Cytotoxicity of Chemicals Present in Agricultural and Household products

Yaseen A. Bokhari

School of Medicine

Faculty of Medicine, Nursing and Health Sciences

Flinders University Adelaide Australia

A thesis submitted in fulfilment of the requirements for the degree of Master of Science

July 1st, 2016

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENT

In the name of Allah, the Gracious, the Merciful.

All Praise be to God (Allah), I praise Him and seek help from Him. I ask forgiveness from Him. I repent to Him and seek refuge in Him from my own evils and bad deeds. I bear witness that there is no God but Allah, the Only One without any partner, and I testify that Muhammad, peace and blessing upon him, is His servant, and His messenger.

Firstly, I would like to thank Almighty Allah, Who allow me mentally and somatically to understand and write this thesis. Praise be to Allah for subjecting technologies to us so that we can know His Majesty within His creations. Allah says in the Holy Quran Chapter 2 verse 164 which translated as {Behold! in the creation of the heavens and the earth; in the alternation of the night and the day; in the sailing of the ships through the ocean for the profit of mankind; in the rain which Allah Sends down from the skies, and the life which He gives therewith to an earth that is dead; in the beasts of all kinds that He scatters through the earth; in the change of the winds, and the clouds which they Trail like their slaves between the sky and the earth;- (Here) indeed are Signs for a people that are wise}.

I then would like to thank my parents for their support, love and prays for me. I thank my sweetheart, my wife, for her support and patient while I was busy, and for her efforts to raise our two little girls, Mariam and Albatool, based on dignified Islamic manners. May Allah glory be to Him grants her paradise. Moreover, I pray to Allah to send mercy and forgive King Abdullah Ben Abdulaziz, who gave me this opportunity to continue my postgraduate Journey. I thank King Salman Ben Abdulaziz for his efforts to make the Kingdom of Saudi Arabia a better place for living and educating.

I thank my kind and excellent supervisor, Dr Barbara Sanderson, for her time

that she spent to help, direct and explain everything that was confusing me. I am pleased to know and work with such a nice supervisor. May Allah heal her and give her strength. Also, special thanks to my Co-supervisor, Professor Chris Franco, for his support and help during the absent of Dr Barbara. Last, but not least, I would like to thank all colleagues and lab mates in the Department of Medical Biotechnology, School of Medicine, Flinders University, for their assistance, advice and encouragement.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Yaseen Abdulmalik Bokhari

July 1st, 2016

ABSTRACT

People can be exposed to agricultural and household products, including daily exposure to multiple products from both fields at the same time. There are two essential ways by which people are exposed to the herbicidal agricultural commodities. The first way is the direct exposure, which occurs during the usage of these chemicals. Indeed, homeowners use greater volume of herbicides in their gardens than the recommended rate, so they are using more herbicides per hectare than farmers. This misuse increases the chance of the exposure to these chemicals. Therefore, the cytotoxicity of 'Once-a-year path weed' and Roundup (R-up), agricultural products, and their constituents on the human skin cells, HaCaT, was studied. Assessing the cytotoxicity of 24h treatment of both products and their components was determined using two extensively used assays, MTT and Crystal violet (CV) assays. 'Once-a-year path weed' showed a significant reduction in the cell viability ($p < 0.001$) of HaCaT at concentrations as low as 0.005% containing 30 µM amitrole, 29 µM ammonium thiocyanate and 22.3 µM simazine. However, this cytotoxicity was not driven by any of the active ingredients, amitrole and simazine, nor the surfactant, ammonium thiocyanate. Simazine showed a significant HaCaT killing at 223 µM, which is ten times the concentration in 0.005% of the commercial products. On the other hand, 0.05% R-up significantly killed ($p < 0.05$) almost half of the human skin cells, but its active ingredient, glyphosate was not toxic at even much higher concentrations. Polyethoxylated tallow amine (POEA) was the surfactant, and it seemed to be highly toxic at very low concentration (13.6 µM). Therefore, it is more likely to be the responsible of R-up cytotoxicity.

The second way of exposure is the indirect exposure, which can take place through food ingestion. Herbicides residues exceed the international tolerance levels in some livestock such as raw bovine milk and farm animal tissues. Via food intake, herbicides residues could reach the blood stream, and it is more likely that

they interact with other substances in the blood such as caffeine. Caffeine is the most consumed psychoactive substance because it is commonly and frequently consumed in many dietary sources. Nowadays, the consumption of the caffeine-containing beverages occupies a significant place in the national cultures for most nations of the world. Accordingly, the second part of this study was to measure the effect of caffeine on the cytotoxicity of the tested commercial products and their constituents on the blood cell line, WIL2-NS. Caffeine increases the toxicity of some chemicals, but that increase was not significant.

1 CHAPTER I: INTRODUCTION:

There is widespread concern that commercial agricultural products have the possibility to interact with a huge number of physiological functions, in exposed populations (Sanderson *et al.*, 2000). The toxicity of selected chemicals used in agricultural products and the food industry are the focus of this study. These two fields have a common factor, which is that people can be exposed to these chemicals, including daily exposure to multiple products from fields at the same time.

The first barrier between the human body and the outside environment is the skin (Gehin *et al.*, 2006). Human skin contains a network of non-enzymatic and enzymatic systems, which oppose any oxidative injury which can occur by the generation of reactive oxygen species in the skin structure (Gehin *et al.*, 2006). Our bodies are frequently exposed to many exogenous agents, including pesticides, atmospheric pollutants, cigarette smoking, or UV light (Amerio *et al.*, 2004; Gehin *et al.*, 2006). Not only synthetic chemicals are toxic, but also natural chemicals are toxic at some doses (Gold *et al.*, 2001b). Individuals are exposed to a huge range of chemicals that are naturally occurring (Gold *et al.*, 2001b).

1.1 Types of hazards posed:

Cytotoxicity, genotoxicity, and ecotoxicity are the three hazards posed by the exposure of the society to pesticides.

1.1.1 Cytotoxicity.

The first hazard posed is cytotoxicity. Within the last decade, the cytotoxicity of sites contaminated with pesticides attracted the attention of researchers because cytotoxic contaminants can be transferred easily to people when they breathe, eat or drink (Berthe-Corti *et al.*, 1998). Cytotoxicity is a complex event causing different effects, such as cell cycle dysregulation, cell damage, and cell death (Freshney, 2005; Marc *et al.*, 2004a). Some contaminants show cytotoxic effects not just on eukaryotic cells but also on prokaryotic cells (Berthe-Corti *et al.*, 1998; Lachance *et*

al., 1999). The cell cycle is a process in eukaryotic cells used to facilitate all living organisms' growth and development (Marc *et al.*, 2004a). The cell cycle has control mechanisms known as cell-cycle checkpoints, which are in response to stress to allow the repair of cellular damage or lead to programmed cell death (PCD) (Marc *et al.*, 2004a).

Cell death is necessary to create functional and complex multi-cellular tissues, for example, to maintain homeostasis of normal cells, and to establish and maintain a suitable balance of each type of cells in tissue. Cell death, also, plays a key role in several pathologies (Danial and Korsmeyer, 2004; Fiers *et al.*, 1999).

There are three main ways by which cells die (Edinger and Thompson, 2004; Fiers *et al.*, 1999). The two principal mechanisms by which cells die are apoptosis and necrosis. The third type of cell death is autophagy or type II PCD. It is, also, suggested as a mechanism of cell death, but some evidence suggests that it is a cell survival strategy (Cloonan and Williams, 2011; Danial and Korsmeyer, 2004). As soon as cells respond to DNA damage, there is a primary protein known as the tumour suppressor protein, p53, and it is found in most cell lines (Bode and Dong, 2007). P53 is expressed in G1 phase when DNA damage occurs (Bode and Dong, 2007). The ataxia telangiectasia mutated (ATM) protein and the AT-related (ATR) homolog phosphorylate p53 and mouse double minute 2 (MDM2), so they prevent p53 degradation and ubiquitination through the association of p53 with MDM2 (Bode and Dong, 2007). That results in p53 stabilization and accumulation (Bode and Dong, 2007). p53 is the essential mediator of cell cycle arrest and the induction of apoptosis (Bode and Dong, 2007).

1.1.1.1 Apoptosis.

Apoptosis is the essential mechanism that leads to cell death in all cell types from embryonic development until adult organisms (Danial and Korsmeyer, 2004). Because it is regulated by numerous cellular signalling pathways such as caspase-

dependent pathways and Bcl-2 protein family, it is known as PCD (Cloonan and Williams, 2011; Edinger and Thompson, 2004). There are different factors characterise apoptotic death, for example, membrane blebbing, the performance of internucleosomal fragments of the DNA, nuclear fragmentation, cell shrinkage, and condensation of the nuclear (Figure 1.1c) (Edinger and Thompson, 2004; Fiers *et al.*, 1999). Apoptosis needs energy (ATPs) for the process to occur (Edinger and Thompson, 2004). Phagocytic cells recognize apoptotic bodies and remove them (Edinger and Thompson, 2004). Apoptosis is the most common form of PCD.

1.1.1.2 Necrosis.

Necrosis was originally thought to be accidently performed as a result of physical damage or toxic attacks (Edinger and Thompson, 2004). In necrosis, the bio-energy (ATP levels) decreases to an insufficient level for cell survival (Edinger and Thompson, 2004). Cells die by swelling, cytoplasm vacuolation, and destruction of the plasma membrane followed by spreading vacuoles and cellular contents outside the cells (Figure 1.1d) (Edinger and Thompson, 2004; Fiers *et al.*, 1999). Necrosis is now known to be a non-apoptosis form of PCD because it is regulated by several cellular signalling pathways (Danial and Korsmeyer, 2004; Edinger and Thompson, 2004). Programmed necrosis takes over as soon as apoptosis is inhibited either genetically (e.g., viral infection) or chemically (Edinger and Thompson, 2004).

1.1.1.3 Autophagy.

Even though autophagy has the same stimuli as necrosis, they are completely different in the way they occur (Edinger and Thompson, 2004). When cells face famine time, there are two ways for autophagy to occur, microautophagy and macroautophagy (Levine and Klionsky, 2004). In microautophagy, lysosomes directly surround organelles in the cytoplasm and degrade them; in contrast, cytoplasmic autophagic vacuoles (autophagosomes), that encapsulate organelles, are formed in macroautophagy pathway. These autophagosomes then fuse with

lysosomes; energy subsequently is provided, and recycling of the contents occurs (Figure 1.1b) (Cloonan and Williams, 2011; Edinger and Thompson, 2004; Levine and Klionsky, 2004). The hypothesis, which says autophagy is more likely to be a survival strategy than cell death mechanism, need to be investigated in further studies before acceptance (Edinger and Thompson, 2004).

1.1.2 Genotoxicity.

A hazard posed by exogenous and endogenous agents is genotoxicity. The genetic material of eukaryotic cells is affected when they are exposed to ionising radiation or carcinogenic chemicals, including causation of chromosome damage (Fenech, 2000). Carcinogenesis involves genotoxic events, and any irregular cell cycle can cause genomic instability and cancer development (CCME, 2008; Marc *et al.*, 2004a). One of the hallmarks of human cancer and tumour cells is cell-cycle dysregulation (Marc *et al.*, 2004a).

1.1.2.1 Carcinogenesis.

Cancer is a major chronic health problem and an extremely complex multi-step s which is caused by multiple mechanisms, including toxicological substances (Barrett, 1993; CCME, 2008). According to Australian Institute of Health and Welfare (AIHW; 2010), cancer is abnormal cells which occur due to a group of diseases and grow randomly to form a mass known as a neoplasm. These cells can attack and damage other tissues around them or worse, can overspread through the lymphatic or bloodstream system to other parts of the body (AIHW, 2010). Not all tumours are invasive, but the uncontrolled spreading of a tumour could cause death (AIHW, 2010).

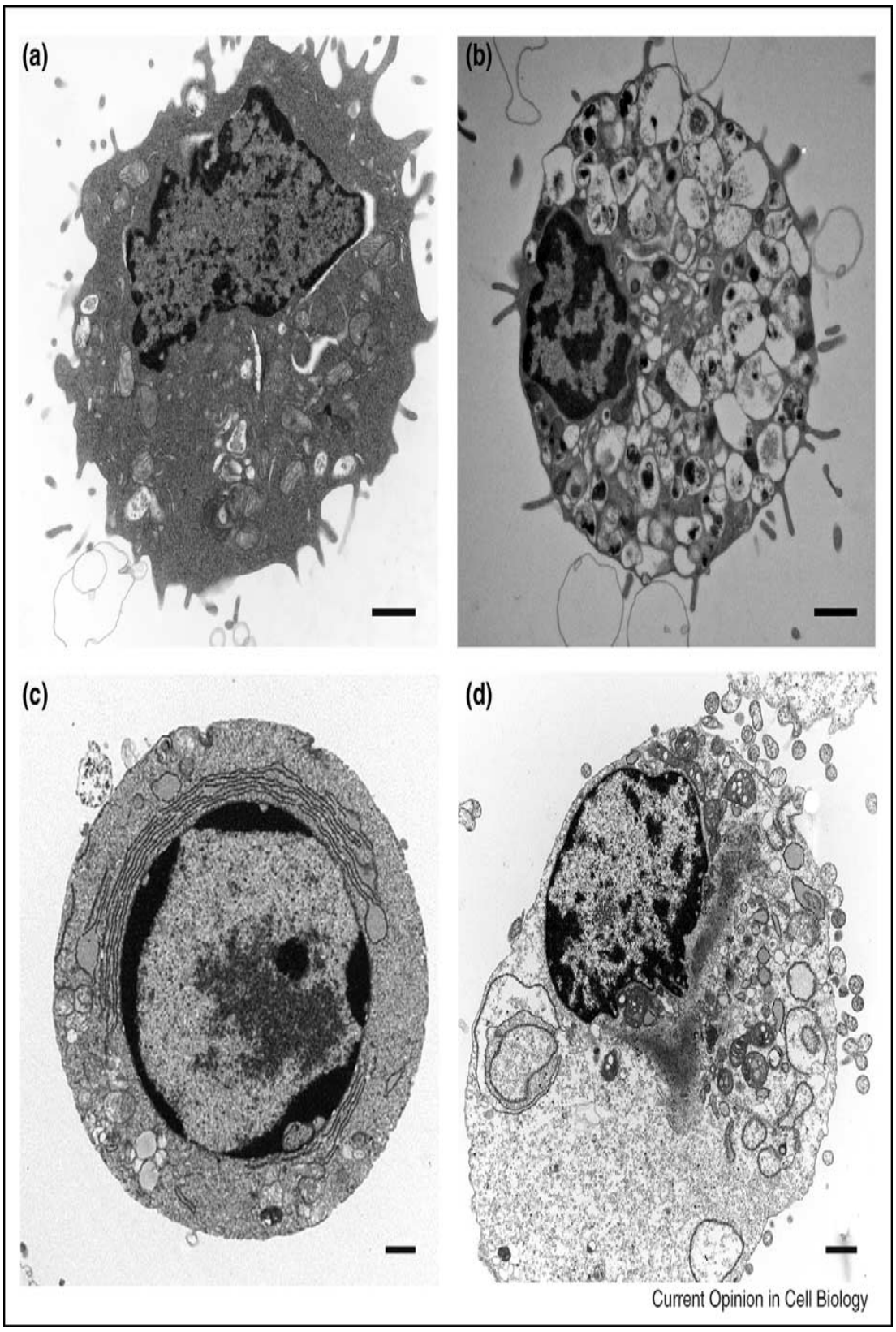

Figure 1.1: Morphological features of normal, autophagic, apoptotic and necrotic cells.
(a) is normal, (b) is autophagic, (c) is apoptotic and (d) is necrotic cell (Edinger and Thompson, 2004).

In 2007, more than 100,000 new cancer cases, which is more than double the number of new cases in 1982, were diagnosed in Australia (AIHW, 2010). The average age at diagnosis was 67 and 64 years for male and female, respectively (AIHW, 2010). Even though cancer is still the second most common cause of death, and many types of cancer deaths involving stomach, uterine, and cervical have reduced, but lung cancer has not (AIHW, 2010; Gold *et al.*, 2001b). Although there is public concern that pesticide residues in food could be cancer hazards, there is evidence that there is a positive effect against many types of cancer as a consequence of high consumption of fruits and vegetables including those containing pesticide residues (Block *et al.*, 1992; Hill *et al.*, 1994). Indeed, fruit and vegetable intake results in twice the cancer rate of several types of cancer such as lung, oral cavity, colon, and bladder, compared to high consumption of these foods (Block *et al.*, 1992; Gold *et al.*, 2001b). However, there is correlative evidence that increasing frequency of cancer is associated with chronic exposures to products involving pesticides (Marc *et al.*, 2004a). In 2007, approximately 5% of cancers diagnosed were related to occupational exposure (AIHW, 2010). The number of cancer cases related to occupations is 5,000 cases a year in Australia in 2007 (AIHW, 2010). Despite the overall decrease in cancer death rates, one of the occupations associated with a high risk of cancer is that of a farmer (Wiklund and Dich, 1995; Gold *et al.*, 2001b; Koller *et al.*, 2012). Some researchers claim that one of the greatest factors that increase the risk of cancer to farmers is exposure to pesticides and other agricultural chemicals (Band *et al.*, 2011; Morrison *et al.*, 1992). Blair and Zahm (1993) suggested that farmers are unlikely to be exposed to the majority of the 180 chemicals that are registered in Australia for agriculture and veterinarian uses. However, they will very likely be exposed to some of them (Blair and Zahm, 1993).

Among the most common type of cancer diagnosed in the United States (US) is skin cancer, which estimated to be 40% of all diagnosed cancer cases (Han

et al., 2011). There are over than one million new cases of skin cancer that are diagnosed every year in the U.S., and the number is increasing each year (Han *et al.*, 2011). In Australia, every year 80% of newly diagnosed cancers are skin cancer, making it one of the five most common cancer in 2007 (www.cancer.org.au, 2012a; AIHW, 2010). Two in three Australians will get skin cancer by the time they reach the age of 70 (www.cancer.org.au, 2012a). The skin cancer type melanoma, the third most common cancer in both genders in Australia, has increased by 50% in the past decade (www.cancer.org.au, 2012a). Over 11,500 people were diagnosed with melanoma in 2009, and as a consequence, more than 1,500 people died due to this type of cancer (www.cancer.org.au, 2012a). The most common type of skin cancer is non-melanoma skin cancer, and 543 died of non-melanoma skin cancer in 2011 (AIHW, 2010; www.cancer.org.au, 2012a).

1.1.3 Ecotoxicity.

The third hazard posed is ecotoxicity. Once contaminants access wherever in the land or marine areas, they effect directly or indirectly on the ecosystem (Bi *et al.*, 2011). Pesticides are used to control one or more target organisms (see section 1.2.1), but they easily escape from the location where they are applied reaching and adversely affecting organisms that present in the new place (Deneer, 2000; Tsui and Chu, 2003). Mainly, they contaminate water courses that located near to agricultural areas (Oropesa *et al.*, 2008). Not only marine ecosystem such as fish, planktons, and mammals are affected, but even birds are affected too (Bi *et al.*, 2011). Different fish species responses differently to simazine, an herbicide (see section 1.2.2.2.1) (Oropesa *et al.*, 2008). The lethal concentration 50% (LC_{50}) for sheepshead minnow (*Cyprinodon variegates*) and Mozambique tilapia (*Tilapia mossambica*) at 96h exposition to simazine were reported as 4.3 mg/L and less than 1000 mg/L, respectively (Oropesa *et al.*, 2008). Because of the various and broad use of simazine as an herbicide including in agriculture, orchards, and parks, there is the possibility to expose and affect many non-targets organisms whether they are

in terrestrial or aquatic areas (Strandberg and Scott-Fordsmand, 2002).

1.2 Agricultural products.

1.2.1 Pesticides.

A pesticide is a substance or mixture of substances formulated naturally or synthetically to resist or control pests including invertebrates (insects), plants (weeds), and microorganisms (bacteria and fungi) (U.S.EPA, 2012; Tadeo, 2008). These pests could spread diseases, compete with people for food, crops, and/or destroy their property (Tadeo, 2008; U.S.EPA, 2012). Pesticides are classified by their type of use (Tadeo, 2008). Herbicide, which is a main group of pesticides, is employed to avoid the growing of undesirable weeds and other plants (see section 1.2.2) (E.P.A, 2005; Tadeo, 2008). Insecticides and fungicides are the second and third main groups of pesticides, and they are used to kill insects and fungi, respectively (Tadeo, 2008). Pesticides are not classified as contaminants; instead, they belong to the residues group (Tadeo, 2008). Residues are substances that intermix with food stuff after intended usage of certain products during food (plant or animal) production (Tadeo, 2008). Pesticide residue monitoring is essential to ensure that consumers are not exposed to more than the maximum residue levels (MRLs) (Tadeo, 2008). Another important term is the admissible daily intake (ADI) of pesticides (Tadeo, 2008). ADI is the acceptable amount of pesticides to be daily ingested without displaying any adverse effect during the whole life (Tadeo, 2008).

Pest control has occurred since the beginning of agriculture (Tadeo, 2008). People were using lead, sulphur, mercury, and other inorganic compounds as pesticides (Tadeo, 2008). Usage of pesticides in agriculture, industry and for domestic application has markedly increased since 1990 (Maroni *et al.*, 2000; Tadeo, 2008). One problem is that homeowners use a greater volume of pesticides in their gardens than the recommended rate, so they are using more pesticides per hectare than farmers (E.P.A, 2005). Five times the recommended rate of pesticides (herbicides) has been applied by Adelaide Hills residents, and this misuse increases

the chance of the exposure to these chemicals (E.P.A, 2005). Pesticide residues, also, exceed the international tolerance levels in some livestock such as raw bovine milk and farm animal tissues (Lioi *et al.*, 1998). According to the annual report 2001-2002 of the European Crop Protection Association (ECPA), North America, Europe, and Asia have the highest percentage of agricultural areas treated with pesticides with 31.9%, 23.8%, and 22.6%, respectively (Tadeo, 2008). At present, over 1100 substances are recorded as pesticides (Tadeo, 2008). More than two million tons of industrial pesticides are used every year even though there are strict regulations for the registration and the utilization of the pesticides universally as a result of their possible toxicity (E.P.A, 2005; Tadeo, 2008). That toxicity occurs from misuse of pesticides, and effects not just human health, but also the environment (E.P.A, 2005). In general, many pesticides are hazardous chemicals, and can cause several reactions, that range from irritations to severe illness, chronic problems appear after a period, or even death (E.P.A, 2005). In addition, there are numerous natural or organic chemical derived pesticides, for example, from pyrethrum, garlic, and essential oils (Meyer *et al.*, 2008; E.P.A, 2005). Clove oil, essential oil, is one type of bio-based pesticides for controlling weeds, nematodes, arthropods, and microbial pathogens (Meyer *et al.*, 2008). Plants produce a diverse array of bio-pesticides to defend themselves against insects, fungi, and other animal predators (Ames *et al.*, 1990a; Gold *et al.*, 2001b). Humans ingest 99.9% of the range of naturally occurring chemicals, and the amount of the natural pesticides in plant foods is greater than that of synthetic pesticides (Ames *et al.*, 1990a; Ames *et al.*, 1990b). Gold *et al.* (2001a) estimated that in the U.S. 1500 mg of natural pesticides is the average daily exposure to such pesticides in the diet and approximately 0.09 mg of synthetic pesticides residues is the daily exposure. Between 5000 and 10,000 different natural pesticides are ingested by humans (Ames *et al.*, 1990a). A small group of natural pesticides present in common foods has undergone carcinogenicity testing, and 37 of 71 of them were rodent carcinogens, and hence possibly human carcinogens (Gold *et al.*, 2001b).

Therefore, they are bound by the same regulation as synthetic pesticides because many of them can be toxic to human and harmful to the environment (E.P.A, 2005). Exposing chick embryo cells to some synthetic pesticides determines an increase in the number of sister chromatid exchanges (SCEs), a genetic damage end-point (Lioi *et al.*, 1998).

The commercial pesticide formulations consist of active ingredient, adjuvants and inert ingredients (Brausch and Smith, 2007). Adjuvants usually aid or modify the action of the active ingredients (Brausch and Smith, 2007). Adjuvants include surfactants which are commonly added in pesticides (Brausch and Smith, 2007). On the other hand, inert ingredients are the chemicals that do not change the action of a pesticide or impair the target pest such as dyes (Brausch and Smith, 2007). There are two essential ways to which people are exposed to pesticides (Tadeo, 2008). The first way is primary (direct) exposure, which occurs during the usage of pesticides (Tadeo, 2008). The second way is secondary (indirect) exposure, which can take place through food ingestion or via the environment (Tadeo, 2008). Water sources are an example of a secondary exposure (Tadeo, 2008). Under s11 of the 2003 Water Quality Policy, a person undertaking an activity should avoid discharge of pesticides' waste into waters or onto lands in a place from which it is likely to enter any aqua system (E.P.A, 2005). Ambient air could be another vehicle through which people are exposed to pesticides because pesticides can travel in the air through their volatilization after applying, and/or through spray drift (Lee *et al.*, 2002). As a consequence of the airborne agricultural pesticides in Californian communities, some associated acute health effects such as irritation of eyes and respiratory and headaches, have been reported (Lee *et al.*, 2002).

1.2.2 Herbicides.

Herbicides are phytotoxic compounds that stop plant growth via blocking the critical metabolic pathways in plants (Mori *et al.*, 1995). They had a major role in the development of farmers' ability to control weeds and cultivate crops (Tadeo, 2008).

Herbicides are classified in three ways. Foliage-applied compounds, absorbed by Leafs, versus soil-applied compounds, absorbed by roots (Tadeo, 2008). They can be categorized as selective herbicides, which control weeds without damage to the crop, versus total herbicides, which kill all vegetation (Tadeo, 2008). Alternately, their classification can be based on their chemical composition, including triazine, amides, benzoic acids, nitriles, organo-phosphorus (OPs), and others (Tadeo, 2008).

1.2.2.1 Organo-phosphorus (OPs) herbicide.

OPs are composed of hydrocarbon complexes which include one or more phosphorus atoms in their molecule (Tadeo, 2008). They have been used on a large scale because of their varied range of lipophilicity, which determines the distribution of a pesticide among fatty tissues of animals (Tadeo, 2008). Also, they only persist in the environment for a short time (Tadeo, 2008). However, OPs present a high acute toxicity to humans (Tadeo, 2008). The toxic action of the OPs pesticides is usually via enzyme inhibition (Tadeo, 2008). They inhibit the normal function of the acetylcholinesterase, a key constituent of the nervous system (Tadeo, 2008). Acetylcholinesterase hydrolyzes acetylcholine, an ester released with the transmissions of the nerve impulse (Tadeo, 2008). Therefore, OPs have the ability to phosphorylate acetyl-cholinesterase, and inhibit its function via rapid removal of acetylcholine (Tadeo, 2008). As a consequence, the nervous system is disrupted (Tadeo, 2008). The most common OP herbicide used over the world is glyphosate (Song *et al.*, 2012a).

1.2.2.1.1 Glyphosate.

N-(phosphonomethyl) glyphosatecine ($C_3H_8NO_5$; Figure 1.2) is known as glyphosate (Tadeo, 2008). It is one of the most extensively used herbicides in the world, and over 907,000 tons were used in 2007 (Koller *et al.*, 2012; Peruzzo *et al.*, 2008). It is a non-selective herbicide and foliage-applied compound (Bradberry *et al.*, 2004; Tadeo, 2008). It is an acid but commonly used in glyphosate-containing herbicides in its salt form, isopropylamine salt (Peruzzo *et al.*, 2008). It is a polar, and binds strongly to the soil particles causing its half-life to be in the soil as much as 3 to 174 days (Tadeo, 2008; Peruzzo *et al.*, 2008). Glyphosate is extensively employed for controlling vegetation in non-crop areas, and controlling weeds in aquatic systems (Tadeo, 2008). It prevents the shikimic acid pathway through which the aromatic ring amino acids, tryptophan, tyrosine, and phenylalanine are synthesized in plants (Tadeo, 2008). Thus, it inhibits the enzyme 5-enolpyruvoyl-shikimate-3-phosphate synthase (Martini *et al.*, 2012; Tadeo, 2008). As a result of the absence of this enzyme in human and animals, Glyphosate is predicted to be

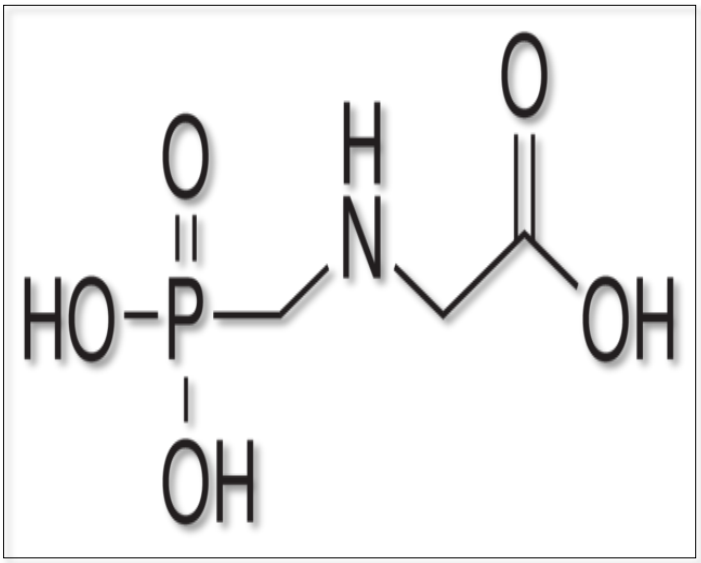

Figure 1.2: The structural formula of glyphosate.
(http://www.sigmaaldrich.com).

non-toxic to them (Martini *et al.*, 2012). Glyphosate was considered previously to be safe within the normal usage or for chronic exposure, and the World Health

Organisation (WHO) classified it as unlikely to present acute hazard in normal use (Gehin *et al.*, 2006; Song *et al.*, 2012a; Williams *et al.*, 2000). In regards of the aquatic organisms, it is observed to be slightly or non-toxic (Wang *et al.*, 2005). In rats, the LD_{50} is 4.32 g/Kg (Lioi *et al.*, 1998). It also causes renal failure, metabolic acidosis, arrhythmia, mental deterioration, death, or other severe effects in people who ingest large volumes of glyphosate (Song *et al.*, 2012a; Bradberry *et al.*, 2004).

1.2.2.1.2 Polyethoxylated tallow amine.

Polyethoxylated tallow amine (POEA) is a predominant non-ionic surfactant used wildly with the advent of glyphosatephosate-based formulations, specifically R-up (Williams *et al.*, 2000; Giesy *et al.*, 2000; Brausch and Smith, 2007). These formulations consist of up to 15% of POEA (Giesy *et al.*, 2000). POEA is belong to alkylamine ethoxylates (ANEOs), which is one of the seven major types of non-ionic surfactants (Brausch and Smith, 2007). Tallow is synthesized from animal-derived fatty acids, and POEA is a mixture of polyethoxylated long-chain alkylamines (Giesy *et al.*, 2000; Brausch and Smith, 2007). It is degradable, and the degradation half-life for POEA in soil is estimated to be within a week (Wang *et al.*, 2005). These surfactants are from a large group of surface-active substances that are employed for a large number of applications, one of which is use in herbicides (Baeurle and Kroener, 2004). The main purpose of POEA is to facilitate and promote the herbicide absorption into plant cuticles and increase the effectiveness of its active ingredients (Martini *et al.*, 2012; Brausch and Smith, 2007). POEA is considered to be moderately toxic to aquatic organisms, and the risk level of these organisms in the shallow water contaminated with POEA is even elevated (Wang *et al.*, 2005). Brausch and Smith (2007) treated an aquatic micro-invertebrate with different doses of POEA in their study, and they concluded that POEA is very toxic and impairs the aquatic organisms in the area where it is applied. Numerous studies evaluated the toxicity of the active ingredients of herbicides, but

narrow information is available on the potential toxicity of POEA alone (Wang *et al.*, 2005; Brausch and Smith, 2007).

1.2.2.2 Triazine herbicide.

Triazine (2-chloro-*s*-triazine) family of herbicides are used widely to control annual grass and broadleaved weeds because they are effective at low concentrations (Tennant *et al.*, 2001; Tadeo, 2008; Sanderson *et al.*, 2001). They were used progressively since the 1960s and are ranked one of the largest herbicide groups that are sold in the U.S. (Kligerman *et al.*, 2000a; Sanderson *et al.*, 2001). Active ingredients as much as 41 to 55 million Kg were used annually (Kligerman *et al.*, 2000a). They are absorbed by roots or by foliage, depending on the time of application, pre- or post-emergence, respectively (Tadeo, 2008; Tennant *et al.*, 2001). Most triazine herbicides starve the treated sensitive plants through electron transport inhibition which leads to a block of photosynthesis (Kaya *et al.*, 2000; Loosli, 1994). The main triazines are amitrole, atrazine, cyanazine, and simazine, and they are usually used as part of wheat, sugar cane, and maize cultivations (Worthing and Walker, 1983; Tadeo, 2008; Kaya *et al.*, 2000). These chemicals are detected in high concentrations in both surface and groundwater in certain parts of USA because of their widespread application (Kligerman *et al.*, 2000a; Tennant *et al.*, 2001; Sanderson *et al.*, 2001). Long-term exposure to this family increases the risk of ovarian and breast cancer (Sanderson *et al.*, 2001). It is estimated that 2-3 million people are exposed to less than 1 µg/L of triazine herbicides as consequences of drinking groundwater as their primary source (Kligerman *et al.*, 2000a; Tennant *et al.*, 2001).

1.2.2.2.1 Simazine.

2-chloro-4,6-bis[ethylamino]-s-triazine or simazine ($C_7H_{12}ClN_5$; Figure 1.3) is one halogenated aromatic herbicide that the currently in widespread use (Haggblom and Milligan, 2000). Simazine is a selective herbicide and belongs to triazine herbicide class (Strandberg and Scott-Fordsmand, 2002; U.S.EPA, 2007; Kearney *et al.*, 1967). It is determined that simazine concentration reached 0.23 mg/L surface water at 30 monitoring stations in Illinois (Kligerman *et al.*, 2000a). Simazine inhibits photosynthesis in the chloroplasts by stopping electron transfer at the photosynthesis complex II (Strandberg and Scott-Fordsmand, 2002). It is phytotoxic to many herb species, which may not be the target for the application, at a lower rate than the recommended application doses, and such effects could remain for one year after usage (Strandberg and Scott-Fordsmand, 2002). Concentrations of 4 µg and 0.68 µg simazine/L were recorded in fresh water and aerial fallout-rain, respectively (Strandberg and Scott-Fordsmand, 2002). The maximum expected simazine concentration in soil is 5 mg/Kg, depending on the application rate in the soil (Strandberg and Scott-Fordsmand, 2002). Waters used for the public supply of some towns in southwest Spain were contaminated with as

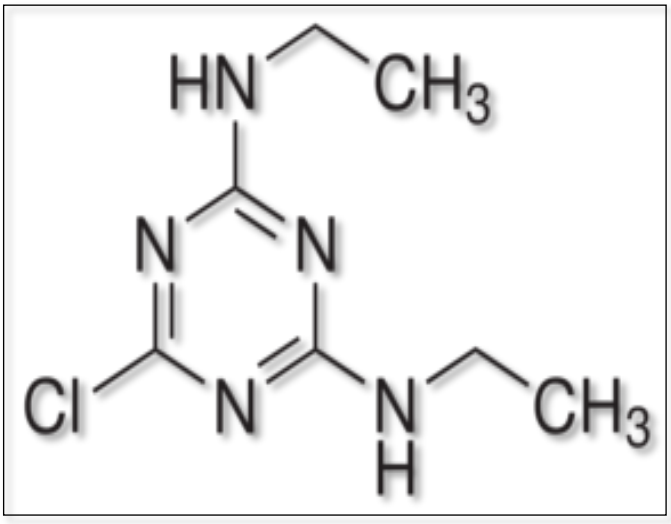

Figure 1.3: The structural formula of simazine.
(http://www.sigmaaldrich.com).

high as 4.5 µg/L of simazine due to its extensive use (Oropesa *et al.*, 2008). That concentration was much higher than the maximum advisory legal limit for drinking water which is 0.1 µg/L (Oropesa *et al.*, 2008). The problem lies in that simazine

remains in aquatic environments for several months after application because simazine is not degraded effectively in the environment. (Oropesa *et al.*, 2008). The major non-biological mechanism appearing to detoxify of simazine in plants and soil is the conversation of simazine to hydroxysimazine with the involvement of benzoxazine in the reaction (Kearney *et al.*, 1967).

1.2.2.2.2 Amitrole.

Amitrole or aminotriazole ($C_2H_4N_4$; 3-amino-1,2,4-triazole; Figure 1.4) is an off-white and non-volatile crystalline powder (Sellamuthu, 2014). It is not a natural chemical and first synthesized in 1898 (Sellamuthu, 2014). It is soluble and stable in water (Sellamuthu, 2014). It is, also, soluble in the polar solvent (Sellamuthu, 2014). It was introduced as a fast-herbicidal active ingredient in many formulations since 1954 (Balkisson *et al.*, 1992; Mattioli *et al.*, 1994; Legras *et al.*, 1996). Because of the misuse of amitrole causing food crisis in 1959, its applications are restricted to non-food crops since then (Ichihara *et al.*, 2005; Sellamuthu, 2014). Herbicides containing amitrole have a wide spectrum of activities against deep-rooting weeds, annual grasses and poison ivy on outdoor

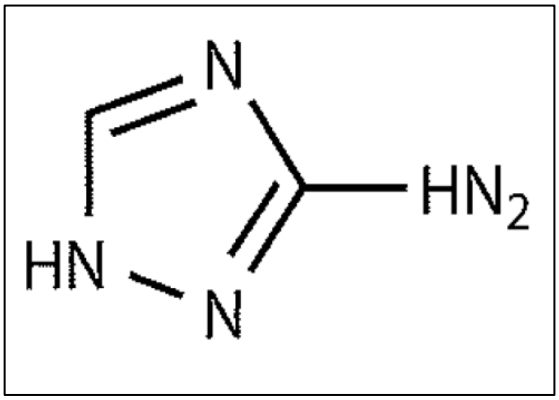

Figure 1.4: The structural formula of amitrole.
(Sellamuthu, 2014).

non-agricultural areas such as houses, cottages, along roads and railway tracks (Balkisson *et al.*, 1992; Furukawa *et al.*, 2010; Sellamuthu, 2014). However, applying amitrole above the recommended level or run off are more likely causing contaminations of soils, surface and groundwater because of its high-water solubility of 280 g/L (Sellamuthu, 2014; Oesterreich *et al.*, 1999). The contamination of these

sites could lead to toxicity of birds, and aquatic species (Sellamuthu, 2014). The half-life of amitrole in soil is 26 days, and 57 days, respectively, under anaerobic condition (Sellamuthu, 2014). Moreover, amitrole is a non-selective triazole herbicide that is taken up through plant leaves and is usually used in formulations together with other chemicals agents some of which are toxic (Furukawa *et al.*, 2010; Legras *et al.*, 1996; Sellamuthu, 2014). Its association with other toxic compounds make it a challenging to study its acute toxicity in human (Legras *et al.*, 1996). Its herbicidal action involves the inhibition of chlorophyll formation and buds regrowth (Balkisson *et al.*, 1992; Legras *et al.*, 1996).

There was a concept that the risk of adverse pulmonary side-effects is rare and tiny because of the low doses applied, and low acute oral toxicity of amitrole tested in the rat (Balkisson *et al.*, 1992; Legras *et al.*, 1996). Because of its restricted usage in non-food crops, the potential human exposure to amitrole is minimal, but the exposure to amitrole occurs to workers during their handling processes in manufacturing units or spraying amitrole-containing herbicides in fields when they do not have a proper protection in the workplace (Sellamuthu, 2014). Thirty percent of amitrole is absorbed through the skin, and the toxicity of the direct contact with amitrole-containing herbicides is thought to be limited to the mucosal membrane of the skin and eyes (Balkisson *et al.*, 1992; Sellamuthu, 2014). The body eliminates amitrole without any metabolic transformation mostly in urine within 24h (Legras *et al.*, 1996; Sellamuthu, 2014). After ingestion by a human, amitrole causes a wide spectrum of responses starting with poisoning to death depending on the dose and the chemicals present in the mixture (Sellamuthu, 2014).

The carcinogenicity of amitrole was supported by studies on experimental animals (Balkisson *et al.*, 1992; Furukawa *et al.*, 2010). Amitrole was classified by the International Agency for Research on Cancer (IARC) as a potential human carcinogen (IARC, 1987). The main target organ for amitrole is the thyroid gland (Sellamuthu, 2014). An *in vivo* study reported that giving rats high doses of amitrole

results in decline in the thyroid hormones T3 and T4 because of the inhibition of thyroid peroxidase enzyme (TPO), and the reduction of these hormones increases the thyroid stimulation hormones (TSH), which are important in thyrocyte proliferation and carcinogenesis (Dellarco and Wiltse, 1998; Furukawa *et al.*, 2010; Sellamuthu, 2014). Therefore, effects of amitrole were not because of its cytotoxicity (Pan *et al.*, 2011). Besides the thyroid tumours, amitrole causes tumours of pituitary, goitre and liver in human after chronic exposure (Sellamuthu, 2014). An *in vivo* study found that a combination of amitrole with sodium nitrite ($NaNO_2$) lacked promotion effects for hepatocarcinogenesis in rats, and that suggested the significant decrease of body weights were from decreases in food and water consumption (Ichihara *et al.*, 2005).

1.2.2.2.3 Ammonium thiocyanate.

Ammonium thiocyanate (NH_4SCN) is a white organic compound (hygroscopic salt) (Bhunia *et al.*, 2000; Van Hoek, 2000). It is used as raw materials or additional supporters in building, chemical industry, fibre production, and agricultural products (Van Hoek, 2000). Large quantities (around 3,000 tons) of ammonium thiocyanate is resumed in agricultural products (Van Hoek, 2000). It is used as a raw material to produce non-selective herbicides controlling unwanted plant, or as an auxiliary (surfactant) which reinforces the activity of amitrole (Balkisson *et al.*, 1992; Van Hoek, 2000; Bhunia *et al.*, 2000). Human exposure to thiocyanate occurs through drinking water and diet whereas it is found naturally in Brussel genus vegetables such as broccoli, Brussel sprouts, kale and others (Horton *et al.*, 2015). Another source of thiocyanate is cigarettes (Horton *et al.*, 2015). Thiocyanate is absorbed swiftly after ingestion, but it does not remain in the body because it is cleared rapidly (4 mL/min) throughout the kidney (Balkisson *et al.*, 1992; Legras *et al.*, 1996). The average half-life elimination of thiocyanate is three days in the normal body having no renal frillier (Legras *et al.*, 1996). This half-life elimination could be dropped to 2.7 – 2.3 days with sodium chloride appearance as it speeds up the excreting of thiocyanate (Legras *et al.*, 1996). It is readily resorbed,

and direct, prolonged skin contact with it can cause skin irritation and rashes (Van Hoek, 2000). However, a high concentration of it could become problematic (Van Hoek, 2000).

1.3 Food industry.

1.3.1 Caffeine.

The chemical and physical properties of caffeine, 1,3,7-trimethylxanthine ($C_8H_{10}N_4O_2$; Figure 1.5), are well studied (Oestreich-Janzen, 2016). It is a nontoxic crystalline alkaloid with white colour, odourless, and bitter taste that perceived throughout the oral cavity (Somogyi, 2010; Green *et al.*, 2010; Oestreich-Janzen, 2016; Traganos *et al.*, 1993). Caffeine, also, has a good character which makes it penetrates biological membranes and parries easily, and that is caffeine is both hydrophilic and lipophilic (Oestreich-Janzen, 2016). Lots of people associate caffeine with coffee, but, indeed, caffeine, comes from different plants belong to different families such as tea leaves, kola nuts, cocoa beans and more than 60 other caffeine plant species at varying concentrations (Somogyi, 2010; Oestreich-Janzen, 2016). Plants containing caffeine are high economic value worldwide, as they spread all over the world (Oestreich-Janzen, 2016). Caffeine serves as an insecticide in those plants where it paralyses or kills insects that feed on the plants

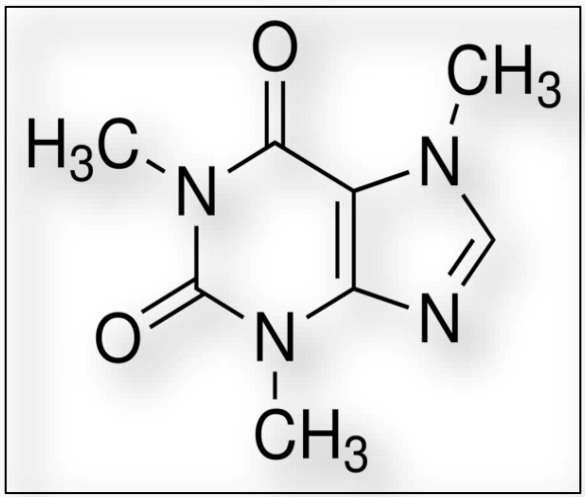

Figure 1.5: Chemical structure of caffeine. (www.segmaaldrich.com).

(Somogyi, 2010). The main plants containing caffeine are coffee (*Coffea canephora* and *Coffea arabica*), cola (*Cola nitride*), cocoa (*Theobroma cacao*), and tea (*Camellia sinensis*) (Bode and Dong, 2007; Chin *et al.*, 2011). It is, also, found in some synthesised medication and pain remedies (Bode and Dong, 2007; Oestreich-Janzen, 2016). Caffeine is the most consumed psychoactive substance all over the world because it is commonly and frequently consumed in many dietary sources, including soft drinks, energy drinks, coffee, tea, and chocolate bars (Bode and Dong, 2007; Han *et al.*, 2011; Somogyi, 2010).

1.3.1.1 Consumption of products.

Caffeine is thought to have been discovered during the third century AD by an Ethiopian shepherd whose goats became agitated after eating coffee berries or beans (IOM, 2001). Early in the sixth century AD, coffee plant cultivation had begun, and coffee as a beverage reached Yemen, where it became a popular social beverage among Muslims during 1000 AD (Green *et al.*, 2010). From there, it reached Europe and the Americas (Green *et al.*, 2010). Nowadays, the conception of the caffeine-containing beverages occupies a significant place in the national cultures for most nations of the world (Fredholm *et al.*, 1999). The global caffeine production is estimated at 10,000-15,000 tons including natural caffeine (over 3,000 tons) was estimated in 1999 (Oestreich-Janzen, 2016). China was the main supplier of synthetic caffeine in 2011 (Oestreich-Janzen, 2016). The Food and Drug Administration (FDA) gave caffeine an interim food additive status for further studies (Deshpande, 2002). Using scientific articles published from 1986 to 1991 that studied the potential health effects of caffeine, the FDA concluded that the consumption of caffeine in cola beverages at 100 mg/person/day showed no hazard on human health (IOM, 2001). The European Food Safety Authority (EFSA) conclude in their publication the scientific opening on the safety of caffeine (2015) that adult daily consumption of up to 400 mg caffeine as a habit intake from all sources do not give rise to safety concerns. Such consumption should be halved for

a pregnant woman (E.F.S.A, 2015).

Coffee comprises an array of compounds produced by the roasting procedure, and some of these compounds may have an anti-tumorigenic or anti-oxidative activity such as cafestol and kahweol, which have been stated to prevent the effect of many carcinogens (Gold *et al.*, 2001b; Gunter *et al.*, 2012). Consequently, it is biologically reasonable that coffee and/or caffeine may be associated with reducing cancer risk (Dubrow *et al.*, 2012). In coffee beans, the content of caffeine relies on the method of preparation and the type of the coffee beans (Somogyi, 2010). Indeed, the roasting process reduces the caffeine content of the beans, so the darker the bean, the less caffeine content (Somogyi, 2010). In general, a single cup of espresso (30 ml) contains 64 mg caffeine, and an ounce cup of automatic drip coffee (237 ml) contains 145 mg (Somogyi, 2010). On average, 256 mg caffeine/day may be consumed (approximately 5-7 mg caffeine/kg body weight/day) since an average of 3.2 cups of coffee *per* day are ingested by coffee drinkers (Mandel, 2002; Green *et al.*, 2010). A cup of tea beverage contains 20-80 mg caffeine (Somogyi, 2010). Seventy percent of soft drinks contain caffeine as an additive ingredient (Green *et al.*, 2010). Soft drinks contain 30-40mg caffeine/ 355ml serving, and energy drinks with added caffeine contain as much as 200mg/ 355ml serving (Somogyi, 2010). A universal survey including 42 countries shows that the estimated consumption of caffeine from all sources was about 70-76 mg/person/day, which equivalent to approximately 1.1 mg/kg (Fredholm *et al.*, 1999).

Caffeine metabolism is well-understood, and the primary organ in which it is metabolised is the liver where the formation of dimethyl- and monomethyl-xanthines, dimethyl and monomethyl uric acid, trimethyl- and dimethyl-allantoin, and uracil derivatives occurs (Fredholm *et al.*, 1999; Green *et al.*, 2010). Caffeine degrades to several metabolites through different biotransformation pathways, but the main pathway for caffeine metabolism is the paraxanthine (1,7-DMX) pathway, which

represents up to 80% of the metabolism of caffeine in healthy humans (Miners and Birkett, 1996; Green *et al.*, 2010). Enzymes that are involved in caffeine degradation are well-characterized except some intermediary steps and enzymes participated in the metabolism of the secondary and tertiary metabolites (McQuilkin *et al.*, 1995). Even though a large number of enzymes and intermediate products are involved in the caffeine metabolism, greater than 95% of the primary step in the metabolic pathway occurs via the cytochrome P450 1A2 (CYP1A2) enzyme (Carrillo and Benitez, 2000; Green *et al.*, 2010). This enzyme drives the dimethyl and monomethyl metabolites (Figure 1.6) (Carrillo and Benitez, 2000; Green *et al.*, 2010). Moreover, 99% of caffeine is absorbed within less than 50 minutes of ingestion (Green *et al.*, 2010). Caffeine is rapidly distributed throughout the body after ingestion and reaches the brain via the blood-brain barrier where it works as a stimulant of the central nervous system (CNS) (Bode and Dong, 2007; Green *et al.*, 2010). The half-life of caffeine for doses less than 10 mg/kg ranges from 0.7 to 1.2h in rats, and from 2.5 to 4.5h in human (Fredholm *et al.*, 1999). Caffeine at doses ranging from 20-200 mg related positively to the mood (Fredholm *et al.*, 1999). Human subjects have been used to study the effect of caffeine on mood, and they reported that they feel energetic, able to concentrate, efficient, self-confident, and motivated to work (Fredholm *et al.*, 1999). It is, also, known that caffeine has well-documented effects on anxiety despite there being no clear relationship between them (Fredholm *et al.*, 1999). Caffeine has effects on sleep and increases wakefulness (Fredholm *et al.*, 1999). This is one of the main reasons why people consume caffeine-containing beverages (Fredholm *et al.*, 1999). In contrast, some people would like to cease their habitual caffeine intake because of the unsatisfactory sleep (Fredholm *et al.*, 1999). Also, caffeine has an efficient ability to scavenge highly reactive radicals to form various radical adducts (Chin *et al.*, 2011; Devasagayam and Kesavan, 1996). Caffeine inhibits two critical oncogene pathways, the serine/threonine kinase (Akt) pathway and the cyclooxygenase-2 (COX-2) pathway (Han *et al.*, 2011; Gunter *et al.*, 2012). Akt is frequently activated

in human cancers, and it promotes cell survival (Bode and Dong, 2003; Datta *et al.*, 1999). Exceeding of COX-2 promotes skin tumorigenesis, so COX-2 inhibition reduces chemical-induced skin tumour progression (Fischer *et al.*, 2007). Overall, the effect of caffeine on cell proliferation and whether it is a carcinogen is ambiguous (Bode and Dong, 2007; Gold *et al.*, 2001b).

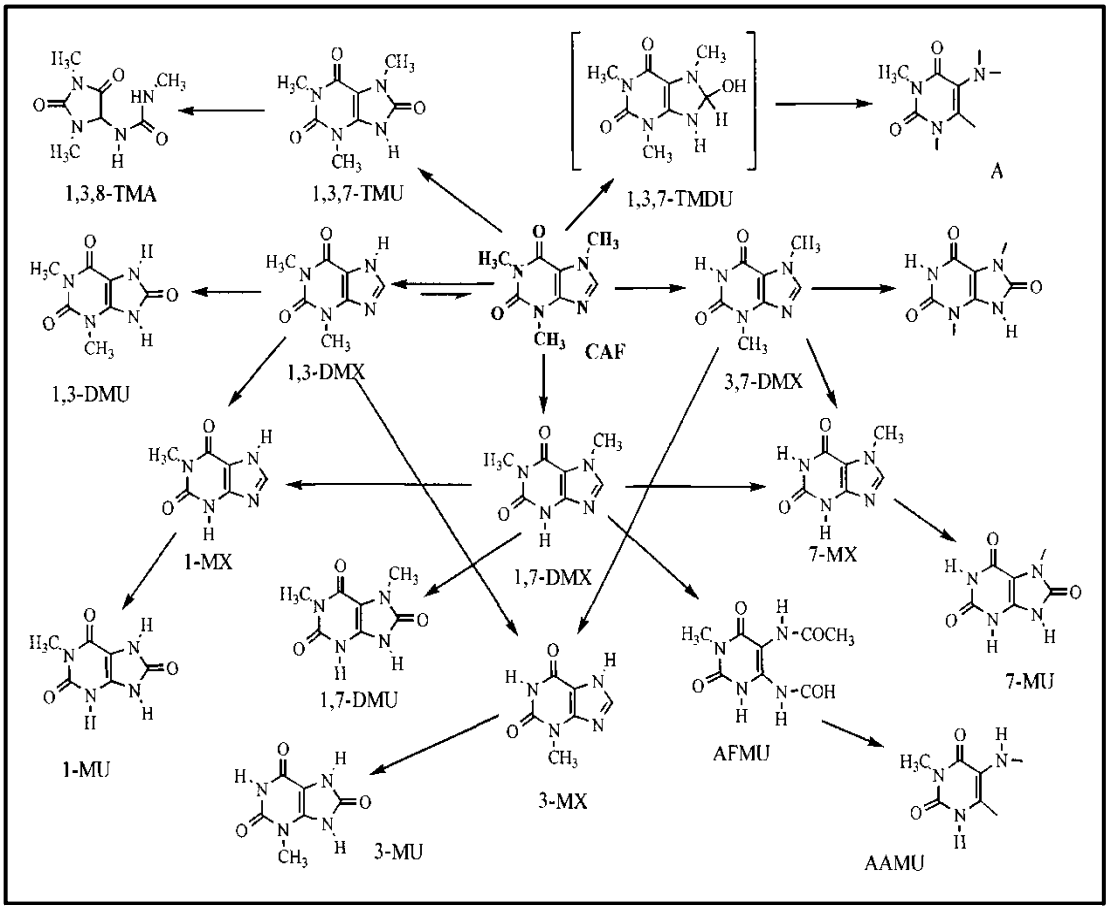

Figure 1.6: Major metabolic pathways of caffeine in mammals.
(Green *et al.*, 2010).

1.4 Human tissue culture.

1.4.1.1 HaCaT.

HaCaT cell line has been derived from a long-term primary culture of human adult skin keratinocytes (Boukamp *et al.*, 1988). This cell line has a transformed phenotype *in vitro* whereas it can make colonies on plastic and in agar (Boukamp *et al.*, 1988). It is considered to be immortal (more than 140 passages) with specific stable marker chromosomes, but it remains non-tumorigenic (Boukamp *et al.*, 1988). HaCaT cells offer a suitable and stable model for keratinization studies because cells retain a remarkable capacity for normal differentiation even after multiple passages (Boukamp *et al.*, 1988).

1.4.1.2 WIL2-NS.

The human non-secreting B lymphocyte, WIL2-NS, is derived from a WI-L2 cell line (ATCC, 2014). WIL-2 was the first cell line that originally isolated from the spleen of a 5-years-old Caucasian boy with hereditary spherocytic anaemia (Levy *et al.*, 1968). The patient, at the time of the culture, was initiated, was free from evidence malignant diseases (Levy *et al.*, 1971; Levy *et al.*, 1968). Cells are duplicated within 30 to 70 hours disbanding on the conditions of the culture (Levy *et al.*, 1968). For example, serum concentration greater than 10% decreases the cell growth (Levy *et al.*, 1968).

1.5 Techniques:

To evaluate the cytotoxicity of chemicals, various assays have been used (Dojindo, 2016). Every assay based on a specific cell function such as cell adherence, enzyme activity, production of co-enzyme or cell membrane penetrability (Dojindo, 2016). Many methods have been established to count the number of live cells after submission to variations of experimental conditions, and some of which are MTT method, crystal violet method, and trypan blue method (Dojindo, 2016; Avelar-Freitas *et al.*, 2014).

1.5.1 MTT assay:

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is one of the enzyme-based assays that is extensively used to assess cytotoxicity (Chiba *et al.*, 1998b; Dojindo, 2016). It quantifies *in vitro* the viable cells whose mitochondria produce the succinate dehydrogenase enzyme converting the yellow soluble MTT to the purple insoluble formazan needle-shaped crystals in the cells (Henriksson *et al.*, 2006; Young *et al.*, 2005; Dojindo, 2016). The reason why tetrazolium salts are used is that their rings are cleaved in active mitochondria; therefore, this reaction does not happen in dead cells (Mosmann, 1983). MTT assay has several advantages. First of all, few materials and reagents are needed for this assay (Mosmann, 1983). The second property is the colorimetric techniques such as MTT assay is one of the best ways by which working with radioactive materials are avoided (Henriksson *et al.*, 2006). The third one is that MTT is a very useful method because it not only analyses a large number of samples at the same time by using 96-well plates, but also, it gives results with a high degree of precision (Henriksson *et al.*, 2006; Mosmann, 1983). The next characteristic is media removal, or washing steps are not required, so the assay's speed is increased, and most importantly, the variability between samples is minimized (Mosmann, 1983; Dojindo, 2016). Finally, it is safe and has wide applicability in proliferation and cytotoxicity tests with so many different types of cell lines (Mosmann, 1983; Dojindo, 2016). As a result of these properties, MTT has been commonly used in cytotoxicity research and drug screening (Mosmann, 1983; Plumb *et al.*, 1989).

1.5.2 Crystal violet (CV) staining assay:

Another simple and reproducible assay of cytotoxicity is CV staining assay, which determines stained cells that refer to growth rate or cell viability (Kueng *et al.*, 1989; Chiba *et al.*, 1998b). CV stain is a triphenylmethane basic dye staining cell nuclei, and it is also known as gentian violet (Vandersickel *et al.*, 2011; Castro-Garza *et al.*, 2007; Gillies *et al.*, 1986). Initially, CV stain was used in the Gram

stain method as the primary stain, and then it became widely used in different applications such as quantifying the number of the monolayer cultured cells, and determining under different conditions the proliferation or viability of cells (Castro-Garza *et al.*, 2007; Vandersickel *et al.*, 2011). There are some essential characteristics of CV staining assay. Firstly, CV staining assay is one of the simplest, most sensitive, most useful and rapid colorimetric assays used for cytotoxicity studies (Gillies *et al.*, 1986; Martin and Clynes, 1993; Castro-Garza *et al.*, 2007; Saotome *et al.*, 1989). Secondly, it is easy to be performed, and more objective than other assays such as colony formation assay (Vandersickel *et al.*, 2011). Also, it is a reliable semi-quantitative assay to measure the cytotoxicity of chemicals (Castro-Garza *et al.*, 2007; Saotome *et al.*, 1989). Comparing with other techniques, CV staining assay is less time consuming (Vandersickel *et al.*, 2011).

1.5.3 Trypan blue (TB) assay.

One of the commonly used assay to determine cell viability and cytotoxicity is the dye exclusion trypan blue (TB) staining assay (Dojindo, 2016; Avelar-Freitas *et al.*, 2014). It is a day It is based on cell membrane permeability, whereas dyes stain the dead cells after penetrating their cytoplasm because of the loss of their membrane integrity (Dojindo, 2016; Avelar-Freitas *et al.*, 2014; Chung *et al.*, 2015). Using a microscope and a hemocytometer the unstained cells (live cells with intact membrane) are counted to determine cell viability (Dojindo, 2016; Avelar-Freitas *et al.*, 2014; Chung *et al.*, 2015).

1.6 Scope and Aims.

The implications of this proof of principle study show the cytotoxic level of two commercial herbicidal products, ‘Once-a-year path weed’ and Roundup (R-up), and their constituents on the people who could be exposed to few droplets while applying these herbicides. Two common assays, MTT and Crystal Violet, were used to assess the cytotoxicity of these chemicals on human skin cells, HaCaT for 24h. Chapter II, The Potential Toxicity of the Commercial Product ‘Once-a-Year

Path Weed' and its Active Ingredients, Amitrole, Ammonium Thiocyanate and Simazine, cytotoxic effects of 'Once-a-year path weed, its components (amitrole, ammonium thiocyanate and simazine) and the lab mixture of 'Once-a-year path weed' were observed on HaCaT cells. In Chapter III, The Secret behind Roundup Toxicity: The Role of Glyphosate and The Surfactant, PoIyethoxylated Tallow Amine (POEA), in The Acute Toxicity of Roundup, toxicity of R-up and its ingredients (glyphosate and POEA) was detected.

A more detailed study was then performed by using β lymphocyte cells, WIL2-NS, because these herbicides could reach the blood stream via penetrating throughout the skin or food ingestion. In the blood stream, herbicides are more likely interact with other substances such as caffeine which is one of most consumed substance in the world (see section 1.3.1). Therefore, combinations of caffeine-herbicides exposed to WIL2-NS cell line for 4h, and the cytotoxicity was determined using MTT and Trypan blue assay (Chapter IV: Effects of caffeine on the cytotoxicity of the tested herbicides).

2 CHAPTER II: THE POTENTIAL TOXICITY OF THE COMMERCIAL PRODUCT ‘ONCE-A-YEAR PATH WEED’ AND ITS ACTIVE INGREDIENTS, AMITROLE, AMMONIUM THIOCYANATE AND SIMAZINE.

2.1 Introduction:

Our skins are the first barrier exposed to compounds in the outside environment including herbicides, of which more than two million tons are applied annually (Gehin *et al.*, 2006; E.P.A, 2005). Many people are use pesticides (herbicides) in their residential houses, and they often exceed the recommended rate, so they are using more pesticides per hectare than farmers (Maroni *et al.*, 2000; E.P.A, 2005). As a result of this misuse, their health, as well as the environment, are badly affected (E.P.A, 2005).

Simazine is a selective herbicide, but it could have an impact on many non-target organisms whether they are in terrestrial or aquatic areas because of its wide usage (Strandberg and Scott-Fordsmand, 2002; U.S.EPA, 2007; Kearney *et al.*, 1967). Simazine induced mammary tumours in female rats and is considered a possible human carcinogen (Kligerman *et al.*, 2000b; Tennant *et al.*, 2001). Simazine at concentration of 45 µg/L showed moderate (30 days treatment) and severe necrosis (45 to 75 days treatment) in kidneys of exposed carp (Oropesa *et al.*, 2008). At the same concentrations of simazine, some necrotic areas were observed in the liver of the fish in the early stages of the exposure, and the severity was increased at day 60 (Oropesa *et al.*, 2008). In vivo, the risk of prostate cancer is elevated among farm workers who are exposed to high levels of simazine compared to workers with low levels of exposure (Mills and Yang, 2003).

Amitrole and simazine are the main triazine herbicides used widely to control undesired weeds because of their effectiveness at low concentrations (Tennant *et al.*, 2001; Tadeo, 2008). 41 to 55 million Kg of active ingredients were annually used causing the contamination of the surface and groundwater with a high concentration of triazine herbicides (Kligerman *et al.*, 2000a; Tennant *et al.*, 2001). Amitrole is a non-selective and a fast-herbicidal active ingredient (Furukawa *et al.*, 2010). It is classified as slightly toxic (Sellamuthu, 2014). It produces benign and

malignant liver and thyroid tumours in rodents by interference with the functioning of thyroid peroxidase (Ichihara *et al.*, 2005; Mattioli *et al.*, 1994; Sellamuthu, 2014). Meretoja *et al.* (1976) found using the mitotic index that amitrole in a concentration of 10 g/L (1% w/v) is a highly cytotoxic dose for human cells in culture. They, also, resulted that 2 g/L amitrole (0.2% w/v) and higher inhibit lymphoblast transformation in blood culture after 72-h exposure (Meretoja *et al.*, 1976). Mattioli *et al.* (1994) concluded in a study of the mechanism of the carcinogenic activity of amitrole that amitrole is likely to be a carcinogen to humans, but it was not genotoxic.

To make amitrole more efficient, a salt called ammonium (amm) thiocyanate (NH_4SCN) is added to the amitrole-containing herbicides (Balkisson *et al.*, 1992). The environment is usually contaminated with thiocyanate via using herbicides and effluents containing thiocyanate (Bhunia *et al.*, 2000). Even though it is reported that thiocyanate converts to cyanide, which is thought to be the cause of some of the toxic effects of thiocyanate, the conversation of the toxicity of amm thiocyanate have not been well known in human (Legras *et al.*, 1996). Ammonium thiocyanate is mildly toxic, but is stable and thus difficult to destroy (Van Hoek, 2000; Budaev *et al.*, 2015). However, there are chronic effects of exposure to it including vomiting, nausea, and dizziness (Balkisson *et al.*, 1992). The mortality of aquatic organisms has a dose-dependent relationship with thiocyanate concentration (Lanno and Dixon, 1996b). The toxicity of ammonium thiocyanate has not been associated with alveolar damage via inhalation, but its effects could be amplified if it is mixed with the active ingredients found in the formulas (Balkisson *et al.*, 1992).

In this chapter, ‘once a year path weed’ product and its individual components were studied in vitro, and it is hypothesised that the1/200 dilution (equivalent to a few droplets) of the commercial product and the individual components are not cytotoxic to HaCaT human skin cell line. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay and crystal violet assay were used to determine the cell viability after treating HaCaT cells.

2.2 Material and methods:

2.2.1 Chemicals:

RPMI1640 medium powder was obtained from Sigma-Aldrich. Foetal bovine serum (FBS) was purchased from Hyclone (Victoria, Australia). Sodium bicarbonate solution obtained from Pfizer (WA, Australia). Penicillin/streptomycin and the L-glutamine were obtained from Gibco® (Life Technology, USA). A 5 mg/ml MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma-Aldrich, USA) solution was prepared and stored at -20°C. 20% SDS (sodium dodecyl sulphate; Sigma-Aldrich, USA) in 0.02M HCl (Merck, Germany) was prepared at room temperature in a fume hood. Crystal violet stain was prepared: 0.5% crystal violet powder (Sigma-Aldrich, USA) in 50% methanol (Sigma-Aldrich, USA) and stored in the fume hood at room temperature. 33% acetic acid (Merck, Germany) was prepared and stored in the fume hood. Phosphate-Buffered-saline (PBS) was prepared and stored at room temperature after it was sterilised. The commercial product, 'once-a-year' path weeds (Yates) was bought from a commercial shop in South Australia. Amitrole, simazine, and ammonium thiocyanate were purchased from Sigma-Aldrich (USA). A lab mixture, which is composed of 5g/L amitrole, 9g/L simazine, and 4.4g/L ammonium thiocyanate, was prepared and stored in -20°C.

2.2.2 Cell culture and medium:

HaCaT (Ha= human adult, Ca= calcium, and T= temperature) is a human keratinocyte cell line which was developed through a long-term culture of normal human adult skin keratinocytes under low calcium concentration and temperature (Boukamp *et al.*, 1988). RPMI1640 medium was used to culture HaCaT cells. Added to it were 10% foetal bovine serum (FBS), 2.5% sodium bicarbonate solution and 1% of both penicillin/streptomycin and L-glutamine. These combined ingredients are referred to as "media" from here onwards. 75cm^2 culture flasks (Sigma-Aldrich, USA) were used to grow HaCaT cells till they reach 70-80% confluence. The

medium was then changed every three days, and cells were subcultured every seven days.

2.2.3 Dosing.

Fresh doses were made just before each experiment. The highest concentration of the commercial product and the lab mixture was 0.005%, which followed by five 1/10 serial dilutions. A 350 µM was the highest concentration of amitrole and ammonium thiocyanate. Five 1/10 serial dilutions were made up from the maximum concentration. The highest concentration of simazine was 223 µM (0.0045% w/v), from which three serial dilutions were prepared.

2.2.4 Preparing plates.

For both MTT and crystal violet assays, HaCaT cells were seeded in 96-well microplates (1 x 10^4 cells/well in RBMI 1640 medium and 10% FBS), and the plates were incubated for 24h in 37°C, 5% CO_2 incubator for cell adherence. After adherence of cells, media was removed from prepared wells. Cells then were exposed to variable concentrations of tested compound for 24h.

2.2.5 Cell viability assays.

2.2.5.1 MTT assay.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) bioassay *in vitro* quantifies the viable cells whose mitochondria produce the succinate dehydrogenase enzyme converting the yellow soluble MTT to the purple insoluble formazan crystals (Henriksson *et al.*, 2006; Young *et al.*, 2005). MTT bioassay was performed as published (Alnuqaydan *et al.*, 2014; Young *et al.*, 2005). Briefly, after the 24h exposure to 'Once-a-year path weed, the lab-mixture of the commercial product, amitrole, simazine or ammonium thiocyanate, the supernatant was removed containing the dead cells. The remaining live cells (attached on the plate) were then washed twice with PBS, and MTT reagent was added to the prepared wells (0.2 ml medium containing 0.5 mg/ml MTT in each well). Cells were incubated for a further 4h allowing the interaction between the tetrazolium MTT and

dehydrogenase enzymes, a mitochondrial enzyme. This interaction occurs only in the living cells and forms purple formazan crystals. The 80 µl of 20% SDS in 0.02 M HCl was added to dissolve the crystals while incubating the cells overnight at the room temperature in the dark. The absorbance values were obtained using µQuant plate reader (Bio-Tek Instruments, INC) with 570 nm measurement wavelength and 630 nm reference wavelength. Relative viability was calculated by comparing to the negative control (contained untreated cells).

2.2.5.2 Crystal violet assay.

CV staining assay is one of the simplest, most useful and rapid colorimetric assays used for cytotoxicity studies (Gillies *et al.*, 1986; Martin and Clynes, 1993; Castro-Garza *et al.*, 2007; Saotome *et al.*, 1989). It stains the nuclei of live cells only. After exposing the cells to variable concentrations of the chemicals ('Once-a-year path weed, the lab-mixture of the commercial product, amitrole, simazine or ammonium thiocyanate,) for 24h, the cellular viability was then assessed using crystal violet assay. Briefly, the supernatant containing dead cells was removed, and the attached cells were washed twice with PBS. The living adherent cells were then stained with crystal violet stain for 10 min. Plates were gently rinsed with water and air-dried overnight at room temperature. After dissolving the stain with 33% acetic acid, plates were ready to be read using µQuant plate reader with a measurement wavelength of 570 nm and a reference wavelength of 630 nm. Cell viability was calculated by comparing to the negative control (contained untreated cells).

2.2.6 Statistics.

Both assays were performed at least in quadruplicate for each chemical, and the data was presented as mean ± standard error of the mean. IMB SPSS software (version 22) was used for statistical analyses. One way ANOVA with Tukey Post-Hoc test were performed to compare data between groups and the level of significance was expressed as $p < 0.05$ or $p < 0.001$.

2.3 Results:-

2.3.1 Cytotoxicity assays:

HaCaT cells were exposed to Once-a-year path weed, lab-mixture, amitrole, simazine or ammonium thiocyanate for 24h. The cytotoxicity of these chemicals was assessed using MTT and Crystal Violet assays.

2.3.1.1 MTT assay:

The commercial product at the concentration of 0.005% showed a significant reducing in cell viability ($p< 0.001$). 34.3% of the cells were killed at 0.005% of the commercial product, but lighter doses showed less than 10% killing (see Figure 2.1a). However, the highest concentration of the lab –mixture (0.005%) presented no significant loss of cell viability whereas only 8.6% was killed (see Figure 2.1b). Amitrole and ammonium thiocyanate have shown almost similar results, and both of them have no significant effects on HaCaT cell proliferation (see Figure 2.1c and d, respectively). They did not impact the cell viability at 350 μM (29 mg/L and 27 mg/L, respectively) or less. On the other hand, simazine induces cytotoxic activity resulting in decreasing the number of live cells to 65.3% at a concentration as low as 45 mg/L (223 μM). Lighter concentrations of simazine did not show any cytotoxic effect on the human skin cells, HaCaT (see Figure 2.1e).

2.3.1.2 Crystal Violet (CV) assay.

Although CV results illustrated some killing of HaCaT cells, none of the chemicals tested did so significantly (see Figure 2.1a-e).

2.4 Discussion.

The highest concentration of 'Once-a-year path weed' product was 0.005% (v/v), and it comprises of 0.45 mg/L, 0.25 mg/L and 0.22 mg/L simazine, amitrole and ammonium thiocyanate, respectively. The commercial product and simazine were the only ones that are displayed cytotoxicity to the human skin cells HaCaT at

the highest tested concentrations (0.005% and 223 μM, respectively) using MTT assay. However, the 0.005% of the commercial product contains 22.3 μM simazine which did not show any toxic effect when it tested by itself (see Figure 2.1 a and e). Simazine shows significant ($p < 0.05$) effects on HaCaT cells at ten times the concentration found in the commercial product. Therefore, it could be the combination of all active ingredients together is the responsible of the toxicity. Another possibility is that other compounds in the product that make simazine or the combination itself more toxic. Surprisingly, the lab mixture containing the same concentrations of the active ingredients as in the commercial product was not toxic to the HaCaT cells using both assays. Thus, the commercial product could contain other ingredients causing the toxicity.

Although the concentration of simazine was as low as 223 μM (45 mg/L), it could influence the environment and people health because of its extensive use (Oropesa *et al.*, 2008). For example, individuals in some towns in Southwest Spain were using contaminated water with 4.5 μg/L of simazine as a public supply, and the maximum advisory legal limit for simazine in drinking water is extremely lower (0.1 μg/L) (Oropesa *et al.*, 2008). Also, 30 μM (6 mg/L) of simazine induces aromatase activity in both H295R adrenocortical and JEG-3 placental cells (Sanderson *et al.*, 2001). Aromatase is an enzyme converting androgen to oestrogen (Acton, 2013). The latter is a major factor that promotes the growth and developments of breast tumours (Acton, 2013). Thus, even a concentration as low as 45 mg/L is harmful to the health and environment.

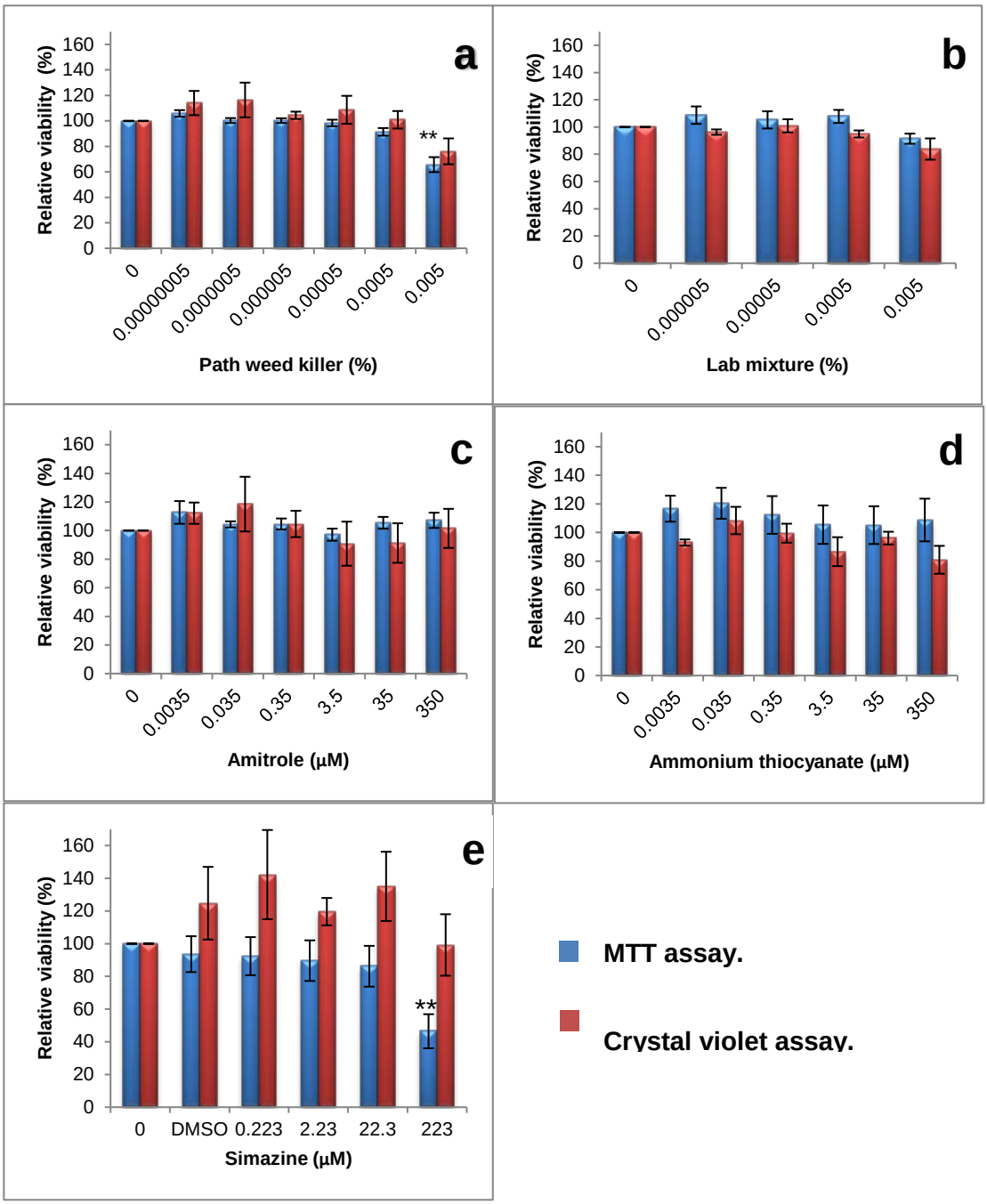

Figure 2.1: Effects of Once-a-year path weed and its active ingredients on the human skin cells, HaCaT.

The data was presented as mean ± stander error of the mean whereas a) is the commercial product itself, b) is the lab mixture (0.005%): 0.297 mM, 0.289 mM and 0.223 mM of amitrole, ammonium thiocyanate and simazine respectively, c) is the active ingredient amitrole, d) is the surfactant ammonium thiocyanate, and e) is the effects of the active ingredient simazine. *=p<0.05, and **=p<0.001

Although some are saying that amitrole is not inducing toxicity to human (Sellamuthu, 2014), it appears that the toxicity of amitrole to mammals is very low (Meretoja *et al.*, 1976). The rapid elimination of amitrole from the body without any metabolic changes could be the reason of the low toxic effect of amitrole (Legras *et al.*, 1996; Sellamuthu, 2014). Meretoja *et al.* (1976) used much higher amitrole concentrations than the tested one in this study, and they fund that amitrole at a concentration of 0.2% (w/v; 2 g/L) and higher inhibited the cell growth of human leucocytes, and cell debris was noticed on slides. The commercial product 'Once-a-year path weed' contains amitrole at a concentration of 5 g/L (0.5% (w/v)) which seems to be critical, but the concentrations tested were much lower than that. The highest concentration of amitrole that was tested individually in this study was 350 μM (29.4 mg/L). The results showed that amitrole had no adverse impact on HaCaT cell line at this concentration, so it is not expected that amitrole will be a part of the cytotoxic effect of the commercial product on human cells. This expectation and results were supported by a previous study found that amitrole at lower than 0.2% (w/v; 2 g/L) had no significant effect on the human leucocyte cell line (Meretoja *et al.*, 1976). Also, another *in vitro* study informed that 1-100 mg/L amitrole did not demonstrate any significant effect on rat thyroid follicular FRTL-5 cell proliferation, which was decreased slightly at 100 mg/L amitrole (Pan *et al.*, 2011).

Ammonium thiocyanate is a slightly toxic, but its toxicity may be marked in people with renal insufficiency (Van Hoek, 2000; Gracia and Shepherd, 2004). However, there is a lack of *in vitro* studies showing the cytotoxic activities of thiocyanate on human cell lines. In the present study, ammonium thiocyanate does not seem to be a part of the commercial product toxicity because it presented no harm on HaCaT proliferation at 350 μM (27 mg/L). This concentration was tenfold higher than its concentration in 0.005% Yates® product, Once-a-year path weed. Contaminating an aquarium with Yates® product containing 4.4 g/L ammonium thiocyanate could become problematic to the aqua-organisms. In water

contaminated with thiocyanate-containing effluents, It is reported that the growth, reproduction, and appetite of fishes have been reduced (Bhunia *et al.*, 2000). 4 mg/L thiocyanate shows a significant effect on fish movement at 28°C (Bhunia *et al.*, 2000). The tested concentration of thiocyanate could impact the feeding rate of aquatic organisms. Half of the feeding rate of fish could be decreased when exposed to 1 mg/L thiocyanate (Bhunia *et al.*, 2000). High concentrations, up to 154 mg/L, of thiocyanate led to fish suffered 100% mortality within 12 weeks (Lanno and Dixon, 1996b). Although mortalities were minimal at low concentrations (35 and 77 mg/L), the level of plasma T_4, a thyroid hormone, was significantly reduced at these concentrations (Lanno and Dixon, 1996b). This depression influences thyroid function (Lanno and Dixon, 1996b).

Crystal violet (CV) assay did not detect any significant reduction in cell viability of HaCaT cells resulting from this study. That is why it is recommended to evaluate cytotoxicity using multiple assays with different endpoint parameters because some chemicals have particular mechanisms of action that may give different results using different assays (Chiba *et al.*, 1998a).

2.5 Conclusion.

In this chapter, the commercial product, 'Once-a-year path weeds' and its active ingredients, simazine, amitrole and ammonium thiocyanate, were tested at the 1/200 dilution for 24h. Their effects on the human skin cells, HaCaT, were varied. Amitrole and ammonium thiocyanate are not harmful at tested concentrations. Simazine elicited a significant effect at higher concentration than in the tested concentration of the commercial product. The lab mixture did not show any effect too. Thus, other compounds in the product could be part of this toxicity.

3 CHAPTER III: THE SECRET BEHIND ROUNDUP TOXICITY: THE ROLE OF GLYPHOSATE AND THE SURFACTANT, POIYETHOXYLATED TALLOW AMINE (POEA), IN THE ACUTE TOXICITY OF ROUNDUP.

3.1 Introduction.

Commercial agricultural products may interact with many physiological functions; in the exposed population, as well as in the environment (Sanderson *et al.*, 2000). Our bodies are frequently exposed to many exogenous agents, including pesticides, atmospheric pollutants, cigarette smoking, or UV light (Amerio *et al.*, 2004; Gehin *et al.*, 2006). Usage of pesticides in agriculture, industry and for domestic application has markedly increased since 1990 (Maroni *et al.*, 2000; Tadeo, 2008). More than 90% of people use pesticides in their houses, so a large proportion of the population exposed to pesticides (E.P.A, 2005; Maroni *et al.*, 2000). Glyphosate (*N*-phosphonomethyl; $C_3H_8NO_5$) is one of the most extensively used herbicides in the world, and over 907,000 tons of it were annually used in 2007 (Koller *et al.*, 2012; Tadeo, 2008). It is a nonselective herbicide that inhibits the pathway for biosynthesis of tryptophan, tyrosine, and other essential aromatic amino acids for plant growth (Williams *et al.*, 2000). This pathway only occurs in plants (Williams *et al.*, 2000). The Environment Protection Authority in South Australia (EPA) (2005) has classified glyphosate as a low toxicity chemical. Several glyphosate-based herbicides were tested by Marc *et al.* (2004a), and they estimated that 10 to 120 µM of glyphosate is the adverse threshold dose that sufficient to provoke dysfunction of at least one cell of the embryo of the sea urchin. Nowadays, over 100 countries are registering a selection of glyphosate-based formulation under different bands (Williams *et al.*, 2000). The application of glyphosate-based formulation has been expanded by farmers in soil construction program to prepare their fields prior to planting (Williams *et al.*, 2000). The recommended concentration of glyphosate used for spraying a formulation product is 40 mM (Marc *et al.*, 2004a). The concentration presented in the sprayed droplets is 500 to 4,000 times higher than the adverse threshold concentration (Marc *et al.*, 2004a). Glyphosate has characteristics retard its penetration into plant tissues, so it is usually mixed with polyethoxylated tallow amine (POEA), and this formulation is commonly known as Roundup (R-up) (Gehin *et al.*, 2006; Koller *et al.*, 2012; Williams *et al.*, 2000).

In 1974, R-up was first introduced for controlling annual and perennial weed in residential, agricultural and industrial settings (Williams *et al.*, 2000). It contains glyphosate as an active ingredient and has been increasingly used over the past two decades (Williams *et al.*, 2000). An *in vivo* (skin of monkeys) and *in vitro* (human skin) studies reported that the dermal penetration of glyphosate formulation including R-up is very slow (Maibach, 1983; Wester *et al.*, 1991). A recent study shows harmful effects, including damaging of the cell membrane and interference of proteins synthesis in a human buccal epithelial cell line (TR146) following the treatment of cells with low concentrations of R-up (> 10mg/L = 0.1%) (Koller *et al.*, 2012). In contrast, there were no such effects resulted from treating TR146 cells with glyphosate up to 200mg/L (Koller *et al.*, 2012). Consequently, Koller *et al.* (2012) agreed with other previous studies that the cytotoxicity of R-up formulations is much higher than glyphosate cytotoxicity (Benachour and Seralini, 2009; Gasnier *et al.*, 2009)

Polyethoxylated tallow amine (POEA) is a commonly used surfactant in the R-up formulation (Williams *et al.*, 2000). The main purpose of POEA is to facilitate the herbicide absorption and increase its effectiveness (Martini *et al.*, 2012). However, this compound likely contributes to the herbicide formulation toxicity (Song *et al.*, 2012a). An *in vivo* study using rabbit showed it to be severely irritating skin and corrosive to the eyes (Williams *et al.*, 2000; WHO, 1996). It is likely commented that the main element responsible for human toxicity in R-up product is not glyphosate, but the surfactants that are present in the formula (Lee *et al.*, 2009; Song *et al.*, 2012a). Song *et al.* (2012a) in their study of the *in vitro* cytotoxicity effect of glyphosate mixtures containing surfactants, treated three types of cells (mouse fibroblast-like cells, alveolar cells, and heart cells) with glyphosate and two common surfactants found in glyphosate herbicide products. They are Polyoxyethylene tallow amine (POEA) and Polyoxyethylene lauryl amine (LN-10). Song *et al.* (2012a) used the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-

diphenyltetrazolium bromide) assay to determine the cellular viability after exposing cells to glyphosate, POEA, and LN-10. They observed that glyphosate did not cause cellular toxicity in the three examined cell lines at 0-100µM concentrations (Song *et al.*, 2012a). However, slopes of POEA and LN-10 were steep when their concentrations were greater than 25µM, and both were cytotoxic at concentrations of 1-100µM (Song *et al.*, 2012a).

In this study, we measured the cytotoxicity of the R-up, its active ingredient (glyphosate), and the surfactant (POEA). These doses are equivalent to few droplets. The implications of this proof of principle study may show the role of glyphosate and POEA in the acute toxicity of R-up.

3.2 Materials and methods.

3.2.1 Chemicals.

RPMI1640 medium powder was obtained from Sigma-Aldrich. Foetal bovine serum (FBS) was purchased from Hyclone (Victoria, Australia). Sodium bicarbonate solution obtained from Pfizer (WA, Australia). Penicillin/streptomycin and the L-glutamine were obtained from Gibco® (Life Technology, USA). 0.05 mM MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma-Aldrich) solution was prepared and stored at -20°C. Sodium dodecyl sulphate (20% SDS; Sigma-Aldrich) in 0.02M HCl was prepared and kept at room temperature in a fume hood. Crystal violet stain was prepared: 0.5% crystal violet powder (Sigma-Aldrich) in 50% methanol (Sigma-Aldrich) and stored in the fume hood at room temperature. Acetic acid (33%) was prepared and stored in the fume hood. Phosphate-Buffered-saline (PBS) was prepared and stored at room temperature after it was sterilised. Regular Roundup® ready to use was purchased from local commercial shops in South Australia. A 50mM glyphosate (Sigma-Aldrich) solution and 10% POEA (Novochem, Australia) were prepared and stored at -20°C.

3.2.2 Cell culture.

HaCaT (Ha= human adult, Ca= calcium, and T= temperature) is a human keratinocyte cell line which developed through a long-term culture of normal human adult skin keratinocytes under low calcium concentration and temperature (Boukamp *et al.*, 1988). RPMI1640 medium was used to culture HaCaT cells. Added to it were 10% foetal bovine serum (FBS), 2.5% sodium bicarbonate solution and 1% of both penicillin/streptomycin and L-glutamine. These combined ingredients are referred to as "media" from here onwards. 75cm^2 culture flasks (Sigma-Aldrich) were used to grow HaCaT cells till they reach 70-80% confluence. The medium was then changed every three days, and cells were subcultured every seven days.

3.2.3 Dosing.

R-up, glyphosate, and POEA doses were made up for each experiment. A 1/20 dilution (0.05%) of R-up containing 21.3 μM glyphosate and 112 μM POEA was made up before seven 1/10 serial delusions were prepared. Six 1/10 serial delusions of 50mM glyphosate were prepared as well. 0.001, 0.0008, 0.0006, 0.0004, and 0.0002% (22.4, 17.9, 13.5, 9, and 4.5μM, respectively) of POEA were prepared.

3.2.4 Preparing plates.

For both MTT and crystal violet assays, HaCaT cells were seeded in 96-well microplates (1 x 10^4 cells/well in RBMI 1640 medium and 10% FBS), and the plates were incubated for 24h in 37°C and a 5% CO_2 incubator for cell adherence. After the cells adhered, the media was removed from the prepared wells. Cells were exposed to a variable concentration of tested compound for 24h.

3.2.5 Cell viability assays.

3.2.5.1 MTT assay.

The commonly used MTT bioassay was performed as published by Young *et al.* (2005) and Alnuqaydan *et al.* (2014). It is described as in section 2.2.5.1.

3.2.5.2 Crystal violet assay.

The Crystal Violet assay was performed as in section 2.2.5.2

3.2.6 Statistics.

Both assays were performed at least in quadruplicate for each chemical, and the data was presented as mean ± stander error of the mean. IMB SPSS software (version 22) was used for statistical analyses. One way ANOVA with Tukey Post-Hoc test was performed to compare data between groups, and $p < 0.05$ was considered significant.

3.3 Result.

3.3.1 Estimating the mitochondrial activity using MTT assay.

The average absorbance was assessed from the negative control wells containing untreated cells. The average absorbance of the control considered as 100% relative survival. The percentage of relative survival resulting from each concentration was obtained by comparing the average absorbance of each concentration with the negative control. One way ANOVA with Tukey Post-Hoc test was then run on all samples to obtain the significant results comparing to the negative control. Glyphosate shows no significant effect on HaCaT cells proliferation. At its highest concentration (5 mM) no significant reduction in cell number was seen (see Figure 3.1b). However, the cell number was significantly decreased ($p < 0.05$) after treating cells with 0.05% R-up, which killed almost half of the cells with respect to the negative control (see Figure 3.1a). Moreover, POEA shows a dose-dependent inhibition of cell proliferation after the 24h. Significant killing of cells ($p < 0.001$) were illustrated at 13.6 µl and higher of POEA (see Figure 3.1c).

3.3.2 Estimating the cell survival using crystal violate assay.

Cell viabilities and significant results were performed as in MTT bioassay (section 3.3.1). No inhibition of cell proliferation was noticed after treating cells with

glyphosate for 24h (see Figure 3.1b). On the other hand, 0.05% R-up shows a significant killing ($p < 0.05$). Thus, over 50% of HaCaT cells were detached from the plate and floated at that concentration resulting in a significant decrease in the survival cell number (see Figure 3.1a). Also, POEA shows similar dose-depending as in MTT assay. The significant killing was occurred at 9 µM POEA and higher ($p < 0.001$; see Figure 3.1c).

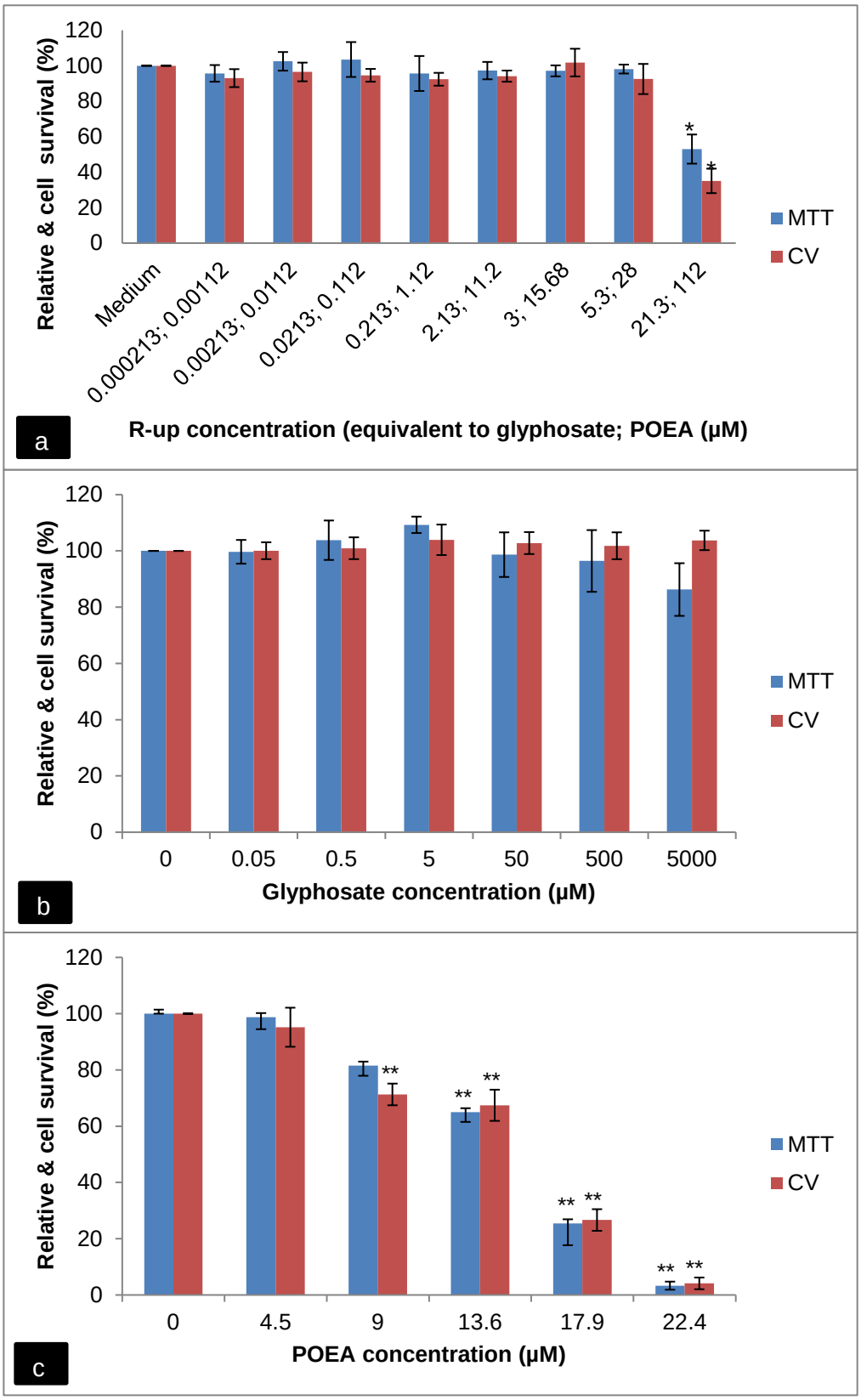

Figure 3.1: Detecting of potential toxicity of HaCaT treatment for 24h.
Percentages of the relative survival after the treatment with a) R-up, b) glyphosate and c) POEA . The percentages are shown as the mean ± the standard error of the mean from a) four experiments, b) seven experiments and c) four experiments. They are expressed relative to the medium (zero control). * $p < 0.05$, and ** $p < 0.001$.

3.4 Discussion.

The MTT assay shows similar results to the Crystal violate assay. The Crystal violet assay displayed a lower cell number than the MTT assay, and that could be due to losing some cells through the washing step. In this study, the results show that glyphosate had a lower cytotoxic effect than Roundup (R-up), and that is consistent with what has been published previously using higher concentrations (Gasnier *et al.*, 2009; Benachour and Seralini, 2009; Koller *et al.*, 2012). Even the highest concentration of glyphosate, 5 mM (845.4 mg/L), did not present any serious loss in viability of HaCaT cells. This result was expected because glyphosate only blocks a particular plant pathway for biosynthesis of some essential aromatic amino acids (Williams *et al.*, 2000). Its acute toxicity is also considered to be low as its dermal and oral median lethal dose (LD_{50}) exceeded the 5000 mg/kg rat's body weight, and it is not irritating rabbit skin (WHO, 1996; Williams *et al.*, 2000). Moreover, up to 200 mg/L (1.18 mM) glyphosate did not show any effects on TR146 cell line using three cytotoxicity assays (Koller *et al.*, 2012). Glyphosate starts affecting TR146 cells in LHDe assay at ≥80 mg/L (0.47 mM) (Koller *et al.*, 2012). In this study, the percentage of the dead cells increased up to 13.7% after treatment with 5 mM glyphosate for 24h using MTT assay. Almost the same result has been detected using trypan blue assay (17 % dead cells) when 3T3-L1 fibroblast cells were treated with 1:2000 dilution (1.24 mM) of glyphosate formulation (Martini *et al.*, 2012).

Although R-up dermal penetration is slow, 24h treatment of 0.05% R-up containing 21.3 μM glyphosate and 112 μM POEA resulted in a significant decrease in HaCat cell viability using both MTT and crystal violet assay ($p < 0.05$). Almost half the cells died (47%) compared to the negative control after 0.05% R-up treatment in MTT assay. The same treatment resulted in an even higher number of dead cells (65%) using crystal violet assay. The LD_{50} of 0.7% R-up is 1.8 times lower than glyphosate at the equivalent concentration (Richard *et al.*, 2005). The

addition of 0.1% R-up increased the toxicity of glyphosate against JEG3 placental cells (Richard *et al.*, 2005). Furthermore, 0.8% R-up containing 8 mM glyphosate induced a delay in entry into M-phase of the cell cycle in sea urchin embryos leading to delay in cell cleavage, whereas no such effect was detected with glyphosate-alone treatment (Marc *et al.*, 2002).

The cellular toxicity of POEA was observed to be dose-dependent (2.2 – 22.4 μM) in HaCaT cell line using MTT and crystal violet assays as well as in other cell lines tested previously by Song *et al.* (2012a)including mouse fibroblast-like cells (L-929), heart (H9C2) and alveolar cells (A549). Nevertheless, glyphosate did not show such toxicity (0 – 100 μM) in this study, nor in the three examined cell lines in the preceding study (Song *et al.*, 2012a). POEA at 22.4 μM provoked a 96.7% and 95.9% reduction of HaCaT cells compared to the negative control ($p < 0.001$) in MTT and crystal violet assay, respectively. This result was quite similar using XTT (2,3-bis(-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide inner salt) assay when L-929 cells were treated with ≥ 25 μM POEA where it resulted in a mitochondrial suppression of up to 96.5% ($p < 0.05$) (Song *et al.*, 2012b). Another study examined several surfactants shows that POEA was the most active surfactant that causes membrane damage which was assessed by lactate dehydrogenase (LDH) assay (Song *et al.*, 2012b). In this study, the POEA toxicity significantly appeared in both assays at the concentration of 13.6 μM, but this concentration within R-up did not show a toxic effect on the human skin cells, HaCaT. Indeed, the toxicity of R-up started when the POEA concentration reaches 112 μM. Therefore, testing a combination of glyphosate and POEA in various concentrations is required in the future to determine whether glyphosate attenuates POEA toxicity or not.

In conclusion, this study found that R-up toxicity does not come from the active ingredient glyphosate, but from POEA, the surfactant.

4 CHAPTER IV: EFFECTS OF CAFFEINE ON THE CYTOTOXICITY OF THE TESTED HERBICIDES

4.1 Introduction.

Caffeine ($C_8H_{10}N_4O_2$) is the most consumed natural psychoactive substance all over the world (Bode and Dong, 2007). It can be found in many dietary sources, including coffee, tea, soft drinks, energy drinks, and chocolate bars (Bode and Dong, 2007; Han *et al.*, 2011; Somogyi, 2010). The estimated consumption of caffeine from all sources is about 70-76 mg/person/day, which is equivalent to approximately 1.1 mg/kg/day (Fredholm *et al.*, 1999). Caffeine is absorbed within the first hour of ingestion and rapidly distributed throughout the body reaching the brain where it works as a stimulant of the central nervous system (CNS) (Bode and Dong, 2007; Green *et al.*, 2010). The half-life of caffeine for doses less than 10 mg/kg ranges from 2.5 to 4.5h in human (Fredholm *et al.*, 1999).

In vivo studies have shown that 5-10 µM caffeine results in mild stimulation of the central nervous system (CNS) (Green *et al.*, 2010). Greater concentrations (50 µM or more) are associated with cardiac stimulation (Green *et al.*, 2010). Consumption of 50-600 mg caffeine/kg leads to 20-57 µM concentration of caffeine in the blood, and ingestion of large doses of caffeine results in toxic effects with an LD_{50} of 200 mg/kg in rats (Fredholm *et al.*, 1999; Green *et al.*, 2010). Cardiac and CNS stimulation could be caused by these concentrations (Green *et al.*, 2010). Caffeine may be associated with reducing cancer risk (Dubrow *et al.*, 2012). Caffeine inhibits cyclooxygenase-2 (COX-2) pathway, a critical oncogene pathway, resulting in reducing the chemicals-induced skin tumour progression (Fischer *et al.*, 2007). An *in vivo* study indicates that caffeine represses tumour progression and inhibits chemical carcinogenesis in rat and mouse skin models, respectively (Nishikawa *et al.*, 1995; Rothwell, 1974). Moreover, caffeine has a potential chemopreventive activity (Chin *et al.*, 2011). It interferes with mutagens and carcinogens such as acridine orange, actinomycin, daunomycin, and others by reducing their binding to DNA, and acting as an intercalation inhibitor (Chin *et al.*, 2011; Davies *et al.*, 2001). During cell cycle progression, caffeine abrogates the

delay periods of G1/S and G2/M checkpoints, so cells with damaged DNA have less time for repair, and eventually they undergo apoptosis (Bode and Dong, 2007; Han *et al.*, 2011; Lu *et al.*, 2008a). Caffeine shows some effects on G1 and G2 phases through both p53-dependent and p53-independent mechanisms (Bode and Dong, 2007). At high concentrations of caffeine (10mM), DNA repair is inhibited during S phase (Auclair *et al.*, 2008). Kastan *et al.* (1991) reported that treating myeloblastic leukaemia (ML-1) cells with 4mM caffeine blocked both the induction of p53 expression and the G1 arrest after ɣ-irradiation (Kastan *et al.*, 1991). Qi *et al.* (2002) found that although 0.5 mM caffeine did not induce apoptosis in the human A549 lung adenocarcinoma cells (even at 96h of treatment). Apoptosis is induced in approximately 15% of the cells at higher concentration (5 mM), and this amount was doubled to 31% when cells treated with ɣ radiation plus 5 mM caffeine (see Figure 4.1) (Qi *et al.*, 2002). They, also, found that 24h treatment with 5 mM caffeine inhibited the activation of cyclin-dependent kinases 2 (Cdk2) resulting in cyclin E/Cdk2 complex inhibition, which leads cells to arrest in G1 phase (Qi *et al.*, 2002; Bode and Dong, 2007). Another study showed that 2mM of caffeine suppressed 75% of G1/S progression (Kaufmann *et al.*, 2003). However, it has no effect on G2/M progression (Bode and Dong, 2007; Kaufmann *et al.*, 2003). Generally, caffeine seems to affect the cell cycle in diverse ways, which appears strongly dependent on caffeine concentration and cell type (Bode and Dong, 2007; Traganos *et al.*, 1993).

Other target proteins of caffeine identified *in vitro* are ataxia telangiectasia mutated (ATM) protein and AT-related (ATR) protein (Bode and Dong, 2007). Both proteins belong to the phosphatidylinositol-3 (PI-3) kinase group, which is associated with critical signalling pathways for tumour development (Krasilnikov, 2000). Caffeine suppresses the activation of ATM or ATR and several of PI-3 kinases at 0.075-1.0 mM concentrations (Brazil *et al.*, 2004; Sarkaria *et al.*, 1999). Moreover, the cytotoxicity of several aromatic compounds interacting with DNA is

diminished in the present of caffeine (Traganos *et al.*, 1993). For example, in a dose-dependent manner, caffeine reduces the cytotoxic effect and apoptotic response induced when HL-60 cell line treated with 0.15 μM camptothecin or topotecan (DNA topoisomerase I inhibitors) (Traganos *et al.*, 1993). In contrast to this potentiating activity, Caffeine tends to increase the cytotoxicity and the toxic effect of a variety of DNA-damaging agents including ionising radiation (IR) and alkylating compounds in human cancer cell lines (Bode and Dong, 2007; Traganos *et al.*, 1993). Also, Gunter et al. (2012) found that there is an inverse relationship between coffee drinking and endometrial cancer regardless of caffeine content and that caffeine may mediate this inverse association (Gunter *et al.*, 2012). The same relationship was observed between caffeine intake and the risk of glioma, a common form of brain cancer (Holick *et al.*, 2010). Although Dubrow *et al.* (2012) support the latter association; they found no evidence of a dose-dependent relationship between caffeine intake.

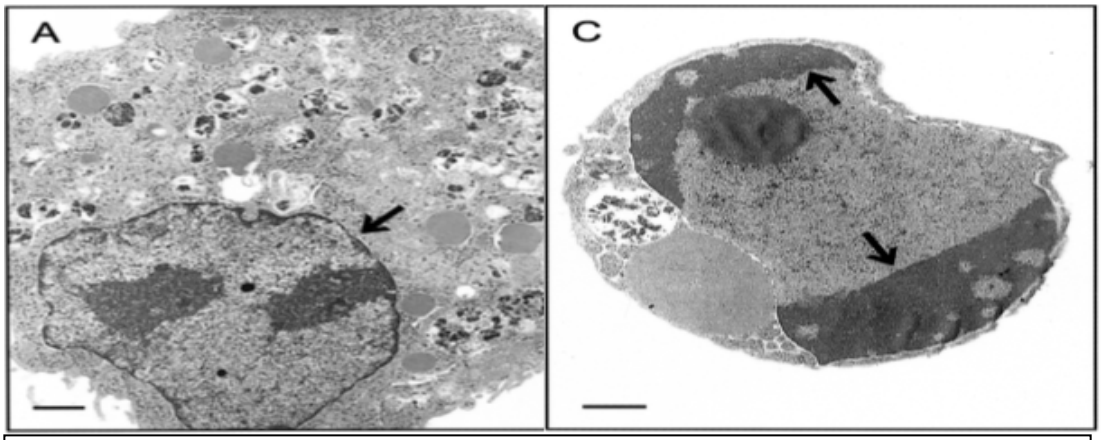

Figure 4.1: Apoptotic cells by microscopy.
Apoptosis induced in A549 cells by 5 mM caffeine is confirmed by electron microscopy. Panel A refers to untreated cells; panel C refers to cells exposed to 5mM caffeine. The arrow in A points to an intact nuclear membrane, and arrows in C point to condensed nuclear chromatin, and the bar at the bottom of both panels represents 0.1μm (Qi *et al.*, 2002).

This chapter will present the effect of caffeine on the proliferation of human blood cell line, WIL2-NS, using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-

diphenyltetrazolium bromide) and trypan blue assays. Caffeine is the most ingested substance and people are frequently exposed to herbicides either directly (applying it) or indirectly (through food) (Bode and Dong, 2007; Tadeo, 2008). Therefore, combinations of caffeine-herbicides will be tested to determine whether or not that caffeine could affect the cytotoxic activities of some commercial herbicides and their active ingredients. Roundup (R-up), once a year weed killer, and/or their individual active ingredients will be mixed with caffeine to treat human blood cell line, WIL2-NS.

4.2 Material and Methods.

4.2.1 Chemicals:

All used chemicals and materials were the same as in section 2.2.1.

4.2.2 Cell culture and medium:

The human non-secreting β lymphocyte cell line, WIL2-NS, was used (see section 1.4.1.2). RPMI1640 medium is the optimum medium to culture WIL2-NS cells. Added to it were 10% foetal bovine serum (FBS), 2.5% sodium bicarbonate solution and 1% of both penicillin/streptomycin and L-glutamine. These combined ingredients are referred to as “media” from here onwards. 25 cm^2 culture flasks (Sigma-Aldrich, USA) were used to grow WIL2-NS cells till they reach 70-80% confluence. The medium was then changed every three days, and cells were subcultured every seven days.

4.2.3 Dosing.

Doses were made freshly before each experiment. For caffeine experiment, five doses were required at the concentrations of 5, 25, 50, 100, 500 μM. Double of these concentrations were prepared because they were diluted (1:2) when cells are seeded.

For the combination (caffeine plus herbicides) experiment, the two highest

concentrations of all chemicals tested in chapter one and two were combined with two doses of caffeine. Table I shows the exact concentrations.

4.2.4 Treating WIL2-NS cells.

4.2.4.1 Caffeine treatment.

WIL2-NS cells were seeded in a six-well plate at a concentration of $5X10^5$ cells/ 0.5 ml/ well. Another 0.5 ml of double the required caffeine concentration was added to each prepared well, so the concentration was diluted to the required dose. Cells were then incubated for 1, 4, and 24h in 37°C, 5% CO_2 incubator. After incubation (treatment), cells in each well were transferred to a 10 ml tube, and washed twice with medium, and centrifuged at 800-900 rpm for 5 min. The supernatant then was discarded, and the pellet resuspended in 0.5 ml medium. At this stage, the concentration of the cells is $1x10^6$ cells/ml.

4.2.4.2 Caffeine-herbicides treatment.

Double the required concentrations of the herbicides and caffeine were prepared. In a 24-well plate, $2x10^5$ cells/ 0.1 ml were added to each well. The volume is then increased to 1 ml by 0.5 ml caffeine and 0.4 ml Herbicides. Cells were then incubated for 4h in 37°C CO_2 (5%) incubator. After incubating the prepared plate, cells in each well were washed and suspended the same way as in section 4.2.5.1.1, but the cell concentration was $2x10^5$ cells/ml.

Table I: Combinations of caffeine and herbicides.

Chemicals	Caffeine (µM)		
	0	**50**	**500**
R-up (%)	0.0125	0.0125	0.0125
	0.05	0.05	0.05
Glyphosate (µM)	500	500	500
	5000	5000	5000
POEA (µM)	13.6	13.6	13.6
	17.9	17.9	17.9
	22.4	22.4	22.4
One a year path weed (%)	0.0005	0.0005	0.0005
	0.005	0.005	0.005
Lab mixture of 'Once-a-year path weed (%)	0.0005	0.0005	0.0005
	0.005	0.005	0.005
Amitrole (µM)	35	35	35
	350	350	350
Amm. thiocyanate (mM)	35	35	35
	350	350	350

Simazine (µM)	22.3	22.3	22.3
	223	223	223

4.2.5 Cell viability assays.

4.2.5.1 MTT assay

4.2.5.1.1 Caffeine treatment.

A 0.1 ml of suspension (prepared in section 4.2.4.1) was added to 0.9 ml medium in a 24 well plate to have $1x10^5$ cells/ml. To prepare MTT-Cell mixture for each dose, a 0.5 ml (contains $5X10^4$ cells) from the previous step was added to 0.22 ml media plus 0.08 ml of 5 g/mg MTT solution. From the prepared MTT-Cell mixture a 0.16 ml was transferred to a 96 well plate. Each dose had four replicates. The loaded plate was then incubated for 4h in 37°C 5% CO_2 incubator. After the 4h incubation, 50 µl of 20% SDS in 0.02 HCl was added to each well & incubated overnight (o/n) at room temperature in the dark. In the next day, a spectrophotometer was used to read the treated plates at 570nm with background absorbance 630nm wavelength.

4.2.5.1.2 The combination treatment

A 50 µl of a suspension containing $1X10^4$ cells was transferred to a 96 well plate. Four replicates were performed for each dose. The volume in the prepared wells was raised up to 0.11 ml using MTT solution (0.5 g/mg). Thus, the total volume of MTT-Cell mixture in each well was 0.16 ml containing $1X10^4$ cells. The prepared plate was then incubated for 4h in 37°C 5% CO_2 incubator. After the 4h incubation, 50 µl of 20% SDS in 0.02 HCl was added to each well & incubated o/n at room temperature in the dark. In the next day, the treated plates were then read using a spectrophotometer (570nm, 630nm wavelength).

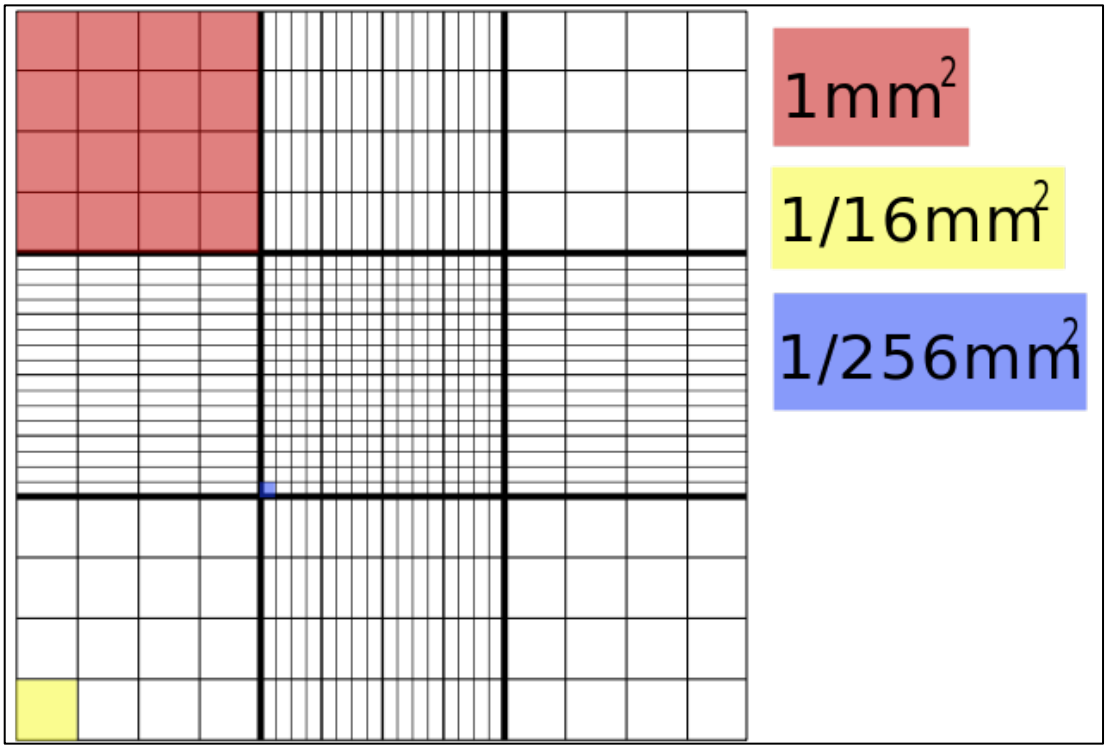

Figure 4.2: Haemocytometer grid.
(Wikimedia, 2015).

Equation 4-1: Calculating cell concentration (cells/ml)

Cell/ml= the average of viable cells X 2 (dilution factor) X 10^4 (correction factor)

Equation 4-2: Evaluating the average of the viable cells.

$$Avrage\ of\ viable\ cells = \frac{\text{total \# of counted cells}}{\text{\# of the counted squares}}$$

4.2.5.2 Trypan blue assay for the combination treatment.

A 50 µl was taken from the cell suspension prepared in section 0 and stained with 50µl Trypan blue making 1:1 dilution. A 10 µl of the stained cells was loaded into each chamber of a haemocytometer. Using light microscopy, viable and dead cells was counted in large squares (1mm^2; see Figure 4.2). The average of

viable cell count was calculated using Equation 4-2**Error! Reference source not found.**. Also, cell concentration (cells/ml) was calculated using Equation 4-1.

4.2.6 Statistics.

The minimum replicates that were performed in both caffeine treatment and caffeine-herbicides treatment were quadruplicate. The data was presented as mean ± standard error of the mean. Also, IMB SPSS software (version 22) was used for statistical analyses. One way ANOVA with Tukey Post-Hoc test were performed to compare data between groups and the level of significance was expressed as $p< 0.05$ or $p< 0.001$.

4.3 Results.

4.3.1 Caffeine treatment.

WIL2-NS cells were exposed to different caffeine concentrations (5, 25, 50, 100 and 500 µM) for various time exposure (1, 4 and 24 hours). All caffeine's concentrations within the three exposure times showed no effects on the proliferation of β lymphocyte cells (see Figure 4.3).

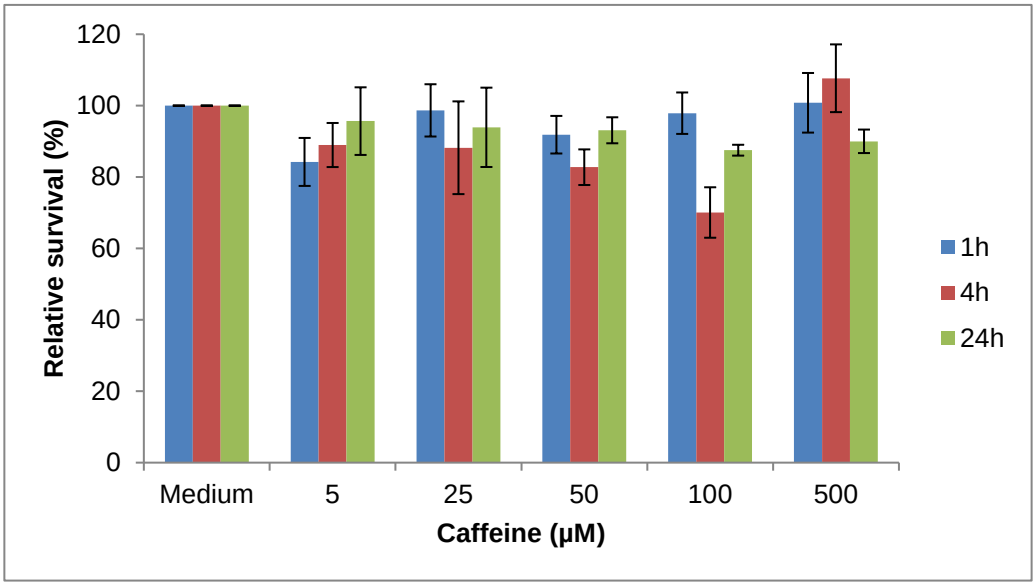

Figure 4.3: The effect of caffeine on WIL2-NS cells after 1, 4, 24h treatments.
The data was presented as mean ± standard error of the mean.

4.3.2 Caffeine-herbicides treatment.

The two products and their active ingredients were mixed with caffeine and the toxicity of the mixtures was examined using MTT and trypan blue assays.

4.3.2.1 Roundup product.

For roundup (R-up) product, two concentrations of R-up and glyphosate and three doses of POEA, the surfactant, were mixed with tow caffeine concentrations. Using the MTT assay, the commercial product showed a significant ($p< 0.05$) reduction in cell viability at 0.05% only when it was mixed with caffeine (see Figure 4.4). Glyphosate results did not show any adverse effect on the WIL2-NS cells (see Figure 4.4). A significant cell killing has been shown in a dose-dependent pattern when cells exposed to the surfactant, POEA. The significant ($p< 0.05$) decrease started at 13.6 μM whether it combined with caffeine or not (see Figure 4.4).

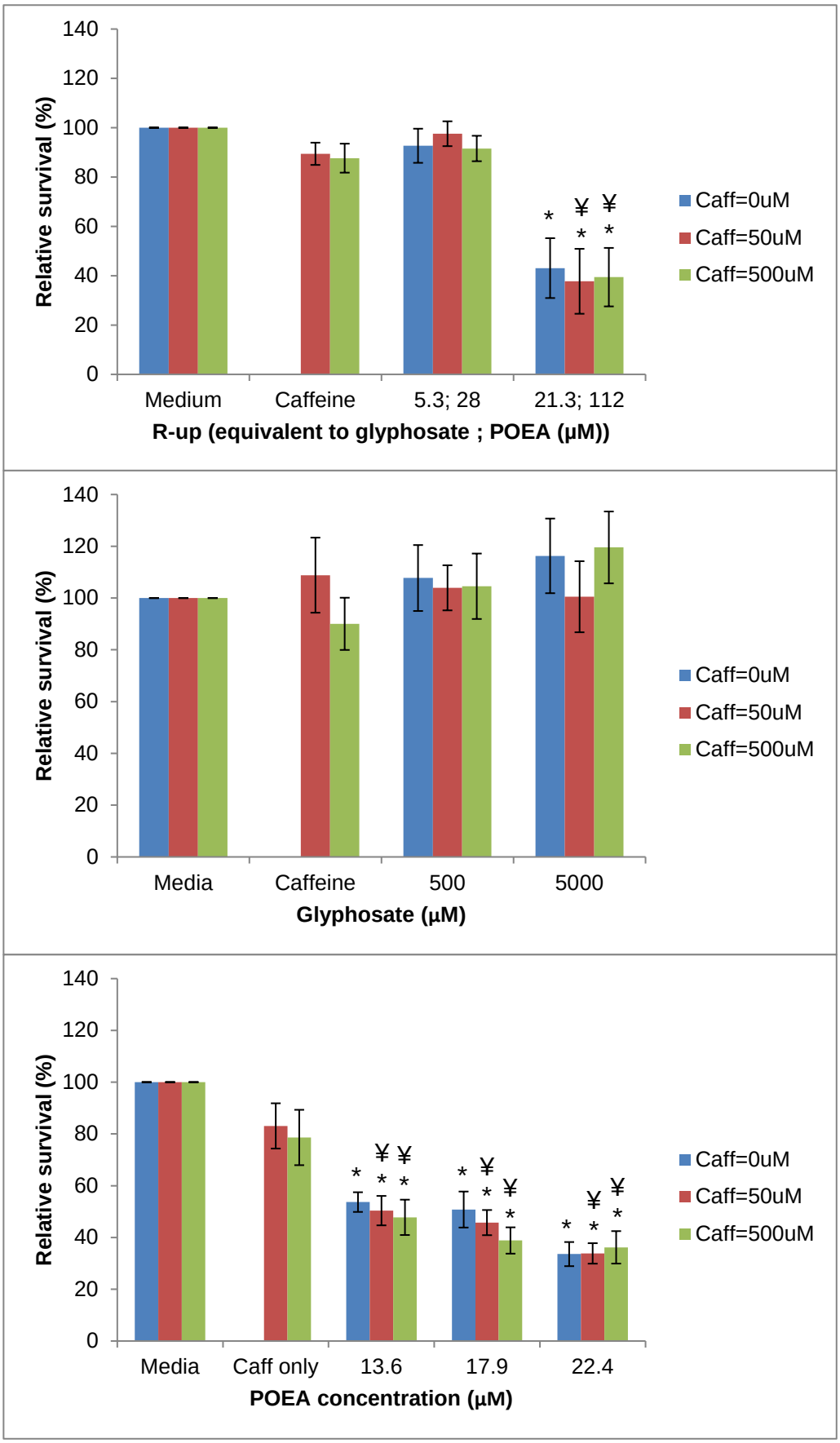

Figure 4.4: Effects of the combination of caffeine-roundup (R-up), caffeine-glyphosate and caffeine-POEA on WIL2-NS exposed for 4h using MTT assay.

The data was presented as mean ± stander error of the mean. The level of significance was expressed as $p < 0.05$ whereas * is comparing data with media (negative control), and ¥ is comparing data with caffeine of the same concentration.

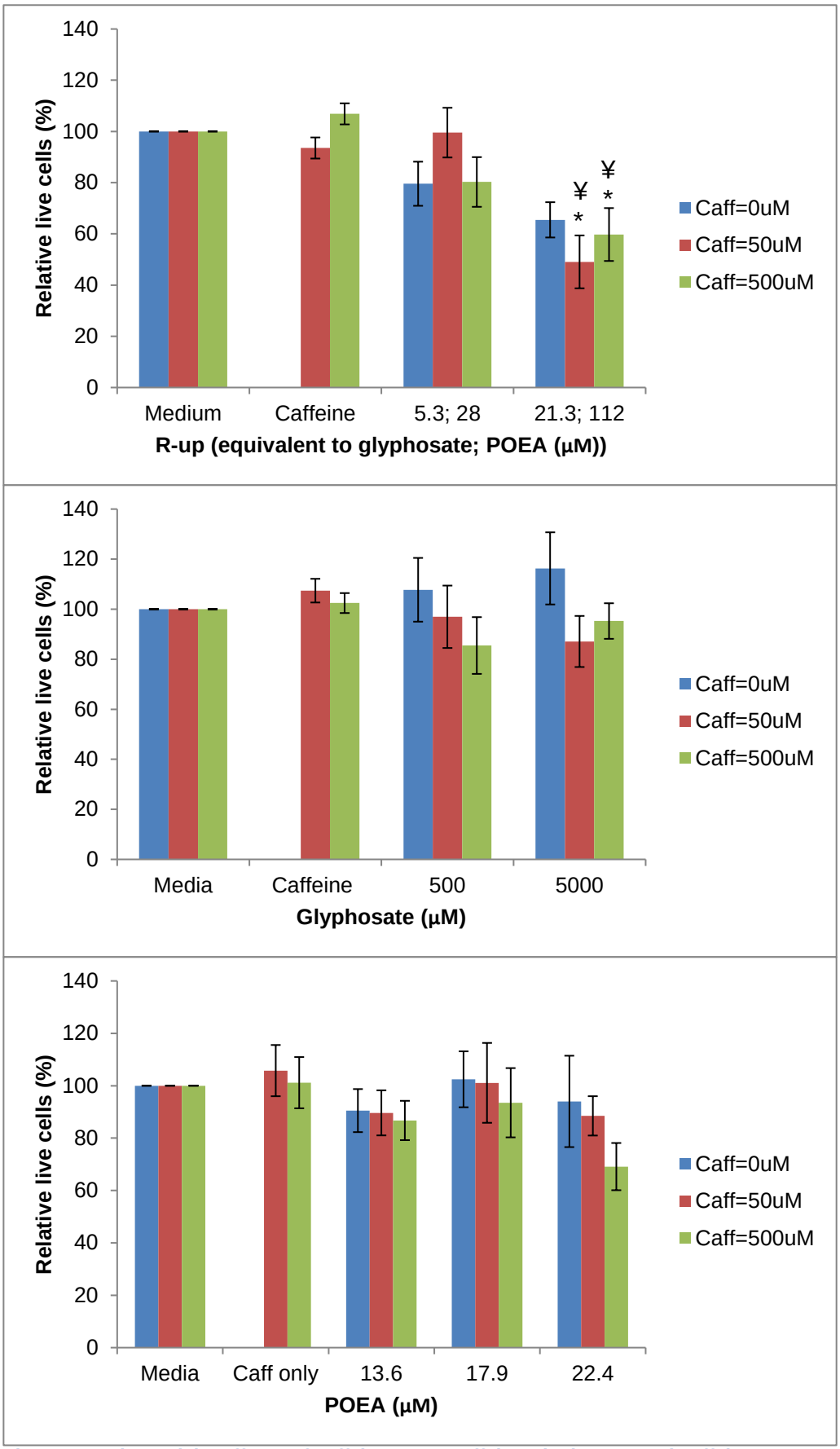

Figure 4.5: The toxicity effects of caffeine-R-up, caffeine-glyphosate and caffeine-POEA combination on the WIL2-NS cells using trypan blue assay.

The data was presented as mean ± stander error of the mean. The level of significance was expressed as *p*< 0.05 whereas * is comparing data with media (negative control), and ¥ is comparing data with caffeine of the same concentration.

Figure 4.5 showed the trypan blue results derived from exposing the WIL2-NS cell line for 4h to R-up-, glyphosate- and POEA- caffeine combinations. Caffeine-glyphosate and caffeine-POEA did not present any significant cell diminishing after exposing the WIL2-NS cells for 4h. Only when roundup was mixed with caffeine, the cell proliferation was significantly impacted.

4.3.2.2 Once a year path weed product.

Treating WIL2-NS cells with caffeine-path weed killer did not result in significant toxic activity after 4h exposure. Caffeine-active ingredients mixtures showed the same thing except at the highest concentration of both caffeine and herbicides whereas they reach over 200% cell viability (see Table II).

The results derived from using trypan blue assay did not detect any toxicity agonist the WIL2-NS cells (see Table II).

4.4 Discussion.

The acute toxicity of caffeine is low, and the risk of accidents (inhalation, contact or ingestion) at worksites is negligible (Oestreich-Janzen, 2016). The European Food Safety Authority (E.F.S.A (2015), concluded in their publication on the safety of caffeine that the adult daily consumption of up to 400 mg caffeine as a habit intake from all sources do not give rise to safety concerns. In this study, it seems that caffeine between 50-500 μM is safe on the β lymphocyte cell line. No significant cell reduction was appraised after 1, 4 or 24h treatment.

No previous studies examined the effect of the combination of caffeine and herbicides. Four hour exposure to 0.05% (500 mg/L) R-up was enough to cause a significant killing ($p< 0.05$) compared to the negative control using MTT assay, but the cell killing did not reach the significance using trypan blue assay. The human cell line TR146 was exposed to different concentrations of R-up for 20 minutes, and

the significant effect was seen at dose level higher than 40 mg/L using XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide) assays (Koller *et al.*, 2012). However, when R-up mixed with caffeine, the cell killing in both assays was slightly higher than R-up alone, and both assays detected significant cell reductions ($p< 0.05$) comparing to the negative control and caffeine treatments. A 0.05% R-up contains 21.3 μM (3.6 mg/L) glyphosate and 112 μM (50 mg/L) PORA. Glyphosate at much higher concentrations (0.5 and 5 mM) was not toxic to the β lymphocyte WIL2-NS cells even when it mixed with caffeine. Glyphosate at 200 mg/L (1.2 mM), also, was not toxic to TR146 cells treated for 20 minutes (Koller *et al.*, 2012). Treating WIL2-NS cells with POEA for 4h showed a dose-dependent effect giving a significant reduction in the cell viability ($p< 0.05$) for the three tested doses (13.6, 17.9 and 22.4 μM) comparing to the negative control using MTT assay. POEA at 25 μM suppressed 96.5% of the mitochondrial function in the L-929 cell line derived from mouse fibroblasts (Song *et al.*, 2012b). On contrast, the results were different using trypan blue assay whereas there were not effects derived from the three doses. The reason for that may because 4h POEA treatment loses some of the cell functions including mitochondrial ones, but it did not affect the membrane integrity. The healthy cells and cells that losing functions but still alive cannot be distinguished using trypan blue staining assay (Dojindo, 2016). Caffeine increased the influence of POEA, but the cell proliferations were not affected significantly comparing to the effect of POEA itself. However, compared to negative and caffeine controls, these influences were significant ($p< 0.05$), and they exhibited in a dose-dependent manner.

The second commercial product, the path weed killer, and its active ingredients were not toxic to the WIL2-NS cells at the tested concentrations for 4h treatment. Even when they are combined with 50 and 500 μM caffeine, there were no toxic effects illustrated in both MTT and trypan blue assays. Regarding amitrole, this result was expected because Its toxicity is considered to be low (Sellamuthu,

2014). Twenty hours exposure of 1.2 mM amitrole (100 mg/L) has no significant impact on the proliferation of the rat thyroid follicular cells (Pan *et al.*, 2011). The highest tested concentration of amm thiocyanate was 27 mg/L (0.35 mM). An *in vivo* study showed that the thyroid hormone L-thyroxine (T_4) have been reduced at 35 mg/L amm thiocyanate leading to thyroid dysfunction, but there were no significant modifications in the morphological and histological characteristics of rainbow trout fishes (Lanno and Dixon, 1996b). However, the effects of thiocyanate rely on the temperature, pH and hardness of the water (Bhunia *et al.*, 2000). Treating WIL2-NS for 4h by 22.3 and 223 µM simazine was not affecting cells proliferation. Another study supported this result when the human ovarian KGN cells exposed to 0.1 to 1 µM simazine for 24h and there was no decrease in its viability and/or proliferation (Park *et al.*, 2014). Surprisingly, mixing the highest concentrations of the lab mixture, amitrole, amm thiocyanate and simazine with 500 µM caffeine resulted in significant increases of cell viabilities ($p< 0.05$) only using MTT assay. The reason why that increase did not appear using trypan blue assay is that caffeine increases, in part, the biogenesis of the mitochondrial (McConell *et al.*, 2010). Caffeine increases the intracellular calcium (Ca^{+2}) which increases the mitochondrial matrix Ca^{+2} (McConell *et al.*, 2010). The latter regulates the activities of some mitochondrial dehydrogenases (Finkel *et al.*, 2015). It, also, increases some markers which activate mitochondrial biogenesis (McConell *et al.*, 2010). Increased cytosolic Ca^{+2} by caffeine, moreover, induce the nitric oxide synthase (NOS) activity which, also, linked with mitochondrial biogenesis progression (see Figure 4.6) (McConell *et al.*, 2010).

4.5 Conclusion.

To sum up, exposing WIL2-NS cell line to Roundup (R-up) and its surfactant, polyethoxylated tallow amine (POEA) for 4h resulted in a significant decrease in cell viabilities. Combining caffeine with herbicides did not significantly increase the effects of the herbicides on WIL2-NS cells, nor did it protect the cells.

Table II: The results of caffeine-Yates® product combination using MTT and trypan blue assays.

		MTT assay			Trypan Blue assay		
Chemicals		Caffeine (µM)			Caffeine (µM)		
		0	50	500	0	50	500
Caffeine (µM)		____	90 %	108 %	____	110 %	99.3 %
Path Weed Killer (%)	0.0005	88.7%	61.8%	69.1%	93.1%	97.3%	110%
	0.005	85.8%	73.6%	94.3%	112.6%	112.7%	108%
Lab Mixture (%)	0.0005	140%	91.5%	130%	108%	91.4%	103%
	0.005	86.4%	116%	292% (* ; i ; ¥)	92%	119%	114%
Amitrole (µM)	35	148%	118%	111%	103%	102%	104%
	350	164%	130%	256% (*)	108%	111%	104%
Ammonium thiocyanate (µM)	35	133.4%	153%	128%	115%	114%	99%
	350	126%	158%	226% (*)	102%	105%	119%
Simazine (µM)	22.3	78.6%	112%	95.4%	94.6%	98.5%	90%
	223	99.3%	123%	220% (*; i)	96.5%	95.4%	102%

The data was presented as mean ± stander error of the mean. The level of significance was expressed as $p < 0.05$ whereas * is comparing data with media (negative control), ¥ is comparing data with caffeine of the same concentration and i is comparing to the chemicals at the same concentration.

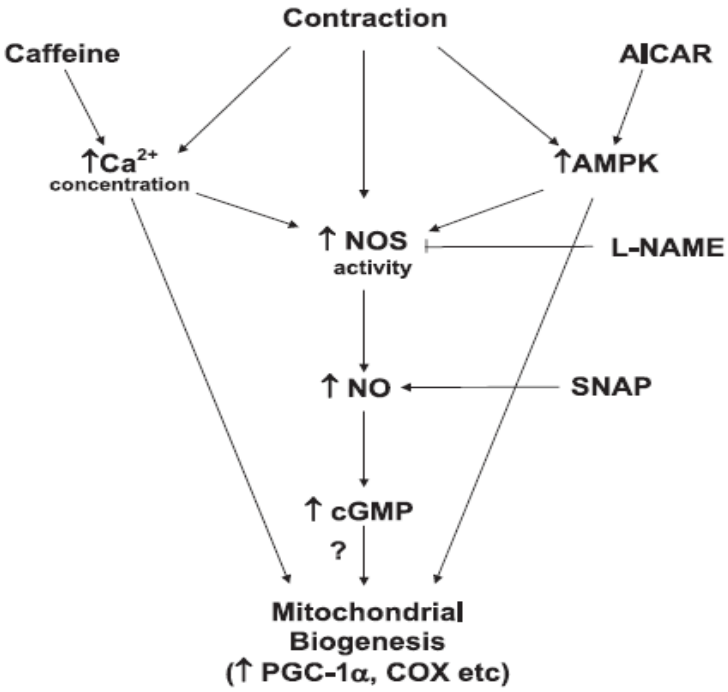

Figure 4.6: the pathways to regulate the mitochondrial biogenesis by caffeine and amide-ribonuleoside (AICAR).
(McConell *et al.*, 2010)

5 CHAPTER V: MAJOR FINDINGS AND GENERAL DISCUSSION.

People are exposed to agricultural products either directly or indirectly (Bi *et al.*, 2011). This work is based on the two principal ways in which humans could be exposed to the agricultural products. The first one is to assess the cytotoxicity of direct contact with droplets of two commercial herbicidal products plus their active ingredients on human skin. Also, this part of the study illustrated how certain chemicals that potentially play key roles of the products' cytotoxicity. The second part is to see if caffeine, the most consumed substance, increase or decrease the level of the toxicity of these chemicals when they reach the blood stream whether through dietary ingestion or penetrating the skin.

In the first part, the human skin cells, HaCaT, was exposed to low doses of the herbicides and their active ingredients for 24h. HaCaT cells are non-tumorigenic human keratinocyte cells with the ability to adhere to plastic surfaces. The cytotoxicity was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and crystal violate assays that have different end points. MTT is a calorimetric assay that based on the mitochondrial function in the viable cells (Henriksson *et al.*, 2006; Young *et al.*, 2005; Dojindo, 2016). Crystal violet staining assay is widely used to stain the adhered living cells in experimental plates (Castro-Garza *et al.*, 2007; Vandersickel *et al.*, 2011). Both commercial herbicides were significantly toxic at a concentration as much as 0.005% of the product. These toxicities were matching the effects of some of their active ingredients and surfactants. The surfactant, polyethoxylated tallow amine (POEA), was the main responsible for the toxicity of Roundup® (R-up) because its cytotoxic events appeared at much lower concentrations than the concentration fund in the commercial product. In once a year path weed product, simazine seems to be the source of the cytotoxicity of the commercial product. However, studying the toxicity of ammonium thiocyanate mixed with amitrole is required because its effects could be amplified when is mixed with the active ingredient amitrole (Balkisson *et al.*, 1992).

The blood β lymphocyte WIL2-NS cell line was used in the second part of this study. They are cells that were originally isolated from the spleen (Levy *et al.*, 1968; ATCC, 2014). These cells are suspension cells, so crystal violet assay has been replaced with trypan blue assay beside the MTT assay. The Trypan blue assay is based on the membrane integrity, so the cytoplasm of the dead cells with damaged membrane will be stained (Dojindo, 2016; Avelar-Freitas *et al.*, 2014; Chung *et al.*, 2015). Two caffeine doses (50 and 500 μM) were mixed with the two commercial herbicides to study the effect of the caffeine on the cytotoxicity of these herbicides. The time exposure was 4 hours as it is the half-life of caffeine in human (Fredholm *et al.*, 1999). The results of these combinations showed that caffeine did not significantly affect the toxic activities of the herbicides even though it's present with some herbicidal chemicals were slightly more toxic.

Figure 5.1 shows comparisons of R-up, Path weed killer, POEA and simazine effects within 4h and 24h treatments using MTT assay. Four-hour treatment was sufficient to increase significantly the cell killing of WIL2-NS cells by R-up and POEA at the concentration of 0.05% (containing 213 μM glyphosate and 112 μM POEA) and 13.6 μM, respectively. Also, 24h treatment of POEA at 22.4 μM resulted in a significant ($p< 0.05$) reduction of the cell viability comparing to its 4h treatment (see Figure 5.1C). Although simazine did not suppress the mitochondrial activities in the 4h experiment, the 24h effect of 223 μM simazine was significant ($p< 0.05$) comparing to both the negative control and its 4h treatment (see Figure 5.1D). The other chemicals including glyphosate, amitrole, amm thiocyanate and the lab mixture did not show any significant results in 4h nor 24h.

In the future, several areas could be addressed to this sort of study. For example, using flow-cytometric analysis, the cell death mechanisms or the mechanism of action that result from the cytotoxicity of the selected chemicals could be determined whereas flow cytometric methods use the changed characteristics such as

alterations in DNA or in membrane integrity to assess apoptosis or necrosis cell death, respectively (Bertho *et. al,* 2000). Western Blotting technique for p53 could, also, be performed to elucidate the carcinogenic effects of samples on the selected cell line. The main role of p53 is that detecting DNA damage or oncogenic stress during a cell cycle then blocking this cycle. Therefore, as soon as it is down-regulated, unchecked proliferation of mutant cells appears (Coutts *et al*, 2009). Genotoxic and cytotoxic effects could be measured by implying a comprehensive assay known as The cytokinesis-block micronucleus (CBMN) assay (Fenech, 2007). It measures the DNA damage events happened in once-divided binucleated cells and include and measures the apoptotic and necrotic cell death (Fenech, 2007; Koller *et al*, 2012).

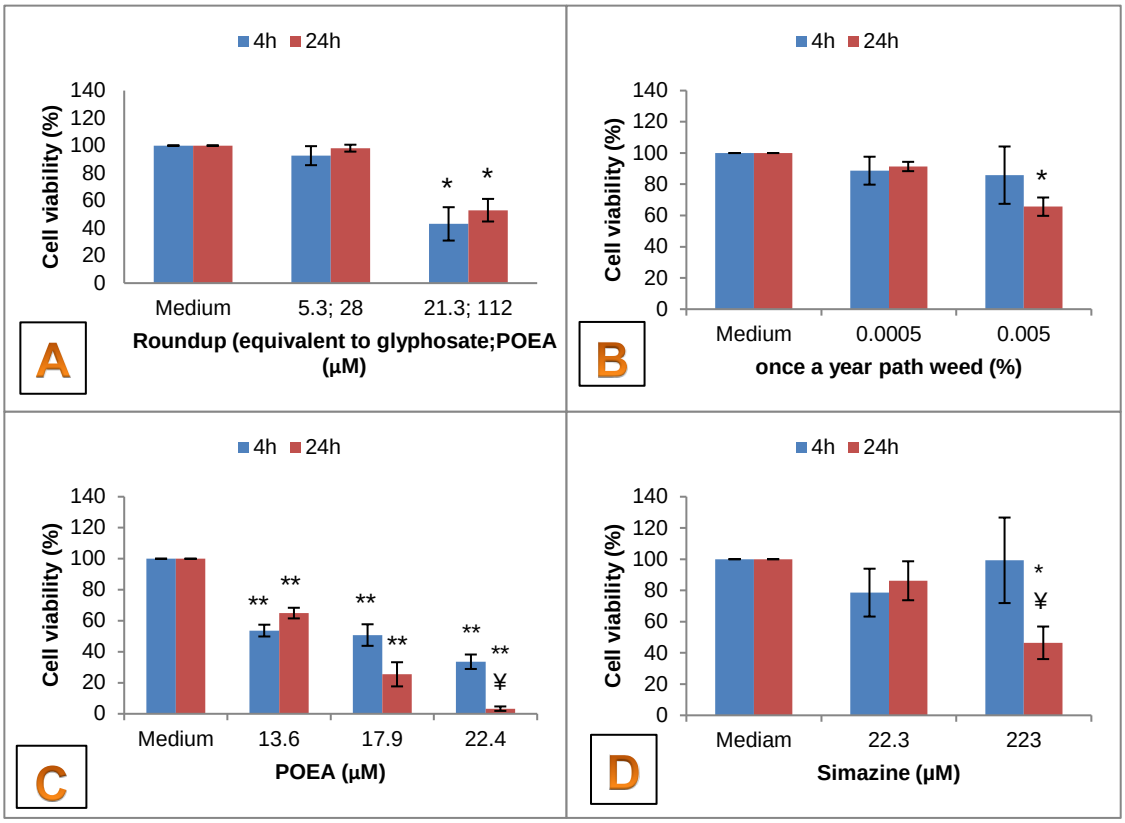

Figure 5.1: Treating WIL2-NS cells for 4h and HaCaT cells for 24h with (A) Roundup, (B) once a year path weed, (C) POEA and (D) simazine.

The data was presented as mean ± standard error of the mean. The level of significance was expressed as * = $p < 0.05$ and ** = $p < 0.001$ and both compared with the negative control Medium. ¥ is a significant result ($p < 0.05$) comparing data with 4h treatment.

6 REFERENCE LIST

Acton, QA 2013, *Testosterone Congeners—Advances in Research and Application,* 2013 edn*,* ScholarlyEditions, Atlanta.

Aihw 2010, Cancer in Australia: an overview, *Australian Institute of Health and Welfare,* 60: 1-227.

Alnuqaydan, AM, Lenehan, CE, Hughes, RR & Sanderson, BJ 2014, Extracts from Calendula officinalis Offer in Vitro Protection Against H O Induced Oxidative Stress Cell Killing of Human Skin Cells, *Phytother Res*.

Amerio, P, Motta, A, Toto, P, Pour, SM, Pajand, R, Feliciani, C & Tulli, A 2004, Skin toxicity from glyphosate-surfactant formulation, *J Toxicol Clin Toxicol,* 42: 317-9.

Ames, BN, Profet, M & Gold, LS 1990a, Dietary pesticides (99.99% all natural), *Proc Natl Acad Sci U S A,* 87: 7777-81.

Ames, BN, Profet, M & Gold, LS 1990b, Nature's chemicals and synthetic chemicals: comparative toxicology, *Proc Natl Acad Sci U S A,* 87: 7782-6.

Atcc 2014, *WIL2-NS (ATCC® CRL-8155™)*, viewed 30/04/2016, <http://www.atcc.org/Products/All/CRL-8155.aspx>.

Auclair, Y, Rouget, R, Affar El, B & Drobetsky, EA 2008, ATR kinase is required for global genomic nucleotide excision repair exclusively during S phase in human cells, *Proc Natl Acad Sci U S A,* 105: 17896-901.

Avelar-Freitas, BA, Almeida, VG, Pinto, MC, Mourao, FA, Massensini, AR, Martins-Filho, OA, Rocha-Vieira, E & Brito-Melo, GE 2014, Trypan blue exclusion assay by flow cytometry, *Braz J Med Biol Res,* 47: 307-15.

Baeurle, S & Kroener, J 2004, Modeling effective interactions of micellar aggregates of ionic surfactants with the Gauss-core potential, *J Math Chem,* 36: 409-21.

Balkisson, R, Murray, D & Hoffstein, V 1992, Alveolar damage due to inhalation of amitrole-containing herbicide, *Chest,* 101: 1174-6.

Band, PR, Abanto, Z, Bert, J, Lang, B, Fang, R, Gallagher, RP & Le, ND 2011, Prostate cancer risk and exposure to pesticides in British Columbia farmers, *Prostate,* 71: 168-83.

Barrett, JC 1993, Mechanisms of multistep carcinogenesis and carcinogen risk assessment, *Environ Health Perspectives,* 100: 9-20.

Benachour, N & Seralini, GE 2009, Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells, *Chem Res Toxicol,* 22: 97-105.

Berthe-Corti, L, Jacobi, H, Kleihauer, S & Witte, I 1998, Cytotoxicity and mutagenicity of a 2,4,6-trinitrotoluene (TNT) and hexogen contaminated soil in S. typhimurium and mammalian cells, *Chemosphere,* 37: 209-18.

Bertho, AL, Santiago, MA & Coutinho, SG 2000, Flow cytometry in the study of cell death, *Mem Inst Oswaldo Cruz,* 95: 429-33.

Bhunia, F, Saha, NC & Kaviraj, A 2000, Toxicity of thiocyanate to fish, plankton, worm, and aquatic ecosystem, *Bull Environ Contam Toxicol,* 64: 197-204.

Bi, H, Rissik, D, Macova, M, Hearn, L, Mueller, JF & Escher, B 2011, Recovery of a freshwater wetland from chemical contamination after an oil spill, *Journal of Environmental Monitoring,* 13: 713-20.

Blair, A & Zahm, SH 1993, Patterns of pesticide use among farmers: implications for epidemiologic research, *Epidemiology,* 4: 55-62.

Block, G, Patterson, B & Subar, A 1992, Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence, *Nutr Cancer,* 18: 1-29.

Bode, AM & Dong, Z 2007, The enigmatic effects of caffeine in cell cycle and cancer, *Cancer Lett,* 247: 26-39.

Bode, AM & Dong, Z 2003, Mitogen-activated protein kinase activation in UV-

induced signal transduction, *Sci STKE,* 2003: RE2.
Boukamp, P, Petrussevska, RT, Breitkreutz, D, Hornung, J, Markham, A & Fusenig, NE 1988, Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line, *J Cell Biol,* 106: 761-71.
Bradberry, SM, Proudfoot, AT & Vale, JA 2004, Glyphosate poisoning, *Toxicol Rev,* 23: 159-67.
Brausch, JM & Smith, PN 2007, Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, Thamnocephalus platyurus, *Arch Environ Contam Toxicol,* 52: 217-21.
Brazil, DP, Yang, ZZ & Hemmings, BA 2004, Advances in protein kinase B signalling: AKTion on multiple fronts, *Trends Biochem Sci,* 29: 233-42.
Budaev, SL, Batoeva, AA & Tsybikova, BA 2015, Degradation of thiocyanate in aqueous solution by persulfate activated ferric ion, *Minerals Engineering,* 81: 88-95.
Carrillo, JA & Benitez, J 2000, Clinically significant pharmacokinetic interactions between dietary caffeine and medications, *Clin Pharmacokinet,* 39: 127-53.
Castro-Garza, J, Barrios-Garcia, HB, Cruz-Vega, DE, Said-Fernandez, S, Carranza-Rosales, P, Molina-Torres, CA & Vera-Cabrera, L 2007, Use of a colorimetric assay to measure differences in cytotoxicity of *Mycobacterium tuberculosis* strains, *Journal of Medical Microbiology,* 56: 733-7.
Ccme 2008, Canadian Soil Quality Guidelines for Carcinogenic and Other Polycyclic Aromatic Hydrocarbons (Environmental and Human Health Effects), *Canadian Council of Ministers of the Environment (CCME)*: 1-218.
Chiba, K, Kawakami, K & Tohyama, K 1998a, Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells, *Toxicol In Vitro,* 12: 251-8.
Chiba, K, Kawakami, K & Tohyama, K 1998b, Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells, *Toxicology In Vitro,* 12: 251-8.
Chin, DH, Li, HH, Kuo, HM, Chao, PD & Liu, CW 2011, Neocarzinostatin as a probe for DNA protection activity-molecular interaction with caffeine, *Mol Carcinog*.
Chung, DM, Kim, JH & Kim, JK 2015, Evaluation of MTT and Trypan Blue assays for radiation-induced cell viability test in HepG2 cells, *International Journal of Radiation Research,* 13: 331-5.
Cloonan, SM & Williams, DC 2011, The antidepressants maprotiline and fluoxetine induce Type II autophagic cell death in drug-resistant Burkitt's lymphoma, *International Journal of Cancer,* 128: 1712-23.
Coutts, A, Adams, C & Thangue, N 2009, p53 ubiquitination by Mdm2: A never ending tail?, *DNA Repair,* 8: 483-490.
Danial, NN & Korsmeyer, SJ 2004, Cell death: critical control points, *Cell,* 116: 205-19.
Datta, SR, Brunet, A & Greenberg, ME 1999, Cellular survival: a play in three Akts, *Genes Dev,* 13: 2905-27.
Davies, DB, Veselkov, DA, Djimant, LN & Veselkov, AN 2001, Hetero-association of caffeine and aromatic drugs and their competitive binding with a DNA oligomer, *Eur Biophys J,* 30: 354-66.
Dellarco, VL & Wiltse, JA 1998, US Environmental Protection Agency's revised guidelines for Carcinogen Risk Assessment: incorporating mode of action data, *Mutat Res,* 405: 273-7.
Deneer, JW 2000, Toxicity of mixtures of pesticides in aquatic systems, *Pest Manag Sci,* 56.
Deshpande, SS 2002, 'Methylxathines', In Deshpande, SS (ed.) *Handbook of food toxicology*, Marcle Dekker, New York 362-4.
Devasagayam, TP & Kesavan, PC 1996, Radioprotective and antioxidant action of caffeine: mechanistic considerations, *Indian J Exp Biol,* 34: 291-7.
Dojindo 2016, *Cell Proliferation Protocol Colorimetric*, viewed 30/05/2016, <https://www.dojindo.com/Protocol/Cell_Proliferation_Protocol_Colorimetric.

pdf>.
Dubrow, R, Darefsky, AS, Freedman, ND, Hollenbeck, AR & Sinha, R 2012, Coffee, tea, soda, and caffeine intake in relation to risk of adult glioma in the NIH-AARP Diet and Health Study, *Cancer Causes Control,* 23: 757-68.
E.F.S.A 2015, Scientific Opinion on the safety of caffeine, *European Food Safety Authority Journal,* 13: 1-120.
E.P.A 2005, EPA Guidelines for Responsible Pesticide Use, *Environment Protection Authority*: 1-87.
Edinger, AL & Thompson, CB 2004, Death by design: apoptosis, necrosis and autophagy, *Current Opinion Cell Biology,* 16: 663-669.
Fenech, M 2000, The in vitro micronucleus technique, *Mutat Res,* 455: 81-95.
Fenech, M 2007, Cytokinesis-block micronucleus cytome assay, Nature Protocols, 2: 1084-104.
Fiers, W, Beyaert, R, Declercq, W & Vandenabeele, P 1999, More than one way to die: apoptosis, necrosis and reactive oxygen damage, *Oncogene,* 18: 7719-30.
Finkel, T, Menazza, S, Holmstrom, KM, Parks, RJ, Liu, J, Sun, J, Liu, J, Pan, X & Murphy, E 2015, The ins and outs of mitochondrial calcium, *Circ Res,* 116: 1810-9.
Fischer, SM, Pavone, A, Mikulec, C, Langenbach, R & Rundhaug, JE 2007, Cyclooxygenase-2 expression is critical for chronic UV-induced murine skin carcinogenesis, *Mol Carcinog,* 46: 363-71.
Fredholm, BB, Battig, K, Holmen, J, Nehlig, A & Zvartau, EE 1999, Actions of caffeine in the brain with special reference to factors that contribute to its widespread use, *Pharmacol Rev,* 51: 83-133.
Freshney, RI 2005, *Culture of Animal Cells: A Manual of Basic Technique,* 5th edn edn, John Wiley & Sons, Hoboken.
Furukawa, A, Oikawa, S, Harada, K, Sugiyama, H, Hiraku, Y, Murata, M, Shimada, A & Kawanishi, S 2010, Oxidatively generated DNA damage induced by 3-amino-5-mercapto-1,2,4-triazole, a metabolite of carcinogenic amitrole, *Mutat Res,* 694: 7-12.
Gasnier, C, Dumont, C, Benachour, N, Clair, E, Chagnon, MC & Seralini, GE 2009, Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines, *Toxicology,* 262: 184-91.
Gehin, A, Guyon, C & Nicod, L 2006, Glyphosate-induced antioxidant imbalance in HaCaT: The protective effect of Vitamins C and E, *Environ Toxicol Pharmacol,* 22: 27-34.
Giesy, JP, Dobson, S & Solomon, KR 2000, 'Ecotoxicological Risk Assessment for Roundup® Herbicide', In Ware, GW (ed.) *Reviews of Environmental Contamination and Toxicology*, Springer-Verlag, New York, 35-120.
Gillies, RJ, Didier, N & Denton, M 1986, Determination of cell number in monolayer cultures, *Analytical Biochemistry,* 159: 109-13.
Gold, LS, Slone, TH & Ames, BN 2001a, 'Pesticide Residues in Food and Cancer Risk: A Critical Analysis', In Krieger, R (ed.) *Handbook of Pesticide Toxicology,* 2nd edn, CA: Academic Press, San Diego, pp 799-843.
Gold, LS, Slone, TH & Ames, BN 2001b, 'Pesticide residues in food and cancer risk: a critical analysis', In Krieger, R (ed.) *Handbook of pesticide toxicology,* 2nd edn, Academic Press, San Diego, 799-843.
Gracia, R & Shepherd, G 2004, Cyanide poisoning and its treatment, *Pharmacotherapy,* 24: 1358-65.
Green, SJ, Holsapple, MP, Saunders, S & Thompson, TN 2010, Dossier in support of the generally recognized as safe (GRAS) status of caffeine as an ingredient in alcoholic beverages, *Fusing Science and Complince*: 1-79.
Gunter, MJ, Schaub, JA, Xue, X, Freedman, ND, Gaudet, MM, Rohan, TE, Hollenbeck, AR & Sinha, R 2012, A prospective investigation of coffee drinking and endometrial cancer incidence, *Int J Cancer,* 131: E530-6.
Haggblom, MM & Milligan, PW 2000, 'Anaerobic Biodegradation of Halogenated

Pesticides: Influence of Alternate Electron Acceptors', In Bollag, J & Stotzky, G (eds.), *Soil Biochemistry*, Marcel Dekker, INC, New York, 1-34.

Han, W, Ming, M & He, YY 2011, Caffeine promotes ultraviolet B-induced apoptosis in human keratinocytes without complete DNA repair, *J Biol Chem,* 286: 22825-32.

Henriksson, E, Kjellen, E, Wahlberg, P, Wennerberg, J & Kjellstrom, JH 2006, Differences in estimates of cisplatin-induced cell kill in vitro between colorimetric and cell count/colony assays, *In Vitro Cellular & Developmental Biology,* 42: 320-3.

Hill, MJ, Giacosa, A & Caygill, CPJ (eds.) 1994, *Epidemiology of diet and cancer* Ellis Horwood, New York.

Holick, CN, Smith, SG, Giovannucci, E & Michaud, DS 2010, Coffee, tea, caffeine intake, and risk of adult glioma in three prospective cohort studies, *Cancer Epidemiol Biomarkers Prev,* 19: 39-47.

Horton, MK, Blount, BC, Valentin-Blasini, L, Wapner, R, Whyatt, R, Gennings, C & Factor-Litvak, P 2015, CO-occurring exposure to perchlorate, nitrate and thiocyanate alters thyroid function in healthy pregnant women, *Environ Res,* 143: 1-9.

Iarc 1987, Overall evaluations of carcinogenicity: an updating of *IARC monographs*, *International Agency for Research on Cancer,* 1-42 (suppl 7): 92-93.

Ichihara, T, Miyashita, K, Kawabe, M, Imaida, K, Asamoto, M, Ogiso, T, Tamano, S, Hirose, M & Shirai, T 2005, Lack of combination hepatocarcinogenicity of harman, norharman and amitrole when given with NaNO2 in the rat, *J Toxicol Sci,* 30: 1-6.

Iom 2001, *Caffeine for the sustainment of mental task performance: Formulations for military operations,* edn*,* National Academy Press, Washington.

Kastan, MB, Onyekwere, O, Sidransky, D, Vogelstein, B & Craig, RW 1991, Participation of p53 protein in the cellular response to DNA damage, *Cancer Res,* 51: 6304-11.

Kaufmann, WK, Heffernan, TP, Beaulieu, LM, Doherty, S, Frank, AR, Zhou, Y, Bryant, MF, Zhou, T, Luche, DD, Nikolaishvili-Feinberg, N, Simpson, DA & Cordeiro-Stone, M 2003, Caffeine and human DNA metabolism: the magic and the mystery, *Mutat Res,* 532: 85-102.

Kaya, B, Yanikoglu, A, Creus, A & Marcos, R 2000, Genotoxicity testing of five herbicides in the Drosophila wing spot test, *Mutat Res,* 465: 77-84.

Kearney, PC, Kaufman, DD & Alexander, M 1967, 'Biochemistry of Herbicide Decomposition in Soils', In Mclaren, AD & Peteson, GH (eds.), *Soil Biochemistry*, Marcel Dekker, INC, New York, 318-42.

Kligerman, AD, Doerr, CL, Tennant, AH & Peng, B 2000b, Cytogenetic studies of three triazine herbicides. II. In vivo micronucleus studies in mouse bone marrow, *Mutat Res,* 471: 107-12.

Kligerman, AD, Doerr, CL, Tennant, AH & Zucker, RM 2000a, Cytogenetic studies of three triazine herbicides. I. In vitro studies, *Mutat Res,* 465: 53-9.

Koller, VJ, Furhacker, M, Nersesyan, A, Misik, M, Eisenbauer, M & Knasmueller, S 2012, Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells, *Arch Toxicol,* 86: 805-13.

Krasilnikov, MA 2000, Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation, *Biochemistry (Mosc),* 65: 59-67.

Kueng, W, Silber, E & Eppenberger, U 1989, Quantification of cells cultured on 96-well plates, *Analytical Biochemistry,* 182: 16-9.

Lachance, B, Robidoux, PY, Hawari, J, Ampleman, G, Thiboutot, S & Sunahara, GI 1999, Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro, *Mutation Researsh,* 444: 25-39.

Lanno, RP & Dixon, DG 1996b, Chronic toxicity of waterborne thiocyanate to rainbow trout (Oncorhynchus mykiss), *Canadian Journal of Fisheries and Aquatic Sciences,* 53: 2137-2146.

Lee, HL, Kan, CD, Tsai, CL, Liou, MJ & Guo, HR 2009, Comparative effects of the formulation of glyphosate-surfactant herbicides on hemodynamics in swine, *Clin Toxicol (Phila),* 47: 651-8.

Lee, S, Mclaughlin, R, Harnly, M, Gunier, R & Kreutzer, R 2002, Community exposures to airborne agricultural pesticides in California: ranking of inhalation risks, *Environ Health Perspect,* 110: 1175-84.

Legras, A, Skrobala, D, Furet, Y, Kintz, P, Forveille, E, Dequin, PF & Perrotin, D 1996, Herbicide: fatal ammonium thiocyanate and aminotriazole poisoning, *J Toxicol Clin Toxicol,* 34: 441-6.

Levine, B & Klionsky, DJ 2004, Development by self-digestion: molecular mechanisms and biological functions of autophagy, *Development Cell,* 6: 463-77.

Levy, JA, Buell, DN, Creech, C, Hirshaut, Y & Silverberg, H 1971, Further characterization of the WI-L1 and WI-L2 lymphoblastoid lines, *J Natl Cancer Inst,* 46: 647-54.

Levy, JA, Virolainen, M & Defendi, V 1968, Human lymphoblastoid lines from lymph node and spleen, *Cancer,* 22: 517-24.

Lioi, MB, Scarfi, MR, Santoro, A, Barbieri, R, Zeni, O, Di Berardino, D & Ursini, MV 1998, Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures in vitro, *Mutat Res,* 403: 13-20.

Loosli, R 1994, Triazines, *Toxicology,* 91: 59-62.

Lu, YP, Lou, YR, Peng, QY, Xie, JG, Nghiem, P & Conney, AH 2008a, Effect of caffeine on the ATR/Chk1 pathway in the epidermis of UVB-irradiated mice, *Cancer Res,* 68: 2523-9.

Maibach, HI 1983. Elimination of 14C-Glyphosate in Rhesus Monkeys Following a Single Dose: Percutaneous Absorption of 14CGlyphosate in Roundup Formulation in Rhesus Monkeys Following a Single Topical Dose. School of Medicine: University of California, San Francisco.

Mandel, HG 2002, Update on caffeine consumption, disposition and action, *Food Chem Toxicol,* 40: 1231-4.

Marc, J, Mulner-Lorillon, O & Belle, R 2004a, Glyphosate-based pesticides affect cell cycle regulation, *Biol Cell,* 96: 245-9.

Marc, J, Mulner-Lorillon, O, Boulben, S, Hureau, D, Durand, G & Belle, R 2002, Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation, *Chem Res Toxicol,* 15: 326-31.

Maroni, M, Colosio, C, Ferioli, A & Fait, A 2000, Biological Monitoring of Pesticide Exposure: a review. Introduction, *Toxicology,* 143: 1-118.

Martin, A & Clynes, M 1993, Comparison of 5 microplate colorimetric assays for in vitro cytotoxicity testing and cell proliferation assays, *Cytotechnology,* 11: 49-58.

Martini, CN, Gabrielli, M & Vila Mdel, C 2012, A commercial formulation of glyphosate inhibits proliferation and differentiation to adipocytes and induces apoptosis in 3T3-L1 fibroblasts, *Toxicol In Vitro,* 26: 1007-13.

Mattioli, F, Robbiano, L, Fazzuoli, L & Baracchini, P 1994, Studies on the mechanism of the carcinogenic activity of amitrole, *Fundam Appl Toxicol,* 23: 101-6.

Mcconell, GK, Ng, GP, Phillips, M, Ruan, Z, Macaulay, SL & Wadley, GD 2010, Central role of nitric oxide synthase in AICAR and caffeine-induced mitochondrial biogenesis in L6 myocytes, *J Appl Physiol (1985),* 108: 589-95.

Mcquilkin, SH, Nierenberg, DW & Bresnick, E 1995, Analysis of within-subject variation of caffeine metabolism when used to determine cytochrome P4501A2 and N-acetyltransferase-2 activities, *Cancer Epidemiol Biomarkers Prev,* 4: 139-46.

Meretoja, T, Gripenberg, U, Bamford, D, Laamanen, I & Sorsa, M 1976, Mutagenicity and toxicity of amitrole. II. Human lymphocyte culture tests, *Mutat Res,* 40: 191-6.

Meyer, SL, Lakshman, DK, Zasada, IA, Vinyard, BT & Chitwood, DJ 2008, Phytotoxicity of clove oil to vegtable crop seedlings and nematotoxicity to root-knot nematodes, *Horttechnology,* 18: 631-8.

Mills, PK & Yang, R 2003, Prostate cancer risk in California farm workers, *J Occup Environ Med,* 45: 249-58.

Miners, JO & Birkett, DJ 1996, The use of caffeine as a metabolic probe for human drug metabolizing enzymes, *Gen Pharmacol,* 27: 245-9.

Mori, I, Fonne-Pfister, R, Matsunaga, S, Tada, S, Kimura, Y, Iwasaki, G, Mano, J, Hatano, M, Nakano, T, Koizumi, S, Scheidegger, A, Hayakawa, K & Ohta, D 1995, A Novel Class of Herbicides (Specific Inhibitors of Imidazoleglycerol Phosphate Dehydratase), *Plant Physiol,* 107: 719-723.

Morrison, HI, Wilkins, K, Semenciw, R, Mao, Y & Wigle, D 1992, Herbicides and cancer, *J Natl Cancer Inst,* 84: 1866-74.

Mosmann, T 1983, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *Journal of lmmunological Methods,* 65: 55-63.

Nishikawa, A, Furukawa, F, Imazawa, T, Ikezaki, S, Hasegawa, T & Takahashi, M 1995, Effects of caffeine on glandular stomach carcinogenesis induced in rats by N-methyl-N'-nitro-N-nitrosoguanidine and sodium chloride, *Food Chem Toxicol,* 33: 21-6.

Oesterreich, T, Klaus, U, Volk, M, Neidhart, B & Spiteller, M 1999, Environmental fate of amitrole: influence of dissolved organic matter, *Chemosphere,* 38: 379-92.

Oestreich-Janzen, S 2016, 'Caffeine: Characterization and Properties', In Caballero, B, Finglas, PM & Toldra, F (eds.), *Encyclopedia of Food and Health*, Elserier Ltd, Waltham, 556-72.

Oropesa, AL, Garcia Cambero, JP & Soler, F 2008, Effect of long-term exposure to simazine on brain and muscle acetylcholinesterase activity of common carp (Cyprinus carpio), *Environ Toxicol,* 23: 285-93.

Pan, H, Sun, Y & Zhang, L 2011, The effects of amitrole on thyroglobulin and iodide uptake in FRTL-5 cells, *Toxicol Ind Health,* 27: 187-92.

Park, S, Kim, S, Jin, H, Lee, K & Bae, J 2014, Impaired development of female mouse offspring maternally exposed to simazine, *Environ Toxicol Pharmacol,* 38: 845-51.

Peruzzo, PJ, Porta, AA & Ronco, AE 2008, Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina, *Environ Pollut,* 156: 61-6.

Plumb, JA, Milroy, R & Kaye, SB 1989, Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay, *Cancer Researsh,* 49: 4435-40.

Qi, W, Qiao, D & Martinez, JD 2002, Caffeine induces TP53-independent G(1)-phase arrest and apoptosis in human lung tumor cells in a dose-dependent manner, *Radiat Res,* 157: 166-74.

Richard, S, Moslemi, S, Sipahutar, H, Benachour, N & Seralini, GE 2005, Differential effects of glyphosate and roundup on human placental cells and aromatase, *Environ Health Perspect,* 113: 716-20.

Rothwell, K 1974, Dose-related inhibition of chemical carcinogenesis in mouse skin by caffeine, *Nature,* 252: 69-70.

Sanderson, JT, Letcher, RJ, Heneweer, M, Giesy, JP & Van Den Berg, M 2001, Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes, *Environ Health Perspect,* 109: 1027-31.

Sanderson, JT, Seinen, W, Giesy, JP & Van Den Berg, M 2000, 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity?, *Toxicol Sci,* 54: 121-7.

Saotome, K, Morita, H & Umeda, M 1989, Cytotoxicity test with simplified crystal violet staining method using microtitre plates and its application to injection drugs, *Toxicology In Vitro,* 3: 317-21.

Sarkaria, JN, Busby, EC, Tibbetts, RS, Roos, P, Taya, Y, Karnitz, LM & Abraham, RT 1999, Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine, *Cancer Res,* 59: 4375-82.

Sellamuthu, R 2014, 'Amitrole', In Wexler, P (ed.) *Encyclopedia of Toxicology,* 3rd edn, Elsevier Science Publishing Co Inc, San Diego, 203-205.

Somogyi, LP 2010, Caffeine intake by the U.S. population, *U.S. Food and Drug Administratin*: 1-83.

Song, HY, Kim, YH, Seok, SJ, Gil, HW & Hong, SY 2012a, In vitro cytotoxic effect of glyphosate mixture containing surfactants, *J Korean Med Sci,* 27: 711-5.

Song, HY, Kim, YH, Seok, SJ, Gil, HW, Yang, JO, Lee, EY & Hong, SY 2012b, Cellular toxicity of surfactants used as herbicide additives, *J Korean Med Sci,* 27: 3-9.

Strandberg, MT & Scott-Fordsmand, JJ 2002, Field effects of simazine at lower trophic levels--a review, *Sci Total Environ,* 296: 117-37.

Tadeo, JL (ed.) 2008, *Analysis of pesticides in food and environmental samples,* CRC Press, Boca Raton.

Tennant, AH, Peng, B & Kligerman, AD 2001, Genotoxicity studies of three triazine herbicides: in vivo studies using the alkaline single cell gel (SCG) assay, *Mutat Res,* 493: 1-10.

Traganos, F, Kapuscinski, J, Gong, J, Ardelt, B, Darzynkiewicz, RJ & Darzynkiewicz, Z 1993, Caffeine prevents apoptosis and cell cycle effects induced by camptothecin or topotecan in HL-60 cells, *Cancer Res,* 53: 4613-8.

Tsui, MT & Chu, LM 2003, Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors, *Chemosphere,* 52: 1189-97.

U.S.Epa 2012, *About pesticides*, U.S. Environmental Protiction Agency, viewed 7/9/2012, <http://www.epa.gov/pesticides/about/>.

U.S.Epa 2007, Nitrates and Nitrites: TEACH Chemical Summary *U.S. environmental protiction agency*: 1-14.

Van Hoek, THJ 2000, 'Thiocyanates, Inorganic', In *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA,

Vandersickel, V, Slabbert, J, Thierens, H & Vral, A 2011, Comparison of the colony formation and crystal violet cell proliferation assays to determine cellular radiosensitivity in a repair-deficient MCF10A cell line, *Radiation Measurments,* 46: 72-75.

Wang, N, Besser, JM, Buckler, DR, Honegger, JL, Ingersoll, CG, Johnson, BT, Kurtzweil, ML, Macgregor, J & Mckee, MJ 2005, Influence of sediment on the fate and toxicity of a polyethoxylated tallowamine surfactant system (MON 0818) in aquatic microcosms, *Chemosphere,* 59: 545-51.

Wester, RC, Melendres, J, Sarason, R, Mcmaster, J & Maibach, HI 1991, Glyphosate skin binding, absorption, residual tissue distribution, and skin decontamination, *Fundam Appl Toxicol,* 16: 725-32.

Who 1996, Glyphosate. Environmental Health Criteria No. 159, *World Health Organization*.

Wikimedia 2015, *Haemocytometer grid*, viewed 04/05/2016, <https://commons.wikimedia.org/wiki/File:Haemocytometer_grid.svg>.

Wiklund, K & Dich, J 1995, Cancer risks among male farmers in Sweden, *Eur J Cancer Prev,* 4: 81-90.

Williams, GM, Kroes, R & Munro, IC 2000, Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans, *Regul Toxicol Pharmacol,* 31: 117-65.

Worthing, CR & Walker, SB (eds.) 1983, *The pesticide manual,* The Lavenham press limited, Lavenham.

Www.Cancer.Org.Au 2012a, *Skin cancer*, www.cancer.org.au, viewed 03/04/2013, <http://www.cancer.org.au/about-cancer/types-of-cancer/skin-cancer.html>.

Young, FM, Phungtamdet, W & Sanderson, BJ 2005, Modification of MTT assay conditions to examine the cytotoxic effects of amitraz on the human lymphoblastoid cell line, WIL2NS, *Toxicology In Vitro,* 19: 1051-9.

APPENDICES

Appendix I: The preparation of the used reagents

The preparation of reagents that were used during this study was as follow.

RPMI 1640 (10% FBS) medium:

1) **Powder**: One litter of stock medium was prepared by dissolving 10.44 g of RPMI 1640 media powder, 0.269 g of L-Glutamine powder, and 10 ml of penicillin/streptomycin in 940 ml of MQ water. After that, pH was adjusted to 7. This solution can be stored at -20ºC to be used later. If it was frozen, it dissolved at 37ºC in a water-bath before adding 100ml of FBS, 25ml of Sodium bicarbonate and 3 ml of BME to the solution. The final solution was then filtered using a 0.22 µm filter, and stored at 4ºC.
2) **Liquid**: Worming the 500 ml medium in the water-bath (37°C) for 10 minutes. FBS (50 ml) and Sodium bicarbonate (18 ml) were filtered using a 0.22 µm filter and added to the worm medium. The final solution was then stored at 4ºC.

Phosphate Buffered Saline (PBS):

Preparing 1X PBS:

NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g) and NH_2PO_4 (0.24 g) were dissolved well by mixing them together in 900 ml of MQ water in a 1 L volumetric flask. The saline was then adjusted to pH 7.4, and the volume increased to 1 L. After that, it was poured in a 1 L glass schott bottle, which was sent to the autoclave, or it directly filtered in the schott bottle using 0.22 µm filter. The sterilized PBS was stored at room temperature on a bench.

Preparing 10X PBS:

It is prepared the same way as 1X PBS, but the amounts of the four chemicals were 10 times higher. They were dissolved in the same volume of MQ water. To prepare 1X PBS from the sterilized 10X PBS, 50 ml of 10X PBS mixed with 450 ml MQ water. Both PBSs were stored on the bench at room temperature.

MTT stock solution:

Thiazolyl Blue Tetrazolium Bromide (MTT; 250 mg) was dissolved in 50 ml of 1X PBS. The solution was then filtered and stored at -20ºC.

Crystal violet staining in 50% methanol solution:

Methanol (50 ml) was added to 50 ml MQ water to prepare 50% methanol. CV powder (0.5 g) was added to the 100 ml of 50% methanol. The solution was stored at the room temperature in a fumes hood.

Acetic acid (33%):

MQ water (77 ml) was transferred to a schott bottle. Acetic acid (33 ml) was added to the measured MQ water in the schott bottle. The solution was stored at room temperature in a fumes hood.

β-Mercaptoethanol (BME):

This solution stores at 4ºC after 30 µm of 2- Mercaptoethanol was added to 100 ml of filtered water.

SDS (20%) in 0.02 M HCl:

One molar HCl was prepared by adding 7.5 ml of 36% HCl to 82.5 ml of MQ water. To make 20% SDS in 0.02 M HCl solution, 20 g of Sodium Dodecyl sulphate was dissolved into 98 ml of MQ water. Two ml of 1 M HCl was then added to the solution which was stored at room temperature.

Trypan Blue (TB):

NaCl (0.9 g) was dissolved in 100 ml of MQ water. Trypan blue powder (0.2 g) was then added. The solution was filtered and stored at 4ºC.

Appendix II: Standard curve derived from MTT and Crystal violet assay.

The same steps were used in both assays. After obtaining the ODs using the spectrophotometer (see Table A 1), the mean, and the standard deviation (SD) of the replicates ODs were assessed. The coefficient variations (Coff. V) were then calculated by dividing the SD by the mean, and multiplying the result by 100 (see Table A 2). A standard curve was then drawn (the mean of the ODs vs. The cell number/well) (see Figure A 1).

Table A 1: The ODs of MTT standard curve 30/04/2015.

Flasks	1	2	3	4	5	6	7	8
A	0.223	0.223	0.239	0.241	0.292	0.375	0.519	0.696
	0.211	0.219	0.234	0.247	0.294	0.375	0.54	0.744
	0.218	0.225	0.228	0.241	0.301	0.374	0.557	0.714
	0.213	0.222	0.231	0.237	0.291	0.373	0.534	0.727

Table A 2: Calculations of the average of the ODs, standard deviation and coefficient variation.

well No.	cells No.	mean A	SD A	Coff. V
1	0	0.21625	0.005377	2.486669
2	625	0.22225	0.0025	1.124859
3	1250	0.233	0.00469	2.013054
4	2500	0.2415	0.004123	1.70729
5	5000	0.2945	0.004509	1.531154
6	10000	0.37425	0.000957	0.255826
7	20000	0.5375	0.015716	2.92395
8	40000	0.72025	0.020304	2.819013

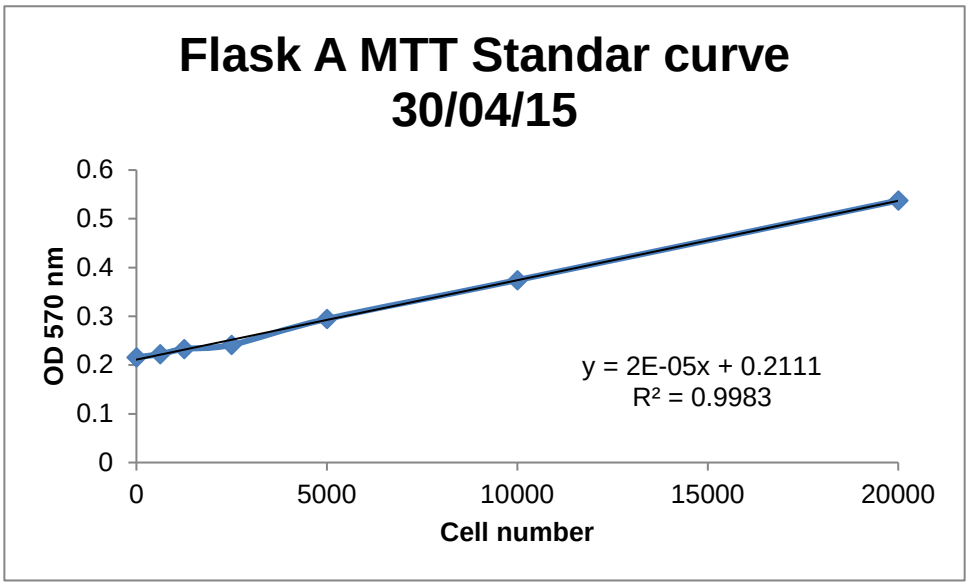

Figure A 1: MTT standard curves.
This curve shows linear relationships indicated to the absorbance corresponded to the cell number until $2x10^4$ cell/well.

Appendix III: Treating Cells with chemicals using MTT Crystal violet and Trypan blue assays.

Using the equation obtained from the standard curve, the cell number in each treated well was then assessed. The percentage of relative survival (with MTT) the relative cell number (with Crystal violet) and the relative live cell (with Trypan blue) were then measured by dividing the cell number in the treated well by the cell number in the media, and multiplying the result by 100 (see Table A 3).

Table A 3: Calculating the percentage of relative survival (with MTT) the relative cell number (with Crystal violet) and the relative live cell (with Trypan blue).

Simazine 18/2/13							
Flask	Medium	0.9% DMSO	4.5E-06	0.000045	0.00045	0.0045	
A	0.478	0.394	0.335	0.35	0.327	0.215	The ODs
	0.492	0.364	0.381	0.341	0.336	0.231	
	0.52	0.348	0.353	0.381	0.379	0.238	
	0.506	0.373	0.377	0.357	0.324	0.212	
Flask	column	mean	S.D	Coff. V	Equ.	cell No.	%
A	Media	0.499	0.018074	3.622029	11236.67	11236.66667	100
	0.09% DMSO	0.3697	0.01919	5.18995	6928.333	6928.333333	61.6582653
	4.5E-06	0.3615	0.021564	5.965106	6653.333	6653.333333	59.21092015
	0.000045	0.35725	0.017134	4.796158	6511.667	6511.666667	57.95016659
	0.00045	0.3415	0.025515	7.471362	5986.667	5986.666667	53.27796222
	0.0045	0.3155	0.03305	10.4755	5446.66	5446.66666	46.1069611

Appendix IV: Cytotoxicity of Roundup, Once-a-year path weed and their constituents using MTT and Crystal violet (CV) assays.

Roundup.

The results of Roundup (R-up), glyphosate and POEA using both assays were presented in section 3.3.1.

Once-a-year path weed.

The results of Once-a-year path weed, the lab mixture of it, amitrol, ammonium thiocyanate and simazine using MTT and CV assays were presented in section 2.3.1.

Appendix V: Cytotoxicity of caffeine-herbicides combinations using MTT and Trypan blue assays.

Mixing caffeine with Roundup, glyphosate and Polyethoxylated tallow amine (POEA).

The results of Roundup (R-up), glyphosate and POEA using MTT and Trypan blue assays were presented in section 4.3.2.1.

Mixing caffeine with Once-a-year path weed, the lab mixture of it, amitrole, ammonium thiocyanate and simazine.

The results of Once-a-year path weed, the lab mixture of it, amitrol, ammonium thiocyanate and simazine using MTT assay were presented in Figure A 2 and using Trypan blue in Figure A 3.

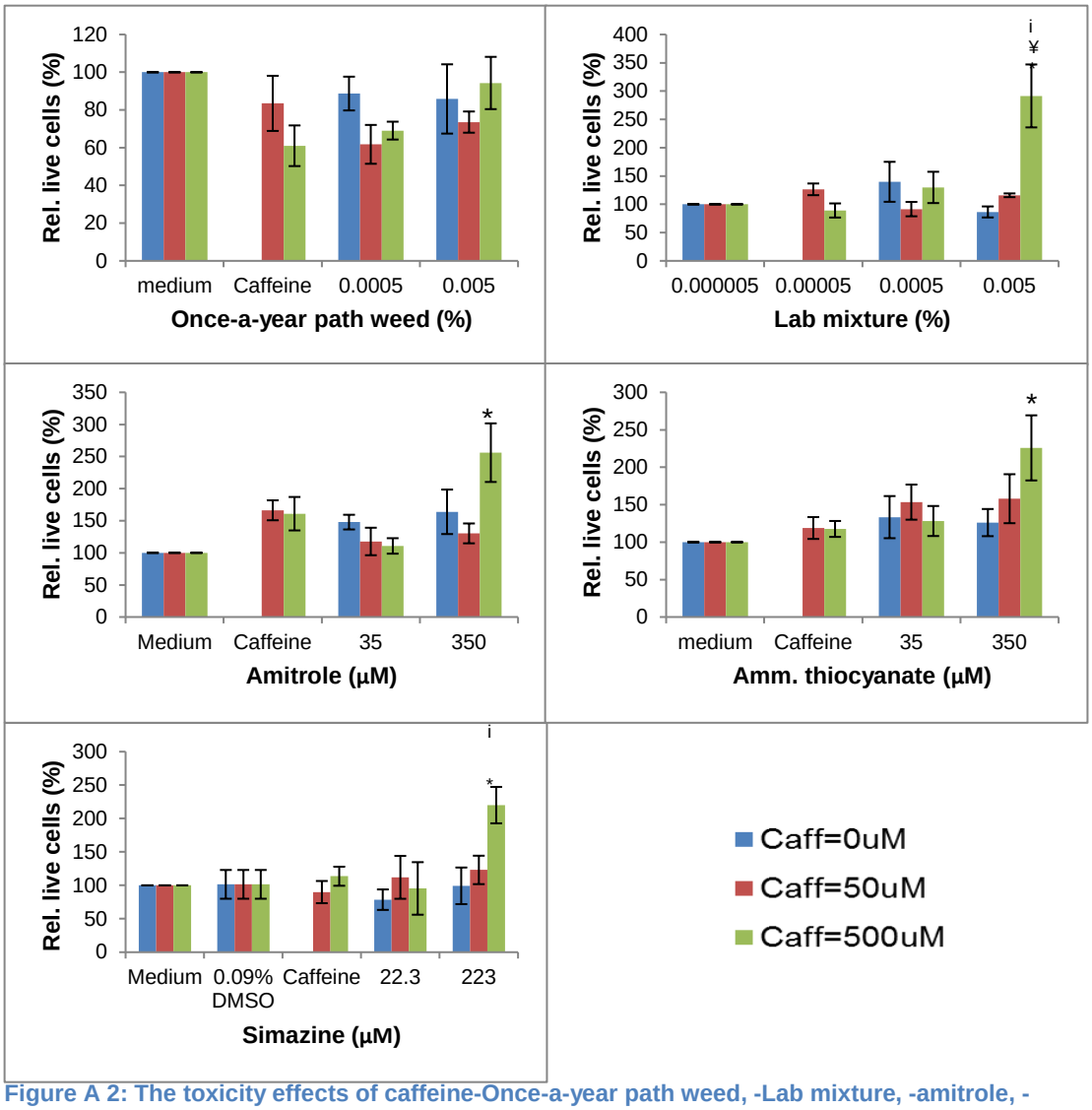

Figure A 2: The toxicity effects of caffeine-Once-a-year path weed, -Lab mixture, -amitrole, -ammonium thiocyanate and -simazine combinations on the WIL2-NS cells using MTT assay. The data was presented as mean ± stander error of the mean. The level of significance was expressed as $p < 0.05$ whereas * is compared data with media (negative control), and ¥ is compared data with caffeine of the same concentration and i is compared data with the chemicals at the same concentration.

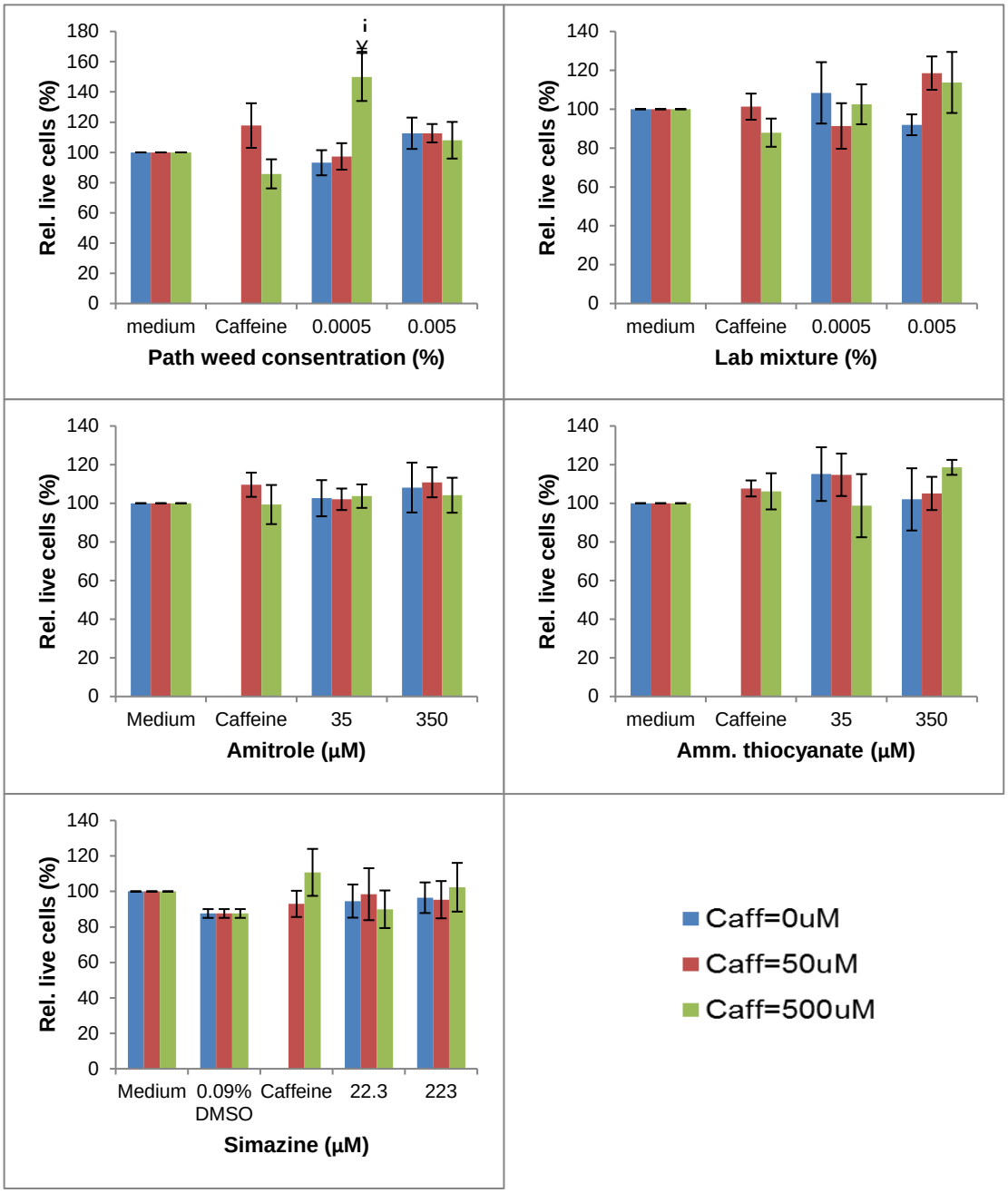

Figure A 3:The toxicity effects of caffeine-Once-a-year path weed, -Lab mixture, -amitrole, -ammonium thiocyanate and -simazine combinations on the WIL2-NS cells using Trypan blue assay.

The data was presented as mean ± stander error of the mean. The level of significance was expressed as $p < 0.05$ whereas * is compared data with media (negative control), and ¥ is compared data with caffeine of the same concentration and i is compared data with the chemicals at the same concentration.