An *in-vitro* assessment of the effects of *Arsenicum album* (30CH and 200CH) on leukocytes previously antagonised by arsenic trioxide.

By

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I, Elaine Ive, do hereby declare that this dissertation is representative of my own work, both in conception and execution.

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DEDICATION

I dedicate this work to my forever supportive and loving parents and family, for all their encouragement and patience through out this study, and for standing by me through out my life; and to my dearest Damian, whose motivation and comfort have guided me through the toughest of times, thank you.

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ABSTRACT

The therapeutic effects of homoeopathic *Arsenicum album* potencies were investigated *in-vitro*, using human cell cultures which were previously antagonised by arsenic trioxide (As_2O_3). Primary cell culture (peripheral blood mononuclear cells) and a continuous cell line (MT4) were treated with succussed and unsuccussed homoeopathic potencies, 6CH, 30CH and 200CH.

This study aimed to verify the homoeopathic law of similars and to determine whether potencies diluted beyond Avogadro's constant had physiological effects on cells; whether various potencies would cause different effects as proposed by the Arndt-Schultz law; whether succussed and unsuccussed homoeopathic potencies had different effects on the cells; and to establish whether a biotechnological method could be used to evaluate the above.

Initial experiments involved isolation and culturing of the peripheral blood mononuclear cells (PBMCs) and the MT4 cell line. Cell titres were determined using the trypan blue dye exclusion assay. The solubilization method of As_2O_3 was optimized through various dissolution experiments, so as to attain a homogenous arsenical solution.

The MTT assay was used to measure the percentage cytotoxicity and the half maximal inhibitory concentration (IC_{50}) caused by the antagonist As_2O_3 on the PBMCs and the MT4 cell line. The two cell cultures were compared with regard to their susceptibility to As_2O_3 and their reliability of response. The homoeopathic potencies of *Arsenicum album* (6CH, 30CH and 200CH) were prepared by initially triturating the As_2O_3, and then either hand succussing 10 times (succussed) or allowing to diffuse for 30 s (unsuccussed) in sterile distilled water, with the final potencies made up in cell culture media, RPMI. The MTT assay was used to determine the percentage cell viability when the As_2O_3-antagonised cells were treated with the *Arsenicum album* potencies. All assays were performed in triplicate.

The As_2O_3 was found to fully dissolve when 396 mg of dry As_2O_3 was added to 100 mL of sterile distilled Milli-Q water, which was left to stand for 10 days at 80°C. The cytotoxicity results showed that the PBMCs were not as reliable as the MT4 cells, which showed significant susceptibility to the As_2O_3. The IC_{50} of As_2O_3 on 1 mL of MT4 cells was found to be 5 µM As_2O_3 (133 µL) for 48 h. The trypan blue dye exclusion assay demonstrated that the viable MT4 cells decreased in number after exposure to the As_2O_3, with an increase in number of the non-viable cells. Microscopically, the cells were fewer in number and displayed signs of possible blebbing and cell shrinkage, showing potential cell death due to apoptosis.

The cell viability results showed that the *Arsenicum album* 6CH resulted in the lowest absorbance readings and the *Arsenicum album* 200CH gave the highest readings; this verified the therapeutic effects of homoeopathic remedies when given according to the law of similars; that potencies diluted beyond Avogadro's constant had stimulating effects; and that the more dilute potencies stimulated recovery in the cells more than the lower potencies, verifying the Arndt-Schultz law. The treatments and the times of exposure were found to be statistically significant determinants of cell viability, whereas succussion did not cause any significant variation in the results.

The study thereby provided evidence that a biotechnological method could be used to scientifically evaluate the physiological effects of homoeopathic potencies on human cells; that the homoeopathic potencies did have therapeutic effects; and that succussion was not required in the potentization method in order to produce a curative remedy.

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THE DEFINITION OF TERMS

Arndt-Schultz Law

This law states that weak stimuli enhance physiologic activity, medium stimuli inhibit physiologic activity, and strong stimuli destroy physiologic activity (Gaier, 1991).

Avogadro's Constant

Avogadro's constant is the number of atoms or molecules in one mole of any substance, and is defined as the number of atoms in 12 grams of carbon-12, equal to 6.0221415×10^{23} (Ebbing and Wentworth, 1995).

Dissolution

The process by which a solute breaks down when placed into a solvent, where ions leave the solute and enter into the solution, causing the solute to dissolve (Ebbing and Wentworth, 1995).

Homoeopathy

A medical art that brings about cure through administration of a medicine, minute in dose and given according to similarity (similia similibus), by lifting the disease without pain or debilitation (O' Reilly, 1996).

Law of Similars

This law forms the basic principle of homeopathy, "similia similibus curentur", or let "like cure like", meaning that any substance that can produce symptoms of disease in a healthy person, can cure those same symptoms in a sick person, when given in very small doses (De Schepper, 2001).

Potentization

In homoeopathy, potentization is the process of deconcentration of a medicinal substance, with succussion or trituration, thereby increasing its physical solubility and therapeutic activity, to produce a homoeopathic potency (Gaier, 1991).

Potency

The medicinal power of a homoeopathic medicine; developed by the method of potentization (Swayne, 2000).

Succussion

The action of shaking a liquid dilution of a homoeopathic medicine vigorously, where each stroke ends with a jolt, usually done by pounding the hand holding the phial of medicine against the other palm (Gaier, 1991).

Trituration

The first stage in the preparation of homoeopathic medicines; prepared by grinding the insoluble solid source material with lactose (Swayne, 2000).

LIST OF ABBREVIATIONS

% – percent

°C – Degree Celsius

µL – Microlitre

µM – Micromole

AFB_1 – Aflatoxin B_1

As_2O_3 – Arsenic trioxide

ATL – Adult T-cell leukemia

Ca^{2+} – calcium ion

$CaCl_2$ – Calcium chloride

CCM – Complete culture media

CH – Centesimal potency scale, dilution 1/100, prepared according to Hahnemannian method

CO_2 – Carbon dioxide

CV – Coefficient of variation

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

ELISA – Enzyme-linked immunosorbent assay

FCS – Fetal calf serum

g – Gram

GHP – German Homoeopathic Pharmacopoeia

h – Hour

HEK 293 – Human embryonic kidney cell line

$HepG_2$ – Human hepatoblastoma cells

HSA – Human serum albumin

HTLV-1 – Human T-cell leukemia virus type 1

IC_{50} – The half maximal inhibitory concentration

IgE – Immunoglobulin E antibody

IgG – Immunoglobulin G antibody

Kg – Kilogram

L – Litre

Mag. – Magnification

Mg^{2+} – Magnesium ion

mL – Millilitre

mM – Millimole

MT4 – ATCC human T-cell lymphotropic virus type I-transformed T-cell line

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaCl – Sodium chloride

NBTS – National Blood Transfusion Services

NF-$_K$B – Nuclear factor kappa-light-chain-enhancer of activated B cells

nm – Nanometre

NMR – Nuclear magnetic resonance

Nux v 30 – Nux vomica 30CH, succussed

Nux v 30u – Nux vomica 30CH, unsuccussed

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate buffered saline

RPMI – Roswell Park Memorial Institute medium

s – Second

SA – South Africa

SCID – Severe combined immunodeficiency

SDS – Sodium dodecyl sulphate

SY-5Y – Human neuroblastoma cell line

THF – Tetrahydrofuran

UV – Ultraviolet

X – Decimal potency scale, dilution 1/10, prepared according to Hahnemannian method

CHAPTER 1

INTRODUCTION

1.1 Introduction to the Study

This *in-vitro* study investigated the effects of homoeopathic *Arsenicum album* 6CH, 30CH and 200CH potencies, on human peripheral blood mononuclear cells (PBMCs) and ATCC human T-cell lymphotropic virus type I-transformed T-cell line (MT4 cell line), which were previously antagonised by the crude extract, arsenic trioxide (As_2O_3).

The 30CH and 200CH potencies chosen in this *in-vitro* study were regarded as high centesimal potencies, as they were diluted past Avogadro's constant, and thus had no particles of the original substance (As_2O_3) present. The therapeutic power of these homoeopathic potencies has been verified predominantly through clinical observation, and hence is regarded as a controversial matter in the scientific world. With the lack of human cell culture research in homoeopathy, it was therefore necessary to do further scientific research to identify whether these homoeopathic potencies would have any effect on a cellular level (Vithoulkas, 1980:102-103).

It was believed that the therapeutic power of such potencies was produced by the successive dilution and succussion (to shake rapidly with a constant force) performed at each stage of the potentization process (method of homoeopathic remedy production), whereby the toxicity of the original substance was nullified through dilution, and the therapeutic power was activated through succussion, adding kinetic energy to the solution. Due to conflicting past study results, an exploration was required into whether succussion was necessary in the potentization method (Vithoulkas, 1980:103).

The defining principle in homoeopathy is the theory that 'like cures like' on which all prescriptions are made. It was therefore necessary to investigate the legitimacy of this principle, by using the same substance in the experiment as the antagoniser and as the treatment, thereby providing evidence that what one substance can cause, it can also treat (O' Reilly, 1996).

1.2 Rationale for the Study

1. This study aimed to provide further evidence that potencies that were diluted beyond Avogadro's constant had physiological effects on cells, despite having no particles of the original substance present.

2. This study set out to determine whether 6CH, 30CH and 200CH would have different effects on the cells as proposed by the Arndt-Schultz law, which states that every stimulus on a living cell elicits an activity which is inversely proportional to the intensity of the stimulus (Gaier, 1991).

3. This study intended to establish whether a biotechnological method could be used to evaluate the physiological effects of homoeopathic potencies compared to the more common use of clinical trials.

1.3 Aim of the Study

The aim of this *in-vitro* assessment was to evaluate the protective effect that homoeopathic potencies of *Arsenicum album* (6CH, 30CH and 200CH) had on cells after antagonisation by the crude substance, namely As_2O_3.

1.4 Statement of the Objectives

1. To culture the PBMCs and MT4 cell line.

2. To determine the optimum solubilization method of As_2O_3.

3. To determine the half maximal inhibitory concentration (IC_{50}) of As_2O_3 on the PBMCs and MT4 cell line, using the MTT assay.

4. To prepare the *Arsenicum album* potencies, namely 6CH, 30CH and 200CH, from As_2O_3 powder.

5. To determine the therapeutic effects of the various *Arsenicum album* potencies (6CH, 30CH and 200CH) on cells after antagonisation with the IC_{50} of As_2O_3.

6. To compare the effects of succussed and unsuccussed potencies after antagonisation with the IC_{50} of As_2O_3.

CHAPTER 2

LITERATURE REVIEW

2.1 Homoeopathy

2.1.1 History

Christian Friedrich Samuel Hahnemann (1842), founder of homoeopathy and German physician, had doubts whilst translating Cullen's edition of *A Treatise on the Materia Medica*, regarding the explanation given for the use of Peruvian bark in the treatment of malaria; hence researched the symptoms of Cinchona on the human body through self-application, and noted them as being characteristic symptoms of malaria (Vithoulkas, 1980:94-96).

Hahnemann subsequently derived a theory, stating that a medicine would cure a particular disease, if able to produce similar symptoms in the healthy as the totality of symptoms in the ill. The above theory, that *like cures like,* was tested using human experimentation, which determined the use for many substances in clinical cases, and became the primary principle for homoeopathic medicine (Vithoulkas, 1980:94-96).

2.1.2 Principles of Homoeopathy

Hahnemann (1842) stated that the ideal cure was the "*rapid, gentle and permanent restoration of health"* and that disease was a "*dynamic mistuning of the life force of the human body*". A homoeopathic medicine that is able to produce a particular set of symptoms in a healthy organism is believed to be able to dynamically alter the condition that displays a similar symptom picture, thereby eliminating the natural disease through similarity (*similia similibus curentur*). This is the first principle in homoeopathy and is now referred to as the law of similars (O' Reilly, 1996).

This study tested self-recovery of cells by applying a homoeopathic dose of the same substance that was responsible for the disease state; in this case *Arsenicum album* was given after the cells had been exposed to As_2O_3 (Van Wijk and Wiegant, 1995). This was done according to the law of similars, where the potentization process caused the remedy to change slightly, and so was only similar and not the same as the original substance (O' Reilly, 1996:98).

Due to the toxic effects of the original substances, only minute doses of potentized remedies were used in homoeopathic practice; it was then established that the process of dilution and succussion enhanced the medicinal powers of the remedies; hence the second principle, referred to as the principle of infinitesimal dose, states that a potentized medicine becomes more curative and helpful the more the dose descends in size. The smaller the dose, the more gentle and curative the medicine, as long as that medicine is chosen due to homoeopathic similarity (O' Reilly, 1996).

The Arndt-Schultz law is the third principle in homoeopathy and states that weak stimuli increase the effective activity of an organ; strong stimuli inhibit it and very strong destroy this activity. Homoeopathic remedies are low concentrations that therefore stimulate, and lower potencies stimulate less than higher potencies (Gaier, 1991).

This study tested and compared three different potencies of vastly different concentrations, in order to identify whether self-recovery of the cells would occur and which potency would stimulate self-recovery of the cells the most, thereby verifying the Arndt-Shultz law.

2.2 Potentization

2.2.1 History

Hahnemann (1842) defined potentization as the process whereby medicinal powers of the original substance were developed through the alteration of its properties, by mechanical action (trituration and succussion) on it's particles with a neutral substance. This process developed the latent dynamic powers of the substance, with activation of its specific medicinal powers, even if the substance had no medicinal powers in its crude state. The end products of potentization are called potencies (O'Reilly, 1996:235).

Toxic effects of the original substances were reduced through dilution. Water or alcohol soluble substances were made directly into mother tinctures, whereas insoluble substances were first triturated until soluble, and then made into mother tinctures. Trituration was the process of grinding a crude insoluble substance with lactose in a mortar and pestle for 3 h, according to Hahnemann's method, raising it to the one-millionth dilution (6X or 3CH), where it was soluble (Vithoulkas, 1980:160-161).

2.2.2 Potency Scales

Four potency scales are commonly used in homoeopathy today. Centesimal potencies are diluted in a 1:100 ratio (e.g.1CH); decimal potencies are diluted in a 1:10 ratio (e.g. 1X), quintamillesimal potencies are diluted in a 1:50 000 ratio (e.g. LM1 or Q1) and Korsakovian potencies are diluted using a different method to the above scales, where the same vial is used at each step of succussion. In this study, the centesimal scale of potencies was used, as these were the original potencies used extensively by Hahnemann, whereas the decimal potencies were introduced later by Constantine Hering (Sukul and Sukul, 2004). See appendix A to illustrate the comparison between dilution and centesimal potencies (Kayne, 1991:50).

2.2.3 Method of Preparation

Hahnemann serially diluted the mother tincture, mixing one part mother tincture to 99 parts solvent, and succussed the solution at each stage of the dilution process 100 times to not lose the effect from high dilution. It was believed that this resulted in more active, stronger remedies each time they were succussed (O' Reilly, 1996:235-243). "*The more the substance is succussed and diluted, the greater the therapeutic effect while simultaneously nullifying the toxic effect*" (Vithoulkas, 1980:102).

2.2.4 Controversy

The quantity of the original substance in the potentized remedy has been a controversial matter in homoeopathy. Avogadro's constant is the number of atoms or molecules in one mole of any substance, and is defined as the number of atoms in 12 grams of carbon-12, equal to 6.0221415×10^{23} (Ebbing and Wentworth, 1995). The limit to the dilution before losing all of the original substance is Avogadro's constant, and corresponds to the homoeopathic potency 12CH (10^{-24} dilution), hence potencies above 12CH have surpassed Avogadro's constant, and thus contain no molecules of the original substance. However, the effectiveness of the remedies is believed not to be decreased, but rather the higher the potency, the higher the therapeutic power (Vithoulkas, 1980:102).

Due to the concerns regarding whether potencies would have an effect when Avogadro's constant was exceeded, this study aimed to further explore the concept of potentization beyond Avogadro's constant by using 30CH and 200CH potencies, as well as 6CH potencies where the original substance was still present. A microbiological study was chosen, as there is insufficient research covering the cellular level affects of homoeopathic remedies; despite the support of clinical trials, homoeopathy has remained controversial due to the lack of scientific evidence.

Research on the cellular level eliminates bias and placebo effect, which is seen as a concern in clinical trials (Van Wijk and Wiegant, 1997).

The ability for the potencies above 12CH to have an effect has been explained by the theory that water molecules organize to store information obtained from the original solute; hence ultra high dilutions contain information of previous molecules in the solution (Schiff, 1995). Succussion in remedy preparation introduces intense turbulence and changes in pressure in the solution (Bellavite and Signorini, 1995). However, most dissolving is believed to be due to the diffusion process: Brownian motion. Flick's law states that if a concentrated substance is put into water, a trace will be found far away from the point of submersion immediately. The substance moves with great velocity from a region of high concentration to a region of low concentration (Auerbach, 1994:129-135).

2.2.5 Succussed and Unsuccussed Potencies

Sukul, De, Dutta, Sukul and Sinhababu (2001) compared succussed and unsuccussed 30CH *Nux vomica* preparations on toads to establish whether succussion was in fact an essential factor in the preparation of homoeopathic potencies.

Adult toads (*Bufo melanostictus)* were treated with *Nux vomica* 30CH, succussed (*Nux v* 30) and unsuccussed (*Nux v* 30u), mixed with sterile distilled water in a ratio of 0.05 mL/1 mL water. These were administered on the tongues of the toads using a micro pipette in the ratio of 0.05 mL/toad. The control consisted of 90% ethanol solution in the same ratio. Four hours after treatment, the toads were injected with 25% ethanol solution intra-peritoneally in a ratio of 8 g/kg body weight. The toads were made immobile as a result within 3-7 s, and laid in the supine position. The duration of ethanol induced sleep time was recorded for each toad, where recovery was defined as righting itself into the erect posture, known as the righting reflex. Ten - twelve toads were tested per day, total number of toads tested added to 279. The experiment was blind

where the researcher was not aware which toads were tested using drugs or controls (Sukul et al., 2001).

The measurement tools used consisted of electronic, infra red and nuclear magnetic resonance (NMR) spectra. The results showed that the toads responded to both the succussed and the unsuccussed potencies, where the toads treated with *Nux v* 30 and *Nux v* 30u regained their righting reflex more quickly than the toads given the control, thus succussion was found to not be an essential factor in producing an effective homoeopathic potency. The spectroscopic data also showed that *Nux v* 30 and *Nux v* 30u were different from their diluent medium 90% ethanol; that the water–ethanol solvent mixture had the capability to absorb certain properties of drug molecules during the potentization process; and that *Nux vomica* acts on toads to reduce ethanol induced sleep time (Sukul et al., 2001).

Numerous studies have been done investigating the value of succussion in potentization, but have conflicting results, thus investigating the effects of succussed potencies opposed to unsuccussed potencies was of great value (Aabel, Fossheim and Rise, 2001; Brizzi, Nani, Peruzzi and Betti, 2000).

2.3 Cell Culture

2.3.1 Cell Types

Mainly primary cells and heteroploid cell lines were used in this study. Primary cell populations are cells that have been obtained from healthy animal tissue and grown for the first time. Heteroploid cell lines are cell populations that can be subcultivated indefinitely, have abnormal chromosome number and morphology, and are distinguishable from normal cells (Bio-Whittaker, 1999/2000:115).

The primary cells used in this study were directly isolated from healthy human tissue, into single cell suspensions (Bio-Whittaker, 1999/2000:138). This was prepared by the researcher, where PBMCs were isolated from freshly isolated peripheral blood by centrifugation using Histopaque 1077. The cells collected were then stored in growth medium. These primary cells had a finite lifespan, lasting a few days before the cells started to die (Gogal, Ahmed and Larsen, 1997).

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The heteroploid cell line used in this study was the ATCC human T-cell lymphotropic virus type I-transformed T-cell line (MT4 cell line). This cell line grew in suspension in the medium and was seen as robust, where it could be propagated indefinitely. Passages twelve and thirteen of the MT4 cell line were used (Bio-Whittaker, 1999/2000:139).

These cell cultures were derived from humans (warm-blooded animals) and thus needed to be grown at 37°C in a medium that consisted of amino acids, vitamins, glucose and inorganic salts; the medium was also supplemented with 10% fetal calf serum (FCS) to enable cell growth. Phenol red, a pH indicator, allowed the researcher to assess growth of the cells and the pH of the medium (Bio-Whittaker, 1999/2000:138).

2.3.2 MT4 Cell Line and PBMC Study

Satou, Nosaka, Koya, Yasunaga, Toyokuni and Matsuoka (2004) set out to identify whether a proteasome inhibitor, namely Bortezomib, had anti-tumour properties against adult T-cell leukemia (ATL), with the ability to suppress the NF-$_k$B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, which has been shown to aid in the proliferation of Human T-cell Leukemia Virus Type 1 (HTLV-1) associated cell lines, with inhibition of apoptosis. ATL is a fatal cancer, derived from CD4-positive T-lymphocytes, with a poor prognosis regardless of intensive chemotherapy. Tax is a protein in the HTLV-1 genome, and contributes to the occurrence of ATL, by activation of viral gene transcription.

The above-mentioned study investigated the effects of Bortezomib on HTLV-1 infected cell lines *in vitro* and *in vivo*, and primary ATL cells *in vitro*. Ten HTLV-1 associated cell lines were used, including ED (tax negative) and MT4 (tax positive) cell lines. Four HTLV-1 non-associated cell lines were used as controls. PBMCs were isolated from patients with acute ATL by ficoll-hypaque. Bortezomib was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C (Satou et al., 2004).

An MTT assay was conducted to assess the inhibitory effects of Bortezomib on cell growth *in vitro*, apoptotic cell death was measured using flow cytometry, and the NF-$_k$B activity was evaluated in the HTLV-1 cell lines by an enzyme-linked immunosorbent assay (ELISA) (Satou et al., 2004).

Severe Combined Immunodeficiency (SCID) mice were inoculated subcutaneously with ED cells (4×10^7/mouse) in 200 µL of Roswell Park Memorial Institute medium (RPMI); when the tumors were measurable in size, the mice received Bortezomib (1 mg/kg) intraperitoneally twice weekly, with the tumors measured every two days to determine the effects of the proteasome inhibitor *in vivo* (Satou et al., 2004).

The results showed that the Bortezomib induced cell death of the HTLV-1 associated cell lines, which were found to be more susceptible to the drug than the HTLV-1 non-associated cell lines. The Bortezomib inhibited NF-$_k$B activation of HTLV-1 associated cell lines, leading to cell death in ATL cells *in vitro*; predominantly the cell death induced was apoptotic, seen in 60.6% of ED cells and 77.9% of MT4 cells, however necrotic cell death was also detected. Anti-tumour activity was seen *in vivo* with Bortezomib treatment (1 mg/kg), where the ED growth was significantly inhibited without adverse effects. The Bortezomib had little effect on healthy PBMCs compared to the apoptotic effect it had on the primary ATL cells (Satou et al., 2004).

The above results indicated that Bortezomib caused apoptosis in ATL cells *in vitro* and *in vivo*, and thus was a potential anti-tumor agent against fatal ATL (Satou et al., 2004). The above study also indicated that MT4 cell lines and PBMCs were effective and reliable sources for testing using the measuring tools mentioned, namely the MTT assay.

2.3.3 Previous Homoeopathic Research

Walach, Jonas, Ives, Van Wijk and Weingartner (2005) discovered that from all the experimental studies performed in homoeopathic research, with positive results in 4 out of 5 experiments, only a few replications had been achieved successfully, where most of the results differed from the original study. It was stated that homoeopathic research lacked credibility due to the fact that no particular model had been widely replicated and that a stable *in-vitro* model was yet to be found. It was noted however that Van Wijk and colleagues had demonstrated, in a series of studies, how the similia principle could be captured biologically, however these studies were carried out using low potencies only.

2.3.4 Similia Principle Study

Van Wijk and Wiegant (1995) completed cell culture research, using an experimental design, to test the homoeopathic similia principle, otherwise known as the law of similars. It was stated that the essence of homoeopathy was the stimulation of self-recovery by application of the similia principle. Self-recovery was defined as the supplementation of protector proteins within the cells, with increased resistance to the disturbing agent and temporary proliferation of cells in order to compensate for previous cell death.

The above-mentioned study described how self-recovery occurred at more than one level in the human body, detected as signs and symptoms due to changes at the organ, cellular and molecular levels. Two processes occurred when there was damage to an organ; 1) processes

that led to cell damage and 2) regeneration capacity which led to restoration of balance. Damage to an organ was therefore due to damage and death of cells exceeding cellular proliferation (Van Wijk and Wiegant, 1995).

Van Wijk and Wiegant (1995) described how self-recovery at the organ level showed a shift of symptom picture over time; a toxin entered a system, damaged an organ, the organ began to regenerate and eliminated the toxin, which then affected a different organ and led to complications with a wide variety in the symptoms over time. It was noted that self-recovery at the cellular and molecular levels had no issue with the above time shift of symptoms. Toxic substances affected the structure of proteins, which led to interactions between abnormal proteins and other molecules, which led to disorder in cell processes, cell damage and finally cell death. However, protective proteins led to cell recovery, by isolating the reactive parts of abnormal proteins and preventing them from interacting with normal proteins, and by restoring abnormal proteins to their original healthy state (Van Wijk and Wiegant, 1995).

The production of protective proteins needed to be increased to a more rapid rate when a cell underwent damage, in order to stimulate cell proliferation. This was induced through signals transmitted to the deoxyribonucleic acid (DNA) in the cell's nucleus, to promote synthesis of protective proteins. This was controlled by an auto-regulating feedback loop of processes; therefore the protective proteins regulated their own production. It was therefore important to determine whether self-recovery could be stimulated by the use of homoeopathy (Van Wijk and Wiegant, 1995).

The ability to use the similia principle effectively in clinical trials is very complex due to the different factors that need to be taken into consideration, for example, the behavioural, emotional and physical systems involved, and the changing of symptom patterns over time. However the symptom shift does not have an effect at a cellular level, and the similia principle can easily be tested using isopathy, where self-recovery is determined by a small dose of the same substance

which was initially responsible for the illness (Van Wijk and Wiegant, 1995). The similia principle was tested in the above-mentioned study by determining the extent to which self recovery was stimulated, by exposing the cells to high concentrations of toxic substances to initially cause cell damage, followed by small doses of the same toxic substances (Van Wijk and Wiegant, 1995).

Van Wijk and Wiegant (2010) described the criteria considered as necessary when investigating the similia principle, specifically on the cellular level.

- The first criterion stated that a disturbed state must be attained, where the proteotoxicity was then studied.
- The second criterion stated that there must be recovery of the cells in response to the disturbed state, this was seen as an increased production of protective proteins in response to proteotoxicity, in order to enable repair of structural damage.
- The third criterion stated that the disease picture needed be taken into account, where the characteristic changes at the molecular and cellular levels were noted after exposure to the stress.
- The fourth criterion stated that the remedy picture should be matched to the disease picture in order to determine the degree of similarity; this was achieved through the cellular responses that were induced by the different stresses, which were then compared through the production of different protective proteins.
- The fifth criterion was to have accepted outcome parameters to evaluate the effects of the treatments on cellular recovery; this was monitored through the cell's survival capacity and the production of protective proteins.

In the above-mentioned study, rat hepatoma cell lines were grown in plastic flasks at 37°C in Leibovitz medium, pH 7.4, and supplemented with antibiotics and FCS. These cell lines were pre-treated with 3 different stressors, namely:

1) Heat shock at 42°C for 30 min, by immersion of the culture flasks in a water bath, heated with Thermomix 1420, providing accuracy in temperature of ± 0.1°C.

2) Sodium arsenite 100 μM for 1 h.

3) Cadmium chloride 10 μM for 1 h (Van Wijk and Wiegant, 1998).

This resulted in a similar depression of overall protein synthesis by 20–30% in the 8 h post-stress period. These stressor conditions led to non-optimal stimulation of protective proteins, therefore allowing enhancement by subsequent low-doses of the stressors. Cells were then re-incubated in fresh medium for 8 h with either:

1) 39.5°C

2) 1 μM arsenite

3) 0.3 μM cadmium (Van Wijk and Wiegant, 1998).

Using gel electrophoresis and a laser scan, results were shown as the mean of three experiments, and showed that synthesis of protective proteins was significantly activated. Cells not pre-treated, and the controls which were re-incubated in fresh medium with no stressors at 37°C, were not affected (Van Wijk and Wiegant, 1998).

The above-mentioned study links homoeopathic knowledge to biomedical research, where self-recovery can be identified through the changes in the molecular level cell processes, thus preventing bias, placebo effects and interactions of different organs in the body seen in clinical trials.

The study did not however test potencies above Avogadro's constant or compare the effects of succussed potencies versus unsuccussed potencies; these were therefore covered in this study,

where high potencies were used to identify whether they had any effects on the cells, and succussed potencies were compared with unsuccussed potencies to identify the importance of succussion in remedy preparation (Van Wijk and Wiegant, 1995).

2.3.5 Chemo-Protective Study

Reddy, Odhav and Bhoola (2006) used an *in-vitro* system to test the chemo-protective effects of β-carotene and lycopene anti-oxidants on human hepatoblastoma cells ($HepG_2$ cells), exposing the cells subsequently to a fungal toxin, namely aflatoxin B_1 (AFB_1), that had been associated with primary hepatocellular carcinoma. Their aim was to determine what cellular and molecular mechanisms these anti-oxidants would inhibit in AFB_1 - infected $HepG_2$ cells.

$HepG_2$ cells were maintained in tissue culture flasks with medium containing FCS and 1% antibiotics. These cells were cultured in a humidified incubator in 5% CO_2 at 37°C and passaged weekly. AFB_1 was dissolved in DMSO before cells were added. Lycopene and β-carotene were dissolved in tetrahydrofuran (THF) and stored at -70°C. The final concentrations of AFB_1, lycopene and β-carotene used for the different experiments were prepared by diluting the stock solutions with the medium (Reddy, Odhav and Bhoola, 2006).

The optimum incubation time and dose response relationship of AFB_1 toxicity was determined using the MTT assay (Satou et al., 2004). The half maximal inhibitory concentration (IC_{50}) and maximum AFB_1 toxicity values were used. The concentrations of lycopene and β-carotene used were those that gave 80% survival of $HepG_2$ cells in the MTT assay. Cell survival was measured using the crystal violet dissolution assay, where pre-treatment with lycopene increased the survival by 14% at an AFB_1 concentration of 11 µg/mL and pre-treatment with β-carotene increased it by 54%, with cell survival decreasing as AFB_1 concentration increased. Flow cytometry was used to confirm these results and analyze the cell survival (Reddy, Odhav and Bhoola, 2006).

In the control group, the $HepG_2$ cells were cultured in the presence of AFB_1, which caused mitochondrial damage, nuclear condensation and loss of intercellular contact with dysfunctional gap junctions, which led to loss of intercellular communication. On the genomic level, AFB_1 formed AFB_1– N7 – guanine adducts which caused apoptosis of the cells and suppression of p53 protein expression (Reddy, Odhav and Bhoola, 2006).

In the experimental group, the cell line was treated first with lycopene or β-carotene, and then exposed to AFB_1. The proliferation and apoptosis of these cells were then determined. The experiments were designed to measure mitochondrial activity and cell survival using enzyme assays and flow cytometry. Morphological changes were examined using phase contrast, light and transmission electron microscopy. An assay for AFB_1– N7 – guanine adducts in $HepG_2$ cells was performed using an ELISA reader, and the levels of p53 tumour suppressor protein were measured using a pantropic ELISA. Apoptosis was determined using a DNA fragmentation assay to detect apoptotic bodies (Reddy, Odhav and Bhoola, 2006).

The results indicated chemo-protective effects of the anti-oxidants β-carotene and lycopene, which showed increased mitochondrial activity, cell survival, p53 protein and intercellular communication and decreased ultra-structural damage, apoptosis and AFB_1– N7 – guanine adducts. Thus the $HepG_2$ cells pre-treated with lycopene and β-carotene were protected from aflatoxin toxicity at cellular and molecular levels (Reddy, Odhav and Bhoola, 2006).

The above-mentioned study was valuable in chemo-protection using anti-oxidants, but differed from this study as the same substance was not used in treatment and infection. This study was performed to compare the homoeopathic approach of treating using the same substance compared to the established practice of using antidotal treatments. The measuring tools utilized in the above-mentioned were of significance to this study, namely the MTT assay, in conjunction with the methods used to culture the cell lines.

2.3.6 Molecular Organization of Water

Benveniste and his co-researchers provided support for the 'water memory' theory, through an *in-vitro* study, where human basophils degranulated after exposure to dilutions of anti–immunoglobulin E (anti-IgE) antibodies, which were diluted past Avogadro's constant (Davenas, Beauvais, Amara, Oberbaum, Robinzon, Miadonna, Tedeschi, Pomeranz, Fortner, Belon, Sainte-Laudy, Poitevin, and Benveniste, 1988).

An allergen enters the body and binds with IgE antibodies, these antibodies then attach to mast cells and basophils. This leads to rupturing of the cells and release of their granules and consequently histamine and other inflammatory mediators, causing an allergic inflammatory reaction, with a change in their staining properties (Guyton and Hall, 1997: 295).

Goat anti-human IgE antiserum was prepared in HEPES-buffered tyrode's solution containing human serum albumin (HSA), and diluted down to 1×10^{60} and then to 1×10^{120} dilutions. Each dilution was vortexed for a minimum of 10 s or pipetted up and down (Davenas et al., 1988).

Venous blood from healthy donors was collected and allowed to sediment. The plasma was removed and washed twice with centrifugation, and resuspended in HEPES-buffered tyrode's solution. Ten microlitres of the human basophil suspensions were deposited in each well of a micro titre plate containing 10 µL calcium chloride ($CaCl_2$) and 10 µL anti-IgE antiserum dilution. The control wells had 10 µL $CaCl_2$ and 10 µL of either tyrode's solution or anti-IgG antiserum dilution. The plates were then incubated at 37°C for 30 min. Staining solution was added to each well and mixed. Non-degranulated basophils stained red, and were counted under light microscopy using a haemocytometer. The percentage of basophil degranulation was then calculated (Davenas et al., 1988).

The results showed that degranulation was induced by the high dilutions of anti-IgE antiserum, seen in 10 experiments on the full range of dilutions, despite the considered absence of anti-IgE molecules. Degranulation (between 40-60%) was found down to the 1×10^{120} dilution (Davenas et al., 1988).

Controls used were goat anti-human immunoglobulin G (IgG) antiserum at a dilution of 1×10^{120} and tyrode's solution with HSA at 1×10^{30}. These were incubated in the same conditions as the experimental groups. The controls induced no degranulation (Davenas et al., 1988).

The importance of agitation in the transmission of information was shown by comparing pipetted dilutions of antibodies with vortexed dilutions. This resulted in the same level of dilution but degranulation only occurred in the vortexed dilutions, after a minimum time of 10 s of vortexing. The basophil degranulation was also seen with substances other than anti-IgE antiserum, such as antigens in allergic patients (Davenas et al., 1988).

Using the molecular weight of immunoglobulins and Avogadro's constant, calculations showed that less than 1 molecule was present in the anti-IgE antiserum at a dilution of 1×10^{14}, and yet significant degranulation occurred down to the 1×10^{120} dilution. Hypotheses therefore stated that due to the fact that none of the starting molecules were present, information was transmitted during the vortexing process, which induced a sub-molecular organization of water, with water acting as a template for the molecule with its hydrogen bonded network, and it's electric and magnetic fields (Davenas et al., 1988).

In an experimental study of this nature, it was crucial to fully incorporate all the principles in homoeopathy, for a true testing of its effects. The above study showed that high potencies that surpass Avogadro's constant do have an effect on cells, but due to controversy regarding the reproducibility of the study, this was an aspect that needed further investigation.

2.4 Arsenic Trioxide

2.4.1 Chemistry

The atomic number of arsenic (As) is 33. Arsenic trioxide (As_2O_3) is a non-metallic inorganic compound that appears as a white powder or transparent crystals. It is odourless, tasteless, stable in air, slowly soluble in water (3.7 g/100 mL at 20°C), and soluble in glycerin. It is non-volatile, and has a molecular weight of 197.84 g/mole (Varma and Vaid, 2002). As_2O_3 has a boiling point of 465°C and a melting point of 315°C. It dissolves slowly in aqueous solutions to form weak acids and it dissolves readily in alkaline solutions to form arsenites (Mallinckrodt Baker Inc., 2008).

2.4.2 Biological Properties

The most common routes of exposure of As_2O_3 are through ingestion and inhalation, where it is quickly absorbed, and through the skin which is slower in absorption and leads to a less severe illness. There are many arsenic compounds found in the environment; As_2O_3 is seen as highly toxic with a half life of 10 h, as opposed to organic arsenic, found mostly in fish and seaweed, which is much less toxic and has a half life of 48 h (Agency for Toxic Substances & Disease Registry, 2009).

As_2O_3 is noted as causing irritation to the skin, eyes and respiratory tract and may be fatal if swallowed or inhaled; it can cause cancer depending on the duration and level of exposure; and it may cause liver and kidney damage. As_2O_3 must be used only with adequate ventilation, respiratory equipment, gloves, goggles and other protective clothing. As_2O_3 must be stored in a closed container, in a cool, dry, well ventilated place, and be well labeled. In disposal, As_2O_3 must be handled as hazardous waste (Mallinckrodt Baker Inc., 2008).

2.4.3 Occurrence

Arsenic is a highly toxic element that occurs naturally in the environment, it is found in food, water, soil and air and does not deteriorate over time. As_2O_3 forms when metallic arsenic is heated to high temperatures. When As_2O_3 is burned, it releases arsine gas which is highly toxic. Arsenic occurs naturally in the earth's crust, where it is dispersed into the environment through mining, ground water, mineral ores and geothermal processes; it is also released into the air from volcanoes (Agency for Toxic Substances & Disease Registry, 2009).

Arsenic can be found in seafood e.g. clams, oysters, mussels, crabs and lobsters; and seaweeds. These are usually organic arsenic compounds that are less toxic. Products that contain arsenic are insecticides, algaecides, desiccants (used in cotton harvesting), glass manufacturing products, herbicides and wood preservatives. Medically, arsenic has been used in chemotherapy for acute promyelocytic leukemia and other cancers, it is used homoeopathically in minute doses, and Fowler's solution (1% As_2O_3) has been used in the past for skin conditions e.g. psoriasis and eczema (Agency for Toxic Substances & Disease Registry, 2009).

2.4.4 Arsenic Trioxide Solubilization Studies

Martindale (1967) states that in order to make an arsenical solution, 1 g of As_2O_3 is to be dissolved in 10 mL potassium hydroxide solution by warming gently, then 50 mL of chloroform water is added and neutralised with dilute hydrochloric acid (2-8 mL), and adjusted to 100 mL with more chloroform water, making up a 1% w/v of As_2O_3.

In a past study, As_2O_3 was dissolved in sodium hydroxide (NaOH) at a concentration of 1 mM/L and further dissolved for use in the study. The researchers stated that the maximum concentration of NaOH in the culture had no influence on the cell growth in the cell lines used (Shim, Kim, Yang, Lee, Choi and Kim, 2002).

Florea, Splettstoesser and Busselberg (2007) dissolved As_2O_3 in methanol and phosphate buffered saline (PBS) that was Ca^{2+} and Mg^{2+} free, to a stock solution of 1 mM; these solvents were used so as not to affect the intracellular calcium levels, which were to be monitored in the study.

The above methods utilized chemicals as the solvents that could potentially affect the cells in culture, hence other solvents and methods needed to be investigated in this study that would lead to full dissolution of the As_2O_3 and have no affect on the cells, so that all effects could be attributed to the As_2O_3 alone.

2.4.5 Arsenic Trioxide Toxicity

Florea et al. (2007) studied the mechanisms of As_2O_3 toxicity on human neuroblastoma (SY-5Y) and embryonic kidney (HEK 293) cell lines, in order to understand the influence of As_2O_3 on intracellular calcium (Ca^{2+}) levels, for potential use of As_2O_3 in the treatment of different forms of cancer, and to evaluate the potential risks of kidney toxicity.

In the above-mentioned study, the SY-5Y cells were maintained in RPMI 1640, supplemented with 10% FCS and 100 IU/mL antibiotics; the HEK 293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) and supplemented as above. The cells were incubated at 37°C in a 5% CO_2 atmosphere. Non-confluent monolayer cells were used (Florea et al., 2007).

Ca^{2+} sensitive dyes were used with laser scanning microscopy and fluorescence activated cell sorting, to measure the Ca^{2+} concentration changes due to As_2O_3 application. The cytotoxicity was evaluated through trypan blue staining and MTT assays, and apoptosis and DNA damage were identified by Hoechst staining (Florea et al., 2007).

In both cell lines, the As_2O_3 increased the Ca^{2+} concentrations and significantly reduced the cell viability (but not to under 50% cell survival) with apoptosis and DNA damage. It was noted that As_2O_3 interacted with DNA repair, thus resulting in carcinogenesis. The results showed that the Ca^{2+} concentration was an important messenger in As_2O_3 induced cell death, where Ca^{2+} concentration disturbances or overloads determined the toxicity and cell death (Florea et al., 2007).

2.5 Measurement Tools

2.5.1 Staining

Hoechst staining was used by Florea et al. (2007) in order to recognize apoptosis and DNA damage by means of staining micro-nucleated cells, condensed nuclei and cells in mitosis. Cells were cultured on chamber slides and incubated overnight, followed by incubation with 1 μM As_2O_3 for 24 h, 48 h and 72 h. The cells were then washed twice with PBS, given methanol and stored overnight at −20 °C for fixation. The slides were then dried and stained using 10 μM Hoechst 33342 for 30 s and covered with cover slips and observed using a fluorescent microscope (Florea et al., 2007).

2.5.2 Trypan Blue

The trypan blue cytotoxicity assay was performed by Florea et al. (2007) in order to identify the cytotoxicity of As_2O_3 on the cells. Cells were exposed to As_2O_3 for 24 h, 48 h and 72 h. Subsequently, the culture media was collected, washed with PBS and the cells trypsinized. The suspension was centrifuged and the supernatant removed. The pellet was then washed with PBS and centrifuged once again. To conclude, 50 μL of cell suspension was mixed with 50 μL trypan blue 0.4% solution. The cells were then counted after 3 min of staining using a haemocytometer

under light microscope, where the bright cells were seen as viable and the blue cells as dead (Florea et al., 2007).

2.5.3 MTT Assay

The MTT assay was conducted by Florea et al. (2007) in an attempt to identify cell viability. The cells were seeded in each well of a 96 well plate, which were incubated overnight to allow the cells to attach. Subsequently, As_2O_3 was added to each of the wells and incubated; after the exposure times, 10 µL of MTT solution was added to the wells and the plate incubated for 4 h. Thereafter, the supernatant was removed and 50 µL sodium dodecyl sulphate (SDS) solution was added, mixed and then incubated for 10 min. The plate was then mixed again, and read at a wavelength of 540 nm (Florea et al., 2007).

2.5.4 Flow Cytometry

Reddy (2005) analysed the results of the cell viability assays using flow cytometry. After treatment of the $HepG_2$ cells with AFB_1, lycopene and beta-carotene, the cells were suspended in PBS and added to propidium iodide. The cells were then gated on the Flow Cytometer at 1×10^4 cells per sample. The fluorescence intensity of the treated $HepG_2$ cultures was measured by the flow cytometer, where the dead cells took up the propidium iodide dye and the viable cells did not.

In this study, the cell cultures were closely monitored for morphological and media changes during incubation and the microscopic examination of cells before and after treatment were recorded. After treatment the cells were counted using the trypan blue dye exclusion assay, and the cell viability was assessed using the MTT assay (Reddy et al., 2006; Florea et al., 2007). These methods of testing have been used in numerous studies and have been proven as reliable.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

This study involved *in-vitro* experiments using human cell cultures. The proposal received ethical approval on May 14th, 2009 by the Faculty of Health Sciences Research Committee, for the use of human cells for the *in-vitro* testing performed in this study.

This research study was an experimental *in-vitro* design. It was conducted in the cell culture laboratory in the department of Biotechnology and Food Technology, at DUT, Durban, South Africa.

The cytotoxic effects due to As_2O_3 exposure and the protective effects of succussed and unsuccussed *Arsenicum album* 6CH, 30CH and 200CH on the cells, were investigated by the MTT cell viability assay and trypan blue dye exclusion assay. All assays were performed in triplicate.

3.2 Media and Reagents

The media and reagents used in this study were of tissue culture grade and were obtained from Sigma (USA), Invitrogen (USA) and Highveld Biological (South Africa). Further details regarding all the media, reagents and equipment used in the experiments are included in Appendix B.

3.3 Aseptic Technique

Aseptic technique is a set of detailed practices and procedures performed under vigilantly controlled conditions with the aim of diminishing contamination by pathogens (Hauswirth and

Sherk, 2004). Aseptic technique was used whilst working with the cell cultures; all work was done under a laminar flow unit (Scientific Engineering Inc, USA), in a closed sterile cell culture room; the work surface was sterilized using ultraviolet germicidal irradiation where ultraviolet (UV) light was used to break down microorganisms; all pipette tips, glass bottles, and distilled water were first autoclaved (D&E International Corp, Taiwan) for sterilization. Latex rubber gloves were worn by the researcher at all times. The work surface, incubator (Heraeus, Germany) and gloves were cleaned continuously using 70% ethanol (Bio-Whittaker, 1999/2000:149).

3.4. Cell Culture

3.4.1 Isolation of PBMCs

Two 20 mL bags of buffy coat blood were obtained from the National Blood Transfusion Services (NBTS), Durban, South Africa. These were maintained at room temperature and used the same day. PBMCs were isolated by density gradient centrifugation of freshly isolated peripheral blood on histopaque 1077 (Sigma, USA) (Gogal, Ahmed and Larsen, 1997).

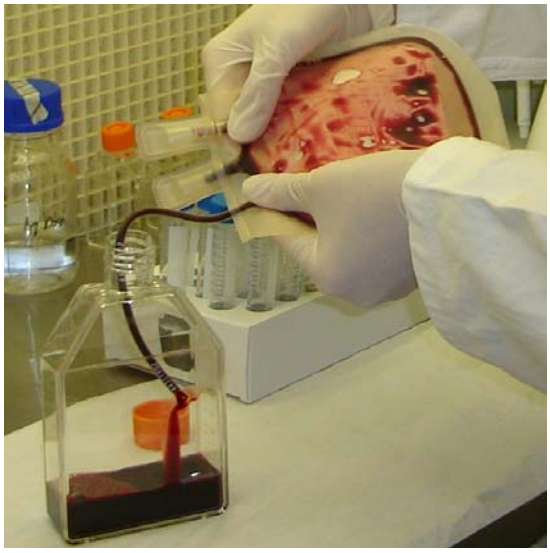


Figure 3.1: Decanting of buffy coat of blood.

Buffy coat blood was decanted into a sterile 75 cm^3 culture flask (Greiner, Germany), the tube of the bag was cut and inserted into the flask opening, and the blood squeezed out. All blood waste was placed into a disposable bag to prevent blood leakage. Five millilitres of the blood and 5 mL of PBS (Invitrogen, USA) were added to 15 mL sterile centrifuge tubes (Greiner, Germany) and mixed well. Nine millilitres of these blood solutions were then added to centrifuge tubes containing 5 mL of histopaque 1077. The blood solutions were poured very slowly with the tubes slanted, to ensure that two layers formed and did not mix, with the blood solution on top of the histopaque (Gogal, Ahmed and Larsen, 1997).

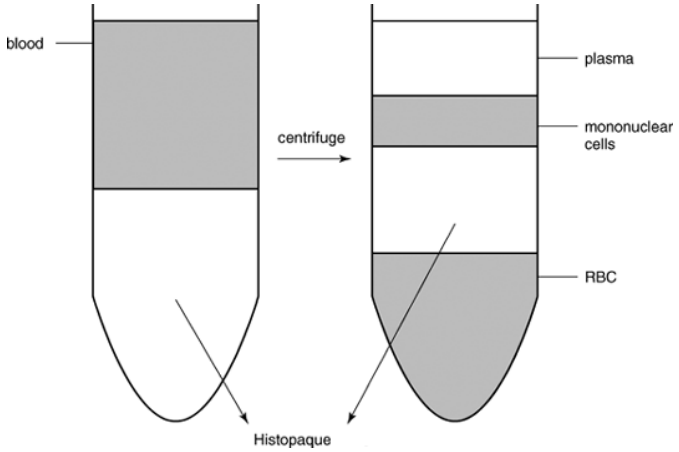


Figure 3.2: Schematic diagram showing separation of blood cells using histopaque 1077 (Cheng, Sirigireddy and Ganta, 2008).

The tightly closed centrifuge tubes were then placed into the centrifuge (Eppendorf, USA), with even weight on opposite sides to prevent an imbalance, and centrifuged at 1 500 rpm for 30 min at 37°C. The centrifuge tubes were then taken out and the distinct layers were noted, as seen in figure 3.3 below.

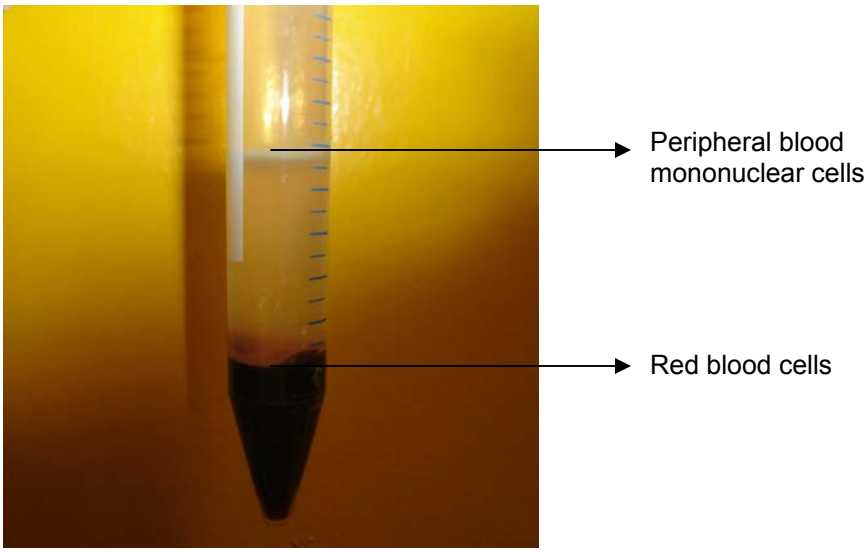


Figure 3.3: Buffy coat showing separation of blood cells after centrifugation (1 500 rpm for 30 min).

Pasteur pipettes were placed just above the dense white PBMC layers, between the plasma and the histopaque, to aspirate out the cells, and were transferred into 50 mL centrifuge tubes containing 20 mL PBS. These were then placed into the centrifuge at 1 700 rpm at 37°C for 10 min. The PBS was then poured out, with the pellet of PBMC left behind, which was white in colour (Gogal, Ahmed and Larsen, 1997).

Twenty millilitres of PBS was then added to the pellet and centrifuged for another 10 min at 1 700 rpm at 37°C. The PBS was poured out once again, leaving the pellet, and another 20 mL of PBS was added and centrifuged for the 3rd time for 10 min at 1 700 rpm at 37°C. The PBS was then poured out and 10 mL of RPMI (Invitrogen, USA) and 2 mL of FCS (Highveld Biological, South Africa) were added into each of the 50 mL centrifuge tubes. The solution was then pipetted up and down to get the pellet into suspension (Gogal, Ahmed and Larsen, 1997).

3.4.2 Culturing of PBMCs

Complete culture medium (CCM) for PBMCs was made up and consisted of 44.5 mL RPMI medium and 5 mL (10%) filtered FCS, supplemented with 0.5 mL (1%) penicillin and streptomycin (Pen Strep) (Highveld Biological, South Africa). One millilitre of the cell suspension was cultured in 25 cm^3 cell culture flasks, containing 5 mL CCM. These flasks were labeled and incubated at 37°C in a 5% CO_2 atmosphere, and allowed to grow. The cells were examined everyday under light microscope (Nikon, USA), observing confluence of the cells and media changes. When the media changed colour, from bright red to light orange, the media had been used up by the cells. Consequently part of the cell suspension was poured into new culture flasks and sub-cultured with fresh CCM (Gogal, Ahmed and Larsen, 1997).

3.4.3 Storage of PBMCs

The PBMCs were stored in cryoprotectant, which consisted of 10 mL DMSO (Sigma, USA) and 40 mL RPMI to produce a 20% solution; 0.9 mL (900 µL) of cell suspension was placed into individual cryovials with 0.9 mL (900 µL) cryoprotectant added to each (Gogal, Ahmed and Larsen, 1997). These were labeled and stored in a 'Mr. Frosty' container (Nalgene Labware, USA) at 4°C for 24 h and subsequently in the biofreezer (Former Scientific, UK) at -70°C.

3.4.4 Culturing of the MT4 Cell Line

Passages 12 and 13 of the ATCC human T-cell lymphotropic virus type I-transformed T-cell line (MT4 cell line) were used in the experiments. The cells were in an actively growing state on arrival; hence they were cultured in CCM immediately and incubated at 37°C in a 5% CO_2 atmosphere. The CCM used for the MT4 cells consisted of 35.8 mL of RPMI, 4 mL (10%) of filtered FCS, and was supplemented with 200 µL (0.5%) of Pen Strep. These cells were cultured in the same manner as the PBMCs above.

3.4.5 Storage of the MT4 Cell Line

The MT4 cells were stored in cryoprotectant that consisted of 1 mL DMSO, 3 mL FCS, 100 µL Pen Strep and 5.9 mL RPMI. MT4 cells were added to CCM in the morning, and centrifuged that afternoon at 1 500 rpm for 15 min. They were washed 3 times with PBS and centrifuged each time at 1 500 rpm for 10 min. Ten millilitres of the cryoprotectant was then added to the pellet and resuspended, with 1 mL transferred into each cryovial. These were labeled and stored in the 'Mr. Frosty' container at 4°C for 24 h and subsequently in the biofreezer at -70°C.

3.5 Cell Counting using the Trypan Blue Dye Exclusion Assay

Principle

This test was performed in order to identify the number of viable cells per millilitre of the cell suspension, and consequently the concentrations and volumes of cells used in each of the experiments. The viable (living) cells were impermeable to the trypan blue dye (Biowhittaker, USA), therefore they appeared translucent, whereas the non-viable (dead) cells took up the dye and therefore stained blue.

Methodology

Fifty microlitres of cell suspension was mixed with 50 µL of the 0.4% trypan blue solution, added to an eppendorf tube and vortexed. With the cover-slip in place, 20 µL of this solution was pipetted into both chambers of a haemocytometer (Reddy, 2005). This was then observed under the phase contrast microscope (Nikon, USA), and the viable cells counted in quadrants A, B, C and D, in order to calculate the cells/mL. The test was done in duplicate, thus the average number of viable cells was calculated.

Calculations and statistics

The total number of viable cells was calculated using the following formula:

Cells/mL = Average number of cells per square x dilution factor (2) x 10^4 (Reddy, 2005).

3.6 The MTT Assay

Principle

The reaction in this assay is due to the reduction of the yellow-coloured MTT (3-(4,5-dimethylthiazol-2-YI)-2,5-diphenyltetrazolium bromide) to purple formazan crystals by the succinate tetrazolium reductase system; this occurs as a result of respiration of mitochondria in viable cells, where mitochondrial dehydrogenase converts MTT into a water-insoluble, darkly coloured formazan derivative. An increase in the number of viable cells therefore results in an increase in overall enzyme activity, and hence leads to a greater quantity of formazan dye being produced (Cole, 1986).

Methodology

MTT reagent was made up by adding 250 mg MTT (Sigma, USA) to 50 mL PBS, the tube was then covered with foil to protect the photo-sensitive MTT from the light, and stored at 4°C overnight (Reddy, 2005). To each culture well of the 96-well plate (150 µL cell culture), 10 µL of MTT reagent was added. For the 24-well plate (1 mL cell culture), 67 µL of MTT reagent was added to each well. The plates were then placed on the shaker for 5 min in a dark room, followed by incubation for 3 h at 37°C in 5% CO_2 atmosphere. Next, 100 µL of DMSO was added to each well of the 96-well plate, and 667 µL of DMSO to each well of the 24-well plate. These were then placed on the shaker for 5 min in a dark room and then incubated for 25 min, to dissolve the

formazan crystals. The amount of mitochondrial dehydrogenase present in the culture was read on the Cary 100 UV-visible spectrophotometer (Varian, USA), firstly by pipetting up and down each culture well to get the cells into suspension, and then by placing 1 mL of the culture in quartz cuvettes and reading at 578 nm. Filtered RPMI was used as the blank to initially zero the machine. All treatments were done in triplicate.

Calculations and statistics

The percentage cell viability and cytotoxicity were determined for the above assay using the following formulae:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100\right)$$

3.7 Storage and Handling of Arsenic Trioxide

Twenty five grams of As_2O_3 99.99% powder (Sigma, USA) was stored in a tightly closed container, in a cool, dry, well ventilated area, at the DUT homoeopathic and cell culture laboratories, as suggested in the Material Safety Data Sheet of As_2O_3 by Mallinckrodt Baker Inc. (2008).

For the preparation of the As_2O_3 solutions, protective measures were taken in order to prevent any dermal exposure or inhalation of the As_2O_3, which could lead to subsequent toxicity. The researcher wore a gas mask, latex gloves and a lab coat, and washed hands, forearms, face and neck when leaving the laboratory (Mallinckrodt Baker Inc., 2008).

3.8 Optimization Studies for Solubilization of Arsenic Trioxide

Solubilization of the As_2O_3 was a fundamental stage in this study; it was necessary to achieve full dissolution of the As_2O_3 in order to attain a homogenous solution where the As_2O_3 entered into the cells and caused cytotoxic effects, and ultimately cell death, with all of the As_2O_3 doses equal in concentration, so as to produce reliable and reproducible results.

Table 3.1: Solubilization methods of As_2O_3

Solvent	Time of Exposure	Stirring	Heat	Degree of Solubility
Distilled water	10 min	Yes	None	0
Distilled water	24 h	Yes	None	+
RPMI	24 h	Yes	None	+
PBS	24 h	Yes	None	+
Normal saline	24 h	Yes	None	+
Distilled water	48 h	Yes	50°C	++
Milli-Q water	10 days	No	80°C	++++

0 = no solubilization
+ = minimal solubilization
++ = moderate solubilization
++++ = full solubilization

The optimum solubilization method of As_2O_3 was determined through a number of dissolution experiments (see appendix D). Initially, 2 mg of As_2O_3 was weighed out on a mass balance and added to 100 mL distilled water, this was placed on a magnetic stirrer with a magnetic rod in the solution for 10 min, to aid in diffusion, and labeled as 1 µM As_2O_3. Fifty millilitres of this solution was then added to 50 mL distilled water and placed on the magnetic stirrer for another 10 min, and labeled as 0.5 µM As_2O_3, the same was then done to make the 0.25 µM As_2O_3 solution.

Further experiments were done, where 4 mg of As_2O_3 was added to 100 mL distilled water, RPMI, PBS and normal saline solution. These solutions were then placed on magnetic stirrers for

24 h and produced the stock solutions of 2 μM As_2O_3 in the respective solvents. Fifty millilitres of the above stock solutions were then added to 50 mL of the respective solvents and placed on the magnetic stirrers for 10 min to form the 1 μM As_2O_3 solutions, and repeated again to form the 0.5 μM As_2O_3 solutions.

In the following experiment, concentrations of 10 μM, 5 μM and 2 μM of As_2O_3 were prepared in distilled water. Two milligrams of As_2O_3 was added to 10 mL distilled water and placed on the magnetic stirrer for 48 h at 50°C to form the stock solution of 10 μM As_2O_3. Five millilitres of the stock was then added to 5 mL distilled water and placed on the magnetic stirrer to form the 5 μM As_2O_3. The stock solution of 2 μM As_2O_3 from the previous experiment was then placed on the magnetic stirrer with heat as above.

The above experiments did not allow for complete dissolution of the As_2O_3, which was said to have a solubility of 37 g/1000 mL water at 20°C (Mallinckrodt Baker Inc., 2008). It was however ultimately established that As_2O_3 was entirely dissolved by adding 396 mg dry As_2O_3 to 100 mL sterile distilled Milli-Q water. These were placed into a conical flask, completely sealed and positioned on a heating plate for 10 days at 80°C, to aid in diffusion. This formed the As_2O_3 aqueous stock solution of 20 mM concentration, as calculated below:

M = mol/L

M (As_2O_3) = 198 g/L

mM = 198 g x 10^{-3}/L

mM = 0.198 g/L

mM = 198 mg/L

mM = 19.8 mg/100 mL

20 mM = 396 mg/100 mL

20 mM = 0.396 g/100 mL (Perfetti, Pokrovski, Ballerat-Busserolles, Majer and Gibert, 2008).

The final As_2O_3 solutions used in the experiments were prepared in RPMI, a medium used for the culture of normal and cancerous human leukocytes. The bottles, lids and pipette tips were first autoclaved for sterility. The 20 mM As_2O_3 stock solution was decanted into the sterile 100 mL bottle, and sealed tightly. In sterile 50 mL centrifuge tubes, 25 µL, 12.5 µL and 2.5 µL of the stock was diluted in RPMI up to 50 mL, each were vortexed, and resulted in 10 µM, 5 µM and 1 µM As_2O_3 solutions respectively, as calculated below:

Table 3.2: Preparation of As_2O_3 standards from stock solution.

As_2O_3 stock concentration = 20 mM ≈ 20 000 µM

Concentration	**Volume**
20 000 µM	50 mL
10 µM	0.025 mL (25 µL)
5 µM	0.0125 mL (12.5 µL)
1 µM	0.0025 mL (2.5 µL)

3.9 Optimization Studies for Arsenic Trioxide Cytotoxicity

To determine the effect of *Arsenicum album* potencies on As_2O_3 antagonized cells, an optimized *in-vitro* experiment had to be initially ascertained, with regard to the dose of As_2O_3; in particular the concentration, volume and time of exposure to As_2O_3. These were determined by using the MTT assay. The half maximal inhibitory concentration (IC_{50}) represents the concentration of a substance, namely As_2O_3 that is needed for 50% inhibition of cells *in-vitro*.

Table 3.3: As_2O_3 treatments of PBMCs and MT4 cells.

Cell Types	Cell Culture Volume	As_2O_3 Doses	Exposure Times	Injection Volumes	Controls
PBMCs	150 µL/well	1 µM; 5 µM; 10 µM	24 h & 48 h	5 µL; 10 µL; 20 µL; 50 µL	DMSO & RPMI
PBMCs & MT4 Cells	1 mL/well	5 µM	48 h & 72 h	33 µL; 67 µL; 133 µL	RPMI

3.9.1 PBMCs used for Optimizing Dose

Principle

To define the correct dosage of As_2O_3 for the stress, the researcher tested different concentrations of As_2O_3 (1 µM, 5 µM and 10 µM), for different exposure times (24 h and 48 h) and used different injection volumes (5 µL, 10 µL, 20 µL and 50 µL), in order to identify the highest sub-lethal dose to cause 50% cell death. This was performed in triplicate using the MTT assay.

Methodology

The experiment was performed in three 96-well, flat-bottomed microtitre plates (Greiner, Germany), the actively growing PBMCs (150 µL/well containing 4 x 10^5 cells/mL) were incubated overnight at 37°C in a 5% CO_2 atmosphere (Reddy, 2005).

Following incubation, 5 µL, 10 µL, 20 µL and 50 µL doses of the controls (DMSO and non-medicated RPMI) and the As_2O_3 antagonisers (1 µM, 5 µM and 10 µM) were added to the culture wells, and incubated for 24 h (plate 1) and 48 h (plate 2 and 3). See appendix E for the schematic diagrams of these plates.

After incubation, the MTT assay was performed as previously described in section 3.6. The absorbance was read for the combined treated cell cultures using the spectrophotometer. The optimum inhibitory dose had a reading close to half of the readings for the RPMI controls, as this was close to the IC_{50}, where 50% of the cells died due to the toxic effects of As_2O_3.

Calculations and statistics

The percentage cytotoxicity was determined as stated in 3.6 above.

3.9.2 PBMCs and MT4 Cells used for Optimizing Injection Volume and Time

Principle

As determined in the previous experiment (section 3.9.1), 5 µM was used as the optimum concentration of As_2O_3 to allow for 50% cell death. It was therefore necessary to determine the optimum injection volume, time of exposure and cell type to ensure the best possible results. This test was done in triplicate using the MTT assay.

Methodology

Using 24-well plates (Greiner, Germany), 1 mL of MT4 cells (4.8×10^5 cells/mL) was added to each well of plate A and B; and 1 mL of PBMCs (4×10^5 cells/mL) was added to each well of plate C and D. These were all incubated overnight at 37°C in 5% CO_2 atmosphere.

The subsequent day, the antagoniser (5 µM As_2O_3) and control (non-medicated RPMI) were added to each of the wells, using injection volumes of 33 µL, 67 µL and 133 µL. This was done in triplicate. Plates A and C were then incubated for 48 h and plates B and D for 72 h.
This was calculated as follows:

Table 3.4: Upscaling of injection volumes of As_2O_3 from 96 well plate to 24 well plate.

Cell culture volume 150 µL	Cell culture volume 1000 µL
Injection volume 5 µL	Injection volume 33 µL
Injection volume 10 µL	Injection volume 67 µL
Injection volume 20 µL	Injection volume 133 µL

After the allocated incubation times, the cell viability was measured by the MTT assay, as previously described in section 3.6. Each treatment was carried out in triplicate.

Calculations and statistics

All assays were performed in triplicate with the mean and standard deviations used.

SPSS version 15.0 was used to analyze the data. A p value of < 0.05 was considered as statistically significant.

Reliability was measured by calculation of the coefficient of variation (CV) percentages from the triplicate absorbencies measured for each condition.

The percentage cytotoxicity was determined as stated in 3.6 above.

3.10 Preparation of *Arsenicum album* Succussed and Unsuccussed Potencies

Principle

The *Arsenicum album* succussed (6CH, 30CH and 200CH) and unsuccussed (1×10^{-12}, 1×10^{-60} and 1×10^{-400}) potencies were prepared by the researcher under sterile conditions in the cell culture laboratory of the Biotechnology and Food technology department. It was decided that potencies that did not surpass Avogadro's constant, namely 6CH and 10^{-12}, would also be utilized in the experiments, in order to compare the effects of high and low potencies on cells. These potencies were used the day of their preparation to prevent contamination, and stored thereafter at 4°C. The remedies were first triturated and then, in sterile distilled water, were either hand succussed 10 times or allowed to diffuse for 30 s, with the final potencies made up in RPMI (to prevent interference with cell cultures), according to method 6 and 8b in the German Homoeopathic Pharmacopoeia (GHP) (British Homoeopathic Association, 1993). Fifteen millilitres of each of the succussed and unsuccussed potencies were prepared.

Methodology

According to method 6 in the GHP, the solid basic drug material, namely As_2O_3, was to be triturated with lactose as the vehicle, by hand, in the ratio of 1 to 100 for centesimal potencies (see appendix C). The 9.9 g of lactose was divided into three equal parts of 3.3 g, with the first part triturated in a porcelain mortar with 0.1 g of As_2O_3 for 6 min, followed by scraping using a spatula for 4 min, and repeated before adding the second part of the lactose. The process was then repeated as before with the second and then the third parts of lactose. The minimum time required to make a single potency was thus 1 h, and was repeated for each successive potency up to 3CH, where 1 part (0.1 g) of the preceding potency was triturated for 1 h with 99 parts (9.9 g) of lactose (British Homoeopathic Association, 1993; O' Reilly, 1996).

According to method 8b in the GHP, aqueous preparations were to be produced from triturations made by method 6. One part (0.1 g) of the 3CH trituration was dissolved in 99 parts (10 mL) of sterile distilled water and either succussed 10 times or allowed to diffuse for 30 s, to produce the 4CH or 1×10^{-8} potencies respectively. Each of the liquid centesimal potencies was made from the previous centesimal potency, using sterile distilled water in a ratio of 1 to 100. The aqueous potencies expired after 3 weeks due to possible contamination, hence the 26CH, 196CH, 1×10^{-52} and the 1×10^{-392} potencies were also made up in 96% ethanol, which could be stored for up to 3 years, for preparation of the final doses on the day of the experiments (British Homoeopathic Association, 1993).

3.11 *Arsenicum album* Potency Study using the MT4 Cell Line

Table 3.5: *Arsenicum album* treatments of MT4 cell line.

Volume Cell Culture	**As_2O_3 Dose**	**Volume Cell Culture**	***Arsenicum album* Dose**
50 mL	• 5 µM • 6.65 mL • 24 h & 48 h	1 mL	• Succussed and unsuccussed 6CH; 30CH; 200CH • 133 µL • 48 h & 72 h

Principle

It was noted in 3.9.2 that the MT4 cell line showed stable and reliable results, which followed a constant and steady trend; whereas the PBMCs were seen as erratic with CV values markedly higher than those for the MT4 cells, thus the PBMCs' values were not considered as reliable. For this reason, it was decided that only the MT4 cell line would be utilized for further experiments, in order to obtain the most dependable results.

This experiment was performed so as to determine the therapeutic effects of the various *Arsenicum album* potencies (6CH, 30CH and 200CH) on cells after antagonisation with the IC_{50} of As_2O_3, and to then compare the effects of the succussed and unsuccussed potencies. The previous experiments (section 3.9) established that the optimum IC_{50} of As_2O_3 was 5 µM with an injection volume of 133 µL.

Methodology

The actively growing MT4 cells were diluted down to attain 2.45 x 10^5 cells/mL; the number of viable cells was calculated by means of the trypan blue dye exclusion assay (section 3.5). Into two sterile 75 cm^3 culture flasks, 50 mL of cell culture was added, each antagonised with 5 µM As_2O_3 (6.65 mL), this was calculated as follows:

Table 3.6: Upscaling of injection volumes of As_2O_3 from 24 well plate to 75 cm^3 flask.

1 mL cell culture	133 µL As_2O_3
50 mL cell culture	6650 µL As_2O_3

The flasks were labeled A and B and incubated at 37°C in 5% CO_2 atmosphere for 24 h and 48 h respectively. After incubation, 8 mL of cell culture was decanted from each flask into a 15 mL centrifuge tube, which was then used later to plate cells untreated by potencies but containing As_2O_3. The remaining culture was then poured into a 50 mL centrifuge tube and centrifuged for 5 min at 3 500 rpm. A pellet of cells formed at the bottom of the tube, and the media (containing As_2O_3) was carefully discarded. Fifty millilitres of freshly made CCM was added to the pellet, which was then resuspended.

Into each well of the two 24-well plates, 1 mL of cell culture (2.35 x 10^5 cells/mL – flask A; 2.25 x 10^5 cells/mL – flask B) was added. Subsequently, 133 µL of the remedies and controls were added to each well and incubated for 48 h (plate A1 and B1) and 72 h (plate A2 and B2) respectively.

After the prescribed incubation times, the MTT assay was performed on each plate in order to identify cell viability (section 3.6). All the above treatments were done in triplicate. The cells were examined under phase contrast light microscope and their morphological changes were monitored. The viable cells were seen as translucent, whereas the cells approaching death increased in density and became darker in colour. The trypan blue stained cells were also examined under phase contrast light microscope, with pictures taken in order to examine the viable and non-viable cells.

Calculations and statistics

All assays were performed in triplicate with the mean and standard deviations used.

SPSS version 15.0 was used to analyze the data. A p value of < 0.05 was considered as statistically significant.

The percentage cell viability was determined using the formula:

$$\% \text{ cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

In order to assess the effect of several conditions on cell viability, univariate ANOVA testing was used with three independent variables, namely plate, succussion and treatment. A full factorial model was used. Bonferroni post hoc tests were performed for treatment and plate in order to assess which treatments and plates were significantly different from each other.

CHAPTER 4

RESULTS

4.1 Solubilization of Arsenic Trioxide

The initial dissolution experiments resulted in minimal solubilization of the As_2O_3; this was measured using the MTT assay on human cells with an ELISA reader (see Table 3.1). The least solubilization was found with 10 min stirring of the As_2O_3 in distilled water, using no heat. The subsequent dissolution experiments tested different solvents (distilled water, RPMI, PBS and saline solution), in which the As_2O_3 was stirred for 24 h using no heat, and resulted in insignificant amounts of solubilization. The succeeding experiment proved more effective, with an increase in the amount of solubilization, where the As_2O_3 was left unstirred on a heat of 50°C, in distilled water, for 48 h. This result was not completely successful however, thus the final dissolution experiment was completed using higher heat and longer time.

The final results regarding the solubility of As_2O_3 established that in order to obtain a fully dissolved 20 mM As_2O_3 solution, it was necessary to add 396 mg of dry As_2O_3 to 100 mL sterile distilled Milli-Q water, and let it stand for a minimum of 10 days at a heat of at least 80°C. This was evaluated by the cytotoxic action of the As_2O_3 solution on human cells; the number of viable cells per millilitre was examined using the trypan blue dye exclusion assay (Table 4.1) and spectrophotometric results were analysed from the MTT assay (Tables 4.2 and 4.3 and Figures 4.1 and 4.6); it was assumed that if cytotoxicity was evident, then the As_2O_3 that was used to antagonise the cells was fully dissolved. Solubilization was also assessed through observation of the macroscopic appearance of the stock solution, which appeared clear, with no visible signs of As_2O_3 granules; and the comparison between the macroscopic appearance of cell cultures with and without As_2O_3, with regard to the consumption of media by the viable cells (Figure 4.2).

Table 4.1: Time response of MT4 cells to As_2O_3 (5 µM) using the trypan blue dye exclusion assay.

Treatment	MT4 viable cell titre per mL
Untreated CCM	$2.65 \times 10^5 \pm 0.2828$
24 h of 5 µM As_2O_3 (133 µL)	$2.55 \times 10^5 \pm 0.2828$
48 h of 5 µM As_2O_3 (133 µL)	$2.25 \times 10^5 \pm 0.00$

Mean ± SD are given of two replicates

4.2 Arsenic Trioxide Cytotoxicity

4.2.1 Arsenic Trioxide Dose Response on Peripheral Blood Mononuclear Cells

Various doses of As_2O_3 were given to healthy PBMCs over 24 h and 48 h in an attempt to measure the percentage cytotoxicity using the MTT assay. Doses of 1 µM, 5 µM and 10 µM were tested in volumes of 5 µL, 10 µL, 20 µL and 50 µL; controls were included, with triplicate wells combined. The MTT assay measured the absorbance, which represented the amount of formazan dye produced by viable cells in the culture, seen in Table 4.2 below. The percentage cytotoxicity was then calculated and represented in Figure 4.1 below.

Table 4.2: Dose response of peripheral blood mononuclear cells (PBMCs) to As_2O_3 using the MTT assay (578 nm).

Dose	As_2O_3 for 24 h	As_2O_3 for 48 h
Untreated	0.1356	0.311
RPMI 5 µL	0.0617	0.1549
RPMI 10 µL	0.0656	0.2262
RPMI 20 µL	0.1144	0.1001
RPMI 50 µL	0.0435	0.0838
1 µM As_2O_3 5µL	0.0334	0.2213
1 µM As_2O_3 10 µL	0.0785	0.1192
1 µM As_2O_3 20 µL	0.0312	0.0947
1 µM As_2O_3 50 µL	0.0487	0.0733
5 µM As_2O_3 5 µL	0.0475	0.1355
5 µM As_2O_3 10 µL	0.0189	0.1281
5 µM As_2O_3 20 µL	0.0069	0.1039
5 µM As_2O_3 50 µL	0.0159	0.0706
10 µM As_2O_3 5 µL	0.043	0.1289
10 µM As_2O_3 10 µL	0.2067	0.1167
10 µM As_2O_3 20 µL	0	0.1092
10 µM As_2O_3 50 µL	0.0554	0.5649
DMSO 5 µL	0.0726	0.2777
DMSO 10 µL	0.1709	0.2167
DMSO 20 µL	0.1217	0.1997
DMSO 50 µL	0.0656	0.2108

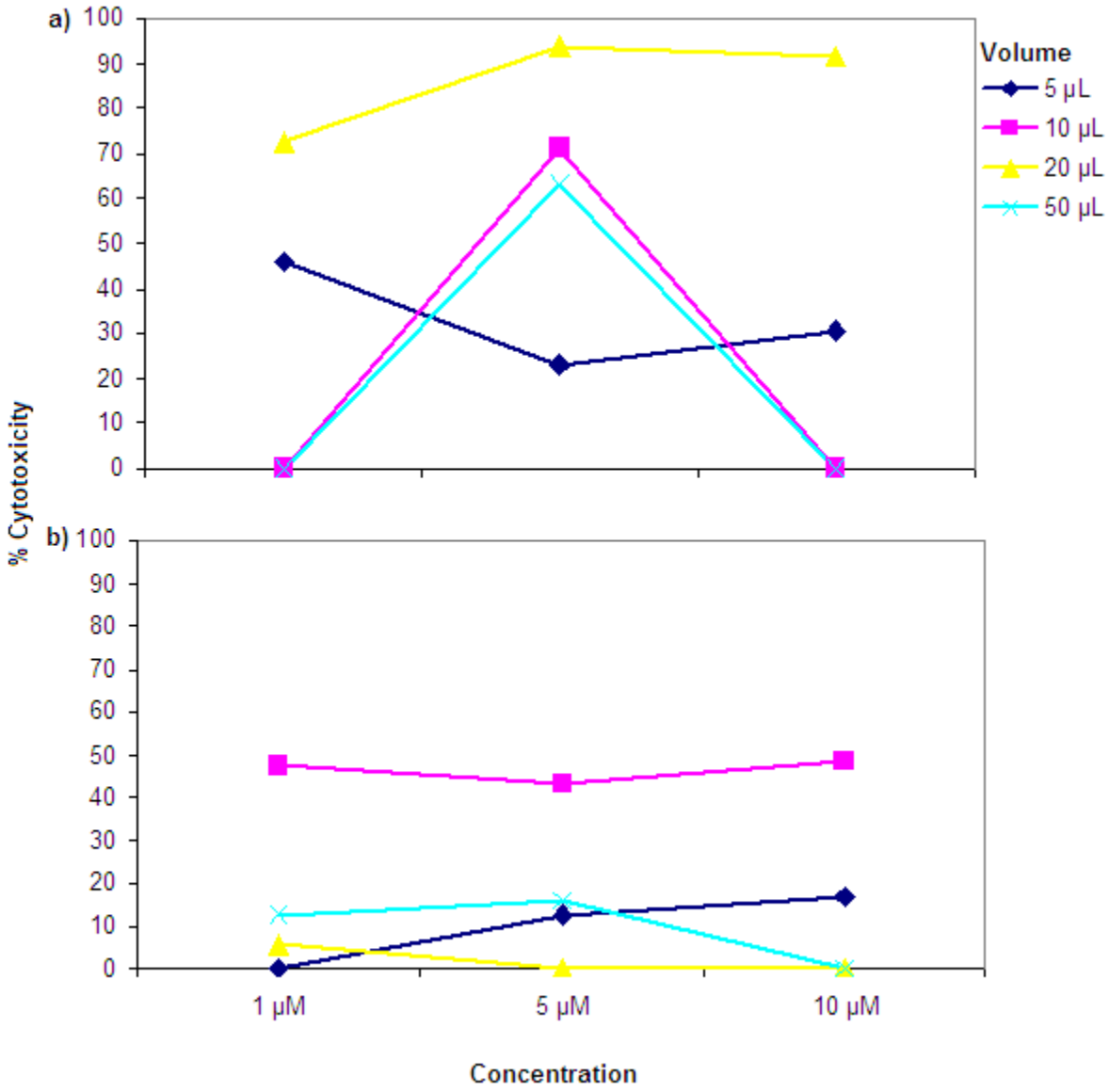


Figure 4.1: Percentage cytotoxicity for the dose response of As_2O_3 (1 – 10 µM; 5 – 50 µL) on PBMCs (a) 24 h exposure (b) 48 h exposure.

The absorbance results (Table 4.2) and hence the cytotoxicity percentages (Figure 4.1) for the PBMCs (4 x 10^5 cells/mL) exposed to As_2O_3 in the 96 well microtitre plate (150 µL/well), did not show a definite trend over both time periods of 24 h and 48 h. However, the 5 µM of As_2O_3 showed the highest cytotoxicity percentages with most of the volumes at 24 h.

4.2.2 Comparison of Arsenic Trioxide Dose Response on the MT4 Cell Line and PBMCs

Analysis of the cytotoxicity results of the above experiment on PBMCs established that the 5 µM As_2O_3 was the most effective concentration of As_2O_3 in causing cell death. The volumes however did not follow any trend; consequently there was need for further investigation. The MT4 cell line was used along with the PBMCs for comparison of reliability and cellular reactions to As_2O_3. The cells were initially cultured, followed by antagonisation with 5 µM As_2O_3 (Figure 4.2). The cells were viewed in suspension (Figure 4.3) as well as stained with trypan blue solution and counted (Figure 4.4). Healthy PBMCs and MT4 cells were plated, antagonised and stained with MTT (Figure 4.5) for testing absorbance (Table 4.3) and percentage cytotoxicity (Figure 4.6). Viable MT4 cells were stained with MTT, and illustrated high concentrations of un-antagonised cells and controls, with decreasing concentrations of cells antagonised with increasing doses of As_2O_3.

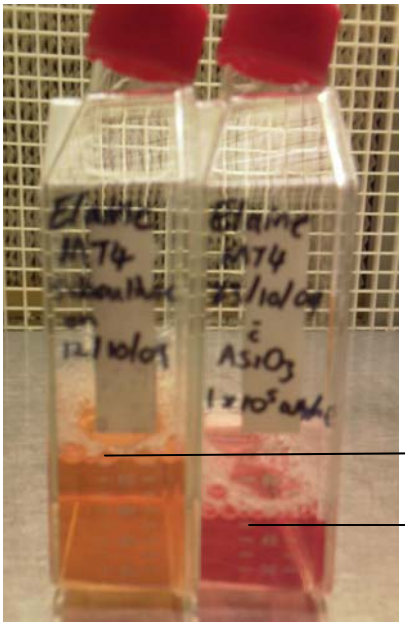


Figure 4.2: MT4 cell cultures (50 mL) with and without 5 µM As_2O_3 (6.65 mL), incubated for 48 h at 37°C in 5% CO_2. Un-antagonised cell culture changed from pink to orange (active growth), whereas the As_2O_3-antagonised cell culture remained pink (slight growth).

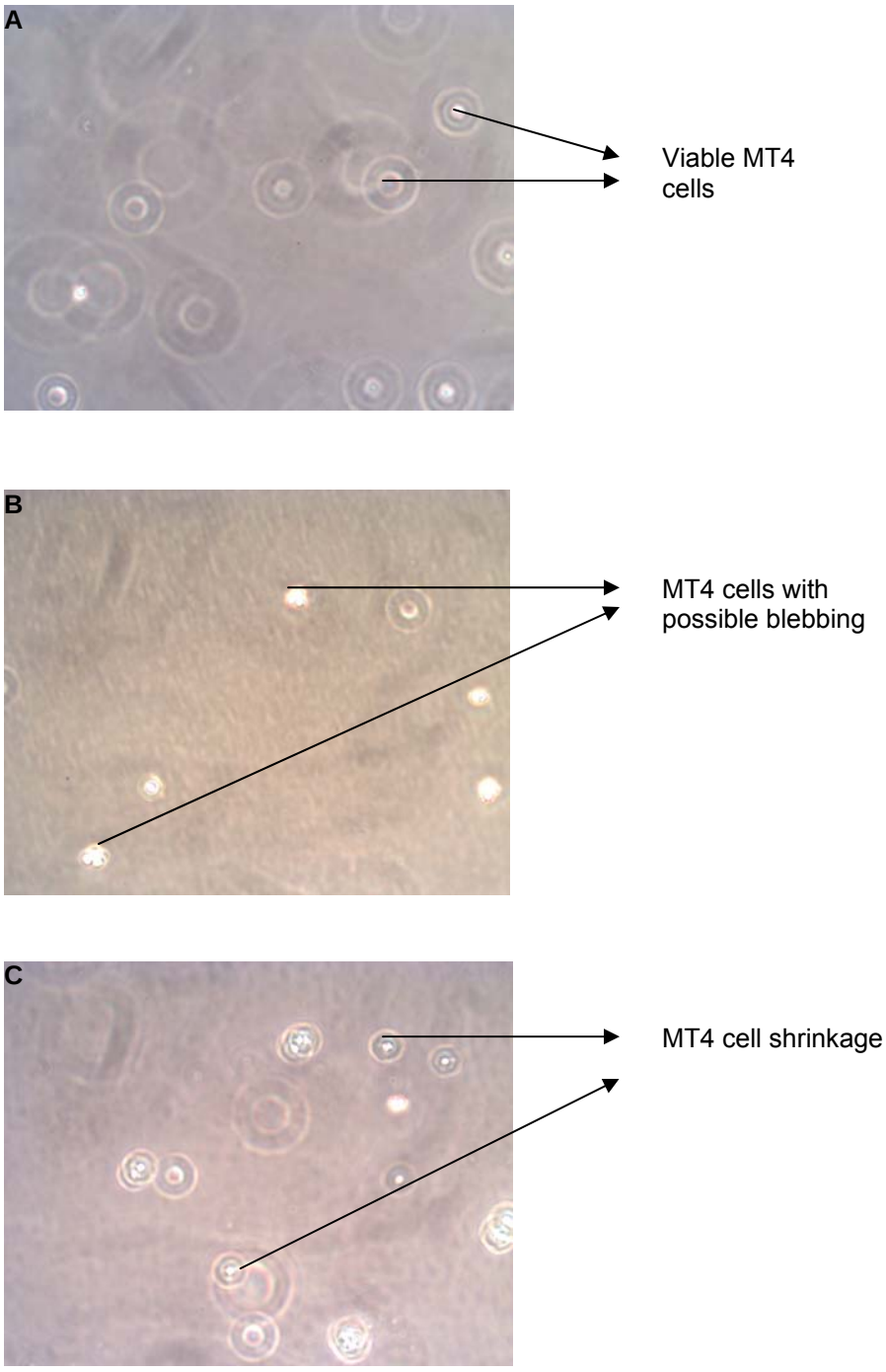


Figure 4.3: MT4 cell morphology viewed on a phase contrast microscope (Nikon)

(A) before antagonised with As_2O_3; (B) 24 h after antagonised with 5 µM As_2O_3

(133 µL); (C) 48 h after antagonised with 5 µM As_2O_3 (133 µL) (mag. 10 X).

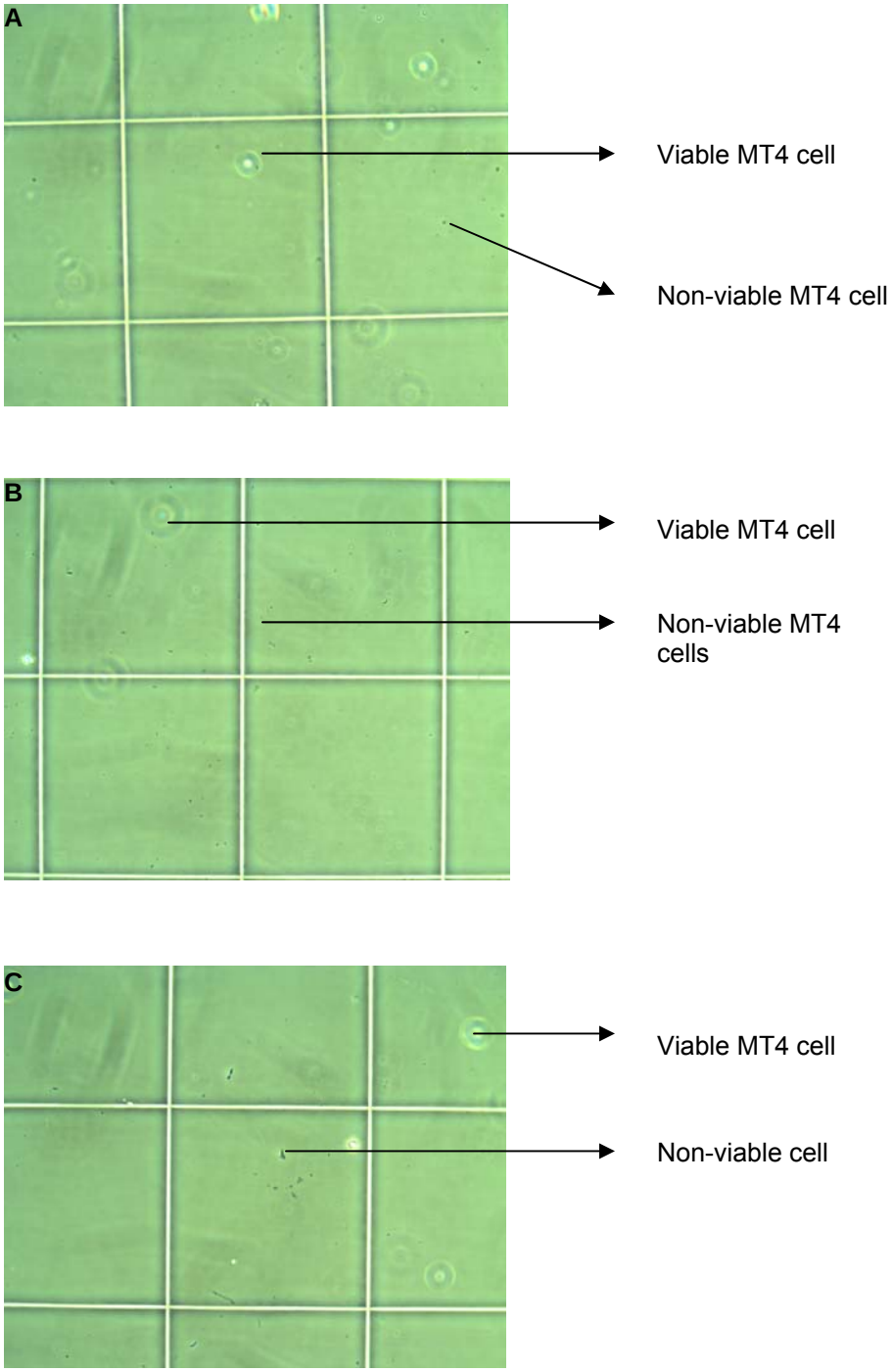


Figure 4.4: Time response of MT4 cells to As_2O_3 (5 µM) using the trypan blue dye exclusion assay (A) 2.85 x 10^5 cells/mL before antagonised with As_2O_3; (B) 2.75 x 10^5 cells/mL after antagonised with 5 µM As_2O_3 (133 µL) for 24 h; (C) 2.25 x 10^5 cells/mL after antagonised with 5 µM As_2O_3 (133 µL) for 48 h (mag. 10 X).

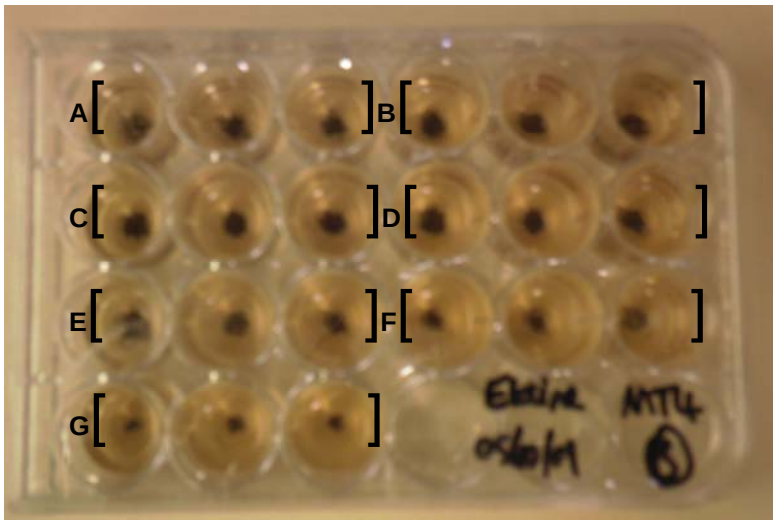


Figure 4.5: MT4 cell culture (1 mL/well), incubated in a 24 well plate at 37°C in 5% CO_2, with controls and As_2O_3 for 72 h. (A) Un-antagonised; (B) RPMI (33 µL); (C) RPMI (67 µL); (D) RPMI (133 µL); (E) 5 µM As_2O_3 (33 µL); (F) 5 µM As_2O_3 (67 µL); (G) 5 µM As_2O_3 (133 µL).

Table 4.3: Dose and time response of MT4 cells and PBMCs to 5 µM As_2O_3 (578 nm).

Cell	Time	Treatment	Mean	N	Std. Deviation	CV%
MT4	48 h	5 µM As_2O_3 133 µL	0.13	3	0.0147	10.91
		5 µM As_2O_3 33 µL	0.22	3	0.0036	1.67
		5 µM As_2O_3 67 µL	0.19	3	0.0246	12.77
		RPMI 133 µL	0.20	3	0.0033	1.64
		RPMI 33 µL	0.25	3	0.0049	2.00
		RPMI 67 µL	0.23	3	0.0133	5.69
		Un-antagonised	0.26	3	0.0050	1.89
	72 h	5 µM As_2O_3 133 µL	0.20	3	0.0176	8.61
		5 µM As_2O_3 33 µL	0.35	3	0.0626	17.76
		5 µM As_2O_3 67 µL	0.28	3	0.0144	5.21
		RPMI 133 µL	0.35	3	0.0328	9.24
		RPMI 33 µL	0.44	3	0.0316	7.17
		RPMI 67 µL	0.39	3	0.0278	7.21
		Un-antagonised	0.43	3	0.0159	3.73
PBMC	48 h	5 µM As_2O_3 133 µL	0.02	3	0.0198	86.66
		5 µM As_2O_3 33 µL	0.15	3	0.0834	56.31
		5 µM As_2O_3 67 µL	0.07	3	0.0486	68.08
		RPMI 133 µL	0.10	3	0.0586	58.70
		RPMI 33 µL	0.37	3	0.4299	114.95
		RPMI 67 µL	0.30	3	0.3907	131.17
		Un-antagonised	0.49	3	0.4143	84.44
	72 h	5 µM As_2O_3 133 µL	2.09	3	0.2131	10.20
		5 µM As_2O_3 33 µL	1.93	3	0.1581	8.20
		5 µM As_2O_3 67 µL	1.79	3	0.3630	20.27
		RPMI 133 µL	1.72	3	0.3832	22.29
		RPMI 33 µL	1.29	3	0.3256	25.19
		RPMI 67 µL	1.84	3	0.3002	16.31
		Un-antagonised	1.45	3	0.2292	15.77

Percentage cytotoxicity by cell type, treatment and time is shown in Figure 4.6 below. The cytotoxic activity in the MT4 cells increased between 48 h and 72 h, but in the PBMCs the cytotoxic activity decreased between these times. The percentage cytotoxicity of the 133 µL dose was highest, followed by the 67 µL and lastly the 33 µL.

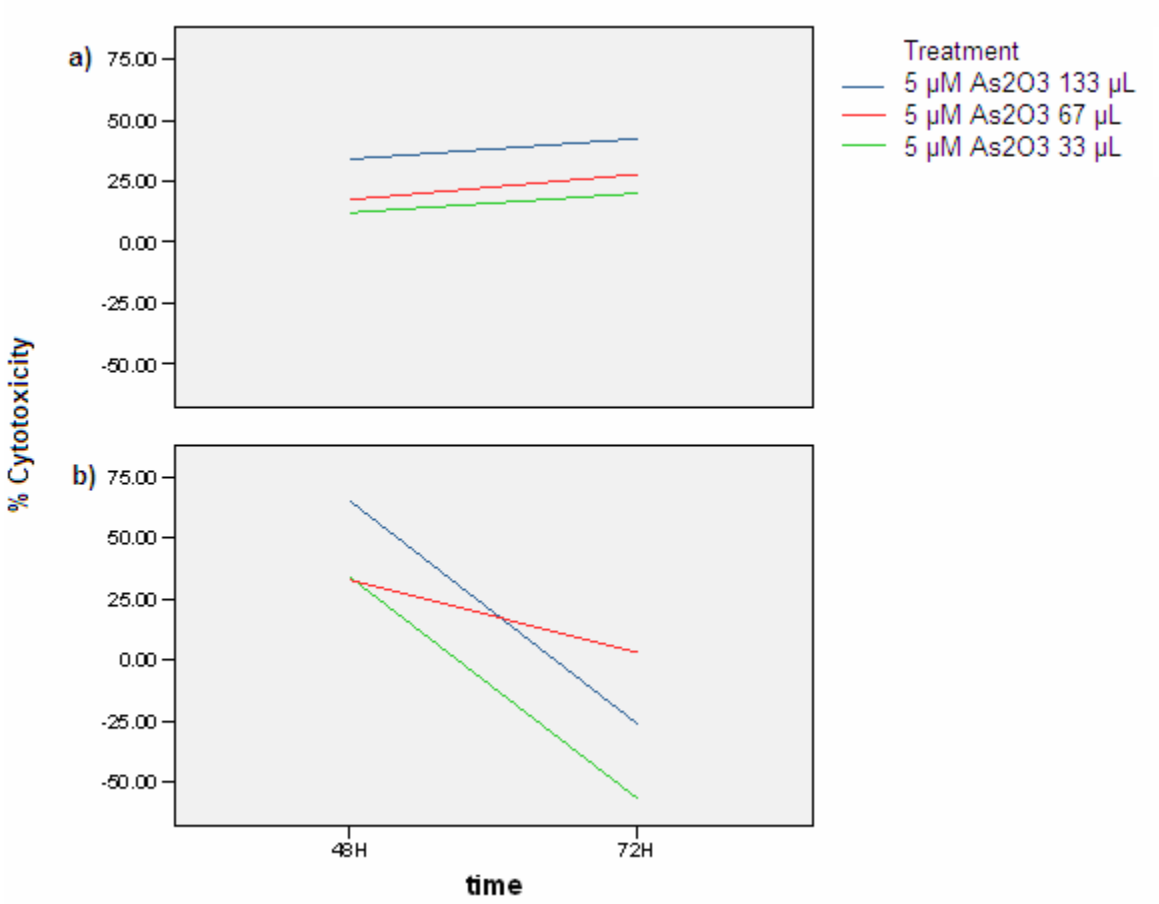


Figure 4.6: Percentage cytotoxicity for the time response of 5 µM As_2O_3 (33 – 133 µL) for 48 h and 72 h; (a) MT4 cell line (b) PBMCs.

The results concerning the cytotoxicity studies of As_2O_3 on MT4 cells and PBMCs showed the MT4 cells to be more stable and hence more reliable when testing with As_2O_3 using the MTT assay; whereas the PBMCs proved to be inconsistent, with CV values (measuring the reliability percentages) considerably higher than those for the MT4 cells; consequently the PBMCs' values were not considered as reliable and hence only the MT4 cells were used for further experiments. This can be seen in Table 4.3 above.

The MT4 cell results showed that the dose closest to the IC_{50} of As_2O_3 was 5 µM with an injection volume of 133 µL for 1 mL of cell culture. This can be seen in Figure 4.6 above, where there is a steady increase in the percentage of cytotoxicity over time and with an increase in dose. The mean and standard deviations (Table 4.3) were calculated from the triplicate results for each treatment (see appendix F for the triplicate absorbance readings of each treatment). The percentage cytotoxicity (Figure 4.6) was calculated according to the relevant RPMI controls which corresponded to the specific treatments with regard to their volumes.

4.3 *Arsenicum album* Potency Study using the MT4 Cell Line

The previous cytotoxicity experiments established that 5 µM As_2O_3 (133 µL for 1 mL cell culture) was the dose closest to the As_2O_3 IC_{50} on the MT4 cell line. This dose was therefore used in the *Arsenicum album* study to antagonise the cells prior to treatment with the various *Arsenicum album* potencies.

The MT4 cell line was cultured in two 75 cm^3 culture flasks (50 mL/flask) and incubated for 24 h and 48 h, each with 5 µM As_2O_3 (6.65 mL). Thereafter the As_2O_3-antagonised media was removed and replaced with fresh media. The cell cultures (2.75 x 10^5 cells/mL after 24 h; 2.25 x 10^5 cells/mL after 48 h) were then plated and treated with 133 µL of *Arsenicum album* succussed and unsuccussed 6CH, 30CH and 200CH potencies and incubated for a further 48 h and 72 h. Images of the treated cells in the wells were captured using the phase contrast microscope (Nikon, USA) which was connected to an image-capturing device (Figures 4.7 to 4.10). The MTT assay was then performed to determine the absorbance of the viable cells (Table 4.4) and cell viability percentages (Table 4.5 and Figure 4.11).

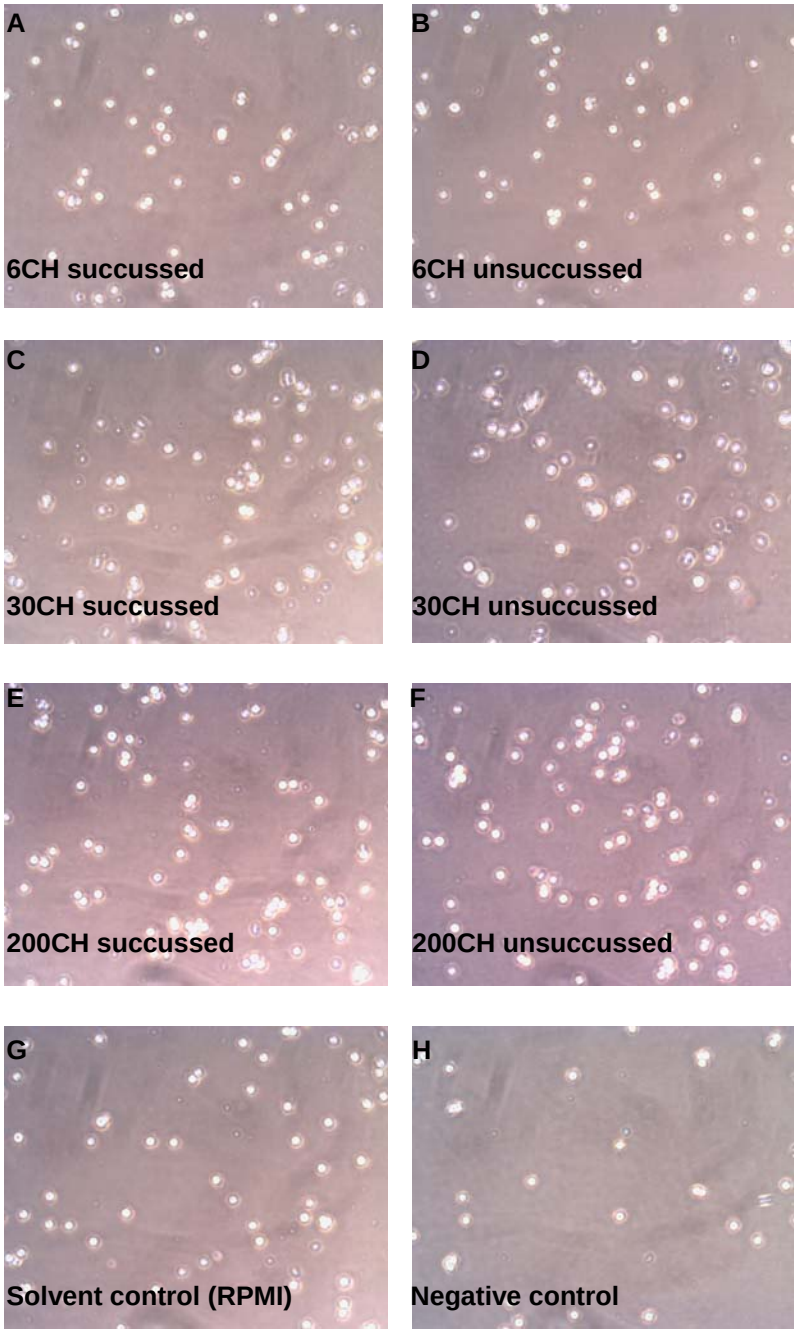


Figure 4.7: Morphology of MT4 cell cultures (Plate A1 – 24 h As_2O_3; 48 h *Arsenicum album*). (A) 6CH succussed (B) 6CH unsuccussed (C) 30CH succussed (D) 30CH unsuccussed (E) 200CH succussed (F) 200CH unsuccussed *Arsenicum album* potencies (G) Solvent control (RPMI) titre of 2.75 x 10^5 cells/mL (H) Negative control (only As_2O_3; no remedy) (mag. 10 X).

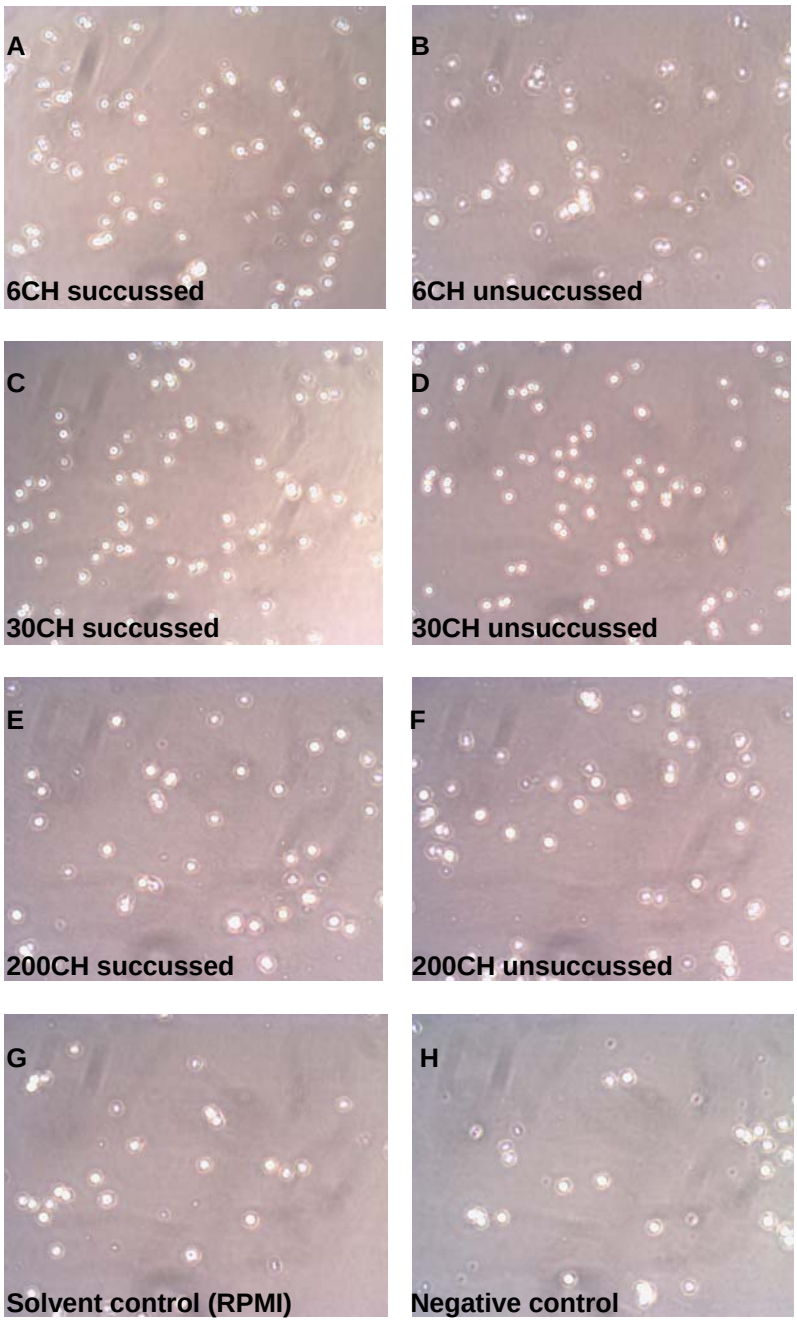


Figure 4.8: Morphology of MT4 cell cultures (Plate A2 – 24 h As_2O_3; 72 h *Arsenicum album*). (A) 6CH succussed (B) 6CH unsuccussed (C) 30CH succussed (D) 30CH unsuccussed (E) 200CH succussed (F) 200CH unsuccussed *Arsenicum album* potencies (G) Solvent control (RPMI) titre of 2.75 x 10^5 cells/mL (H) Negative control (only As_2O_3; no remedy) (mag. 10 X).

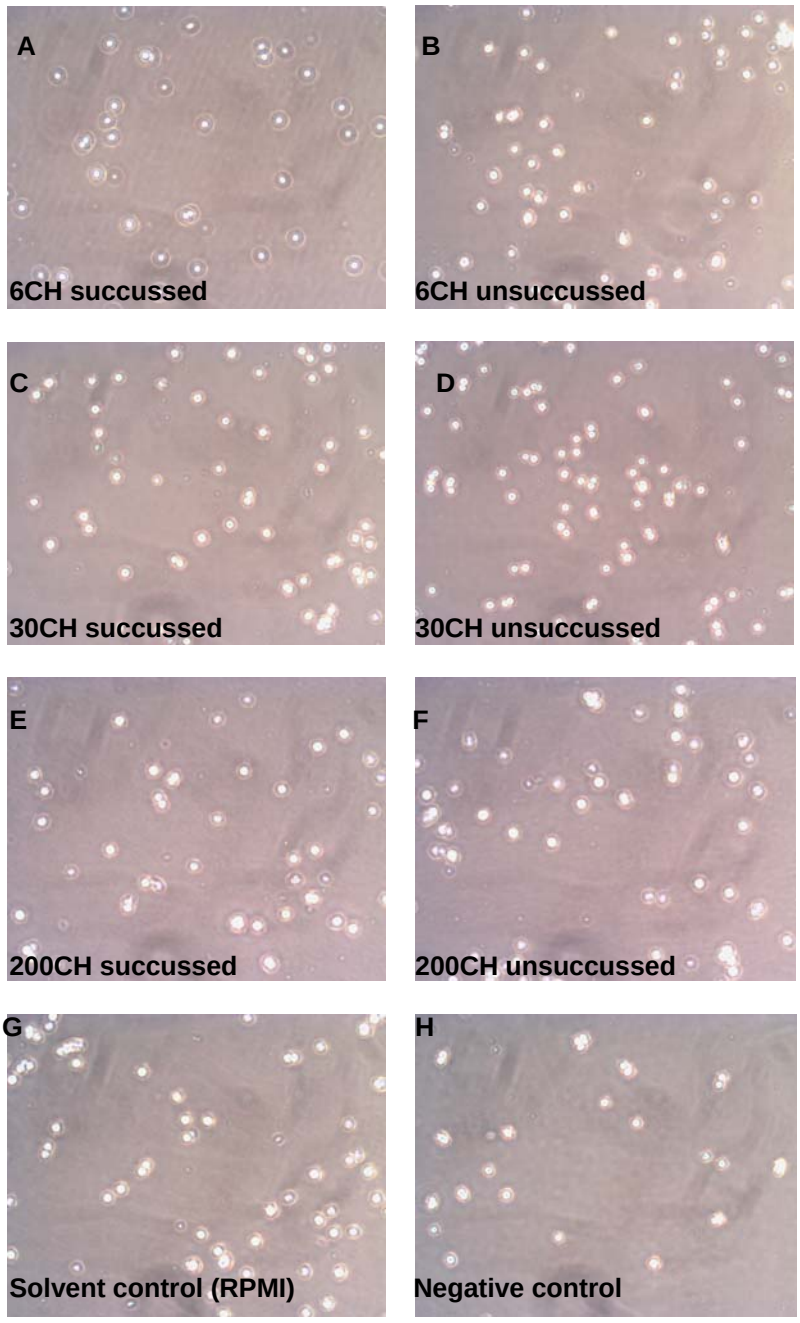


Figure 4.9: Morphology of MT4 cell cultures (Plate B1 – 48 h As_2O_3; 48 h *Arsenicum album*). (A) 6CH succussed (B) 6CH unsuccussed (C) 30CH succussed (D) 30CH unsuccussed (E) 200CH succussed (F) 200CH unsuccussed *Arsenicum album* potencies (G) Solvent control (RPMI) titre of 2.75 x 10^5 cells/mL (H) Negative control (only As_2O_3; no remedy) (mag. 10 X).

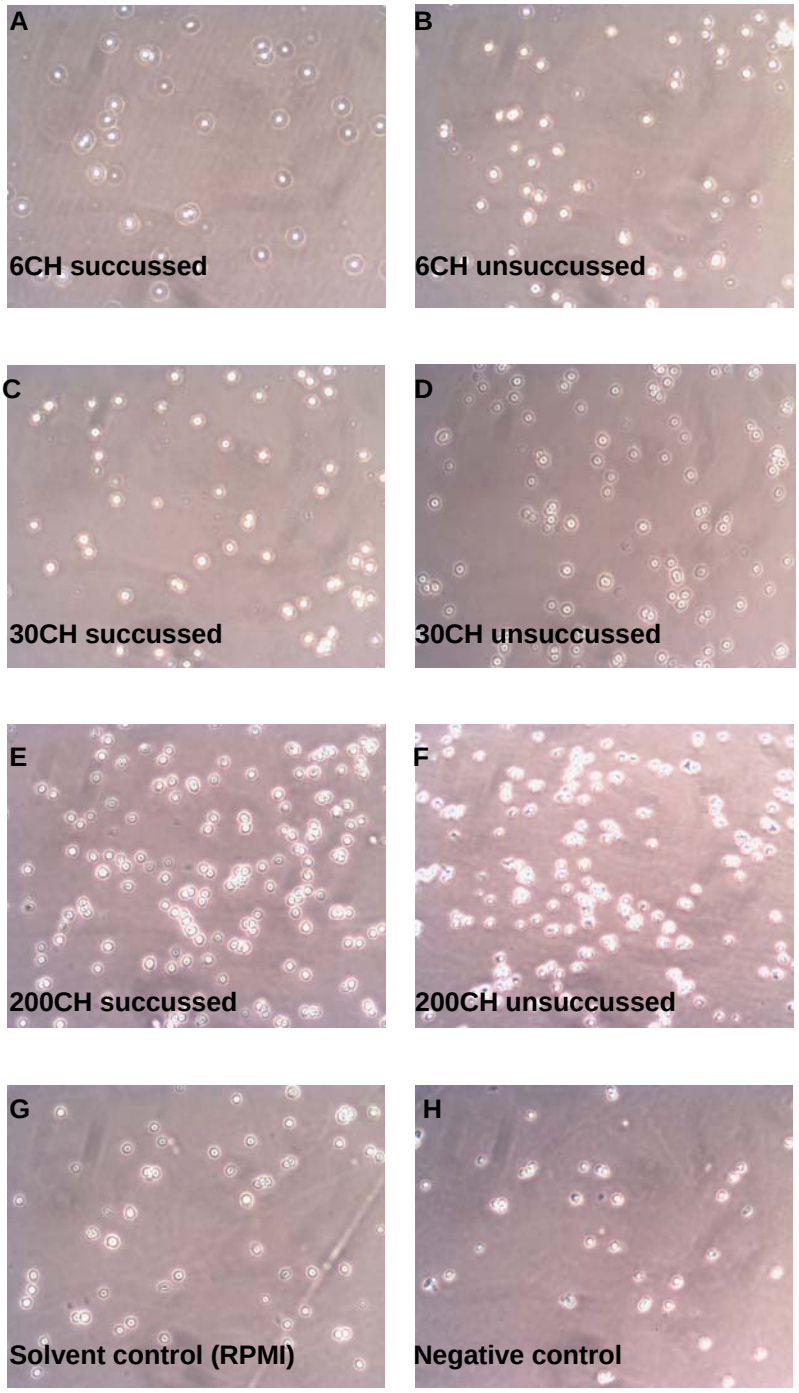


Figure 4.10: Morphology of MT4 cell cultures (Plate B2 – 48 h As_2O_3; 72 h *Arsenicum album*). (A) 6CH succussed (B) 6CH unsuccussed (C) 30CH succussed (D) 30CH unsuccussed (E) 200CH succussed (F) 200CH unsuccussed *Arsenicum album* potencies (G) Solvent control (RPMI) titre of 2.75 x 10^5 cells/mL (H) Negative control (only As_2O_3; no remedy) (mag. 10 X).

Table 4.4: Potency response of *Arsenicum album* on the MT4 cell line after antagonisation with 5 µM As_2O_3 (133 µL) using the MTT assay (578 nm).

Treatments		24 well plates (1 mL cell culture/well)							
		*A1		*A2		*B1		*B2	
		Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
Negative control		0.0593	0.0124	0.0365	0.0046	0.0458	0.0046	0.0471	0.0127
Solvent control (RPMI)		0.0306	0.0028	0.0240	0.0093	0.0332	0.0068	0.0378	0.0138
6CH	Succussed	0.0323	0.0019	0.0168	0.0004	0.0192	0.0023	0.0285	0.0029
	Unsuccussed	0.0249	0.0044	0.0175	0.0077	0.0178	0.0086	0.0349	0.0082
30CH	Succussed	0.0329	0.0072	0.0234	0.0014	0.0166	0.0025	0.0357	0.0018
	Unsuccussed	0.0376	0.0083	0.0268	0.0053	0.0242	0.0041	0.0373	0.0081
200CH	Succussed	0.0459	0.0038	0.0304	0.0043	0.0367	0.0134	0.0681	0.0035
	Unsuccussed	0.0476	0.0023	0.0333	0.0070	0.0361	0.0082	0.0609	0.0035

*Plate A1 = 24 h As_2O_3 followed by 48 h treatment
*Plate A2 = 24 h As_2O_3 followed by 72 h treatment
*Plate B1 = 48 h As_2O_3 followed by 48 h treatment
*Plate B2 = 48 h As_2O_3 followed by 72 h treatment

Table 4.5: Percentage cell viability of MT4 cell cultures treated with *Arsenicum album* potencies (6 – 200CH) after antagonisation with 5 µM As_2O_3 (133 µL).

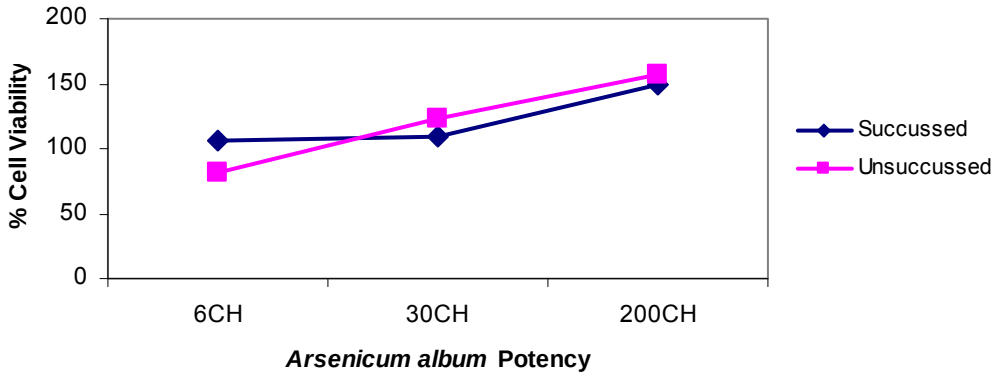
Arsenicum album Treatments		24 well plates (1 mL cell culture/well)			
		*A1	*A2	*B1	*B2
6CH	Succussed	105.90	76.71	60.53	81.09
	Unsuccussed	81.33	72.13	52.07	96.88
30CH	Succussed	109.25	107.51	52.33	102.68
	Unsuccussed	122.35	117.42	74.94	102.80
200CH	Succussed	149.99	139.83	112.00	193.97
	Unsuccussed	156.58	147.62	110.08	175.66

*Plate A1 = 24 h As_2O_3 followed by 48 h treatment
*Plate A2 = 24 h As_2O_3 followed by 72 h treatment
*Plate B1 = 48 h As_2O_3 followed by 48 h treatment
*Plate B2 = 48 h As_2O_3 followed by 72 h treatment

a)

Plate A1

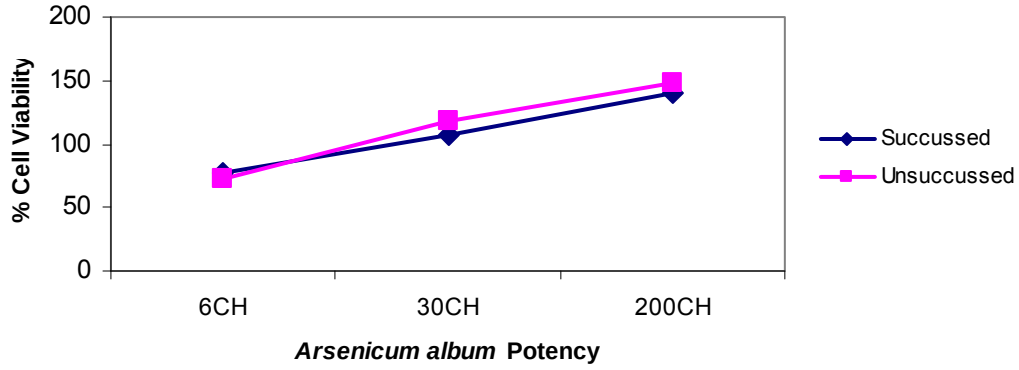
24 h As_2O_3 followed by 48 h treatment with *Arsenicum album*



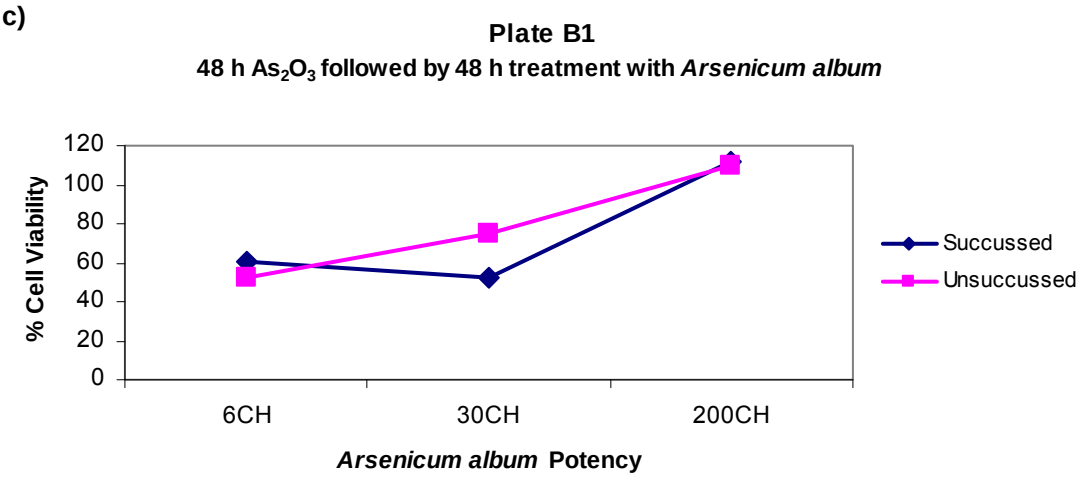
b)

Plate A2

24 h As_2O_3 followed by 72 h treatment with *Arsenicum album*



c)



d)

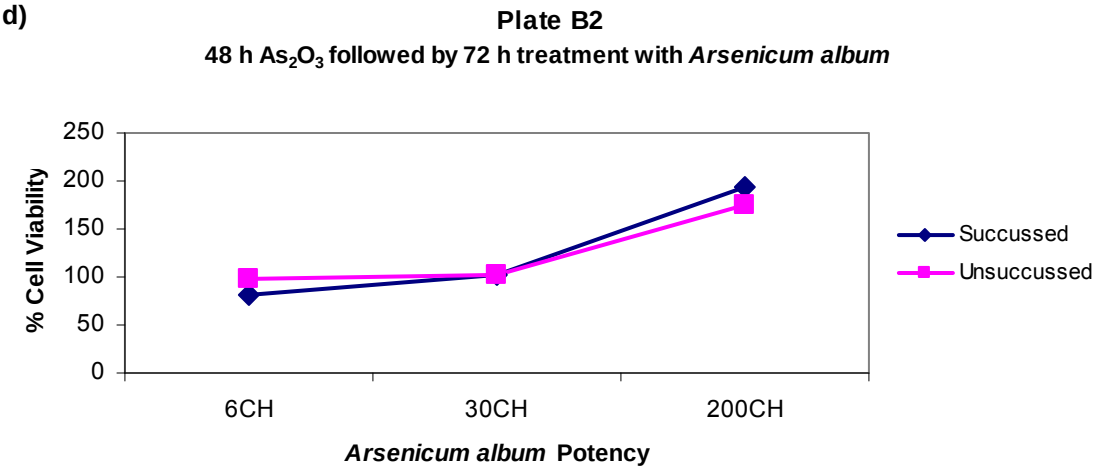


Figure 4.11: Percentage cell viability of MT4 cell cultures treated with *Arsenicum album* potencies (6 – 200CH) comparing succussed and unsuccussed treatments; (a) plate A1 (24 h As_2O_3 followed by 48 h *Arsenicum album*) (b) plate A2 (24 h As_2O_3 followed by 72 h *Arsenicum album*) (c) plate B1 (48 h As_2O_3 followed by 48 h *Arsenicum album*) (d) plate B2 (48 h As_2O_3 followed by 72 h *Arsenicum album*).

The results obtained from this experiment verified the therapeutic effects of the various succussed and unsuccussed *Arsenicum album* potencies (6CH, 30CH and 200CH) on human cancer cells after antagonisation with the IC_{50} of As_2O_3, by increasing the percentage of cell viability from 65.95% after exposure to As_2O_3 for 48 h and 57.51% after 72 h, to a maximum of 193.97% cell viability when treated thereafter with succussed *Arsenicum album* 200CH for 72 h (Table 4.5). It was also noted that the results for the succussed and unsuccussed potencies did not vary to an extent where they could be distinguishable (Figure 4.11). This empirical evidence has been shown as reliable due to its consistent, precise and repeatable nature, and is therefore seen as valuable in the scientific and homoeopathic worlds.

4.4 Statistical Analysis

SPSS version 15.0 was used to analyze the data. A p value of < 0.05 was considered as statistically significant. Below is the univariate ANOVA table (Table 4.6) which examines the effects of plate, succussion and treatment (the 3 between subjects' effects) on their own (as main effects) as well as in combination (as interaction effects). The dependent variable used is the percentage cell viability. The 'sig' column shows if the p value is < 0.05 and hence whether that effect is significant.

Table 4.6: Statistical analysis (ANOVA) of *Arsenicum album* succussed and unsuccussed potencies over exposure time.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	97339.867(a)	23	4232.168	4.250	0.000
Intercept	846069.182	1	846069.182	849.640	0.000
Plate (exposure time)	25902.627	3	8634.209	8.671	0.000
Succussion	40.764	1	40.764	0.041	0.841
Treatment	62025.353	2	31012.676	31.144	0.000
Plate * Succussion	134.746	3	44.915	0.045	0.987
Plate * Treatment	6158.033	6	1026.339	1.031	0.417
Succussion * Treatment	935.248	2	467.624	0.470	0.628
Plate * Succussion * Treatment	2143.096	6	357.183	0.359	0.901
Error	47798.274	48	995.797		
Total	991207.323	72			
Corrected Total	145138.141	71			

a R Squared = 0.671 (Adjusted R Squared = 0.513)

It was found that the main effects of plate and treatment were statistically significant determinants of cell viability in the model ($p < 0.001$). There were no significant interaction effects, and succussion was also seen as not being significant ($p > 0.05$). In order to discover which exposure times (plates A1, A2, B1 and B2) were significantly different from each other and which treatments (*Arsenicum album* 6CH, 30CH and 200CH) were significantly different from each other, the Bonferroni post hoc tests were used (Table 4.7 and 4.8), with the percentage cell viability as the dependent variable.

Table 4.7: Bonferroni multiple comparisons test of As_2O_3 antagonisation exposure with potency treatment exposure.

(I) plate	(J) plate	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
A1 (**24 h As_2O_3 followed by 48 h *Arsenicum album***)	**A2**	10.7005	10.51875	1.000	-18.2473	39.6484
	B1	43.9124(*)	10.51875	0.001	14.9646	72.8603
	B2	-4.6116	10.51875	1.000	-33.5594	24.3363
A2 (**24 h As_2O_3 followed by 72 h *Arsenicum album***)	**A1**	-10.7005	10.51875	1.000	-39.6484	18.2473
	B1	33.2119(*)	10.51875	0.017	4.2641	62.1598
	B2	-15.3121	10.51875	0.912	-44.2600	13.6357
B1 (**48 h As_2O_3 followed by 48 h *Arsenicum album***)	**A1**	-43.9124(*)	10.51875	0.001	-72.8603	-14.9646
	A2	-33.2119(*)	10.51875	0.017	-62.1598	-4.2641
	B2	-48.5240(*)	10.51875	0.000	-77.4719	-19.5762
B2 (**48 h As_2O_3 followed by 72 h *Arsenicum album***)	**A1**	4.6116	10.51875	1.000	-24.3363	33.5594
	A2	15.3121	10.51875	0.912	-13.6357	44.2600
	B1	48.5240(*)	10.51875	0.000	19.5762	77.4719

Based on observed means.
* The mean difference is significant at the 0.05 level.

Table 4.8: Bonferroni multiple comparisons test of *Arsenicum album* treatments (6CH, 30CH and 200CH).

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
6CH	**30CH**	-20.3302	9.10951	0.091	-42.9289	2.2685
	200CH	-69.8861(*)	9.10951	0.000	-92.4848	-47.2874
30CH	**6CH**	20.3302	9.10951	0.091	-2.2685	42.9289
	200CH	-49.5559(*)	9.10951	0.000	-72.1546	-26.9572
200CH	**6CH**	69.8861(*)	9.10951	0.000	47.2874	92.4848
	30CH	49.5559(*)	9.10951	0.000	26.9572	72.1546

Based on observed means.
* The mean difference is significant at the 0.05 level.

Table 4.7 shows that plate A1 and B1 were significantly different in relation to percentage cell viability, as well as A2 and B1, and B1 and B2. In terms of treatments, Table 4.8 shows that the 200CH group was significantly different from both the 6CH and 30CH groups. The Figure 4.12 below represents the estimated marginal means of cell viability, where the succussed and unsuccussed potency results have been averaged; this shows that the 200CH with plate B2 provided the highest level of cell viability.

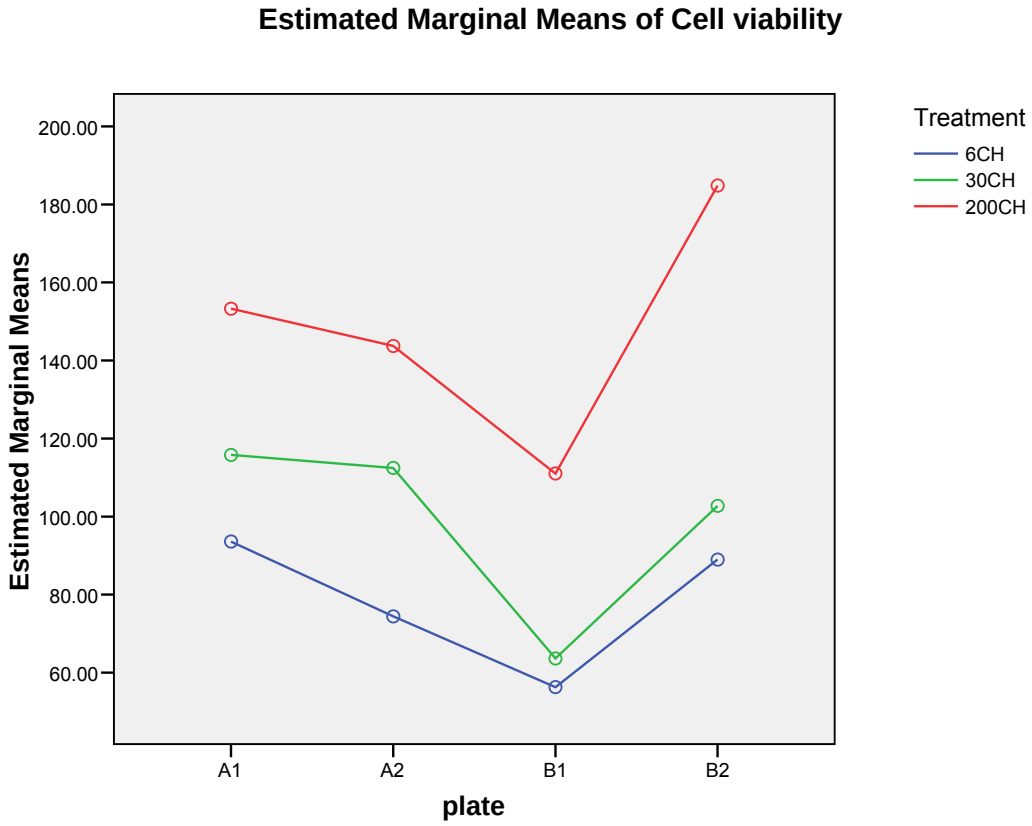


Figure 4.12: Estimated marginal means of cell viability for various *Arsenicum album* potencies (6 – 200CH) and exposure times (24 h, 48 h) on the MT4 cell line.

CHAPTER 5

DISCUSSION

5.1 Arsenic Trioxide Solubilization

Various methods and solvents were used in an attempt to dissolve As_2O_3 with minimal interaction between the solvents and the cells in culture (Table 3.1). Many of the methods stated in previous literature mention using chemicals that could potentially affect the health of the cells in the culture, such as potassium hydroxide solution, chloroform water and hydrochloric acid used by Martindale (1967) and sodium hydroxide (NaOH) which was used by Shim et al. (2002), who stated that the maximum concentration of NaOH in the culture had no influence on the cell growth in the cell lines used.

The use of the above chemicals, despite the statements made, have the ability to negatively affect the cells, thus resulting in uncertainty as to what caused the cell death; for this reason only neutral solvents were investigated in this study, that would cause minimal harm to the cells, namely sterile distilled Milli-Q water, RPMI, PBS and saline solution, with final doses further dissolved in filtered RPMI.

It was noted that the above solvents, when exposed to As_2O_3 for 24 h, with continuous stirring but no heat, resulted in negligible solubilization (Table 3.1); this was identified through cytotoxicity tests of the As_2O_3 solutions on human cells. However, the ultimate dissolution method used in this study showed that the ideal solvent was sterile distilled Milli-Q water, which had no effect on the cells in the culture once the As_2O_3 stock solution was diluted in the RPMI for the final arsenical doses. It was also noted that 80°C was the minimum amount of heat required to allow for full dissolution of the As_2O_3, and was necessary for at least a 10 day period (Table 3.1).

The established optimum solubilization method was determined through macroscopic and microscopic evaluation of the stock and treated cell cultures, and resulted in a completely dissolved 20 mM As_2O_3 solution. Macroscopically, the arsenical solution appeared translucent with no visible signs of As_2O_3 granules. Furthermore, the cell cultures antagonised with As_2O_3 did not noticeably use up their media, this was as a result of too few viable cells in the suspension, attributable to As_2O_3 cytotoxicity; in comparison to the cell cultures with no As_2O_3, which used up their media rapidly due to proliferation of the viable cells (Figure 4.2). These results illustrate the ability for the solubilized As_2O_3 to enter into the cells causing cytotoxicity and ultimately cell death, evidence that the As_2O_3 was fully dissolved in solution.

Microscopically, the viable cells were monitored by the trypan blue dye exclusion assay, which showed that the number of viable cells decreased with exposure to 5 µM As_2O_3 133 µL over 24 h and 48 h (Table 4.1 and Figure 4.4). It was also noted that the percentage of cytotoxicity increased with rising doses of As_2O_3, measured using the MTT assay on the MT4 cell line (Figure 4.6); thus showing that the As_2O_3 was in a fully dissolved homogenous solution, which was then absorbed into the cells, caused toxic changes and eventually cell death. These results were considered reliable, repeatable and consistent in nature.

5.2 Arsenic Trioxide Cytotoxicity

In order to ascertain the approximate IC_{50} of As_2O_3, various doses were given to healthy PBMCs and MT4 cells over a range of time periods, with results measured using the MTT assay. Florea et al. (2007) investigated whether As_2O_3 caused significant cytotoxicity on HEK and neuroblastoma cell lines using the trypan blue cytotoxicity assay and the MTT assay; these measuring tools were thereby established as reliable, showing significant cytotoxicity ($p < 0.05$) after exposure of the cells to 1 µM As_2O_3 for 2 h, 24 h, 48 h and 72 h. Shim et al. (2002) investigated the cytotoxic effects of As_2O_3 on a human leukemia cell line (K562) for 24 h and 48 h, also using the MTT assay. It was found that cell death increased with increasing doses of

As_2O_3, with an IC_{50} value of 10 µM. The cytotoxicity of K562 cells was therefore shown at higher concentrations of As_2O_3 than that of the HEK and neuroblastoma cells in the study by Florea et al. (2007). These studies verified the use of the MTT assay as a reliable measuring tool, and identified the various concentrations of As_2O_3 that cause cell death in different cell lines over various time periods.

After exposure of the PBMCs to the various doses of As_2O_3, the absorbance (Table 4.2) and percentage cytotoxicity (Figure 4.1) results showed no trend in relation to the volumes of As_2O_3 given; however the concentration of 5 µM As_2O_3 resulted in the highest cytotoxicity readings at 24 h (Figure 4.1). Due to the above mentioned, further experiments utilized the MT4 cell line in conjunction with the PBMCs, in order to compare the reliability of each when using the MTT assay, and to determine the effects of As_2O_3 on cancerous and healthy cells respectively. The concentration of 5 µM As_2O_3 was used for further experiments as the optimum concentration, with various injection volumes investigated so as to identify the approximate IC_{50} of As_2O_3.

On evaluation of the two cell types, various volumes (33 µL, 67 µL and 133 µL) of 5 µM As_2O_3 were given to the MT4 cells and PBMCs in order to determine the optimum IC_{50} of As_2O_3, and to establish the cell type that was most susceptible to the As_2O_3 and followed a reliable trend. The results demonstrated that the PBMCs were not reliable, with CV percentages significantly higher than those of the MT4 cells (Table 4.3). This may be attributed to the very sensitive nature of the PBMCs as primary blood cells; this has been noted in a prior study by Goodyear, Piper, Khan, Starczynski, Mahendra, Pratt, and Moss (2005), where the freshly isolated PBMCs were seen as unreliable when compared to the T-cell line, due to a low frequency of responding cells when tested using the ELISPOT assay. However, the MT4 cell line responded significantly to the As_2O_3 doses, with the results regarded as reliable. This may be attributable to the susceptibility of cancer cells to a carcinogenic substance, namely As_2O_3. From these results it was deduced that in further experiments only the MT4 cell line would be used, as the PBMCs' results could not be regarded as accurate or dependable.

The optimum concentration, injection volume and exposure time variables of As_2O_3 were established using the MT4 cell line, where the dose with a result closest to 50% cytotoxicity was used as the IC_{50}. Table 4.3 shows the mean absorbance results for the MTT assay on MT4 cells and PBMCs, where the dose that gave the most reliable results in combination with the lowest absorbance readings was the MT4 cell line after 48 h antagonised with 5 µM As_2O_3 (133 µL). This was therefore the dose used in further experiments as the IC_{50} of As_2O_3. The percentage cytotoxicity for this dose was calculated as follows: 100 – (0.13 ÷ 0.2 x 100), with a result of 35% cytotoxicity.

When further analysed however, the percentage cytotoxicity was highest for the MT4 cells after 72 h given 5 µM As_2O_3 (133 µL) (Figure 4.6), calculated as: 100 – (0.2 ÷ 0.35 x 100) with a resultant cytotoxicity of 42.86%. An increase in the cytotoxic activity of the MT4 cells can be seen between 48 h and 72 h, as opposed to the PBMCs where the cytotoxic activity decreased over time with erratic results (Figure 4.6).

The results of the above experiments clearly show that the As_2O_3 induced cytotoxic changes in the MT4 cell line in a dose dependent manner, with 133 µL having the highest percentage of cytotoxicity, followed by the 67 µL, and the 33 µL dose causing the least cytotoxicity. In Figure 4.5 it is possible to identify the gradual decrease in number of stained viable MT4 cells, from the untreated (A) and RPMI control wells (B), (C) and (D) with high concentrations of cells, to the 33 µL (E), 67 µL (F) and 133 µL (G) doses of 5 µM As_2O_3 treated wells, with much fewer cells the higher the volume of the As_2O_3; showing the dose dependent cytotoxic action of As_2O_3 on the MT4 cells.

The trypan blue dye exclusion assay demonstrated that the viable MT4 cells decreased in number (Table 4.1), with an increase in number of the non-viable cells, after exposure to 5 µM As_2O_3 (133 µL) over time (24 h and 48 h). This is illustrated in Figure 4.4, where the translucent

cells are noted as viable, as opposed to the small blue cells which are non-viable. On counting of the viable cells, it was calculated that there were 2.85 x 10^5 cells/mL before antagonisation with As_2O_3, 2.75 x 10^5 cells/mL after 24 h antagonisation with 5 µM As_2O_3 (133 µL) and 2.25 x 10^5 cells/mL after 48 h antagonisation with 5 µM As_2O_3 (133 µL). These results confirm that the cytotoxicity of As_2O_3 was dose and time dependent.

In Figure 4.3, the MT4 cells are in suspension and can be seen as confluent and healthy in shape, size, number and colour before giving the As_2O_3 (A). However, after antagonisation with 5 µM As_2O_3 (133 µL) for 24 h (B), the cells are much fewer in number, evidence of growth inhibition and cell death, and display signs of possible blebbing, where the cells exhibit irregular bulges, possibly due to the separation of the plasma membrane from the cytoskeleton with resultant bulging outward, which would then be evidence of potential apoptosis (Dai and Sheetz, 1999). These allegations can only be confirmed however with further molecular testing. Cell shrinkage can be seen in the cells antagonised with 5 µM As_2O_3 (133 µL) for 48 h (C); this could lead to irreversible cell injury, along with possible bleb formation and apoptotic cell death. Apoptosis has been proven by several investigations as the lead cause of As_2O_3-induced cell death Florea et al. (2007).

5.3 Treatment of MT4 cells with *Arsenicum album* potencies

The effects of various homoeopathic potencies on human cells were investigated after antagonisation with the IC_{50} dose of As_2O_3, namely 5 µM As_2O_3 (133 µL), using an *in-vitro* design to investigate the law of similars. There has been a vast lack of homoeopathic *in-vitro* research done in the past, despite the necessity for reputable scientific evidence on the actions of homoeopathic potencies. The homoeopathic similia principle was investigated using an *in-vitro* experimental design by Van Wijk and Wiegant (1995). The degree of stimulation for self recovery was measured by exposing cells to high concentrations of toxic substances, to initially cause cell damage, followed by small doses of the same toxic substances. The results showed that the

synthesis of protective proteins was significantly activated, thus leading to self recovery of the cells. The above study therefore demonstrated the ability to investigate the similia principle using homoeopathic doses in an *in-vitro* model.

Van Wijk and Wiegant (2010) stated the criteria required when investigating the similia principle on the cellular level, as previously described in chapter 2.

- The first criterion states that a disturbed state must be attained, which in this research study was achieved after antagonisation with As_2O_3, where the cytotoxicity was monitored.
- The second criterion states that there must be recovery of the cells in response to the disturbed state.
- The third criterion states that the disease picture needs to be taken into account, which in this study was observed as a decrease in cell number, with possible bleb formation and cell shrinkage.
- The fourth criterion states that the remedy picture should be matched to the disease picture in order to determine the degree of similarity, which was attained in this study by the use of the same substance in antagonisation and treatment, with previous provings done on the toxic and therapeutic effects of arsenic trioxide and *Arsenicum album* respectively.
- The fifth criterion is to have accepted outcome parameters to evaluate the effects of the treatments on cellular recovery, in this study the cell viability percentages were monitored to determine the therapeutic power of the remedies.

The potencies tested in this study were both below (6CH) and above (30CH and 200CH) Avogadro's constant, with succussed and unsuccussed potencies compared, using human cells. This differs from Van Wijk and Wiegant (2010) in that they utilized rat hepatoma cell lines and did not test homoeopathic potencies above Avogadro's constant, nor did they compare succussed and unsuccussed potencies.

Succussion has been investigated in numerous past studies, with varying results; Sukul et al. (2001) performed *in-vivo* investigations comparing succussed and unsuccussed *Nux vomica* 30CH potencies on toads. The results showed that the toads responded to both the succussed and the unsuccussed potencies, thus succussion was found to not be an essential factor in producing an effective homoeopathic potency. Similarly, Aabel, Fossheim and Rise (2001) found that *Sulphur* potencies, both succussed and unsuccussed, had identical results when comparing NMR spectra. However, Brizzi et al. (2000) used a wheat germination *in-vitro* model and noted that succussed As_2O_3 potencies had significant and reproducible effects, whereas the unsuccussed As_2O_3 did not show any significant effects. These conflicting study results confirmed the necessity for further investigation into the need for succussion in homoeopathic potency preparation.

After antagonisation with the As_2O_3, resulting in approximately 50% MT4 cell death, and subsequent treatment with the various *Arsenicum album* potencies, the mean absorbance readings from the MTT assay demonstrated that the treatments had stimulating effects on the MT4 cells in comparison to the controls, for the various exposure times represented by each plate (Table 4.4). The absorbance readings represent the amount of formazan dye produced as a result of mitochondrial respiration in the viable cells, therefore the higher the readings, the higher the number of viable cells in the culture.

The *Arsenicum album* 6CH resulted in the lowest absorbance readings, with higher readings from the *Arsenicum album* 30CH and the highest readings from the *Arsenicum album* 200CH (Table 4.4). These results show absorbance as potency dependent, where each potency caused stimulation of the cells, increasing in amount as the potencies increased in dilution; thereby verifying the Arndt-Schultz law, which states that every stimulus on a living cell elicits an activity which is inversely proportional to the intensity of the stimulus (Gaier, 1991). The more the potencies were diluted beyond Avogadro's constant, the more stimulating they were on the cells.

The viable MT4 cells seen in suspension in Figures 4.7, 4.8, 4.9 and 4.10, increase in number from A (unsuccussed *Arsenicum album* 6CH) to C (unsuccussed *Arsenicum album* 30CH) to E (unsuccussed *Arsenicum album* 200CH); with no notable differences in the number of cells between unsuccussed and succussed potency treatments, seen comparing A with B, C with D and E with F. The cells given RPMI as the control (G) are much fewer in number than the *Arsenicum album* treated cells and there is a marked decrease in cell number in the untreated cells (H) where As_2O_3 is still present. These results demonstrate the similia principle, whereby the cells are stimulated when treated with homoeopathic doses of the same substance that caused cell death, namely *Arsenicum album*, but to a less extent when given the RPMI control.

In accordance with the above results, the cell viability percentages were potency dependent (Table 4.5 and Figure 4.11). In all of the plates (A1, A2, B1 and B2), which vary in time of exposure to As_2O_3 and treatment, the least amount of cell viability was noted when treated with the *Arsenicum album* 6CH, whereas cell viability was significantly highest in the cells treated with the *Arsenicum album* 200CH. It was discovered that the percentage of cell viability increased from 65% after exposure to As_2O_3 for 48 h and 57.14% after 72 h, to a maximum of 193.97% cell viability after treatment with succussed *Arsenicum album* 200CH for 72 h (Table 4.5).

With cell viability as the dependent variable, univariate ANOVA was used to determine the significance ($p < 0.05$) of the effects of the various plates, succussion and treatments, which were referred to as the three between subjects' effects. These were analysed on their own as main effects, as well as in combination as interaction effects (Table 4.6). On observation of these statistics (Table 4.6), it was ascertained that the treatments and the plates were statistically significant determinants of cell viability when analysed on their own as main effects, both having p values of < 0.001, thereby showing that the potencies did cause relevant therapeutic stimulation of the cells, and that different times of exposure to As_2O_3 and the treatments significantly

influenced the cell viability percentages. There were no significant interaction effects of the plates, succussion and treatment groups when analysed, with p values > 0.05.

The Bonferroni post hoc test was used in an attempt to determine which plates were significantly different from each other using cell viability as the dependent variable (Table 4.7). The results showed that plate A1 and B1 were significantly different in terms of cell viability, as well as A2 and B1, and B1 and B2. These statistics show the significant difference in the results of plate B1 and the other plates, with evidently lower cell viability readings in plate B1, represented in Table 4.5. This can also be observed in Figure 4.12 where there is a marked dip in the estimated marginal means of cell viability for plate B1. This may be explained by exposing the MT4 cells to the antagoniser (As_2O_3) and the treatments (*Arsenicum album* potencies) for equal lengths of time, whereas all of the other plates were exposed to the treatments for at least 24 h longer than the antagoniser.

The Bonferroni post hoc test was also used in order to ascertain which treatments were significantly different from each other. Table 4.8 shows that the *Arsenicum album* 200CH group was significantly different from both the *Arsenicum album* 6CH and 30CH groups. From these statistics it can be assumed that the *Arsenicum album* 200CH treatment caused a significant increase in cell viability when compared to the other treatment groups, displaying evidence that potencies beyond Avogadro's constant can cause physiological effects on cells, despite having no particles of the original substance present. These statistics also established that each potency caused significantly different effects on the cells, as stated by the Arndt-Schultz law.

Figure 4.12 illustrates the estimated marginal means of cell viability percentages for the different plates and potency treatments, where succussed and unsuccussed potency results have been averaged. It can be noted that the *Arsenicum album* 200CH with plate B2 provided the highest percentage of cell viability, whereas the *Arsenicum album* 6CH with plate B1 resulted in the least percentage of cell viability. The mean cell viability percentages can be clearly detected as

potency dependent, with the regular increases in the percentages from *Arsenicum album* 6CH with the lowest cell viability to the *Arsenicum album* 200CH with the highest level of cell viability. A trend was also seen in the plates, where plate B2 (48 h As_2O_3 followed by 72 h treatment) resulted in the highest cell viability percentages, followed by plate A1 (24 h As_2O_3 followed by 48 h treatment), then plate A2 (24 h As_2O_3 followed by 72 h treatment) and lastly plate B1 (48 h As_2O_3 followed by 48 h treatment) which resulted in the lowest cell viability percentages; this was noted for all treatment groups.

Succussion did not cause any significant variation in the results (Figure 4.11), observed in the line graphs which did not diverge significantly between succussed and unsuccussed potencies in each plate. When analysed statistically using univariate ANOVA (Table 4.6), the succussion was not found as significant, with a p value of 0.841, thus establishing that succussion does not cause any significant change to the potency when testing on the cellular level; therefore the method of allowing 1 part of the preceding potency to diffuse for 30 s in 99 parts of distilled water was sufficient in remedy preparation, as opposed to the established practise of a minimum of 10 hand succussions to produce an effective aqueous potency.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study was undertaken to investigate the protective effect that homoeopathic potencies of *Arsenicum album* (6CH, 30CH and 200CH) had on cells after antagonisation by the crude substance, namely As_2O_3, in an *in-vitro* system. The study thereby provided evidence that a biotechnological method could be used to evaluate the physiological effects of homoeopathic potencies on human cells.

In this study, it was necessary to successfully culture the PBMCs and the MT4 cell line; the cells were investigated concerning their susceptibility to As_2O_3 through extensive experimentation and analysis of the results with regard to reliability. As_2O_3 is known to be highly toxic and carcinogenic in nature, with severe systemic effects after acute and chronic exposure. It has been used in chemotherapy for several cancers, with significant effects on acute promyelocytic leukemia. The MT4 cell line utilised in this study is a human T-cell lymphotropic virus type I-transformed T-cell line, and therefore responded well to the cytotoxic effects of the As_2O_3, and the therapeutic effects of the *Arsenicum album*, according to the homoeopathic law of similars.

The optimum solubilization method of As_2O_3 was successfully determined, with negligible influence of the solvent on the cells in culture; as a result all affects on the cells were attributed to the As_2O_3 in the solution. Through many trials, the optimum IC_{50} of As_2O_3 was established as 5 µM As_2O_3 (133 µL) for 48 h on 1 mL of MT4 cells.

Arsenicum album potencies were successfully prepared under sterile conditions; these included 6CH, 30CH and 200CH, succussed and unsuccussed potencies. These *Arsenicum album* potencies showed therapeutic effects on the cells, therefore the method of preparation

successfully liberated the innate therapeutic power of the substance, namely As_2O_3, with dilution nullifying the toxic effects. The results verified that succussion in the potentization method was not required in order to produce a curative remedy, where the potencies that were allowed to dissolve resulted in the same therapeutic affects on the cells as those that had been succussed.

The therapeutic effects of the *Arsenicum album* potencies on the MT4 cells after antagonisation with the IC_{50} of As_2O_3 were determined through the MTT assay, which identified an increase in the percentage cell viability to almost 200%. These results confirm the protection of the MT4 cells against the toxic effects of the As_2O_3 by the homoeopathic remedy, namely *Arsenicum album*; thereby verifying the law of similars, which states that what a substance can cause it can also treat.

The results showed that the potencies diluted beyond Avogadro's constant, namely the 30CH and 200CH, had physiological effects on cells, despite having no particles of the original substance present; and verified the Arndt-Schultz law by confirming that different potencies have different effects on cells, with the more dilute potencies having the most stimulatory effects. This was observed where the 200CH caused the highest level of cell viability when compared to the 6CH, which caused the least.

6.2 Recommendations

This research investigated the physical appearance, number and the mitochondrial activity of viable MT4 cells after antagonisation with As_2O_3 and treatment with *Arsenicum album* potencies, through the use of light and phase contrast microscopy, the trypan blue dye exclusion assay and the MTT assay. It did not however investigate on a molecular level. Further research should involve investigating the structural changes of the cells using transmission electron microscopy, where the cells are stained so as to identify the organelles at a much higher magnification. The molecular effects within the cells should also be investigated, including DNA fragment end-

labelling of apoptotic bodies, in order to identify whether the cells died due to apoptosis, which is the most common result of As_2O_3 toxicity; and measurement of the p53 protein, a tumour suppressor gene which protects humans against cancer (Reddy, 2005).

This study investigated the chemo-therapeutic effects of *Arsenicum album* potencies on human cells, after antagonisation with As_2O_3; further research can explore the chemo-protective action of the *Arsenicum album* potencies on cells before antagonisation with the As_2O_3, thereby investigating the controversial subject of prophylaxis in homoeopathy. This was investigated in this study, but with inconclusive results (see appendix G).

Using similar methodology, other substances may be used in future work, to further verify the law of similars, using the same substance to antagonise and treat the cells. This scientific research will give increased recognition and legitimacy to the practice of homoeopathy.

This study or previous research may be repeated under similar conditions, in order to identify whether the results could be easily replicated, and therefore validated, giving them more credit scientifically.

In future research, the true IC_{50} of As_2O_3, namely 5 µM As_2O_3 (133 µL) for 72 h on the MT4 cell line, may be used to determine the differences in results when given the *Arsenicum album* potencies, compared to the dose given in this study, namely 5 µM As_2O_3 (133 µL) for 48 h.

REFERENCES

Aabel, S., Fossheim, S. and Rise, F. 2001. Nuclear Magnetic Resonance (NMR) Studies of Homeopathic Solutions. *British Homoeopathic Journal*, 90(1): 14-20.

Agency for Toxic Substances & Disease Registry (ATSDR). 2009. *Case Study in Environmental Medicine – Arsenic Toxicity.* Course: WBCBDV1576. [online] Available: http://www.atsdr.cdc.gov/csem/arsenic/what.html, [Accessed 18 February 2010].

Auerbach, D. 1994. *Mass, fluid and wave motion during the preparation of ultra high dilutions. In: Ultra High Dilution,* Eds. P.C. Endler, J. Schulte. Kluwer, Dordrecht.

Bellavite, P. and Signorini, A. 1995. *Homoeopathy: A Frontier in Medical Science.* California: North Atlantic Books.

Bio-Whittaker Product Catalog and Technical Manual. 1999/2000. pp. 115-149.

British Homeopathic Association. 1993. *German Homoeopathic Pharmacopoeia*: Deutscher Apotheker Verlag Stuttgart. Translation of the 5th supplement 1991 to the first edition 1978.

Brizzi, M., Nani, D., Peruzzi, M. and Betti, L. 2000. Statistical analysis of the effect of high dilutions of arsenic in a large dataset from a wheat germination model. *British Homoeopathic Journal*, 89(2): 63-67.

Cheng, C., Sirigireddy, K. and Ganta, R. 2008. Isolation and molecular detection of Ehrlichia from vertebrate animals. *Current Protocols in microbiology*, Chapter 3: Unit 3A.3.

Cole, S. 1986. Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. *Cancer Chemotherapy Pharmacology* 17(3): 259-263.

Dai, J. and Sheetz, M. 1999. Membrane Tether Formation from Blebbing Cells. *Biophysical Journal*, 77(6): 3363-3370.

Davenas, E., Beauvais, F., Amara, J., Oberbaum, M., Robinzon, B., Miadonna, A., Tedeschi, A., Pomeranz, B., Fortner, P., Belon, P., Sainte-Laudy, J., Poitevin, B. and Benveniste, J. 1988. Human basophil degranulation triggered by very dilute antiserum against IgE. *Nature*, 333:816-818.

De Schepper, L. 2001. *Hahnemann Revisited*. Santa Fe: Full of Life Publications.

Ebbing, D. and Wentworth, R.A.D. 1995. *Introductory Chemistry.* Boston: Houghton Mifflin Company.

Florea, A-M., Splettstoesser, F. and Busselberg, D. 2007. Arsenic trioxide (As_2O_3) induced calcium signals and cytotoxicity in two human cell lines: SY-5Y neuroblastoma and 293 embryonic kidney (HEK). *Toxicology and Applied Pharmacology*, 220: 292-301.

Gaier, H. 1991. *Thorson's Encyclopaedic Dictionary of Homeopathy.* London. Thorsons.

Gogal, R.M., Ahmed, A. and Larsen, C.T. 1997. Analysis of Avian lymphocyte proliferation by a new, simple non-radioactive assay. *Avian diseases V*, 41: 714-725.

Goodyear, O., Piper, K., Khan, N., Starczynski, J., Mahendra, P., Pratt, G. and Moss, P. 2005. CD8_T cells specific for cancer germline gene antigens are found in many patients with multiple myeloma, and their frequency correlates with disease burden. *Blood*, 106(13): 4217-4224.

Guyton, A. and Hall, J. 1997. *Human Physiology and Mechanisms of Disease.* 6th ed. Pennsylvania: W.B. Saunders Company.

Hauswirth, K. and Sherk, S. 2004. *Gale Encyclopedia of Surgery: A Guide for Patients and Caregivers*. [online] Available: http://www.encyclopedia.com/doc/1G2-3406200050.html, [Accessed 25 March 2010].

Kayne, S. 1991. An agricultural application of homoeopathy. *British Homoeopathic Journal*, 80(3):157-160.

Mallinckrodt Baker Inc. 2008. *Material Safety Data Sheet: Arsenic Trioxide*. [online] Available: http://www.jtbaker.com/msds/englishhtml/a7512.htm, [Accessed 18 February 2010].

Martindale. 1967. *Extra Pharmacopoeia.* 25th ed. London: Pharmaceutical Society of Great Britain.

O' Reilly, W. (ed.) 1996. *Organon of the Medical Art.* 6th ed. Adapted from the 6th edition of the *Organon der Heilkunst* by Samuel Hahnemann in 1842. California: Birdcage Books.

Perfetti, E., Pokrovski, G., Ballerat-Busserolles, K., Majer, V. and Gibert, F. 2008. Densities and heat capacities of aqueous arsenious and arsenic acid solutions to 350 °C and 300 bar, and revised thermodynamic properties of $As(OH)_3°(aq)$, $AsO(OH)_3°(aq)$ and iron sulfarsenide minerals. *Geochimica et Cosmochimica Acta*, 72(3):713-731.

Reddy, L., Odhav, B. and Bhoola, K. 2006. Aflatoxin B_1 – Induced Toxicity in HepG2 Cells Inhibited by Carotenoids: Morphology, Apoptosis and DNA Damage. *The Journal of Biological Chemistry*, 387: 87-93.

Reddy, L. 2005. *Chemoprotective Action of Natural Products on Cultured Human Epithelial Cells Exposed to Aflatoxin B_1*. Doctor of Technology: Biotechnology, Durban University of Technology.

Satou, Y., Nosaka, K., Koya, Y., Yasunaga, J-I., Toyokuni, S. and Matsuoka, M. 2004. Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both *in vivo* and *in vitro*. *Leukemia*, 18: 1357-1363.

Schiff, M. 1995. *The Memory of Water.* London: Thorsons.

Shim, M., Kim, H., Yang, S., Lee, I. and Kim, T. 2002. Arsenic Trioxide Induces Apoptosis in Chronic Myelogenous Leukemia K562 Cells: Possible Involvement of p38 MAP Kinase. *Journal of Biochemistry and Molecular Biology*, 35(4): 377-383.

Sukul, N.C. and Sukul, A. 2004. *High dilution effects: physical and biochemical basis*. Netherlands: Kluwer Academic Publishers.

Sukul, NC., De, A., Dutta, R., Sukul, A. and Sinhababu, SP. 2001. *Nux vomica* 30 prepared with and without succussion shows antialcoholic effect on toads and distinctive molecular association. *British Homoeopathic Journal*, 90(2): 79-85.

Swayne, J. 2000. *Churchill Livingstone's International Dictionary of Homeopathy*. London: Churchill Livingstone, an imprint of Harcourt Publishers Ltd.

Van Wijk, R. and Wiegant, F.A.C. 2010. The similia principle: Results obtained in a cellular model system. *Homeopathy,* 99(1): 3-14.

Van Wijk, R. and Wiegant, F.A.C. 1998. Stressor-specific enhancement of hsp induction by low doses of stressors in conditions of self- and cross-sensitization. *Toxicology*, 127(1-3): 107-119.

Van Wijk, R. and Wiegant, F.A.C. 1997. *The Similia Principle in Surviving Stress: Mammalian Cells in Homeopathic Research.* Utrecht: Utrecht University.

Van Wijk, R. and Wiegant, F.A.C. 1996. Experimental Work to Prove Similia Principle. *British Homoeopathic Journal*, 85(3): 176.

Van Wijk, R and Wiegant, F.A.C. 1995. Stimulation of Self Recovery by Similia Principle? *British Homoeopathic Journal*, 84(3): 131-139.

Varma, P. and Vaid, I. 2002. *Encyclopaedia of Homoeopathic Pharmacopoeia.* 3rd ed. New Delhi: B. Jain Publishers.

Vithoulkas, G. 1980. *The Science of Homeopathy.* New Delhi: B. Jain Publishers (P) Ltd.

Walach, H., Jonas, W.B., Ives, J., Van Wijk, R. and Weingartner, O. 2005. Research in Homeopathy: State of the Art. *The Journal of Alternative and Complementary Medicine*, 11(5): 813-829.

APPENDICES

List of Appendices

APPENDIX A

Comparison between Dilution, Concentration and Homoeopathic Centesimal Potency

Dilution	Concentration	Centesimal potency
1:100	10^{-2}	1CH
1:10 000	10^{-4}	2CH
1:1 000 000	10^{-6}	3CH
$1:10^{12}$	10^{-12}	6CH
$1:10^{30}$	10^{-30}	15CH
$1:10^{60}$	10^{-60}	30CH
$1:10^{400}$	10^{-400}	200CH
$1:10^{2000}$	10^{-2000}	1000CH or 1M
$1:10^{20\,000}$	$10^{-20\,000}$	10 000CH or 10M

Homoeopathic centesimal potency (Kayne, 1991:50)

APPENDIX B

Media, Reagents and Equipment for Cell Culture

1. 70% Ethanol – Merck Chemicals, South Africa
2. As_2O_3 (Arsenic trioxide) – Sigma, USA
3. Autoclave – D&E International Corp, Taiwan
4. Biofreezer – Former Scientific, UK
5. Cell culture flasks ($25cm^2$ and $75cm^2$) – Greiner, Germany
6. Centrifuge – Eppendorf, USA
7. Centrifuge tubes – Greiner, Germany
8. Complete Culture Medium (CCM) for the MT4 cell line consisted of RPMI medium containing 10% filtered FCS and supplemented with 0.5% Pen Strep.
9. CCM for PBMCs consisted of RPMI medium containing 10% filtered FCS and supplemented with 1% Pen Strep.
10. Culture plates – Greiner, Germany
11. DMSO (dimethyl sulfoxide) – Sigma, USA
12. ELISA (Enzyme-linked immunosorbent assay) reader – Digital and Analog Systems, Italy.
13. FCS (fetal calf serum) – Highveld Biological, South Africa
14. Histopaque 1077 – Sigma, USA
15. Humidified incubator – Heraeus, Germany
16. Laminar flow – Scientific Engineering Inc, USA
17. Light microscope – Nikon, USA
18. Mr. Frosty – Nalgene Labware, USA
19. MT4 Cell Line – Durban University of Technology Bio-bank, Department of Biotechnology and Food Technology, Durban, South Africa.
20. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent – Sigma, USA
21. NaCl (sodium chloride) – Merck Chemicals, SA

22. PBS (phosphate buffered saline) – Invitrogen, USA
23. Penicillin/Streptomycin (Pen Strep) Mixture - Highveld Biological, South Africa
24. RPMI (Roswell Park Memorial Institute) medium - Invitrogen, USA
25. Cary 100 UV-Visible Spectrophotometer – Varian, USA
26. Trypan blue solution – Biowhittaker, USA

APPENDIX C

Potentization of *Arsenicum album*

Aim: To potentise Arsenic trioxide crude substance to the third centesimal potency.

Apparatus: Mass balance (accurate and calibrated); 12 pieces of clean paper; lactose monohydrate; arsenic trioxide crude substance; face mask; gloves; stainless steel spatulas (small and large); mortar and pestle (unglazed porcelain); distilled water; paper towelling; 96% alcohol; lighter; clock; 3 × no.10 glass vials (40ml); 3 × labels.

Method:

a) Cleaned laminar flow and laboratory counters with 96% alcohol.
b) Washed mortar, pestle and spatulas with water and soap, rinsed with distilled water, and dried with paper towelling.
c) Flamed above using 96% alcohol.
d) Allowed above to cool.
e) Lined laminar flow counter with layer of paper towelling.
f) Massed 3 × 3.3 g lactose on 3 separate pieces of paper using mass balance and small spatula.
g) Massed 0.1 g arsenic trioxide on paper using mass balance, wearing a face mask and gloves.
h) Combined 0.1 g arsenic trioxide and 3.3 g lactose in mortar.
i) Triturated using the pestle for 6 min.
j) Scraped using the large spatula for 4 min.
k) Repeated the 6 min triturating and 4 min scraping.
l) Added the next 3.3 g of lactose, and repeated the above 20 min cycle.

m) Added the final 3.3 g of lactose, and repeated the 20 min cycle.

n) Decanted the triturate into the no.10 glass vial labelled '*Arsenicum album* 1CH triturate'.

o) Washed and flamed mortar, pestle and spatulas.

p) Repeated as above for the 2CH and 3CH *Arsenicum album* triturates, using 0.1 g of the previous potency with 9.9 g of lactose (British Homoeopathic Association, 1993)

APPENDIX D

Optimization of Solubilization Method of Arsenic Trioxide

1. Calculations and Preparation of Stock Solutions of Arsenic Trioxide using Distilled Water as the Solvent

Calculations: M = mol/L

M (As_2O_3) = 198 g/L

= 198 x 10^3 mg/L

µM = 198 x 10^3 mg x 10^{-6}/L

µM = 0.198 mg/L

For 100 µL cell culture in micro titre plate:

µM = 0.198 mg/L x 100

= 19.8 mg/L

= 2 mg/100 mL

Antagonisers: L1 = 1 µM (2 mg/100 mL stock) of As_2O_3

L2 = 0.5 µM of As_2O_3

L3 = 0.25 µM of As_2O_3

Method:

1) Researcher wore gas mask, latex gloves and protective lab coat.
2) Weighed 2 mg of As_2O_3 using a mass balance.
3) Measured 100 mL of distilled water.
4) Added 2 mg of As_2O_3 to the distilled water.
5) Placed on a magnetic stirrer to aid in diffusion (10 min).

6) Labelled: L1
7) Poured out 50 mL of L1 into a new flask, and added 50 mL distilled water.
8) Placed on a magnetic stirrer.
9) Labelled: L2
10) Poured out 50 mL of L2 into a new flask, and added 50 mL distilled water.
11) Placed on a magnetic stirrer.
12) Labelled: L3

2. Preparation of Stock Solutions of Arsenic Trioxide using:

2.1 Distilled Water as the Solvent

Antagonisers: A4 = 2 μM (4 mg/100 mL Aq dist) of As_2O_3

A1 = 1 μM of As_2O_3 in Aq dist

A2 = 0.5 μM of As_2O_3 in Aq dist

Method:

1) Researcher wore gas mask, latex gloves and protective lab coat.
2) Weighed out 4 mg of As_2O_3 using a mass balance.
3) Measured out 100 mL distilled water.
4) Placed on a magnetic stirrer overnight to aid in diffusion.
5) Labelled: A4 (stock)
6) Poured out 50 mL of A4 into a new flask, and added 50 mL distilled water.
7) Placed on a magnetic stirrer (10 min).
8) Labelled: A1
9) Poured out 50 mL of A1 into a new flask, and added 50 mL distilled water.
10) Placed on a magnetic stirrer.
11) Labelled: A2

2.2. RPMI as the Solvent

Antagonisers: R4 = 2 μM (4 mg/100 mL RPMI) of As_2O_3

R1 = 1 μM of As_2O_3 in RPMI

R2 = 0.5 μM of As_2O_3 in RPMI

Method:

1) Weighed out 4 mg of As_2O_3 using a mass balance.
2) Measured out 100 mL RPMI.
3) Placed on a magnetic stirrer overnight.
4) Labelled: R4 (stock)
5) Poured out 50 mL of R4 into a new flask, and added 50 mL RPMI.
6) Placed on a magnetic stirrer.
7) Labelled: R1
8) Poured out 50 mL of R1 into a new flask, and added 50 mL RPMI.
9) Placed on a magnetic stirrer.
10) Labelled: R2

2.3. Normal Saline solution (NS) as the Solvent

Antagonisers: N4 = 2 μM (4 mg/100 mL Normal saline) of As_2O_3

N1 = 1 μM of As_2O_3 in Normal saline

N2 = 0.5 μM of As_2O_3 in Normal saline

Method:

1) Weighed out 1.8 g sodium chloride using a mass balance.
2) Measured 200 mL of distilled water.
3) Placed on a magnetic stirrer for 5 min.
4) Labelled: Normal Saline solution (NS).
5) Weighed out 4 mg of As_2O_3 using a mass balance.

6) Measured out 100 mL NS.
7) Placed on a magnetic stirrer overnight.
8) Labelled: N4 (stock)
9) Poured out 50 mL of N4 into a new flask, and added 50 mL NS.
10) Placed on a magnetic stirrer.
11) Labelled: N1
12) Poured out 50 mL of N1 into a new flask, and added 50 mL NS.
13) Placed on a magnetic stirrer.
14) Labelled: N2

2.4. PBS as the Solvent

Antagonisers: P4 = 2 μM (4 mg/100 mL PBS) of As_2O_3

P1 = 1 μM of As_2O_3 in PBS

P2 = 0.5 μM of As_2O_3 in PBS

Method:

1) Weighed out 4 mg of As_2O_3 using a mass balance.
2) Measured out 100 mL PBS.
3) Placed on a magnetic stirrer overnight.
4) Labelled: P4 (stock)
5) Poured out 50 mL of P4 into a new flask, and added 50 mL PBS.
6) Placed on a magnetic stirrer.
7) Labelled: P1
8) Poured out 50 mL of P1 into a new flask, and added 50 mL PBS.
9) Placed on a magnetic stirrer.
10) Labelled: P2

3. Preparation of Stock Solutions of Arsenic Trioxide using Distilled Water, Heat and Stirring

Antagonisers: A4 = 2 μM (4 mg/100 mL Aq dist) of As_2O_3

A5 = 10 μM (2 mg/10 mL Aq dist) of As_2O_3

A6 = 5 μM of As_2O_3

Method:

1) Researcher wore gas mask, latex gloves and protective lab coat.
2) Weighed out 4 mg of As_2O_3 using a mass balance.
3) Measured out 100 mL distilled water.
4) Placed on a magnetic stirrer for 48 h at 50°C to aid in diffusion.
5) Labelled: A4
6) Weighed out 2 mg of As_2O_3 using a mass balance.
7) Measured out 10 mL distilled water.
8) Placed on a magnetic stirrer for 48 h at 50°C.
9) Labelled: A5
10) Poured out 5 mL of A5 into a new bottle, and added 5 mL distilled water.
11) Placed on a magnetic stirrer.
12) Labelled: A6

4. Final Solubilization Method for Arsenic Trioxide

Calculations: M = mol/L

M (As_2O_3) = 198 g/L

mM = 198 g x 10^{-3} /L

= 0.198 g/L

= 198 mg/L

= 19.8 mg/100 mL

20 mM = 396 mg/100 mL

= 0.396 g/100 mL

Antagonisers: A1 = 1 µM of As_2O_3

A5 = 5 µM of As_2O_3

A10 = 10 µM of As_2O_3

Method:

1) Researcher wore gas mask, latex gloves and protective lab coat.
2) Weighed out 396 mg of As_2O_3 using a mass balance.
3) Measured out 100 mL sterile distilled Milli-Q water.
4) Placed into a flask and on a heating plate at 80°C to aid in diffusion for 10 days.
5) Labelled: stock 20 mM As_2O_3
6) Decanted 100 mL stock into sterile 100 mL bottle with lid, using mask and gloves.

   Arsenic was fully dissolved.
7) Using 50 mL centrifuge tubes:
8) Made arsenical dilutions:

-» For 50 mL of each dilution:

-» 20 mM = 20 000 µM

-» 20 000 µM: 50 mL

10 µM: 0.025 mL (25 µL)

-» 20 000 µM: 50 mL

5 µM: 0.0125 mL (12.5 µL)

-» 20 000 µM: 50 mL

1 µM: 0.0025 mL (2.5 µL)

-» A10: diluted 25 µL stock (20 mM) in RPMI up to 50 mL = 10 µM; placed onto vortex.

-» A5: diluted 12.5 µL stock in RPMI up to 50 mL = 5 µM; place onto vortex.

-» A1: diluted 2.5 µL stock in RPMI up to 50 mL = 1 µM; place onto vortex.

9) Stored arsenical dilutions in fridge.

APPENDIX E

Schematic Diagrams of Microtitre Culture Plates

1. Schematic representation of 96-well, flat-bottomed microtitre plates used in the As_2O_3 cytotoxicity assay (method section 3.9.1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	U	U	U		An	An	An		Bn	Bn	Bn	
B					Cn	Cn	Cn		Dn	Dn	Dn	
C												
D	A1	B1	C1	D1	A5	B5	C5	D5	A10	B10	C10	D10
E	A1	B1	C1	D1	A5	B5	C5	D5	A10	B10	C10	D10
F	A1	B1	C1	D1	A5	B5	C5	D5	A10	B10	C10	D10
G												
H	Ad	Ad	Ad	Bd	Bd	Bd	Cd	Cd	Cd	Dd	Dd	Dd

A = 5 µL inj. Vol.
B = 10 µL inj. Vol.
C = 20 µL inj. Vol.
D = 50 µL inj. Vol.

1 = 1 µM As_2O_3
5 = 5 µM As_2O_3
10 = 10 µM As_2O_3

d = DMSO
n = Blank (RPMI)

2. Schematic representations of 24-well plates used in the As_2O_3 cytotoxicity assay (method section 3.9.2).

	1	2	3	4	5	6
A	U	U	U	An	An	An
B	Bn	Bn	Bn	Cn	Cn	Cn
C	A5	A5	A5	B5	B5	B5
D	C5	C5	C5			

U = Untreated
A = 33 µL inj. Vol.
B = 67 µL inj. Vol.
C = 133 µL inj. Vol.
n = Blank (RPMI)
5 = 5 µM As_2O_3

3. Schematic representations of 24-well plates used in the *Arsenicum album* potency study using the MT4 Cell Line (method section 3.11).

	1	2	3	4	5	6
A	UA	UA	UA	B	B	B
B	6	6	6	10^{-12}	10^{-12}	10^{-12}
C	30	30	30	10^{-60}	10^{-60}	10^{-60}
D	200	200	200	10^{-400}	10^{-400}	10^{-400}

UA = Untreated + As_2O_3
B = Blank (RPMI)
6 = *Arsenicum album* 6CH (succ)
10^{-12} = *Arsenicum album* 6CH (unsucc)
30 = *Arsenicum album* 30CH (succ)
10^{-60} = *Arsenicum album* 30CH (unsucc)
200 = *Arsenicum album* 200CH (succ)
10^{-400} = *Arsenicum album* 200CH (unsucc)

APPENDIX F

Results

1. Arsenic Trioxide Cytotoxicity – PBMCs

Table 1: Percentage cytotoxicity for the dose response of As_2O_3 (1 – 10 µM; 5 – 50 µL) on PBMCs over 24 h and 48 h exposure (results section 4.2.1).

Dose	As_2O_3 for 24 h	As_2O_3 for 48 h
1 µM As_2O_3 5 µL	45.87	-42.86
1 µM As_2O_3 10 µL	-19.66	47.31
1 µM As_2O_3 20 µL	72.73	5.39
1 µM As_2O_3 50 µL	-11.95	12.53
5 µM As_2O_3 5 µL	23.01	12.52
5 µM As_2O_3 10 µL	71.19	43.37
5 µM As_2O_3 20 µL	93.97	-3.8
5 µM As_2O_3 50 µL	63.45	15.75
10 µM As_2O_3 5 µL	30.31	16.79
10 µM As_2O_3 10 µL	-215.09	48.41
10 µM As_2O_3 20 µL	91.7	-9.09
10 µM As_2O_3 50 µL	-27.36	-574.11

2. Arsenic Trioxide Cytotoxicity – MT4 cells and PBMCs

Table 2.1: Triplicate readings (578 nm) for the dose and time response of MT4 cells and PBMCs to 5 µM As_2O_3 (results section 4.2.2).

Doses	MT4 – 48 h	MT4 – 72 h	PBMC – 48 h	PBMC – 72 h
Un-antagonised	0.2605	0.4259	0.0599	1.6528
	0.2679	0.4436	0.8863	1.2032
	0.2585	0.4118	0.5259	1.5056
RPMI 33 µL	0.2502	0.4552	0.8662	1.304
	0.2412	0.4051	0.0719	1.6124
	0.2492	0.4636	0.1839	0.9614
RPMI 67 µL	0.2484	0.4051	0.0622	1.8794
	0.2334	0.3535	0.7488	2.1192
	0.2218	0.3972	0.0825	1.5227
RPMI 133 µL	0.204	0.3905	0.0588	1.5713
	0.1999	0.3259	0.167	2.1539
	0.2065	0.3484	0.0738	1.4314
5 µM As_2O_3 33 µL	0.221	0.4246	0.2053	1.7484
	0.2142	0.322	0.0524	1.9842
	0.2154	0.3111	0.1865	2.0487
5 µM As_2O_3 67 µL	0.1765	0.2676	0.0271	2.0095
	0.18	0.2702	0.0636	1.9912
	0.2207	0.2938	0.1233	1.3718
5 µM As_2O_3 133 µL	0.1195	0.2239	0.0336	1.917
	0.1344	0.198	0	2.0217
	0.1488	0.1904	0.035	2.3272

Table 2.2: Percentage cytotoxicity for the time response of 5 µM As_2O_3 (33 – 133 µL) for 48 h and 72 h on the MT4 cell line and PBMCs (results section 4.2.2).

	MT4 - 48 h	MT4 - 72 h	PBMC - 48 h	PBMC - 72 h
5 µM As2O3 33 µL	12.15	20.1	60.4	-49.09
5 µM As2O3 67 µL	17.95	28.06	76.06	2.69
5 µM As2O3 133 µL	34.05	42.49	77.08	-21.51

3. *Arsenicum album* Potency Study using the MT4 Cell Line

Table 3: Triplicate readings (578 nm) for the potency response of *Arsenicum album* on the MT4 cell line after antagonisation with 5 µM As_2O_3 (133 µL) using the MTT assay (results section 4.3).

Treatment	Plate A1*	Plate A2*	Plate B1*	Plate B2*
Untreated + As_2O_3	0.0736	0.0405	0.0478	0.0617
	0.0518	0.0315	0.0405	0.0392
	0.0524	0.0374	0.049	0.0404
Blank (RPMI)	0.0274	0.017	0.0258	0.0261
	0.0316	0.0205	0.0391	0.0343
	0.0328	0.0346	0.0346	0.053
6CH Arsenicum album	0.0302	0.0171	0.0213	0.0286
	0.0329	0.0169	0.0168	0.0256
	0.0339	0.0163	0.0194	0.0313
6CH unsuccussed	0.0222	0.0132	0.0086	0.0258
	0.0226	0.0128	0.019	0.0419
	0.03	0.0264	0.0257	0.0369
30CH Arsenicum album	0.0402	0.0238	0.0176	0.0345
	0.0259	0.0245	0.0138	0.0378
	0.0325	0.0218	0.0185	0.0348
30CH unsuccussed	0.0314	0.0209	0.0224	0.0279
	0.0344	0.0287	0.0213	0.042
	0.0471	0.0309	0.0289	0.0419
200CH Arsenicum album	0.0416	0.0347	0.0279	0.066
	0.0473	0.0262	0.0301	0.0662
	0.0487	0.0303	0.0522	0.0721
200CH unsuccussed	0.048	0.0343	0.0292	0.0593
	0.0452	0.0259	0.0339	0.0649
	0.0497	0.0397	0.0451	0.0586

*Plate A1 = 24 h As_2O_3 followed by 48 h treatment
*Plate A2 = 24 h As_2O_3 followed by 72 h treatment
*Plate B1 = 48 h As_2O_3 followed by 48 h treatment
*Plate B2 = 48 h As_2O_3 followed by 72 h treatment

APPENDIX G

Chemotherapeutic and Chemoprotective Study using *Arsenicum album*

1. Method

The chemotherapeutic and chemoprotective effects of *Arsenicum album* potencies (succussed and unsuccussed) were investigated on MT4 cells using the MTT assay. The final potencies and antagonisers were made up in filtered RPMI. The previous experiments (section 3.9.2) established that the optimum IC_{50} of As_2O_3 was 5 µM with an injection volume of 133 µL.

After determining the number of viable MT4 cells, by means of the trypan blue dye exclusion assay (section 3.5), 1 mL of MT4 cells (3×10^5 cells/mL) was added to each culture well of four sterile 24-well plates, and incubated overnight at 37°C in 5% CO_2 atmosphere.

The following day, 133 µL of the 5 µM As_2O_3 was given to plates A and C for testing chemotherapeutics, and 133 µL of the treatments (namely succussed and unsuccussed 30CH and 200CH *Arsenicum album* potencies and the RPMI control) were given to plates B and D for testing chemoprotection. All four plates were then incubated at 37°C in 5% CO_2 atmosphere. After 1 h, plates A and B were given 133 µL doses of treatments and antagonisers respectively and incubated for a further 47 h. Plates C and D were given 133 µL doses of treatments and antagonisers respectively after 24 h of incubation, and then incubated for an additional 24 h.

Schematic Representation of Plate A (antagonised by As_2O_3 for 1 h, followed by treatments for 47 h) and Plate C (antagonised by As_2O_3 for 24 h, followed by treatments for 24 h)

U	U	U	UA	UA	UA
B	B	B	30	30	30
200	200	200	10^{-60}	10^{-60}	10^{-60}
10^{-400}	10^{-400}	10^{-400}			

U = un-antagonised (no As_2O_3)
UA = un-antagonised (with As_2O_3)
B = RPMI

30 = *Arsenicum album* 30CH (succ)
200 = *Arsenicum album* 200CH (succ)
10^{-60} = *Arsenicum album* 30CH (unsucc)
10^{-400} = *Arsenicum album* 200CH (unsucc)

Schematic Representation of Plate B (pre-treated for 1 h, followed by antagonisation with As_2O_3 for 47 h) and Plate D (pre-treated for 24 h, followed by antagonisation with As_2O_3 for 24 h)

U	U	U	UT_{30}	UT_{30}	UT_{30}
UT_{200}	UT_{200}	UT_{200}	B	B	B
(30)	(30)	(30)	(200)	(200)	(200)
(10^{-60})	(10^{-60})	(10^{-60})	(10^{-400})	(10^{-400})	(10^{-400})

U = un-antagonised
UT_{30} = 30CH *Arsenicum album* (no As_2O_3)
UT_{200} = 200CH *Arsenicum album* (no As_2O_3)
B = RPMI

(30) = *Arsenicum album* 30CH (succ)
(200) = *Arsenicum album* 200CH (succ)
(10^{-60}) = *Arsenicum album* 10^{-60} (unsucc)
(10^{-400}) = *Arsenicum album* 10^{-400} (unsucc)

After the set incubation times, the cell viability was measured by the MTT assay, as previously described in section 3.6. Each treatment was carried out in triplicate.

2. Results

2.1 Triplicate readings (578 nm) for the (a) chemotherapeutic and (b) chemoprotective effects of *Arsenicum album* on the MT4 cell line when antagonised with 5 µM As_2O_3 (133 µL) using the MTT assay.

a)

Treatment	*Plate A	*Plate C
Un-antagonised	0.3878	0.4203
	0.3392	0.3803
	0.362	0.3575
Un-antagonised + As_2O_3	0.1934	0.2353
	0.1962	0.2326
	0.2126	0.2327
Blank - RPMI	0.1869	0.2063
	0.1928	0.202
	0.1879	0.2104
30CH *Arsenicum album* (succ)	0.1886	0.1946
	0.2013	0.2088
	0.1968	0.2044
200CH *Arsenicum album* (succ)	0.1943	0.1943
	0.1994	0.1976
	0.2008	0.2061
30CH *Arsenicum album* (unsucc)	0.1957	0.2009
	0.203	0.2193
	0.9587	0.225
200CH *Arsenicum album* (unsucc)	0.2064	0.2168
	0.1997	0.2155
	0.2066	0.2083

*Plate A = antagonised by As_2O_3 for 1 h, followed by treatments for 47 h
*Plate C = antagonised by As_2O_3 for 24 h, followed by treatments for 24 h

b)

Treatment	*Plate B	*Plate D
Un-antagonised	0.2713	0.2659
	0.2766	0.2705
	0.255	0.2737
Un-antagonised + 30CH	0.2219	0.2476
	0.2243	0.2286
	0.2312	0.2424
Un-antagonised + 200CH	0.2407	0.2332
	0.236	0.2206
	0.2208	0.199
Blank - RPMI	0.1483	0.1886
	0.1587	0.1645
	0.1619	0.2062
30CH *Arsenicum album* (succ)	0.3796	0.178
	0.1926	0.1995
	0.1822	0.1696
200CH *Arsenicum album* (succ)	0.1586	0.1751
	0.1546	0.1837
	0.1597	0.1925
30CH *Arsenicum album* (unsucc)	0.172	0.1982
	0.1737	0.2509
	0.1633	0.2421
200CH *Arsenicum album* (unsucc)	0.154	0.2431
	0.1676	0.2437
	0.1595	0.233

*Plate B = pre-treated for 1 h, followed by antagonisation with As_2O_3 for 47 h
*Plate D = pre-treated for 24 h, followed by antagonisation with As_2O_3 for 24 h

2.2 Means for the (a) chemotherapeutic and (b) chemoprotective effects of *Arsenicum album* on the MT4 cell line when antagonised with 5 µM As_2O_3 (133 µL) using the MTT assay.

a)

Treatment	*Plate A	*Plate C
Un-antagonised	0.363	0.386
Un-antagonised + As_2O_3	0.2007	0.2335
Blank - RPMI	0.1892	0.2062
30CH *Arsenicum album* (succ)	0.1956	0.2026
200CH *Arsenicum album* (succ)	0.1982	0.1993
30CH *Arsenicum album* (unsucc)	0.4525	0.2151
200CH *Arsenicum album* (unsucc)	0.2042	0.2135

*Plate A = antagonised by As_2O_3 for 1 h, followed by treatments for 47 h
*Plate C = antagonised by As_2O_3 for 24 h, followed by treatments for 24 h

b)

Treatment	*Plate B	*Plate D
Un-antagonised	0.2676	0.27
Un-antagonised + 30CH	0.2258	0.2395
Un-antagonised + 200CH	0.2325	0.2176
Blank - RPMI	0.1563	0.1864
30CH *Arsenicum album* (succ)	0.2515	0.1824
200CH *Arsenicum album* (succ)	0.1576	0.1838
30CH *Arsenicum album* (unsucc)	0.1697	0.2304
200CH *Arsenicum album* (unsucc)	0.1604	0.2399

*Plate B = pre-treated for 1 h, followed by antagonisation with As_2O_3 for 47 h
*Plate D = pre-treated for 24 h, followed by antagonisation with As_2O_3 for 24 h

2.3 Percentage cell viability of MT4 cell cultures treated with *Arsenicum album* potencies (6 – 200CH) and antagonised with 5 µM As_2O_3 (133 µL).

Treatment	Plate A	Plate C	Plate B	Plate D
30CH *Arsenicum album* (succ)	103.38	99.25	160.91	97.85
200CH *Arsenicum album* (succ)	104.76	96.65	100.83	98.61
30CH *Arsenicum album* (unsucc)	239.16	104.32	108.57	123.61
200CH *Arsenicum album* (unsucc)	107.93	103.54	102.62	128.7