	4.172147	16.25103
Reading 7	2.196275	2.182456	1.960938	3.481939	1.939686	2.569863	5.915464	5.492188	16.14583
Reading 8	2.699438	1.878755	1.478398	2.892922	1.863229	2.567989	5.532692	4.627027	16.55497
Reading 9	2.490331	2.132517	1.519547	3.21836	2.052195	2.570336	5.726899	3.938503	10.91038
Average	1.769668	2.166636	2.10295	3.436064	1.836617	2.590937	4.659721	4.464615	13.20311
Stdev	0.641409	0.167127	0.565605	0.318984	0.332089	0.596769	1.029694	0.52305	3.089183
st error	0.213803	0.055709	0.188535	0.106328	0.110696	0.198923	0.343231	0.17435	1.029728
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.31772	1.613307	1.565885	2.558541	1.367571	1.929247	3.469693	3.324415	9.831216
Stdev	0.641409	0.167127	0.565605	0.318984	0.332089	0.596769	1.029694	0.52305	3.089183
st error	0.213803	0.055709	0.188535	0.106328	0.110696	0.198923	0.343231	0.17435	1.029728

Data for figure 67

NBT 72 hours - HL60 Day 12 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.911	2.1985	1.2165	1.0155	0.642	1.2325	1.6145	1.008	1.582
Reading 2	0.92	1.5165	0.996	0.909	0.871	0.7895	0.963	1.245	1.6985
Reading 3	0.662	2.274	1.018	0.955	0.626	1.094	0.924	1.041	1.053
Reading 4	0.929	1.125	1.467	0.853	1.063	1.23	0.844	1.022	1.115
Reading 5	0.848	2.403	1.17	0.986	0.418	1.574	2.181	0.792	1.684
Reading 6	0.974	1.994	1.263	1.045	0.866	0.891	1.048	1.224	1.48
Reading 7	0.866	1.039	0.729	0.773	0.876	0.688	0.878	1.266	0.917
Reading 8	0.911	2.1985	1.2165	1.0155	0.642	1.2325	1.6145	1.008	1.582
Reading 9	0.8885	1.764	1.3185	0.9195	0.7405	1.402	1.5125	0.907	1.8995
Average	0.878833	1.834722	1.154944	0.941278	0.749389	1.125944	1.286611	1.057	1.445667
Stdev	0.089137	0.506099	0.214784	0.087743	0.190708	0.289764	0.46395	0.160438	0.336909
st error	0.029712	0.1687	0.071595	0.029248	0.063569	0.096588	0.15465	0.053479	0.112303
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	2.087679	1.314179	1.071054	0.852709	1.281181	1.463999	1.202731	1.644984
Stdev	0.089137	0.506099	0.214784	0.087743	0.190708	0.289764	0.46395	0.160438	0.55692
st error	0.029712	0.1687	0.071595	0.029248	0.063569	0.096588	0.15465	0.053479	0.18564

MTT 72 hours - HL60 Day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.6325	0.7995	0.7015	0.2985	0.71	0.606	0.561	0.386	0.18
Reading 2	0.915	0.776	0.699	0.251	0.659	0.584	0.584	0.356	0.242
Reading 3	0.714	0.835	0.676	0.281	0.762	0.585	0.551	0.425	0.221
Reading 4	0.55	0.801	0.702	0.281	0.664	0.657	0.588	0.443	0.155
Reading 5	0.71	0.781	0.709	0.273	0.758	0.592	0.567	0.426	0.17
Reading 6	0.555	0.818	0.694	0.324	0.662	0.62	0.555	0.346	0.19
Reading 7	0.704	0.773	0.711	0.282	0.816	0.514	0.53	0.345	0.161
Reading 8	0.6325	0.7995	0.7015	0.2985	0.71	0.606	0.561	0.386	0.18
Reading 9	0.63	0.791	0.7055	0.277	0.711	0.6245	0.5775	0.4345	0.1625
Average	0.671444	0.797111	0.699944	0.285111	0.716889	0.598722	0.563833	0.394167	0.184611
Stdev	0.109963	0.02007	0.01033	0.02028	0.053431	0.039127	0.017997	0.039216	0.02921
st error	0.036654	0.00669	0.003443	0.00676	0.01781	0.013042	0.005999	0.013072	0.009737
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.187159	1.042446	0.424624	1.067682	0.891693	0.839732	0.587043	0.274946
Stdev	0.109963	0.02007	0.01033	0.02028	0.053431	0.039127	0.017997	0.039216	0.02921
st error	0.036654	0.00669	0.003443	0.00676	0.01781	0.013042	0.005999	0.013072	0.009737

NBT/MTT 72 hours - HL60 Day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.440316	2.749844	1.734141	3.40201	0.904225	2.033828	2.877897	2.611399	8.788889
Reading 2	1.005464	1.954253	1.424893	3.621514	1.3217	1.351884	1.648973	3.497191	7.018595
Reading 3	0.927171	2.723353	1.505917	3.398577	0.821522	1.870085	1.676951	2.449412	4.764706
Reading 4	1.689091	1.404494	2.089744	3.035587	1.600904	1.872146	1.435374	2.306998	7.193548
Reading 5	1.194366	3.076825	1.650212	3.611722	0.551451	2.658784	3.846561	1.859155	9.905882
Reading 6	1.754955	2.437653	1.819885	3.225309	1.308157	1.437097	1.888288	3.537572	7.789474
Reading 7	1.230114	1.344114	1.025316	2.741135	1.073529	1.338521	1.656604	3.669565	5.695652
Reading 8	1.440316	2.749844	1.734141	3.40201	0.904225	2.033828	2.877897	2.611399	8.788889
Reading 9	1.410317	2.230088	1.868887	3.319495	1.041491	2.244996	2.619048	2.087457	11.68923
Average	1.343568	2.296719	1.650348	3.306373	1.058578	1.871241	2.280844	2.736683	7.95943
Stdev	0.281655	0.617083	0.305362	0.278143	0.313683	0.44085	0.814044	0.66897	2.118599
st error	0.093885	0.205694	0.101787	0.092714	0.104561	0.14695	0.271348	0.22299	0.7062
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.709418	1.228333	2.46089	0.787886	1.39274	1.697602	2.036877	5.924099
Stdev	0.281655	0.617083	0.305362	0.278143	0.313683	0.44085	0.814044	0.66897	2.747489
st error	0.093885	0.205694	0.101787	0.092714	0.104561	0.14695	0.271348	0.22299	0.91583

Data for figure 68

NBT 120 hours - HL60 Day 12 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.0545	1.103	1.2055	0.99	1.069	1.605	1.4005	1.0005	1.024
Reading 2	1.175	0.789	1.685	2.037	1.315	1.322	1.07	1.081	1.216
Reading 3	1.14	1.203	1.199	1.193	1.392	1.235	1.078	1.109	1.102
Reading 4	0.895	1.242	1.267	1.163	1.021	1.06	1.196	1.125	1.5
Reading 5	2.265	1.085	1.257	1.202	3.823	1.103	1.144	1.065	1.091
Reading 6	0.959	1.103	1.254	1.163	1.067	2.08	1.044	1.116	1.099
Reading 7	1.15	1.103	1.157	0.817	1.071	1.13	1.757	0.885	0.949
Reading 8	1.1575	0.996	1.442	1.615	1.3535	1.2785	1.074	1.095	1.159
Reading 9	1.0545	1.103	1.2055	1.167	1.069	1.605	1.4005	1.0005	1.024
Average	1.205611	1.080778	1.296889	1.260778	1.4645	1.379833	1.240444	1.053	1.129333
Stdev	0.408644	0.130022	0.166533	0.359551	0.896161	0.329312	0.237454	0.078036	0.159543
st error	0.136215	0.043341	0.055511	0.11985	0.29872	0.109771	0.079151	0.026012	0.053181
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.371831	1.229787	1.475694	1.434604	1.666414	1.570074	1.411467	1.198179	1.285037
Stdev	0.408644	0.130022	0.166533	0.359551	0.896161	0.329312	0.237454	0.078036	0.159543
st error	0.136215	0.043341	0.055511	0.11985	0.29872	0.109771	0.079151	0.026012	0.053181

MTT 120 hours - HL60 Day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.6075	0.718	1.046	0.86	1.279	1.175	1.0165	0.7625	0.292
Reading 2	1.777	0.629	1.013	0.8	1.506	1.208	0.909	0.718	0.313
Reading 3	1.774	0.774	1.042	0.855	1.476	1.182	0.95	0.698	0.316
Reading 4	1.714	0.771	1.021	0.735	1.53	1.268	1.045	0.817	0.293
Reading 5	1.534	0.729	0.918	0.755	1.477	1.133	0.992	0.805	0.283
Reading 6	1.473	0.779	1.126	0.785	1.541	1.24	1.06	0.773	0.288
Reading 7	1.742	0.657	0.966	0.935	1.017	1.11	0.973	0.752	0.296
Reading 8	1.7755	0.7015	1.0275	0.8275	1.491	1.195	0.9295	0.708	0.3145
Reading 9	1.6075	0.718	1.046	0.86	1.279	1.175	1.0165	0.7625	0.292
Average	1.667167	0.719611	1.022833	0.823611	1.399556	1.187333	0.987944	0.755111	0.298611
Stdev	0.11487	0.052055	0.057474	0.061911	0.174633	0.048775	0.051727	0.041256	0.012469
st error	0.03829	0.017352	0.019158	0.020637	0.058211	0.016258	0.017242	0.013752	0.004156
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	2.482955	1.071736	1.523333	1.226626	2.084395	1.768327	1.471372	1.124607	0.444729
Stdev	0.11487	0.052055	0.057474	0.061911	0.174633	0.048775	0.051727	0.041256	0.012469
st error	0.03829	0.017352	0.019158	0.020637	0.058211	0.016258	0.017242	0.013752	0.004156

NBT/MTT 120 hours - HL60 Day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.655988	1.536212	1.152486	1.151163	0.835809	1.365957	1.377767	1.312131	3.506849
Reading 2	0.661227	1.254372	1.663376	2.54625	0.873174	1.094371	1.177118	1.505571	3.884984
Reading 3	0.642616	1.554264	1.150672	1.395322	0.943089	1.044839	1.134737	1.588825	3.487342
Reading 4	0.52217	1.610895	1.24094	1.582313	0.66732	0.835962	1.144498	1.376989	5.119454
Reading 5	1.476532	1.48834	1.369281	1.592053	2.588355	0.973522	1.153226	1.322981	3.855124
Reading 6	0.651052	1.415918	1.113677	1.481529	0.692408	1.677419	0.984906	1.443726	3.815972
Reading 7	0.660161	1.678843	1.197723	0.873797	1.053097	1.018018	1.805755	1.176862	3.206081
Reading 8	0.651929	1.419815	1.403406	1.951662	0.90778	1.069874	1.15546	1.54661	3.685215
Reading 9	0.655988	1.536212	1.152486	1.356977	0.835809	1.365957	1.377767	1.312131	3.506849
Average	0.730851	1.49943	1.271561	1.547896	1.044094	1.160658	1.256804	1.398425	3.785319
Stdev	0.283069	0.124406	0.178445	0.479428	0.591103	0.25937	0.239636	0.133172	0.546017
st error	0.094356	0.041469	0.059482	0.159809	0.197034	0.086457	0.079879	0.044391	0.182006
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	0.543963	1.116006	0.946406	1.152079	0.777105	0.863862	0.935423	1.04083	2.817363
Stdev	0.283069	0.124406	0.178445	0.479428	0.591103	0.25937	0.239636	0.133172	0.546017
st error	0.094356	0.041469	0.059482	0.159809	0.197034	0.086457	0.079879	0.044391	0.182006

Data for figure 69

NBT 72 hour HL60 Day 12 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.486	1.426	2.964	1.431	2.861	3.071	1.522	2.662	2.98
Reading 2	1.524	1.357	3.465	3.581	1.612	1.551	3.277	2.267	3.199
Reading 3	1.181	1.567	3.146	1.979	1.582	1.497	3.576	1.485	3.322
Reading 4	1.464	1.643	3.26	3.534	1.586	3.36	2.99	2.859	1.513
Reading 5	1.91	2.012	2.894	1.999	1.916	1.659	1.452	3.3	3.215
Reading 6	3.577	3.307	3.944	1.882	3.635	3.188	3.494	3.231	2.656
Reading 7	1.505	1.3915	3.2145	2.506	2.2365	2.311	2.3995	2.4645	3.0895
Reading 8	1.3225	1.605	3.203	2.7565	1.584	2.4285	3.283	2.172	2.4175
Reading 9	2.7435	2.6595	3.419	1.9405	2.7755	2.4235	2.473	3.2655	2.9355
Average	1.857	1.885333	3.278833	2.401	2.198667	2.387667	2.7185	2.634	2.814167
Stdev	0.791884	0.671673	0.310616	0.756266	0.741067	0.714667	0.809218	0.606963	0.565695
st error	0.263961	0.223891	0.103539	0.252089	0.247022	0.238222	0.269739	0.202321	0.188565
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	1.015258	1.765661	1.292946	1.183989	1.285766	1.46392	1.418417	1.515437
Stdev	0.791884	0.671673	0.310616	0.756266	0.741067	0.714667	0.809218	0.606963	0.565695
st error	0.263961	0.223891	0.103539	0.252089	0.247022	0.238222	0.269739	0.202321	0.188565

MTT 72 hour HL60 Day 12 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.064	0.321	0.269	0.932	0.58	0.527	0.896	0.772	0.369
Reading 2	1.066	0.356	0.329	1.194	0.884	0.545	0.514	0.662	0.345
Reading 3	2.252	0.313	0.35	1.042	1.359	1.028	0.707	0.705	0.335
Reading 4	2.154	0.3	0.349	0.975	1.315	1.149	0.842	0.699	0.386
Reading 5	2.26	0.335	0.371	0.671	1.378	1.273	0.87	0.827	0.475
Reading 6	2.12	0.383	0.351	0.79	1.373	1.201	0.96	0.815	0.473
Reading 7	1.065	0.3385	0.299	1.063	0.732	0.536	0.705	0.717	0.357
Reading 8	2.203	0.3065	0.3495	1.0085	1.337	1.0885	0.7745	0.702	0.3605
Reading 9	2.19	0.359	0.361	0.7305	1.3755	1.237	0.915	0.821	0.474
Average	1.819333	0.334667	0.3365	0.934	1.148167	0.953833	0.798167	0.746667	0.397167
Stdev	0.567389	0.027499	0.032648	0.171193	0.321867	0.321866	0.139102	0.062592	0.059344
st error	0.18913	0.009166	0.010883	0.057064	0.107289	0.107289	0.046367	0.020864	0.019781
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.18395	0.184958	0.513375	0.631092	0.524276	0.438714	0.410407	0.218303
Stdev	0.283694	0.027499	0.032648	0.171193	0.321867	0.321866	0.139102	0.062592	0.059344
st error	0.094565	0.009166	0.010883	0.057064	0.107289	0.107289	0.046367	0.020864	0.019781

NBT/MTT 72 hour HL60 Day 12 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.396617	4.442368	11.01859	1.535408	4.932759	5.827324	1.698661	3.448187	8.075881
Reading 2	1.429644	3.811798	10.53191	2.999162	1.823529	2.845872	6.375486	3.424471	9.272464
Reading 3	0.524423	5.00639	8.988571	1.899232	1.164091	1.456226	5.057992	2.106383	9.916418
Reading 4	0.679666	5.476667	9.340974	3.624615	1.206084	2.924282	3.551069	4.090129	3.919689
Reading 5	0.845133	6.00597	7.800539	2.979136	1.390421	1.303221	1.668966	3.990326	6.768421
Reading 6	1.687264	8.634465	11.23647	2.382278	2.647487	2.654455	3.639583	3.964417	5.615222
Reading 7	1.413146	4.110783	10.75084	2.357479	3.055328	4.311567	3.403546	3.437238	8.654062
Reading 8	0.600318	5.236542	9.164521	2.733267	1.184742	2.231052	4.238864	3.094017	6.705964
Reading 9	1.25274	7.408078	9.470914	2.6564	2.017812	1.959175	2.702732	3.977467	6.193038
Average	1.092105	5.57034	9.811481	2.574109	2.158028	2.834797	3.592989	3.503626	7.235684
Stdev	0.430837	1.576755	1.138315	0.622341	1.240263	1.438494	1.517745	0.627647	1.912469
st error	0.143612	0.525585	0.379438	0.207447	0.413421	0.479498	0.505915	0.209216	0.63749
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	5.100552	8.984005	2.357015	1.976025	2.595718	3.289965	3.208139	6.625445
Stdev	0.861674	1.576755	1.138315	0.622341	1.240263	1.438494	1.517745	0.627647	1.912469
st error	0.287225	0.525585	0.379438	0.207447	0.413421	0.479498	0.505915	0.209216	0.63749

Data for figure 70

NBT 120 hour HL60 Day 12 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.214	1.49	1.68	1.674	1.376	1.645	1.509	3.856	1.245
Reading 2	2.112	3.899	1.777	2.296	1.503	1.599	1.434	1.75	1.672
Reading 3	1.498	1.597	1.461	1.515	1.272	4.054	1.603	1.531	2.343
Reading 4	2.691	1.937	1.47	3.461	1.372	1.402	1.533	1.149	1.725
Reading 5	1.119	1.724	4.07	1.966	1.341	2.388	2.422	2.117	1.722
Reading 6	1.404	2.151	1.438	1.324	1.321	1.715	1.755	1.513	1.733
Reading 7	1.663	2.6945	1.7285	1.985	1.4395	1.622	1.4715	2.803	1.4585
Reading 8	2.0945	1.767	1.4655	2.488	1.322	2.728	1.568	1.34	2.034
Reading 9	1.2615	1.9375	2.754	1.645	1.331	2.0515	2.0885	1.815	1.7275
Average	1.673	2.133	1.982667	2.039333	1.364167	2.133833	1.709333	1.986	1.74
Stdev	0.41659	0.493689	0.680815	0.424118	0.065396	0.557578	0.331894	0.74634	0.287954
st error	0.138863	0.164563	0.226938	0.141373	0.021799	0.185859	0.110631	0.24878	0.095985
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	0.900915	1.148627	1.067672	1.098187	0.734608	1.149076	0.920481	1.069467	0.936995
Stdev	0.41659	0.493689	0.680815	0.424118	0.065396	0.557578	0.331894	0.74634	0.287954
st error	0.138863	0.164563	0.226938	0.141373	0.021799	0.185859	0.110631	0.24878	0.095985

MTT 120 hour HL60 Day 12 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.295	0.174	0.196	0.911	1.249	1.201	1.076	0.992	0.561
Reading 2	1.237	0.168	0.212	1.003	1.275	1.174	1.042	0.973	0.586
Reading 3	1.325	0.17	0.203	1.092	1.309	1.165	1.107	0.995	0.543
Reading 4	1.358	0.168	0.235	1.086	1.279	1.229	1.076	0.967	0.551
Reading 5	1.339	0.159	0.228	1.147	1.292	1.282	1.12	0.974	0.556
Reading 6	1.49	0.174	0.239	1.306	1.335	1.214	1.111	0.751	0.535
Reading 7	1.266	0.171	0.204	0.957	1.262	1.1875	1.059	0.9825	0.5735
Reading 8	1.3415	0.169	0.219	1.089	1.294	1.197	1.0915	0.981	0.547
Reading 9	1.4145	0.1665	0.2335	1.2265	1.3135	1.248	1.1155	0.8625	0.5455
Average	1.340667	0.168833	0.218833	1.090833	1.289833	1.210833	1.088667	0.942	0.555333
Stdev	0.07641	0.004514	0.015835	0.125425	0.026854	0.037209	0.027211	0.082068	0.016021
st error	0.02547	0.001505	0.005278	0.041808	0.008951	0.012403	0.00907	0.027356	0.00534
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.7369	0.0928	0.120282	0.599579	0.708959	0.665537	0.598388	0.517772	0.30524
Stdev	0.07641	0.004514	0.015835	0.125425	0.026854	0.037209	0.027211	0.082068	0.016021
st error	0.02547	0.001505	0.005278	0.041808	0.008951	0.012403	0.00907	0.027356	0.00534

NBT/ MTT 120 hour HL60 Day 12 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.14749	8.195402	15.12245	1.570801	2.290633	2.557036	1.414498	2.683468	5.311943
Reading 2	1.232013	8.077381	16.34434	3.570289	1.264314	1.321124	3.144914	2.329908	5.459044
Reading 3	0.891321	9.217647	15.49754	1.812271	1.208556	1.284979	3.230352	1.492462	6.117864
Reading 4	1.078056	9.779762	13.87234	3.254144	1.240031	2.73393	2.77881	2.956567	2.745917
Reading 5	1.426438	12.65409	12.69298	1.742807	1.482972	1.294072	1.296429	3.38809	5.782374
Reading 6	2.400671	19.00575	16.50209	1.441041	2.722846	2.62603	3.144914	4.302264	4.964486
Reading 7	1.313586	15.75731	8.473039	2.07419	1.14065	1.365895	1.389518	2.852926	2.543156
Reading 8	1.561312	10.45562	6.691781	2.284665	1.021638	2.279031	1.436555	1.365953	3.718464
Reading 9	0.891835	11.63664	11.79443	1.341215	1.013323	1.64383	1.872255	2.104348	3.166819
Average	1.326969	11.64218	12.999	2.121269	1.487218	1.900658	2.189805	2.608443	4.423341
Stdev	0.460997	3.666118	3.478278	0.792536	0.604376	0.635345	0.863281	0.920893	1.382844
st error	0.153666	1.222039	1.159426	0.264179	0.201459	0.211782	0.28776	0.306964	0.460948
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.215056	10.66031	11.9027	1.942367	1.36179	1.740362	2.005123	2.388453	4.050288
Stdev	0.460997	3.666118	3.478278	0.792536	0.604376	0.635345	0.863281	0.920893	1.382844
st error	0.153666	1.222039	1.159426	0.264179	0.201459	0.211782	0.28776	0.306964	0.460948

Data for figure 71

NBT 72 hours - K562 day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.091	2.308	1.917	1.564	1.836	1.816	3.578	3.799	3.426
Reading 2	1.85	3.186	3.923	2.434	1.915	2.57	2.345	2.031	2.203
Reading 3	1.764	2.098	2.095	1.555	3.255	3.325	2.961	4.158	4.075
Reading 4	1.134	1.734	1.792	1.994	3.248	1.857	4.021	4.188	3.969
Reading 5	1.213	1.655	1.52	2.028	2.471	2.318	1.668	1.522	4.217
Reading 6	1.367	1.621	1.31	2.126	2.674	2.375	3.369	2.574	4.016
Reading 7	1.581	1.418	1.512	2.185	1.755	1.628	1.777	1.724	1.724
Reading 8	1.398	1.691	2.043	1.468	1.616	1.702	1.468	1.58	1.985
Reading 9	1.597	1.495	1.441	1.423	1.429	1.765	1.374	1.434	1.376
Average	1.443889	1.911778	1.950333	1.864111	2.244333	2.150667	2.506778	2.556667	2.999
Stdev	0.271921	0.55494	0.790085	0.36694	0.69273	0.553928	1.000917	1.173864	1.157604
st error	0.09064	0.18498	0.263362	0.122313	0.23091	0.184643	0.333639	0.391288	0.385868
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	1.324048	1.35075	1.291035	1.554367	1.489496	1.736129	1.770681	2.07703
Stdev	0.271921	0.55494	0.790085	0.36694	0.69273	0.553928	1.000917	1.173864	1.157604
st error	0.09064	0.18498	0.263362	0.122313	0.23091	0.184643	0.333639	0.391288	0.385868

MTT 72 hours - K562 day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.05	0.311	0.272	0.707	1.166	1.022	0.853	0.605	0.402
Reading 2	0.988	0.299	0.276	0.854	1.225	0.804	0.835	0.619	0.388
Reading 3	1.13	0.301	0.258	0.61	1.071	1.055	0.911	0.685	0.402
Reading 4	1.098	0.282	0.259	0.782	1.205	1.015	0.878	0.715	0.403
Reading 5	1.024	0.23	0.265	0.879	1.148	1.186	0.971	0.644	0.401
Reading 6	1.465	0.294	0.212	0.987	0.842	0.942	0.854	0.677	0.386
Reading 7	1.384	0.241	0.24	0.841	0.982	0.788	0.728	0.536	0.329
Reading 8	1.051	0.264	0.245	0.503	0.927	0.723	0.568	0.615	0.307
Reading 9	1.059	0.26	0.251	0.563	0.907	0.795	0.757	0.643	0.226
Average	1.138778	0.275778	0.253111	0.747333	1.052556	0.925556	0.817222	0.637667	0.360444
Stdev	0.168134	0.02846	0.019406	0.162132	0.142108	0.155696	0.118735	0.052562	0.061484
st error	0.056045	0.009487	0.006469	0.054044	0.047369	0.051899	0.039578	0.017521	0.020495
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.24217	0.222266	0.656259	0.924285	0.812762	0.717631	0.559957	0.316519
Stdev	0.168134	0.02846	0.019406	0.162132	0.142108	0.155696	0.118735	0.052562	0.061484
st error	0.056045	0.009487	0.006469	0.054044	0.047369	0.051899	0.039578	0.017521	0.020495

NBT/ MTT 72 hours - K562 day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.039048	7.421222	7.047794	2.212164	1.574614	1.776908	4.194607	6.279339	8.522388
Reading 2	1.87247	10.65552	14.21377	2.850117	1.563265	3.196517	2.808383	3.281099	5.677835
Reading 3	1.561062	6.9701	8.120155	2.54918	3.039216	3.151659	3.250274	6.070073	10.13682
Reading 4	1.032787	6.148936	6.918919	2.549872	2.695436	1.829557	4.579727	5.857343	9.848635
Reading 5	1.18457	7.195652	5.735849	2.307167	2.152439	1.954469	1.717817	2.363354	10.51621
Reading 6	0.933106	5.513605	6.179245	2.154002	3.175772	2.521231	3.944965	3.802068	10.40415
Reading 7	1.142341	5.883817	6.3	2.598098	1.787169	2.06599	2.440934	3.216418	5.240122
Reading 8	1.330162	6.405303	8.338776	2.918489	1.743258	2.35408	2.584507	2.569106	6.465798
Reading 9	1.508026	5.75	5.741036	2.527531	1.575524	2.220126	1.815059	2.230171	6.088496
Average	1.289286	6.882684	7.621727	2.518513	2.145188	2.341171	3.037364	3.963219	8.100049
Stdev	0.306455	1.5633	2.644858	0.262123	0.655996	0.529286	1.026867	1.656295	2.21626
st error	0.102152	0.5211	0.881619	0.087374	0.218665	0.176429	0.342289	0.552098	0.738753
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	5.33837	5.911589	1.953418	1.663858	1.815867	2.35585	3.073965	6.282587
Stdev	0.306455	1.5633	2.644858	0.262123	0.655996	0.529286	1.026867	1.656295	2.21626
st error	0.102152	0.5211	0.881619	0.087374	0.218665	0.176429	0.342289	0.552098	0.738753

Data for figure 72

NBT 120 hours - K562 day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.51975	1.01775	1.01975	1.03925	0.42275	1.027	1.04525	1.01775	1.076
Reading 2	0.52025	0.348	0.3285	1.04025	0.2915	1.04875	0.6375	0.32275	1.01875
Reading 3	0.524625	1.01525	0.29675	1.032	0.4115	0.455	1.02275	0.4825	0.444
Reading 4	0.52875	0.354	1.04	1.05475	0.5545	0.383	0.44025	0.40375	0.362
Reading 5	0.522625	0.362	0.32775	1.068	0.98975	1.0525	0.3715	0.3355	0.3295
Reading 6	0.623625	0.26275	0.28825	1.275	0.35675	0.346	0.417	0.2905	0.317
Reading 7	0.628	0.28475	0.28275	0.3155	0.2675	1.01875	0.949	0.24675	0.1615
Reading 8	0.51	0.3315	0.27425	0.86275	1.0235	0.457	0.99775	0.79075	0.15875
Reading 9	0.5136	0.2795	0.96175	0.96325	0.973	1.0045	0.342	0.362	0.76275
Average	0.543469	0.472833	0.535528	0.961194	0.587861	0.754722	0.691444	0.472472	0.514472
Stdev	0.047025	0.310242	0.354786	0.26519	0.316816	0.328835	0.308404	0.260024	0.350678
st error	0.015675	0.103414	0.118262	0.088397	0.105605	0.109612	0.102801	0.086675	0.116893
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	0.376393	0.327472	0.370893	0.665698	0.407137	0.522701	0.478876	0.327222	0.35631
Stdev	0.047025	0.310242	0.354786	0.26519	0.316816	0.328835	0.308404	0.260024	0.350678
st error	0.015675	0.103414	0.118262	0.088397	0.105605	0.109612	0.102801	0.086675	0.116893

MTT 120 hours - K562 day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.448	0.251	0.182	0.4044	0.491	0.37	0.3415	0.329	0.1955
Reading 2	0.4112	0.218	0.18	0.2892	0.4915	0.4415	0.376	0.3235	0.202
Reading 3	0.406	0.226	0.202	0.4252	0.475	0.4065	0.4285	0.337	0.1925
Reading 4	0.4232	0.212	0.203	0.4144	0.5185	0.4165	0.2945	0.32	0.196
Reading 5	0.4228	0.201	0.213	0.3644	0.4785	0.455	0.39	0.333	0.201
Reading 6	0.5104	0.158	0.262	0.374	0.476	0.4335	0.3785	0.3335	0.1995
Reading 7	0.3968	0.177	0.198	0.3372	0.4505	0.3845	0.3685	0.3125	0.196
Reading 8	0.4012	0.182	0.171	0.3888	0.4855	0.4515	0.3675	0.3235	0.197
Reading 9	0.4044	0.183	0.17	0.3236	0.5	0.4035	0.3845	0.327	0.1945
Average	0.424889	0.200889	0.197889	0.369022	0.485167	0.418056	0.369944	0.326556	0.197111
Stdev	0.03565	0.02882	0.028405	0.04521	0.018838	0.029696	0.036506	0.007618	0.00312
st error	0.011883	0.009607	0.009468	0.01507	0.006279	0.009899	0.012169	0.002539	0.00104
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	0.37311	0.176407	0.173773	0.324051	0.426042	0.367109	0.324861	0.28676	0.17309
Stdev	0.03565	0.02882	0.028405	0.04521	0.018838	0.029696	0.036506	0.007618	0.00312
st error	0.011883	0.009607	0.009468	0.01507	0.006279	0.009899	0.012169	0.002539	0.00104

NBT/MTT 120 hours - K562 day 9 proteins									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.160156	4.054781	5.603022	2.569857	0.860998	2.775676	3.060761	3.093465	5.503836
Reading 2	1.265199	1.59633	1.825	3.596992	0.593082	2.375425	1.695479	0.997682	5.043317
Reading 3	1.29218	4.492257	1.469059	2.427093	0.866316	1.119311	2.386814	1.431751	2.306494
Reading 4	1.249409	1.669811	5.123153	2.545246	1.069431	0.919568	1.494907	1.261719	1.846939
Reading 5	1.236105	1.800995	1.538732	2.930845	2.068443	2.313187	0.952564	1.007508	1.639303
Reading 6	1.221836	1.662975	1.100191	3.409091	0.749475	0.798155	1.101717	0.871064	1.588972
Reading 7	1.582661	1.608757	1.42803	0.935647	0.593785	2.649545	2.575305	0.7896	0.82398
Reading 8	1.271186	1.821429	1.603801	2.219007	2.108136	1.012182	2.714966	2.444359	0.805838
Reading 9	1.27003	1.527322	5.657353	2.976669	1.946	2.489467	0.889467	1.107034	3.921594
Average	1.283196	2.248295	2.816482	2.623383	1.206185	1.828057	1.874665	1.444909	2.608919
Stdev	0.118672	1.15716	1.997854	0.778144	0.643934	0.836606	0.826195	0.791722	1.77199
st error	0.039557	0.38572	0.665951	0.259381	0.214645	0.278869	0.275398	0.263907	0.590663
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	0.995277	1.74383	2.184529	2.034757	0.935545	1.417884	1.454033	1.120705	2.023538
Stdev	0.118672	1.15716	1.997854	0.778144	0.643934	0.836606	0.826195	0.791722	1.77199
st error	0.039557	0.38572	0.665951	0.259381	0.214645	0.278869	0.275398	0.263907	0.590663

Data for figure 73

NBT 72 hours - K562 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.855	2.37	1.794	2.031	1.879	1.963	1.715	1.703	1.853
Reading 2	1.457	2.278	3.196	2.687	1.96	2.116	3.86	1.742	1.68
Reading 3	1.693	1.945	2.747	3.396	2.009	1.892	4.006	3.965	3.005
Reading 4	2.011	1.945	1.572	3.175	1.837	1.765	1.937	3.984	2.019
Reading 5	3.676	3.054	1.953	3.924	1.933	3.326	2.448	1.882	1.819
Reading 6	2.906	3.881	2.374	3.866	3.91	3.946	3.725	3.687	3.876
Reading 7	2.413	1.554	1.529	2.367	1.802	2.566	1.492	3.578	3.591
Reading 8	2.402	1.648	1.502	2.884	1.424	1.578	2.524	3.43	3.56
Reading 9	3.527	1.7	1.578	3.874	1.625	1.411	1.616	1.67	1.284
Average	2.437778	2.263889	2.027222	3.133778	2.042111	2.284778	2.591444	2.849	2.520778
Stdev	0.788916	0.76284	0.612106	0.693276	0.723803	0.847229	1.017165	1.058821	0.982547
st error	0.262972	0.25428	0.204035	0.231092	0.241268	0.28241	0.339055	0.35294	0.327516
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	0.928669	0.831586	1.285506	0.837694	0.937238	1.063036	1.168687	1.034047
Stdev	0.973682	0.76284	0.612106	0.693276	0.723803	0.847229	1.017165	1.058821	0.982547
st error	0.324561	0.25428	0.204035	0.231092	0.241268	0.28241	0.339055	0.35294	0.327516

MTT 72 hours - K562 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.797	0.698	0.527	1.486	1.605	1.409	1.272	1.098	0.67
Reading 2	1.831	0.682	0.662	1.528	1.644	1.39	1.253	1.1	0.651
Reading 3	1.796	0.706	0.642	1.399	1.51	1.166	1.008	1.033	0.664
Reading 4	1.673	0.688	0.645	1.511	1.244	1.37	1.182	1.021	0.652
Reading 5	1.936	0.713	0.593	1.472	1.648	1.208	1.214	0.984	0.607
Reading 6	1.476	0.771	0.559	1.324	1.635	1.379	1.293	1.067	0.659
Reading 7	1.405	0.387	0.352	0.816	1.249	1.089	0.864	0.739	0.407
Reading 8	1.319	0.41	0.37	0.844	1.268	1.061	0.902	0.798	0.385
Reading 9	1.271	0.429	0.317	0.933	1.107	1.013	0.884	0.754	0.406
Average	1.611556	0.609333	0.518556	1.257	1.434444	1.231667	1.096889	0.954889	0.566778
Stdev	0.247035	0.152989	0.136696	0.302345	0.215094	0.157927	0.180241	0.148747	0.126988
st error	0.082345	0.050996	0.045565	0.100782	0.071698	0.052642	0.06008	0.049582	0.042329
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.378103	0.321773	0.779992	0.890099	0.764272	0.68064	0.592526	0.351696
Stdev	0.247035	0.152989	0.136696	0.302345	0.215094	0.157927	0.180241	0.148747	0.126988
st error	0.082345	0.050996	0.045565	0.100782	0.071698	0.052642	0.06008	0.049582	0.042329

NBT/MTT 72 hours - K562 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.032276	3.395415	3.404175	1.366756	1.170717	1.393187	1.34827	1.551002	2.765672
Reading 2	0.79574	3.340176	4.827795	1.758508	1.192214	1.522302	3.080607	1.583636	2.580645
Reading 3	0.94265	2.754958	4.278816	2.427448	1.330464	1.622642	3.974206	3.838335	4.525602
Reading 4	1.202032	2.827035	2.437209	2.101257	1.476688	1.288321	1.638748	3.902057	3.096626
Reading 5	1.89876	4.28331	3.293423	2.665761	1.172937	2.753311	2.016474	1.912602	2.996705
Reading 6	1.968835	5.033722	4.246869	2.91994	2.391437	2.861494	2.880897	3.455483	5.881639
Reading 7	1.717438	4.015504	4.34375	2.900735	1.442754	2.35629	1.726852	4.841678	8.823096
Reading 8	1.821077	4.019512	4.059459	3.417062	1.123028	1.487276	2.798226	4.298246	9.246753
Reading 9	2.77498	3.962704	4.977918	4.152197	1.467931	1.392892	1.828054	2.214854	3.162562
Average	1.572643	3.736926	3.985491	2.634407	1.418686	1.853079	2.365815	3.066432	4.786589
Stdev	0.634571	0.729285	0.805964	0.84933	0.39067	0.624409	0.861225	1.258275	2.624431
st error	0.211524	0.243095	0.268655	0.28311	0.130223	0.208136	0.287075	0.419425	0.87481
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	2.376207	2.534262	1.675146	0.902103	1.178322	1.504356	1.949859	3.043659
Stdev	0.697941	0.729285	0.805964	0.84933	0.39067	0.624409	0.861225	1.258275	2.624431
st error	0.232647	0.243095	0.268655	0.28311	0.130223	0.208136	0.287075	0.419425	0.87481

Data for figure 74

NBT 120 hours - K562 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.953	1.494	1.367	1.833	2.084	1.989	2.101	1.846	2.22
Reading 2	1.798	3.028	1.518	2.044	2.635	2.374	2.535	3.584	3.881
Reading 3	1.763	2.21	1.798	1.919	1.76	1.574	4.236	4.039	4.043
Reading 4	1.929	1.82	1.427	2.046	2.55	3.429	3.87	2.039	2.158
Reading 5	1.751	1.354	2.182	1.776	1.749	2.593	4.06	1.955	0.74
Reading 6	3.153	4.223	1.162	3.167	2.2	1.997	4.12	2.85	2.376
Reading 7	3.842	3.727	1.308	1.69	3.146	3.207	4.127	2.66	1.598
Reading 8	1.706	1.291	1.768	1.355	3.115	3.756	1.391	1.193	1.17
Reading 9	1.393	1.244	1.914	1.205	3.901	3.19	4.227	1.271	1.199
Average	2.143111	2.265667	1.604889	1.892778	2.571111	2.678778	3.407444	2.381889	2.153889
Stdev	0.803053	1.127492	0.330262	0.558038	0.716608	0.752081	1.093015	0.982476	1.161536
st error	0.267684	0.375831	0.110087	0.186013	0.238869	0.250694	0.364338	0.327492	0.387179
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	0.879125	0.929398	0.658341	0.776436	1.054695	1.098861	1.397767	0.977074	0.883546
Stdev	0.803053	1.127492	0.330262	0.558038	0.716608	0.752081	1.093015	0.982476	1.161536
st error	0.267684	0.375831	0.110087	0.186013	0.238869	0.250694	0.364338	0.327492	0.387179

MTT 120 hours - K562 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.127	0.219	0.189	0.956	0.973	0.829	0.763	0.671	0.392
Reading 2	1.012	0.221	0.214	0.72	0.992	0.868	0.769	0.687	0.383
Reading 3	1.035	0.223	0.212	0.95	0.857	0.861	0.785	0.675	0.422
Reading 4	1.009	0.204	0.228	0.87	0.772	0.752	0.78	0.699	0.377
Reading 5	0.879	0.211	0.223	0.509	0.981	0.927	0.758	0.635	0.402
Reading 6	1.145	0.224	0.249	0.52	0.995	0.867	0.775	0.658	0.335
Reading 7	1.417	0.475	0.473	0.834	1.134	1.051	0.875	0.737	0.469
Reading 8	1.387	0.499	0.516	0.899	1.212	1.123	0.892	0.722	0.47
Reading 9	1.558	0.45	0.491	0.699	1.059	0.961	0.696	0.618	0.379
Average	1.174333	0.302889	0.310556	0.773	0.997222	0.915444	0.788111	0.678	0.403222
Stdev	0.227689	0.129564	0.138401	0.171688	0.132251	0.114789	0.060184	0.038481	0.044164
st error	0.075896	0.043188	0.046134	0.057229	0.044084	0.038263	0.020061	0.012827	0.014721
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	0.728696	0.187948	0.192705	0.479661	0.618795	0.56805	0.489038	0.420712	0.250207
Stdev	0.227689	0.129564	0.138401	0.171688	0.132251	0.114789	0.060184	0.038481	0.044164
st error	0.075896	0.043188	0.046134	0.057229	0.044084	0.038263	0.020061	0.012827	0.014721

NBT/MTT 120 hours - K562 Day 9 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.732919	6.821918	7.232804	1.917364	2.141829	2.399276	2.753604	2.751118	5.663265
Reading 2	1.77668	13.70136	7.093458	2.838889	2.65625	2.735023	3.296489	5.216885	10.13316
Reading 3	1.703382	9.910314	8.481132	2.02	2.053676	1.828107	5.396178	5.983704	9.580569
Reading 4	1.911794	8.921569	6.258772	2.351724	3.303109	4.55984	4.961538	2.917024	5.724138
Reading 5	1.992036	6.417062	9.784753	3.489194	1.782875	2.797195	5.356201	3.07874	1.840796
Reading 6	2.753712	18.85268	4.666667	6.090385	2.211055	2.303345	5.316129	4.331307	7.092537
Reading 7	2.711362	7.846316	2.765328	2.026379	2.77425	3.05138	4.716571	3.609227	3.407249
Reading 8	1.229993	2.587174	3.426357	1.50723	2.570132	3.344613	1.559417	1.652355	2.489362
Reading 9	0.894095	2.764444	3.898167	1.723891	3.683664	3.319459	6.073276	2.056634	3.163588
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Average	1.856219	8.646981	5.956382	2.662784	2.575204	2.926471	4.381045	3.510777	5.454963
Stdev	0.604259	5.143495	2.411636	1.42082	0.614912	0.785714	1.498141	1.431506	3.020848
st error	0.20142	1.714498	0.803879	0.473607	0.204971	0.261905	0.49938	0.477169	1.006949
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1.180318	5.498375	3.787497	1.69319	1.637501	1.860861	2.785784	2.232405	3.468659
Stdev	0.604259	5.143495	2.411636	1.42082	0.614912	0.785714	1.498141	1.431506	3.020848
st error	0.20142	1.714498	0.803879	0.473607	0.204971	0.261905	0.49938	0.477169	1.006949

Data for figure 75

NBT 72 hours - K562 day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.618	1.276	4.161	1.37	2.075	1.666	1.434	1.679	1.911
Reading 2	2.012	1.596	1.562	1.339	0.977	1.38	2.449	1.792	1.813
Reading 3	1.7	1.366	0.953	2.12	3.164	1.32	1.71	1.561	2.085
Reading 4	1.919	1.719	4.035	2.085	3.841	1.454	3.497	1.694	1.387
Reading 5	1.728	1.769	3.877	1.867	1.584	1.357	1.907	1.467	1.514
Reading 6	1.822	1.677	1.627	1.675	1.777	1.792	1.747	2.04	1.505
Reading 7	1.659	1.321	2.557	1.745	2.6195	1.493	1.572	1.62	1.998
Reading 8	1.917	1.6365	1.5945	1.507	1.377	1.586	2.098	1.916	1.659
Reading 9	1.6935	1.545	3.217	1.806	2.10175	1.425	1.7395	1.5435	1.756
Average	1.785389	1.545056	2.620389	1.723778	2.168472	1.497	2.017056	1.701389	1.736444
Stdev	0.137335	0.181462	1.237603	0.282352	0.904567	0.156195	0.630253	0.185635	0.23921
st error	0.045778	0.060487	0.412534	0.094117	0.301522	0.052065	0.210084	0.061878	0.079737
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	0.865389	1.467685	0.965491	1.214566	0.838473	1.129757	0.952951	0.972586
Stdev	0.137335	0.181462	1.237603	0.282352	0.904567	0.156195	0.630253	0.185635	0.23921
st error	0.045778	0.060487	0.412534	0.094117	0.301522	0.052065	0.210084	0.061878	0.079737

MTT 72 hours - K562 day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.545	0.477	0.457	0.777	1.433	1.347	3.172	1.11	0.663
Reading 2	1.358	0.492	0.527	0.735	1.42	1.39	1.068	0.632	0.7
Reading 3	1.751	0.445	0.493	0.805	1.449	1.296	1.221	1.046	0.708
Reading 4	1.34	0.456	0.519	0.833	1.408	1.255	1.309	1.145	0.69
Reading 5	1.603	0.523	0.47	0.872	1.547	1.141	1.306	1.084	0.691
Reading 6	1.522	0.464	0.46	0.611	1.314	1.24	1.338	1.141	0.702
Reading 7	1.648	0.461	0.475	0.791	1.441	1.3215	2.1965	1.078	0.6855
Reading 8	1.44	0.478	0.4935	0.673	1.367	1.315	1.203	0.8865	0.701
Reading 9	1.6255	0.492	0.4725	0.8315	1.494	1.23125	1.75125	1.081	0.68825
Average	1.536944	0.476444	0.485222	0.769833	1.430333	1.281861	1.618306	1.022611	0.692083
Stdev	0.13732	0.023596	0.024865	0.083505	0.067346	0.073895	0.677393	0.165275	0.013244
st error	0.045773	0.007865	0.008288	0.027835	0.022449	0.024632	0.225798	0.055092	0.004415
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.309995	0.315706	0.500886	0.930634	0.834032	1.052937	0.665353	0.450298
Stdev	0.13732	0.023596	0.024865	0.083505	0.067346	0.073895	0.677393	0.165275	0.013244
st error	0.045773	0.007865	0.008288	0.027835	0.022449	0.024632	0.225798	0.055092	0.004415

NBT/MTT 72 hours - K562 day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.047249	2.675052	9.105033	1.763192	1.448011	1.236823	0.452081	1.512613	2.882353
Reading 2	1.481591	3.243902	2.963947	1.821769	0.688028	0.992806	2.293071	2.835443	2.59
Reading 3	0.970874	3.069663	1.933063	2.63354	2.183575	1.018519	1.400491	1.492352	2.944915
Reading 4	1.43209	3.769737	7.774566	2.503001	2.727983	1.158566	2.671505	1.479476	2.010145
Reading 5	1.077979	3.382409	8.248936	2.141055	1.023917	1.189308	1.460184	1.353321	2.191027
Reading 6	1.197109	3.614224	3.536957	2.741408	1.352359	1.445161	1.30568	1.787905	2.143875
Reading 7	1.006675	2.86551	5.383158	2.206068	1.817835	1.129777	0.715684	1.502783	2.914661
Reading 8	1.33125	3.42364	3.231003	2.239227	1.007315	1.206084	1.743973	2.161309	2.366619
Reading 9	1.041833	3.140244	6.808466	2.171978	1.406794	1.15736	0.993291	1.427845	2.551398
Average	1.176294	3.242709	5.442792	2.246804	1.517313	1.170489	1.44844	1.728116	2.510555
Stdev	0.193148	0.348746	2.635795	0.334079	0.635946	0.131389	0.712063	0.482527	0.354588
st error	0.064383	0.116249	0.878598	0.11136	0.211982	0.043796	0.237354	0.160842	0.118196
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	2.756716	4.627066	1.91007	1.289909	0.995065	1.231358	1.469119	2.134291
Stdev	0.193148	0.348746	2.635795	0.334079	0.635946	0.131389	0.712063	0.482527	0.354588
st error	0.064383	0.116249	0.878598	0.11136	0.211982	0.043796	0.237354	0.160842	0.118196

Data for figure 76

NBT 120 hours - K562 day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.604	2.272	1.947	2.181	1.638	1.503	1.2	1.355	1.15
Reading 2	3.341	1.782	1.943	3.679	2.124	1.878	3.632	1.342	3.532
Reading 3	4.129	1.546	1.814	3.962	3.596	1.549	1.376	1.058	0.975
Reading 4	3.837	2.236	3.553	1.447	2.071	2.284	1.923	1.179	1.079
Reading 5	2.033	2.263	1.764	1.827	1.773	2.125	1.584	1.196	1.186
Reading 6	1.794	3.71	1.163	1.162	1.821	1.804	2.162	2.166	1.088
Reading 7	2.8665	1.909	1.8805	3.0715	2.617	1.526	1.288	1.2065	1.0625
Reading 8	2.5675	2.746	1.553	2.4205	1.9725	1.841	2.897	1.754	2.31
Reading 9	3.35175	2.0725	2.71675	2.25925	2.344	1.905	1.6055	1.19275	1.07075
Average	2.835972	2.281833	2.037139	2.445472	2.217389	1.823889	1.963056	1.38325	1.494806
Stdev	0.902517	0.635588	0.700369	0.958212	0.597569	0.26922	0.817995	0.35404	0.866332
st error	0.300839	0.211863	0.233456	0.319404	0.19919	0.08974	0.272665	0.118013	0.288777
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.588434	1.27806	1.141006	1.369714	1.241964	1.021564	1.099511	0.774761	0.837244
Stdev	0.902517	0.635588	0.700369	0.958212	0.597569	0.26922	0.817995	0.35404	0.866332
st error	0.300839	0.211863	0.233456	0.319404	0.19919	0.08974	0.272665	0.118013	0.288777

MTT 120 hours - K562 day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	2.478	0.501	0.649	1.343	2.15	2.015	1.814	1.663	1.182
Reading 2	2.219	0.492	0.645	1.214	2.035	1.988	1.889	1.666	1.063
Reading 3	2.443	0.459	0.575	1.248	2.063	1.93	1.781	3.219	1.002
Reading 4	1.997	0.526	0.626	1.138	2.328	1.886	1.754	1.55	1.093
Reading 5	2.231	0.503	0.582	1.281	2.142	1.999	1.752	1.738	1.092
Reading 6	2.135	0.428	0.561	1.433	2.004	1.945	1.763	1.542	0.879
Reading 7	2.4605	0.48	0.612	1.2955	2.1065	1.9725	1.7975	2.441	1.092
Reading 8	2.177	0.46	0.603	1.3235	2.0195	1.9665	1.826	1.604	0.971
Reading 9	2.22875	0.503	0.619	1.21675	2.21725	1.92925	1.77575	1.9955	1.0925
Average	2.26325	0.483556	0.608	1.276972	2.118361	1.959028	1.794694	1.935389	1.051833
Stdev	0.164431	0.02998	0.030639	0.085859	0.105056	0.040414	0.043709	0.559658	0.088411
st error	0.05481	0.009993	0.010213	0.02862	0.035019	0.013471	0.01457	0.186553	0.02947
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1.472565	0.314621	0.39559	0.830851	1.378294	1.274625	1.167703	1.259245	0.684367
Stdev	0.164431	0.02998	0.030639	0.085859	0.105056	0.040414	0.043709	0.559658	0.088411
st error	0.05481	0.009993	0.010213	0.02862	0.035019	0.013471	0.01457	0.186553	0.02947

NBT/MTT 120 hours - K562 day 12 Proteins									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.647296	4.53493	3	1.623976	0.76186	0.745906	0.661521	0.814793	0.972927
Reading 2	1.505633	3.621951	3.012403	3.030478	1.043735	0.944668	1.92271	0.805522	3.322672
Reading 3	1.690135	3.368192	3.154783	3.174679	1.743093	0.802591	0.7726	0.328674	0.973054
Reading 4	1.921382	4.250951	5.675719	1.271529	0.889605	1.211029	1.096351	0.760645	0.987191
Reading 5	0.911251	4.499006	3.030928	1.42623	0.827731	1.063032	0.90411	0.688147	1.086081
Reading 6	0.840281	8.668224	2.073084	0.810886	0.908683	0.927506	1.226319	1.404669	1.23777
Reading 7	1.165007	3.977083	3.072712	2.370899	1.242345	0.773638	0.716551	0.494265	0.972985
Reading 8	1.179375	5.969565	2.575456	1.828863	0.976727	0.936181	1.586528	1.093516	2.378991
Reading 9	1.50387	4.120278	4.388934	1.856791	1.057165	0.98743	0.904125	0.59772	0.980092
Average	1.262692	4.778909	3.331558	1.932703	1.050105	0.932442	1.087868	0.776439	1.43464
Stdev	0.422922	1.63426	1.070572	0.790856	0.296006	0.147797	0.425945	0.319693	0.841346
st error	0.140974	0.544753	0.356857	0.263619	0.098669	0.049266	0.141982	0.106564	0.280449
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1.073449	4.062681	2.832248	1.643044	0.892723	0.792695	0.924827	0.660072	1.219627
Stdev	0.422922	1.63426	1.070572	0.790856	0.296006	0.147797	0.425945	0.319693	0.841346
st error	0.140974	0.544753	0.356857	0.263619	0.098669	0.049266	0.141982	0.106564	0.280449

Data for figure 77

NBT 72 hours Day 12 K562 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.486	1.426	2.964	1.431	2.861	3.071	1.522	2.662	2.98
Reading 2	1.524	1.357	3.465	3.581	1.612	1.551	3.277	2.267	3.199
Reading 3	1.181	1.567	3.146	1.979	1.582	1.497	3.576	1.485	3.322
Reading 4	1.464	3.643	3.26	3.534	1.586	3.36	2.99	2.859	1.513
Reading 5	1.91	2.012	2.894	1.999	1.916	1.659	1.452	3.3	3.215
Reading 6	3.577	3.307	3.944	1.882	3.635	3.188	3.494	3.231	2.656
Reading 7	1.3335	1.4965	3.055	1.705	2.2215	2.284	2.549	2.0735	3.151
Reading 8	1.717	1.6845	3.1795	2.79	1.764	1.605	2.3645	2.7835	3.207
Reading 9	2.5205	3.475	3.602	2.708	2.6105	3.274	3.242	3.045	2.0845
Average	1.857	2.218667	3.278833	2.401	2.198667	2.387667	2.7185	2.634	2.814167
Stdev	0.754573	0.964145	0.336661	0.787085	0.710679	0.828105	0.805145	0.592842	0.623739
st error	0.251524	0.321382	0.11222	0.262362	0.236893	0.276035	0.268382	0.197614	0.207913
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	1.194759	1.765661	1.292946	1.183989	1.285766	1.46392	1.418417	1.515437
Stdev	0.754573	0.964145	0.336661	0.787085	0.710679	0.828105	0.805145	0.592842	0.623739
st error	0.251524	0.321382	0.11222	0.262362	0.236893	0.276035	0.268382	0.197614	0.207913

MTT 72 hours Day 12 K562 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.532	0.321	0.269	0.732	0.58	0.527	0.896	0.772	0.369
Reading 2	1.533	0.356	0.329	0.94	0.884	0.545	0.514	0.662	0.345
Reading 3	1.126	0.313	0.35	0.642	1.359	1.028	0.707	0.705	0.335
Reading 4	1.077	0.212	0.349	0.775	1.315	1.149	0.842	0.699	0.386
Reading 5	1.13	0.335	0.371	0.671	1.378	1.273	0.87	0.827	0.475
Reading 6	1.06	0.383	0.351	0.79	1.373	1.201	0.96	0.815	0.473
Reading 7	1.329	0.317	0.3095	0.687	0.9695	0.7775	0.8015	0.7385	0.352
Reading 8	1.3315	0.3455	0.35	0.7325	1.131	0.909	0.692	0.7445	0.41
Reading 9	1.0685	0.2975	0.35	0.7825	1.344	1.175	0.901	0.757	0.4295
Average	1.243	0.32	0.3365	0.750222	1.148167	0.953833	0.798167	0.746667	0.397167
Stdev	0.193908	0.047919	0.030506	0.087952	0.282582	0.282378	0.138637	0.053758	0.053107
st error	0.064636	0.015973	0.010169	0.029317	0.094194	0.094126	0.046212	0.017919	0.017702
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.257442	0.270716	0.603558	0.923706	0.767364	0.642129	0.600697	0.319523
Stdev	0.193908	0.047919	0.030506	0.087952	0.282582	0.282378	0.138637	0.053758	0.053107
st error	0.064636	0.015973	0.010169	0.029317	0.094194	0.094126	0.046212	0.017919	0.017702

NBT/MTT 72 hours Day 12 K562 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.969974	4.442368	11.01859	1.954918	4.932759	5.827324	1.698661	3.448187	8.075881
Reading 2	0.994129	3.811798	10.53191	3.809574	1.823529	2.845872	6.375486	3.424471	9.272464
Reading 3	1.048845	5.00639	8.988571	3.082555	1.164091	1.456226	5.057992	2.106383	9.916418
Reading 4	1.359331	17.18396	9.340974	4.56	1.206084	2.924282	3.551069	4.090129	3.919689
Reading 5	1.690265	6.00597	7.800539	2.979136	1.390421	1.303221	1.668966	3.990326	6.768421
Reading 6	3.374528	8.634465	11.23647	2.382278	2.647487	2.654455	3.639583	3.964417	5.615222
Reading 7	1.003386	4.72082	9.870759	2.481805	2.291387	2.937621	3.180287	2.807718	8.951705
Reading 8	1.289523	4.875543	9.084286	3.808874	1.559682	1.765677	3.416908	3.738751	7.821951
Reading 9	2.358914	11.68067	10.29143	3.460703	1.942336	2.786383	3.598224	4.022457	4.853318
Average	1.565433	7.373554	9.795948	3.168871	2.10642	2.72234	3.576353	3.510315	7.243896
Stdev	0.813968	4.449916	1.101684	0.82526	1.168989	1.336234	1.474011	0.66738	2.086992
st error	0.271323	1.483305	0.367228	0.275087	0.389663	0.445411	0.491337	0.22246	0.695664
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	4.710233	6.25766	2.024278	1.345583	1.739033	2.284577	2.242393	4.627407
Stdev	0.813968	4.449916	1.101684	0.82526	1.168989	1.336234	1.474011	0.66738	0.683702
st error	0.271323	1.483305	0.367228	0.275087	0.389663	0.445411	0.491337	0.22246	0.227901

Data for figure 78

NBT 120 hours Day 12 K562 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.214	1.49	1.68	2.674	1.376	1.645	1.509	3.856	1.245
Reading 2	4.112	3.899	1.777	2.296	1.503	1.599	1.434	1.75	1.672
Reading 3	1.498	1.597	1.461	2.515	1.272	4.054	1.603	1.531	2.343
Reading 4	2.691	1.937	1.47	2.461	1.372	1.402	1.533	1.149	1.725
Reading 5	1.119	1.724	4.07	1.966	1.341	2.388	2.422	2.117	1.722
Reading 6	1.404	2.151	1.438	1.324	1.321	1.715	1.755	1.513	1.733
Reading 7	1.356	1.5435	1.5705	2.5945	1.324	2.8495	1.556	2.6935	1.794
Reading 8	2.6155	2.8115	2.9235	2.131	1.422	1.9935	1.928	1.9335	1.697
Reading 9	2.0475	2.044	1.454	1.8925	1.3465	1.5585	1.644	1.331	1.729
Average	2.006333	2.133	1.982667	2.206	1.364167	2.133833	1.709333	1.986	1.74
Stdev	0.630758	0.638668	0.816865	0.356959	0.051333	0.656841	0.194415	0.682766	0.049427
st error	0.210253	0.212889	0.272288	0.118986	0.017111	0.218947	0.064805	0.227589	0.016476
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.080416	1.148627	1.067672	1.187938	0.734608	1.149076	0.920481	1.069467	0.936995
Stdev	0.630758	0.638668	0.816865	0.356959	0.051333	0.656841	0.194415	0.682766	0.049427
st error	0.210253	0.212889	0.272288	0.118986	0.017111	0.218947	0.064805	0.227589	0.016476

MTT 120 hours Day 12 K562 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.295	0.174	0.196	0.911	1.249	1.201	1.076	0.992	0.561
Reading 2	1.237	0.168	0.212	1.003	1.275	1.174	1.042	0.973	0.586
Reading 3	1.325	0.17	0.203	1.092	1.309	1.165	1.107	0.995	0.543
Reading 4	1.358	0.168	0.235	1.086	1.279	1.229	1.076	0.967	0.551
Reading 5	1.339	0.159	0.228	1.147	1.292	1.282	1.12	0.974	0.556
Reading 6	1.49	0.174	0.239	1.306	1.335	1.214	1.111	0.751	0.535
Reading 7	1.31	0.172	0.1995	1.0015	1.279	1.183	1.0915	0.9935	0.552
Reading 8	1.288	0.1635	0.22	1.075	1.2835	1.228	1.081	0.9735	0.571
Reading 9	1.424	0.171	0.237	1.196	1.307	1.2215	1.0935	0.859	0.543
Average	1.340667	0.168833	0.218833	1.090833	1.289833	1.210833	1.088667	0.942	0.555333
Stdev	0.076108	0.00495	0.016867	0.116631	0.02467	0.035605	0.023467	0.082865	0.015676
st error	0.025369	0.00165	0.005622	0.038877	0.008223	0.011868	0.007822	0.027622	0.005225
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.078573	0.135827	0.176053	0.877581	1.037678	0.974122	0.875838	0.757844	0.446769
Stdev	0.076108	0.00495	0.016867	0.116631	0.02467	0.035605	0.023467	0.082865	0.015676
st error	0.025369	0.00165	0.005622	0.038877	0.008223	0.011868	0.007822	0.027622	0.005225

NBT/MTT 120 hours Day 12 K562 Terpenes									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.14749	8.195402	15.12245	1.570801	2.290633	2.557036	1.414498	2.683468	5.311943
Reading 2	1.232013	8.077381	16.34434	3.570289	1.264314	1.321124	3.144914	2.329908	5.459044
Reading 3	0.891321	9.217647	15.49754	1.812271	1.208556	1.284979	3.230352	1.492462	6.117864
Reading 4	1.078056	21.68452	13.87234	3.254144	1.240031	2.73393	2.77881	2.956567	2.745917
Reading 5	1.426438	12.65409	12.69298	1.742807	1.482972	1.294072	1.296429	3.38809	5.782374
Reading 6	2.400671	19.00575	16.50209	1.441041	2.722846	2.62603	3.144914	4.302264	4.964486
Reading 7	1.035115	8.973837	7.87218	2.590614	1.035184	2.408707	1.425561	2.711122	3.25
Reading 8	2.030668	17.19572	13.28864	1.982326	1.107908	1.623371	1.783534	1.986133	2.971979
Reading 9	1.437851	11.95322	6.135021	1.582358	1.030222	1.27589	1.503429	1.549476	3.184162
Average	1.408847	12.99528	13.0364	2.17185	1.486963	1.902793	2.191382	2.599943	4.420863
Stdev	0.498502	5.100757	3.683423	0.782184	0.603921	0.6576	0.856852	0.897585	1.355819
st error	0.166167	1.700252	1.227808	0.260728	0.201307	0.2192	0.285617	0.299195	0.45194
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.899973	8.301399	8.327662	1.38738	0.949873	1.215506	1.399857	1.660846	2.824051
Stdev	0.498502	5.100757	3.683423	0.782184	0.603921	0.6576	0.856852	0.897585	1.355819
st error	0.166167	1.700252	1.227808	0.260728	0.201307	0.2192	0.285617	0.299195	0.45194

Part K: Statistics for Ester and Ether.

Cell Line	Day Since Test	Shapiro Wilk (p-value)	Normality Interpretation	Kruskall Wallis (p-value)	Pairwise Comparison Test
HL60	3	0.000	Not Normal	0.000	Dunn's Test
HL60	5	0.000	Not Normal	0.000	Dunn's Test
K562	3	0.000	Not Normal	0.000	Dunn's Test
K562	5	0.000	Not Normal	0.000	Dunn's Test

The following represent p values. Red is statistical significance. 3 represents 72 hours exposed to treatment and 5 represents 120 hours exposed to treatment. T represents <5KDa.

Cell Line	K562
Day Since Test	3

	Complete	T	Ester	Ether
Complete		0.196	0.042	0.206
T	0.196		0.000	0.000
Ester	0.042	0.000		1.000
Ether	0.206	0.000	1.000	

Cell Line	K562
Day Since Test	5

	Complete	T	Ester	Ether
Complete		0.000	0.000	1.000
T	0.000		0.000	0.001
Ester	0.000	0.000		0.000
Ether	1.000	0.001	0.000	

Cell Line	HL60
Day Since Test	3

	Complete	T	Ester	Ether
Complete		0.000	0.022	0.000
T	0.000		0.000	0.000
Ester	0.022	0.000		0.021
Ether	0.000	0.000	0.021	

Cell Line	HL60
Day Since Test	5

	Complete	T	Ester	Ether
Complete		0.000	0.014	0.000
T	0.000		0.000	0.076
Ester	0.014	0.000		0.000
Ether	0.000	0.076	0.000	

Part L: Data for Ester and Ether.

NBT 72 hours HL60 Day 9 Ester									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.394	1.537	1.718	1.618	1.835	2.823	2.0765	1.599	3.531
Reading 2	1.726	2.032	1.72	1.893	1.022	1.56	2.143	1.996	3.767
Reading 3	1.83	1.979	1.905	1.978	1.97	1.733	2.204	2.393	2.4515
Reading 4	1.62	2.02	2.285	1.889	1.603	4.04	1.618	1.639	3.868
Reading 5	1.412	2.934	1.716	1.788	1.975	1.945	2.8	1.858	2.191
Reading 6	1.718	2.335	2.075	1.672	1.986	2.06	2.095	1.641	1.78
Reading 7	1.075	1.465	0.876	1.923	1.938	1.682	2.469	2.263	3.123
Reading 8	1.489	1.388	1.146	2.408	1.108	1.954	2.282	2.739	1.498
Reading 9	0.857	1.803	0.983	1.945	1.108	2.083	1.871	1.357	1.307
Average	1.457889	1.943667	1.602667	1.901556	1.616111	2.208889	2.173167	1.942778	2.612944
Stdev	0.319983	0.483714	0.492515	0.226634	0.4201	0.776841	0.337104	0.44636	0.990989
st error	0.106661	0.161238	0.164172	0.075545	0.140033	0.258947	0.112368	0.148787	0.33033
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalised	1	1.333206	1.099306	1.304321	1.108528	1.515128	1.490626	1.332597	1.79228
Stdev	0.319983	0.483714	0.492515	0.226634	0.4201	0.776841	0.337104	0.44636	0.990989
st error	0.106661	0.161238	0.164172	0.075545	0.140033	0.258947	0.112368	0.148787	0.33033

MTT 72 hours HL60 Day 9 Ester									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.888	0.994	0.888	0.549	0.746	0.686	0.783	0.674	0.743
Reading 2	0.813	0.95	0.939	0.316	0.714	0.771	0.692	0.767	0.761
Reading 3	0.795	0.928	0.868	0.524	0.748	0.723	0.749	0.758	0.756
Reading 4	0.79	0.94	0.912	0.537	0.787	0.861	0.772	0.711	0.7
Reading 5	0.897	0.895	0.921	0.357	0.594	0.75	0.732	0.656	0.699
Reading 6	0.861	0.916	0.888	0.559	0.949	0.719	0.83	0.695	0.834
Reading 7	0.628	0.938	0.883	0.54	0.726	0.837	0.704	0.731	0.813
Reading 8	0.877	0.899	0.919	0.549	0.739	0.751	0.745	0.793	0.657
Reading 9	0.674	0.719	0.906	0.548	0.893	0.756	0.752	0.702	0.734
Average	0.802556	0.908778	0.902667	0.497667	0.766222	0.761556	0.751	0.720778	0.744111
Stdev	0.095196	0.077066	0.022472	0.092458	0.10332	0.055848	0.04153	0.045193	0.055818
st error	0.031732	0.025689	0.007491	0.030819	0.03444	0.018616	0.013843	0.015064	0.018606
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	1.132355	1.12474	0.620102	0.954728	0.948913	0.935761	0.898103	0.927177
Stdev	0.102149	0.077066	0.022472	0.014478	0.10332	0.055848	0.04153	0.045193	0.055818
st error	0.03405	0.025689	0.007491	0.004826	0.03444	0.018616	0.013843	0.015064	0.018606

NBT/MTT 72 hours HL60 Day 9 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.56982	1.546278	1.934685	2.947177	2.459786	4.11516	2.65198	2.372404	4.752355
Reading 2	2.123001	2.138947	1.831736	5.990506	1.431373	2.023346	3.096821	2.602347	4.950066
Reading 3	2.301887	2.132543	2.1947	3.774809	2.63369	2.396957	2.94259	3.156992	3.242725
Reading 4	2.050633	2.148936	2.505482	3.517691	2.036849	4.692218	2.095855	2.305204	5.525714
Reading 5	1.574136	3.278212	1.863192	5.008403	3.324916	2.593333	3.825137	2.832317	3.134478
Reading 6	1.995354	2.549127	2.336712	2.991055	2.092729	2.86509	2.524096	2.361151	2.134293
Reading 7	1.711783	1.561834	0.992072	3.561111	2.669421	2.009558	3.507102	3.095759	3.841328
Reading 8	1.697834	1.543938	1.247008	4.386157	1.499323	2.601864	3.063087	3.453972	2.280061
Reading 9	1.271513	2.50765	1.084989	3.54927	1.240761	2.755291	2.488032	1.933048	1.780654
Average	1.810662	2.156385	1.776731	3.969576	2.154316	2.894758	2.910522	2.679244	3.515742
Stdev	0.327432	0.574225	0.551091	0.99532	0.685801	0.915694	0.53733	0.490322	1.341559
st error	0.109144	0.191408	0.183697	0.331773	0.2286	0.305231	0.17911	0.163441	0.447186
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.190937	0.98126	2.192333	1.189795	1.598729	1.607435	1.479704	1.941688
Stdev	1.183375	1.079663	0.551091	1.675434	1.445637	1.41825	1.117271	0.97326	1.490911
st error	0.394458	0.359888	0.183697	0.558478	0.481879	0.47275	0.372424	0.32442	0.49697

NBT 120 hours HL60 Day 9 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.852	0.981	0.908	0.955	1.149	0.935	1.023	1.364	1.323
Reading 2	0.829	1.482	0.913	0.833	0.873	0.983	0.918	1.006	1.34
Reading 3	0.661	0.88	0.871	0.877	1.017	0.997	1.837	1.235	1.743
Reading 4	0.812	0.813	0.796	0.772	0.84	1.011	1.095	1.313	1.144
Reading 5	0.976	0.746	0.731	0.796	0.673	0.654	1.087	0.174	2.041
Reading 6	0.834	0.86	1.017	0.763	1.007	0.827	1.051	0.962	1.31
Reading 7	0.8405	1.2315	0.9105	0.894	1.011	0.959	0.9705	1.185	1.3315
Reading 8	0.7365	0.8465	0.8335	0.8245	0.9285	1.004	1.466	1.274	1.4435
Reading 9	0.905	0.803	0.874	0.7795	0.84	0.7405	1.069	0.568	1.6755
Average	0.827333	0.960333	0.872667	0.832667	0.9265	0.901167	1.1685	1.009	1.4835
Stdev	0.090219	0.241861	0.081105	0.064677	0.138478	0.130103	0.294592	0.397593	0.280942
st error	0.030073	0.08062	0.027035	0.021559	0.046159	0.043368	0.098197	0.132531	0.093647
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	0.567487	0.658715	0.598582	0.571145	0.635508	0.618131	0.801501	0.692097	1.017567
Stdev	0.090219	0.241861	0.081105	0.064677	0.138478	0.130103	0.294592	0.397593	0.280942
st error	0.030073	0.08062	0.027035	0.021559	0.046159	0.043368	0.098197	0.132531	0.093647

MTT 120 hours HL60 Day 9 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.054	0.757	0.757	0.575	1.073	1.032	0.758	1.049	1.052
Reading 2	1.072	0.725	0.736	0.571	1.014	1.025	0.996	1.08	0.963
Reading 3	1.185	0.72	0.682	0.549	1.148	1.14	1.059	1.079	1.098
Reading 4	1.112	0.748	0.812	0.513	1.188	1.184	1.082	1.095	1.078
Reading 5	1.173	0.8	0.761	0.678	1.075	1.165	1.111	1.121	1.142
Reading 6	1.066	0.661	0.792	0.582	1.079	1.075	1.093	1.046	0.96
Reading 7	1.167	0.697	0.729	0.568	0.858	1.009	0.852	0.952	0.968
Reading 8	1.008	0.702	0.707	0.579	1.077	1.106	1	0.992	1.079
Reading 9	1.191	0.743	0.698	0.547	0.701	1.064	1.012	1.013	0.635
Average	1.114222	0.728111	0.741556	0.573556	1.023667	1.088889	0.995889	1.047444	0.997222
Stdev	0.067238	0.040076	0.043229	0.044755	0.152107	0.063499	0.118137	0.053738	0.150808
st error	0.022413	0.013359	0.01441	0.014918	0.050702	0.021166	0.039379	0.017913	0.050269
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.388343	0.907241	0.923993	0.714661	1.275509	1.356777	1.240897	1.305136	1.242558
Stdev	0.067238	0.040076	0.043229	0.044755	0.152107	0.063499	0.118137	0.053738	0.150808
st error	0.022413	0.013359	0.01441	0.014918	0.050702	0.021166	0.039379	0.017913	0.050269

NBT/MTT 120 hours HL60 Day 9 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.808349	1.295905	1.199472	1.66087	1.070829	0.906008	1.349604	1.300286	1.257605
Reading 2	0.773321	2.044138	1.240489	1.458844	0.860947	0.959024	0.921687	0.931481	1.391485
Reading 3	0.557806	1.222222	1.277126	1.59745	0.885889	0.874561	1.734655	1.144578	1.587432
Reading 4	0.730216	1.086898	0.980296	1.504873	0.707071	0.853885	1.012015	1.199087	1.061224
Reading 5	0.832055	0.9325	0.960578	1.174041	0.626047	0.561373	0.978398	0.155219	1.787215
Reading 6	0.782364	1.301059	1.284091	1.310997	0.933272	0.769302	0.961574	0.919694	1.364583
Reading 7	0.720223	1.766858	1.248971	1.573944	1.178322	0.950446	1.139085	1.244748	1.375517
Reading 8	0.730655	1.20584	1.178925	1.424007	0.862117	0.907776	1.466	1.284274	1.337813
Reading 9	0.759866	1.080754	1.252149	1.425046	1.198288	0.695959	1.056324	0.560711	2.638583
Average	0.743873	1.326242	1.180233	1.458897	0.924753	0.830926	1.179927	0.97112	1.533495
Stdev	0.07912	0.355136	0.123641	0.1503	0.195782	0.131976	0.278045	0.387571	0.460923
st error	0.026373	0.118379	0.041214	0.0501	0.065261	0.043992	0.092682	0.12919	0.153641
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.410829	0.732462	0.651824	0.805725	0.510727	0.458907	0.651655	0.536334	0.846925
Stdev	0.07912	0.355136	0.123641	0.1503	0.195782	0.131976	0.278045	0.387571	0.460923
st error	0.026373	0.118379	0.041214	0.0501	0.065261	0.043992	0.092682	0.12919	0.153641

NBT 72 hours HL60 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.057	1.883	1.903	1.496	2.275	2.184	1.728	2.129	1.98
Reading 2	1.99	1.863	1.888	0.868	1.827	2.18	2.043	1.831	2.143
Reading 3	1.706	1.6	1.796	1.675	1.835	1.877	1.62	1.538	2.031
Reading 4	2.066	1.96	2.031	1.701	2.005	3.991	1.913	1.319	1.736
Reading 5	1.715	1.743	1.71	1.045	1.855	1.808	1.882	1.633	1.749
Reading 6	1.737	2.476	2.434	1.156	1.702	1.862	1.237	1.738	2.005
Reading 7	1.435	1.576	1.851	0.919	3.64	2.011	1.47	2.751	1.47
Reading 8	1.662	1.573	1.678	1.636	1.629	1.303	1.797	1.548	2.468
Reading 9	1.772	1.484	1.86	2.028	1.412	1.876	1.89	2.033	2.743
Average	1.793333	1.795333	1.905667	1.391556	2.02	2.121333	1.731111	1.835556	2.036111
Stdev	0.207842	0.303737	0.224272	0.406944	0.653071	0.747511	0.251841	0.425992	0.386453
st error	0.069281	0.101246	0.074757	0.135648	0.21769	0.24917	0.083947	0.141997	0.128818
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	1.001115	1.062639	0.77596	1.126394	1.1829	0.965304	1.023544	1.135378
Stdev	0.207842	0.303737	0.224272	0.406944	0.653071	0.747511	0.251841	0.425992	0.386453
st error	0.069281	0.101246	0.074757	0.135648	0.21769	0.24917	0.083947	0.141997	0.128818

MTT 72 hours HL60 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.521	0.791	0.798	0.324	0.527	0.416	0.46	0.467	0.54
Reading 2	0.554	0.812	0.905	0.205	0.504	0.475	0.414	0.527	0.469
Reading 3	0.562	0.724	0.807	0.283	0.343	0.353	0.433	0.471	0.542
Reading 4	0.508	0.813	0.797	0.377	0.385	0.362	0.409	0.469	0.475
Reading 5	0.561	0.825	0.827	0.223	0.347	0.418	0.448	0.505	0.545
Reading 6	0.63	0.835	0.867	0.273	0.478	0.499	0.405	0.547	0.568
Reading 7	0.642	0.78	0.851	0.224	0.361	0.356	0.367	0.437	0.383
Reading 8	0.597	0.726	0.849	0.254	0.457	0.494	0.431	0.476	0.473
Reading 9	0.625	0.868	0.824	0.318	0.513	0.589	0.44	0.559	0.438
Average	0.577778	0.797111	0.836111	0.275667	0.435	0.440222	0.423	0.495333	0.492556
Stdev	0.048269	0.048017	0.035614	0.056307	0.075685	0.080305	0.027803	0.041358	0.060583
st error	0.01609	0.016006	0.011871	0.018769	0.025228	0.026768	0.009268	0.013786	0.020194
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.379615	1.447115	0.477115	0.752885	0.761923	0.732115	0.857308	0.8525
Stdev	0.048269	0.048017	0.035614	0.056307	0.075685	0.080305	0.027803	0.041358	0.060583
st error	0.01609	0.016006	0.011871	0.018769	0.025228	0.026768	0.009268	0.013786	0.020194

NBT/MTT 72 hours HL60 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	3.948177	2.380531	2.384712	4.617284	4.316888	5.25	3.756522	4.558887	3.666667
Reading 2	3.592058	2.294335	2.086188	4.234146	3.625	4.589474	4.934783	3.474383	4.569296
Reading 3	3.035587	2.209945	2.225527	5.918728	5.349854	5.31728	3.741339	3.265393	3.747232
Reading 4	4.066929	2.410824	2.548306	4.511936	5.207792	11.02486	4.677262	2.812367	3.654737
Reading 5	3.057041	2.112727	2.067715	4.686099	5.345821	4.325359	4.200893	3.233663	3.209174
Reading 6	2.757143	2.965269	2.807382	4.234432	3.560669	3.731463	3.054321	3.177331	3.52993
Reading 7	2.235202	2.020513	2.175088	4.102679	10.0831	5.648876	4.00545	6.295195	3.83812
Reading 8	2.78392	2.166667	1.976443	6.440945	3.564551	2.637652	4.169374	3.252101	5.217759
Reading 9	2.8352	1.709677	2.257282	6.377358	2.752437	3.185059	4.295455	3.636852	6.262557
Average	3.145695	2.252276	2.28096	5.013734	4.867346	5.078892	4.092822	3.74513	4.188386
Stdev	0.604062	0.340668	0.262284	0.953731	2.165229	2.447823	0.551342	1.071058	0.985747
st error	0.201354	0.113556	0.087428	0.31791	0.721743	0.815941	0.183781	0.357019	0.328582
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.715987	0.725105	1.59384	1.547304	1.614553	1.301087	1.190557	1.331466
Stdev	0.604062	0.340668	0.262284	0.953731	2.165229	2.447823	0.551342	1.071058	0.985747
st error	0.201354	0.113556	0.087428	0.31791	0.721743	0.815941	0.183781	0.357019	0.328582

NBT 120 hours HL60 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.601	1.527	1.49	1.311	1.587	1.431	2.702	1.474	1.621
Reading 2	3.862	1.415	1.606	1.422	1.357	3.739	1.347	1.254	1.367
Reading 3	1.431	1.483	1.438	2.03	1.362	1.425	1.439	2.687	1.484
Reading 4	1.296	1.302	1.394	1.385	1.296	1.302	1.404	1.341	1.312
Reading 5	1.225	1.209	1.891	2.429	1.267	1.415	1.329	1.792	1.268
Reading 6	1.307	1.239	3.37	1.257	1.392	1.352	1.206	1.228	1.328
Reading 7	2.892	2.331	2.197	2.252	1.882	1.829	1.927	1.976	2.003
Reading 8	2.015	2.155	2.412	2.189	2.276	1.862	1.999	1.734	2.128
Reading 9	2.249	2.042	2.114	1.771	1.979	2.571	1.953	2.259	2.078
Average	1.986444	1.633667	1.990222	1.782889	1.599778	1.880667	1.700667	1.749444	1.621
Stdev	0.89322	0.426145	0.633234	0.454347	0.361001	0.804208	0.483601	0.493976	0.353738
st error	0.29774	0.142048	0.211078	0.151449	0.120334	0.268069	0.1612	0.164659	0.117913
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.107683	0.910967	1.109789	0.994176	0.892069	1.048699	0.948327	0.975527	0.903903
Stdev	0.89322	0.426145	0.633234	0.454347	0.361001	0.804208	0.483601	0.493976	0.353738
st error	0.29774	0.142048	0.211078	0.151449	0.120334	0.268069	0.1612	0.164659	0.117913

MTT 120 hours HL60 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.813	0.608	0.552	0.814	0.848	0.875	0.997	0.752	0.807
Reading 2	0.883	0.647	0.558	0.873	0.958	0.925	1.023	0.992	0.912
Reading 3	0.938	0.619	0.554	0.648	0.969	0.979	1.018	1.079	0.826
Reading 4	0.819	0.602	0.57	0.741	0.849	0.936	0.918	1.019	0.87
Reading 5	0.91	0.609	0.582	0.748	0.903	0.971	0.866	0.86	0.814
Reading 6	0.696	0.632	0.504	0.882	0.85	0.811	0.764	0.784	0.741
Reading 7	0.988	0.678	0.605	0.638	1.001	0.919	0.871	0.991	0.886
Reading 8	0.9	0.706	0.662	0.711	1.088	0.975	1.098	0.996	0.901
Reading 9	0.939	0.483	0.661	0.714	0.991	1.043	1.006	0.921	0.743
Average	0.876222	0.620444	0.583111	0.752111	0.939667	0.937111	0.951222	0.932667	0.833333
Stdev	0.08786	0.06224	0.052018	0.088359	0.08337	0.066885	0.103746	0.111781	0.063926
st error	0.029287	0.020747	0.017339	0.029453	0.02779	0.022295	0.034582	0.03726	0.021309
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1.516538	1.073846	1.009231	1.301731	1.626346	1.621923	1.646346	1.614231	1.442308
Stdev	0.08786	0.06224	0.052018	0.088359	0.08337	0.066885	0.103746	0.111781	0.063926
st error	0.029287	0.020747	0.017339	0.029453	0.02779	0.022295	0.034582	0.03726	0.021309

NBT/MTT 120 hours HL60 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.96925	2.511513	2.699275	1.610565	1.871462	1.635429	2.71013	1.960106	2.008674
Reading 2	4.373726	2.187017	2.878136	1.628866	1.416493	4.042162	1.316716	1.264113	1.498904
Reading 3	1.525586	2.3958	2.595668	3.132716	1.405573	1.455567	1.413556	2.490269	1.79661
Reading 4	1.582418	2.162791	2.445614	1.869096	1.526502	1.391026	1.529412	1.315996	1.508046
Reading 5	1.346154	1.985222	3.249141	3.247326	1.403101	1.457261	1.534642	2.083721	1.55774
Reading 6	1.877874	1.960443	6.686508	1.42517	1.637647	1.667078	1.578534	1.566327	1.792173
Reading 7	2.927126	3.438053	3.631405	3.529781	1.88012	1.990207	2.2124	1.993946	2.260722
Reading 8	2.238889	3.052408	3.643505	3.078762	2.091912	1.909744	1.820583	1.740964	2.36182
Reading 9	2.395101	4.227743	3.198185	2.480392	1.996973	2.465005	1.941352	2.452769	2.79677
Average	2.248458	2.657888	3.447493	2.444742	1.692198	2.001497	1.784147	1.874246	1.953495
Stdev	0.93505	0.76849	1.288329	0.823894	0.272095	0.836694	0.446113	0.44388	0.445479
st error	0.311683	0.256163	0.429443	0.274631	0.090698	0.278898	0.148704	0.14796	0.148493
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	0.714773	0.844929	1.09594	0.777171	0.537941	0.636266	0.567171	0.595813	0.621006
Stdev	0.93505	0.76849	1.288329	0.823894	0.272095	0.836694	0.446113	0.44388	0.445479
st error	0.311683	0.256163	0.429443	0.274631	0.090698	0.278898	0.148704	0.14796	0.148493

NBT 72 hours HL60 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.012	1.783	1.841	2.375	1.247	1.334	1.709	1.409	1.301
Reading 2	1.657	2.903	2.156	2.075	1.635	3.325	3.467	1.657	2.624
Reading 3	1.668	1.586	2.183	2.596	1.494	3.906	1.622	1.411	2.367
Reading 4	1.94	1.584	4.095	1.635	4.051	3.538	1.85	3.377	4.028
Reading 5	1.684	3.91	1.589	2.83	1.687	2.154	1.458	1.686	3.412
Reading 6	2.726	1.853	1.566	1.891	1.584	2.609	1.58	1.411	1.194
Reading 7	2.592	2.784	3.725	2.671	3.912	2.825	3.133	2.464	3.047
Reading 8	2.086	2.273	2.284	2.524	3.508	2.897	1.63	1.892	2.753
Reading 9	2.244	2.552	2.195	2.666	2.934	2.244	2.067	2.096	2.509
Average	2.067667	2.358667	2.403778	2.362556	2.450222	2.759111	2.057333	1.933667	2.581667
Stdev	0.392864	0.767526	0.898359	0.406329	1.14082	0.786653	0.730395	0.647309	0.911462
st error	0.130955	0.255842	0.299453	0.135443	0.380273	0.262218	0.243465	0.21577	0.303821
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	1.140738	1.162556	1.142619	1.185018	1.334408	0.995002	0.935193	1.248589
Stdev	0.723251	0.767526	0.898359	0.681618	1.14082	0.786653	0.730395	0.647309	0.911462
st error	0.241084	0.255842	0.299453	0.227206	0.380273	0.262218	0.243465	0.21577	0.303821

MTT 72 hours HL60 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.864	0.918	0.913	0.41	0.679	0.705	0.711	0.826	0.713
Reading 2	0.556	0.92	0.916	0.298	0.62	0.654	0.717	0.678	0.758
Reading 3	0.824	0.743	0.918	0.476	0.68	0.64	0.644	0.735	0.665
Reading 4	0.754	0.88	0.885	0.274	0.64	0.615	0.683	0.663	0.697
Reading 5	0.694	0.853	0.969	0.371	0.637	0.711	0.69	0.666	0.62
Reading 6	0.732	0.878	0.946	0.459	0.647	0.651	0.811	0.697	0.748
Reading 7	0.713	0.755	0.936	0.367	0.685	0.55	0.599	0.839	0.592
Reading 8	0.912	0.889	0.906	0.578	0.617	0.613	0.643	0.622	0.657
Reading 9	0.737	0.826	0.808	0.398	0.534	0.567	0.608	0.582	0.59
Average	0.754	0.851333	0.910778	0.403444	0.637667	0.634	0.678444	0.700889	0.671111
Stdev	0.104416	0.064973	0.045554	0.092971	0.04653	0.05482	0.065181	0.086198	0.062822
st error	0.034805	0.021658	0.015185	0.03099	0.01551	0.018273	0.021727	0.028733	0.020941
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.129089	1.207928	0.535072	0.845712	0.840849	0.899794	0.929561	0.890068
Stdev	0.104416	0.064973	0.045554	0.063763	0.04653	0.05482	0.065181	0.086198	0.062822
st error	0.034805	0.021658	0.015185	0.021254	0.01551	0.018273	0.021727	0.028733	0.020941

NBT/MTT 72 hours HL60 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.328704	1.942266	2.016429	5.792683	1.836524	1.892199	2.403657	1.705811	1.824684
Reading 2	2.980216	3.155435	2.353712	6.963087	2.637097	5.084098	4.835425	2.443953	3.461741
Reading 3	2.024272	2.13459	2.377996	5.453782	2.197059	6.103125	2.518634	1.919728	3.559398
Reading 4	2.572944	1.8	4.627119	5.967153	6.329688	5.752846	2.708638	5.093514	5.779053
Reading 5	2.426513	4.583822	1.639835	7.628032	2.648352	3.029536	2.113043	2.531532	5.503226
Reading 6	3.724044	2.110478	1.655391	4.119826	2.448223	4.00768	1.948212	2.02439	1.596257
Reading 7	3.635344	3.687417	3.979701	7.277929	5.710949	5.136364	5.230384	2.93683	5.146959
Reading 8	2.287281	2.556805	2.520971	4.366782	5.685575	4.725938	2.534992	3.041801	4.190259
Reading 9	3.044776	3.089588	2.716584	6.698492	5.494382	3.957672	3.399671	3.601375	4.252542
Average	2.780455	2.784489	2.654193	6.029752	3.887539	4.40994	3.076962	2.810993	3.923791
Stdev	0.603988	0.928077	1.01679	1.236726	1.848614	1.339925	1.184865	1.045888	1.490879
st error	0.201329	0.309359	0.33893	0.412242	0.616205	0.446642	0.394955	0.348629	0.49696
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.001451	0.95459	2.168621	1.398166	1.58605	1.10664	1.010983	1.411205
Stdev	0.949899	0.928077	1.01679	2.779242	1.848614	1.339925	1.184865	1.045888	1.490879
st error	0.316633	0.309359	0.33893	0.926414	0.616205	0.446642	0.394955	0.348629	0.49696

NBT 120 hours HL60 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.177	1.981	1.871	1.657	1.706	1.901	3.03	2.305	1.697
Reading 2	1.456	1.333	1.361	1.157	2.845	1.563	1.361	1.699	2.137
Reading 3	1.593	1.926	1.718	1.223	2.904	1.396	3.25	3.674	3.925
Reading 4	1.137	2.721	1.325	1.398	1.618	1.407	1.496	3.506	4.075
Reading 5	1.217	1.407	1.7	1.471	1.547	1.569	4.279	4.257	4.013
Reading 6	1.119	2.183	4.199	1.281	4.247	1.992	1.426	1.413	1.493
Reading 7	1.779	3.94	2.047	1.084	1.654	2.439	2.286	1.811	3.842
Reading 8	1.331	2.26	1.876	1.3	1.81	2.193	3.092	3.668	3.889
Reading 9	1.658	1.627	1.738	1.936	2.054	2.103	1.878	3.402	1.382
Average	1.385222	2.153111	1.981667	1.389667	2.265	1.840333	2.455333	2.859444	2.939222
Stdev	0.246154	0.799613	0.863779	0.267354	0.90447	0.373195	1.014411	1.050494	1.216296
st error	0.082051	0.266538	0.287926	0.089118	0.30149	0.124398	0.338137	0.350165	0.405432
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	0.669945	1.041324	0.958407	0.672094	1.095438	0.890053	1.18749	1.382933	1.421516
Stdev	0.246154	0.799613	0.863779	0.267354	0.90447	0.373195	1.014411	1.050494	1.216296
st error	0.082051	0.266538	0.287926	0.089118	0.30149	0.124398	0.338137	0.350165	0.405432

MTT 120 hours HL60 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.212	0.951	0.97	0.582	1.41	0.944	1.027	1.019	0.833
Reading 2	1.493	0.928	0.928	0.549	1.425	1.044	1.18	0.946	0.876
Reading 3	1.469	0.954	0.918	0.549	0.46	0.97	1.285	1.134	1.014
Reading 4	1.345	0.89	0.889	0.57	1.17	1.16	1.162	1.152	0.857
Reading 5	1.417	0.815	0.927	0.537	1.261	1.161	1.128	1.026	0.89
Reading 6	1.144	0.89	0.916	0.645	0.726	0.802	0.658	1.205	0.869
Reading 7	0.688	0.589	0.607	0.429	0.926	0.844	0.944	0.929	0.836
Reading 8	0.7	0.644	0.641	0.384	0.953	0.82	0.9	0.823	0.839
Reading 9	0.56	0.62	0.581	0.459	0.813	0.832	0.838	0.817	0.739
Average	1.114222	0.809	0.819667	0.522667	1.016	0.953	1.013556	1.005667	0.861444
Stdev	0.368295	0.149939	0.159579	0.082442	0.326491	0.142204	0.19738	0.139914	0.071877
st error	0.122765	0.04998	0.053193	0.027481	0.10883	0.047401	0.065793	0.046638	0.023959
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.477748	1.072944	1.087091	0.693192	1.34748	1.263926	1.344238	1.333775	1.142499
Stdev	0.368295	0.149939	0.159579	0.082442	0.326491	0.142204	0.19738	0.139914	0.071877
st error	0.122765	0.04998	0.053193	0.027481	0.10883	0.047401	0.065793	0.046638	0.023959

NBT/MTT 120 hours HL60 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.971122	2.08307	1.928866	2.847079	1.209929	2.013771	2.950341	2.262022	2.037215
Reading 2	0.975218	1.436422	1.466595	2.107468	1.996491	1.497126	1.15339	1.795983	2.439498
Reading 3	1.084411	2.018868	1.87146	2.227687	6.313043	1.439175	2.529183	3.239859	3.870809
Reading 4	0.845353	3.057303	1.490439	2.452632	1.382906	1.212931	1.287435	3.043403	4.754959
Reading 5	0.858857	1.72638	1.833873	2.739292	1.226804	1.351421	3.79344	4.149123	4.508989
Reading 6	0.978147	2.452809	4.584061	1.986047	5.849862	2.483791	2.167173	1.172614	1.718067
Reading 7	2.585756	6.689304	3.372323	2.526807	1.786177	2.88981	2.42161	1.949408	4.595694
Reading 8	1.901429	3.509317	2.926677	3.385417	1.899265	2.67439	3.435556	4.456865	4.63528
Reading 9	2.960714	2.624194	2.991394	4.217865	2.526445	2.527644	2.24105	4.164015	1.870095
Average	1.462334	2.844185	2.496187	2.721144	2.68788	2.010007	2.442131	2.91481	3.381178
Stdev	0.813995	1.579797	1.047603	0.704269	1.971707	0.648638	0.879589	1.184764	1.33178
st error	0.271332	0.526599	0.349201	0.234756	0.657236	0.216213	0.293196	0.394921	0.443927
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.525933	1.022921	0.897762	0.978669	0.966705	0.722906	0.878321	1.048321	1.216052
Stdev	0.813995	1.579797	1.047603	0.704269	1.971707	0.648638	0.879589	1.184764	1.33178
st error	0.271332	0.526599	0.349201	0.234756	0.657236	0.216213	0.293196	0.394921	0.443927

NBT 72 hours HL60 Day 12Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.197	1.324	2.498	1.256	1.457	1.098	1.143	1.186	1.156
Reading 2	1.233	1.241	1.316	1.191	1.001	0.96	1.034	0.898	0.952
Reading 3	1.378	1.215	1.043	1.923	1.253	1.122	1.196	0.923	0.857
Reading 4	1.312	1.118	0.87	1.992	1.062	1.128	1.499	1.011	1.175
Reading 5	1.219	1.171	1.043	1.332	1.146	1.141	1.503	1.184	0.902
Reading 6	1.072	2.004	1.104	2.041	1.116	1.068	1.325	1.199	0.978
Reading 7	1.149	1.509	1.593	1.347	1.542	1.426	1.743	1.476	1.71
Reading 8	1.45	1.475	1.32	1.346	1.188	1.643	1.544	1.603	1.334
Reading 9	1.469	1.678	1.385	1.476	1.161	1.464	1.224	1.825	1.572
Average	1.235167	1.3455	1.312333	1.6225	1.1725	1.086167	1.283333	1.066833	1.003333
Stdev	0.104547	0.329922	0.598325	0.401716	0.163057	0.066976	0.192867	0.13979	0.132465
st error	0.034849	0.109974	0.199442	0.133905	0.054352	0.022325	0.064289	0.046597	0.044155
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	1.089327	1.062475	1.313588	0.949265	0.879369	1.038996	0.863716	0.812306
Stdev	0.104547	0.329922	0.598325	0.401716	0.163057	0.066976	0.192867	0.13979	0.132465
st error	0.034849	0.109974	0.199442	0.133905	0.054352	0.022325	0.064289	0.046597	0.044155

MTT 72 hours HL60 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.64	0.49	0.558	0.232	0.65	0.757	0.722	0.645	0.55
Reading 2	0.466	0.496	0.554	0.241	0.624	0.656	0.629	0.657	0.566
Reading 3	0.501	0.506	0.501	0.339	0.629	0.631	0.651	0.618	0.571
Reading 4	0.713	0.496	0.526	0.435	0.587	0.569	0.556	0.58	0.466
Reading 5	0.476	0.509	0.538	0.231	0.52	0.671	0.63	0.569	0.496
Reading 6	0.512	0.505	0.541	0.39	0.56	0.765	0.569	0.617	0.467
Reading 7	0.386	0.513	0.537	0.202	0.641	0.553	0.619	0.625	0.534
Reading 8	0.457	0.511	0.528	0.219	0.506	0.551	0.605	0.603	0.495
Reading 9	0.505	0.508	0.536	0.303	0.563	0.467	0.63	0.519	0.5
	RPMI	PMA10	PMA100	DMSO	4%	8%	12%	16%	32%
Average	0.517333	0.503778	0.535444	0.288	0.586667	0.624444	0.623444	0.603667	0.516111
Stdev	0.099381	0.007902	0.016674	0.083359	0.052882	0.099306	0.048039	0.042311	0.040316
st error	0.033127	0.002634	0.005558	0.027786	0.017627	0.033102	0.016013	0.014104	0.013439
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.973797	1.035009	0.556701	1.134021	1.207045	1.205112	1.166881	0.997637
Stdev	0.099381	0.007902	0.016674	0.083359	0.052882	0.099306	0.048039	0.042311	0.040316
st error	0.033127	0.002634	0.005558	0.027786	0.017627	0.033102	0.016013	0.014104	0.013439

NBT/MTT 72 hours HL60 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.870313	2.702041	4.476703	5.413793	2.241538	1.450462	1.583102	1.83876	2.101818
Reading 2	2.645923	2.502016	2.375451	4.941909	1.604167	1.463415	1.643879	1.366819	1.681979
Reading 3	2.750499	2.401186	2.081836	5.672566	1.992051	1.77813	1.837174	1.493528	1.500876
Reading 4	1.840112	2.254032	1.653992	4.57931	1.809199	1.982425	2.696043	1.743103	2.521459
Reading 5	2.560924	2.300589	1.938662	5.766234	2.203846	1.700447	2.385714	2.080844	1.818548
Reading 6	2.09375	3.968317	2.040665	5.233333	1.992857	1.396078	2.328647	1.943274	2.094218
Reading 7	2.976684	2.94152	2.96648	6.668317	2.405616	2.578662	2.815832	2.3616	3.202247
Reading 8	3.172867	2.886497	2.5	6.146119	2.347826	2.981851	2.552066	2.658375	2.694949
Reading 9	2.908911	3.30315	2.583955	4.871287	2.062167	3.134904	1.942857	3.516378	3.144
Average	2.535554	2.806594	2.513083	5.476985	2.073252	2.051819	2.198368	2.111409	2.306677
Stdev	0.489969	0.553419	0.833866	0.662503	0.258403	0.676075	0.45933	0.662485	0.618969
st error	0.163323	0.184473	0.277955	0.220834	0.086134	0.225358	0.15311	0.220828	0.206323
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.106896	0.991138	2.160075	0.817672	0.809219	0.867017	0.832721	0.909733
Stdev	0.489969	0.553419	0.833866	0.662503	0.258403	0.676075	0.45933	0.662485	0.618969
st error	0.163323	0.184473	0.277955	0.220834	0.086134	0.225358	0.15311	0.220828	0.206323

NBT 120 hours HL60 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.013	2.448	2.168	2.294	1.93	2.047	1.676	2.33	1.905
Reading 2	1.971	1.83	1.831	2.134	2.113	1.827	1.795	1.89	1.799
Reading 3	1.303	1.853	1.972	2.226	2.169	1.891	1.89	2.282	1.665
Reading 4	1.129	1.902	2.029	2.33	1.89	2.216	2.163	1.858	2.327
Reading 5	2.472	2.128	2.104	2.193	2.343	2.057	1.819	1.824	1.894
Reading 6	1.764	2.144	2.09	2.976	2.641	2.188	1.787	1.828	1.9
Reading 7	2.041	1.824	1.502	2.592	1.438	3.437	1.809	1.977	1.989
Reading 8	1.931	2.399	3.634	3.381	4.045	1.622	1.618	1.963	1.924
Reading 9	1.487	2.254	2.447	2.333	2.1	3.242	3.319	2.431	1.903
Average	1.775333	2.050833	2.032333	2.358833	2.181	2.037667	1.855	2.002	1.915
Stdev	0.494184	0.237669	0.119209	0.310363	0.27939	0.155406	0.165922	0.23716	0.222021
st error	0.164728	0.079223	0.039736	0.103454	0.09313	0.051802	0.055307	0.079053	0.074007
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.437323	1.66037	1.645392	1.909729	1.765754	1.64971	1.501822	1.620834	1.550398
Stdev	0.494184	0.237669	0.119209	0.310363	0.27939	0.155406	0.165922	0.23716	0.222021
st error	0.164728	0.079223	0.039736	0.103454	0.09313	0.051802	0.055307	0.079053	0.074007

MTT 120 hours HL60 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	0.892	0.384	0.408	0.686	0.903	0.737	0.5	0.53	0.326
Reading 2	0.918	0.434	0.387	0.5775	0.999	0.768	0.448	0.542	0.349
Reading 3	0.932	0.399	0.443	0.518	0.997	0.849	0.501	0.553	0.342
Reading 4	0.924	0.374	0.405	0.588	0.975	0.917	0.61	0.548	0.311
Reading 5	0.854	0.399	0.433	0.7525	0.861	0.893	0.582	0.563	0.325
Reading 6	0.798	0.418	0.431	0.5565	0.894	0.688	0.328	0.541	0.384
Reading 7	0.725	0.362	0.39	0.539	0.751	0.65	0.595	0.535	0.321
Reading 8	0.845	0.426	0.388	0.581	0.764	0.768	0.636	0.583	0.333
Reading 9	0.849	0.413	0.411	0.539	0.764	0.685	0.576	0.581	0.383
Average	0.859667	0.401	0.410667	0.593056	0.878667	0.772778	0.530667	0.552889	0.341556
Stdev	0.067192	0.024223	0.020911	0.076883	0.100932	0.095102	0.097433	0.019127	0.026268
st error	0.022397	0.008074	0.00697	0.025628	0.033644	0.031701	0.032478	0.006376	0.008756
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	0.46646	0.477705	0.689867	1.022102	0.898927	0.617294	0.643143	0.397312
Stdev	0.067192	0.024223	0.020911	0.076883	0.100932	0.095102	0.097433	0.019127	0.026268
st error	0.022397	0.008074	0.00697	0.025628	0.033644	0.031701	0.032478	0.006376	0.008756

NBT/MTT 120 hours HL60 Day 12Ether									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	2.256726	6.375	5.313725	3.344023	2.13732	2.777476	3.352	4.396226	5.843558
Reading 2	2.147059	4.21659	4.731266	3.695238	2.115115	2.378906	4.006696	3.487085	5.154728
Reading 3	1.398069	4.64411	4.451467	4.297297	2.175527	2.227326	3.772455	4.126582	4.868421
Reading 4	1.221861	5.085561	5.009877	3.962585	1.938462	2.416576	3.545902	3.390511	7.482315
Reading 5	2.894614	5.333333	4.859122	2.914286	2.721254	2.303471	3.12543	3.239787	5.827692
Reading 6	2.210526	5.129187	4.849188	5.347709	2.954139	3.180233	5.448171	3.378928	4.947917
Reading 7	2.815172	5.038674	3.851282	4.808905	1.91478	5.287692	3.040336	3.695327	6.196262
Reading 8	2.285207	5.631455	9.365979	5.819277	5.294503	2.111979	2.544025	3.367067	5.777778
Reading 9	1.751472	5.457627	5.953771	4.328386	2.748691	4.732847	5.762153	4.184165	4.968668
Average	2.108967	5.212393	5.376187	4.279745	2.666643	3.046279	3.84413	3.696187	5.674149
Stdev	0.570171	0.609282	1.602014	0.933788	1.055884	1.167043	1.08785	0.428348	0.832856
st error	0.190057	0.203094	0.534005	0.311263	0.351961	0.389014	0.362617	0.142783	0.277619
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	4.076612	10.0755	10.39211	8.272703	5.154594	5.888425	7.430663	7.14469	10.96807
Stdev	0.570171	0.609282	1.602014	0.933788	1.055884	1.167043	1.08785	0.428348	0.832856
st error	0.190057	0.203094	0.534005	0.311263	0.351961	0.389014	0.362617	0.142783	0.277619

NBT 72 hours K562 Day 12Ester									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.619	1.823	1.978	1.959	2.201	2.04	2.399	1.677	1.863
Reading 2	2.226	1.549	2.044	1.797	2.062	1.457	1.913	2.224	1.897
Reading 3	2.021	1.888	1.84	1.955	2.046	1.957	1.883	2.022	2.019
Reading 4	2.013	1.851	1.821	1.982	2.139	1.806	1.998	1.979	1.71
Reading 5	2.874	3.318	1.998	2.064	2.469	2.041	1.573	2.315	2.006
Reading 6	1.136	1.795	1.787	1.957	1.719	2.019	1.836	1.64	1.706
Reading 7	1.498	1.285	1.445	1.29	2.469	1.718	1.115	1.32	1.753
Reading 8	2.674	1.344	1.557	1.493	1.957	1.607	1.318	1.57	1.246
Reading 9	1.711	1.48	1.866	1.982	2.271	1.781	1.86	1.849	2.279
Average	1.974667	1.814778	1.815111	1.831	2.148111	1.825111	1.766111	1.844	1.831
Stdev	0.558037	0.607435	0.200003	0.263536	0.240422	0.207556	0.381658	0.323135	0.285182
st error	0.186012	0.202478	0.066668	0.087845	0.080141	0.069185	0.127219	0.107712	0.095061
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalised	1	0.91903	0.919199	0.927245	1.087835	0.924263	0.894384	0.933828	0.927245
Stdev	0.580101	0.607435	0.200003	0.488684	0.240422	0.207556	0.381658	0.323135	0.285182
st error	0.193367	0.202478	0.066668	0.162895	0.080141	0.069185	0.127219	0.107712	0.095061

MTT 72 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Reading 1	1.466	0.668	0.595	0.909	1.398	1.278	1.371	1.322	1.351
Reading 2	1.32	0.658	0.544	0.955	1.142	1.233	1.24	1.317	1.289
Reading 3	1.208	0.571	0.532	0.836	1.275	1.204	1.276	1.286	1.272
Reading 4	1.237	0.582	0.545	0.911	1.241	1.162	1.185	1.115	1.037
Reading 5	1.226	0.489	0.52	0.688	1.232	1.282	1.039	1.142	1.178
Reading 6	1.175	0.55	0.542	0.845	1.146	1.119	1.087	1.4	1.062
Reading 7	1.44	0.539	0.613	1.039	1.188	0.693	1.456	1.33	1.578
Reading 8	1.367	0.602	0.535	1.033	1.403	1.14	1.329	1.287	1.533
Reading 9	1.259	0.582	0.558	0.69	1.341	1.205	1.376	1.399	1.308
Average	1.299778	0.582333	0.553778	0.878444	1.262889	1.146222	1.262111	1.288667	1.289778
Stdev	0.104439	0.05614	0.030626	0.128559	0.099657	0.179082	0.138729	0.09992	0.185371
st error	0.034813	0.018713	0.010209	0.042853	0.033219	0.059694	0.046243	0.033307	0.06179
	RPMI	PMA10	PMA100	1.6% DMS	2%	4%	6%	8%	10%
Normalise	1	0.448025	0.426056	0.675842	0.971619	0.88186	0.971021	0.991452	0.992306
Stdev	0.104439	0.05614	0.030626	0.128559	0.099657	0.179082	0.138729	0.09992	0.185371
st error	0.034813	0.018713	0.010209	0.042853	0.033219	0.059694	0.046243	0.033307	0.06179

NBT/MTT 72 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.104366	2.729042	3.32437	2.155116	1.574392	1.596244	1.749818	1.268533	1.378979
Reading 2	1.686364	2.354103	3.757353	1.881675	1.805604	1.181671	1.542742	1.688686	1.471683
Reading 3	1.673013	3.30648	3.458647	2.338517	1.604706	1.625415	1.475705	1.572317	1.587264
Reading 4	1.627324	3.180412	3.341284	2.175631	1.72361	1.554217	1.686076	1.774888	1.648987
Reading 5	2.344209	6.785276	3.842308	3	2.004058	1.592044	1.513956	2.027145	1.702886
Reading 6	0.966809	3.263636	3.297048	2.315976	1.5	1.80429	1.689052	1.171429	1.606403
Reading 7	1.040278	2.384045	2.357259	1.241578	2.078283	2.479076	0.765797	0.992481	1.1109
Reading 8	1.956108	2.232558	2.91028	1.445305	1.394868	1.409649	0.991723	1.219891	0.812785
Reading 9	1.359015	2.542955	3.344086	2.872464	1.693512	1.478008	1.351744	1.321658	1.742355
Average	1.528609	3.197612	3.292515	2.158474	1.708782	1.635624	1.418513	1.448559	1.45136
Stdev	0.456382	1.407156	0.4434	0.581098	0.224881	0.35917	0.334478	0.33538	0.307988
st error	0.152127	0.469052	0.1478	0.193699	0.07496	0.119723	0.111493	0.111793	0.102663
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	2.091844	2.153928	1.41205	1.117867	1.070008	0.927976	0.947632	0.949464
Stdev	0.53957	1.407156	0.4434	0.937123	0.224881	0.35917	0.334478	0.33538	0.307988
st error	0.179857	0.469052	0.1478	0.312374	0.07496	0.119723	0.111493	0.111793	0.102663

NBT 120 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.108	1.061	0.92	1.163	1.104	1.304	1.319	1.22	1.233
Reading 2	0.862	0.795	0.852	1.163	1.014	1.149	1.128	1.217	1.246
Reading 3	1.95	0.832	1.069	1.296	1.55	1.142	1.049	0.914	1.138
Reading 4	0.843	0.953	0.898	1.045	1.102	0.968	0.948	0.907	1.004
Reading 5	1.022	0.839	0.918	1.061	1.144	1.276	0.964	0.886	0.659
Reading 6	0.817	0.81	0.986	0.885	1.026	1.149	0.773	0.858	0.905
Reading 7	1.114	1.07	1.103	0.925	1.116	1.168	1.115	1.137	0.814
Reading 8	0.837	0.913	1.003	0.475	1.076	0.939	1.202	1.146	1.233
Reading 9	1.042	0.584	0.925	1.059	0.891	0.883	1.126	1.04	1.028
Average	1.066111	0.873	0.963778	1.008	1.113667	1.108667	1.069333	1.036111	1.028889
Stdev	0.35246	0.1494	0.082783	0.235553	0.180333	0.147284	0.159184	0.147751	0.206558
st error	0.117487	0.0498	0.027594	0.078518	0.060111	0.049095	0.053061	0.04925	0.068853
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	0.539894	0.4421	0.488071	0.510466	0.563977	0.561445	0.541526	0.524702	0.521044
Stdev	0.35246	0.1494	0.082783	0.235553	0.180333	0.147284	0.159184	0.147751	0.206558
st error	0.117487	0.0498	0.027594	0.078518	0.060111	0.049095	0.053061	0.04925	0.068853

MTT 120 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.535	0.463	0.262	1.229	1.339	1.112	1.131	1.244	1.324
Reading 2	1.281	0.34	0.346	1.143	1.316	1.167	0.971	1.182	1.093
Reading 3	1.056	0.376	0.324	1.042	1.229	1.006	0.931	1.17	1.101
Reading 4	1.232	0.391	0.298	1.048	1.206	1.178	1.074	1.203	1.082
Reading 5	1.369	0.315	0.281	0.856	1.063	1.157	1.133	1.212	1.188
Reading 6	1.377	0.306	0.31	0.955	1.223	1.232	1.251	1.146	1.196
Reading 7	1.464	0.561	0.42	1.442	1.581	1.585	1.394	1.424	1.532
Reading 8	1.429	0.568	0.433	1.399	1.401	1.574	1.189	1.46	1.612
Reading 9	1.457	0.59	0.505	1.487	1.439	1.591	1.581	1.361	1.542
Average	1.355556	0.434444	0.353222	1.177889	1.310778	1.289111	1.183889	1.266889	1.296667
Stdev	0.146135	0.113938	0.081564	0.225459	0.152147	0.228949	0.204056	0.117078	0.213173
st error	0.048712	0.037979	0.027188	0.075153	0.050716	0.076316	0.068019	0.039026	0.071058
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.042913	0.334245	0.271756	0.906223	1.008463	0.991793	0.910839	0.974697	0.997606
Stdev	0.146135	0.113938	0.081564	0.225459	0.152147	0.228949	0.204056	0.117078	0.213173
st error	0.048712	0.037979	0.027188	0.075153	0.050716	0.076316	0.068019	0.039026	0.071058

NBT/MTT 120 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.721824	2.291577	3.51145	0.946298	0.824496	1.172662	1.166225	0.980707	0.931269
Reading 2	0.672912	2.338235	2.462428	1.017498	0.770517	0.984576	1.161689	1.029611	1.139982
Reading 3	1.846591	2.212766	3.299383	1.243762	1.261188	1.135189	1.126745	0.781197	1.033606
Reading 4	0.684253	2.43734	3.013423	0.997137	0.913765	0.821732	0.882682	0.753948	0.927911
Reading 5	0.74653	2.663492	3.266904	1.239486	1.076199	1.102852	0.850838	0.731023	0.554714
Reading 6	0.593319	2.647059	3.180645	0.926702	0.838921	0.93263	0.617906	0.748691	0.756689
Reading 7	0.760929	1.907308	2.62619	0.64147	0.705882	0.736909	0.799857	0.798455	0.531332
Reading 8	0.585724	1.607394	2.316397	0.339528	0.768023	0.596569	1.010934	0.784932	0.764888
Reading 9	0.715168	0.989831	1.831683	0.712172	0.61918	0.554997	0.712207	0.764144	0.666667
Average	0.814139	2.121667	2.834278	0.896006	0.864241	0.893124	0.925454	0.81919	0.811895
Stdev	0.392018	0.541388	0.55555	0.290608	0.196958	0.229788	0.201505	0.108075	0.210865
st error	0.130673	0.180463	0.185183	0.096869	0.065653	0.076596	0.067168	0.036025	0.070288
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.532601	1.387972	1.854155	0.586158	0.565377	0.584272	0.605422	0.535905	0.531133
Stdev	0.392018	0.541388	0.55555	0.290608	0.196958	0.229788	0.201505	0.108075	0.210865
st error	0.130673	0.180463	0.185183	0.096869	0.065653	0.076596	0.067168	0.036025	0.070288

NBT 72 hours K562 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.476	2.903	1.605	3.987	1.764	1.506	1.874	2.277	2.856
Reading 2	2.023	2.652	1.632	1.522	2.16	4.061	2.667	1.93	1.669
Reading 3	1.498	2.933	1.368	1.49	1.6	3.928	1.597	3.96	1.568
Reading 4	2.599	1.434	1.253	1.195	1.94	4.13	4.169	1.945	3.402
Reading 5	1.809	1.35	1.176	1.476	2.335	3.435	3.771	1.781	3.805
Reading 6	3.582	2.002	2.322	1.399	4.132	2.851	2.062	3.926	2.597
Reading 7	1.357	1.416	1.565	2	1.825	1.776	1.898	1.68	3.26
Reading 8	1.568	1.402	1.451	2.121	2.237	2.018	2.023	1.751	1.704
Reading 9	1.727	1.538	1.482	1.607	2.018	1.636	1.784	1.869	1.646
Average	1.959889	1.958889	1.539333	1.866333	2.223444	2.815667	2.427222	2.346556	2.500778
Stdev	0.714601	0.684722	0.331769	0.84623	0.753352	1.102523	0.92756	0.920893	0.876961
st error	0.2382	0.228241	0.11059	0.282077	0.251117	0.367508	0.309187	0.306964	0.29232
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	0.99949	0.785419	0.952265	1.134475	1.436646	1.238449	1.19729	1.275979
Stdev	0.714601	0.684722	0.331769	0.84623	0.753352	1.102523	0.92756	0.920893	0.876961
st error	0.2382	0.228241	0.11059	0.282077	0.251117	0.367508	0.309187	0.306964	0.29232

MTT 72 hours K562 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMSC	2%	4%	6%	8%	10%
Reading 1	1.239	0.605	0.602	0.892	1.265	1.204	1.246	1.182	0.994
Reading 2	1.219	0.67	0.563	0.925	1.421	1.523	1.351	1.499	1.194
Reading 3	1.302	0.671	0.629	0.972	1.508	1.364	1.514	1.493	1.26
Reading 4	1.266	0.686	0.641	1.013	1.513	1.532	1.425	1.283	1.32
Reading 5	1.204	0.697	0.658	1.069	1.489	1.578	1.425	1.311	0.641
Reading 6	1.211	0.679	0.612	0.914	1.282	1.372	1.408	1.275	1.21
Reading 7	1.253	0.646	0.622	0.953	1.389	1.368	1.336	1.233	1.157
Reading 8	1.212	0.684	0.611	0.997	1.455	1.551	1.388	1.405	0.918
Reading 9	1.257	0.675	0.621	0.943	1.395	1.368	1.461	1.384	1.235
Average	1.240167	0.668	0.6175	0.964167	1.413	1.428833	1.394833	1.3405	1.103167
Stdev	0.032287	0.027531	0.026551	0.055178	0.091237	0.123465	0.077631	0.111627	0.215506
st error	0.010762	0.009177	0.00885	0.018393	0.030412	0.041155	0.025877	0.037209	0.071835
	RPMI	PMA10	PMA100	1.6% DMSC	2%	4%	6%	8%	10%
Normalise	1	0.538637	0.497917	0.777449	1.139363	1.15213	1.124714	1.080903	0.889531
Stdev	0.032287	0.027531	0.026551	0.055178	0.091237	0.123465	0.077631	0.111627	0.215506
st error	0.010762	0.009177	0.00885	0.018393	0.030412	0.041155	0.025877	0.037209	0.071835

NBT/MTT 72 hours K562 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.191283	4.798347	2.666113	4.469731	1.394466	1.250831	1.504013	1.926396	2.873239
Reading 2	1.659557	3.958209	2.898757	1.645405	1.520056	2.666448	1.974093	1.287525	1.397822
Reading 3	1.150538	4.371088	2.174881	1.532922	1.061008	2.879765	1.054822	2.652378	1.244444
Reading 4	2.052923	2.090379	1.954758	1.179664	1.282221	2.695822	2.925614	1.515978	2.577273
Reading 5	1.502492	1.936872	1.787234	1.38073	1.568167	2.176806	2.646316	1.358505	5.936037
Reading 6	2.957886	2.948454	3.794118	1.530635	3.223089	2.077988	1.464489	3.079216	2.146281
Reading 7	1.083433	2.193648	2.518101	2.099738	1.313895	1.298246	1.421191	1.363083	2.817632
Reading 8	1.294263	2.051207	2.37674	2.127382	1.537457	1.301516	1.457493	1.246263	1.857221
Reading 9	1.374453	2.278519	2.388396	1.704136	1.446595	1.195906	1.221081	1.350434	1.332794
Average	1.585203	2.958525	2.506566	1.963371	1.594106	1.949259	1.741012	1.753309	2.464749
Stdev	0.595854	1.120707	0.591603	0.988803	0.630927	0.698627	0.64553	0.670554	1.446254
st error	0.198618	0.373569	0.197201	0.329601	0.210309	0.232876	0.215177	0.223518	0.482085
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	1.866338	1.581227	1.238561	1.005616	1.229659	1.09829	1.106047	1.554848
Stdev	0.595854	1.120707	0.591603	0.988803	0.630927	0.698627	0.64553	0.670554	1.446254
st error	0.198618	0.373569	0.197201	0.329601	0.210309	0.232876	0.215177	0.223518	0.482085

NBT 120 hours K562 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.208	1.013	1.392	1.403	1.822	1.535	4.227	1.734	3.97
Reading 2	1.424	1.335	1.398	3.602	1.347	4.04	3.632	3.549	1.714
Reading 3	2.824	2.03	1.423	1.709	1.277	1.455	1.35	1.579	1.657
Reading 4	1.287	1.314	1.401	2.248	1.335	1.601	1.58	1.497	1.599
Reading 5	3.018	1.363	1.483	1.284	1.323	1.269	1.461	1.457	1.666
Reading 6	1.403	3.827	1.427	2.391	3.966	1.652	1.425	1.351	1.534
Reading 7	1.153	1.127	1.371	2.851	1.18	1.108	1.011	1.184	1.324
Reading 8	3.189	1.218	1.366	1.851	1.045	0.852	1.256	1.205	1.323
Reading 9	1.142	1.162	0.966	1.177	0.905	1.029	1.265	1.153	1.237
Average	1.960889	1.598778	1.358556	2.057333	1.577778	1.615667	1.911889	1.634333	1.780444
Stdev	0.852104	0.884496	0.15129	0.800678	0.930426	0.949771	1.164314	0.744004	0.839158
st error	0.284035	0.294832	0.05043	0.266893	0.310142	0.31659	0.388105	0.248001	0.279719
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.00051	0.815749	0.69318	1.049719	0.805034	0.824366	0.975509	0.833891	0.908442
Stdev	0.852104	0.884496	0.15129	0.800678	0.930426	0.949771	1.164314	0.744004	0.839158
st error	0.284035	0.294832	0.05043	0.266893	0.310142	0.31659	0.388105	0.248001	0.279719

MTT 120 hours K562 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.815	0.301	0.329	0.906	0.662	0.825	0.938	0.859	0.253
Reading 2	0.917	0.387	0.353	1.303	1.024	1.121	1.091	1.136	0.944
Reading 3	0.909	0.429	0.427	1.368	1.257	1.262	1.451	1.159	0.895
Reading 4	0.942	0.403	0.385	1.29	1.209	1.475	1.333	1.124	0.923
Reading 5	0.855	0.397	0.421	1.351	1.16	1.323	1.324	1.166	0.89
Reading 6	0.668	0.378	0.36	1.173	0.921	0.991	1.014	0.91	0.825
Reading 7	0.794	0.38	0.398	0.996	0.811	1.078	1.006	0.918	0.81
Reading 8	0.845	0.466	0.456	1.17	0.975	1.172	1.122	0.875	0.811
Reading 9	0.997	0.45	0.425	1.092	0.897	1.037	1.048	0.898	0.796
Average	0.860222	0.399	0.394889	1.183222	0.990667	1.142667	1.147444	1.005	0.794111
Stdev	0.09659	0.048166	0.041495	0.161456	0.194474	0.192937	0.177888	0.135655	0.209985
st error	0.032197	0.016055	0.013832	0.053819	0.064825	0.064312	0.059296	0.045218	0.069995
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.693634	0.321731	0.318416	0.954083	0.798817	0.921382	0.925234	0.810375	0.640326
Stdev	0.09659	0.048166	0.041495	0.161456	0.194474	0.192937	0.177888	0.135655	0.209985
st error	0.032197	0.016055	0.013832	0.053819	0.064825	0.064312	0.059296	0.045218	0.069995

NBT/MTT 120 hours K562 Day 9 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.709202	3.365449	4.231003	1.548565	2.752266	1.860606	4.506397	2.018626	15.6917
Reading 2	1.55289	3.449612	3.96034	2.76439	1.31543	3.603925	3.329056	3.12412	1.815678
Reading 3	3.106711	4.731935	3.332553	1.249269	1.015911	1.152932	0.930393	1.362381	1.851397
Reading 4	1.366242	3.260546	3.638961	1.742636	1.104218	1.085424	1.185296	1.331851	1.732394
Reading 5	3.529825	3.433249	3.522565	0.950407	1.140517	0.959184	1.103474	1.249571	1.87191
Reading 6	2.100299	10.12434	3.963889	2.038363	4.306189	1.667003	1.405325	1.484615	1.859394
Reading 7	1.452141	2.965789	3.444724	2.86245	1.454994	1.027829	1.00497	1.28976	1.634568
Reading 8	3.773964	2.613734	2.995614	1.582051	1.071795	0.726962	1.11943	1.377143	1.631319
Reading 9	1.145436	2.582222	2.272941	1.077839	1.008919	0.992285	1.207061	1.283964	1.55402
Average	2.304079	4.058542	3.484732	1.75733	1.685582	1.452906	1.7546	1.613559	3.293598
Stdev	1.001559	2.36081	0.588469	0.685558	1.124373	0.882297	1.268161	0.613067	4.65073
st error	0.333853	0.786937	0.196156	0.228519	0.374791	0.294099	0.42272	0.204356	1.550243
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.453491	2.560266	2.198288	1.108584	1.063322	0.916542	1.106862	1.017888	2.077713
Stdev	1.001559	3.85796	0.588469	1.051945	1.124373	0.882297	1.268161	0.613067	4.65073
st error	0.333853	1.285987	0.196156	0.350648	0.374791	0.294099	0.42272	0.204356	1.550243

NBT 72 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.378	2.122	2.047	4.226	2.472	1.763	3.854	1.902	2.869
Reading 2	2.695	2.158	2.116	1.813	1.986	1.604	2.002	2.315	1.868
Reading 3	2.058	1.648	2.832	2.671	2.192	2.54	3.901	1.996	2.143
Reading 4	1.868	2.057	2.172	4.063	2.167	2.038	2.419	1.683	1.695
Reading 5	1.997	4.11	3.521	4.177	1.737	1.409	1.642	1.662	2.305
Reading 6	1.874	1.359	2.016	4.275	1.257	1.52	2.018	1.538	1.643
Reading 7	1.564	1.986	2.7	1.742	1.926	1.813	1.914	3.019	1.73
Reading 8	1.834	2.283	2.496	2.138	2.02	1.629	1.811	1.952	2.103
Reading 9	1.842	1.908	1.786	1.712	3.508	1.956	3.782	2.142	1.909
Average	1.901111	2.181222	2.409556	2.979667	2.140556	1.808	2.593667	2.023222	2.029444
Stdev	0.364209	0.776463	0.538906	1.18015	0.613503	0.340676	0.961889	0.446234	0.385766
st error	0.121403	0.258821	0.179635	0.393383	0.204501	0.113559	0.32063	0.148745	0.128589
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	1.147341	1.267446	1.567329	1.12595	0.951023	1.36429	1.064231	1.067504
Stdev	1.0385	1.008825	1.106159	1.18015	1.055617	0.821891	1.070024	0.763517	1.153706
st error	0.346167	0.336275	0.36872	0.393383	0.351872	0.273964	0.356675	0.254506	0.384569

MTT 72 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.182	0.498	0.451	0.766	1.169	0.948	1.089	1.125	1.114
Reading 2	1.18	0.572	0.574	0.926	1.218	1.217	1.27	1.177	1.233
Reading 3	1.1	0.619	0.559	0.794	1.319	1.24	1.526	1.351	1.325
Reading 4	1.184	0.633	0.545	0.832	1.41	1.268	1.354	1.396	1.208
Reading 5	1.107	0.579	0.518	0.911	1.268	1.236	1.383	1.302	1.319
Reading 6	1.051	0.567	0.486	0.687	1.092	1.14	1.114	1.143	1.091
Reading 7	1.284	0.729	0.655	0.938	1.465	1.349	1.432	1.458	1.525
Reading 8	1.295	0.551	0.665	1.086	1.562	1.413	1.454	1.429	1.514
Reading 9	1.307	0.748	0.683	0.956	1.321	1.308	1.433	1.371	1.141
Average	1.187778	0.610667	0.570667	0.877333	1.313778	1.235444	1.339444	1.305778	1.274444
Stdev	0.092206	0.082227	0.081928	0.119653	0.148002	0.133623	0.152366	0.126772	0.161261
st error	0.030735	0.027409	0.027309	0.039884	0.049334	0.044541	0.050789	0.042257	0.053754
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.514125	0.480449	0.738634	1.10608	1.040131	1.127689	1.099345	1.072965
Stdev	0.092206	0.082227	0.081928	0.119653	0.148002	0.133623	0.152366	0.126772	0.161261
st error	0.030735	0.027409	0.027309	0.039884	0.049334	0.044541	0.050789	0.042257	0.053754

NBT/MTT 72 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.165821	4.261044	4.538803	5.516971	2.114628	1.859705	3.539027	1.690667	2.575404
Reading 2	2.283898	3.772727	3.686411	1.957883	1.630542	1.317995	1.576378	1.966865	1.515004
Reading 3	1.870909	2.662359	5.06619	3.36398	1.661865	2.048387	2.556356	1.477424	1.617358
Reading 4	1.577703	3.249605	3.985321	4.883413	1.536879	1.607256	1.786558	1.205587	1.403146
Reading 5	1.803975	7.098446	6.797297	4.585071	1.369874	1.139968	1.187274	1.276498	1.747536
Reading 6	1.783064	2.396825	4.148148	6.222707	1.151099	1.333333	1.81149	1.345582	1.505958
Reading 7	1.218069	2.72428	4.122137	1.857143	1.314676	1.343958	1.336592	2.070645	1.134426
Reading 8	1.416216	4.143376	3.753383	1.968692	1.293214	1.152866	1.24553	1.36599	1.389036
Reading 9	1.409334	2.550802	2.614934	1.790795	2.655564	1.495413	2.639218	1.562363	1.673094
Average	1.614332	3.651052	4.301403	3.571851	1.636482	1.477653	1.964269	1.551291	1.617885
Stdev	0.356964	1.470332	1.148314	1.763801	0.475108	0.31075	0.791016	0.303955	0.401875
st error	0.118988	0.490111	0.382771	0.587934	0.158369	0.103583	0.263672	0.101318	0.133958
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	2.261648	2.664509	2.212587	1.013721	0.915334	1.216769	0.960949	1.002201
Stdev	1.044434	2.136915	2.462692	1.763801	0.661701	0.651227	0.99445	0.710985	0.940459
st error	0.348145	0.712305	0.820897	0.587934	0.220567	0.217076	0.331483	0.236995	0.313486

NBT 120 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.34	1.59	1.68	2.22	2.24	2.34	2.154	1.87	1.654
Reading 2	1.16	1.25	1.52	1.13	2.48	2.55	2.587	1.92	1.35
Reading 3	1.19	1.87	1.37	1.09	2.59	2.6	2.624	1.47	1.845
Reading 4	1.04	1.08	1.56	1.57	2.687	1.95	2.158	1.52	1.405
Reading 5	1.39	1.001	1.32	1.52	2.584	1.09	2.411	1.62	1.317
Reading 6	1.47	1.03	1.22	2.41	2.784	1.011	2.65	1.257	1.358
Reading 7	1.32	1.46	1.74	1.646	2.01	2.802	2.873	2.544	2.395
Reading 8	1.466	1.2	1.26	2.312	2.992	1.685	2.445	1.59	1.734
Reading 9	1.321	1.034	1.151	1.206	2.161	1.237	1.499	1.665	1.397
Average	1.299667	1.279444	1.424556	1.678222	2.503111	1.918333	2.377889	1.717333	1.606111
Stdev	0.144177	0.300989	0.209127	0.516966	0.315392	0.693847	0.403113	0.368817	0.352309
st error	0.048059	0.10033	0.069709	0.172322	0.105131	0.231282	0.134371	0.122939	0.117436
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	0.683635	0.672998	0.749328	0.882759	1.316657	1.009059	1.250789	0.903331	0.844828
Stdev	0.676919	0.606136	0.692305	1.164326	1.223871	1.31192	1.188886	1.00377	0.95525
st error	0.22564	0.202045	0.230768	0.388109	0.407957	0.437307	0.396295	0.33459	0.318417

MTT 120 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	0.662	0.243	0.242	0.808	0.856	0.689	0.781	0.777	0.693
Reading 2	0.742	0.299	0.291	0.847	0.888	0.925	0.879	0.689	0.666
Reading 3	0.656	0.302	0.284	0.981	0.964	0.878	1.002	0.919	0.941
Reading 4	0.85	0.341	0.295	0.957	1.154	0.945	0.93	1.027	0.918
Reading 5	0.804	0.324	0.312	0.98	0.91	1.087	1.148	0.983	0.927
Reading 6	0.697	0.287	0.253	0.724	0.95	0.832	0.83	0.853	0.845
Reading 7	0.419	0.228	0.25	0.623	0.854	0.544	1.32	0.651	0.697
Reading 8	0.49	0.278	0.263	0.696	0.954	0.581	1.35	0.741	0.855
Reading 9	0.622	0.254	0.244	0.614	0.844	0.592	0.469	0.535	0.557
Average	0.660222	0.284	0.270444	0.803333	0.930444	0.785889	0.967667	0.797222	0.788778
Stdev	0.138462	0.03735	0.02555	0.147691	0.095602	0.191513	0.277435	0.162279	0.138254
st error	0.046154	0.01245	0.008517	0.04923	0.031867	0.063838	0.092478	0.054093	0.046085
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.555847	0.239102	0.227689	0.676333	0.783349	0.661646	0.814687	0.671188	0.664079
Stdev	0.138462	0.03735	0.02555	0.147691	0.095602	0.191513	0.277435	0.162279	0.138254
st error	0.046154	0.01245	0.008517	0.04923	0.031867	0.063838	0.092478	0.054093	0.046085

NBT/MTT 120 hours K562 Day 12 Ester									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.024169	6.54321	6.942149	2.747525	2.616822	3.396226	2.758003	2.406692	2.386724
Reading 2	1.563342	4.180602	5.223368	1.33412	2.792793	2.756757	2.943117	2.786647	2.027027
Reading 3	1.814024	6.192053	4.823944	1.111111	2.686722	2.961276	2.618762	1.599565	1.96068
Reading 4	1.223529	3.167155	5.288136	1.640543	2.328423	2.063492	2.32043	1.480039	1.530501
Reading 5	1.728856	3.089506	4.230769	1.55102	2.83956	1.00276	2.100174	1.648016	1.420712
Reading 6	2.109039	3.58885	4.822134	3.328729	2.930526	1.215144	3.192771	1.473623	1.607101
Reading 7	3.150358	6.403509	6.96	2.642055	2.35363	5.150735	2.176515	3.907834	3.436155
Reading 8	2.991837	4.316547	4.790875	3.321839	3.136268	2.900172	1.811111	2.145749	2.02807
Reading 9	2.123794	4.070866	4.717213	1.964169	2.560427	2.089527	3.196162	3.11215	2.508079
Average	2.080994	4.616922	5.310954	2.182346	2.693908	2.615121	2.568561	2.284479	2.100561
Stdev	0.630229	1.389876	0.978154	0.847802	0.263591	1.24503	0.496312	0.850403	0.621758
st error	0.210076	0.463292	0.326051	0.282601	0.087864	0.41501	0.165437	0.283468	0.207253
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.799083	1.772857	2.039359	0.838001	1.034437	1.004183	0.986305	0.87722	0.806597
Stdev	0.630229	1.389876	0.978154	0.847802	0.263591	1.24503	0.496312	0.850403	0.621758
st error	0.210076	0.463292	0.326051	0.282601	0.087864	0.41501	0.165437	0.283468	0.207253

NBT 72 hours K562 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.966	2.01	2.026	1.878	1.06	1.51	1.729	1.55	1.852
Reading 2	2.009	2.199	1.899	2.082	1.55	1.753	1.895	2.072	1.659
Reading 3	2.897	2.205	2.222	2.094	1.47	1.817	1.895	2.037	1.531
Reading 4	1.856	1.928	1.734	1.815	1.886	1.658	1.724	2.656	1.734
Reading 5	1.925	1.938	2.083	1.726	2.029	1.667	1.496	1.719	1.497
Reading 6	1.957	1.752	1.757	1.594	2.017	1.768	1.788	1.731	1.414
Reading 7	1.9875	2.1045	1.9625	1.98	1.684	1.6315	1.812	1.811	1.7555
Reading 8	2.3765	2.0665	1.978	1.3775	1.9195	1.7375	1.8095	2.3465	1.6325
Reading 9	1.941	1.845	1.92	1.66	2.023	1.7175	1.642	1.725	1.4555
Average	2.101667	2.005333	1.9535	1.800722	1.737611	1.6955	1.7545	1.960833	1.6145
Stdev	0.33287	0.154665	0.15235	0.237351	0.329462	0.091034	0.126488	0.35502	0.149638
st error	0.110957	0.051555	0.050783	0.079117	0.109821	0.030345	0.042163	0.11834	0.049879
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1	0.954163	0.9295	0.856807	0.826778	0.806741	0.834814	0.93299	0.7682
Stdev	0.33287	0.154665	0.15235	0.237351	0.329462	0.091034	0.126488	0.35502	0.149638
st error	0.110957	0.051555	0.050783	0.079117	0.109821	0.030345	0.042163	0.11834	0.049879

MTT 72 hours K562 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.129	0.245	0.241	0.525	1.5	1.42	0.988	0.785	0.646
Reading 2	1.1	0.235	0.235	0.604	1.45	1.53	0.952	0.776	0.666
Reading 3	1.099	0.241	0.254	0.586	1.423	1.029	1.046	0.819	0.729
Reading 4	1.106	0.254	0.258	0.584	1.031	1.021	0.997	0.934	0.693
Reading 5	0.814	0.262	0.246	0.53	1.088	1.111	1.044	0.847	0.741
Reading 6	0.99	0.262	0.244	0.581	1.032	0.999	1.067	0.821	0.781
Reading 7	1.1145	0.24	0.238	0.5645	1.475	1.475	0.97	0.7805	0.656
Reading 8	1.1025	0.2475	0.256	0.585	1.227	1.025	1.0215	0.8765	0.711
Reading 9	0.902	0.262	0.245	0.5555	1.06	1.055	1.0555	0.834	0.761
Average	1.039667	0.249833	0.246333	0.568333	1.254	1.185	1.015667	0.830333	0.709333
Stdev	0.112602	0.010518	0.008078	0.026913	0.206539	0.221403	0.040669	0.051083	0.047694
st error	0.037534	0.003506	0.002693	0.008971	0.068846	0.073801	0.013556	0.017028	0.015898
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	0.240301	0.236935	0.54665	1.206156	1.139788	0.976916	0.798653	0.68227
Stdev	0.112602	0.010518	0.008078	0.026913	0.524463	0.518673	0.040669	0.051083	0.047694
st error	0.037534	0.003506	0.002693	0.008971	0.174821	0.172891	0.013556	0.017028	0.015898

NBT/MTT 72 hours K562 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.741364	8.204082	8.406639	3.577143	0.706667	1.06338	1.75	1.974522	2.866873
Reading 2	1.826364	9.357447	8.080851	3.44702	1.068966	1.145752	1.990546	2.670103	2.490991
Reading 3	2.636033	9.149378	8.748031	3.573379	1.033029	1.765792	1.811663	2.487179	2.100137
Reading 4	1.678119	7.590551	6.72093	3.107877	1.829292	1.623898	1.729188	2.843683	2.502165
Reading 5	2.364865	7.396947	8.46748	3.256604	1.86489	1.50045	1.43295	2.029516	2.020243
Reading 6	1.976768	6.687023	7.20082	2.743546	1.954457	1.76977	1.675726	2.108404	1.810499
Reading 7	1.783311	8.76875	8.245798	3.507529	1.141695	1.106102	1.868041	2.320307	2.676067
Reading 8	2.155556	8.349495	7.726563	2.354701	1.564385	1.695122	1.771415	2.677125	2.296062
Reading 9	2.151885	7.041985	7.836735	2.988299	1.908491	1.627962	1.555661	2.068345	1.912615
Average	2.034918	8.060628	7.937094	3.1729	1.45243	1.477581	1.731688	2.353243	2.297295
Stdev	0.320566	0.939311	0.647197	0.419824	0.46909	0.291543	0.164802	0.326764	0.361932
st error	0.106855	0.313104	0.215732	0.139941	0.156363	0.097181	0.054934	0.108921	0.120644
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1	3.961156	3.900449	1.559227	0.713754	0.726113	0.850986	1.156431	1.128937
Stdev	0.320566	0.939311	0.647197	0.419824	0.46909	0.291543	0.164802	0.326764	0.361932
st error	0.106855	0.313104	0.215732	0.139941	0.156363	0.097181	0.054934	0.108921	0.120644

NBT 120 hours K562 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.351	2.19	1.934	2.168	1.47	2.541	1.799	2.008	1.769
Reading 2	1.838	2.088	2.025	2.173	1.51	2.023	2.545	3.95	1.879
Reading 3	2.49	2.019	2.206	2.004	1.61	1.843	2.569	2.414	2.075
Reading 4	2.288	2.193	2.241	2.733	2.577	1.85	1.825	2.081	2.047
Reading 5	1.977	2.104	2.088	2.38	1.983	2.611	1.955	2.244	2.157
Reading 6	1.834	2.73	2.419	2.191	1.292	2.029	2.753	2.17	2.08
Reading 7	1.998	1.593	2.735	2.58	1.594	2.561	2.781	2.011	1.966
Reading 8	2.038	2.006	2.515	2.165	2.168	2.032	2.061	2.183	2.216
Reading 9	2.319	1.827	1.994	2.315	1.608	2.462	1.961	2.086	2.278
Average	2.125889	2.083333	2.239667	2.301	1.756889	2.216889	2.249889	2.349667	2.051889
Stdev	0.240091	0.307873	0.268637	0.230158	0.406568	0.320223	0.405285	0.613348	0.161148
st error	0.08003	0.102624	0.089546	0.076719	0.135523	0.106741	0.135095	0.204449	0.053716
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalised	1.011525	0.991277	1.065662	1.094845	0.83595	1.054824	1.070526	1.118002	0.976315
Stdev	0.240091	0.307873	0.268637	0.230158	0.406568	0.320223	0.405285	0.613348	0.161148
st error	0.08003	0.102624	0.089546	0.076719	0.135523	0.106741	0.135095	0.204449	0.053716

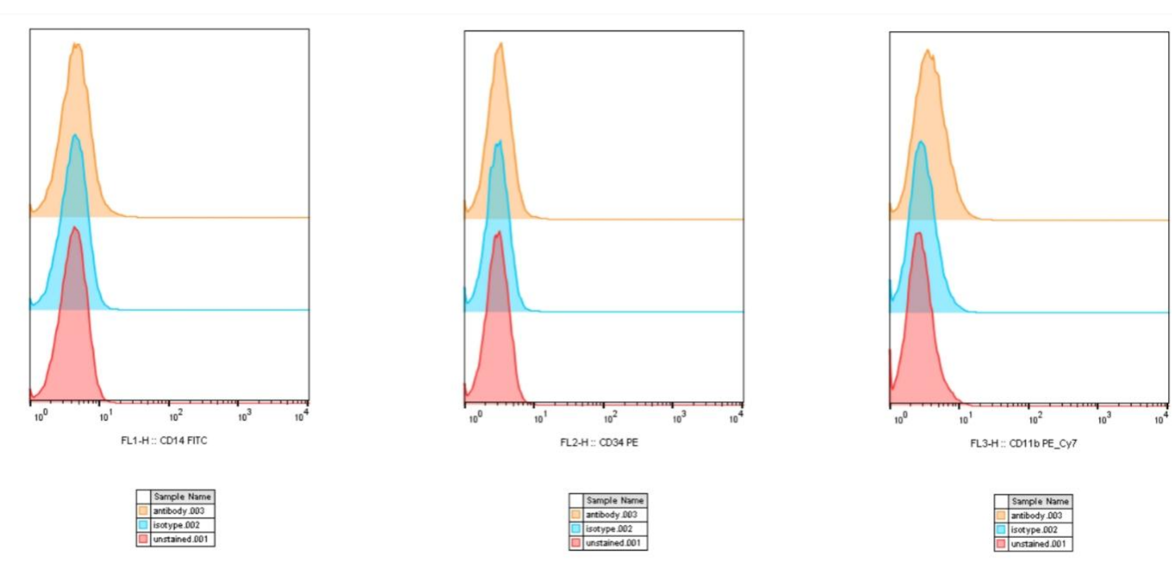
MTT 120 hours K562 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	1.05	0.205	0.198	0.847	1.398	0.845	0.837	0.454	0.534
Reading 2	1.036	0.226	0.186	0.89	1.451	0.903	0.886	0.753	0.457
Reading 3	1.066	0.247	0.198	0.994	1.526	0.919	0.809	0.74	0.504
Reading 4	1.016	0.23	0.224	1.158	1.041	0.986	0.881	0.77	0.499
Reading 5	1.031	0.228	0.228	1.226	1.083	0.867	0.871	0.781	0.466
Reading 6	1.067	0.25	0.218	1.17	1.091	0.898	0.891	0.818	0.507
Reading 7	1.126	0.271	0.239	0.469	1.462	1.205	0.883	0.894	0.476
Reading 8	0.317	0.334	0.26	0.531	1.045	1.024	0.944	0.716	0.481
Reading 9	1.123	0.244	0.261	0.532	1.004	1.034	0.751	0.778	0.469
Average	0.981333	0.248333	0.223556	0.868556	1.233444	0.964556	0.861444	0.744889	0.488111
Stdev	0.252046	0.037084	0.026777	0.297055	0.218045	0.112213	0.055651	0.120494	0.024629
st error	0.084015	0.012361	0.008926	0.099018	0.072682	0.037404	0.01855	0.040165	0.00821
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	0.943892	0.238859	0.215026	0.835417	1.186385	0.927755	0.828578	0.716469	0.469488
Stdev	0.252046	0.037084	0.026777	0.297055	0.218045	0.328046	0.055651	0.120494	0.024629
st error	0.084015	0.012361	0.008926	0.099018	0.072682	0.109349	0.01855	0.040165	0.00821

NBT/MTT 120 hours K562 Day 12 Ether									
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Reading 1	2.239048	10.68293	9.767677	2.559622	1.051502	3.007101	2.149343	4.422907	3.312734
Reading 2	1.774131	9.238938	10.8871	2.441573	1.040662	2.24031	2.87246	5.245684	4.111597
Reading 3	2.335835	8.174089	11.14141	2.016097	1.055046	2.005441	3.175525	3.262162	4.117063
Reading 4	2.251969	9.534783	10.00446	2.360104	2.475504	1.876268	2.07151	2.702597	4.102204
Reading 5	1.917556	9.22807	9.157895	1.941272	1.831025	3.011534	2.244546	2.873239	4.628755
Reading 6	1.718838	10.92	11.09633	1.87265	1.184235	2.259465	3.089787	2.652812	4.102564
Reading 7	1.774423	5.878229	11.44351	5.501066	1.090287	2.125311	3.14949	2.249441	4.130252
Reading 8	6.429022	6.005988	9.673077	4.077213	2.074641	1.984375	2.183263	3.048883	4.607069
Reading 9	2.065004	7.487705	7.639847	4.351504	1.601594	2.381044	2.611185	2.681234	4.857143
Average	2.500647	8.572303	10.09015	3.013456	1.489388	2.321205	2.616346	3.237662	4.21882
Stdev	1.491048	1.835284	1.21154	1.299797	0.533667	0.419888	0.464681	0.969571	0.447778
st error	0.497016	0.611761	0.403847	0.433266	0.177889	0.139963	0.154894	0.32319	0.149259
	RPMI	PMA10	PMA100	1.6% DMS(	2%	4%	6%	8%	10%
Normalise	1.228869	4.212603	4.958502	1.480873	0.731916	1.140687	1.285725	1.591053	2.073214
Stdev	1.491048	1.835284	1.21154	1.299797	0.533667	0.419888	0.464681	0.969571	0.447778
st error	0.497016	0.611761	0.403847	0.433266	0.177889	0.139963	0.154894	0.32319	0.149259

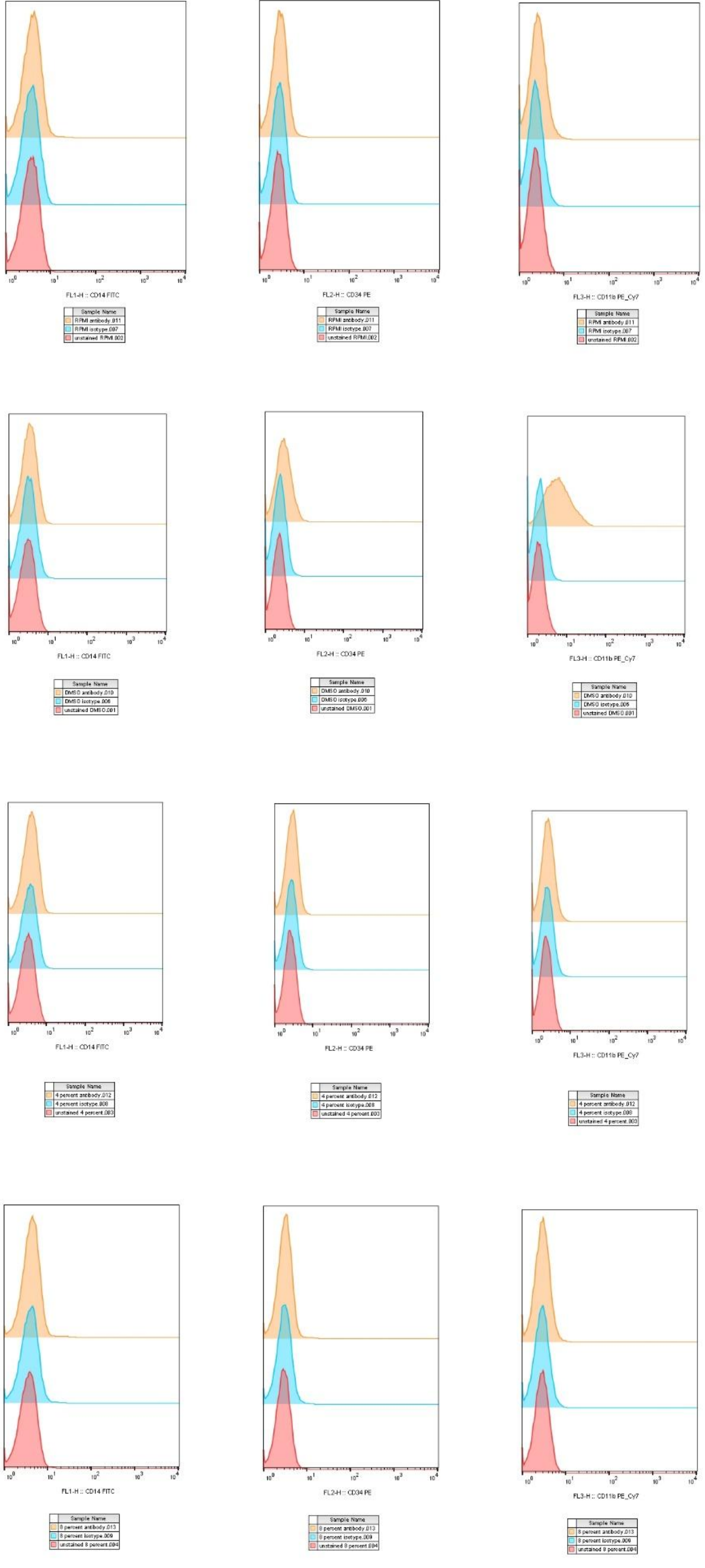
Appendix 6
Flow

Flow

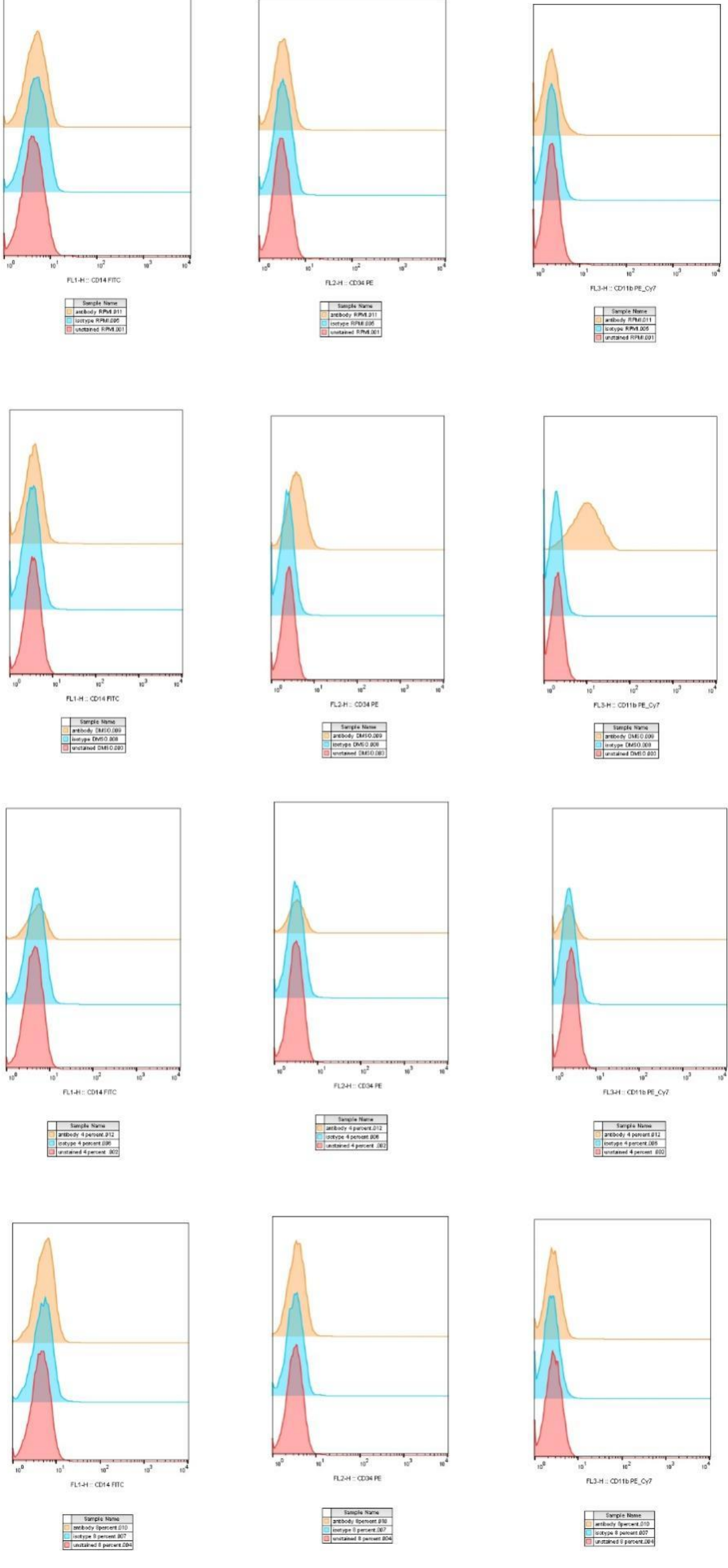
Day 0



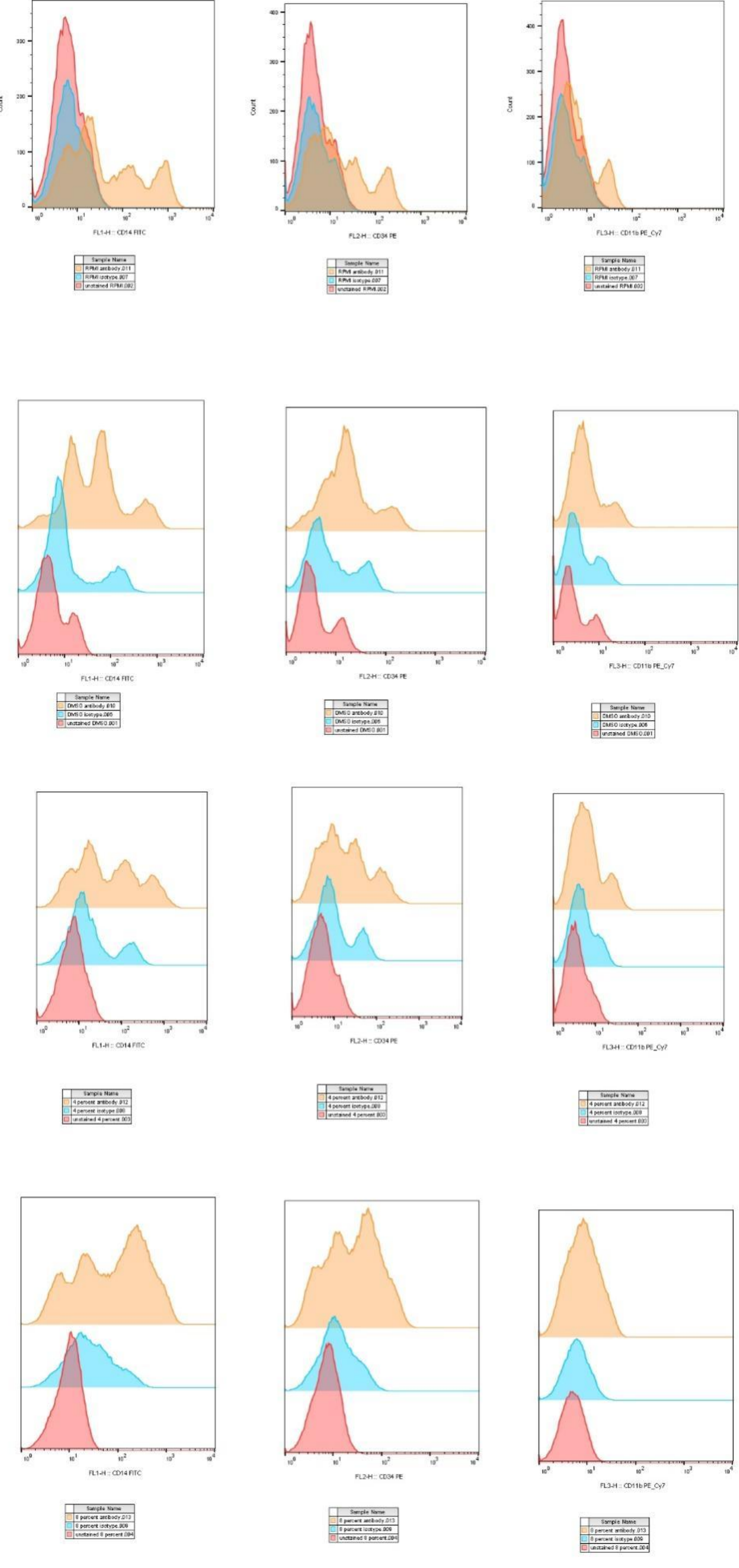
Population 1 – Day 3



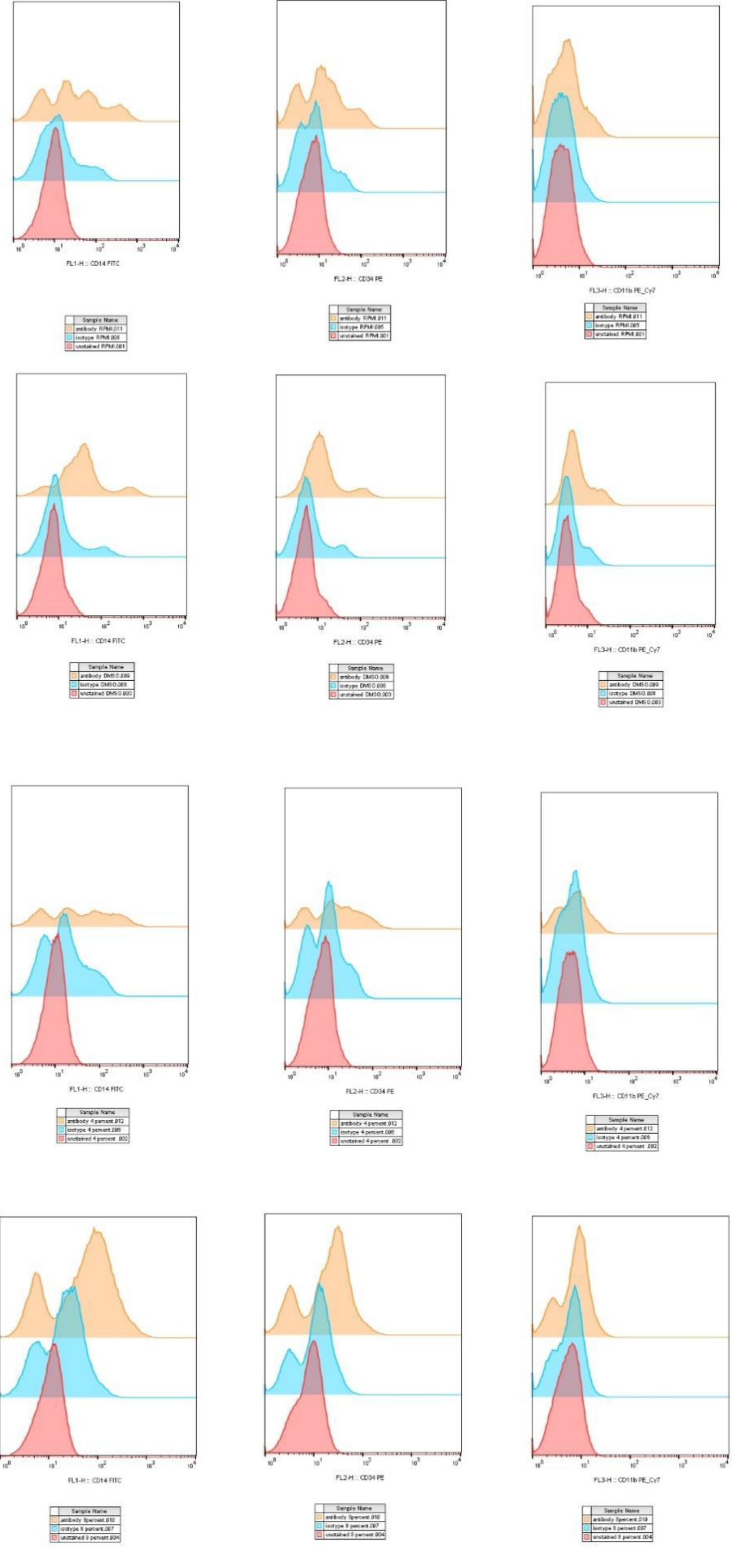
Population 1 – Day 5



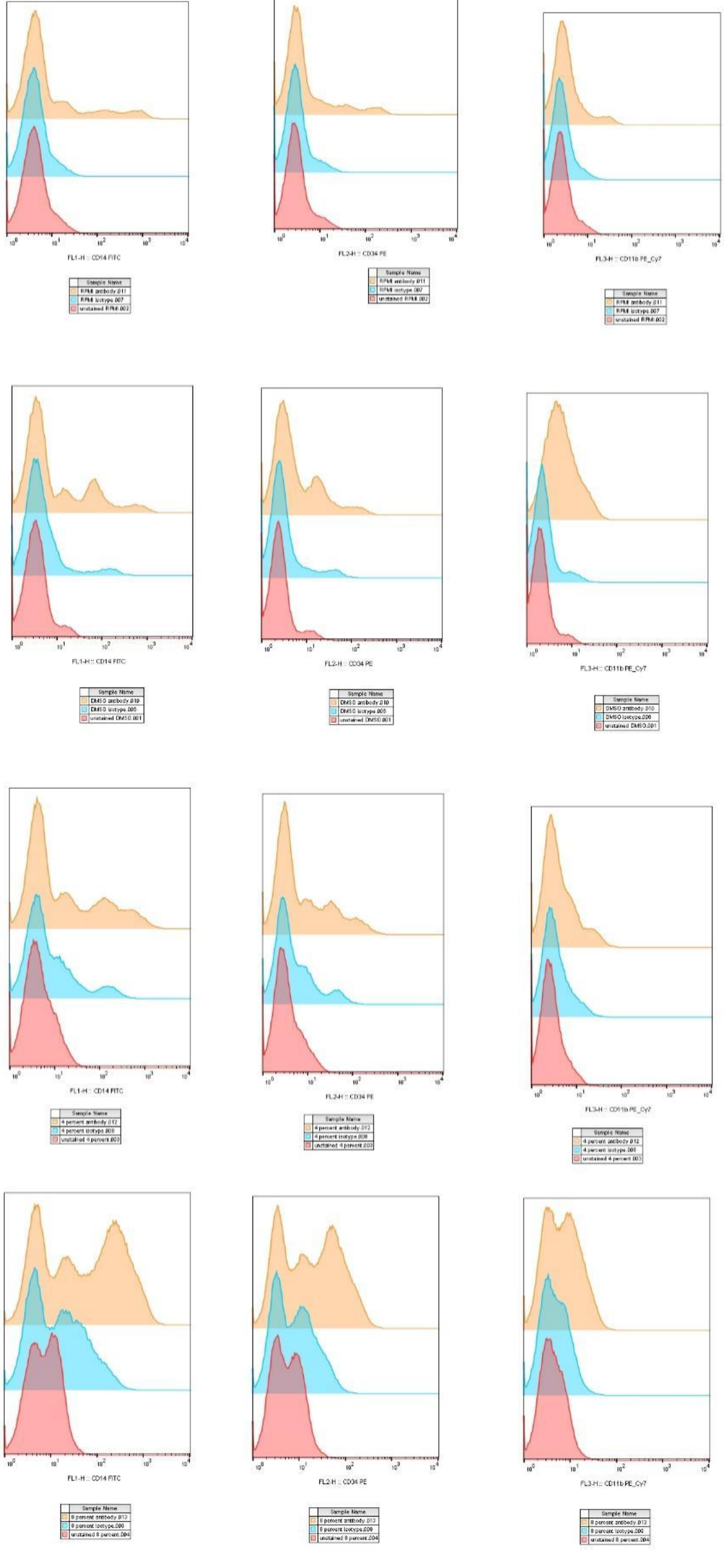
Population 2– Day 3



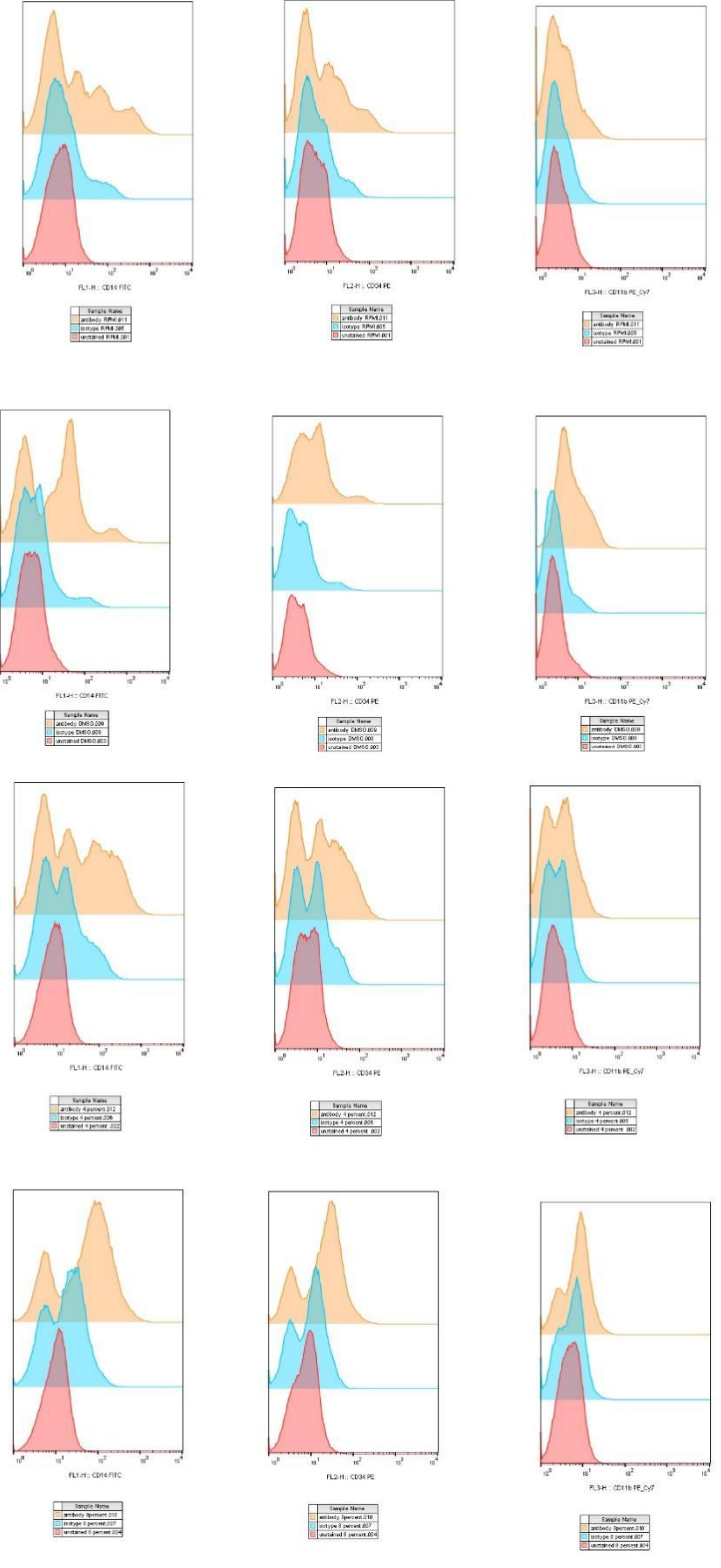
Population 2 – Day 5



Population 3 – Day 3



Population 3 – Day 5



Appendix 7
Letter from University of Palermo about the month of internship