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AN ASSESSMENT OF AGENTS OF HEPATOTOXICITY AND NEUROTOXICITY

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ABSTRACT

In recent years there is growing concern about the neurotoxicity of alcohol, acetaldehyde, H_2O_2 and organophosphate pesticides on neuronal cells. An assessment of the neurotoxicity of these four commonly encountered neuro-toxicants was conducted using a human neuroblastoma SH-SY5Y cell line. Cells were cultured at four different media concentrations: Culture media without (0%) foetal bovine serum (FBS), 3% FBS, 10% FBS, 15% FBS and with EMEM. Passage 12-14 SH-SY5Y cells were seeded in 96 well plates and challenged with neuro-toxicants prepared at serial dilutions in respective growth media. Cell viability was assessed using a (3- [4, 5-dimethylthiazol -2-yl] 2-5 diphenyl tetrazolium bromide) (MTT) assay. Ethanol, Acetaldehyde, H_2O_2, and the pesticides Azamethiphos-oxon, and Chlorpyrifos-oxon reduced SH-SY5Y cell viability in a dose-dependent manner. Cells grown in low serum media showed higher cell cytotoxicity to all neurotoxic chemicals, suggesting serum proteins may limit release or action of the toxic moieties of test compounds. Western blot analysis was performed using cell lysates of SH-SY5Y cells treated with 50, 100, and 200 mM ethanol to examine tubulin protein expression after 24-hour exposure. There were no significant changes in tubulin protein levels in ethanol exposed cells over the range of used concentration for 24 hours. Collectively, the results obtained determined concentrations of the neuro-toxicants that inhibited cell viability by 50% (IC_{50}s), and these will provide an insight to the health effects associated with moderate to high neuro-toxicant exposures in humans.

All the neuro-toxicants studied in here were bio-transformed in the human liver and the metabolic products damaged to the liver cells. Natural plant extracts are used in the pharmaceutical industry due to their useful primary and secondary metabolites. These secondary metabolites can act as free radical scavengers

at the cellular level. An assessment of the hepatotoxicity of certain plant extracts from African medicinal plants were evaluated *in vitro* by assessing their cytotoxicity to a human hepatocellular carcinoma (HepG2) cell line. The majority of plant extracts did not reduce HepG2 cell viability and may therefore be useful for further drug development.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of Variance
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity
EMEM	Eagle's Minimal Essential Medium
FBS	Foetal Bovine Serum
GSH	Glutathione
H_2O_2	Hydrogen peroxide
IC_{50}	Half maximal inhibitory concentration
kg	Kilogram
mg	Milligram
µg	Microgram
µl	Microliter
ml	Millilitre
µM	Micro molar
mM	Milli molar
M	Molar
nM	Nano molar
nm	Nano meter
OPs	Organophosphate pesticides
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen specie
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid

1 INTRODUCTION

Neurotoxicants were widely studied during the past decades for their neurotoxic effects on human neuronal cells. Among them, pesticides and alcohol played a major role as they were considered as neurotoxic substances which may lead to neurodegeneration and neuronal death. Pesticides and alcohol are bio-transformed in the human liver and the toxicity of each are compromised from the toxic metabolites produced in the liver (Nakamura *et al.,* 2000; Rintala *et al.,* 2001; Upadhya and Ravindranath, 2002, Mangas *et al.,* 2017). The mechanism of neurotoxicity of both pesticides and alcohol were found to be similar in the literature as both kill neuronal cells in a dose-dependent fashion through apoptosis or necrosis (Spampinato *et. al.,* 2005, Erdozain *et al.*, 2014, Ramlochansingh *et al.,* 2011; Slotkin *et al*., 2016). Acetaldehyde is one of the toxic metabolites formed by alcohol and was found to be toxic to neuronal cells (Yan *et al.,* 2016). H_2O_2 is a free radical that is formed during the cellular metabolism of pesticides and alcohol and is a cause of redox stress in cells (Kim *et al.,* 2015).

The safety testing of these neurotoxicants including their metabolic products were heavily studied using animal dosing and is therefore extremely time consuming and expensive and has some species differences. Recently, primary cell lines were used to study the mechanism of toxicity of these neurotoxicants which has many advantages over animal-based experiments such as less time consuming and can used for high throughput screening studies with minimal species differences. Human neuroblastoma cell line and Human hepatocellular carcinoma cell line are currently using for neurotoxicological studies and hepatoxicity studies to mimic the effect of environmental toxic chemicals on cell viability, proliferation and differentiation.

There have been several studies done to find the influences of these neurotoxicants on neuronal cells and their mode of action. It has been recorded that serum albumin plays a major role in the binding environmental and can reduce toxicity of pesticides on cells (Peeples *et al*, 2005). Therefore, there is an immense need to investigate the role of serum albumins in culture media on cytotoxicity of these neurotoxicants to standardise experimental protocols to mimic *in vivo* human toxicity.

The liver is the primary target of these neurotoxic chemicals and their metabolic products are also cytotoxic to the liver cells. Plant antioxidants are widely studied due to their ability act as free radical scavengers at the cellular level. Traditional medical plants have been highly studied for their ability to act as free radical scavenging activities.

Therefore, this study was carried out to determine the half maximal cell death concentrations of neurotoxicants: two commonly employed organophosphate pesticides, alcohol, acetaldehyde and H_2O_2 and to determine the effect of commonly used traditional African medical plants on cell viability. Two useful cell models were employed: human neuroblastoma cells and HepG2 cells as models of neurons and hepatocytes, respectively.

The study provides an insight to future standardisation protocols for rapid screening of neurotoxicological studies of common neurotoxicants and the development of pharmaceutical therapies from traditional medicinal plants.

1.1 Rationale of the study

In vitro neurotoxicological and hepatotoxicological studies are useful to mimic the cellular mechanism of neurotoxic and hepatotoxic or hepatoprotective compounds. Use of a wide concentration of compounds with small quantities is cost effective for toxicological studies than using large number of rodents for

predicting the effect of results on humans. The safe limit of these neurotoxic compounds should be studied to prevent harm associated with using them. Understanding the potential health impact of neurotoxic compounds is important to prevent neuronal damage in humans. To explore the biological basis of neurotoxic compounds, the effects of alcohol, acetaldehyde, and OP pesticides on SH-SY5Y cell viability were studied. These neurotoxic compounds target cellular proteins and morphological impairments are common due to the formation of new adducts. This can be further explored by detecting the level of certain proteins after treating cells with neurotoxic compounds using *in vitro* conditions. Although these plant extracts are used as medicinal plants in Africa their biochemical basis of action has not been assessed. However, there is a gap in the literature regarding their hepatotoxicity. An assessment of the hepatotoxicity of these medicinal plants is necessary for efficient and safe use of plants for daily use as well as for use of these plants as drugs to cure many liver related diseases. It may also support data generated through *in vivo* studies.

1.2 Hypothesis

The research hypothesis therefore is that the toxic action of alcohol, acetaldehyde, H_2O_2 and commonly employed two OP pesticides can be assessed using neuroblastoma cells also some of plants extracts from African medicinal plants that have been used as therapeutics to cure many diseases which influence cell viability would be better understood when assessed by *in vitro* cell culture.

1.3 Aims of the study

1. Neurotoxicity evaluation of alcohol, acetaldehyde and selected OP pesticides.

2. Determine the doses producing 50 % cell death (IC_{50}s).
3. Determine the cellular protein levels that change arising from exposure to alcohol and H_2O_2.
4. Hepatotoxicity evaluation of selected plant extracts by assessing cell viability.
5. Determine the doses producing 50 % cell death (IC_{50}s).
6. Conclude whether the selected plant extracts are hepatotoxic or hepatoprotective and could be used as potential natural medicine to treat liver diseases.

2 BACKGROUND OF THE STUDY

2.1 Neurotoxicity

Neurotoxicity is defined as “any adverse effect on chemistry, structure and function of the nervous system during development or at maturity, induced by chemical or physical influences”. The change can be affected on morphology, neurochemicals, neurologic or behavioural aspects of the nervous system. Therefore, any treatment that interferes with normal function of the nervous system can be led to neurotoxicity. The effect can be transitory or persistent (Costa, 1998).

2.1.1 Nervous system

The nervous system is regarded as the main governor of all body functions. It consists of the brain, spinal cord and nerves. The functional unit of the system is neuronal cells. Brain and the spinal cords belong to central nervous system. The parasympathetic nervous system is the connector in between the stimuli and the brain. It is composed of two parts: Autonomic nervous system which governs the involuntary actions such as breathing and digestion, and the somatic nervous system which regulates the voluntary body actions and reflexes (Bear *et al*., 2001). Apart from the neurones, glial cells also can be found within nervous system and two types of glial cells can be found in the nervous system: large bodied cells as macroglia and small bodied cells as microglia (**Figure 2-1**). Astrocytes and oligodendrocytes are two major macroglia found in the central nervous system (Bear *et al*., 2001; Franklin, 2017).

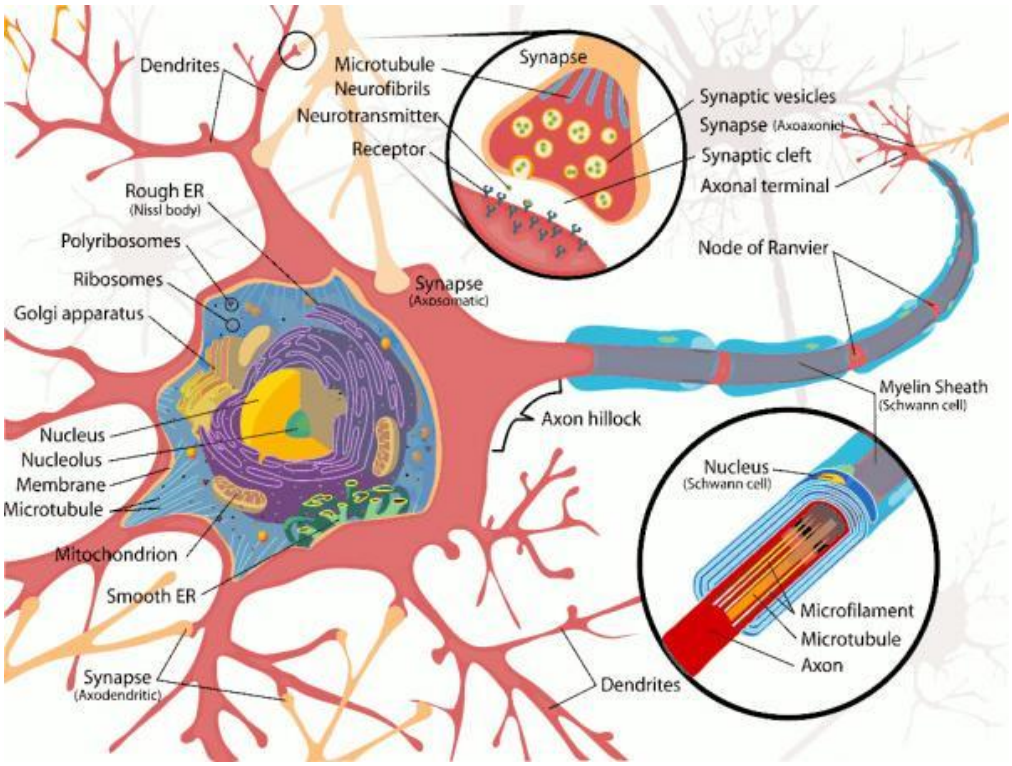


Figure 2-1 Structure of a multipolar neuron that showing its internal organelles and structure of a synapse (adapted from Flagg, 2017).

2.1.2 Mechanism of neurotoxicity

Recently, many mechanisms have been proposed that contribute to neurotoxicity. Notably, several mechanisms have been proposed to predict the cell death or cell injury caused by an apoptosis or necrosis process. Investigations related to neurotoxicity on cells under *in vitro* conditions suggest four mechanisms of neurotoxicity. These include glutamate- induced excitotoxicity that contributes to intracellular free calcium (Ca^{2+}) overload, oxidative stress and mitochondrial or endoplasmic dysfunction and reticulum (ER) stress, and calpain activation. At least one of these mechanisms is observed after exposing cells to exogenous neurotoxic chemicals (Erdozain *et al*., 2014; Gupta *et al*., 2011; Han *et al*., 2012; Rajendran *et al*., 2016; Saravi and Dephpour, 2016;). According to the literature it can be suggested that each

of these mechanisms are interconnected. For example, intracellular free radical overdose can be due to oxidative stress and mitochondrial dysfunction (**Figure 2-2**).

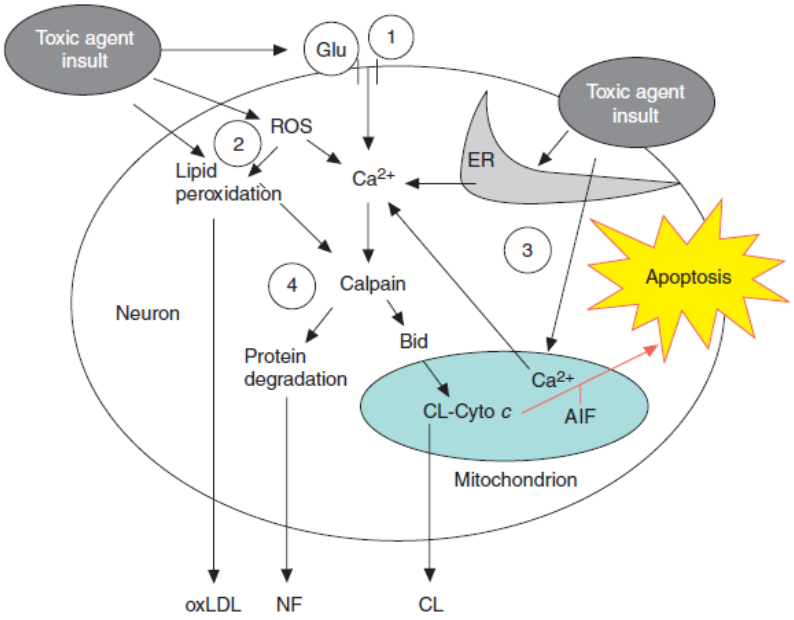


Figure 2-2 The diagram illustrates common neurotoxicity mechanisms.

1 - Neurotoxins interfere with glutamate which is the main neurotransmitter in the central nervous system. Glutamate uptake into the cells may be limited due to the action of neurotoxic compounds or they can interfere with the excess production of glutamate. This triggers the entry and accumulation of Ca^{2+} in the cytoplasm. Excess productions of glutamate also trigger the release of reactive oxygen species (ROS). 2 – The activity of neurotoxic compounds may interfere the production of ROS, and/ or reduce the normal levels of antioxidant enzymes, effecting energy production of the cell. Excessive ROS causes lipid peroxidation and mitochondrial dysfunction. 3 – Direct activity of toxic compounds on endoplasmic reticulum and mitochondria trigger the formation of cell apoptosis factors that activates caspases and causes cell damage and cell death. 4 – The enhanced generation of Ca^{2+} because of exogenous compound results in inactivation of intercellular calpain. This reaction mediated cytoskeleton protein degradation of cells and triggers the apoptosis process (Greenberg and Jin, 2007; Schaumburg and Spencer 2000; Vosler *et al*., 2008; Xu *et al*., 2005).

Neurotoxicity is mainly caused by exposing cells to natural or artificial toxic compounds such as organophosphate pesticides, heavy metals and some secondary metabolites present in plants as natural toxic compounds. This neurotoxicity can be direct or an indirect effect on the nervous system. Thus because of damage to hepatic, renal or pancreatic structures this can result in indirect neurotoxicity to humans (Costa, 1998). Alcohol intake, pesticide exposure, heavy metals, and exogenous antioxidants are reported as main sources of neurotoxicity in humans (Boyes, 2012; Grandjean and Lnadrigan, 2014; Slotkin*, et al*., 2016).

2.1.3 Alcohol neurotoxicity

Excessive alcohol consumption can lead to many health problems. The current statistics indicate there is a rapid increase in alcohol related health problems around the world. 20-30% of hospital admissions are due to alcoholism (D' Onofrio *et al*., 1998; Erdozain *et al*., 2014; Fiellin *et al*., 2000). Sullivan *et al*. (2011) has pointed out there is correlation between alcohol consumption and brain weight in humans. It is further supported by the findings related to brain atrophy. Alcohol consumption has a strong relationship to the gradual reduction of effectiveness of the affected area of the brain tissue. The reason for this is documented as certain metabolites can effect a decrease of cell size due to loss of cytoplasmic protein inside the cells. This finding is further demonstrated by the investigations related to alcohol metabolism and cell damage. Altogether, studies suggest that the toxic metabolites of alcohol and their intermediates produced during the metabolism cause severe damage to neurons (Nakamura *et al*., 2000; Rintala *et al.,* 2001; Upadhya and Ravindranath, 2000). This is mediated by the effect on neurotransmitters, receptors and electrolytes (Spampinato *et al*., 2005). In support of these results, Weiss *et al*., (1993) have demonstrated that the effect is dose-dependent. Investigations have further

suggested that acute toxicity interferes with the system transmission process by affecting neurotransmitters. On the other hand, chronic toxicity also changes neuronal transmission processes via sensitization, tolerance, withdrawal and dependence.

According to McCaskill *et al*. (2011) the first toxic metabolite of alcohol metabolism is acetaldehyde. Acetaldehyde forms adducts with cellular proteins. These adducts mainly target cellular α-tubulin, which mainly functions in microtubule polymerization. α and β-tubulin are major components of microtubules and are non-covalent polymers which contribute to the cell shape, mobility and stability. They are giant supports for mitosis and vesicular transportation in neuronal cells. Hence, adducts that form in alcohol metabolism have an influence on α and β tubulin that finally effect on the cell shape and the mitosis process. The study of Erdozain *et al*. (2014) documented an effect of alcohol of α and β tubulin in cells. This may affect mitosis and have an effect on cell proliferation. This idea is further supported by the results of Luo and Miller, (1997) who showed that SH-SY5Y lose their viability in a dose-dependent manner when treated with alcohol. This result is further clarified by the concept presented in Luo and Miller (1997) that stated that the ethanol related reduction of the number of viable cells can be due to the inhibition of the proliferation of neuronal precursors or may be due to neuronal death. Therefore, there is still a gap in the literature as to what the mechanism is that inside cells triggers a loss of viability with increasing ethanol concentration.

2.1.4 Acetaldehyde neurotoxicity

Acetaldehyde neurotoxicity is an unexploited area of neurotoxicological investigations. Acetaldehyde is an aldehyde which is systemically named as ethanal. The literature has revealed that it is a strong toxic metabolite formed

from alcohol metabolism. It is formed from the metabolism of alcohol in three pathways, alcohol dehydrogenases, and catalase and from the microsomal ethanol oxidizing system (**Figure 2-3**). Acetaldehyde is regarded as mutagenic and genotoxic (Rajendram *et al.,* 2016). Other than alcohol consumption, humans are exposed to exogenous acetaldehydes from the diet. Alcoholic beverages such as coffee, ethanol containing beverages, tobacco smoke, yogurt and ripe fruits contain acetaldehyde (Miyake and Shibamoto, 1993). Metabolism of these alcoholic beverages produces acetaldehyde inside the body. It is a highly toxic metabolite which forms adducts with cellular DNA and proteins (Nakamura *et al.*, 2003, 2000; Niemela, 2007, Upadya and Ravindranath, 2002). Acetaldehyde toxicity is mainly found in liver because most of the alcohol consumed is metabolised in the liver, but it may also have effects on neurons. This toxic metabolite is reported as a strong inhibitor of cell proliferation that can cause cell death. It was further supported by the Wieslander *et al*. (1995) that showed a reduction of cell growth in mouse fibroblasts. This toxic metabolite affects synaptic function under accumulation of higher concentrations (Yan *et al*., 2016). The observations in the rodent models showed that acetaldehyde can inhibit the cell growth via an apoptosis process (Holownia *et al*., 2009). However, given the extensive evidences on mechanisms behind the cell growth inhibition there is also a correlation between mitochondrial dysfunction and the reactive oxygen species formation during metabolism and this effect on cell death (**Figure 2-3**); (Farfan Labbonne *et al*., 2009; Gomez –Quiroz *et al*., 2003). This was supported by the studies undertaken with rat brain neuronal cells that have been tested for cell viability against acetaldehyde treatment and lead to the postulate that the cytotoxic effect was due to the activity of the caspase cascade (Clavijo-Cornejo 2014; Jung *et al*., 2006; Tong *et al*., 2011).

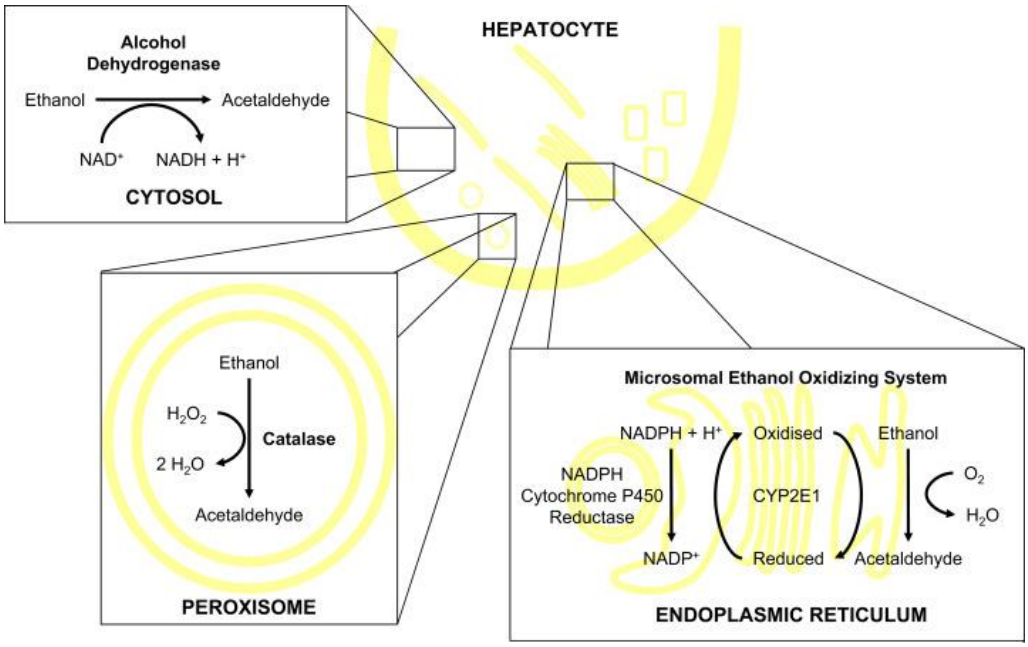


Figure 2-3 Pathways of acetaldehyde formation from alcohol (adapted from Rajendram et al., 2005).

However, this observation was challenged by the fact that acetaldehyde treatment to rat cerebellar cell cultures showed a strong reduction of cell viability. The studies further reported this is due to mitochondrial dysfunction and oxidative stress (Jung *et al*., 2006). In a study done with oligodendrocytes treated with acetaldehyde, cell death occurred when the concentration of acetaldehyde increased from 0.5 mmol/l. In addition to this there is a reduction of serum transferases and reductase due to acetaldehyde toxicity (Morgan *et al*., 2002).

Still there is no clear understanding about the mechanism behind cytotoxicity of neuronal cells by acetaldehyde. Yan *et al.* (2016) showed that the neurotoxicity of acetaldehyde was due to apoptosis, inhibition of cell survival pathway and due to the oxidative stress by using SH-SY5Y cells as a model. Erdozain *et al*., (2014) pointed out that acetaldehyde forms cellular protein adducts that caused cell injury. This is may be due to α and β tubulin targeted adduct formation.

Therefore, it is vital to study about these proteins and acetaldehyde toxicity to provide an insight into the mechanism behind the acetaldehyde mediated neurotoxicity.

2.1.5 H_2O_2 neurotoxicity

Reactive oxygen species (ROS) formation is a major metabolite formed in endogenous and exogenous metabolism in the body. Formation of ROS is a concern in with neurological diseases like Alzheimer's disease and Parkinson's disease. Hydrogen peroxide is a nonradical form of ROS which can be converted to the hydroxyl radical that is toxic to the cell. This toxic radical causes damage to the cell and gradually the cell dies. ROS damage is prominent in neuronal cells. However, the most common free radical that causes neuronal and glial cell is H_2O_2 (Chetsawang *et al.*, 2006; Takuma *et al*., 2003). It is further supported by the evidences that have showed that H_2O_2 is able to activate many caspase cascades which may trigger cell death (Lee *et al*., 2005). These mechanisms have not been investigated too deeply to conclude which mechanisms are behind this process and how H_2O_2 is involved. It has been widely studied that many kinase activations and signalling pathways are involved in H_2O_2 related cell death (Sugden and Clerk, 2006). Chetsawang *et al*. (2010) showed ROS signalling pathway of H_2O_2 mediated toxicity in neuronal cells by using SH-SY5Y cells. This study further showed that the H_2O_2 greatly affected the cell viability on neuronal cells (**Figure 2-4**).

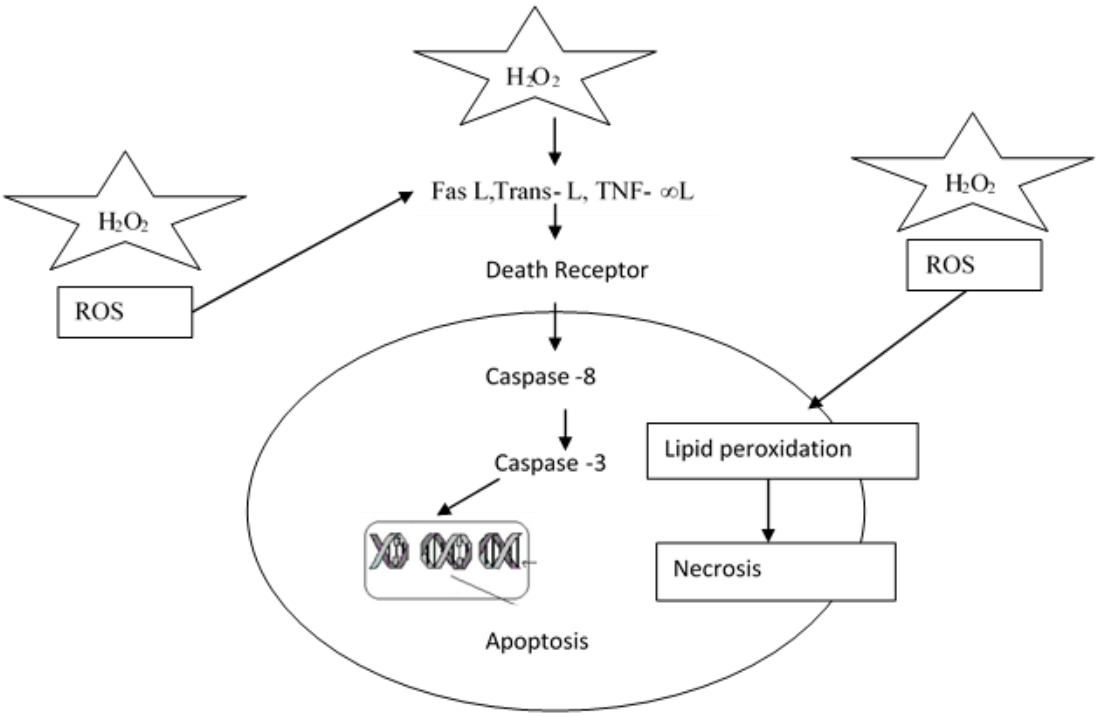


Figure 2-4 H_2O_2 induced toxicity in neuronal cells.

H_2O_2 can activate death ligands like FAS - L and Trans -L receptors in cells and these ligands act upon the death receptors. These death receptors activate the caspase enzymes which targets the cellular DNA and protein. Damage to these molecules ultimately causes cell death via apoptosis. On the other hand, H_2O_2 induces lipid peroxidation and releasing LDH causes necrosis (Garcimartin et al., 2014)

However, many studies have reported the H_2O_2 effect on cell viability, but the threshold H_2O_2 level which causes cell death was not discovered. In support of this idea recent findings summarize H_2O_2 as an agent that can damage cells via apoptosis and redox changes when exposed to cells. But to induce the toxicity there should be a threshold level of H_2O_2 according to the studies done by Cai *et al*. (2008) and Veal *et al*. (2007). Exogenous H_2O_2 induced neurotoxicity in neuronal like cells can be used to mimic H_2O_2 induced cell death (Cañas *et al.*, 2007; Ismail *et al*., 2014).

2.1.6 Organophosphate pesticide neurotoxicity

Organophosphates (OPs) are synthetic in origin and are esters, amides or thiol derivatives of phosphoric, phosphorothioic or phosphonothioic acids.

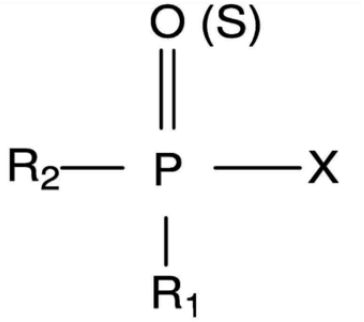


Figure 2-5 General chemical structure of organophosphates.

R1 and R2 are most commonly alkyl or aryl groups. X group can be any one of the wide varieties of substituted or branched aliphatic, aromatic or heterocyclic groups linked to phosphorous atom through a liable bond usually O or S and is referred to as the leaving group (adapted from Sogorb and Vilanova, 2002).

There are three main groups: phosphates (without a sulphur atom), phosphoric (with sulphur atom) acid and phosphorodithioates (with two sulphur atoms, **Figure 2-5**). The X group is bond to the phosphorous atom through an oxygen or sulphur (Sogorb and Vilanova, 2002). OPs are less persistent in the environment as they are decomposed by biological and chemical reactions (Zacharia, 2011). OPs are very toxic to both vertebrates and invertebrates since they act upon the nervous system. Their target site is the synapse of the neurone and inhibits the activity of cholinesterase while completely overlaying the neurotransmission process.

2.1.6.1 Mechanism of organophosphate neurotoxicity

Pesticide neurotoxicity happens in three ways. Neuronopathies – caused by damage to the neuronal cells; Axonopathies - caused by damage to the axon of the neuronal cells and that interferes with the signal transmission process;

Myelinopathies – which interfere with the neurotransmission process. Organophosphate pesticides target the neuronal cells and interfere with the acetylcholine activity (**Figure 2-6**; Costa *et al*., 2008).

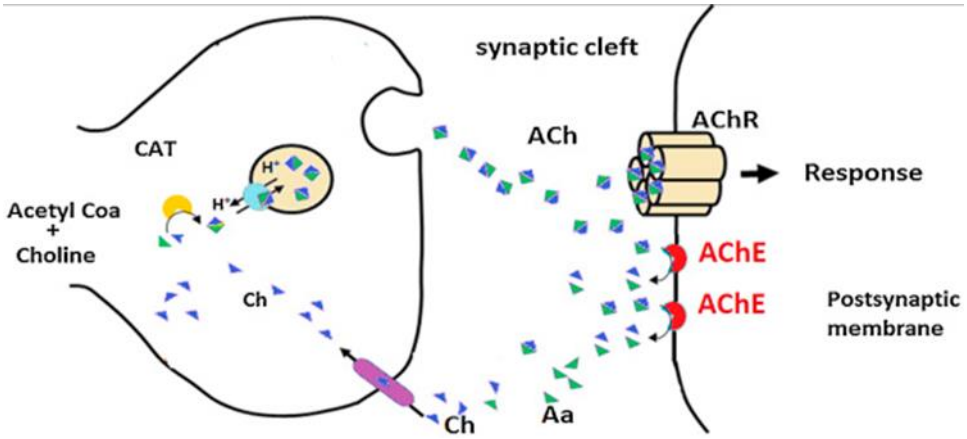


Figure 2-6 Synaptic cleft showing the function of acetylcholinesterase (AChE) (adapted from Vargas-Bernal et al., 2012).

The acetylcholine released from the pre-synaptic neuron into the synaptic cleft and the molecules interacts with the receptors in postsynaptic membrane. Then the molecule disassociates to acetic acid (Aa) and choline (Ch) by the catalytic action of acetylcholinesterase (AChE). Then Ch enters back into the presynaptic neuronal cells for resynthesize (Mangas *et al.,* 2017). Inhibition of AChE by OPs lead to excess accumulation of acetylcholine (ACh) in synapses resulting in overstimulation of receptors which causes cholinergic symptoms.

In OP neurotoxicity, organophosphorylation of AChE happens and this covalent bond is not easily cleaved (Estévez *et al*., 2012; Mangas *et al*., 2017; Sogorb and Vilanova, 2002). OP neurotoxicity is reported in high mortality rates in recent decades. The neurotoxic effect of exposure to OP is dependent on the dose, level of exposure, time of exposure, type of OP pesticide and inherited characteristic of the person who is exposed like sensitivity, age etc. (Muñoz-Quezada *et al.,* 2013).

OPs are heavily studied for their ability to cause neurotoxicity in humans. Mechanisms behind the cell injury caused by OPs are greatly discussed in developmental toxicology. The thion group of OPs has less toxic on humans but phosphorothioates can produce the oxon by oxidation. The enzymes which catalyze this reaction is present in liver and it was reported that UV light and air also contribute to trigger the reaction spontaneously (Galloway and Handy, 2003; Sorgrob and Vilanova, 2002; Vilanova and Sorgorb, 1999). The oxon is the reactive metabolite produced from the thion oxidation. The oxon metabolites of OPs can rapidly react with certain proteins to form adducts. The oxon binds with these proteins covalently and the proteins increase their molecular weight due to these adducts. This is the governing property of OPs in humans that they can form adducts by organophosphorylation of a protein with covalent modifications (Mangas *et al*., 2017). The literature has revealed that most OPs target the serine hydrolase protein family and organophosphorylation is at serine residues. Other than these targets it was reported tyrosines can also bind with OPs (Sorgorb and Vilanova, 2002). Noncovalent binding of OPs found to albumin protein in blood has also been reported (Mangas *et al*., 2017). The following figure (**Figure 2-7**) depicted the two oxon derivatives of two OPs used for the current study.

A – Azamethiphos-oxon B - Chlorpyrifos-oxon

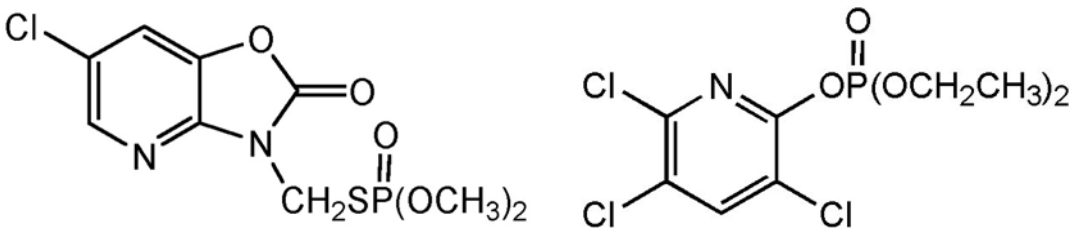


Figure 2-7 Chemical structure of pesticides.

These pesticides have been extensively studied for their ability to cause neurotoxicity in humans. Chlorpyrifos-oxon and methyl oxon was reported as a

strong inhibitor of cannabinoid receptors in both *in vivo* and *in vitro* studies using rodent models (adapted from Quistad *et al.,* 2002).

Axelrad *et al*. (2002) reported that chlorpyrifos-oxon has an IC_{50} of 26 ± 14 µmol/l in commercial formulation studied on NB2a neuroblastoma cells. They further mentioned that this cell line behaves in a dose-dependent manner when treated individually. After differentiation, they also stated that neurite growth was reduced with increasing concentration. However, with combination with other pesticides chlorpyrifos-oxon gives a synergistic effect. Veronesi and Ehrich, (1993) reported that the different cell lines were having differential cytotoxic sensitivity to OPs. Study further reports according to the species diversity this is varied such as mouse neuroblastoma cells were more sensitive than the human neuroblastoma cells. This is because species specific neuronal targets after metabolism. Metabolism of their oxon moiety may influence the lethality of cells. Other than these studies, OPs are heavily studied for their protein adducts. However, the direct OP effect on cells is more important for studying of the mechanism of OP toxicity in humans. Human derived cell lines used for the neurotoxicological studies pave the way for mimicking the cellular mechanism *in vivo* in a cost effective and less time-consuming manner. High throughput screening of wide concentrations of OP pesticides has recently been used in developmental neurotoxicological studies. This provides the base line studies for mimicking the mechanism of OP toxicity in the human body and these studies are further useful for future proteomic studies to detect the molecular targets of OPs.

2.2 Hepatotoxicity

Hepatotoxicity is defined as “injury to the liver or impairment of the liver function caused by exposure to xenobiotics such as drugs, food additives, alcohol, chlorinated solvents, peroxidised fatty acids, fungal toxins, radioactive isotopes, environmental toxicants and even some medicinal plants” (Manfo *et al.,* 2014). This definition is further supported by the evidences from studies done by Manfo *et al.* (2014) and Verma and Kaplowitz, (2013) working with the liver and illustrate that it is the major biotransformation unit of toxic compounds that enter the body. Many drugs recommended for human diseases are toxic to the liver if they consumed in overdoses or sometimes even within a therapeutic range (Mbaveng *et al.,* 2014; Moseley, 2013; Núñez, 2006; Reuben, 2013; Verma and Kaplowitz, 2013). Symptoms of hepatotoxicity are stomach pain, nausea (upset stomach), unusual tiredness, dark-colored urine, light or clay-colored stools, jaundice (yellow skin and sclera of eyes), loss of appetite and fever. The degree of symptoms can be varied with the exposure level (Lammert *et al.,* 2008; Lucena *et al.,* 2009), formulation of drug and the inheritance characteristics of the person such as age (Jinjuvadia *et al.,* 2007), gender (Franks *et al.,* 1989), nutrition level and genetic disorders for instance gene polymorphism (encoding histocompatibility class II) (Sharma *et al.,* 2002).

2.2.1Anatomy and physiology of human liver

The human liver has two lobes and each lobe consists of multiple lobules and sinusoids. The portal vein supplies ~75% of the liver blood supply. Portal vein collects blood with metabolic products from small intestine, stomach, pancreas and spleen and transports them to the liver. The remaining 25% of the blood supply to the liver is coming from arteries which arise from the hepatic vein (Jonson, 1991). The blood coming through these two veins are unloaded to the sinusoids present in the liver and these get expand with incoming blood and spread the blood all over the liver. Blood coming from the hepatic artery and portal vein are mixed at the point of entry to the liver sinusoids and mixed blood leaks to the endothelial cell lining to contact the hepatocytes (Duffus and Worth, 2006). The blood with metabolic products in liver collected by hepatic veins that connected to the inferior vena cava and return to the heart (**Figure 2-8**).

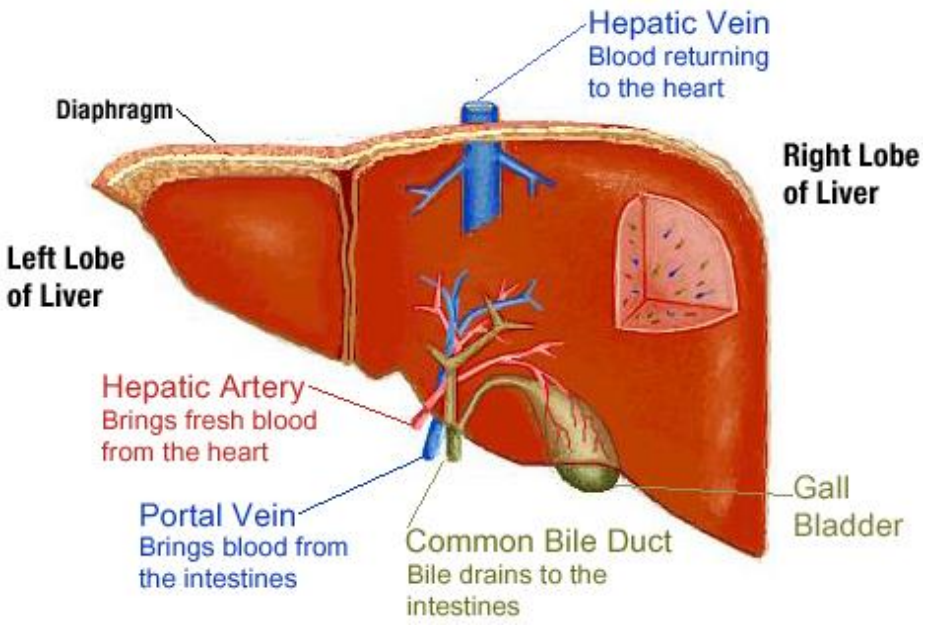


Figure 2-8 Anatomical structure of the human liver (adapted from Lipski, 2005).

The functional cells of the liver are hepatocytes; also known as parenchymal cells which construct the liver lobules. The supporting cells known as endothelial

cells (nonparenchymal cells) construct the lining of the wall of sinusoids. Other than endothelial cells a special type of cells called Kupffer cells (reticuloendothelial cells), hepatic stellate cells (Perisinusoidal or Ito cells) and intrahepatic lymphocytes for instance pit cells, which are known as liver specific natural killer cells, are present in the liver. Hepatocytes secrete bile into the cuniculus in liver and they release collected bile into bile duct, which connects to the duodenum. The whole liver is covered by a capsule of connective tissue. This capsule also houses blood vessels, lymphatic vessels and bile duct **(Figure 2-8).** The main functions of the human liver are digestion, defence and metabolism (Guyton and Hall, 2006; Jonson, 1991).

2.2.1.1 Role of liver in the metabolism of exogenous compounds

The liver metabolises exogenous chemical compounds in two phase reactions.

2.2.1.1.1 Phase I

Phase I reactions include oxidation, hydrolysis and reduction. Enzymes belonging to the cytochrome P450 group such as monooxygenase system or Flavin – containing monooxygenase, catalyse these reactions. Phase I results in more polarized hydroxylated compounds from initial pass lipophilic compounds in xenobiotics (Usmani *et al.,* 2004; Smith and Hillman, 1999).

2.2.1.1.2 Phase II

The resulted products from Phase I with functional groups such as hydroxyl, amino, carboxyl, epoxide or halogen are combined with endogenous metabolites as glucuronic acids, sugars, amino acids, glutathione, sulphates and acetates. They make conjugates in this Phase and produce less toxic compounds that can be easily eliminated from the body (Lüllmann *et al.,* 2011; Usmani *et al.,* 2004).

2.2.2 Hepatotoxic compounds

Many drugs used for the cure of diseases such as tuberculosis (Verma and Kaplowitz, 2013) and Alzheimer's disease (Ballard *et al.,* 2011; Mufson *et al.,* 2008; Parihar and Hemnani, 2004) are reported to be hepatotoxic in nature while alcohol, pesticides, heavy metals, environmental pollutants, food contaminants and herbal products are also regarded as hepatotoxicants (Kumar *et al*., 2013; Mukazayire *et al.,* 2010; Saoudi and Feki, 2012).

2.2.3 Mechanism of hepatotoxicity

Hepatotoxicity occurs within the liver in the context of physiology, biology and metabolism. In considering the liver physiology bile acid induced cell injury, mitochondrial dysfunction, formation of reactive oxygen species (ROS) and neutrophils mediated liver injury are leading mechanisms of hepatotoxicity. Fromenty and Pessayre, (1995) reported that bile acid synthesis during metabolism can become toxic to hepatocytes. This is further supported by Faubion and Gores, (1999); Faubion *et al.,* (1999); Miyoshi *et al.,* (1999) that also describe bile acid induced cell injury and apoptosis.

Mitochondrial dysfunction also causes hepatotoxicity as mitochondria are the main energy factory in cells. The excessive ROS produced during the exogenous compound metabolism in cells causes oxidative stress in cells. This has been widely studied with drugs like acetaminophen (APAP) and ibuprofen administered to rats and rabbits as experimental models. The free radicals generated through the metabolism of these drugs depletes the mitochondrial proteins and cellular glutathione (GSH) gradually causing oxidative stress and dysfunction of mitochondria (**Figure 2-9**) (Jaeschke *et al.,* 2013; Koop *et al.,* 2006; Du *et al.,* 2016; Manthripragada *et al.*, 2011). GSH act as intracellular free radical hunter and acts to reduce the toxicity of xenobiotics by making

glutathione conjugates (Ramappa and Aithal, 2013). Chen *et al.* (2001); Dai *et al.* (1993); Guengerich *et al.* (1990); and Koop, (1992) have provided evidence of the generation of ROS via cytochrome P450 metabolism in hepatocytes. Therapeutic drugs, ethanol and methanol increase P450 induced oxidative stress in mitochondria and toxicity cause mitochondrial dysfunction.

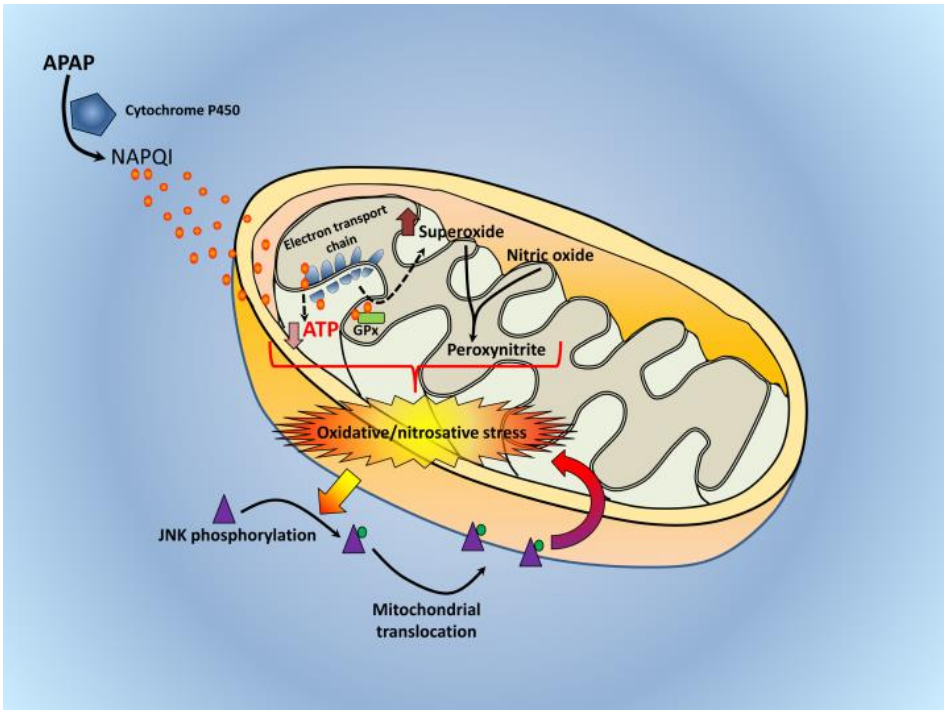


Figure 2-9 ROS production through mitochondrial dysfunction by Acetaminophen (APAP) hepatotoxicity.

Overuse of APAP causes hepatotoxicity. APAP produces a toxic intermediate from the Phase I reaction. NAPQI (N-acetyl-p-benzoquinone imine) is a toxic metabolite formed during metabolism of APAP in humans. Intermediate forms target the mitochondrial proteins and reduces the activity of ATPsynthase. Hence, it impairs the electron transport chain and gradually leads to cell death due to the oxidative stress. This concept is supported by the data generated through the study of positional isomers of APAP by Xie *et al*., (2014) in human hepatocytes.

2.2.4 The relationship between hepatotoxicity and cell proliferation

Hepatotoxicity has profound effects on the proliferation of hepatocytes (Jaeschke, 2002). As discussed above, hepatotoxicity is significantly triggered

by mitochondrial dysfunction. Additionally, Ca^{2+} ions influence the membrane potential of the cell and an altered concentration is harmful to cells (Nieminen *et al.,* 1995). Cytotoxicity of hepatotoxic chemicals leads to cellular apoptosis through death receptor pathway or mitochondrial pathway (Faubion and Gores, 1999; Faubion *et al.,* 1999; Miyoshi *et al.,* 1999; Sodeman *et al.,* 2000). Neutrophils mediated necrosis can also lead to cell death (Jaeschke, 2002; Maher *et al.,* 1997; Richter and Kass, 1991). Kupffer cells activated indirectly or directly by hepatotoxic compounds may release biologically active cytokines, growth regulators as well as ROS and nitrogen species which can interfere with cell proteins and nucleotide synthesis (Jaeschke, 2002). Hence, this can directly influence proliferation and differentiation of hepatocytes. *In vitro* cell models assess the cell viability of hepatocytes to mimic the cell proliferation and differentiation effects.

2.2.5 Herbal medicines

Herbal medicines play a major role in ayurvedic medicine to treat many diseases. The phytochemical compounds present in herbal medicines such as polyphenols, flavonoids, terpenoids and alkaloids have been used for the treatment of hepatic disorders in ancient Chinese and Indian medicine (Adams *et al.,* 2007; Gurib–Fakim, 2006; Howes and Houghton, 2003; Kiran *et al.,* 2012). These secondary metabolites present in the medicinal plants when extracted into organic solvents could be used as pharmaceutical therapeutics. The secondary metabolites present in medicinal plants rich in antioxidant activities have been used for treatments to oxidative stress related diseases like neurotoxicity and hepatotoxicity (Nn, 2015).

In support to this, many research studies have showed that medicinal plants have the ability to reduce high serum aspartate aminotransferase, alanine

aminotransferase, alkaline phosphatase and bilirubin levels in relation to hepatic damage. Studies have been determined using human cell lines or rats as a test for pharmacologically active agents (Abdel–Azeem *et al.,* 2013; Adebajo *et al.,* 2006; Eleyinmi, 2007; Fakurazi *et al.,* 2008; Hamza, 2010; Antai *et al.,* 2009; Johnkennedy and Adamma, 2011; Menut *et al.,* 1993). For example, studies from Adebajo *et al.* (2006); Fakurazi *et al.* (2008) and Hamza (2010) have showed that a significant reduction of lipid peroxidised products that are highly chemotoxic to the liver cells have been achieved using *Murray akoenigii* and hydroalcoholic (80% ethanol: 20% distilled water) extract of the plant *Moringa oleifera* Lam. (Moringaceae), respectively. The plant *Aloe vera* have been widely utilized in the herbal medical industry in African countries as a prominent hepatoprotective plant. This plant can be also hepatoprotective since it alleviated serum transaminase activities in response to paracetamol (Nayak *et al*., 2011). Most of African plants used as folk medicines are not scientifically studied for their toxicities (Manfo *et al.,* 2014). Recent evidences suggest that many plants extracts may be hepatotoxic rather than hepatoprotective. This is further supported by the studies of same plant for their gastro-protective or anti-diarrheal properties. For instance, *Pteleopsis hylodendron* Mildbr is medicinal plant used in West and Central Africa for the treatment of several diseases like measles, chickenpox, and sexually transmitted diseases. However, the plant is assessed as hepatotoxic as it is cause vascular congestion and inflammation in the liver tissue, as well as increased serum activities (Nana *et al.,* 2011; Manfo *et al.,* 2014).

The chemical constituents present in a medically important plant that are responsible for triggering hepatotoxicity, can target either one or multiple types of cell in liver or subcellular structures (Teschke *et al.*, 2014). In addition to that, the plant part and solvent used for extraction can also have a significant effect

on the activity of secondary metabolites in plants. Studies by Omotayo *et al.,* (2011) and Oduola *et al* (2010) showed that *Morinda Lucida* (l.) Benth extracts used for diabetes, typhoid fever and malaria treatment was not hepatotoxic whereas an aqueous extract from the roots of same plant was hepatotoxic in rats. This highlights the need to study about different plant parts extracted in different solvents for consideration of liver toxicity. The solvents used in extraction can have effects on quantity of secondary metabolites extracted to the solvent. A study of the comparative evaluation of different extracts of leaves of *Psidium guava* Linn. for hepatoprotective activity, markedly highlights this theory, since a methanolic extract of the plant significantly reduced liver serum levels of serum transaminase compared to the aqueous, ethyl, petroleum ether and chloroform extracts (Roy and Das, 2010).

2.2.6 Plants examined in this dissertation

The *Carpolobia lutea* belongs to the family Polygalaceae and is a perinnial herb. It is native to West and Central Tropical Africa. It is widely spread in rainforests and Guinea savannah of Sierra Leone, Cameroon and Nigeria. Plant extracts of this plant have been reported to exhibit anti-diarrheal, anti–nociceptive (Nwidu, *et al.*, 2011) and gastro protective (Nwidu *et al.,* 2014) properties. However, this plant is not scientifically studied for its ability to cause liver injury.

Spondium mombin is a fructiferous tree that belongs to the family Anacardiaceae, with a habitat in the West Indies, Southern Mexico, Peru, Brazil, and many tropical African countries such Equatorial Guinea, Cote D'evoir, Nigeria and Sierra-Leone (Irvine, 1961). Plant parts of this tree have been used as traditional folk medicine to treat dysentery, haemorrhoids, gonorrhoea and leucorrhoea. The plant is reported as its antimicrobial, antibacterial, antifungal,

antiviral and beta-lactamase inhibitory properties (Abo *et al.,* 1999; Ajao *et al.,* 1985; Coates *et al.,* 1994; Corthout *et al.,* 1994; Rodrigues *et al.,* 2000).

Gongronema latifolium and Moringa oleifera are native to India; Trichosanthes cucumerina, Artocarpus communis, Capsicum annum are plants used as vegetables in Asian and African countries. These plants have been reported as medicinal plants having anti-inflammatory properties. Various plant parts of *M. oleifera* have been used for antitumor, antipyretic, antiseptic, anti-inflamatory, anti-ulcer, antidiuretic, antihypersensitive, antioxidant properties (Anwar *et al.,* 2007, Fakurazi *et al.*, 2008; Hamza, 2010; Kar *et al.,* 2003; Sharifudin *et al.,* 2013). Interestingly, hydroalcoholic extracts of the plant have been used for treatment of APAP treated rats and showed significant reduction in serum aspartate aminotransferase and alanine aminotransferase levels. The study observed an increase of GSH level in rats administered with *M. oleifera* (Fakurazi *et al.,* 2008). This hepatoprotective observation was also observed by the study of Hamza, (2010) which reported that cadmium-induced liver injury was reduced by oral administration of methanolic extracts of *M. oleifera*.

Musanga cecropioides is a deciduous evergreen plant that belongs to the family Urticaceae and is considered as African medicinal plant. Plant leaf extracts exhibit anti-inflammatory and anti-nociception properties (Adeneye *et al.,* 2006, 2007; Dongmo *et al.,* 1996; Kamanyi *et al.,* 1992, 1996; Sowemimo *et al.,* 2015; Uwah *et al.,* 2013). Bark aqueous extracts are reported as a treatment for hepatic injuries. This was supported by a study in carbontetrachloride ($CCL_{4)}$ induced liver injury in rats when treated with aqueous extracts of plant showed restoration of liver enzymes (Adeneye and Agbaje, 2007).

Terminalia catappa is used as landscaping plant and is famous as an Indian olive. The plant prevented liver injury that were stimulated by using 1, 1-

diphenyl-2-picrylhydrazyl and superoxide (O_2^-) anion in both *in vivo* and *in vitro* conditions. However, an *in vivo* study also showed that rats treated with plant extracts exhibit strong elevation of high serum transferases (Kinoshita *et al.,* 2007).

Millettia thonningii Bak. family Leguminosae has a wide range of biological activities such as anti-tumour, anti-inflammatory, anti-viral, bactericidal, insecticidal and pest-destroying properties (Banzouzi *et al.,* 2008). However, hepatoxicity or hepatoprotective ability of plant extracts of *Millettia* genus has not been reported.

Peltophorum pterocarpum belongs to the family Leguminosae and is native to North America. The plant shows antioxidant and anti-glycemic properties as reported by Manaharan *et al.* (2011). A study of bark and leaf extracts interfered with the free radical scavenging activity suggesting that the plant has potential use for oxidative stress related hepatotoxicity treatments (Manaharan *et al.,* 2011). *Glyphae brevis* shrub (Spreng) Monachino of the family Tiliaceae has been selected for many studies of hepatotoxicity. Ojelabi *et al.* (2012) showed that the ethanol extract of plants had strong inhibition of certain enzymes responsible for ROS production in hepatocytes for cadmium poisoned rats.

Parquetina nigrescens is herbaceous, perennial twine belonging to the family *Asclepiadaceae*. The plant has many secondary metabolites like cardenolides, glycosides and alkaloids (Burkill, 1985; Marks, 1975). The plant is widely used to cure anemia and increase milk production in breast feeding women in Africa (Agbor and Odetola, 2001, 2005).

The studies demonstrate the application of plants as hepatoprotective agents but there is still a gap in the literature related to their hepatoprotective ability in different solvents with different plant extracts.

2.3 *In vitro* evaluation of neurotoxicity and hepatotoxicity using human derived cell lines

Potential neurotoxicity studies have been performed using *in vitro* studies which has more advantageous over the older *in vivo* methods. This includes the fact that a large number of chemicals can be tested at different time points with high accuracy and in a cheaper way (Bal-Price *et al*., 2012). Immortal neuronal cell lines (Mundy *et al*., 2010), transformed neuronal precursor cells (Krug *et al.,* 2013), primary central nervous system (CNS) cultures (Harrill *et al.,* 2013), co-culture systems (Anderl *et al.,* 2009), and stem cell-derived neuroprogenitor cells (Breier *et al.,* 2010; Harrill *et al.,* 2010; Sison-Young *et al.,* 2012) are the most commonly used cell lines for neurotoxicological studies. CNS cultures are the best cells for mimicking the neurotoxicity, although CNS cells are expensive to obtain from a human as well as they are not suitable for screening purposes since they have variability in their quality and composition (Betts, 2010). Stem cell derived progenitor cells like hN2TM cell line derived from human embryonic stem cells have promising characteristics of the developing brain. Therefore, they are used for developmental neurotoxicity testing studies. Neuroblastoma cells are one of the tumours derived immortal cell line which is easy to maintain and expand and can differentiated into mature neurones with well-defined protocols (Betts, 2010).

Hepatotoxicity testing with challenged chemicals were done in recent years using primary and immortalized cell lines. Among them Fa2N-4, HepG2, Hep3B, PLc/PRFs Huh7, HBG, and HepaRG are the most promising cell lines. All of these are liver-derived immortalized cell lines and phenotypically different from the liver tissue. The last one, HEpaRG cell line was recently developed and retained more functional proteins and enzymes as in liver cells with stable

karyotype. They also can differentiate into hepatocyte or biliary lineages with high proliferative ability.

2.3.1.1 Cell lines used for the study

Human neuroblastoma cell was used in this study as they widely used for the neurotoxicity screening studies. SH-SY5Y cells were derived from metastatic bone tumour biopsy, cells are a subline of the parental line SK-N-SH. SK-N-SH were subcloned three times; first to SH-SY, then to SH-SY5, and finally to SH-SY5Y. SH-SY5Y were deposited to the ATCC (®) in 1970 by June L. Biedler. The most important characteristics to consider the cell for this study are,

1. Cultures have both adherent and floating cell which viable and adherent cells are were used for the studies while floating cells were discarded with media change.
2. Cell can express proteins, protein isoforms and enzymes expressed by catecholminergic neurones.
3. Large scale proliferation ability in undifferentiated state.
4. Low cost culture.
5. No ethical concerns as may be the case for primary cells (Breier *et al.,* 2010).

The most prominently used cell line for hepatotoxicity studies is the human hepatocellular carcinoma (HepG2) cell line. These cells were first isolated from a Caucasian adolescent about twenty years ago (Aden *et al.*, 1979; Knowles *et al.,* 1980). This cell line is often used to investigate hepatotoxicity mechanisms of ethanol induced liver damages. It has also been used for drug screening and an investigation of (Neuman *et al.,* 1993) hepatotoxicity mechanisms (Bajt, 2004; Essani *et al.,* 1995; 1997; Faubion and Gores, 1999, Koop, 2006; McGill *et al.,* 2012).

Characteristics of Hep G2 cells for use as an *in vitro* model,

1. Expression and ability to secrete relevant drug metabolized enzymes under *in vitro* conditions (Knasmuller *et al.,* 1998, 2004).
2. Ideal source to determine the effect of novel molecular entities since hepatocytes can generate reactive metabolites and form protein adducts that damage mitochondria (Bai and Cederbaum, 2004).
3. Expression of nuclear transcription factors that provide a means to investigate mechanisms of hepatotoxicity (Motomura *et al.,* 2006).
4. Ability to predict phospholipidosis, mitochondrial toxicity and oxidative stress (Bova *et al.,* 2005; Kessova and Cederbaum, 2005; Sawada *et al.,* 2005).

2.4 Strategies used in the study

2.4.1.1 Cell viability evaluation

Cell viability has been commonly measured using veriety of tetrazolium compounds such as MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and WST-1(water-soluble tetrazolium salts). MTT is a positively charged molecule and easily penetrates through the cell membrane of eukaryotic cells while others are negatively charged and act as intermediate electron acceptors from the cytoplasm or plasma membranes to facilitate the reaction. MTT is the homogenous cell viability assay which was developed for 96 well format and highly recommended for high throughput screening studies (Riss *et al.*, 2004).

MTT is used to assess the cell viability after treating with test compounds. The principle behind the assay is that most cells are viable due to constant mitochondrial activity. An increase or decrease in the number of viable cells is directly proportional to the mitochondrial activity of the cells (Berridge *et al*., 1993, 1995; Mosmann, 1983). This is reflected by the conversion of the MTT tetrazolium salt into formazan crystals by enzymatic reduction. The solubilised crystals are purple in colour and the rate of reduction produce a colour change that can be quantified optically using a plate reader at 540 nm (Riss *et al.,* 2004).

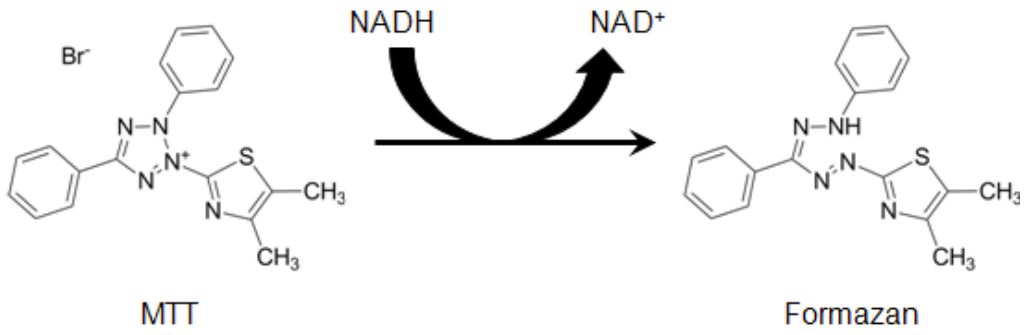


Figure 2-10 Enzymatic reduction of MTT.

When the cells die, they lose the ability to convert MTT into formazan. Therefore, the intensity of colour change is an important marker for the activity of viable cells. The exact mechanism of activity of MTT at the cellular level is controversial, although it is assumed that the NADPH transfers electrons to MTT to reduce the molecule (Goodwin, 1995).

MTT reagent is normally dissolved in a physiologically balanced solution such as PBS or RPMI without phenol red (to prevent the interference of optical density signal). 0.5 mg/ml of MTT was normally added to cell culture and incubated for 1-4 hours. Formazan crystals formed (**Figure 2-10**) by the reaction are deposited inside the cells and near to the cell medium and as well as in the culture medium. This is an irreversible reaction with the resultant formazan

insoluble in nature. There are several methods to dissolve the formazan crystals to give stable colour without any disturbances of the phenol and other culture component to enhance the optical density as well as to reduce evaporation. Acidified isopropanol, DMSO, dimethylformamide, sodium dodecyl sulphate, and combinations of detergent and organic solvent are among the reagents used (Denizot and Lang, 1986; Hansen *et al*., 1989; Mosmann, 1983; Tada *et al*., 1986). Acidification of the solubilizing solution has the benefit of changing the colour of phenol red to yellow colour that may have less interference with absorbance readings. The pH of the solubilization solution can then be adjusted to provide a maximum absorbance if sensitivity is an issue.

2.4.1.2 Quantification protein expression levels

Gel electrophoresis is a widely used technique for analysis of biological samples and for quantification of proteins, RNA and DNA. The theory behind this is that charged molecules will migrate through an electric field and this is widely used to study the protein changes in tissues, cell or other biological samples in clinical sciences, molecular biology and biochemistry (Anderson and Anderson, 1996). Usually samples were run in a supported matrix such as a polyacrylamide gel to separate the proteins. In this technique proteins in the biological samples were denatured by heating at high temperature to break the disulphide bonds in proteins. The negatively charged proteins can be then separated according to their size. The biological samples were prepared in low conductivity buffer and were loaded in higher conductive buffer and the stacking gel. Separated proteins were transferred solid support by western blotting and visualized by marking with primary antibodies and then a secondary antibody (Mahmood and Yang, 2012). PVDF membranes were used to transfer the proteins in this study as they provide mechanical support and allow reblotting and storage for future references.

3 METHODOLOGY

3.1 Chemicals and reagents

Thiazolyl Blue Tetrazolium Bromide (MTT), (M5655, Sigma) was prepared in Roswell Park Memorial Institute (RPMI-1640) medium without phenol red (11435-063, Gibco) to prepare 5 mg/ml MTT stock solution. SH-SY5Y cell culture media was prepared using Minimum Essential Medium Eagle (EMEM) (M4655, Sigma), Ham's F12 Nut mix (1x) (217665-029, Gibco) supplemented with 1% Non-Essential Amino Acid Solution (NEAA) (100X), heat inactivated Foetal Bovine Serum (FBS) (F9665, Sigma), 2 mM glutamine and 1% penicillin–streptomycin solution containing 10,000 IU penicillium and 10 mg /ml streptomycin (p/s) (p4333, Sigma). HepG2 cell culture media was prepared using 88% Minimum Essential Medium Eagle (EMEM) (M4655, Sigma), supplemented with 1% Non-Essential Amino Acid Solution (NEAA) (100x), 10% heat-inactivated Foetal Bovine Serum (FBS) (F9665, sigma), 2 mM glutamine and 1% penicillin–streptomycin solution containing 10,000 IU penicillium and 10 mg /ml streptomycin (p4333, Sigma). Formazan crystals formed from MTT assay were dissolved in 1:1 100% Dimethyl sulfoxide (DMSO) (D8418, Sigma) and 2-propanol (P/7490/17, Fisher Scientific). Subculture and trypsinisation of cells were done using trypsin-ethylenediaminetetraacetic acid (trypsin–EDTA) with phenol red (0.05%) (25300-062, Gibco) and Hanks' Balanced Salt Solution (HBSS) (14170-088, Gibco). 50 µM Pyrazinamide (pyrazinecarboxamide; P7136, Sigma) prepared from 500 µM stock was used as a positive control to compare the cell viability of treated cells 70% ethanol for disinfection was prepared from 99% stock ethanol (CHE 1040, Scientific Laboratory Supplies).

Lowry protein assay was done by using 2 ml Bovine serum albumin protein (500-027, Bio Rad); Triss buffer (10x), Reagent A (500-0113, Bio Rad); Reagent

B (500-0113, Bio Rad). For ethanol treatment 99.5% pure ethyl alcohol (459844, Sigma) was used. Acetaldehyde (402788, Sigma) was used for acetaldehyde treatment and 30% H_2O_2 in H_2O (H1009, Sigma) used for H_2O_2 treatments. Azamethiphos-oxon (S-6-Chloro-2, 3–dihydro-2-oxo-1, 3-oxazolo (4, 5b) pyridine-3-ylmethyl O,O-dimethyl., $C_9H_{10}ClN_2O_5PS$) was purchased (10340000, Dr, Ehrenstorfer-Schafers) with a purity at 95-99.5% and Chlorpyrifos–oxon (Diethyl-,5,6-trichloro-2-pyridinyl phosphate, $C_9H_{11}C_{l3}NO4P$), purity at 97.2-99.1% from Greyhound laboratories, Birkenhead, UK and used for pesticide treatments.

Cell lysis was performed with protease inhibitor cocktail tablets (05 892 791 001, Roche), Radioimmuno-precipitation assay buffer (RIPA lysis buffer) (10x) (92590, Temecula), Phosphatase inhibitor, (P0044, Sigma), Phosphate buffered saline (pH 7.2, P5119, Sigma).

Sample preparation for gel electrophoresis was done by using 10 mM Tris –HCl buffer pH 7.5, 10x Novex reducing agent (NP0009, Invitrogen), 4x LDS sample buffer (NP0007, Invitrogen).

Gel electrophoresis and Western blotting was performed by using Running buffer 20x (3-(N-Morpholino) propanesulfonic (MOPS) acid (NP0001, Invitrogen), Antioxidant (NP0005, Invitrogen), Pre stained molecular weight marker (26616, 26612, Invitrogen), Simply BlueTM Safe stain (46-5044, Novex), Colloidal Blue staining kit (LC6025, Invitrogen), Fixative solution (50% methanol, 10% Acetic acid, 40% distilled water), Methanol (M/3950/17, Fisher Scientific, Transfer buffer (NP0006, Invitrogen), PVDF membranes (ISEQ20200, Immobilon, Millipore), PBS-T (Phosphate buffered saline tablet (P4417, Sigma) one tablet dissolved in 200 ml of deionized water, 0.05 (v/v) Tween® 20 (P9416, Sigma), Marvel dried skimmed milk, ClarityTM Western

ECL substrate peroxide solution, Coomassie blue safe stain (46-5034, Invitrogen).

All the plant extracts used for the study (**Table 3-1**) were obtained from Dr Lucky Nwidu, University of Port Harcourt. Nigeria. The extracts were imported to U.K and stored at 4^{o}C until required.

Table 3-1 Plant extracts used for the experiment.

Extract No.	Name of extract	Abbreviation
4	*Carpolobia. lutea* hot butanolic leaf extract	CLHBLE
11	*Carpolobia. lutea* ethyl acetate leaf fraction	CLEALF
21	*Parquetina nigrescens* stem extract	PNSTE
22	*Parquetina nigrescens* leaf extract	PNLE
28	*Milletia thorningii* leaf extract	MTLE
33	*Herbalin* complex extract	HCE
38	*Glyphae brevis* root extract	GBRE
40	*Musanga cecropioides* leaf extract	MCLE
42	*Peltoforum pterocarpum* stem extract	PPSTE
48	*Spondias mombin* stem extract	SMSTE
55	*Terminalia catappa* seed extract	TCSE
57	*Tricosanthes cucumerina* extract	TCE
58	*Capsicum annum* seed extract	CASE
63	*Moringa oleifera* bie extract	MOBE
69	*Atrocarpus communis* fruit extract	ACFE
73	*Gongrenema latifolium* leaf extract	CLLE
76	*Moringa oleifera* ethanolic leaf extract	MOELE

3.2 Cell culture

SH-SY5Y (ECACC-94030304) were obtained from European Collection of Authenticated Cell Culture (ECACC) and all cells were used between passages 12 -14. Cells were maintained in 41% EMEM supplemented with 41% F12 nut mix, 10% FBS, 1% NEAA, 2 mM glutamine and 1% P/S.

HepG2 cells (ECACC-85011430) were obtained from European Collection of Authenticated Cell Culture (ECACC) and all cells were used between passages

16-18. Cells were maintained in 88% EMEM supplemented with 10% FBS, 1% NEAA, 2 mM glutamine and 1% P/S.

3.2.1 Seeding cells that had been stored in liquid nitrogen

A 1 ml cryovial (379189, NUNC) of 1 X 10^6 SH-SY5Y cells or HepG2 cell was removed from the liquid nitrogen and placed in a laboratory Dewar contained liquid nitrogen and immediately thawed in a 37ºC water bath (GLS Aqua 18 plus, GRANT). After minute cells were transferred into a canted neck, blue vented capped T75 flask (RE 353136, Falcon) containing 14 ml of pre-warmed respective cell culture media under Class II Microbiological Safety Cabinet (Microflow – peroxide Advance Biosafety cabinet class 2, ABS 1000 UK/C) and placed in the incubator (Sanyo Biomedical) at 37ºC with an atmosphere of 5% CO_2 and 95% humidity. The media was changed every other day after resuscitation until the cells attained 80% confluence. The flask was observed using a microscope (CKX53, Olympus) every day until the next passage.

3.2.2 Subculture of cells

At 80% confluence, the cells were subcultured in a class II microbiological safety cabinet which had been disinfected beforehand with 10% trigene and 70% ethanol diluted in distilled water. Spent culture media of the T75 flask was aspirated carefully using an aspiration vacuum and adhered cell monolayer was rinsed with 15 ml of HBSS. 5 ml of trypsin-EDTA was gently pipetted onto the cell monolayer and the flask was incubated for two minutes in the incubator at 37ºC with an atmosphere of 5% CO2 and 95% humidity. After that cells were observed under an inverted microscope to confirm the detachment of cells. Immediately after detachment, 5 ml of FBS was added to inhibit the trypsinisation process, and the base of the flask was gently washed with HBSS in order to gain the maximum cell yield. The cell suspension was then

transferred into a 15 ml centrifuge tube (339651, Falcon) and centrifuged in an Eppendorf centrifuge (E5804000064, Scientific Laboratory Supplies) for 5 minutes at 1000 rpm for SH-SY5Y cells and for 7 minutes at 800 rpm for HepG2 cells at room temperature. Supernatant was discarded after centrifugation and the resultant cell pellet was suspended in 15 ml of HBBS and centrifuged again. Following this, the supernatant was discarded, and 5 ml of respective growth media was added to resuspend the pellet. Cells were counted using a TC 10 cell counter (1450010, BioRad) and plated on 96 well plates or maintained in T75 flask for future use accordingly (1x 10^6 cells to a T75; 2.5 x 10^5 cells to at T25; 3 x 10^4 cells per well in 96 well plates). Cell culture medium and cell suspension was checked for microbial contamination before preservation and further passaging. Results of microbial contamination analysis indicated there were no microbial contaminations in both cell suspension and respective media.

3.2.3 Freezing cells

At 80% confluent in a T75 flask HepG2 cells were passaged as described above and the cell pellet was resuspended in the appropriate volume of the respective media and 10% DMSO (freezing medium) added to falcon tube after counting the cells to achieve 1 x 10^6 cell/ ml. 1 ml of cell suspension in freezing medium was aliquoted into cryovials. Vials were then placed in an ice box prepared in advanced for 20 min and then transferred into a CryoTM freezing container at 1^{o}C (12 x 1-2 ml, BCS-405G, Sigma) and stored in -80^{o}C freezer overnight. The rate of cooling was approximately -1^{o}C / minute, and then the following day the cryovials were transferred into liquid nitrogen for long term storage at approximately -200^{o}C.

3.3 Cell density standard curve

MTT standard curve was generated in order to establish the relationship between cell number and linearity of signal produced to determine the optimum number of cell density that should be plated on 96 well microtiter culture plates. Cells were counted using TC10 automated cell counter and in a microbiological safety cabinet, cells were serially diluted in SH-SY5Y cell growth media to a range of cell densities of 1×10^3, 2.5×10^3, 5×10^3,1×10^4, 2.5×10^4, 5×10^4 and 7.3×10^4. SH-SY5Y cells were then seeded in replicates of three wells per seeding at the densities in four separated 96 well plates, with each comprising a total working volume of 100 µl. Three wells of each plate were left cell free for background controls. Culture plates were then transferred to a humidified CO_2 incubator at 37^{o}C. After 12 hours cells were checked for attachment using an inverted microscope. Cell viability was assessed by using MTT assay in four different time points in order to establish the relationship between cell number and signal produced at 0.5 hours, 1 hour, 2 hours and 4 hours after addition of MTT reagent into media. The background corrected optical density (OD) readings obtained were used to generate the cell density standard curve and determine the linearity of signal.

3.4 Cell viability assays

3×10^4 SH-SY5Y or HepG2 cells per well were seeded in 96 well plates with respective culture media (Perkin Elmer, 6005181) under MSC Class II cabinet. Several wells are left as cell free for back ground controls and plates were incubated at 37^{o}C with an atmosphere of 5% CO_2 and 95% humidity in an humidified incubator. After 24 hours, spent media was carefully aspirated and fed with fresh respective growth media. At 80 – 90% confluence levels cells were treated. Each neurotoxicant tested was serially diluted in growth media

supplemented with 0% FBS, 3% FBS and 15% FBS. SH-SY5Y cells were treated with ethanol. Azamethiphos-oxon and Chlorpyrifos-oxon was prepared in ethanol (99.5%) as 50 mM stocks and stored at 4^{o}C until used. At 80 – 90% confluence levels HepG2 cells were treated with plant extracts or fractions. 100 µl of each plant extracts or fraction was added in concentrations of 0.1, 1, 10 and 100 µg/ ml in triplicates. Dilution of plant extracts and fractions was performed in cell culture media as serial dilutions in the wells of the respective plate. 50µM of pyrazinamide which was reported as a strong hepatotoxic compound (Verma and Kaplowitz, 2013; Lacroix *et al.,* 1987; Facino and Carini, 1980; Shibata, *et al.,* 2001; Rao *et al.,* 2012) prepared from a 500 µM stock (prepared in advance in DMSO) was added as positive control to the respective growth media with cells. Cell free raw of wells were served as background controls. Plates were incubated for 24 hours in the incubator at 37^{o}C with an atmosphere of 5% CO_2 and 95% humidity.

Thereafter, cell viability was assessed after 24 hours by replacing neurotoxicant treated cells and plant extract or fraction treated media of 0.5 mg/ml MTT containing cell growth media prepared in advanced under dark conditions inside the MSC class II biological safety cabinet. Plates were protected with aluminium foil to prevent the photodegrading of MTT and placed inside the incubator at 37^{o}C for four hours and then spent media with MTT was replaced with 1:1 (v/v) DMSO: isopropanol. Each well with DMSO: isopropanol was resuspended until the formazan crystals had been dissolved thoroughly by mixing using multichannel pipette. Plates were then incubated for 15 minutes at room temperature. Following incubation, the optical density (OD) was measured using a spectramax plate reader (Multiskan Spectrum, Thermo Electron Corporation). Readings were imported to Microsoft Excel (2016) and average OD values obtained after subtracting cell free control wells to correct for

background OD values. Corrected OD readings for each treatment was normalized to the mean of the negative control as a minimum value and corrected positive control for the highest value (only for plant extract treated plates), providing the cell viability percentage as a percentage relative to the negative control.

3.5 Low dose pesticide responses

3 x 10^4 cells per well seeded in 3 separate 96 well plates were used for assessing the viability of undifferentiated SH-SY5Ycell viability by treating with low pesticide doses. Experiments were undertaken in both 10% and 15% FBS media for three different time points. Cells dosed for 24 hours were then allowed to recover for another 24 hours and thereafter they were again treated with low pesticide dose as before and incubated for another 24 hours. The viability was assessed using an MTT assay as previous at three time points of 24 hours, 48 hours and 72 hours to assess whether the cells had recovered after the treatment period. IC_{20} and IC_{50} values of each pesticide were determined and interpolated using the respective dose response curve.

3.6 Cell lysis

SH-SY5Y cells were grown in T25 flasks in 2.5 x 10^5 cells as required for the experiment. Media was replaced with low serum media after 24 hours of seeding and incubated for 18 hours. Neurotoxic agents (Ethanol or H_2O_2) were diluted into low serum media and added to each flask with a 20 minutes time gap between each treatment. Spent media with treatment was removed after 24 hours of incubation. The cell monolayer was then washed with cold PBS for three times. After that 0.5 ml of RIPA buffer containing protease inhibitors (1:100 diluted stock) and 1:100 diluted phosphatase inhibitor cocktail (prepared in advance and kept on ice) was added and flasks were placed and agitated on

ice for 5 minutes. Cells were scraped into RIPA buffer and collected to a prelabelled Eppendorf tube and vortexed thoroughly. A 28g needle was used for passage of the cell lysate 25 times. Samples were stored at -20^{o}C until required.

3.7 Protein quantification and determination

To determine the amount of protein in all treated cell samples, a modification of the Lowry assay (Lowry *et al.,* 1951) was used. Bovine serum albumin protein was used as a standard. Lowry assay was performed in 96 well plates by using 5 concentrations of standard from 1 mg/ml to 0. 05 mg/ml. Every time fresh standards were prepared along with the samples and then Lowry protein assay performed.

Frozen cell lysates were thawed at room temperature and centrifuged at 500 x g for 10 minutes at 4^{o}C in a bench top centrifuge to pellet the nuclear fraction. The supernatants were then centrifuged at 21,300 x g for 40 min at 4^{o}C to produce crude cytosolic preparations. Each fraction was stored at -80^{o}C until used.

The cytosolic fractions were thawed at room temperature and vortexed. Then they were heated in a block heater at 70^{o}C for 10 minutes and allowed to cool at room temperature. Samples were pulsed in a centrifuge at room temperature for 5 seconds at 5000 x g before undertaking the assay. Cell lysates were diluted 1:5 or 1:8 in the study with 10 mM Triss buffer for loading into a 96 well plate as duplicates along with standards. Then 20 µl of Reagent A was added to each well and 160 µl of Reagent B was added to each well. After 15 minutes the plate was agitated in a Spectramax plate reader (Multiskan Spectrum, Thermo Electron Corporation) for 5 minutes before taking the readings at 740 nm. The protein yield is expressed as mg/ml and data was imported to Microsoft Excel

(2016) for analysis. A standard curve was plotted on Microsoft Excel (2016) in order to calculate the amount of protein concentration in each sample from linearity of the equation of line of best fit.

3.8 Gel electrophoresis and western blotting

Cell lysates were prepared to a concentration of 1µg/µl. Then samples were mixed with 10 mM Triss buffer, Novex reducing agent and 4x LDS sample buffer and heated at 70^{o}C for 10 minutes on a block heater (Stuart Scientific). Samples were allowed to cool at room temperature, and then pulsed in a centrifuge for 5 seconds to collect the material for loading.

Xcell surelock Mini-cell was fixed with precast gels (10% Bis – Tris Gel 1.0mm x 10 well (NP0301BOX) and 4-12% Bis – Tris Gel 1.0 mm x 12 well (NP0322BOX) were used in this study. The inner chamber of the Mini-cell was filled with running buffer (MOPS). 0.5 ml of antioxidant was added to the inner chamber and then buffer filled to the top. The outer chamber was filled with gel running buffer to the second marked line. Samples prepared were loaded into wells as required (typically 15-25 µl loaded per gel well) using gel loading pipette tips. The first lane of the gel was loaded with 10 µl of a pre -stained protein molecular weight marker and unused wells were loaded with 1x loading buffer. Thereafter, the complete Mini–cell was connected to a power supply and gel was ran for 100 minutes at 125 V constant voltage. Then gel plates were carefully separated by using a purposely made gel knife and placed in fixative solution for staining. The gel ridge and comb were carefully removed using a scalpel. After 15 minutes gels were stained with Colloidal Blue staining Kit overnight. The next day gels were destained by washing with double distilled water for one hour on a rocking platform. When they were fully destained pictures were taken using a ChemiDoc imaging system (BioRad).

8.5 x 8.5 cm polyvinyl difluoride (PVDF) (Millipore) membranes were cut and soaked in 100% methanol for 5 minutes on a rocking platform. The membrane was taken to equilibrium by placing in transfer buffer of 50% methanol solution and shaken on a rocking platform for 5 minutes for three times until the membrane was fully equilibrated in 100% transfer buffer for 15 minutes.

A Bio Rad Mini Trans-Blot cell was placed on ice within a polystyrene container and a frozen block of transfer buffer has inserted into the end compartment. A transfer sandwich was prepared on a supporting grid as sponge, 3 of 8.5 x 8.5 cm squares of filter paper, SDS–PAGE gel, and PVDF membrane, and then 3 of 8.5 x 8.5 cm squares of filter paper and sponge again. The transfer sandwich was built on the black negative plate. Then it was inserted into the cell and filled with cold transfer buffer to the top and ran for 120 minutes at 80 V constant voltage.

Thereafter, the PVDF membrane was removed and washed in 20 ml of Coomassie blue safe stain for 30 minutes for visualize the protein bands. Then the PVDF membrane was placed on cling film overnight to dry at room temperature.

The following day the membrane was destained as previous in a fixative for 5 minutes and placed on a rocking platform for shaking with a wash buffer of PBS –T until equilibrated. This was done for three times for 10 minutes each time. After that membranes were blocked with blocking solution of PBS-T with 5% (w/v) milk protein. Then membranes were incubated for overnight with primary antibody (Beta tubulin (Rabbit polyclonal, H-235, SC-9104, Santa Cruz biotechnology) and alpha tubulin (Mouse monoclonal SC-8035, Santa Cruz biotechnology) at a 1:500 dilution in blocking buffer in a sealed packet attached to a rotary mixer at 4^{o}C. The following day membranes were washed in PBS-T

for three times as previous for 10 minutes each. 1:1000 diluted horseradish conjugated anti-species secondary antibody (Polyclonal goat, Anti-rabbit Immunoglobulins/ HRP P0448, Dako) was applied to the membrane inside a sealed polythene packet and incubated for an hour at room temperature as before attached to a rotary mixer. After one hour they were washed in washing buffer for three times as previous and placed on a cling film. 4 ml of 1:1 Clarity Western Enhanced Chemiluminescence substrate reagent A and B was poured over the membrane and left for 5 minutes. Images were captured by placing them on the imaging platform in a ChemiDoc imaging system. Membranes were stored for later use by washing in wash buffer as previous and dried overnight on cling film

3.9 Statistical analysis

The cell viability spectrophotometric measurement used to generate the standard curve were transformed to a logarithmic scale and modelled by means of non-linear regression using GraphPad Prism 7 (GraphPad Prism, San Diego, California USA). Cell viability and cytotoxicity readings were analysed by normalizing the values to the respective control treatment OD values or vehicle control OD values. Comparison between controls and treatments were performed using one-way analysis of variance (one-way ANOVA) with Dunnett's multiple comparisons test. Result of assessing optimal seeding density were analysed by using two way-ANOVA and were considered significant at $p< 0.05$. Comparison between controls and treatments of low dose response treatment were performed using one-way analysis of variance (one-way ANOVA) with Turkey's multiple comparison test and were considered significant at $p< 0.05$.

Lowry assay reading were analysed by using Microsoft Excel (2016) and a standard curve was plotted to determine the sample protein concentration.

Statistical comparisons between means of protein molecular weights were done by using Student –t test in GraphPad prism 7.0 software and results were expressed as means ± SEM. A $p< 0.05$ was regarded as statistically significant.

4 RESULTS

4.1 Optimal cell line seeding densities

Figure 4-1 (a) depicts the standard curve for SH-SY5Y cell line determined by MTT based cell viability assay. In both Figures (**Figure 4-1 (a) and Figure 4-1 (b)**), it can be observed that seeding cells at lower cell densities shows an exponential or logarithmic increase in MTT signal intensity. However, as observed in **Figure 4-1 (a)** in higher seeding densities the intensity eventually plateaus. In the range of 10,000-30,000 cells per well a linear correlation between the optical signal (OD signal at 570 nm) and the cell number according to the **Figure 4-1 (b)** was observed. Therefore, lag phase observed in both **Figures 4-1 (a)** and **(b)** at 10,000-30,000 cell densities can be attributed to an adequate number of viable cells able to produce a detectable or quantifiable signal and the best signal intensity was given after 4 hours incubation period according to both **Figures 4-1 (a)** and **(b)**. Two-way ANOVA confirmed that there was significant interaction between the cell number and time taken to develop the signal ($p \leq 0.0001$). Also, there was significant difference in all time points with 0.5 hours ($p \leq 0.0001$). According to the results revealed from two-way ANOVA and multiple mean comparison by Tukey's multiple mean comparison test all the treatment above 1×10^4 cells/well produced a significant interaction between means of cell number and time taken to develop the signal. But there was no significant mean difference among 2 hours and 4 hours points in 3×10^4 cell/ well treatment when compared to 0.5 hours (**Figure 4-2**).

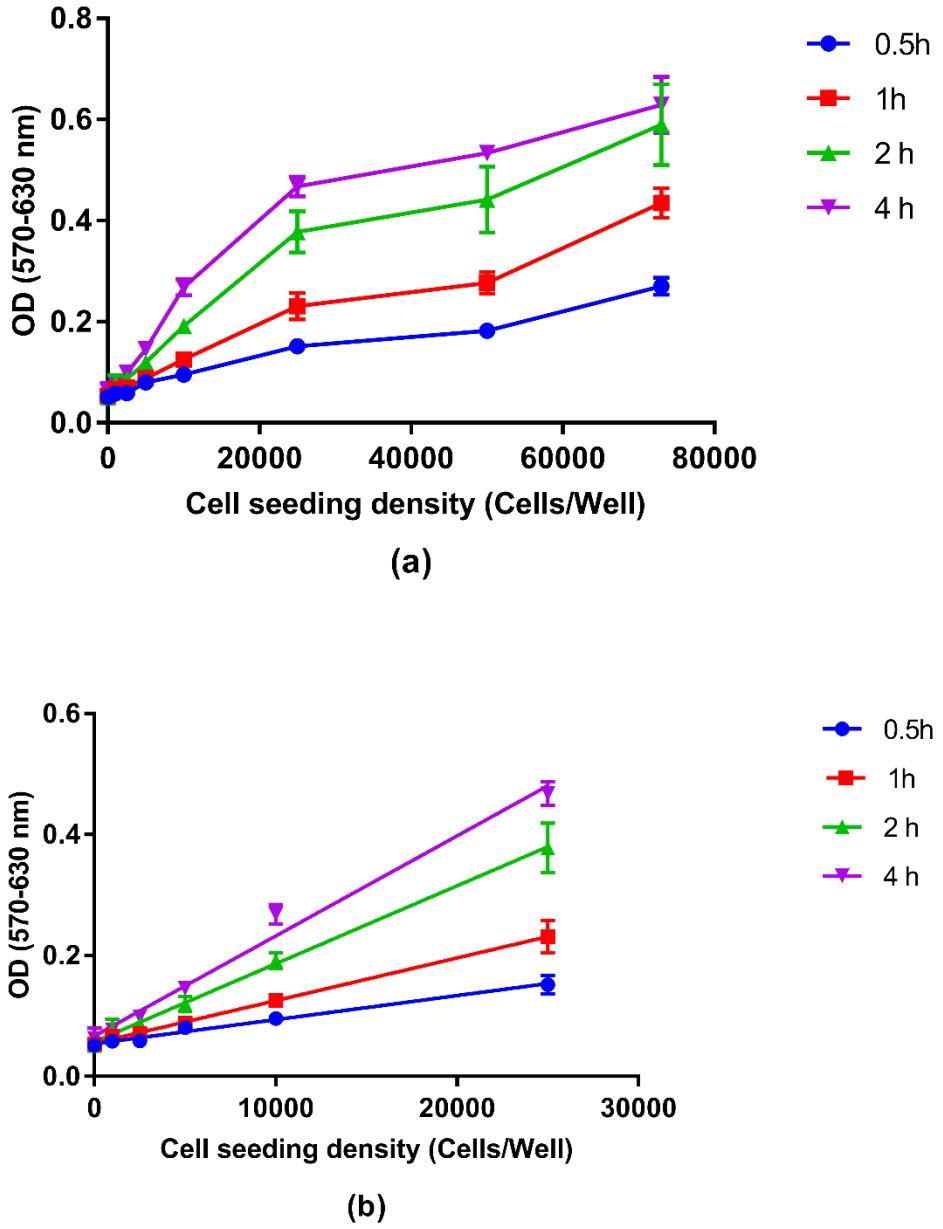


Figure 4-1 Direct correlation of absorbance and SH-SY5Y cell number.

Cell viability readings for SH-SY5Y standard curve were determined using a MTT assay. Cell viability readings for each time point was obtained from one plate (a) SH-SY5Y cells were seeded at different cell concentrations and MTT assay was performed to establish the linearity of signal at 4 different time points. (b) The graph illustrates the best signal can be obtained in log phase of cell growth phases after 4-hour incubation period (R^2= 0.9812). Viability readings were obtained from total of 5 repeated plates for

each time point (n= 15). Results are expressed as the mean ± SD. n, number of repeats; SD, standard deviation; OD Optical density.

Quantification of linear range of MTT intensity for SH-SY5Y cell line was important for optimising parameters for cells that would be subjected to neurological treatments. According to the graphs in **Figure 4-1** it can be concluded that 3 x 10^4 cells per well was the optimal seeding density for treatment plates for SH-SY5Y cells. Optimal seeding density selection at the beginning of experiments of the most linear range of the standard curve ensured that the cells be ran over 24 hours and still be within the detectable region for MTT without reaching the saturation point.

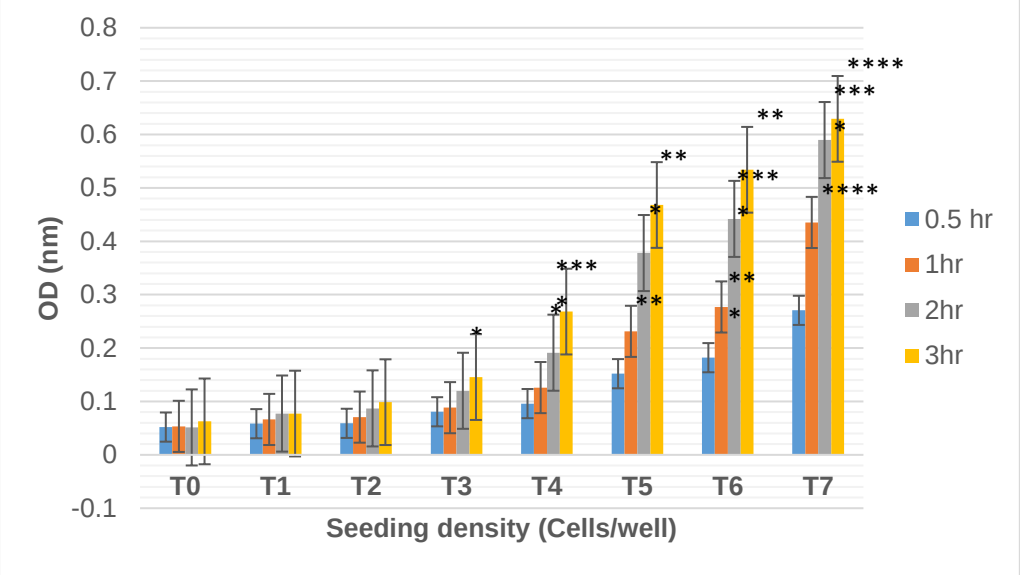


Figure 4-2 Mean comparison of two-way ANOVA results of SH-SY5Y cell number and MTT signal development with time.

T0-Media only; T1-1 x 10^3; T2-2.5 x 10^3; T3-5 x 10^3; T4-1 x 10^4; T5-2.5 x 10^4; T6-5 x 10^4; T7–7.3 x 10^4. $p \leq 0.1$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$ (****)

4.2 Ethanol reduces SH-SY5Y cell viability in dose-dependent fashion

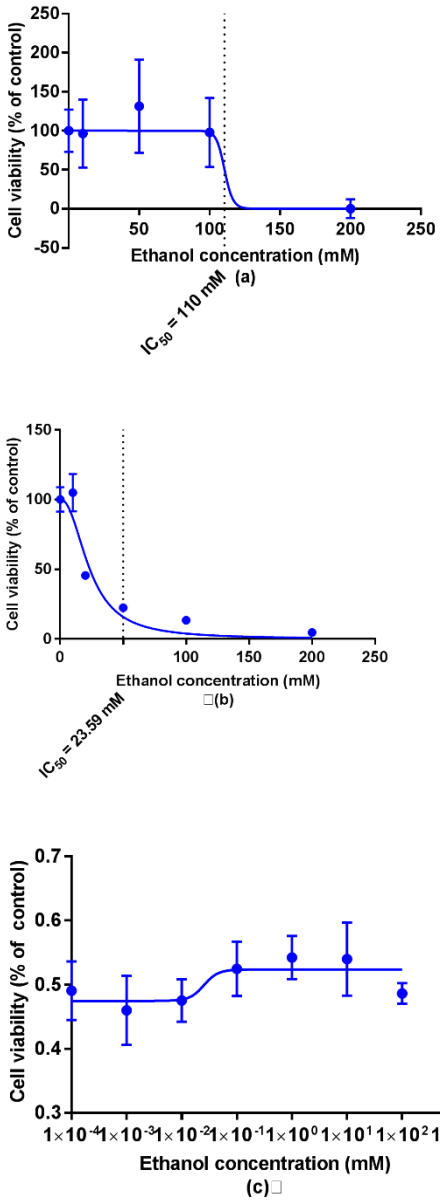


Figure 4-3 Cell viability inhibition dose response curves for SH-SY5Y in (a) 3% FBS, (b) 10% FBS and (c) EMEM only media.

Viability was assessed using MTT assay. Readings were normalized to the viability of control, providing cell viability percentage, as a percentage value to the control. Cell viability readings including control and treatments were performed as triplicates in 5 individual experiments. Results are expressed as the mean ± SD. SD, standard deviation.

Figure 4- 3. Shows the behaviour of cell viability inhibition dose response curve for SH-SY5Y cell following treatment with ethanol for 24 hours in 3% FBS, 10 % FBS and EMEM only media. After 24-hour treatment, the results confirm that ethanol influences SH-SY5Y cell viability, with curve exhibiting varying degrees of deviation from 100% cell viability. As ethanol concentration increases the cell viability decrease in 10% FBS media. Although there was a significant reduction (p<0.05) in cell viability in SH-SY5Y cells cultured on 10% FBS media, one- way ANOVA revealed that there was a significant difference in each treatment means with control mean apart from the 10 mM treatment (**Figure 4-3 (a); Table 4-1**). In contrast to that 3% FBS media SH-SY5Y cell viability did not changed significantly up to 100mM concentration and starts to decrease significantly after that (**Figure 4-3 (b); Table 4-1**). Cell viability increases in EMEM media according to the results without any significant mean differences among the treatments and with control as revealed by one-way ANOVA over the treatment range within 24 hours. Therefore, cells were treated in the range of micro molar concentration to observe the effect of ethanol on SH-SH5Y cells. Although micro molar concentration showed no increment or reduction of SH-SY5Y cell viability up to 100 µM and then gradually increased without any significant difference compared to the control (**Figure 4-3 (c)**).

Table 4-1 Results of one-way ANOVA for SH-SY5Y cell line following treatment of ethanol in different media.

Media used	10 mM	20 mM	50 mM	100 mM	200 mM
3% FBS	NS	NS	NS	NS	NS
10% FBS	NS	***	****	****	****
EMEM	NS	NS	NS	NS	NS

p> 0.05 (NS); p≤0.001(***); p≤0.0001(****)

4.2.1 Ethanol is more toxic to SH-SY5Y cells in 10% FBS media for 24 hours treatment

When comparing the effect of ethanol in both 3% and 10% FBS media, it can be concluded that the SH-SY5Y cells are more sensitive to ethanol in 10% FBS media (**Figure 4-4 (a) and (b), Table 4-1**) than the 3% FBS (**Figure 4-4 (a) and (b), Table 4-1**) media as IC_{50} values of 10% FBS media is lower than that of the 3% FBS media. But in EMEM, SH-SY5Y cells exhibit no significant decrease in cell viability within treated concentration range after 24 hours.

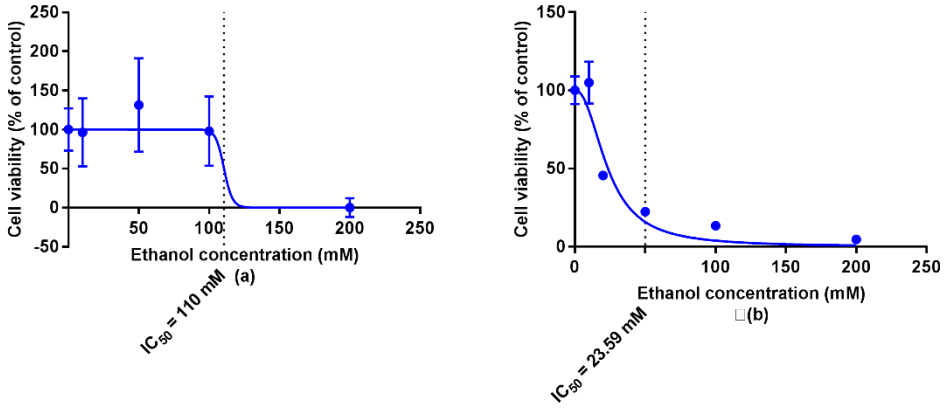


Figure 4-4 Cell viability inhibition dose response curves for SH-SY5Y in 3% FBS and 10% media.

Viability was assessed using MTT assay. Readings were normalized to the viability of control, providing cell viability percentage, as a percentage value to the control. Cell viability readings including control and treatments are performed as triplicates in 5 individual experiments as repeats. Results are expressed as the mean ± SD. SD, standard deviation.

4.2.2 Determination of IC_{50} of ethanol

The IC_{50} values were determined for SH-SY5Y cells and were interpolated for each media from the corresponding ethanol dose response curve. The IC_{50} for the 3% and 10% FBS media shows a trend line of decreasing cell viability within

the used concentration range. **Table 4-2** summarize the IC_{50} values providing an indication of how well the regression model fits to the data provided. Therefore, a prediction of the IC_{50} values for ethanol within higher R^2 values can be obtained. However, IC_{50} value for EMEM only media has very low R^2 value since the data in each tested concentration showed higher variability. Therefore, it was not possible to reliably predict the IC_{50} value for ethanol on the viability of SH-SY5Y cell in EMEM media only with only the P values of analysis as cell viability increases with treatment of ethanol.

Table 4-2 Summary statistics for the SH-SY5Y cells following ethanol treatment in 3% and 10% FBS media after 24 hours treatment.

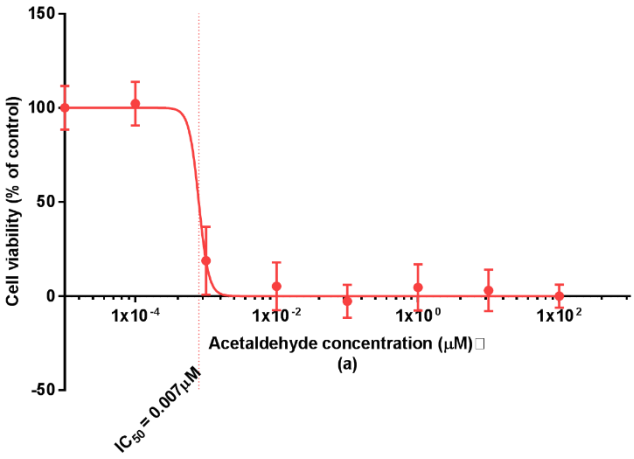
Media FBS concentration	IC_{50} (mM)	R^2
3% FBS	110.6±14.96	0.4926
10% FBS	15.26±11.57	0.9022
EMEM	-	0.3658

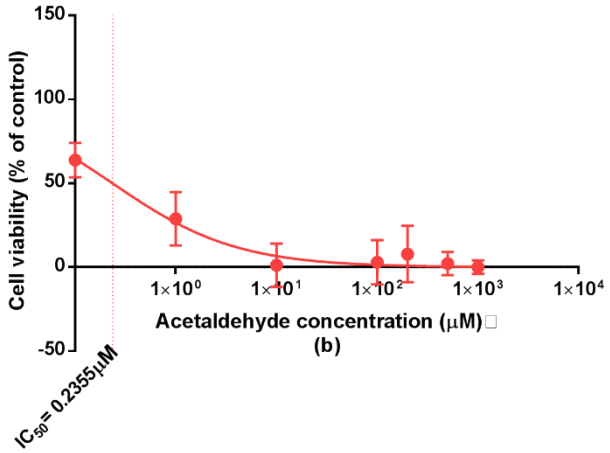
IC_{50} values were interpolated from their corresponding dose response curve. Results are expressed as means ±95% confidence interval. IC_{50}= Concentration of the ethanol that result 50% reduction in cell viability

4.3 Effect of acetaldehyde on viability of SH-SY5Y cells

Figure 4-5 illustrates the cell viability inhibition dose response curves for SH-SY5Y cells in 3%, 15% FBS and EMEM only media following treatment with acetaldehyde for 24 hours. The results confirm that acetaldehyde had an effect on SH-SY5Y cell viability, as each curve exhibited varying degrees of deviation from 100% cell viability. SH-SY5Y cells experience dose-dependent inhibition in all tested media with FBS and EMEM. Therefore, when increasing the concentration of acetaldehyde, the viability of cells was decreased within a 24 hours period. However, when increasing the concentration of treatments, mean differences between the viability of controls and treated cells at 10 and 100 µM

(p≤0.1), and 1, 10 and 100 mM (p≤0.01) showed significant decreases in cell viability of SH-SY5Y cells in 3% FBS media (**Figure 4-5**; **Table 4-3**). In contrast, cells exhibited significant cell viability reduction at 1 (p≤0.001),10 and 100 mM ((p≤0.0001) treatments when their mean differences were compared with respective controls as revealed by ANOVA in15% FBS media (**Figure 4-5**; **Table 4-3**). EMEM media showed decreasing pattern of SH-SY5Y cell viability and significant differences in each treatment means with controls was found as revealed by one-way ANOVA (**Figure 4-5; Table 4-3**). Therefore, with an increase of the concentration of acetaldehyde in all media the cell viability reduces in a dose-dependent manner as illustrated in (**Figure 4-5**).





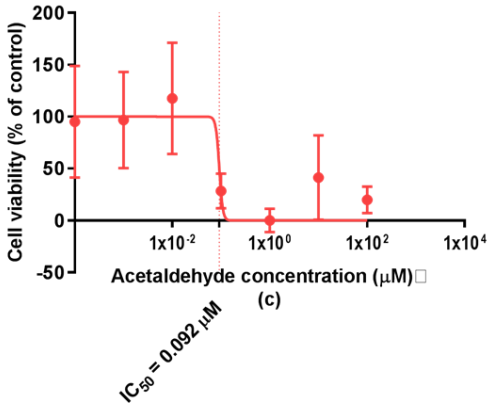


Figure 4-5 Cell viability inhibition dose response curves for SH-SY5Y in (a) 3% FBS media, (b) 15% FBS and (c) EMEM.

Viability was assessed using MTT assay. Readings were normalized to the viability of control, providing cell viability percentage, as a percentage value to the control. Cell viability readings including control and treatments are performed as triplicates in 5 individual experiments. Results are expressed as the mean ± SD. SD, standard deviation.

Table 4-3 Results of one-way ANOVA for SH-SY5Y cell line following treatment of acetaldehyde in different media.

Media used	0.1μM	1 μM	10 μM	100 μM	1 mM	10 mM	100 mM
3% FBS	NS	NS	*	*	**	**	**
15% FBS	NS	NS	NS	NS	***	****	****
EMEM	NS	NS	NS	*	**	**	**

p> 0.05 (NS); *p*≤0.01(**); *p*≤0.001(***); *p*≤0.0001(****)

4.3.1 Acetaldehyde is more cytotoxic on SH-SY5Y cells in 3% FBS media than 15% FBS media and EMEM

When comparing the effect of acetaldehyde in both 3% and 15% FBS media, it can be seen that the SH-SY5Y cells are more sensitive to acetaldehyde in 3% FBS media (**Figure 4-5, Table 4-4**) than the 15% FBS (**Figure 4-5, Table 4-4**)

media and EMEM as IC_{50} values of 15% FBS media is higher than that of the 3% FBS media and EMEM media.

4.3.2 Determination of IC_{50} of acetaldehyde

The IC_{50} values were interpolated from their respective dose response curves of different media for SH-SY5Y cells. IC_{50} values were determined for acetaldehyde in 3% and 10% FBS and EMEM which showed a reduction of cell viability within the used treatment concentration range (**Figure 4-5**). The IC_{50} for the 3% and 10% FBS and EMEM media was predicted as it shows a trend line of decreasing cell viability within the used concentration range. **Table 4-4** summarize the IC_{50} values providing an indication of how well the regression model fits to the data provided. Therefore, it can precisely predict the IC_{50} for acetaldehyde within higher R^2 values.

Table 4-4 Summary statistics for the SH-SY5Y cells following acetaldehyde treatment in 3% and 10% FBS and EMEM media after 24 hours treatment.

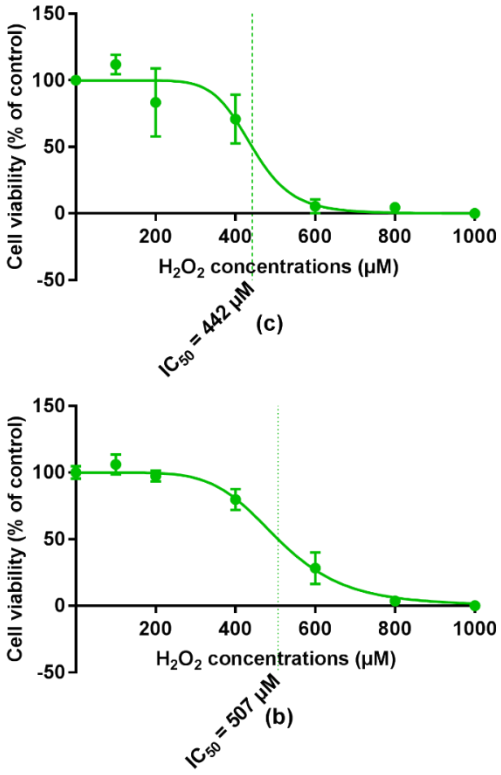
Media FBS concentration	IC_{50} (µM)	R^2
3% FBS	0.00729±2.792	0.9527
15% FBS	0.2355±0.1910	0.9230
EMEM	0.0928±0.1032	0.4971

IC_{50} values were interpolated from their corresponding dose response curve. Results are expressed as means±95% confidence interval. IC_{50}= Concentration of the acetaldehyde that result 50% reduction in cell viability.

4.4 Effect of H_2O_2 on viability of SH-SY5Y cells

Figure 4-6 shows the cell viability dose response curves for SH-SY5Y cell line following treatment with H_2O_2 in 0% FBS, 3% FBS and 15% FBS media for 24 hours. The result indicate that H_2O_2 has significant effect on SH-SY5Y cell

viability with each curve showing varying degrees deviating from 100% cell viability within the used concentration ranges. As shown in **Table 4-5** comparison between each treatment of H_2O_2 in respective media one-way ANOVA revealed a significant reduction in cell viability of SH-SY5Y cells in all three media. According to the results it can be seen that 10 mM ($p \leq 0.0001$),1 mM ($p \leq 0.0001$),800 µM ($p \leq 0.0001$) and 600 µM ($p \leq 0.0001$) treatments have significant effect on SH-SY5Y cell viability compared to the control hence as concentration increasing, mean differences between the viability of control and treated cells became more significant and viability gradually decreased in SH-SY5Y cells. 400 µM ($p \leq 0.1$) treatment showed a significant reduction in cell viability of SH-SY5Y cells in both 3% FBs and 0% FBS media as revealed by one-way ANOVA. Therefore, as shown in **Figure 4-6** and **Table 4-5** H_2O_2 exhibits reduction of cell viability of SH-SY5Y cells in a dose-dependent manner, such that with an increasing concentration of H_2O_2, there is a corresponding cell viability decrease in SH-SY5Y cells.



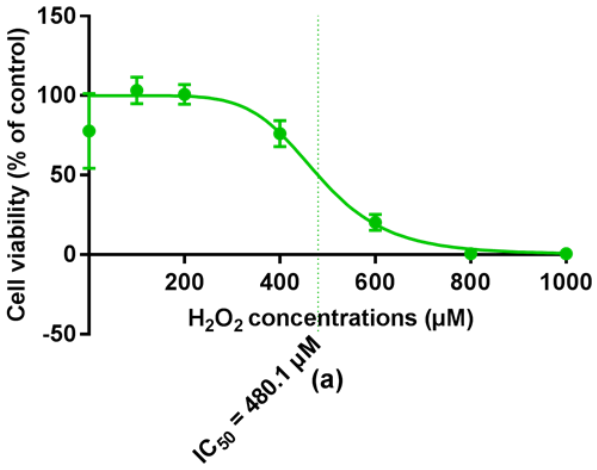


Figure 4-6 Cell viability inhibition dose response curves for SH-SY5Y in (a) 0% FBS (b) 3%FBS media and (c) 15% FBS following treatment of H_2O_2.

Viability was assessed using MTT assay. Readings were normalized to the viability of control, providing cell viability percentage, as a percentage value to the control. Cell viability readings including control and treatments are performed as triplicates in 5 separate plates as repeats. Results are expressed as the mean ±SD. SD, standard deviation.

Table 4-5 Results of one-way ANOVA for SH-SY5Y cell line following treatment of H_2O_2 in different media.

Media used	10 mM	1 mM	800 μM	600 μM	400 μM	200 μM	100 μM
0% FBS	****	****	****	****	*	NS	NS
3% FBS	****	****	****	*****	*	NS	NS
15% FBS	****	****	****	****	NS	NS	NS

p> 0.05 (NS); *p*≤0.1(*); *p*≤0.01(**); *p*≤0.001(***); *p*≤0.0001(****)

4.4.1 H_2O_2 is more cytotoxic on SH-SY5Y cells in 0% FBS media than 3% FBS media and 15% FBS

Media FBS concentration has an effect on susceptibility of cells to H_2O_2 as depicted in **Figure 4-7** and **Table 4-5** Although the curves of respective media

FBS shows a dose-dependent manner, their IC_{50} values were slightly varying without any significant mean differences (P≥ 0.05) following H_2O_2 treatments after 24 hours. The highest IC_{50} value was reported in 3% media and lowest by 0% media (**Table 4-6**) following the H_2O_2 treatment.

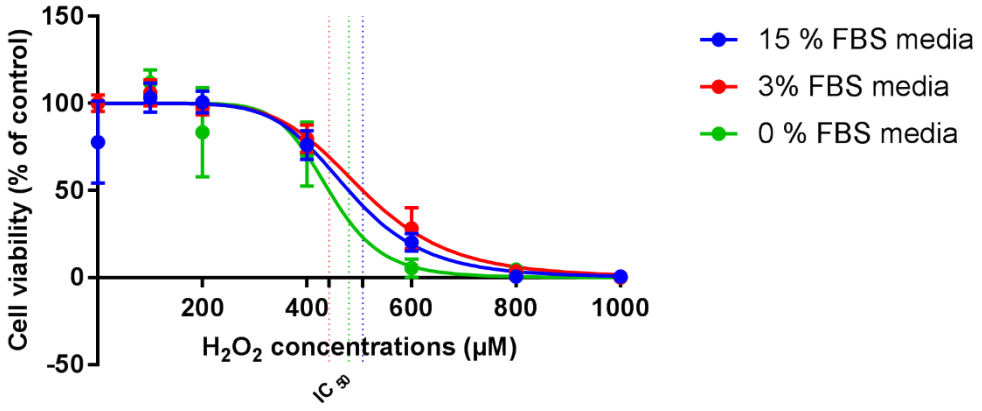


Figure 4-7 Cell viability inhibition dose response curve for SH-SY5Y in 0%, 3% and 15% FBS media.

Viability was assessed using MTT assay. Readings were normalized to the viability of control, providing cell viability percentage, as a percentage value to the control. Cell viability readings including control and treatments are performed as triplicates in 5 individual experiments. Results are expressed as the mean ± SD. SD, standard deviation.

Table 4-6 Summary statistics for the SH-SY5Y cells following H_2O_2 in 3% and 10% FBS and EMEM media after 24 hours treatment.

Media FBS concentration	IC_{20} (μM)	IC_{50} (μM)	IC_{80}(μM)	R^2
0% FBS	377.56	442.3	518.12	0.9843
3% FBS	402.01	507	639.39	0.9345
15 % FBS	387.74	480.1	594.44	0.9337

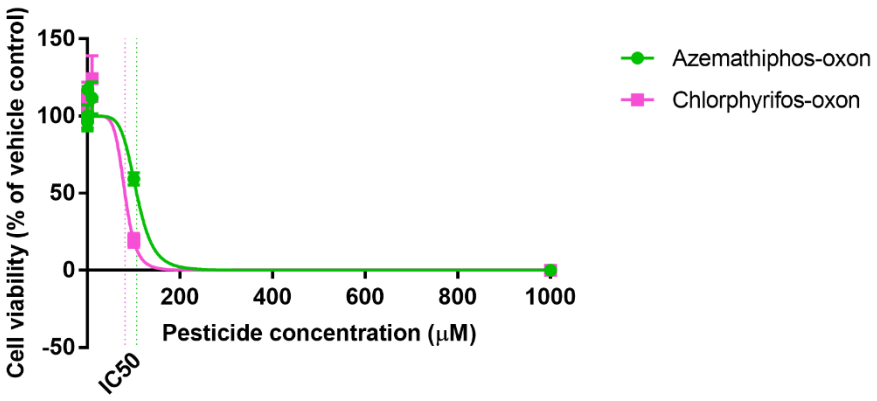
IC_{50} values were interpolated from their corresponding dose response curve. IC= Inhibition concentration producing the stated level of reduction in cell viability.

4.4.2 Determination of IC_{50} of H_2O_2

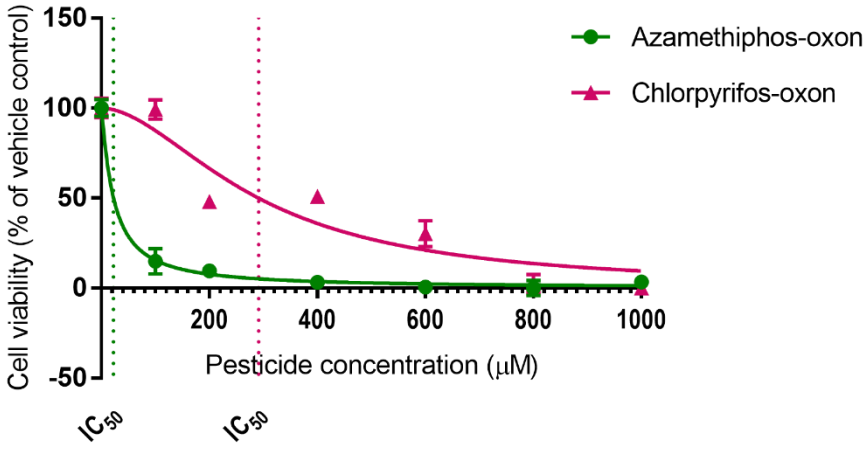
IC_{50} values for SH-SY5Y cell line following H_2O_2 treatment were interpolated from respective dose response curve for different media with FBS concentrations. **Table 4-6** summarizes the IC_{50} values for H_2O_2 treatments in media with different FBS concentrations that have a significant effect on cell viability of SH-SY5Y cells. IC_{50} values could be precisely predicted within the three media, showed high R^2 values in analysis. When it comes to 450 µM all three media showed 20% cell death according to the graphs (**Figure 4-7**). According to the **Figure 4-7** and **Table 4-6** values three media have slightly different IC_{50} values but the mean differences were not significant ($P \geq 0.05$). The lowest value was observed in 0% FBS media and the highest was from 3% FBS media.

4.5 Effect of pesticides on viability of SH-SY5Y cells

Cell viability was measured *in vitro* to evaluate the effect of Azamethiphos-oxon and Chlorpyrifos-oxon on cell viability of SH-SY5Y cells and to determine the IC_{50} values for each pesticide in different media FBS concentrations. The viability of each of the pesticide concentrations was checked in triplicates throughout the study as 5 individual experiments using passage 13 of SH-SY5Y cells. **Figure 4-8** shows the cell viability inhibition dose response curves following pesticide treatments for SH-SY5Y cells in different media FBS concentrations for 24 hours. The results confirm that both Azamethiphos-oxon and Chlorpyrifos-oxon have an effect on SH-SY5Y cell viability with each curve showing varying degrees of deviation from 100% cell viability. As shown in **Table 4-7**, mean comparisons using one-way ANOVA revealed significant differences between both pesticides and their corresponding vehicle control across SH-SY5Y cell line following 24 hours treatment.


Cell viability (% of vehicle control)
150
100
50
0
-50
200
400
600
800
1000
Pesticide concentration (μM)
IC50
Azemathiphos-oxon
Chlorphyrifos-oxon

(a)


Cell viability (% of vehicle control)
150
100
50
0
-50
200
400
600
800
1000
Pesticide concentration (μM)
IC50
IC50
Azamethiphos-oxon
Chlorpyrifos-oxon

(b)

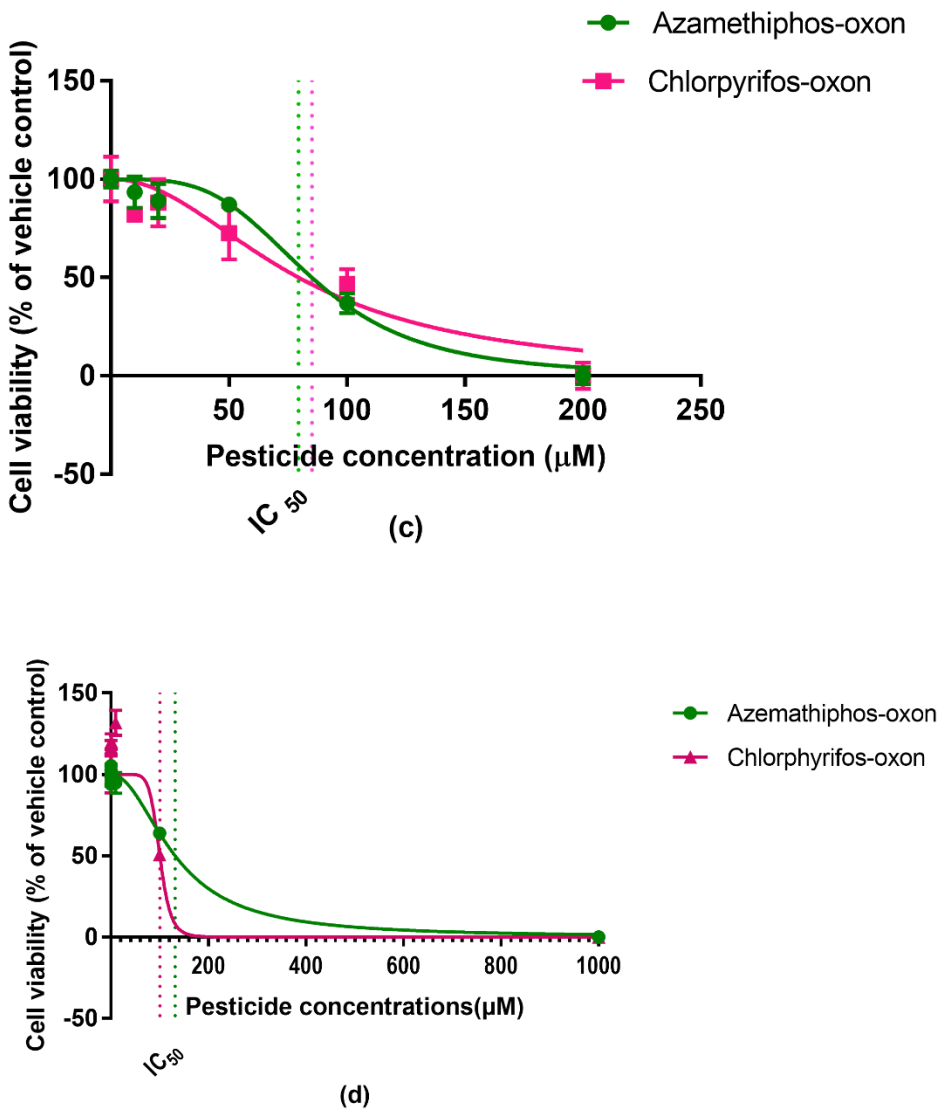


Figure 4-8 Cell viability inhibition dose response curves for SH-SY5Y in 0%, 3% 10% and 15% FBS media ((a)-(d)).

Viability was assessed using MTT assay. Cell viability readings were normalized to the working concentration of vehicle of control, providing cell viability percentage, as a percentage value to the control. (a)-0% FBS media; (b)-3% FBS media; (c)-10% FBS media; (d)-15% FBS media. Cell viability readings including vehicle control and treatments are performed as triplicates in 5 separate plates as repeats. Results are expressed as the mean ± SD. SD, standard deviation.

Table 4-7 Results of one-way ANOVA for SH-SY5Y cell line following treatment Azamethiphos-oxon and Chlorpyrifos-oxon of in different media.

Media used	10 µM	20 µM	50 µM	100 µM	200 µM
Azamethiphos-oxon					
0% FBS	NS	NS	***	****	****
3% FBS	NS	*	***	****	****
10% FBS	NS	*	***	****	*****
15% FBS	NS	**	****	****	****
Chlorpyrifos-oxon					
0% FBS	NS	NS	***	***	****
3% FBS	NS	NS	***	****	****
10% FBS	NS	NS	NS	**	****
15% FBS	NS	NS	NS	NS	**

$p > 0.05$ (NS); $p \leq 0.1$(*); $p \leq 0.01$(**); $p \leq 0.001$(***); $p \leq 0.0001$(****)

4.5.1 Azamethiphos-oxon and Chlorpyrifos-oxon reduce cell viability of in a SH-SY5Y dose-dependent manner

Figure 4-8. shows the cell viability inhibition dose response curve for SH-SY5Y cell line following 24 hours treatment with both pesticides in different media FBS concentrations. From observing the dose response curves in **Figure 4-8** it was possible to determine that cell viability deviates in dose-dependent manner, with increasing concentration of both pesticides causing subsequent decrease in cell viability.

The treatment with lowest concentration of both pesticides (10 µM) caused no significant decrease in the viability of SH-SY5Y cells within 24 hours of exposure. 20 µM in Azamethiphos-oxon showed significant reduction in cell viability after 24 hours of exposure in 3%, 10% and 15% FBS media. In contrast, 20 µM did not significantly reduce viability of cells with Chlorpyrifos-oxon in all

media. However, as the concentration of both pesticides increased, mean differences between the viability of vehicle control and treated cells became increasingly more significant (*) and viability gradually decreased in cells (**Figure 4-8, Table 4-7**)**.** It was further proved by the results from ANOVA and dose response curves. Averagely, more than 20% cell death has obtained in 50 µM treatment in all media with Azamethiphos-oxon treated cells.

4.5.2 Azamethiphos-oxon is more cytotoxic than Chlorpyrifos-oxon on SH-SY5Y cells

When comparing the effect of both pesticides across the cell line, if can be seen that Azamethiphos-oxon is more cytotoxic than Chlorpyrifos-oxon on SH-SY5Y cells (**Figure 4-8, Table 4-7**). As shown in the graphs Azamethiphos-oxon is more toxic on cells in 3% FBS media. 20% cell death can be observed at 25 µM treatment in Azamethiphos-oxon treated cells in 15% FBS media. Higher concentrations were significantly cytotoxic such that 80% cells death can be seen at 290 µM in 15 % FBS media. This was reflected by the IC_{50} values in each media that has been obtained from respective dose response curves (**Table 4-8**). In contrast, Chlorpyrifos-oxon is more cytotoxic in 10% FBS media (**Table 4-8**). This was further proved by the results of one-way ANOVA and multiple mean comparisons in treatment means with their respective vehicle controls. In 10% FBS media Chlorpyrifos-oxon showed 20% reduction in cell viability when treated with 12 µM while 250 µM treatment showed 80% cell viability reduction.

4.5.3 Determination of IC_{50} of Azamethiphos-oxon and Chlorpyrifos-oxon

The IC_{20} and IC_{50} values for both Aazamethiphos-oxon and Chlorpyrifos-oxon were determined for SH-SY5Y cells following 24-hours treatment were 24 hours

Table 4-8 Summary of IC_{20} and IC_{50} values for undifferentiated SH-SY5Ycells following pesticide treatment for 24 hours incubation.

Media FBS concentration (%)	Pesticide	IC_{50}(µM)	R^2
0%	Azamethiphos-oxon	50.08±7.82	0.9406
	Chlorpyrifos-oxon	150.81±3.14	0.9110
3%	Azamethiphos-oxon	18.09±2.08	0.9859
	Chlorpyrifos-oxon	151.76±7.85	0.9805
10%	Azamethiphos-oxon	73.65±1.79	0.9110
	Chlorpyrifos-oxon	55.79±8.74	0.9683
15%	Azamethiphos-oxon	92.93±1.84	0.9828
	Chlorpyrifos-oxon	191.62±3.9	0.8541

IC_{50} values were interpolated from their corresponding dose response curves. Results are expressed as means ±95% confidence interval. IC= Inhibition concentration producing the stated level of reduction in cell viability.

4.6 Repeated dose responses of low pesticide doses to undifferentiated SH-SY5Y cells

3 x10^4 cells per well were seeded in 3 separate 96 well plates for assessing the viability of undifferentiated SH-SY5Y cell viability by treating low pesticide doses for three different time points at 24 hours, then allowing the cells to recover for another 24 hours and thereafter they were again treated with low pesticide dose as before. The viability was assessed by using MTT assay as previous in three time points including 24 hours, 48 hours and 72 hours to assess cell were recovery after treating low dose within a 24-hour time period. IC_{20} and IC_{50} values of each pesticide were determined and interpolated using the respective dose response curve. The results were analysed using GraphPad prism 7. Comparison between each treatment was performed using one-way ANOVA with Turkey's multiple mean comparison. Results of each treatment are

presented in **Table 4-9**. Interestingly, it was observed that the in low dose treatment with IC_{20}, significantly increased the cell viability after recovery period in 10% FBS media in both pesticides (**Figure 4-9**).

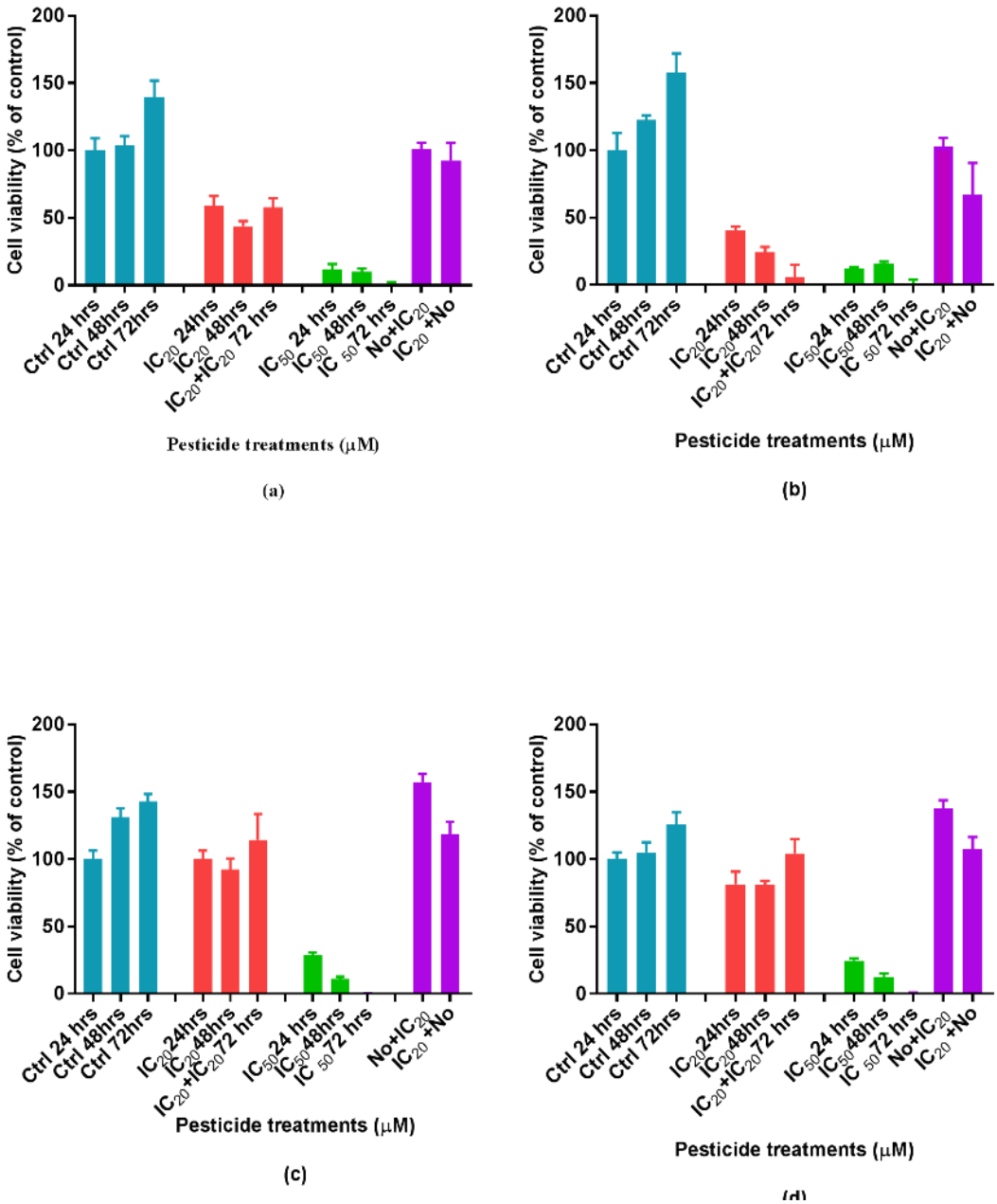


Figure 4-9 SH-SY5Y cells treated with low pesticide doses with recovery period.

(a)-15% FBS: Chlorpyrifos-oxon, (b)-10% FBS: Chlorpyrifos-oxon, (c)-10% FBS: Azamethiphos-oxon, (d)-15% FBS: Azamethiphos-oxon. Viability was assessed using MTT assay. Cell-free wells were subtracted from spectrophotometric readings for samples, which were then normalised to vehicle control readings and histograms are

displayed relative to control values of 100%. Cell viability readings, including vehicle control and treatments, were performed in triplicate and the experiment was repeated twice (n=3). Results analysed using one-way ANOVA with Dunnett's multiple comparisons and expressed as the mean ± standard error of the mean (SEM), with significant changes from control marked with asterisks. For significance: * = $p \leq 0.05$. Ctrl– vehicle control, IC_{20}- 20% inhibition concentration, IC_{50}- 50% inhibition concentration, IC – inhibition concentration.

Table 4-9 Summary statistics results of one-way ANOVA for SH-SY5Y cell line following repeated dose with recovery period. (*Comparisons were made between the control and each concentration of pesticide)

Media FBS concentration	**Pesticide**	**First treatment (24 h)**			**Recovery period (24 h) Added media**			**Second treatment (24 h)**			
		IC_{20}	**IC_{50}**	**No treatment**	**IC_{20}**	**IC_{50}**	**No treatment**	**IC_{20}**	**IC_{50}**	**No treatment + IC_{20}**	**IC_{20}+ No treatment**
10%	Azamethiphos-oxon	NS	****	Control	**	****	Control	**	****	NS	*
15%	Azamethiphos-oxon	NS	*	Control	NS	**	Control	NS	**	NS	NS
10%	Chlorpyrifos-oxon	NS	*	Control	**	**	Control	*	**	NS	*
15%	Chlorpyrifos-oxon	NS	*	Control	*	**	Control	*	*	NS	**

p>0.05(NS); p≤0.05(*); p≤0.01(**); p≤0.001(***); p≤0.0001(****), NS-not significant; h-hours; IC-Inhibition concentration

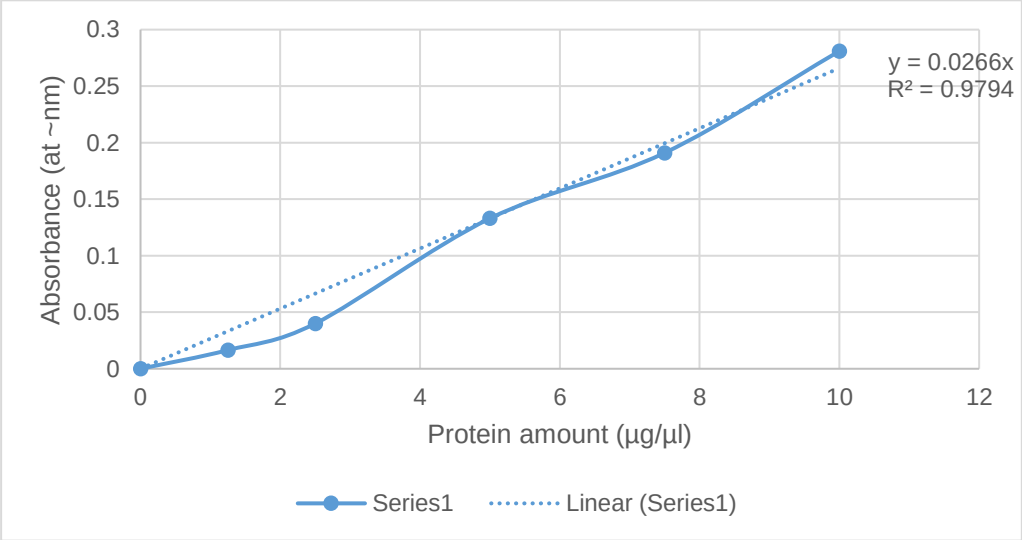
4.7 Gel electrophoresis and Western blotting experiments to determine the level of protein expression

4.7.1 Protein quantification

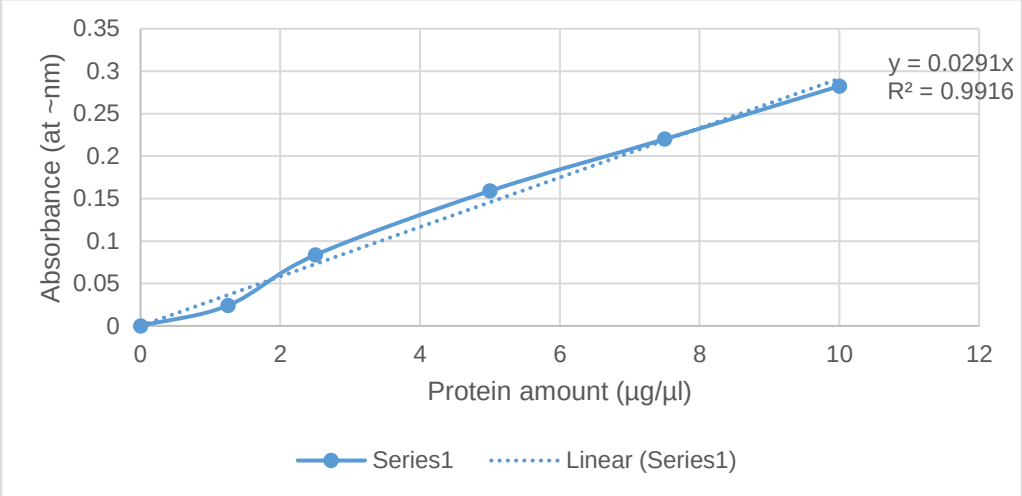
Ethanol and H_2O_2 treated cell lysates were used for the quantification and modified Lowry protein assays were conducted in duplicate as individual experiments.

The unknown samples of SH-SY5Y cells were diluted using 10 mM Tris buffer into 1:4, 1:5, 1:7 and 1:8 to find the best dilution ratio at first that was suitable for protein quantification using standard curve. According to the results it was found that 1:8 was the best dilution ratio for SH-SY5Y cells. Throughout these experiments R^2 was 0.95-0.99 and used for unknown protein quantification.

Figure 4-10 showed the standard curves for ethanol and H_2O_2 treated SH-SY5Y cells and **Table 4-10** shows the protein concentration for each sample used in experiments**.** These concentrations were used to determine the amount of protein that was used for gel electrophoresis.



(a)



(b)

Figure 4-10 Standard curves for ethanol and H_2O_2 treated cells as determined using a modified Lowry assay. (a)-Ethanol; (b) -H_2O_2

Table 4-10 Protein concentration of ethanol and H_2O_2 treated SH-SY5Y cell lysates following 24 hours treatment.

Treatment Ethanol	**Protein concentration (µg/µl)**
200 mM	0.85
100 mM	1.02
50 mM	0.93
Control	1.01
Treatment H_2O_2	**Protein concentration (µg/µl)**
Ctrl R1	1.202749
Ctrl R2	1.264605
IC_{20} R1	1.137457
IC_{20} R2	1.103093
IC_{50} R1	0.975945
IC_{50} R2	0.845361
IC_{80} R1	1.013746
IC_{80} R2	0.95189
500uM R1	0.680412

500uM R2	0.66323

Ctrl –control, IC_{20}- 20% inhibition concentration, IC_{50}- 50% inhibition concentration, IC_{80}- 80% inhibition concentration, IC –inhibition concentration

4.7.2 Effect of ethanol and H_2O_2 on expression of tubulin proteins in SH-SY5Y cells treated for 24 hours

The results from MTT assay showed that high concentrations of ethanol and H_2O_2 reduces cell viability of SH-SY5Y cells that justified the running of a gel for a protein assay, to examine specific proteins of interest in SH-SY5Y cells which may be affected by both neurotoxicants. However, following interpolation of unknown samples via the standard curve generated from Lowry assay, the protein concentrations for 50mM and 200 mM were very low and there were no significant protein concentration changes at higher concentrations of ethanol. This is inconsistent with the result observed in the MTT assay, where cell viability decreased dramatically at higher concentrations of ethanol.

SH-SY5Y cells were resolved by SDS-PAGE and Western blotting was performed with a panel of antibodies. **Figure 4-11** shows the gel following SDS-PAGE and displays cytosolic fraction. According to the gel, all band are in equally expressed in over the used treatment range at approximately 50 kDa. The observation was validated and quantify this protein level changes due to different concentrations of ethanol, a Western blot was performed on major cytoskeleton proteins α- and β-tubulin.

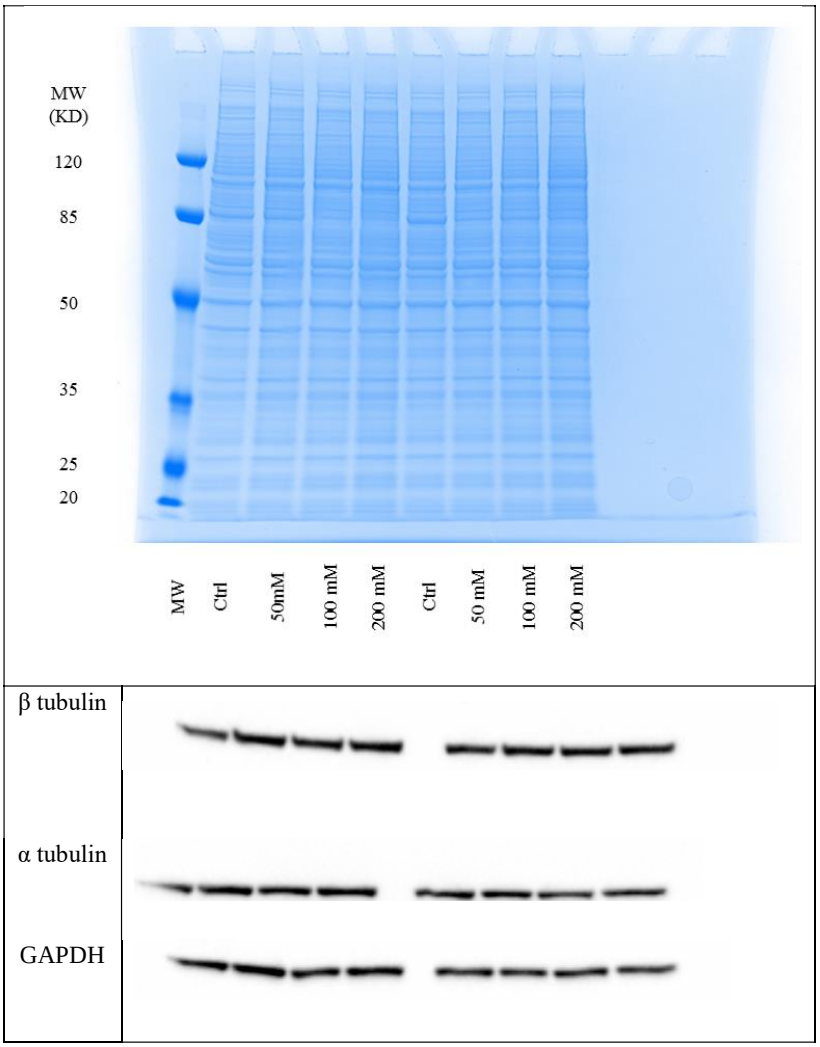


Figure 4-11Commasssie blue stained gel image following treatment of SH-SY5Y cells with ethanol for 24 hours.

Protein levels were visualized by protein staining (upper panel) and then microtubule proteins visualised by Western blotting (lower panel). GAPDH-glyceradehyde 3-phosphate dehydrogenase.

4.7.3 No significant changes in tubulin protein expression in ethanol treated SH-SY5Y cells

Quantification of these blots showed there was no significant reduction in the expression of α- and β-tubulin compared to control (**Figure 4-12**), performed via one-way ANOVA with Dunnett's multiple comparisons test.

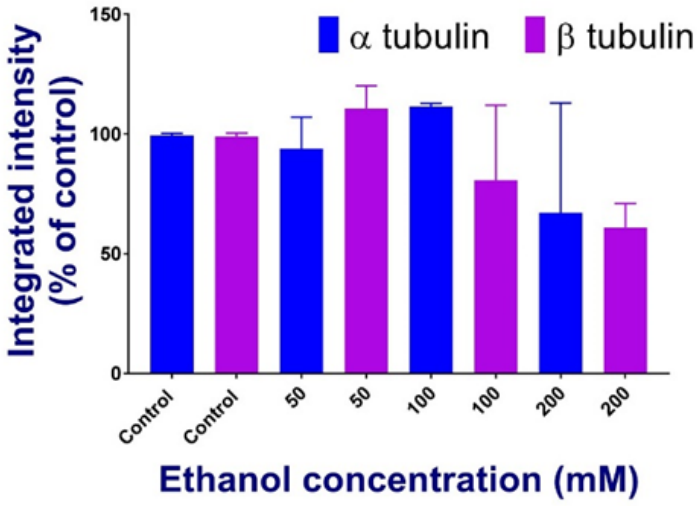


Figure 4-12 Quantification of protein level of SH-SY5Y cells following treatment with ethanol for 24 hours.

Protein levels for proteins of interest were quantified by Western blotting and normalised to total proteins, then expressed as a ratio of the control protein level for each protein of interest. Western blots were performed in duplicate for each protein of interest and a mean value generated. Western blots were repeated once on a separate set of samples (n = 2). Results analysed using one-way ANOVA with Dunnett's multiple comparisons. For significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \geq 0.001$. GAPDH- glyceraldehyde 3-phosphate dehydrogenase.

4.7.4 Quantification of protein expression in H_2O_2 treatment

Figure 4-13 shows the gel following SDS-PAGE and displays cytosolic fraction. According to the gel, no bands were expressed at higher concentrations of H_2O_2 treated cells such as IC_{50} and IC_{80} concentrations at 50kDa.This protein

expression changes were validated by Western blot performed on major cytoskeleton proteins α- and β-tubulin.

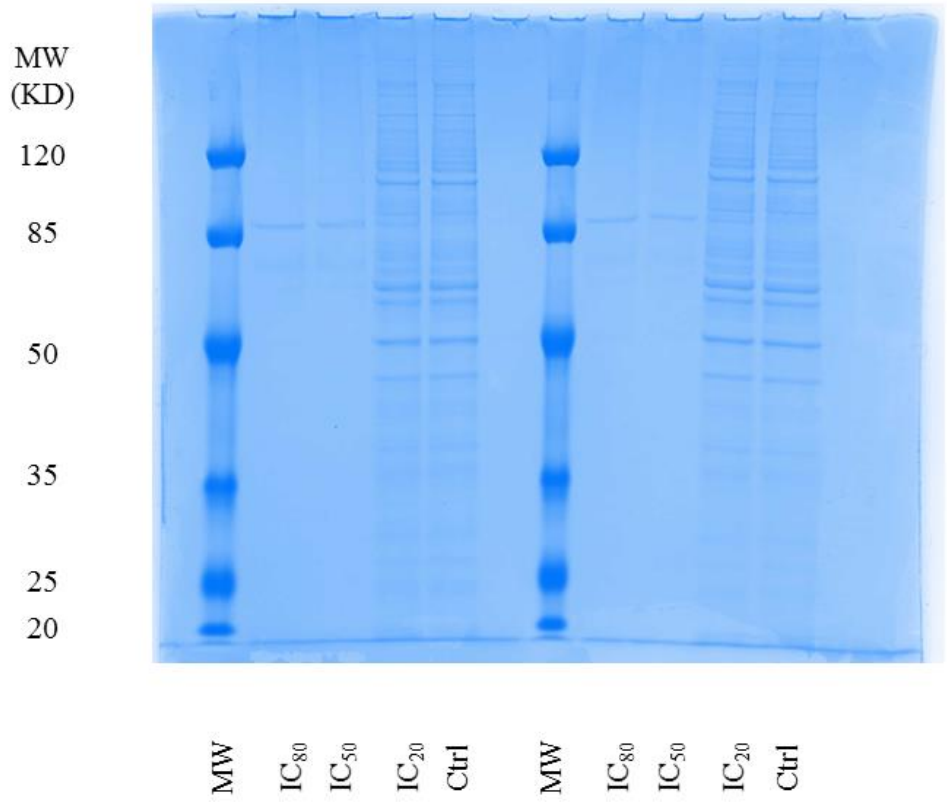


Figure 4-13 Commasssie blue stained gel image following treating of SH-SY5Y cells with H_2O_2 for 24 hours. Protein levels were visualized by Commasssie staining.

Ctrl –control, IC_{20}- 20% inhibition concentration, IC_{50}- 50% inhibition concentration, IC_{80}- 80% inhibition concentration, IC –inhibition concentration

There were no any bands found for β tubulin while only bands were expressed in control and IC_{20} treatment and no quantification was performed for the tubulin expression of H_2O_2 treatment within used concentration range.

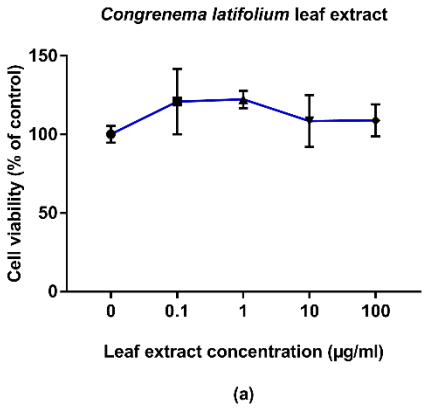
4.8 Effect of plant extracts on HepG2 cell viability

Figure 4-14 and **4-15** show the cell viability dose response curves for HepG2 cell line following treatment with different plant extracts. The results indicate that all plant extracts have some effect on HepG2 cell viability with each curve exhibiting from varying degrees deviating from 100% cell viability within the treated range. As shown in **Table 4-11** mean comparison between each treatment of plant extracts of respective control using one-way ANOVA revealed that CLEALF, TCE, ACFE, CASE, MCLE, TCSE, PPSTE, MTLE and SMSTE have no significant reduction or increase of HepG2 cell viability within the used concentration range (0.1, 1, 10, 100 µg/ml) when compared to the controls. But MELE (p = 0.0001, MOBE (p=0.05), HCE (p= 0.001), GBRE (p=0.01), PNLE (p = 0.0001), PNSE (p=0.05), and CLHBLE (p=0.001) have significant differences between 100 µg/ml plant extracts with their corresponding controls on cell viability of HepG2 cells (**Table 4-11, Figure 4-14, Figure 4-15).**

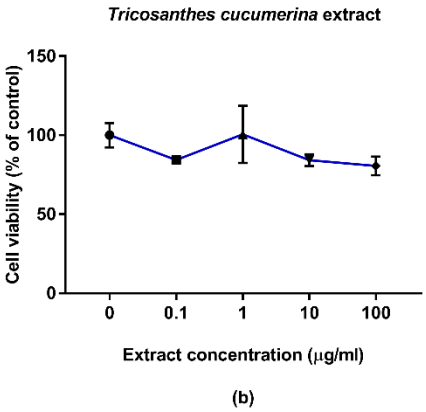
Table 4-11Results of one-way ANOVA for HepG2 cell line following treatment of different plant extracts for a 24 hours incubation period.

Plant Extract	Abbreviation	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml
Congrenema latifolium leaf extract	CLLE	NS	NS	NS	NS
Moringa olicifera ethanolic leaf extract	MOELE	NS	NS	NS	****
Tricosanthes cucumerina extract	TCE	NS	NS	NS	NS
Atrocarpus communis fruit extract	ACFE	NS	NS	NS	NS
Capsicum annum seed extract	CASE	NS	NS	NS	NS
Moringa oleifera bie extract	MOBE	NS	NS	NS	*
Musanga cecropioides leaf extract	MCLE	NS	NS	NS	NS
Terminalia catappa seed extract	TCSE	NS	NS	NS	NS
Herbalin complex extract	HCE	NS	NS	NS	***
Peltoforumptero carpum stem extract	PPSTE	NS	NS	NS	NS
Milletia thorningii leaf extract	MTLE	NS	NS	NS	NS
Glyphae brevis root extract	GBRE	NS	NS	NS	**
Parquetina nigrescens leaf extract	PNLE	NS	NS	NS	****
Parquetina nigrescens stem extract	PNSE	NS	NS	NS	*
Carpolobia. lutea hot butanolic leaf extract	CLHBLE	NS	NS	NS	***
Carpolobia. lutea ethyl acetate leaf fraction	CLEALF	NS	NS	NS	NS
Spondis mobin stem extract	SMSTE	NS	NS	NS	NS

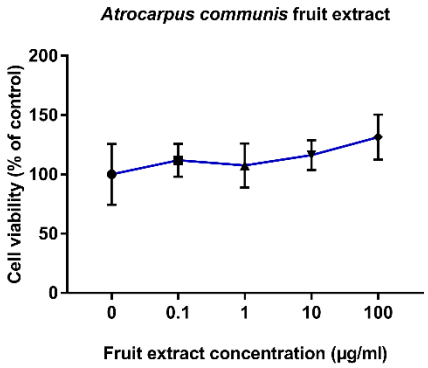
$p > 0.05$(NS); $p \leq 0.05$(*); $p \leq 0.01$(**); $p \leq 0.001$ (***); $p \leq 0.0001$(****); NS, not significant


Congrenema latifolium leaf extract
Cell viability (% of control)
150
100
50
0
0
0.1
1
10
100
Leaf extract concentration (µg/ml)

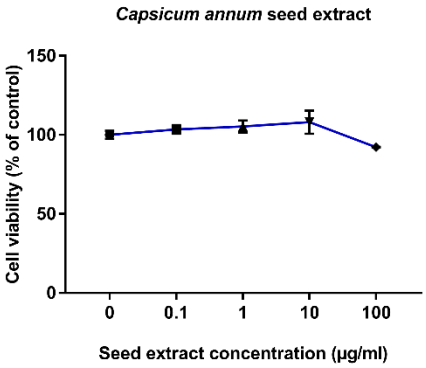
(a)


Tricosanthes cucumerina extract
Cell viability (% of control)
150
100
50
0
0
0.1
1
10
100
Extract concentration (µg/ml)

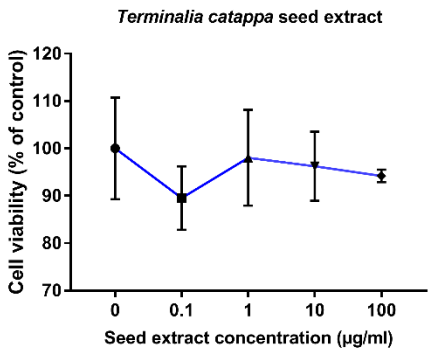
(b)


Atrocarpus communis fruit extract
Cell viability (% of control)
200
150
100
50
0
0
0.1
1
10
100
Fruit extract concentration (µg/ml)

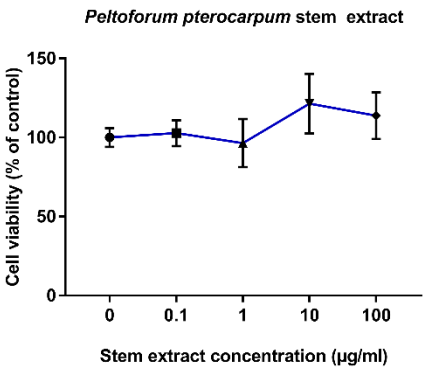
(c)


Capsicum annum seed extract
Cell viability (% of control)
150
100
50
0
0
0.1
1
10
100
Seed extract concentration (µg/ml)

(d)


Terminalia catappa seed extract
Cell viability (% of control)
120
110
100
90
80
70
0
0.1
1
10
100
Seed extract concentration (µg/ml)

(e)


Peltoforum pterocarpum stem extract
Cell viability (% of control)
150
100
50
0
0
0.1
1
10
100
Stem extract concentration (µg/ml)

(f)

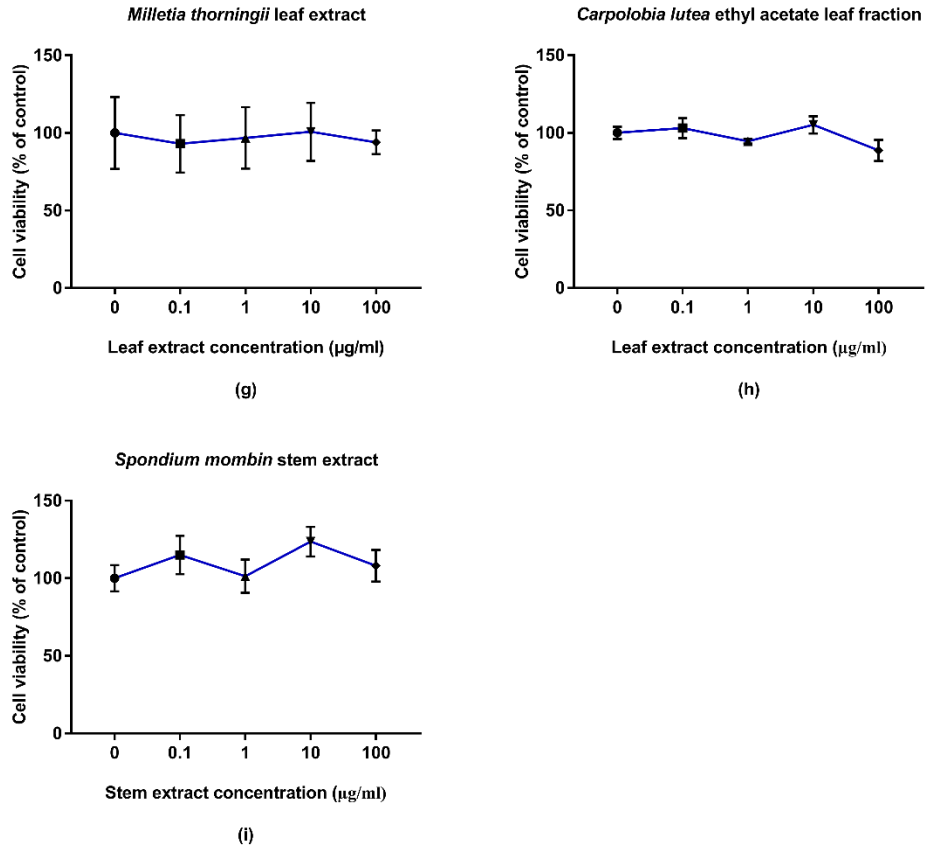


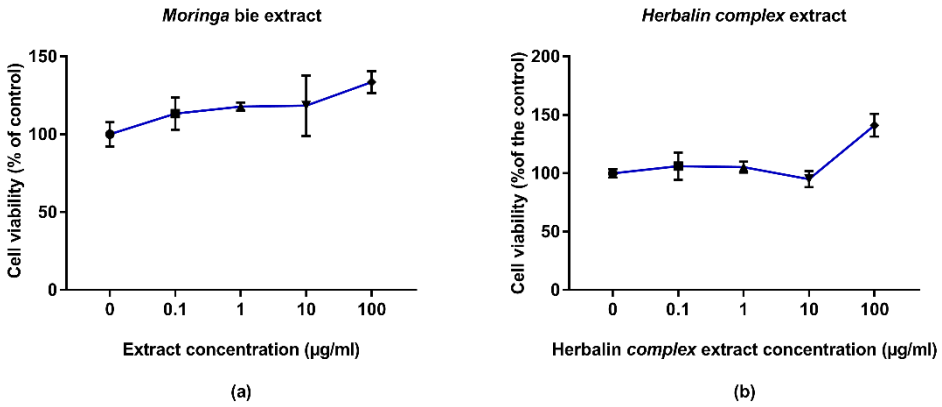
Figure 4-14 Cell viability inhibition dose response curves for CLLE, TCE, ACFE, CASE, TCSE MCLE, PPSTE, MTLE, CLEALF and SMSTE (a –i).

Cell viability was analysed using MTT assay. Cell viability readings were normalized to the mean of negative control as minimum value and corrected positive control (50µM pyrazinamide) for the highest value, providing the cell viability percentage, as a percentage relative to the negative control. Cell viability readings including negative control (cells without extracts) treated wells with plant extracts and positive control were performed as triplicates. Results were expressed as the mean ± SD, SD – standard deviation.

4.9 Some of the plant extracts reduced cell viability in a dose-dependent manner

Figure 4-14 illustrates the cell viability inhibition as a dose response curve. The dose response curves of plant extracts behave in a dose response manner

within the concentration range used with increasing concentration of plant extract of MOELE (*p* = 0.0001), MOBE (*p*=0.05), GBRE (*p*=0.01), PNLE (*p* = 0.0001), PNSE (*P*=0.05) and CLHLE (p=0.001) there was a subsequent decrease in cell viability. But mean comparison with control of 0.1, 1, 10 µg/ml was not significantly reduced cell viability according to the ANOVA results (**Table 4-11**) in the above-mentioned extracts. However, as the concentration increased, mean differences between the viability of controls and treated cells of MOELE (*p* = 0.0001), MOBE (*p*=0.05), GBRE (*p*=0.01), PNLE (*p* = 0.0001), PNSE (*p*=0.05) and CLHLE (*p*=0.001) became increasingly more significant and viability gradually decreased in HepG2 cells (**Table 4-11; Figure 4-15**). When considering the CLLE, TCE, ACFE, CASE, MCLE, TCSE, PPSTE, MTLE, SMSTE, and CLEALF they behaved in a dose-dependent manner, but there was no significant effect of decreasing or increasing cell viability within the range of concentrations used in the study (**Table 4-11**; **Figure 4-14**).



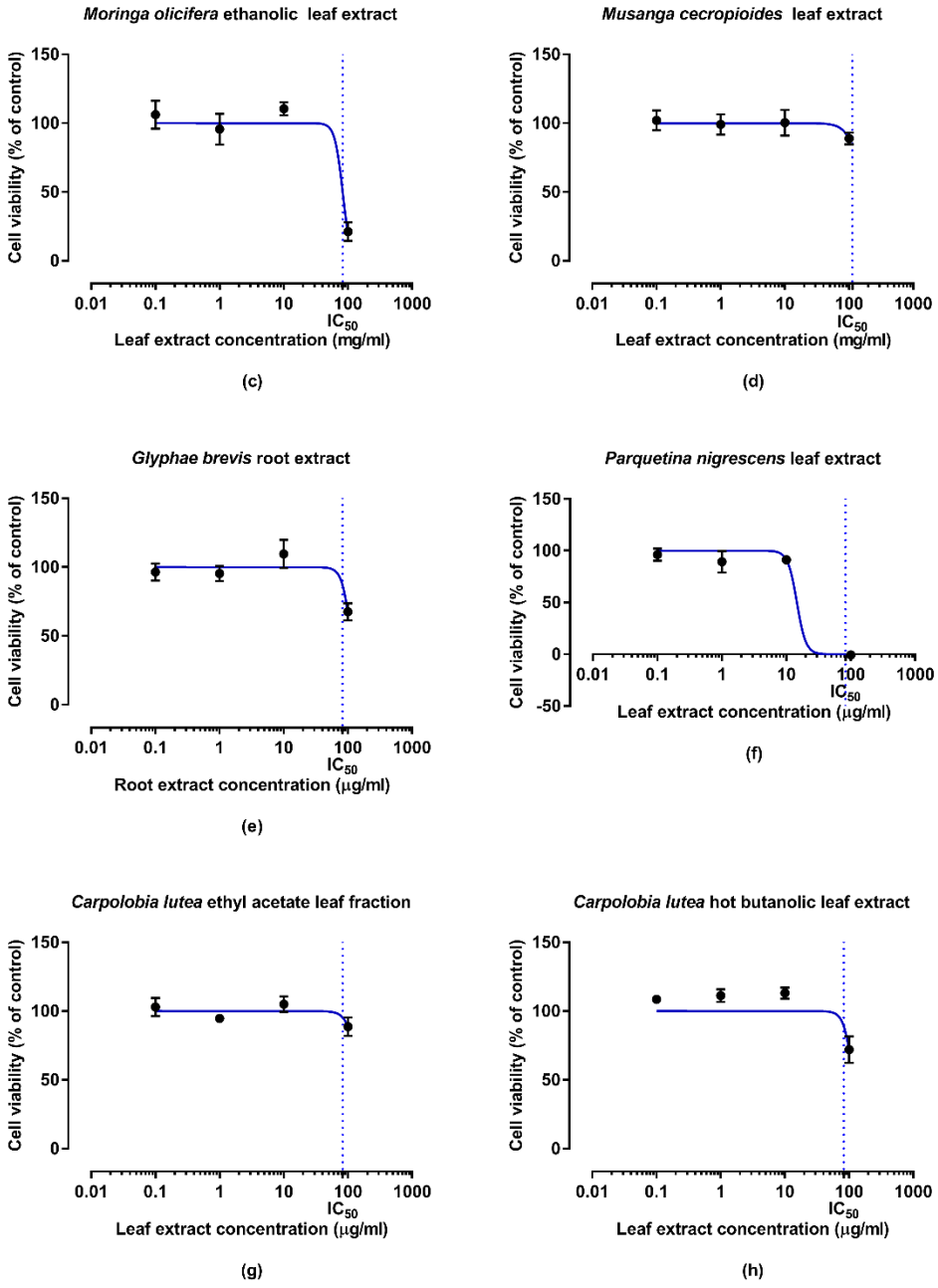


Figure 4-15 Cell viability dose response curves for HepG2 cells following incubation with plant extracts.

Cell viability was assessed using an MTT assay. The readings were normalized to the mean of negative control as minimum value and corrected positive control (50 µM pyrazinamide) for the highest value, providing the cell viability percentage as a percentage to the negative control. Cell viability data used was transformed to a logarithmic scale and modelled by nonlinear regression using GraphPad prism 7.0. (a)-GBRE and (b)-MCLE, showing increasing the cell viability on HepG2 cells. c, d, e, f, g

and h are MOELE, MCLE, GBRT, PNLE, CLHLE and CLEALF respectively showing decreasing cell viability on HepG2cells.

4.10 Determination of IC_{50} of plant extracts

The IC_{50} values were determined for HepG2 cells and were interpolated from each extract corresponding dose response curve. IC_{50} was determined for MOELE (p = 0.0001), MCLE (p=0.05), GBRE (p=0.01), PNLE (p = 0.001), CLHLE (p = 0.001) which showed a significant reduction of cell viability within the used treatment concentration range (**Figure 4-15**). The IC_{50} for the MCLE and CLEALF was predicted as it shows the trend line of decreasing viability within the used concentration. Though the conventional IC_{50} was determined for these plant extracts, GBRE (p=0.01), MCLE (p = 0.0001) increased in cell viability with increasing extract concentration **(Figure 4-15). Table 4-12** summarizes the IC_{50} for each extract that have significant effect on viability of HepG2 cells with their R^2 values providing an indication of how well the regression model fits to the data provided **(Table 4-12)**. Therefore, it can precisely predict the IC_{50} for particular plant extracts with higher R^2 values. However, IC_{50} of CLLE, TCE, ACFE, CASE, MCLE, TCSE, PPSTE, MTLE and SMSTE were not provided as it is not possible to reliably predict what concentration would affect the viability of HepG2 cells **(Figure 4-14)**.

Table 4-12 Summary statistics for HepG2 cells following treatment of plant extracts for a 24 hours incubation.

Plant extract/ fraction	IC_{50} (µM)	R^2
MCLE	~171.3	0.3948
MOESE	~82.83	0.9243
GBRE	~122.5	0.7249
PNLE	~14.61	0.9562
CLEALF	~148.6	0.4235
CLHLE	~116.7	0.6252

IC_{50} values (µM) for plant extracts were interpolated from their corresponding dose response curve. IC_{50} = Concentration of the plant extract / fraction that results in a 50% reduction in cell viability.

5 DISCUSSION

Neurotoxicity in humans may arise due to exposure to several agents such as heavy metals, pesticides, alcohol and acetaldehyde. In this study, the effect of ethanol, acetaldehyde, H_2O_2 and organophosphate pesticide as neurotoxic agents have been studies under *in vitro* conditions using SH-SY5Y cells. Also, the effect of some of the African medicinal plant extracts on viability of HepG2 cells were studied in this study.

Cells lose their viability as a loss of cell membrane integrity once they expose to exogenous toxic compounds. Therefore, this is critical for the maintenance of homeostasis and maintenance of cellular metabolism. Significant disruption of metabolic activity in cells, triggers cell cycle arrest and the intrinsic apoptotic cascade and then triggers cell death due to apoptosis or necrosis (Berridge *et al*, 1993, 1995).

All four neurotoxicant used in this study showed dose-dependent reduction of cell vibility of SH-SY5Y cells in all media and FBS concentrations used for study. But ethanol and acetaldehyde when tested on EMEM only showed reduction of vibility for acetaldehyde and increased the vibility of cell after treatment with ethanol. FBS plays an important role in culture media providing hormones, water-insoluble nutrients, growth factors, and protease inhibitors to cells to maintain *in vitro* conditions. EMEM only provides salt, non-essential amino acids, and sodium pyruvate to cell culture media. Therefore, the cell might not reach log phase when they are treated. This might be a reason for showing nonsignificant reduction of cell viability at lower concentrations. Ethanol does not readily effect cell viability in EMEM as it can be dissolved in EMEM and could form other compounds and these could have a positive effect on cell growth and metabolism. On the other hand, EMEM does not contain serum

protein thus it increases the toxicity of acetaldehyde since there is no other binding places for toxic moieties (Arora, 2017) and therefore, reduce the viability of cells growing in EMEM at higher concentration.

In the neurotoxicant studies performed herein it was observed that the IC_{50} values are different in different media as well as the same chemical treatment exerted different effects on cell viability in SH-SY5Y cells. Low serum media or serum free media showed the greatest variation in treatment effect than in 10% and 15% media. The variability of the IC_{50} values of all four neurotoxicants in the study related to the serum concentration of the culture media.

According to the dose metrics in toxicology, nominal concentration of chemicals (measured concentration of chemical in the exposure medium) have been used, and this nominal concentration can degrade over time and bind to plastic as well as to serum proteins, and lipids in the media. Therefore, the actual concentration uptake by cells is likely to be significantly different from the nominal or total concentration (analytically measured concentration of the test chemical). Furthermore, the physical properties of test chemical as well as assay condition also effect the actual amount of chemical uptake by the cells dossing *in vitro* assay conditions (Groothuis *et al*., 2013). Therefore, the freely dissolved unbound concentration of a chemical is only available for cells to cause toxicity (Duffus and Worth 2006). Serum proteins commonly present in cell culture medium therefore play a vital role in reducing the bioavailability of test chemical during *in vitro* cell culture assays (Kramer *et al*., 2015). This might be reason for the variability of IC_{50} values of same neurotoxicant in 0%, 3%, 10% and 15% FBS media. The study also showed higher IC_{50} values in low serum media while higher the media FBS concentration the lower the IC_{50} values. Because media FBS can bind the toxic moieties of neurotoxicant the actual chemical effect is very low to the cells. As aforementioned, studies have

reported that a nominal concentration of phenanthrene was reduced in low serum media and freely available concentration to cells in media also decreased and cytotoxicity induced by the test chemical has a dependence on this (Groothuis *et al*., 2013). Therefore, higher cytotoxicity end points may be observed in cells under *in vitro* conditions when there is a decrease in the serum proteins concentration (Groothuis *et al*., 2013). Similar observations were found in the current study such that when the serum level decreased the IC_{50} values also decreased and lower concentration of treatments showed a more significant reduction in cell viability compared to controls. However, test chemicals can also bind to the plastic of well plates as well as other plastic equipment used to prepare the test chemicals for dosing. This condition is more favoured by the lipophilic chemicals as mentioned by the Groothuis *et al*. (2013). Evaporation of test chemicals also effects toxicity studies *in vitro* (Kramer *et al*., 2015). In this study, acetaldehyde, may evaporate over time and therefore, the freely available concentration to the cell may reduce over time. Therefore, it can be concluded that effect of foetal bovine serum in SH-SY5Y culture media reduces agent cytotoxicity.

In this study, it has been showed that cell viability was decreased after increasing the alcohol concentration in both 3% and 10% FBS media. Similar observations were made by Ramlochansingh *et al.,* (2011) that the SH-SY5Y cells cultured in 10% FBS media greatly reduced their viability when treated with higher concentrations of alcohol. The cells exposed to higher concentration of ethanol showed cell shrinkage in glioblastoma cells and SH-SY5Y cells and before losing their viability. When increasing the concentration of ethanol, the viability of cells decreased because of higher concentration directly effecting cellular membrane integrity and leakage of extracellular chemicals due to activation of apoptosis signalling. This idea was further supported by the study

related to ethanol treatment and three neuroblastoma cell lines suggesting that ethanol reduced growth of SH-SY5Y cell in a dose-dependent manner, but did not reduce cell viability, thus reducing cell proliferation (Luo and Miller, 1997). The study also reported that 200 mg/dl significantly reduced SH-SY5Y cell viability in 10% foetal calf serum media. The initial concentrations of acetaldehyde such as 0.1µM and 1µM did not show a remarkable reduction of cell viability of SH-SY5Y cells, with changes not significant. A similar observation was made by Yan *et al.* (2016) that showed that there was no significant reduction of cell viability in SH-SY5Y cells following treatment of acetaldehyde up to 5 mM with compared to control in 10% FBS media. In contrast, the current study showed even with 1 mM treatment it can observed that there was a significant ($p \leq 0.1$) reduction in SH-SY5Y cell viability in 15% FBS media. Acetaldehyde treatment does not show cytostatic properties under *in vitro* conditions. The dose response curves of acetaldehyde in EMEM also exhibits a curve that becomes a plateau on the X axis showing cell viability approaches 0% compared with control after reaching to half maximal concentration. This idea was further supported by Yan *et al.* (2016) that showed that 10 mM treatment of acetaldehyde for 24 hours induced apoptosis in SH-SY5Y cell by Hoechst 33528 assay.

H_2O_2 is a non-radical form of ROS which is readily convereted to the hydroxl radical in cells. This hydroxl radical causes damage to neuronal cells by damaging cellular damages and altering cellular metabolic pathways. The effects of H_2O_2 on brain damage have been widely studied as it plays an important role in nerurological diseases such as Alzheimer's disease and Parkinson's disease. Therefore, the current study focused on the evaluation of the effect of H_2O_2 on cell viability of SH-SY5Y cells. Interestingly, results indicate even at low doses SH-SY5Y cells respond to H_2O_2. The viability of SH-SY5Y

cells was reduced in a concentration dependent manner (**Figure 4-6; Table 4-5**). At higher concentrations, H_2O_2 is more toxic to cells and could lead to cell death following 24 hours treatment. This is supported by the evidence that H_2O_2 toxicity induces Ras signaling pathway in human neuroblastoma SH-SY5Y cultured cells. SH-SY5Y cells treated with H_2O_2 of various concentrations (10-200 µM) displayed reduction of cell viability in a dose-depenedant manner in all three FBS media following 24 hour treatment. Higher concentration of H_2O_2 effect cell vibility as they induce oxidative stress in cells and then cause celllar apoptosis. Simillar observations were made by Chetsawang *et al.* (2010) that observed that treatment of SH-SY5Y with H_2O_2 with 100 µM to 1 mM showed significant reduction on SH-SY5Y viability in dose-dependent manner in 10% FBS media. However, this study reported 73% decrease in cell viability at a treatment concentration of 1 mM. This is simillar to the findings of this study that 80% cell viabilitiy has reduced at a concentration of 0.5 mM in 15% FBS media. No studies of yet have used H_2O_2 in low serum media, hence it was hard to compare the results of the current study with the 3% media and 0% FBS or no serum media with others. Herein the study shows that SH-SY5Y cells had cell viability reduction following 24 hours treatment in a dose-dependent manner and with lower IC_{50} values in no serum media. Although 3% FBS media showed higher IC_{50} values it can be concluded the SH-SY5Y media without serum is more sensitive to ROS damage than cells in more concentrated media (**Table 4-6**). H_2O_2 is cytotoxic to SH-SY5Y cells by oxidative stress (Ismail *et al*., 2014). Therefore, it can be suggested that the half maximal concentration that can cuase cell death in SH-SY5Y cell after . treatment would be an insight into the future studies related to H_2O_2 induced neurotoxicity studies on mechanisms of cytotoxicity of H_2O_2 neurotoxicity, with an exmination of the production of ROS and induction of apoptosis considered.

Organophosphate pesticide neurotoxicity is heavily studied in developmental neurotoxicology related to human neuronal cells. These studies are important for developing treatments and prevention of occupational pesticide exposure in humans. The current study focused on two organophosphate pesticides that are widely used in agricultural, domestic and industral settings. The results from this study clearly demonstrate that both Chlorpyrifos-oxon and Azamethiphos – oxon reduce cell viability or growth of SH-SY5Y cell following treatments for 24 hours in a dose-dependent manner. All three media showed a dose-dependent reduction in cell viability in SH-SY5Y cells (**Figure 4-8; Table 4-8**). According to the study it can be also concuded that Azamethiphos-oxon induced more cytotoxicity than Chlorpyrifos-oxon in SH-SY5Y cells in all media after acute exposure upto half maximal inhibition concentration and higher concnetrations such as 100 mM and 200 mM of Chlorpyrifos-oxon showe higher toxic insult than Azamethiphos-oxon for neuronal cells. 10 µM treatment did not significantly reduce cell viability in SH-SY5Y cell in all media, but 20 µM showed significant reduction in cell viability in 3%, 10% and 15% media apart from 0% media for azamethiphos-oxon. 50 µM-200 µM treatements significantly reduced SH-SY5Y cell viability in all media.

Chloropyrifos-oxon reduced cell vibility in a dose-dependent manner with a concentration range of 10-50 µM, and was significant in all media except for 50 µM in low serum media. Therefore, it clearly demonstrated that low serum media is more sensitive to the toxic effects of pesticide than serum containing media. Arora (2017) showed that FBS can bind the toxic moieties of chemicals in media. Therefore, it can be suggested that FBS media can reduce the toxicity of pesticides that effect cells to some extent. Eaton *et al* (2008) reported that Chlorpyrifos–oxon is more toxic than Chlorpyrifos-thion *in vitro* when applied to neuronal cells. The study further stated that the effective range is from micro

molar level. Similarly, herein, the most effective concentration range for Chlorpyrifos-oxon was 10 µM to 200 µM from preliminary studies. Slotkin *et al*., (2016) showed Chlopyrifos-oxon treated on PC12 cell reduced cell viability in a dose-dependent manner. Furthermore, it also showed IC_{50} for Chlorpyrifos in cerebellar granular neuronal cells was 12 µM and for Chlorpyrifos-oxon it was 2.5 µM (Eaton *et al.,* 2008). But for PC12 cell it was recorded as 290 µM. Eaton *et al.,* (2008) reported that Chlopyrifos reduced SH-SY5Y cell viability in a dose and time dependent manner with an IC_{50} interpolated as 313 µM following 24 hours treatment in 15% FBS media. This study also showed that the IC_{50} gradually decreased with exposure time. This is coincident with the current study that IC_{50} for Chlorpyrifos–oxon in 15% FBS media was interpolated as 191.62 µM, so close to the figure determined by Eaton *et al.* (2008). While no studies of yet have used low serum media for pesticide treatment in literature, however, this study found that the IC_{50} value greatly varied with media serum level.

There is a deficit in the literature about how low dose pesticides act on neuronal cells and cellular mechanisms. Low dose pesticide treatment of SH-SY5Ycells allowed an examination of the ability of cells to recover from the treatment. This showed some interesting result indicating that after 24 hours cell treated with IC_{20} of each pesticide were fully recovered and their viability increased compared to the viability of vehicle control (**Figure 4-9, Table 4-9**). There was not any similar study yet reported in the literature. This may cause due to cells can confluent more than 80% within 24 hours of recovery period which was not affected by the pesticide. In contrast, IC_{10} chlorpyrifos-oxon treated cells, had a reduced cell viability after 24 hours compared to the vehicle control cells. Therefore, low pesticide dose may have positive effect on cellar proliferation

that the low does like IC_{20} can trigger the cell growth as a stress response of cells.

When considering the expression of damaged tubulin proteins through Western blot experiments there was not any significant reduction in tubulin proteins in ethanol treated cells. This contradicts the ideas suggested by Erdozain *et al.* (2014) that mentioned alcohol neuronal toxicity on neuronal cells has an effect on tubulin proteins in cells which compromises the cell architecture. Microtubules play a major role on keeping cell shape and the mitosis process in cells. So, cell shrinkage and shape lost during the ethanol treatment may have an effect on cellular tubulin proteins. This may be due to the study being conducted using undifferentiated (as opposed to differentiated) SH-SY5Y cells and the cells are further proliferating and proteins were synthesise readily in the cells when they undergoing cellular mitotic division. Furthermore, it was not possible to successfully separate protein using gel electrophoresis from H_2O_2 treated cells. Therefore, the current study does not show any results from H_2O_2 treated cells for protein expression study. This relates to the extensive protein loss and probably apoptosis that occurs at high H_2O_2 dosing.

Natural plant extracts are widely use in the pharmaceutical industry due to their ability to reduce cytotoxicity effects to cells. They are rich in antioxidants like alkaloids, flavonoid and polyphenols which can act as free radical scavengers in ROS related toxicity conditions. The results of this study show some the plant extracts / fractions have the ability to reduce cell viability while most of them were not have significantly toxic to reduce cell viability. However, two plant extracts including *H. complex* extract and *M. oleifera* bie extract increased the cell viability of HepG2 cells.

Plant antioxidants interfere with the cell signaling pathways of cells and increase cell proliferation. The plants which reduced the cell viability of HepG2 cells were

M. oleifera leaf extract, *M. cecropioides* leaf extract, *P. nigrescens* leaf extract, *G. brevis* root extract and *C. lutea* hot butanolic extract, *C. lutea* ethyl acetate leaf fraction. Literature has widely reported plant parts of *M.oleifera* as a medicinal plant with anti-tumour, anti-pyretic, anti-epileptic, anti-inflammatory, anti-ulcer, diuretic, anti-hypersensitive, antioxidant, anti-diabatic, antibacterial, antifungal and hepatoprotective properties (Anwer *et al.,* 2007; Kar *et al.,* 2003; Sharifudin *et al.,* 2013). Also, the literature has widely reported different plant extracts of different plant parts are hepatoprotective in nature to cells (Fakurazi *et al.,* 2008; Hamza, 2010; Sharifudin *et al.,* 2013). However, scarce information is available on ethanolic leaf extracts. But controversially, *M. oleifera* essential seed oil has been reported as hepatotoxic on HepG2 cells within the range of 0.15 to 1 mg/ml for a 24 hours incubation period. IC_{50} was recorded as 751.9 µg/ml for essential oil seed extract (Elsayed *et al.,* 2015). Controversially, this study reported that increasing the concentration of ethanolic leaf extract greatly decreased the cell viability of HepG2 cells depending on the concentration applied after 24 hours. IC_{50} for ethanolic leaf extract was 82.83 µg/ml from this study for 24 hours incubation period (**Figure 4.10**). The results are in accordance with the results obtained by Jafarain, *et al.* (2014) that also obtained a dose-dependent response of different concentrations of leaf extracts on Hela cells. The Hela cells showed highest decrease in cell viability in 500 µg/ ml and 50% reduction of cell viability observed at 260 µg/ ml. The result obtained from this study is consistent with that of Jafarain, *et al.,* (2014) that leaf extracts of *M. oleifera* are cytotoxic. Generally cytotoxic effect of *M. oleifera* leaf extract is likely due to the presence of phenolic compounds (Fukai *et al.,* 2000; Nomura *et al.,* 2003; Sabzevari *et al,* 2004; Sakagami *et al.,* 2000; Smejkal *et al.,* 2010).

The study of *M. oleifera* bie extract showed an increased the cell viability within 24 hours incubation period (**Figure 4.10, Graph a**) suggesting it is

hepatoprotective. Results of the study show that the increment of cell viability started from concentration 10 µg/ml. In accordance with the studies (Fakurazi *et al.,* 2008; Hamza, 2010) of many plant parts from this plant are hepatoprotective. Two plant extracts from same plant, extracted into different solvents show different behavior in their toxicity and protectivity on HepG2 cells, and this confirmed that there is an effect of different plant parts and their extracted solvent on hepatotoxicity.

M.cecropioides, Glyphae brevis and *P. nigresense* also behave as hepatotoxic plants in this study. The information regarding hepatotoxic effect of these plants is scarce in the literature. But *M.cecropioides* reported as hypotensive properties in its leaf extracts in *in vitro* studies due to the presence of aponins, flavonoids and procyanidins. In accordance to that study the *M.cecropioides* leaves showed inhibitory action (Dongmo *et al.,* 2002). Konan, *et al.* (2014) showed that n-butanol extract from *Glyphaea brevis* roots are cytotoxic to glioma cells. Dose-dependent response was showed after treating with 48 hours was observed for proliferation of glioma cells and EC_{50} was recorded as > 1 mg/ml. This is consistent with our study that *Glyphaea brevis* root extracts have an IC_{50} of 122.5 µg/ml with HepG2 cells. Their study further pointed outs that the inhibitory action was due to the presence of phenolic compounds like protocatechuic acid (PCA) by HPLC analysis. Interestingly, *P. nigresense* was not studied for its cytotoxic ability yet. Although, it was widely studied for its anti-inflammatory properties (Owoyele *et al.,* 2008), although toxicity studies are rare. The plant biochemical profile analysis showed that it is rich with alkaloids, saponins, flavonoids, cardiac glycosides, steroids, tannins, phlobatannins, cardenolides, phenolics, anthraquinones and triterpenes with alkaloids. These secondary metabolites could have profound effect on the cytotoxic ability of leaf extract on HepG2 cells (Kayode and Yakubu, 2017). *C. lutea* hot butonolic leaf

extract plant extracts also exhibit same dose-dependent cell viability reduction on HepG2 cell that coincides with the results observed by Nwidu *et al.,* (2017) and the observation in the current study showed that *C. lutea* ethyl acetate leaf fraction is not hepatotoxic in statistical analysis but can observe dose-dependent response within the used concentration range.

According to the results of the study *Herbalin complex* extract and *M.oleifera* bie exract showed both cytostatic and possibly hepatoprotective properties.

However, *G.latifolium* leaf extract, *T. cucumerina* exract, *A. communis* fruit extract, *C. anum* seed extract, *T.catappa* seed extract, *P.pterocarpum* stem extract, *M. thorningii* leaf extract, and *S. mombin* stem extract did not show any significance reduction or increment of cell viability within the used concentration range. Thus, these plants are used as vegetables and seed plants in African and Asian countries it can be concluded that they may be safe to consume daily since there is no hepatotoxic effect according to the current study. However, the ability to increase cell viability of HepG2 cell by *G. latifolium* in *in vitro* condition suggested it could be hepatoprotective in nature. This was supported by study conducted using Wistar rats by oral administration of 200 and 400 mg/kg aqueous leaf extract showed protection against APAP, which is conflicting compared to current study as our results indicate there is no significant effect on viability of HepG2 cells.

On the other hand, it could be suggested that plant extracts itself has some oxidative properties that can oxidize the MTT reagent in media. This is consistent with the study that showed polyphenols, such as (-)-epigallocatechin-3-gallate (EGCG) present in the botanical treatments on the cell culture media underestimate the viability of cells in MTT assay (Peng *et al.,* 2005; Srivastava *et al.,* 2000)

In conclusion, the current study shows that ethanol, acetaldehyde, H_2O_2 and organophosphate pesticides reduce viability of SH-SY5Y cells in a dose-dependent manner. There was an effect exerted on toxicity of each neurotoxic chemicals by the media FBS concentration. Finally, cellular tubulin protein level was not changed by even up to 200 mM ethanol concentration. However, the data generated through this study is important for future research when using standardisation protocols on high throughput screening studies in development neurotoxicology. Also, future studies will provide an insight into the structural and functional damages experienced by neuronal cells and to determine their mechanism under *in vitro* conditions.

The current study shows *M. oleifera* leaf extract, *M. cecropioides* leaf extract, *P. nigrescens* leaf extract, *G. brevis* root extract and *C. lutea* hot butnolic extract and *C. lutea* ethyl acetate leaf fraction are potentially hepatotoxic and *Herbalin complex* extract and *M. oleifera* bie extract are hepatoprotective, Neither hepatotoxic or hepatoprotective properties were observed for *G. latifolium* leaf extract, *T. cucumerina* extract, *A. communis* fruit extract, *C. anum* seed extract, *T. catappa* seed extract, *P. pterocarpum* stem extract, *M. thorningii* leaf extract and *S. mombin* stem extract according to the current study within the used concentration range. This study can be used as preliminary data for further investigation of possible mechanisms and role of different plant extracts and different solvent extracts of same plant on other *in vitro* cell lines

5.1 Draw backs in present study

The present study was conducted with SH-SY5Y *in vitro* cultures and a major problem encountered was the treatment of SH-SY5Y cells with acetaldehyde. Acetaldehyde is a volatile compound and its boiling point is 20^{o}C. It was stored in the cold room until used. When preparing serial dilution in cell growth media

it can be evaporated over time. Therefore, the total concentration would be different from that calculated when applied to cells. In order to prevent this, dilutions were prepared inside a Styrofoam ice box. However, with time acetaldehyde could still be evaporated from the media as well as from the plate. Indeed, initial experiments showed that all cells died due to evaporation of the chemical even into control wells.

Ethanol and acetaldehyde experiments when conducted in the same 96 well plate also resulted in reduced cell viability, of ethanol treated cells due to evaporation of acetaldehyde. The best possible remedy for this is repeat the assay inside a cold room if the cell culture facilities were available or prepared the dilution serious inside the cold room on ice and immediately treat the cells.

When plating cells in 96 well plate the outer most rows of well also needed to be discarded as they are effected by absorbance edge effects, and this can lead to improper absorbance values. When plates were placed inside the incubator medium the outermost wells were evaporated and therefore a treatment effect to those cells can be lower than the intermediate wells use as replicates due to changes in media concentrations, volume and evaporation rates. The effect was minimized using outer most wells filled only with respective culture media only without cells. This was first experienced for the experiments and the repeated experiments were done without seeding cells on the outer most wells of the plate. One row of inner most wells were used as the blanks to minimize the effect on viability readings.

Finally, it was impossible to compare results with other literature for low serum media experiments since most of the studies were conducted culture SH-SY5Y cells in 10% or 15% media.

5.2 Proposal for the future work

Acetaldehyde treatment should be repeated to determine the precise IC_{50} values in a well-planned way to reduce an effect of evaporation. All assays should be repeated with standardisation protocols with fixed cell concentrations as well as with serum free assays to avoid the effect of serum binding effect to chemicals. Partition – controlled dosing systems can be employed in *in vitro* culture assays to reduce the loss of a test chemical in cell culture media. As observed in the pesticide experiments serum effect can be minimised if pesticides are diluted into PBS for cell treatment.

Repeated dose experiment with low doses should be repeated with lower IC values such as IC_{10} in order to examine how well the cells recovered when exposed to low pesticide doses. Furthermore, chronic toxicity of low dose pesticide exposure should be done in long term culture condition to assess the products of oxidatively damage proteins and their pesticide adducts.

It is important to do LDH assays to confirm that their neurotoxic agents were cytotoxic other than just cytostatic to SH-SY5Y cells. MTT assay will not work with plant extract since availability of polyphenols can oxidise the MTT. This will affect the cell viability assay and will not give a precise reading. It is important to repeat the plant extract assays with ATP assay as it does not change the assay chemistry and would be a precise way to measure the cell viability and MTT assay. Therefore, more precise IC_{50} values can be obtained for all environmental toxicants than MTT assay for future studies.

Altered protein expression due to the effect of each neurotoxicant should be repeated to future validate the results. In current study, protein purification by gel electrophoresis should be repeated to do electroblotting of H_2O_2 cell lysates

to generate more results and assess protein changes such as to tubulin proteins.

6 REFERENCES

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