The antioxidant and cellular effects of *Withania somnifera* alone and in combination with selenium

Claire Lynne Venter

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by

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Degree: MSc Cell Biology

Abstract

The respiratory system is a target for environmental oxidants from airborne pollution and other contaminants. Chronic exposure may result in inflammatory conditions such as allergies and asthma. The ability of the body to counteract oxidation is dependent on the oxidative status of the individual, and can also benefit from the use of medicinal plant preparations such as *Withania somnifera* (WS), which is accredited with antioxidant and anti-inflammatory properties. Selenium is an important cofactor of antioxidant enzymes such as glutathione peroxidase. A deficiency of Se will result in decreased antioxidant enzymes levels and a poor response to oxidative damage. The aim of this study was to determine the antioxidant content and activity of WS, as well as its *in vitro* effects with seleno-L-methionine (Se-Met), the major component of dietary selenium, in a physiologically relevant cell model system. Little is known about the combinational effects of non-enzymatic and enzymatic antioxidants i.e., WS in combination with Se-Met, and these effects were also quantified.

The antioxidant content (total phenolic (TPC) and flavonoid content (TFC)) and activity (2,2-diphenyl-2-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC)) and oxygen radical absorbance capacity (ORAC) of a water extract of WS, as traditionally prepared and used, was determined. The intracellular and total protection of WS alone (0-33.33 mg/ml) and in combination with Se-Met (0-33.33 pg/ml) (range: ½-100x RDA) was evaluated in a standard cell line, the SC-1 fibroblast cell line and in physiologically relevant primary cultures of chick embryo lung cells (CELC) using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay.

Water extracts of WS, as traditionally consumed, were found to possess significant antioxidant content and activity. For TPC and TFC, WS was found to contain 4.84 g CE/100 g DW, and 355 mg CE/100 g DW respectively. For DPPH, the IC_{50} of WS was 56.34 mg/ml, and the antioxidant activity of WS was 274.8 µmol TE/g. For TEAC, the IC_{50} of WS was 60.3 mg/ml, and the antioxidant activity of WS was 2.2 mmol TE/g. Using ORAC, the antioxidant activity of WS was found to be 15.05 µM TE/g.

WS and Se-Met exhibited both pro-oxidant and antioxidant effects in the DCFH-DA assay and this may be a function of concentration, as well as combinations of WS and Se-Met ratios. Data indicates that both WS and Se-Met, alone and in combination, elicit protection via the intracellular (at all concentrations) and extracellular (at higher concentrations)

compartment of the SC-1 cell. A synergistic interaction is observed extracellularly between WS and Se-Met, with mostly additive effects occurring intracellularly.

For CELC, WS and Se-Met alone both demonstrated total protective effects at almost all concentrations, and intracellular protective effects at higher concentrations. The combination of WS and Se-Met provided total and intracellular protection to the CELC at the highest concentration combination. Antagonism between WS and Se-Met was observed at low concentrations in the total protection assay, with weak synergism and antagonism occurring at higher concentrations. Synergism between WS and Se-Met was observed at low concentrations intracellularly, with antagonistic interactions occurring at higher concentrations.

In conclusion, WS and Se-Met, alone and in combination at non-cytotoxic concentrations, displayed significant cellular protection against oxidative damage and this was a function of cell line, concentration and ratios between WS and Se-Met.

Declaration

I, Claire Venter, declare that the dissertation, which I hereby submit for the degree MSc Cell Biology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed:

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Table of contents

List of Tables

List of Figures

List of Abbreviations

α	Alpha
β	Beta
%	Percentage
℃	Degrees Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar

A

Aβ	Beta amyloid
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt
$AlCl_3$	Aluminium chloride
AO	Antioxidant
ATP	Adenosine triphosphate
AUC	Area under curve

C

CAT	Catalase
Cd	Cadmium
CE	Catechin equivalent
CELC	Chick embryo lung cells
cm^2	Centimetres squared
CO_2	Carbon dioxide
CV	Crystal Violet

D

DCFH-DA	Dichlorofluorescein diacetate
ddH_20	Double distilled water
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid

DPBS	Dulbecco's phosphate buffer saline
DPPH	2,2-diphenyl-2-picrylhydrazyl
DW	Dry weight

E

e.g.	For example
EMEM	Eagle's minimum essential medium
EDTA	Ethylene diamine tetraacetic acid
EP	Expected protection
et. al.	*et alia* / and others

F

FC	Folin-Ciocalteu
FCS	Fetal calf serum
Fe^{2+}	Iron

G

G	Gram
GAE	Gallic acid equivalent
GPx	Glutathione peroxidase
GPx-1	Glutathione peroxidase-1
GSH	Glutathione
GST	Glutathione-*S*-transferase

H

H_20	Water
H_20_2	Hydrogen peroxide
HO^-	Hydroxyl
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography mass spectrometry

I

IC_{50}	Half maximal inhibitory concentration
i.e.	That is

I/	Interaction index
IP	Intracellular protection

K

KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate

L

L	Litre
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LSD	Least significant difference

M

M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
MTT	3- (4,5- Dimethylthiazol- 2- yl) -2,5- diphenylftetrazolium bromide

N

Na_2CO_3	Sodium carbonate
Na_2HPO_3	Sodium hydrogen phosphite
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
nM	Nanomolar
Nm	Nanometer
NO^-	Nitric oxide
NR	Neutral Red
Ns	Not significant

O

$^1O_2$	Singlet oxygen
O_2	Oxygen
$O_2^{\bullet -}$	Superoxide
OP	Observed protection
ORAC	Oxygen radical absorbance capacity

P

PBS	Phosphate buffer saline
pg	Picogram
pH	Measure of hydrogen ion concentration (acidity/alkalinity) of a solution
PO	Pro-oxidant

R

R^2	Coefficient of determination
RNS	Reactive nitrogen species
RO^-	Alkoxyl
ROO^-	Peroxyl
ROS	Reactive oxygen species
RP-HPLC	Reverse-phase high performance liquid chromatography

S

SA	South Africa
Se	Selenium
SEM	Standard error of means
Se-Met	Seleno-L-methionine
SC-1	Embryonic *Mus musculis* (mouse) fibroblast cells
SOD	Superoxide dismutase

T

TE	Trolox equivalent
TEAC	Trolox equivalent absorbance capacity
TFC	Total flavonoid content
TLC	Thin layer chromatography

TP	Total protection
TPC	Total phenolic content
TRx	Thiodine reductase

V

vs.	Versus

W

w/v	Weight/volume
WS	*Withania somnifera*
WW	Wet weight

X

x g/RCF	Relative centrifugal force

Z

Zn	Zinc

Chapter 1: Introduction

An old German proverb says “The garden is the poor man’s apothecary”. The World Health Organisation has estimated that approximately 80% of third-world populations make use of traditional medicines derived from plants for primary health care (Ranilla *et al*., 2010) due to their potent pharmacological activities, low toxicity and economic viability (Auddy *et al*., 2003).

Withania somnifera (WS) is such a plant and is commonly referred to as Ashwagandha or Winter Cherry. The origins of WS lie in Ayurvedic medicine, the traditional and holistic medical system that originated in India over five thousand years ago (Mishra *et al*., 2000; Visavadiya *et al*., 2007). The entire plant is credited with medicinal properties and used as a home remedy throughout the world for a wide variety of diseases (Owais *et al*., 2005).

WS possesses multiple biological properties that have been ascertained through extensive pharmacological studies, and some examples include antioxidant, anti-inflammatory, antitumour, antistress, immunostimulatory, haemopoetic and rejuvenating properties. It is also reported to have a positive effect on the endocrine, cardiopulmonary and central nervous systems (Dhuley, 1997; Davis *et al*., 1998; Chaurasia *et al*., 1999; Archana *et al*., 1999; Kulkarni *et al*., 2008; Rasool *et al*., 2006; Visavadiya *et al*., 2007; Panda *et al*., 1999).

Commercially, WS extracts and specific WS-derived molecules are available to the public and these include Withaferin A, an angiogenesis inhibitor, and extracts of WS sold as neutraceutical products. Traditionally, indigenous populations prepared a water extract, infusion or decoction of dry powders (Di Stasi *et al*., 2002; Widodo *et al*., 2011), which was used to treat a variety of ailments including tuberculosis, asthma, stress, inflammation, arthritis and conjunctivitis.

Several different strategies can be used to evaluate the properties of medicinal plants. One such approach involves the isolation and characterisation of the major compounds of the plant. Using this strategy, Pal and co-workers (2012), identified alkaloid, hydrobenzene, terpene ansteroid, saponin, organic acids and flavones as the major constituent molecules of WS with thin layer chromatography (TLC). Reverse-phase high performance liquid chromatography (RP-HPLC) was further employed to identify and quantify four polyphenols responsible for antioxidant activity in WS, namely epicatechin, quercetin-3-rhamnoside, gallic acid and rutin hydrate (Pal *et al*., 2012). In a separate study, eight polyphenols were

identified in WS using high performance liquid chromatography (HPLC), namely gallic, syringic, vanillic, benzoic and p-coumaric acids, catechin, kaempferol and naringenin (Alam *et al*., 2011). The strategies employed provide very little information specifically related to the activity, efficacy and possible toxicity of extracts as traditionally used (Tabart *et al*., 2009). The purpose of this study is to address this limitation in the scientific literature.

Selenium (Se) is known as an essential micronutrient (Li *et al*., 2008, Rudolf *et al*., 2008; Zhou *et al*., 2009) and is a known cofactor of antioxidant enzymes such as glutathione peroxidase (GPx) and Se is primarily recognised for its antioxidant activity and chemopreventive, anti-inflammatory and antiviral properties (Papp *et al*., 2007). Se deficiencies have been linked to diabetes and a number of cardiovascular and neurodegenerative disorders (Brenneisen *et al*., 2005). Humans take up Se naturally through the consumption of grains, cereals, vegetables and meat (Tapiero *et al*, 2003), and many other foods are enriched with Se. Seleno-methionine (Se-Met) is an organic form of Se, and the major component of dietary Se, which is more bio-available than inorganic forms, and may therefore provide enhanced protection (Xiong *et al*., 2007). For these reasons, Se-Met was chosen for this study.

Se-Met plays a pivotal role in the cellular antioxidant defence system (Wojewoda *et al*., 2010) and is an essential component of antioxidant enzymes like GPx, thioredoxin reductase (TRx), iodothyronine deiodinases, selenophosphate synthetase, selenoprotein P and other selenoproteins(Li *et al*., 2008; Zhou *et al*., 2009). Glutathione peroxidase and thioredoxin reductase have been associated with antioxidant activity, anticancer effect and other physiological functions (Li *et al*., 2008; Zhou *et al*., 2009). GPx protects membrane lipids and macromolecules against oxidative damage caused by peroxides (Zhou *et al*., 2009).

There has been an overwhelming increase in interest and focus on antioxidant research over the past few decades. Clinical trials and epidemiological studies have established an inverse relationship between the consumption of dietary antioxidants and the occurrence of disease (Ou *et al*., 2002; Thaipong *et al*., 2006; Tabart *et al*., 2009; Amin *et al*., 2006; Huang *et al*., 2005). It is understood that antioxidants fight reactive oxygen species (ROS) and reactive nitrogen species (RNS), the harmful by-products generated during normal cell aerobic respiration. These damaging derivatives are a factor in the pathogenesis of ageing and disease. Antioxidants play a very important role in the fight against, amongst others, cardiovascular disease, cancer and ageing (Thaipong *et al*., 2006; Amin *et al*., 2006; Huang *et al*., 2005).

In addition, the organism's antioxidant defence system comprises a network of enzymatic (e.g., Se) and non-enzymatic (e.g., polyphenolics) antioxidants (Stahl & Sies, 2003). It is believed that interactions between enzymatic and non-enzymatic antioxidants, with variable structure and antioxidant activity, can significantly improve the protection against oxidative stress provided by individual antioxidants (Stahl & Sies, 2003; Chen & Tappel, 1995).

In this study, the antioxidant activity of a water extract of WS was measured alone and in combination with Se-Met, and the cellular antioxidant effects in *in vitro* cell culture models were determined. In a further part of this study, the interaction between WS extracts and Se-Met was explored.

Chapter 2: Literature review

Introduction

There has been an increase in the integration of traditional and modern health research and practices on a global scale. Despite man-made drugs having taken control of the international pharmaceutical industry of late, herbal medicine continues to stand its ground. It is the foundation of health care in many developing countries. The use of traditional medicines promotes primary health care, better cultural acceptability and fewer side effects (Jaleel, 2009).

A major criticism of traditional medicines is that these products are not subjected to scientific evaluation and clinical study as pharmaceutical drugs are. The main focus of ethnopharmacological research is to subject these plants and their extracts to scientific evaluation and isolate and identify the bioactive components. The effect of the plant extract, as used traditionally, can also be evaluated for antibacterial, antiviral, anti-inflammatory and antioxidant activity as there may be synergistic beneficial or toxic effects between the various components of the extract.

Oxidative stress

The process of oxidation forms an essential part of metabolism, and can be defined as the transfer of electrons from one atom to another (Pietta, 2000). The electron transport system, found within cells, is a series of membrane-bound carriers that pass electrons from one carrier to the next. High-energy electrons enter and low-energy electrons leave the system, and the energy that is released is used to synthesize ATP. Oxygen (O_2) is the final electron acceptor in the system (Mader, 2001).

When the electron flow becomes uncoupled, unpaired electrons are transferred, and a chemical species known as free radicals is generated. These free radicals are highly reactive, unstable and potentially damaging to cells, tissues and organs, and attempt to extract electrons from other molecules in order to achieve stability. Oxygen-centred free radicals are known as ROS and examples include superoxide ($O_2^{\bullet -}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and hydroxyl ($HO^{\bullet}$). In addition to these ROS radicals, there are other non-radicals that exist in the living organism, such as the singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Nitrogen-centred free radicals are known as reactive nitrogen species (RNS), for example nitric oxide ($NO^{\bullet}$) (Pietta 2000; Ali *et al.*, 2008).

The process described above illustrates the production of free radicals within the body during normal metabolic functions (Honzel *et al*., 2008). Approximately 1% of the all the oxygen consumed by mitochondria is transformed into ROS (Silva *et al*., 2005). Free radicals can also be introduced from the external environment in the form of pollution, smoke, sunlight, redox cycling xenobiotics and radiation (Brenneisen *et al*., 2005; Honzel *et al*., 2008).

In small amounts, ROS are involved in energy production, phagocytosis, cell growth regulation, intercellular signalling and the synthesis of biologically important compounds (Brenneisen *et al*., 2005, Atmani *et al*., 2009). However, ROS also has the ability to play a negative role *in vivo,* and oxidative stress may occur when the generation of ROS exceeds a system's ability to neutralize and eradicate these molecules. The imbalance may be caused by a disturbance in the production or distribution of antioxidants, or by an excess of ROS, either from endogenous sources or external environmental stressors (Brenneisen *et al*., 2005, Honzel *et al.,* 2008). If a proper equilibrium is not maintained, ROS are able to inhibit signal transduction pathways and normal cellular function by attacking lipids found in cell membranes, proteins in tissues, enzymes, carbohydrates and DNA, and oxidise low density lipoproteins. Membrane and DNA damage, and protein and enzyme modifications are a result.

This damage is believed to play a role in the ageing process, as well as in degenerative diseases, including cardiovascular disease, cardiac failure, cognitive dysfunction and cataracts (Brenneisen *et al*., 2005; Amin *et al*., 2006, Huang *et al*., 2005; Pietta, 1999; Atmani *et al*., 2009). The most devastating diseases associated with ROS and oxidative stress are neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, Creutzfeldt-Jakob disease and meningoencephalitis. Cancer is brought about by a multitude of causes, of which RNS/ROS is a component (Ali *et al*., 2008). It is believed that free radicals contribute to more than one hundred disorders in humans (Pourmorad *et al*., 2006) and that approximately 95% of pathologies occurring in people over the age of thirty-five are associated with the production and build-up of free radicals in the body (Silva *et al*., 2005).

Antioxidants

Humans have various natural defence mechanisms in place to protect the body from damage caused by ROS. These involve preventative and repair mechanisms, as well as physical and antioxidant defences. Endogenous antioxidants possess both enzymatic and non-enzymatic defences (Pietta, 2000; Ali *et al*., 2008), which play a vital role in

counteracting oxidative damage caused by free radicals within the body (Honzel *et al*., 2008).

Antioxidants can be described as natural or synthetic molecules that either prevent the uncontrolled formation of ROS, or inhibit its reaction with biological structures (Huang *et al*., 2005). In biochemical or medical terms, antioxidants are characterized as “enzymes or other organic substances, such as vitamin E or β-carotene, that are capable of counteracting the damaging effects of oxidation in animal tissues” (Huang *et al*., 2005).

The Institute of Medicine has defined a dietary antioxidant as “a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans” (Huang *et al*., 2005). A dietary antioxidant can either act sacrificially to scavenge ROS/RNS to stop radical chain reactions, or it can play a preventative role by inhibiting the reactive oxidants from initially being formed (Huang *et al*., 2005).

Dietary antioxidants can include antioxidant enzyme cofactors. A good illustration of this is selenium, a cofactor of a selenoprotein, e.g., GPx, which has the ability to reduce peroxides to alcohols and water. Selenium itself does not function as a ROS/RNS scavenger. Therefore results achieved in an *in vitro* antioxidant capacity assay will not reflect the role selenium plays in a biological system.

Autoxidation in a non-biological system occurs by radical chain reactions, while oxidation in a biological system is mediated by a multitude of redox reactions. Nevertheless, non-enzymatic lipid autoxidation by radical chain reaction may still occur and lead to oxidative stress. For these reasons, biological antioxidants include enzymatic antioxidants (e.g., superoxide dismutase (SOD), catalase (CAT) and GPx, and non-enzymatic antioxidants such as oxidative enzyme (e.g., cyclooxygenase) inhibitors, antioxidant enzyme cofactors, ROS/RNS scavengers and transition metal chelators (Huang *et al*., 2005).

There are multiple strategies employed by antioxidants, including enzymatic and non-enzymatic defences. Non-enzymatic compounds include α-tocopherol (vitamin E), carotenoids, ascorbic acid (vitamin C), glutathione (GSH), ubiquinols and flavonoids. Micronutrient elements such as selenium (Se) and zinc have also shown to be essential components of protective enzymes via special amino acids, for example, seleno-cysteine and Se-Met, or structural components, for example, Zn fingers and Zn-metallothionein. These low molecular mass antioxidant molecules are essential for the enzymatic defence

system provided by SOD, CAT, thioredoxin reductase (TRx) and GPx (Brenneisen *et al*., 2005; Ali *et al*., 2008).

Table 2.1: A summary of enzymatic and non-enzymatic biological antioxidants

Enzymatic antioxidants	Non-enzymatic antioxidants (small molecules)
• Superoxide dismutase • Catalase • Glutathione peroxidase	• Oxidative enzyme inhibitors e.g., aspirin, ibuprofen • Transition metal chelators e.g., EDTA • Polyphenolics e.g., gallic acid • Vitamins e.g., Vitamin C & E

Enhancing the body's natural antioxidant defences or supplementing the diet with proven dietary antioxidants makes it possible to reduce the risks of chronic diseases and prevent disease progression, which has far reaching consequences for health care. This is one of the primary reasons why discovery and production of antioxidants, as well as the research into naturally occurring antioxidants has become a leading field in recent times (Huang *et al*., 2005).

Antioxidants that are obtained from fruits, vegetables, spices and cereals are very effective and tend not to interfere with the body's ability to use free radicals constructively. Natural antioxidants are mainly found in plants in the form of phenolic compounds (namely flavonoids and phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), ascorbic acid and carotenoids (Kanner *et al*., 1994) and are used for medicinal purposes. Medicinal plants are only occasionally used and provide an additional source of antioxidants that can protect against the oxidative stressors of disease. One such product is *Pelargonium sidoides*, also known as EPs 7630, a medicinal plant that has been clinically shown to improve the symptoms of dysentery, fever and respiratory disease, e.g., bronchitis. Some of the main constituents of the plant are polyphenolic compounds, i.e., catechin, gallocatechin and gallic acid, and the plant is known for its antimicrobial and immune modulatory effects (Theisen & Muller 2012; Matthys *et al*., 2003).

The role of antioxidants in lung disease

South Africa is subject to many sources of air pollution. The burning of coal, oil and natural gas in industrial processes, power generation and vehicles, the burning of wood and kerosene, cigarette smoke, insecticides and household materials all contribute to the problem. Some of the more common air pollutants that can have a major impact on health are sulphur dioxide, oxides of nitrogen, particulate matter, lead, ozone, carbon monoxide and volatile organic compounds, for example benzene. Chronic exposure to these pollutants can lead to associated inflammatory respiratory conditions such as wheeze, shortness of

breath, sinusitis, rhinitis, bronchitis, pneumonia (Matooane *et al*., 2004). A central theme is an increase in ROS generation in the respiratory system (Dworski, 2000), which has adverse effects on lung growth and pulmonary function, resulting in associated inflammatory diseases (Wichmann *et al.,* 2008, Girard-Lalancette *et al.,* 2009).

The respiratory system and the lungs in particular are a specific target for toxicity, as these organs are directly exposed to the environment (Cross *et al.,* 1998). All eukaryotic cells have the ability to utilize energy produced from the reduction of oxygen to water. With each reduction, a small number of oxygen intermediates, such as $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, and $^1O_2$ are produced, which are highly unstable and reactive. As the cells of the lungs are exposed to the highest concentration of oxygen, these are a specific target for oxidant injury (Martin *et al.,* 1981).

The consumption of foods rich in polyphenols is associated with a decrease in the incidence of asthma symptoms, which exert anti-allergic, anti-inflammatory and bronchodilatory effects in the respiratory system (Franova *et al*., 2011). The flavonoid quercetin has shown ability to inhibit mast cell degranulation and the subsequent release of histamine in the treatment of asthma (Taur & Patil, 2011).

Withania somnifera

Withania somnifera (WS) is an herbal plant that is also commonly referred to as Ashwagandha or Winter Cherry. Belonging to the family *Solanaceae*, this green subtropical shrub is found throughout the drier parts of India, Pakistan, Sri Lanka, South Africa, Egypt and Morocco, to name but a few locations (Chaurasia *et al*., 2000).

The origins of WS lie in Ayurvedic medicine, the Indian traditional and holistic medical system (Mishra *et al*., 2000; Visavadiya *et al*., 2007) and is highly regarded by its practitioners (Kulkarni *et al*., 2008; Rasool *et al*., 2006; Bhattacharya *et al*., 2001). It is listed in the Indian Pharmacopeia, the official drug compendium for all prescription and over-the-counter drugs, as well as other health care products manufactured or sold in India (Dhuley, 1998; Rasool *et al*., 2006; Owais *et al*., 2005). The entire plant is credited with medicinal properties and is used as a home remedy for a number of diseases and disorders in India and throughout the world (Owais *et al*., 2005).

Essentially, Ayurveda can be described as the knowledge of healthy living, with the word “ayur” meaning life and “veda” translated as knowledge, and therefore encompasses, but is

not confined to, the treatment of illness and disease with nutrition, hygiene and rejuvenation (Dhuley, 1997). Within the branches or specialities of Ayurveda, the plant is classified as a rasayana, which prevents disease and counteracts ageing by maintaining homeostasis (Dhuley, 1997; Auddy *et al*., 2003; Surveswaran *et al*., 2007). Rasayanas are reported to possess strong antioxidant activity (Auddy *et al*., 2003) and are believed to have the ability to enhance physical and mental health, invigorate the body in debilitated conditions, and promote a long life (Kulkarni *et al*., 2008; Bhattacharya *et al*., 2001). Various parts of WS have been reported to treat an array of disorders over many centuries (Kulkarni *et al*., 2008).

WS has been classified as an adaptogen, which is a natural metabolic that can "increase the ability of an organism to adapt to environmental factors and to avoid damage from such factors" (Panossian *et al*., 1999). It is able to cause adaptive reactions to a variety of unrelated diseases, and produce an increased resistance to the pathological effects of physical, chemical and biological agents (Mishra *et al*., 2000).

Active biochemical constituents of *Withania somnifera*

WS is believed to possess such an array of medicinal properties due to the diversity of the biochemical constituents present in different parts of the plant. Alkaloids (including ashwagandin, cuscohygrine, anahygrine and topine), steroidal compounds (including withanolides A-Y (Figure 2.1C), withaferin A (Figure 2.1B), withasomniferin A, withasomnidienone, withasomnierose A-C and withanone), saponins containing an additional acyl group (sitoindosides VII and VIII), and withanolides with a glucose at C27 (sitoindoside IX and X) have been isolated from the plant, and the structure of these molecules have been elucidated. The withanolides, which comprise of steroidal alkaloids and lactones, have a C28 steroidal nucleus with C9 side chain, and a six-membered lactone ring and bear a structural resemblance to ginsenosides, the active constituents in the plant *Panax ginseng*, consequently earning the plant its alias "Indian ginseng". (Kulkarni *et al*., 2008; Bhatnagar *et al*., 2009).

The withanolides Withaferin A and 3-ß-hydroxy-2,3-dihydrowithanolide F isolated from WS are known to have antibacterial, antitumour, immunomodulatory and anti-inflammatory effects (Rasool *et al*., 2006). The glycowithanolides Withafurin A and sitoindosides VII – X, which have been isolated from the roots of the plant, have been known to reverse ibotenic acid-induced cognitive defects in Alzheimer's disease. 5-dehydroxywithanolide-R and withasomniferin A have been identified in the aerial part of the plant (Archana *et al*., 1999).

Withaferin A, associated with anti-inflammatory and immunosuppressive properties, has also shown to be an inhibitor of angiogenesis, causing it to be protective against certain cancers. In a study on both old and young rats, two glycowithanolides showed an increase in learning acquisition and memory retention, which demonstrated the antistress activity of WS (Kulkarni *et al*., 2008). Sitoindisides VII-X and Withaferin A have shown significant antistress and antioxidant effect in a study of the rat frontal cortex and striatum (Pal *et al*., 2011).
The root of WS is considered the most active in therapeutic terms. It is regarded as a tonic and an aphrodisiac, and has been used in the treatment of consumption (tuberculosis), emaciation, incapacity, dyspepsia and rheumatism. A decoction of the root bark is prepared and administered for asthma, and the root itself is used for colds and chills. The plant has also been used for the treatment of syphilis (Archana *et al*., 1999; Rasool *et al*., 2006).

WS is known to be used as a home remedy for a number of ailments (Rasool *et al*., 2006; Archana *et al*., 1999) and it is known to be the chief constituent of various formulations prescribed for common diseases of the respiratory and reproductive tracts (Visavadiya *et al*., 2007). While WS has been used and recognised as an important medicinal plant in India for centuries, it has only recently become the focus of modern science (Jaleel, 2009).

WS has been the subject of extensive pharmacological studies, and is said to possess antioxidant, anti-inflammatory, antitumour, antistress, immunostimulatory, haemopoetic, and rejuvenating properties. It is also reported to have a positive effect on the endocrine, cardiopulmonary and central nervous systems (Dhuley, 1997, Davis *et al*., 1998, Chaurasia *et al*., 2000, Archana *et al*., 1999, Kulkarni *et al*., 2008, Rasool *et al*., 2006, Visavadiya *et al*., 2007, Panda *et al*., 1999).

Even though it is believed to be innocuous, and is reportedly safely used by males and females of all ages, as well as pregnant women (Archana *et al*., 1999), it is important to determine the effects of WS in order to determine if it can be effectively used in place of or in conjunction with commonly used pharmaceuticals.

Many of the claims made with regard to WS's uses and properties have not yet been scientifically validated, and therefore some scepticism can be expected, especially since it is supposedly useful in so many conditions (Mishra *et al*., 2000). As the antioxidant activities of WS will be investigated in this study, activity related to plant-derived antioxidants will be discussed in greater detail.

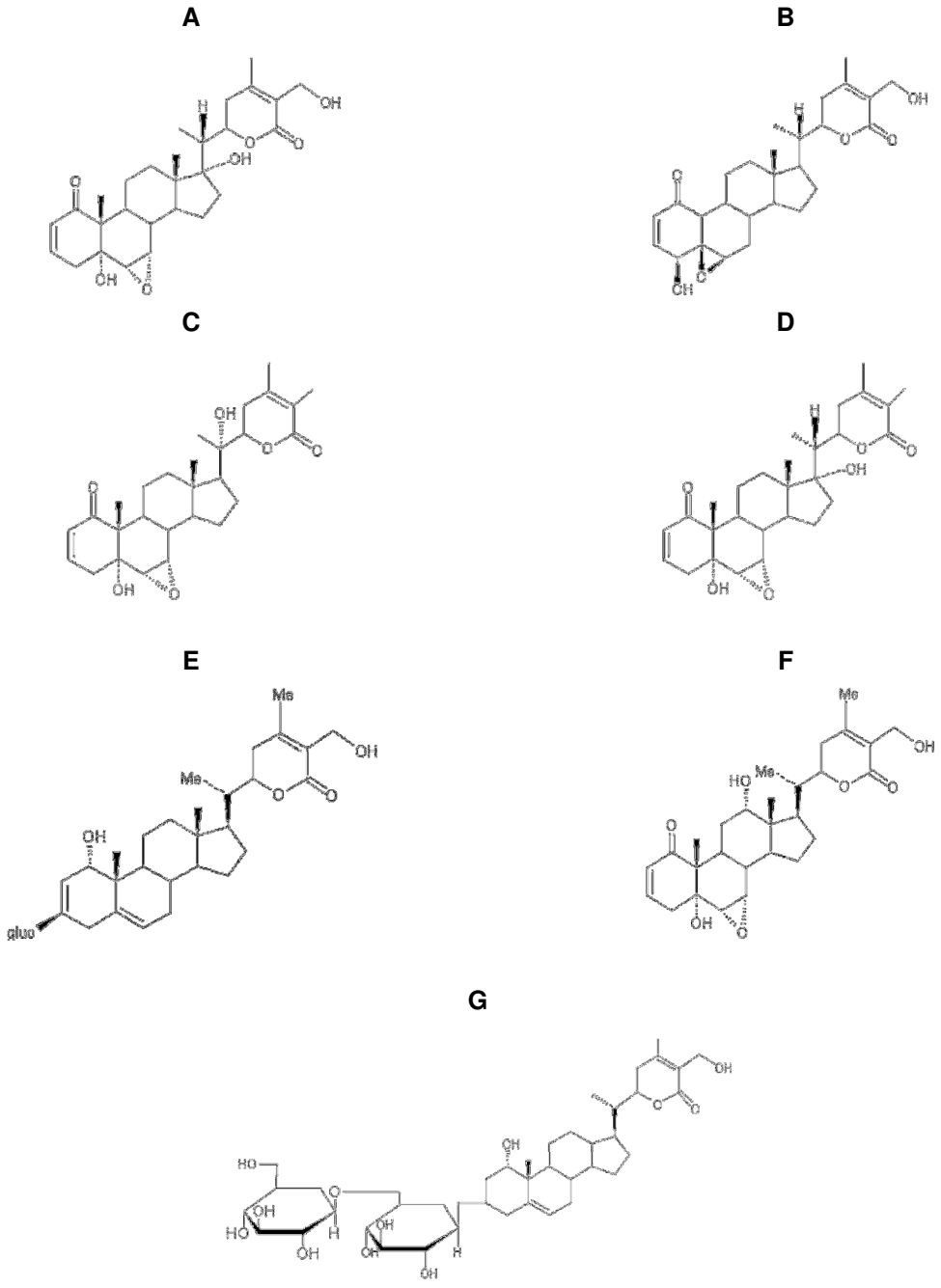


Figure 2.1: The chemical structures of the main active compounds that have been isolated from the roots and leaves of WS. 27-hydroxywithanone (A), Withaferin A (B), Withanolide A (C), Withanone (D), Physagulin-D (E), Withastramonolide (F), Withanoside-IV (G) (Dhar *et al*., 2006).

Non-enzymatic antioxidants: Plant-derived antioxidants related to *Withania somnifera*

The root extract of WS possesses a free radical-scavenging ability, which has been largely attributed to the presence of phenolic compounds (Bhatnagar *et al*., 2009). It was shown as being capable of modulating the oxidative stress markers of the body by being able to significantly reduce the lipid peroxidation and increase SOD and CAT activity (Dhuley, 1998). Withanolides and sitoindosides (VII-X) enhance CAT and GPx activities in rat frontal cortex and striatum (Bhatnagar *et al*., 2009). Antioxidants that have been identified in WS are alkaloid, hydrobenzene, terpene ansteroid, saponin, organic acids and flavones. The main individual polyphenolic compounds revealed the presence of epicatechin, quercetin-3-rhamnoside, gallic acid and rutin hydrate, as well as some unidentified polyphenolic compounds (Pal *et al*., 2012). Alam and co-workers identified five phenolics by HPLC analysis, namely gallic, syringic, vanillic, benzoic and p-coumaric acids (Alam *et al*., 2011).

The phenolic compounds present in the roots of WS contribute to the antioxidant activity of the plant. Of importance in this study are the non-enzymatic plant-derived antioxidants such as those found in WS, and these included gallic acid, epicatechin, rutin hydrate and quercetin-3- rhamnoside found in methanolic root extracts of WS (Pal *et al*., 2012).

Gallic acid has antioxidant, anti-inflammatory, antimutagenic and anticancer properties. In both *in vitro* cancer cell lines and animal models it has an inhibitory effect on cancer cell growth. Its effect is mediated by the modulation of genes which encode for cell cycle, metastasis, angiogenesis and apoptosis (Verma *et al*., 2013). Epicatechin is known for its powerful antioxidant properties and is relevant in biological, pharmacological and medicinal fields (Iacopini *et al*., 2008). It protects pancreatic islet cells against exposure to streptozotocin. Quercetin-3-rhamnoside is a flavonoid that has been reported to have an inhibitory effect on viruses, in particular influenza (Choi *et al*., 2009), as well as on human platelet aggregation *in vitro*. Quercetin has also showed potential anticancer properties by the inhibition of the protein tyrosine kinase (Iacopini *et al*., 2008), and the ability to inhibit mast cell degranulation and the subsequent release of histamine in the treatment of asthma (Taur & Patil, 2011).

As the specific focus of this study is the antioxidant activity of WS, alone and in combination with Se-Met, the role of Se-Met as a component of antioxidant enzymes will be discussed in greater detail.

Selenium

Selenium (Se) is known as an essential micronutrient (Li *et al*., 2008, Rudolf *et al*., 2008): a trace mineral that is only required in small amounts by the body, but is vital to good health (Zhou *et al*., 2009). Se is primarily recognised for its antioxidant activity and its chemopreventive, anti-inflammatory and antiviral properties (Papp *et al*., 2007). Se is involved in the maintenance of homeostasis within the cell. It is believed that Se is necessary for the functioning of the immune system and it has also been linked to the delay of the ageing process. Se deficiencies have been linked to diabetes and a number of cardiovascular and neurodegenerative disorders (Brenneisen *et al*., 2005). Humans take up Se primarily through the consumption of grains, cereals, vegetables and meat (Tapiero *et al*., 2003).

In the body selenocysteine, an amino acid, is a component of selenoproteins, some of which have important enzymatic functions (Rayman, 2000). Se, as selenocysteine, forms part of the active site of many Se-dependent antioxidant enzymes; glutathione peroxidase (GPx), thioredoxin reductase (TRx), iodothyronine deiodinases and selenophosphate synthetase (Santamaria *et al*., 2003; Rayman, 2000). When TRx reduces nucleotides in DNA synthesis, or when iodothyronine deiodinases produce active thyroid hormone from its inactive precursor, Se functions as a redox centre. Another example of the maintenance of the intracellular redox state is the reduction of hydrogen peroxide and lipid and phospholipid hydroperoxides to water and alcohols by the family of Se-dependent GPxs. This results in the maintenance of membrane integrity, protection of prostacyclin production, and the minimalizing of further oxidative damage to lipids, lipoproteins and DNA, as well as the risk of associated conditions such as atherosclerosis and cancer (Rayman, 2000; Li *et al*., 2008; Zhou *et al*., 2009; Messarah *et al*., 2010; Zhou *et al*., 2009).

Se is known to have beneficial physiological activity and toxic effects, depending on the concentration used. Research has shown that when used at low concentrations, selenium has anticarcinogenic effects, while at concentrations higher than necessary for nutritional purposes selenium has shown genotoxic and carcinogenic effects (Valdiglesias *et al*., 2010). In 2004, Hamilton reported that three levels of biological activity of Se exist, and they include trace concentrations necessary for normal growth and development, moderate concentrations which can be stored while homeostatic functions are maintained and elevated concentrations which can cause toxic effects (Hamilton 2004).
The recommended daily dietary allowance (RDA) for selenium is dependent a number of factors, including health regulation bodies and selenium intake. The RDA in the United States of America is 55 µg/day; while in the United Kingdom it is 75 µg/day for adult males,

and 60 µg/day for adult females (Papp *et al*., 2007). The Estimated Safe and Adequate Daily Intake, as set by the US Food and Nutrition Board is 50 – 200 µg, with a recommended dietary allowance of 55µg for men and woman. Some of the diseases that are associated with selenium deficiency are Keshan disease (cardiomyopathy of children and young women), cardiomyopathy in patients on total parenteral nutrition, muscle weakness and pain, nail changes, T and B cell dysfunction, cancer and coronary heart disease (Tapiero *et al*., 2003).

Se plays an important role in decreasing oxidative stress. Oxidative stress and free radicals contribute to the degeneration of nerve cells. Se has been found to be effective in decreasing nerve cell degeneration and also increased GPx activity, which reduces toxic effects on cells. It is believed that Se could slow the progression of diseases like Huntington's and Parkinson's, which are characterised by damage to nerve cells by free radicals and oxidative stress (Santamaria *et al*., 2003, Zafar *et al*., 2003).

Seleno-methionine

Seleno-L-Methionine (Se-Met) is the major component of dietary selenium. It is an organic form of selenium that is more bio-available than inorganic forms, and may therefore provide enhanced protection. However, very few studies have been done on the effect of Se-Met on oxidative stress. Se-Met has been shown to regulate p53 and ERK, thus demonstrating anticancer properties. It is also known to increase GPx protein and activity, and thereby decrease free radical generation (Xiong *et al*., 2007).

(A) L-Methionine **(B) Seleno-L-Methionine**

Figure 2.2: The chemical structure of (A) L-Methionine (Conn & Stumpf, 1976) and (B) Seleno-L-Methionine.

We know that WS is highly regarded as an anti-inflammatory, antitumour, antistress, antioxidant, immunostimulatory, haemopoetic, and rejuvenating natural product, and literature also tells us that selenium is a mineral with powerful antioxidant and anti-inflammatory properties. The question that now arises is will these two products, both separately and in combination, have a positive or adverse effect on cells in culture? This will ultimately give us an idea of the effect that they will have on the body.

The role of selenium in lung disease

According to Qujeq and co-workers, selenium is known to modulate the immunological mechanisms of asthma. Asthmatics are known to have a reduced circulatory Se status, and therefore reduced activity of the selenium-dependent enzyme glutathione peroxidase, which plays an extremely important role in the cellular elimination of free radicals, particularly hydroperoxides. It can be said that glutathione peroxidase protects cells from oxidative damage caused by free radicals. Low levels of Se may influence the asthmatic inflammatory process by reducing the activity of glutathione peroxidase (Qujec *et al*., 2003).

In a study of twenty-four asthmatics, those who received Se supplementation for a period of fourteen weeks showed a significant improvement in symptoms compared to those who received placebo. Even with these findings, it was documented that more studies must be undertaken in order to determine whether selenium supplementation is safe for patients with a respiratory condition like asthma (Allam & Lucane, 2004).

Interactions between antioxidants types

Most balanced diets contain multiple antioxidants, and, as mentioned in the literature, the body contains a multitude of endogenous antioxidants that are made up of enzymatic and non-enzymatic antioxidants (Stahl & Sies 2003; Pietta, 2000; Ali *et al*., 2008). It is believed that interactions between these structurally different compounds can provide additional protection against increased oxidative stress. Conducting research on the interactions of antioxidants is both practically and theoretically significant (Stahl & Sies, 2003).

Antioxidant research is starting to shift away from *in vitro* testing of single, purified compounds, and focusing more on the interactions that occur naturally between antioxidants, which seldom work independently. Various types of interactions may result from the combination of two or more antioxidants. An additive effect refers to the sum of the effects of the individual components; a synergistic effect occurs when the effect is greater than the sum of the individual components; and an antagonistic effect is when the sum of the effects is less than the predicted sum of the individual components (Wang *et al*., 2011).

These effects can occur between the polyphenolics in the same extract i.e., for WS epicatechin and gallic acid. Antioxidant effects can be enhanced if there is a synergistic effect between non-enzymatic and enzymatic antioxidants such as polyphenolics and GPx. For example GPx activity is dependent on Se, a cofactor for this enzyme. By optimising Se levels, optimal GPx functioning occurs and possibly increased synergism between GPx and polyphenolics.

Aims

The aims of this study were therefore to:

1. Determine the non-enzymatic antioxidant content of a water extract of WS by measuring the total phenolic content (TPC) and total flavonoid content (TFC).

2. Optimise the 2,2-diphenyl-2-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC) and ORAC assays in order to determine the antioxidant properties of water extracts of WS and Se-Met.

3. Determine the effect of WS and Se-Met on the cellular growth of SC-1 (mouse fibroblast) cell line, using the combined Neutral Red (NR)/Crystal Violet (CV) assay and evaluate the effects of WS and Se-Met, alone and in combination, on SC-1 cellular morphology with light and phase contrast microscopy.

4. Determine the protective effects of WS and Se-Met against the oxidative damage on the SC-1 (mouse fibroblast) cell line, using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay and determine the type of interaction, if any, between WS and Se-Met using the DCFH-DA cell-based assay on the SC-1 cell line.

5. Determine the effect of WS and Se-Met on the cellular growth of chick embryo lung primary cell cultures (CELC), using the combined NR/CV assay and evaluate the effects of WS and Se-Met, alone and in combination, on chick embryo lung primary cell culture morphology with light and phase contrast microscopy.

6. Determine the protective effects of WS and Se-Met against the oxidative damage on the CELC, using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay and determine the type of interaction, if any, between WS and Se-Met using the DCFH-DA cell-based assay on the CELC.

Hypotheses

Hypothesis 1:

A water extract of WS will have high TPC and TFC levels, and show significant antioxidant activity in the DPPH, TEAC and ORAC assays.

Hypothesis 2:

Water extracts of WS and Se-Met, alone and in combination, will not show cytotoxicity in the SC-1 cells and CELC, and will protect these cells against AAPH-induced oxidative damage in the DCFH-DA assay.

Hypothesis 3:

WS, a non-enzymatic antioxidant, and Se-Met, a cofactor of antioxidant enzymes, will act synergistically to protect the SC-1 and CELC against AAPH-induced oxidative damage.

Chapter 3: Antioxidant content and capacity of *Withania somnifera* and selenium alone and in combination

3.1 Introduction

WS, an Ayurvedic medicinal plant (Mishra *et al.*, 2000), possesses a multitude of pharmacological properties (Auddy *et al.*, 2003), namely antioxidant, anti-inflammatory, antitumour, antistress, immunostimulatory, haemopoetic and rejuvenating properties (Dhuley, 1997, Davis *et al.*, 1998, Chaurasia *et al.*, 1999, Archana *et al.*, 1999, Kulkarni *et al.*, 2008, Rasool *et al.*, 2006, Visavadiya *et al.*, 2007, Panda *et al.*, 1999). A medicinal plant's antioxidant activity is largely due to the presence of polyphenolics and flavonoids (Amaral *et al.*, 2009, Pal *et al.*, 2011).

The Ayurvedic medicinal system makes use of dry powders or crude extracts of medicinal plants (Widodo *et al.*, 2011). Simple infusions and decoctions were traditionally freshly prepared from medicinal plants and used to treat a wide range of conditions (Di Stasi *et al.*, 2002). A more modern approach to the use of traditional medicinal plants, which forms part of a large, rapidly growing alternative health industry, is to prepare single molecules or pure compounds based on identified beneficial effects (Widodo *et al.*, 2011). However, the activity of complex mixtures such as those used by indigenous populations is neglected. Although the concentration and identity of specific molecules in the mixture used in this study is unknown, it is known that synergism can occur between antioxidants e.g., polyphenolics and antioxidant enzymes.

Various strategies can be employed in the evaluation of the properties of medicinal plants. These may involve the isolation and characterisation of the plant's major constituents. Using TLC, Pal *et al.* (2012) identified alkaloid, hydrobenzene, terpene ansteroid, saponin, organic acids and flavones in WS. RP-HPLC was further employed to identify and quantify four polyphenols in WS, namely epicatechin, quercetin-3-rhamnoside, gallic acid and rutin hydrate (Pal *et al.*, 2012). In a separate study, eight polyphenols were identified in WS using HPLC, namely gallic, syringic, vanillic, benzoic and p-coumaric acids, catechin, kaempferol and naringenin (Alam *et al.*, 2011). Although important information related to the molecules present in medicinal plants is provided in these studies, the processes are time-consuming and provide very little information specifically related to the activity, efficacy and possible toxicity of extracts as they were traditionally used (Tabart *et al.*, 2009). In this study, an infusion or a decoction prepared from the roots, stems and leaves of WS was prepared with water as a solvent.

A bio-informatics study to investigate and elucidate the mechanisms of action of a crude extract and purified components of WS leaves was undertaken by Widodo and co-workers in 2011. These authors reported that Withaferin A (an isolated, purified component) induced cellular pathways that signified higher toxicity, while the crude extract was less toxic. This could be an indication that the crude extract may be a safer option in therapeutic terms (Widodo *et al*., 2011). This may also be due to the lower concentration of withaferin A in the crude extracts, and/or the beneficial effects of other components such as polyphenolics.

Selenium (Se) is an important cofactor of antioxidant enzymes such as GPx (Papp *et al*., 2007; Li *et al*., 2008; Zhou *et al*., 2009). A low Se intake will result in low GPx activity and a low enzymatic antioxidant status, and possibly lead to poor interaction between enzymatic and non-enzymatic antioxidants such as those present in WS. Se-Met is an organic form of selenium, which is more bio-available than inorganic forms, and may therefore provide enhanced protection (Xiong *et al*., 2007). For these reasons, Se-Met was chosen for this study.

The primary aim of this chapter is to determine the antioxidant content and activity of a water extract of WS. Although Se-Met is not expected to show activity in the assays used, it will indirectly serve as a negative control.

The specific objectives of this chapter are:

1. To measure the TPC and TFC content of a water extract of WS.
2. To optimize the DPPH, TEAC and ORAC assays in order to determine the antioxidant activity of a water extract of WS and Se-Met, alone and in combination.
3. Use the best of the above techniques to quantify the antioxidant activity of WS.

3.2 Materials

Preparation of extracts of *Withania somnifera*

Plant material was collected in the Pretoria area, South Africa (SA) and was identified as WS by botanists from the Department of Botany, University of Pretoria, SA. The leaves, stems and roots of the plant were fragmented and a water extract of 100 mg/ml was made using double distilled water (ddH_20). An antibiotic stock solution was prepared by mixing 10 000 µg/ml penicillin G (sodium salt), 10 000 µg/ml streptomycin sulphate and 25 µg/ml Amphotericin B in 0.85% saline. A volume of 2.66 ml of the antibiotic stock solution was added to 66.6 ml WS extract (1:25) and the extract was filtered. were added and the solution was filtered, divided into aliquots and stored at -70℃.

Preparation of seleno-methionine

Seleno-L-Methionine (Se-Met) was obtained from Sigma-Aldrich, Johannesburg, SA and prepared as described in Chapter 4 with ddH_2O.

For the combination studies, equal volumes of WS and Se-Met were added and the final concentrations are specified in Table 3.1.

Reagents, equipment and disposable plastic ware

Folin-Ciocalteu's reagent, sodium carbonate anhydrous, catechin, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt (ABTS) and fluorescein sodium salt were obtained from Sigma-Aldrich Company, Atlasville, SA. The organic solvent methanol was of analytic grade and was purchased from Merck, Johannesburg, SA.

Equipment used included a BioTek plate reader purchased from Analytical and Diagnostic Products (ADP) Johannesburg, SA and a FLUOstar OPTIMA plate reader from BMG Labtechnologies, Offenburg, Germany. A Hermle Z300 centrifuge, a Crison GLP 21 pH Meter and Eppendorf pipettes from Eppendorf AG Hamburg, Germany were all supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, SA.

Disposable plastic ware included 96-well plates, 15 ml and 50 ml tubes, and pipette tips (10, 25, 100, 200, and 1000 µl), which were obtained from Greiner Bio-One, supplied by LASEC, Cape Town, SA.

Laboratory facilities

All research was conducted in the research facilities of the Departments of Anatomy and Pharmacology in the School of Medicine, Faculty of Health Sciences, University of Pretoria.

3.3 Methods

Total phenolic content

The total phenolic content (TPC) of WS was determined using the Folin-Ciocalteu (FC) method of Amin *et al.* (2006), which was modified to a 96-well plate method. The FC assay is based on the ability of a phenolate ion to reduce the phosphotungstic-phosphomolybdic complex (FC reagent) to a blue coloured solution. An extract with a high concentration of phenolic compounds will cause an increase in the reduction of the FC reagent, a darker colour and therefore a higher absorbency (Arbianti *et al.*, 2007). A catechin stock solution of

0.1 µg/µl was used, from which a serial dilution of 0.004, 0.008, 0.013, and 0.017 µg/µl in ddH_2O was used to prepare the standard curve. For the determination of total TPC in WS, 15 and 20 µl of a 100 mg/ml stock solution was used. To the above, 50 µl of FC reagent and 50 µl of 7.5% Na_2CO_3 were added to each well. The colour development was determined spectrophotometrically at 630nm using a BioTek plate reader. The TPC of WS was expressed as catechin equivalent (CE) in grams per 100 grams (g/100 g) wet (WW) or dry weight (DW). Data was converted to gallic acid equivalents (GAE) to enable comparison of results to other studies.

Total flavonoid content

The total flavonoid content (TFC) was determined using the modified aluminium chloride ($AlCl_3$) method of Amaral *et al.* (2009). The principle of this method is based on $AlCl_3$ forming acid stable complexes with the C-4 keto group, and the C-3 or C-5 hydroxyl groups of flavones and flavonols. $AlCl_3$ also forms acid labile complexes with the orthodihydroxyl groups in the A- or B-rings of flavonoids (Chang *et al.*, 2002). A serial dilution of a 1 µg/µl catechin stock solution was used as a standard. The concentrations used to prepare the standard curve were 0.03, 0.06, 0.09, 0.12, 0.15 and 0.18 µg/µl. Volumes of 25 and 30 µl of a 100 mg/ml WS solution were used to determine the TFC of WS. To the standards/samples in the wells of a 96-well plate, 20 µl of 2.5% $NaNO_2$, 20 µl of 1.25% $AlCl_3$, and 100 µl of 2% NaOH was added. Colour development was determined spectrophotometrically at 450 nm. The TFC of WS was expressed as CE in grams per 100 grams (g/100 g) wet (WW) or DW.

2,2-Diphenyl-1-picrylhydrazyl radical-scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a free radical that is stable at room temperature and produces a violet solution in methanol (Katsube, 2004). DPPH is reduced in the presence of antioxidants, resulting in a loss of colour. The DPPH radical-scavenging assay was carried out according to a modified method of Awika *et al.* (2003). Stock solutions of DPPH were prepared by dissolving 24 mg DPPH in 100 ml methanol. The solution was shaken in a sonicator for 20 minutes. The working solution was prepared by diluting 20 ml of stock solution with 80 ml methanol. A concentration range of 0 – 800 µM of a 1M solution of trolox, or 25 mg/ml, was used to prepare a standard curve. The concentrations of WS and Se-Met alone and in combination, as used are shown in Table 3.1. A 15 µl volume of each sample was added to 285 µl of DPPH in a 96-well plate. To eliminate the possible effects of interference, each sample served as its own control i.e., all components without DPPH. The plate was read immediately at 570 nm using a BioTek plate reader and thereafter at 15, 30, 60, 90 and 120 minutes exposure. The effect of time on the decomposition of DPPH was determined and the antioxidant activity was expressed as µM TE per g WW or DW of the

sample and also as a percentage of the control, where the control is 100%. The IC_{50} of the samples, the concentration of the extract required to scavenge or inhibit the formation of the DPPH radicals by 50% (Bertonelj *et al.*, 2007; Lee *et al.*, 2003) was calculated using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

Trolox equivalent antioxidant capacity assay

The trolox equivalent antioxidant capacity (TEAC) assay, according to Awika *et al.* (2003), was modified to be measured with a BioTek plate reader. The 2,2'-azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt ($ABTS^{\bullet+}$) was freshly generated by adding 3 mM potassium peroxodisulfate ($K_2S_2O_8$) solution to 8 mM ABTS and the mixture was left to react in the dark at room temperature for at least 12 hours. The working solution was prepared by diluting the ABTS stock solution with 0.2 M phosphate buffer, pH 7.4. Trolox was used as a standard, concentration range 0 – 1000 µM. The concentrations of WS and Se-Met, alone and in combination, are shown in Table 3.1. A 290 µl volume of the working solution was added to 10 µl of each of the samples. The reaction mixtures were left to stand at room temperature and the absorbance readings were taken at 630 nm after 15 and 30 minutes. Each sample served as its own control i.e., all components in the absence of ABTS. The results were expressed as µM TE/g WW or DW of sample, and as a percentage of the control, where the control is 100%. The IC_{50} of the samples was also calculated.

Table 3.1: WS and Se-Met concentrations alone and in combination for DPPH and TEAC assays

Sample	Concentrations and combinations
WS	3.13, 6.25, 12.50, 25.00, 50.00 & 100.00 mg/ml
Se-Met	0.0156, 0.03125, 0.0625, 0.125, 0.25 & 0.50 pg/ml ($7.96x10^{-5}$ – $2.55x10^{-4}$ nM)
WS + Se-Met	1.60 mg/ml WS + 0.0078 pg/ml Se-Met 3.13 mg/ml WS + 0.0156 pg/ml Se-Met 6.25 mg/ml WS + 0.03125 pg/ml Se-Met 12.50 mg/ml WS + 0.0625 pg/ml Se-Met 25.00 mg/ml WS + 0.125 pg/ml Se-Met 50.00 mg/ml WS + 0.25 pg/ml Se-Met

Oxygen radical absorbance capacity assay

The ORAC assay was based on a modified method of Ou *et al.* (2002). AAPH was used as a peroxyl radical generator, trolox was used as a standard (0 – 1000 µM), and fluorescein as a fluorescent probe. Phosphate buffered saline (PBS) (0.2 M Na_2HPO_4, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl, pH=7.4) was used as a blank. To 160 µl volume of 0.139 nM fluorescein working solution, 40 µl of PBS, trolox (serial dilution), WS, Se-Met or a combination of WS and Se-Met was added. The concentrations of the samples used are indicated in Table 3.2.

This was followed by the addition of 40 µl of a 0.11 µM AAPH solution. Samples were mixed well and the microplate was placed into the plate reader and incubated at 37°C. The fluorescence was measured every 5 minutes for 4 hours. The assay protocol included: measurement start time of 0.0 seconds, 10 flashes per cycle, 300 seconds cycle time, 485 nm for the excitation filter and 520 nm for the emission filter. The final ORAC values of the samples were calculated by using the net area under the decay curves (AUC). The results were expressed as µmol TE per g WW or DW sample. For the control samples, PBS replaced the WS and Se-Met samples.

The highest concentration of Se-Met that was used in the DPPH and TEAC assays was used (0.50 pg/ml) for the ORAC assay. To this concentration, increasing concentrations of WS were added (3.13 – 100 mg/ml) as shown in Table 3.2.

Table 3.2: WS and Se-Met concentrations alone and in combination for ORAC assay

Sample	Concentration range and combinations
WS	3.13, 6.25, 12.50, 25.00, 50.00 & 100.00 mg/ml
Se-Met	0.50 pg/ml ($2.55x10^{-4}$ nM)
WS + Se-Met	3.13 mg/ml WS + 0.50 pg/ml Se-Met 6.25 mg/ml WS + 0.50 pg/ml Se-Met 12.50 mg/ml WS + 0.50 pg/ml Se-Met 25.00 mg/ml WS + 0.50 pg/ml Se-Met 50.00 mg/ml WS + 0.50 pg/ml Se-Met 100.00 mg/ml WS + 0.50 pg/ml Se-Met

Data management and statistical analysis

All data is an average of at least three experiments and each measurement was done at least in triplicate, thereby generating a minimum of nine data points. The results are expressed as mean ± standard error of mean (SEM). Fisher's least significant difference (LSD) test was used for comparison of means using StatSoft, Inc. (2007) STATISTICA (data analysis software system), version 8.0. The IC_{50} of the samples for the DPPH and TEAC assays was calculated using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

3.4 Results and discussion

Total phenolic content

Phenolic compounds are present in both edible and non-edible plants, and possess many biological properties such as antioxidant activity (Kähkönen *et al.*, 1999). Anti-inflammatory action, antimicrobial activity, and inhibition of platelet aggregation are other important

properties displayed by phenolic compounds (Kanner *et al.*, 1994). These molecules are essential for the normal growth and development of the plant, as well as for protection against infection and injury. The antioxidant activity of phenolics is largely due to their redox properties, allowing them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kähkönen *et al.*, 1999). Polyphenols have the ideal chemical structure to act as antioxidants, and studies have shown them to be more effective *in vitro* than vitamins E and C (Rice-Evans *et al.*, 1997).

Determination of the TPC is a rapid method that involves the measurement of the colour that develops via the interaction between the FC reagent and polyphenols. Catechin was used as a standard to derive the calibration curve that was linear from 0 – 0.017 µg/µl, with an R^2 value of 0.99.

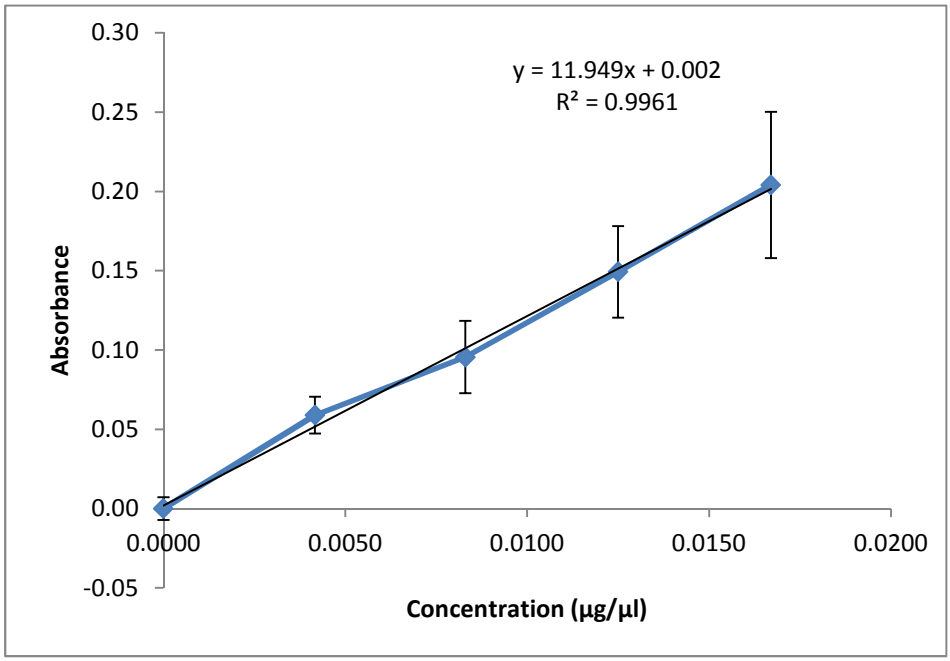


Figure 3.1: Catechin standard curve for FC assay. Concentration range 0–57.59 µM, equivalent to 0–0.0167 µg/µl CE or 0–0.61µg/µl GAE. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM.

The TPC of WS was found to be 4.84 g CE/100 g DW (2.90 g GAE/100 g DW) or 0.011 g CE/100 g WW (0.0066 g GAE/100 g WW).

Pal *et al.* (2011) measured the TPC content of six different extracts of WS, including a water extract. A TPC of 66.72 µg GAE/mg DW was found for the water extract. For purposes of comparison, this value was converted to g GAE/100 g, which is 6,67 g GAE/100 g extract. These findings by Pal *et al.* (2011) is double the TPC determined in this study, i.e., 2.90

g/100 g for the WS water extract. Polyphenolic content of a plant is a function of season, climatic conditions and geographical location. The WS used by Pal *et al*. (2011) was obtained from Mysore, India, and in this study, WS from the Highveld region of SA was used. Furthermore, although water extracts were used in both studies, the temperature and time of extraction may have differed.

Another study was carried out on the TPC of 80% aqueous methanol extracts of the roots, fruit and leaves of WS. The roots were found to have a TPC of 17.80 ± 5.80 mg GAE/g (DW) (1.80 ± 0.58 g GAE/100 g DW), the fruits had a TPC of 22.29 ± 1.99 mg GAE/g (DW) (2.22 ± 0.2 g GAE/100 g (DW)) and the leaves of WS had the highest phenolic content of 32.58 ± 3.16 mg GAE/g (DW) (3.2 ± 0.32 g GAE/100 g (DW) (Alam *et al*., 2011). This is similar to the data in the present study where a TPC of 2.90 g GAE/100 g DW was found, indicating that most polyphenolics in WS may be water soluble. The yield of biologically active compounds found in plants, for example, polyphenols, is dependent on both the solvent used and the method of extraction (Tabart *et al*., 2007). The antioxidant concentration relative to the plant material can be greatly increased by selecting a suitable extraction technique and solvent (Schwarz *et al*., 2001), and the method of extraction must enable complete extraction of the compounds without chemical modification (Tabart *et al*., 2007).

In 2012, Pal *et al.* used TLC to identify the main compounds of a methanolic extract of WS as alkaloid, hydrobenzene, terpene ansteroid, saponin, organic acids and flavones. Alam and co-workers (Alam *et al*., 2011) identified five phenolics by HPLC analysis and these were gallic, syringic, vanillic, benzoic and p-coumaric acids. Pal *et al.* (2012) identified the main polyphenolic compounds as epicatechin, quercetin-3-rhamnoside, gallic acid and rutin hydrate using RP-HPLC. The variations in the results between the two studies may be due to extraction methods and experimental conditions, such as the type of column used.

The TPC of WS can be compared to the TPC of other medicinal plants to determine if levels are within the range found for medicinal plants. Huang *et al.* (2010) conducted an investigation into the TPC of thirteen medicinal fruit plant species in Hong Kong, using the FC method. The results of the tested samples ranged from 0.2 – 16.2 g of GAE/100 g DW. Cai *et al.* (2004) measured the antioxidant activity and phenolic content of 112 traditional Chinese medicinal plants that are associated with anticancer properties and concentrations were found to range from 0.19 – 50.20 g GAE/100 g DW for aqueous extracts, with an average value of 2.93 GAE g/100 g DW. The TPC of WS as determined in this study, 4.84g CE/100 g DW, is similar to that found in other medicinal plants.

Four plants belonging to the same family as WS, Solanaceae, were amongst the plants evaluated in the study by Cai *et al.* (2004). An aqueous extract of the seeds of *Hyoscyamus niger* L. yielded a TPC of 0.58 g GAE/100 g DW, while the root bark of *Lycium barbarum* L. was found to have 0.76 g GAE/100 g DW and the fruit of the same plant had 0.70 GAE g/100 g DW. The TPC of the aerial parts of the plant *Solanum nigrum* L. was 0.90 g GAE/100 g DW. The yield of 2.9 g GAE/100 g DW for WS roots, stems and leaves in this study was greater than that measured for the seeds, bark and fruit of medicinal plants of the same family.

Total flavonoid content

Flavonoids are phenolic derivatives that are synthesized in considerable amounts, widely distributed in plants, and are known to have antioxidant and anti-inflammatory properties (Vinson *et al.*, 1995; Pourmorad *et al.*, 2006). These polyphenolics have the ability to scavenge free radicals and inhibit hydrolytic and oxidative enzymes (Pourmorad *et al.*, 2006).

Flavonoids present in medicinal plants have the ability to activate antioxidant enzymes, and inhibit enzymes like myeloperoxidase, NADPH oxidase and xanthine oxidase, which synthesize free radicals. These molecules are also known for their cardioprotective effect, possessing the ability to prevent LDL peroxidation (Atmani *et al.*, 2009; Amaral *et al.*, 2009).

Catechin was used as a standard to derive the calibration curve, which was linear from 0 – 0.2 µg/µl with an R^2 value of 0.99. The TFC for WS was found to be 355 mg CE/100 g DW or 0.87 mg CE/100 g WW which is 7.33% of the TPC, DW. This indicates that the majority of polyphenolics present in WS are probably not flavonoids. However, the yield of flavonoids will be influenced by the method of extraction, and as well as the solvent used, and the results may vary according to this. The antioxidant activity of WS is therefore possibly derived from other polyphenolics such as phenolic acids, stilbenes, tannins, ligans and lignin (Kähkönen *et al.*, 1999). Identified polyphenolics specifically in WS are epicatechin, quercetin-3-rhamnoside, gallic acid and rutin hydrate, of which epicatechin, quercetin-3-rhamnoside and rutin hydrate are flavonoids (Pal *et al.*, 2012; Alam *et al.*, 2011).

A study by Alam and co-workers (2011) investigated the TFC of 80% aqueous methanol extracts of the roots, fruit and leaves of WS. The roots were found to have a TFC of 15.49 ± 1.20 mg CE/g (DW), the fruits had a TFC of 21.15 ± 5.32 mg CE/g (DW), and the leaves of WS had the highest flavonoid content of 31.58 ± 5.07 mg CE/g (DW) (Alam *et al.*, 2011). The TFC of WS obtained in the present study, 3.55 mg CE/g (DW) is much lower than the results

obtained by Alam and co-workers. In the same study, three flavonoids were identified in WS using HPLC analysis and these were catechin, kaempferol and naringenin. Of these, catechin concentration was the highest in the leaves of WS (Alam *et al.*, 2011).

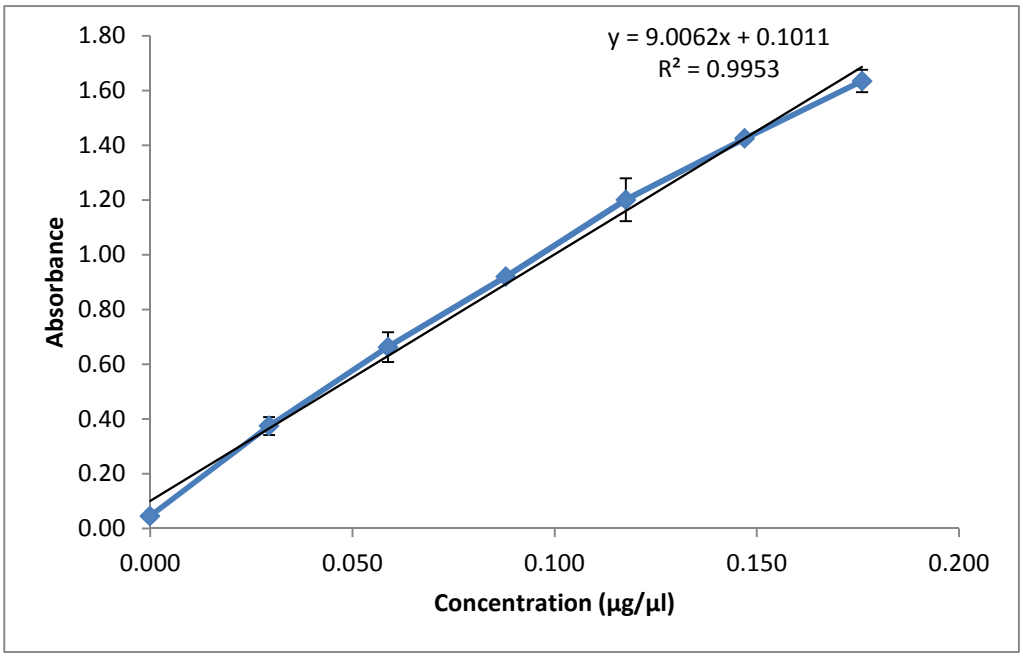


Figure 3.2: Catechin standard curve for determination of flavonoid content. Concentration range 0–575.9 µM, equivalent to 0–0.167 µg/µl CE. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM.

Table 3.3: Summary of the antioxidant content of WS

Assay	DW	WW
TPC	4.84g CE/100g 2.90 g GAE/100g	0.011g CE/100g 0.0066g GAE/100g
TFC	355 mg CE/100g	0.00872 mg CE/100g

Water extracts of WS have been shown to contain significant amounts of polyphenolics and flavonoids. It must be determined whether these levels translate into significant antioxidant activity. For this purpose, the DPPH, TEAC and ORAC assays were used, and the results are discussed in detail below.

2,2-Diphenyl-1-picrylhydrazyl radical-scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a free radical that is used in the DPPH assay. It is stable at room temperature and produces a violet solution in methanol. In the presence of antioxidants, DPPH is reduced, resulting in a loss of absorption and colour. The DPPH assay is widely used *in vitro* due to its stability, simplicity and reproducibility (Katsube, 2004; Awika *et al.*, 2003), but the relevance of the assay to biological systems is undefined. In this study,

the DPPH assay was used to determine the antioxidant activity of WS alone and in combination with Se-Met.

Trolox was used as a standard to derive the calibration curve for the DPPH assay. The standard curve was linear from 0 – 800 µM with an R^2 value of 0.99 (Figure 3.3). Data generated from the DPPH assay can be reported as IC_{50}, the concentration of the samples at which 50% of the DPPH is scavenged or as µmol TE/g, the standard method of data expression for the DPPH assay, and other antioxidant assays such as the TEAC and ORAC assays.

The reaction between trolox and DPPH was rapid (Figure 3.4A and 3.5A). Se-Met served indirectly as the negative control. Se-Met is considered an important cofactor of antioxidant enzymes, and therefore antioxidant activity is only expected to be observed within a physiological system where Se-Met can associate with antioxidant enzymes like GPx. No change in the colour of DPPH was observed over all Se-Met concentrations and incubation times (Figure 3.4C and 3.5C).

WS showed a delay in DPPH decolouration (Figure 3.4B and 3.5B), which did not stabilize and the time-related decreases in absorbance between T0 – T120 were significantly different (Figure 3.5B). A possible explanation for this observation is that the WS extracts are a complex mixture of molecules, whereas trolox and Se-Met are single purified compounds. Interestingly, lower concentrations of WS, as seen for the WS + Se-Met combination, measured decolourisation after 15, 30 and 60 minutes that were not statistically different (Figure 3.5D). These findings were substantiated in literature, where it was reported by Bondet and co-workers (1997) that most phenolic antioxidants react slowly with DPPH, taking up to six hours to reach stability, and therefore antioxidant activity using DPPH should be measured over a long period of time. In this study, the concentration used seems to be critical and therefore must be optimized for each extract.

In spite of the fact that decolouration was not complete for WS after 120 minutes, the IC_{50} (Figure 3.6B) and µmol TE/g for WS was calculated and was found to be 274.8 µM TE/g, or 56.34 mg/ml. Russo *et al.* (2001) evaluated the DPPH free radical-scavenging ability of a methanol extract of the roots of WS. The authors found that 400 µg/ml was equivalent to 25 µM trolox i.e., 62.5 µM TE/g. The DPPH-scavenging potential of 80% aqueous methanol extracts of the roots, fruit and leaves of WS was investigated by Alam and co-workers in 2011. These authors found the roots of WS to inhibit DPPH by 59.16 ± 1.20%, the fruits of WS by 70.38 ± 0.84% and the leaves by 91.84 ± 0.38% (Alam *et al.*, 2011).

Kintzios *et al.* (2010) evaluated the antioxidant activity of four medicinal plants traditionally grown in Slovenia. Water and methanolic extracts of the plants were evaluated using the DPPH assay. Percentage inhibition was measured after 30 minutes, and the methanolic extracts demonstrated a higher DPPH-scavenging activity than the aqueous extracts. The aqueous extracts showed inhibition ranging from approximately 15% – 70%, and the methanolic extracts results ranged from approximately 18% – 85%. Although scavenging activity was found, data reported as a percentage is difficult to compare with other studies, therefore the µM TE/g and the IC_{50} are the methods of choice for reporting data.

Lee *et al.* (2003) measured the DPPH free radical-scavenging activity of nine medicinal plant extracts traditionally used in Chinese medicine. The extract with the highest DPPH radical-scavenging activity was *Areca catechu* var. *dulcissima*, which showed an IC_{50} value of 1.8 µg/ml. All the medicinal plants tested showed DPPH free radical-scavenging activity in a dose-dependent manner. In this study, the IC_{50} of WS is 141.6 mg/ml (Figure 3.6). The lower the IC_{50}, the greater the scavenging activity of the plant.

The DPPH assay has provided tentative information on antioxidant activity in this study, because the µM TE/g and IC_{50} were calculated from data where decolouration was not complete. Furthermore Sharma and Bhat (2009) claimed that it is not possible to compare results obtained from the DPPH assay across laboratories. Protocols differ so widely that even results obtained for standard antioxidants like ascorbic acid and butylated hydroxytoluene vary greatly. Factors that have not been standardised include the concentration of DPPH, the incubation time, the reaction solvent and the pH of the reaction mixture (Sharma & Bhat, 2009). For this reason, other antioxidant assays, such as the TEAC and ORAC assays, are undertaken to confirm antioxidant activity.

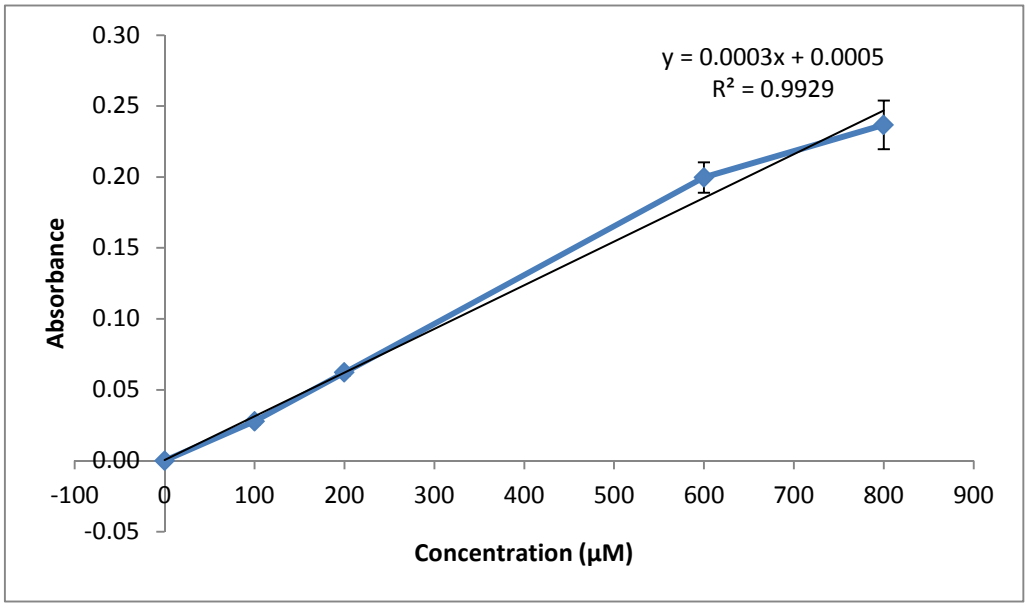


Figure 3.3: Trolox standard curve for determination of antioxidant activity, DPPH assay. Concentration range 0–800 µM, equivalent to 0–200 µg/µl TE. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM.

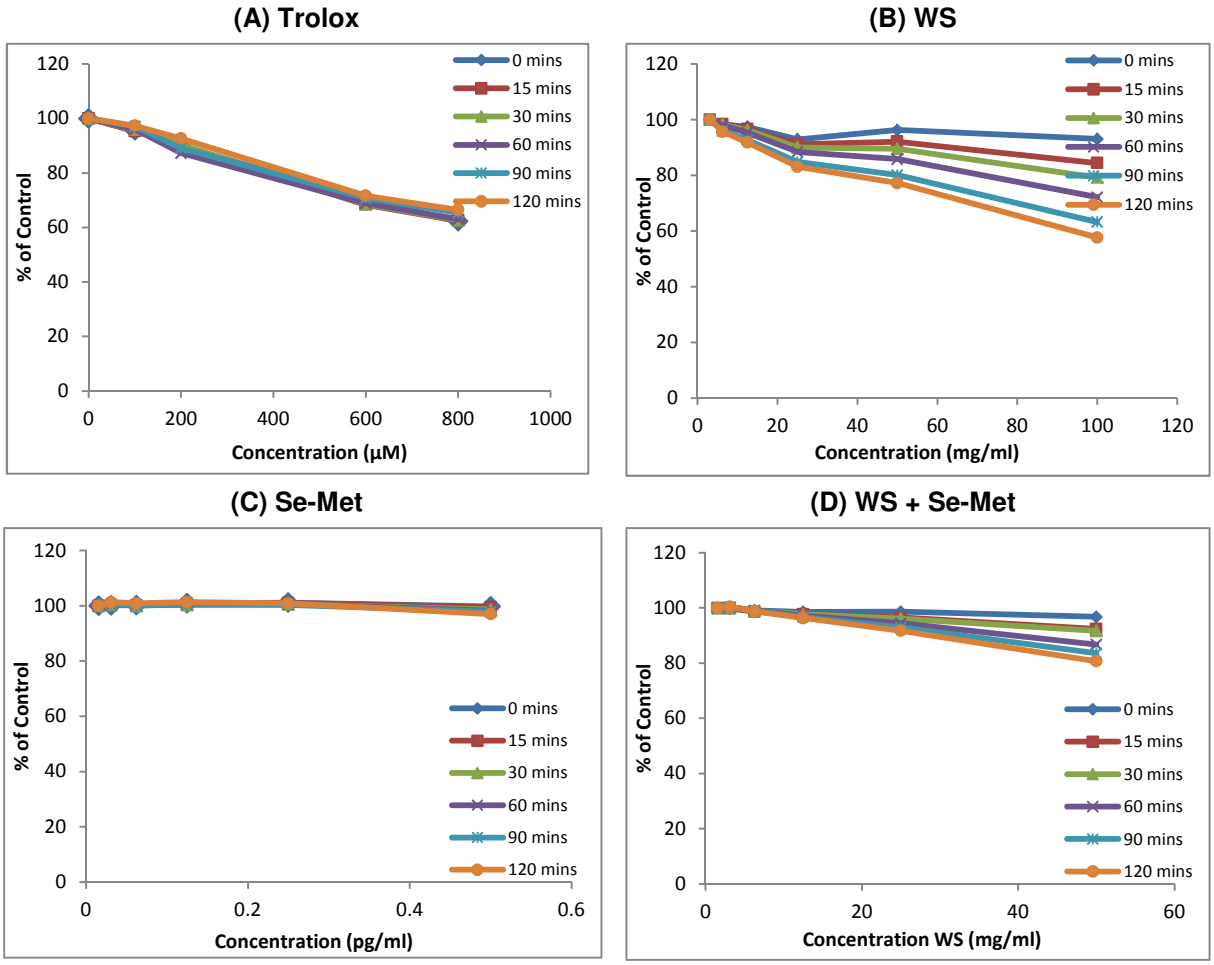


Figure 3.4: Time-related (0, 15, 30, 60, 90 and 120 minutes) decrease in absorbance of DPPH due to the presence of (A) Trolox (0–800µM), (B) WS (3.13–100 mg/ml), (C) Se-Met (0.0156–0.5 pg/ml) and (D) WS + Se-Met (1.60 mg/ml + 0.0078 pg/ml–50 mg/ml + 0.25 pg/ml).

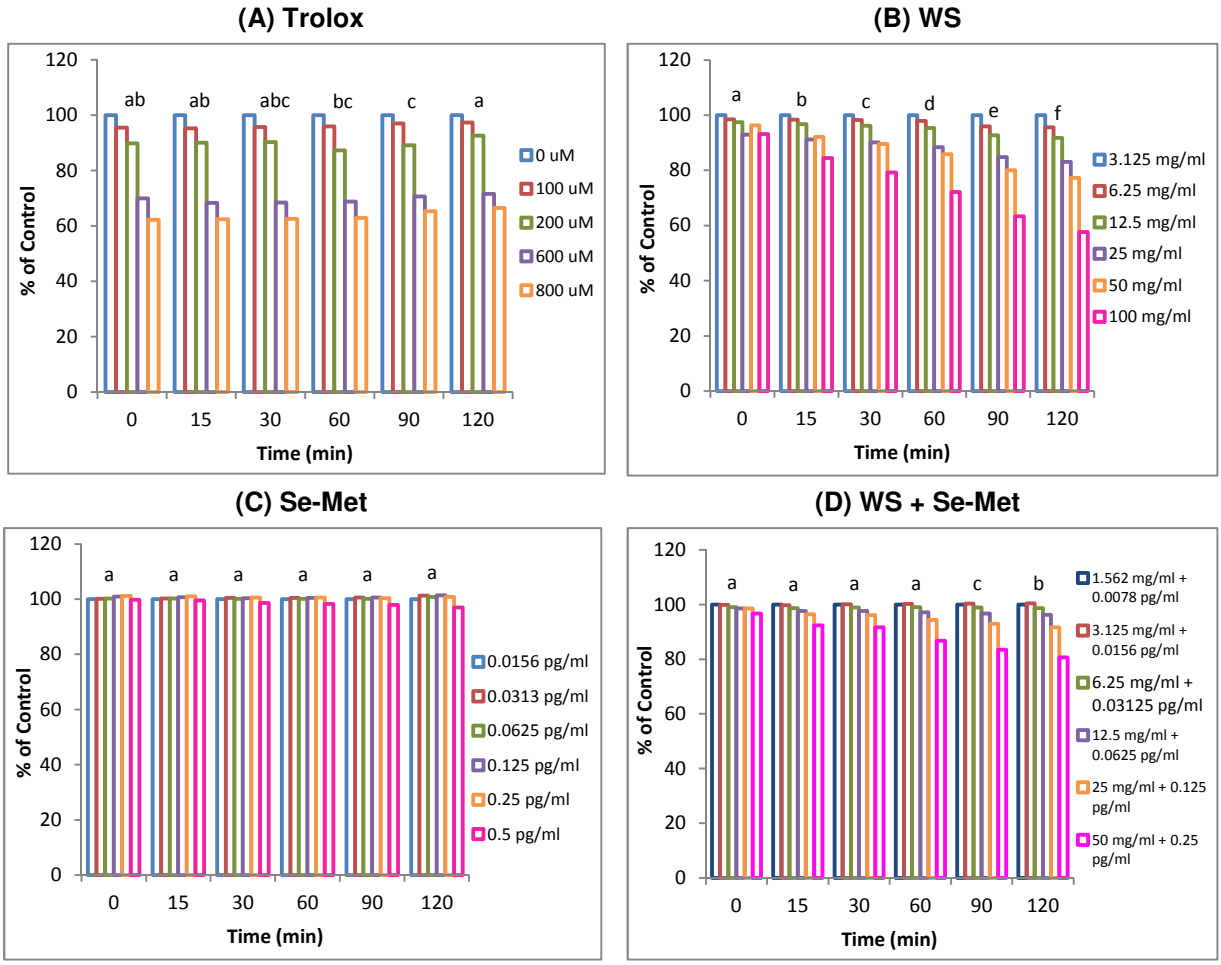


Figure 3.5: Time-related (15, 30, 60, 90 and 120 minutes) decrease in absorbance of DPPH due to the presence of (A) Trolox (0–800µM), (B) WS (3.13–100 mg/ml), (C) Se-Met (0.0156–0.5 pg/ml) and (D) WS + Se-Met (1.60 mg/ml + 0.0078 pg/ml–50 mg/ml + 0.25 pg/ml). Gradients of samples with different letters are significantly different.

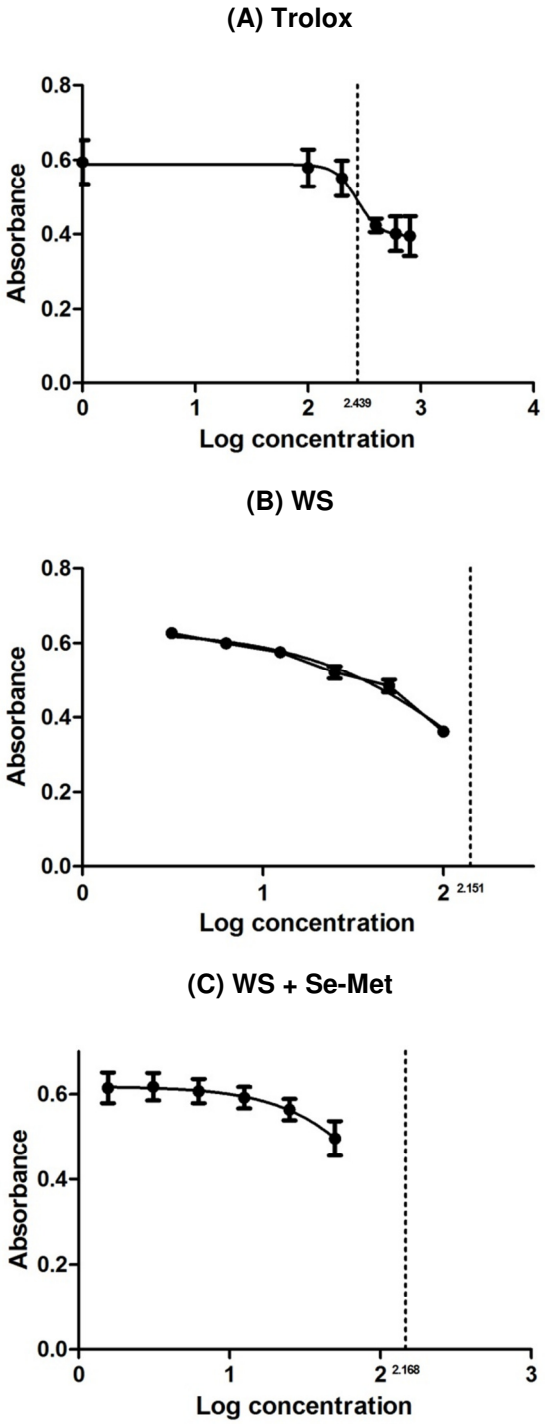


Figure 3.6: Log plot demonstrating the decrease in absorbance of (A) Trolox (0–800µM), (B) WS (3.13–100 mg/ml) and (C) WS + Se-Met (1.562 mg/ml + 0.0078 pg/ml–50 mg/ml + 0.25 pg/ml) in the DPPH assay after 120 minutes. The log IC_{50} is displayed as a dotted line in each graph.

Trolox equivalent antioxidant capacity assay

The TEAC assay is an electron transfer assay that measures the ability of an antioxidant to scavenge $ABTS^{\bullet+}$ (2,2'-azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diammonium salt), a free radical, as compared to a trolox standard. The $ABTS^{\bullet+}$ is generated by reacting a strong oxidising agent with the ABTS salt. In this study, potassium peroxodisulfate was used as an oxidising agent. The reduction of the $ABTS^{\bullet+}$ radical by the antioxidant is measured by the decrease of its characteristic long wave absorption spectrum (Awika *et al*, 2003). The TEAC assay can be used over a wide range of pH values and in both aqueous and organic solvent systems and for these reasons it is employed to determine the antioxidant activity of many different compounds and food samples (Huang *et al.*, 2005; Tabart *et al.*, 2009). Although this method has been established as a valuable tool in the evaluation of antioxidant activity *in vitro,* the method, much like DPPH, has not yet been correlated to biological systems (Awika *et al*, 2003).

The TEAC assay is usually classified as an end-point assay, and does not take the different reaction rates between antioxidants and oxidants into account. Therefore, in this study, the TEAC assay was modified to a 96-well assay, enabling the samples to be read at different time intervals. The effect of time on the antioxidant activity was evaluated as described for DPPH.

To calculate the µM TE/g, a standard curve derived from trolox was linear from 0 – 1000 µM (Figure 3.7), with an R^2 value of 0.99 was used. From this curve the antioxidant activity of WS measured using the TEAC assay was 2.2 mmol/100 g DW and 0.0054 mmol/100 g WW.

The decrease in absorbance, and hence the decolouration of ABTS by trolox (Figure 3.8A and 3.9A), WS (Figure 3.8B and 3.9B), Se-Met, (Figure 3.8C and 3.9C) (negative control) and a combination of WS and Se-Met (Figure 3.8D and 3.9D) over time (0 – 30 minutes) was measured. A dose-dependent decrease in absorbance was observed, which was rapid for trolox and constant for WS after 15 minutes (Figure 3.9B). No change in absorbance was found for Se-Met (Figure 3.9C). The µM TE/g or IC_{50} for WS was calculated after 30 minutes. Compared to the DPPH assay, the TEAC assay is rapid and generates reproducible results after only 15 minutes.

The IC_{50} of trolox, which was calculated after 30 minutes (Figure 3.10A), is 428.5 µM (219.68 mg/ml). The IC_{50} of WS (Figure 3.10B) alone was calculated to be 60.3 mg/ml. Kumar and co-workers (2008) measured the antioxidant activity of a methanolic extract of WS using the

TEAC assay, and obtained an IC_{50} value of 214.78 µg/ml, or 0.215 mg/ml sample. This is considerably higher than the IC_{50} obtained in the present study, which indicates that methanol will extract polyphenolic components with high antioxidant activity more efficiently.

The TEAC value of twenty-two samples from thirteen edible and medicinal fruit plant species in Hong Kong were evaluated and the values ranged from 2.4 – 213.4 mmol trolox/100g DW, with a mean value of 53.9 mmol trolox per 100g DW (Huang *et al.*, 2010).

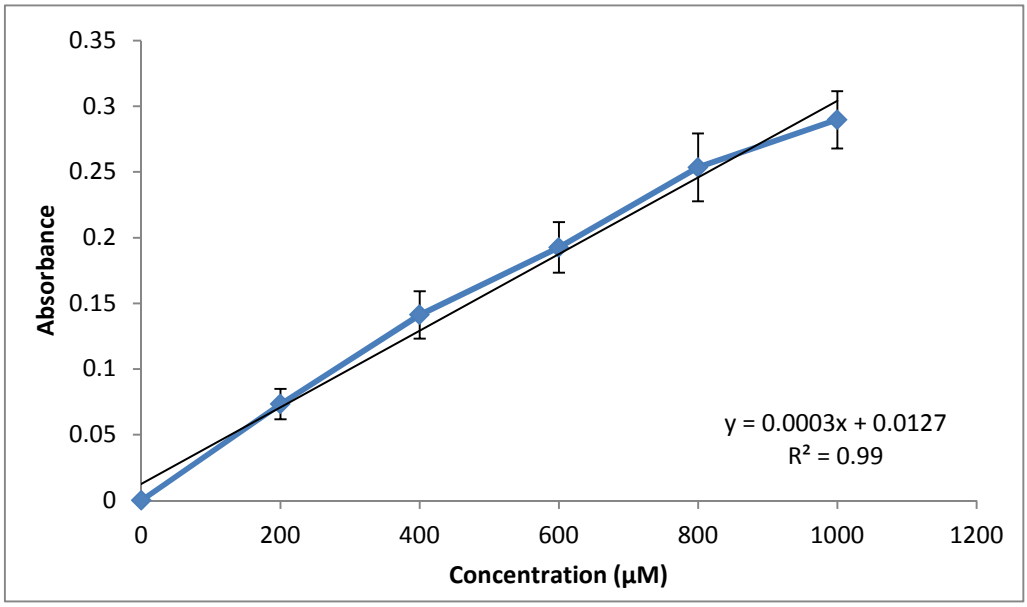


Figure 3.7: Trolox standard curve for determination of antioxidant activity, TEAC assay. Concentration range 0–1000 µM, equivalent to 0–250.29 µg/µl TE. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM.

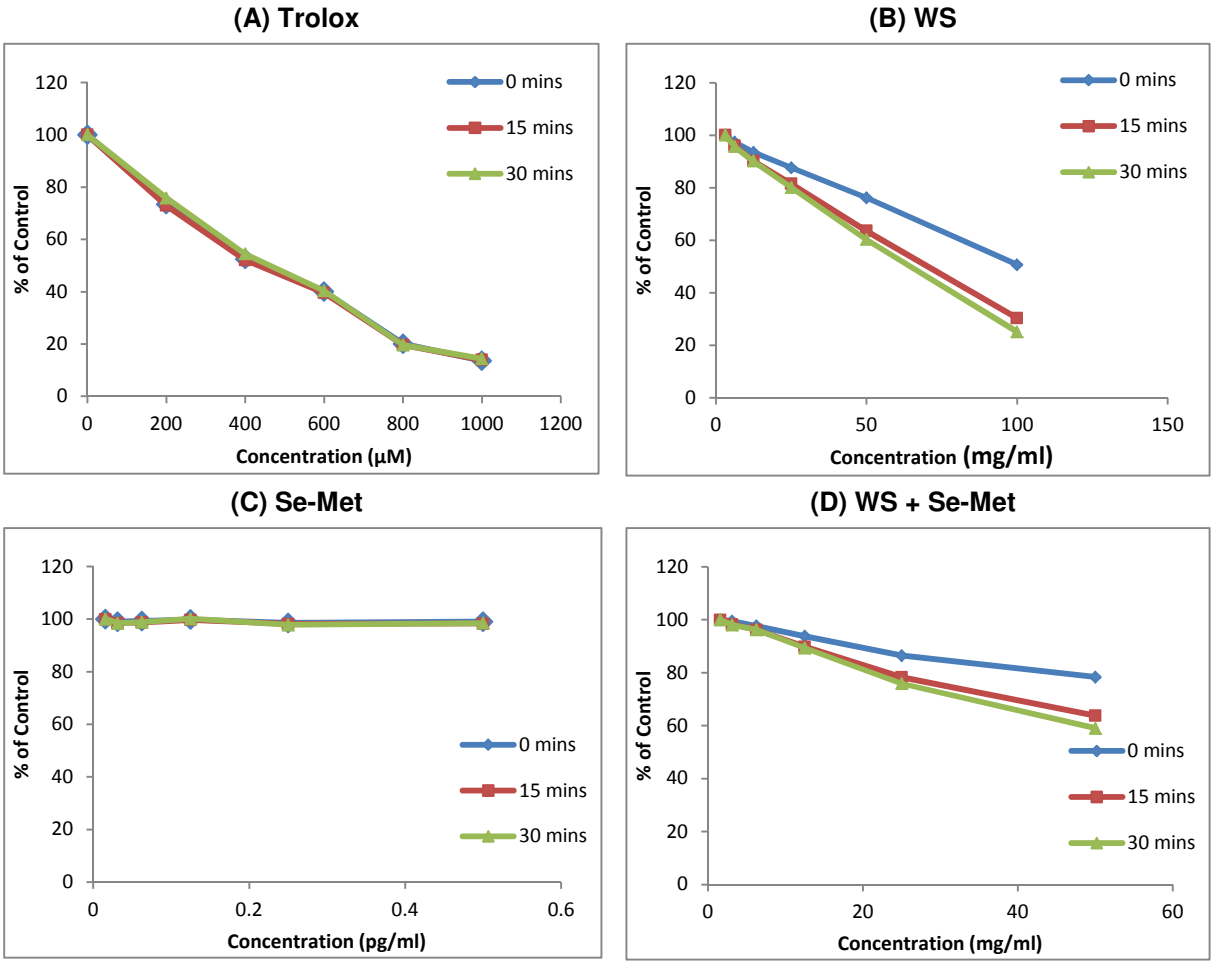


Figure 3.8: Time-related (0, 15 and 30 minutes) decrease in absorbance of ABTS due to the presence of (A) Trolox (0–1000µM), (B) WS (3.13–100 mg/ml), (C) Se-Met (0.0156–0.5 pg/ml) and (D) WS + Se-Met (1.562 mg/ml + 0.0078 pg/ml–50 mg/ml + 0.25 pg/ml).

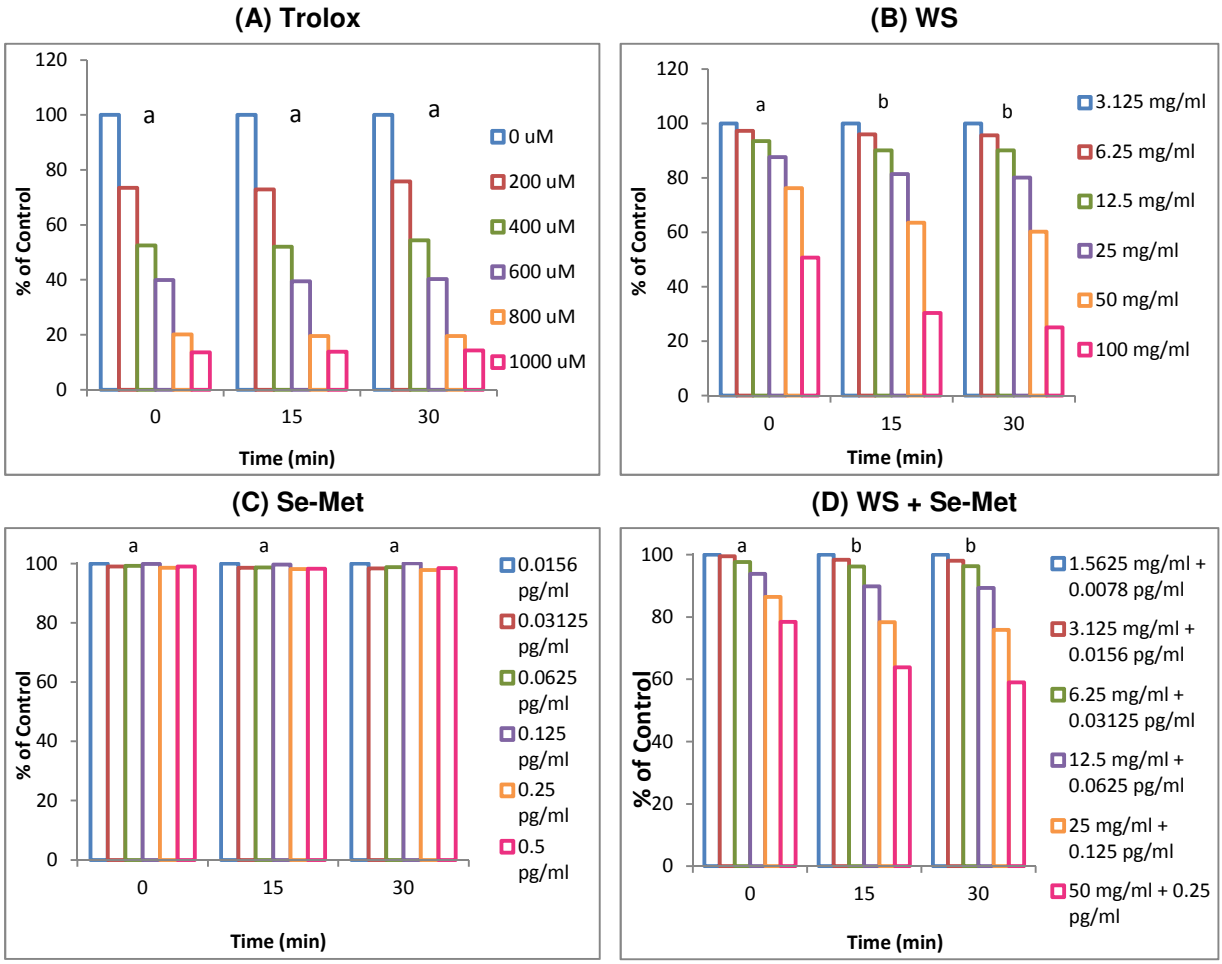


Figure 3.9: Time-related (0, 15 and 30 minutes) decrease in absorbance of ABTS due to the presence of (A) Trolox (0–1000µM), (B) WS (3.13–100 mg/ml), (C) Se-Met (0.0156–0.5 pg/ml) and (D) WS + Se-Met (1.562 mg/ml + 0.0078 pg/ml–50 mg/ml + 0.25 pg/ml). Gradients of samples with different letters are significantly different.

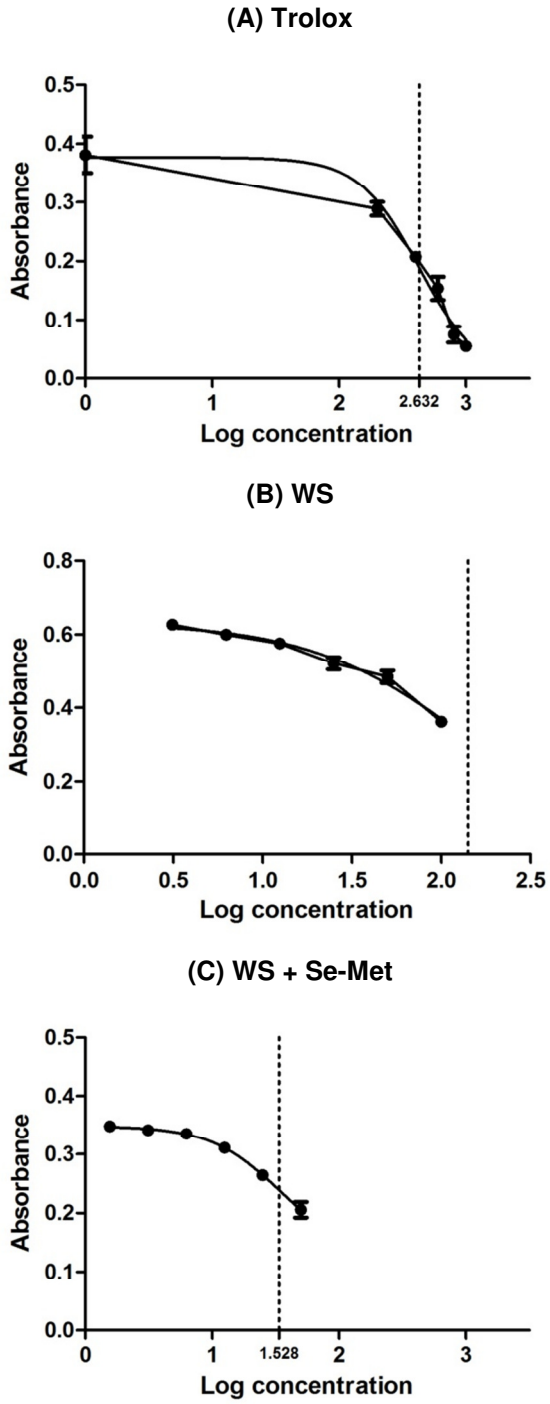


Figure 3.10: Log plot demonstrating the decrease in absorbance of (A) trolox (0–1000µM), (B) WS (3.13–100 mg/ml) and (C) WS + Se-Met (1.562 mg/ml + 0.0078 pg/ml–50 mg/ml + 0.25 pg/ml) in the TEAC assay after 30 minutes. The log IC_{50} is displayed as a dotted line in each graph.

Oxygen radical absorbance capacity assay

The ORAC assay is recognised far and wide as a standard technique to measure antioxidant activity in the neutraceutical, pharmaceutical, and food industries (Huang *et al.*, 2002). ORAC's applications in the assessment of free radical-scavenging capacity also encompasses human plasma, proteins, DNA, pure antioxidant compounds and antioxidant plant and food extracts (Dávalos *et al.*, 2004). This method is highly regarded because the free radicals that are used are more biologically relevant than in other assays, and has the ability to mimic the antioxidant activity of phenols in a biological system much more efficiently (Awika *et al.*, 2003). At present, ORAC is the only assay that takes free radical action to completion, and uses an AUC technique for quantification, combining the degree of inhibition and the length of the inhibition time of the free radical action by antioxidants into a single entity (Ali *et al.*, 2008; Huang *et al.*, 2002).

Figure 13.11 illustrates the effect of the AAPH radical on fluorescein. There is an increase in radical formation with time, resulting in a decrease in fluorescence. Trolox and WS counteract the effect of AAPH. Se-Met did not inhibit AAPH-induced degradation of fluorescein, or enhance/inhibit the antioxidant effect of WS.

Data obtained from the ORAC assay is interpreted by calculating the AUC, and thereafter the net AUC ($AUC_{sample} - AUC_{blank}$). Using trolox, a standard curve, linear from 0 – 1000µM (Figure 3.12) with an R^2 value of 0.97, was derived from which the antioxidant activity of the samples was calculated.

The AUC was determined for 0 – 100 mg/ml of the WS extract and a linear dosage effect with an R^2 value of 0.99 was obtained. No effect was obtained for Se-Met (data not shown). The ORAC values of WS ranged from 301 µM TE for 20 mg/ml to 1718 µM TE for 100 mg/ml with an average of 17.8 µM TE/g WW. The antioxidant activity of a methanolic extract of WS was measured using the ORAC assay, and a result of 443.5 µmol TE/g was obtained (Kumar *et al.*, 2008).

Wojchikowski and co-workers (2007) tested the antioxidant activity of three different extracts of WS root. The results obtained were as follows: ethyl acetate extract: 1.48 ± 0.18 µmol TE/g DW; methanol extract: 47.58 ± 5.12 µmol TE/g DW; aqueous methanol (1:1): 35.80 ± 3.45 µmol TE/g DW. The methanolic extract is by far the highest of the three results, and therefore may be considered the best solvent to be used for WS. This was also confirmed by the results obtained for antioxidant content and antioxidant activity measured with the TEAC assay.

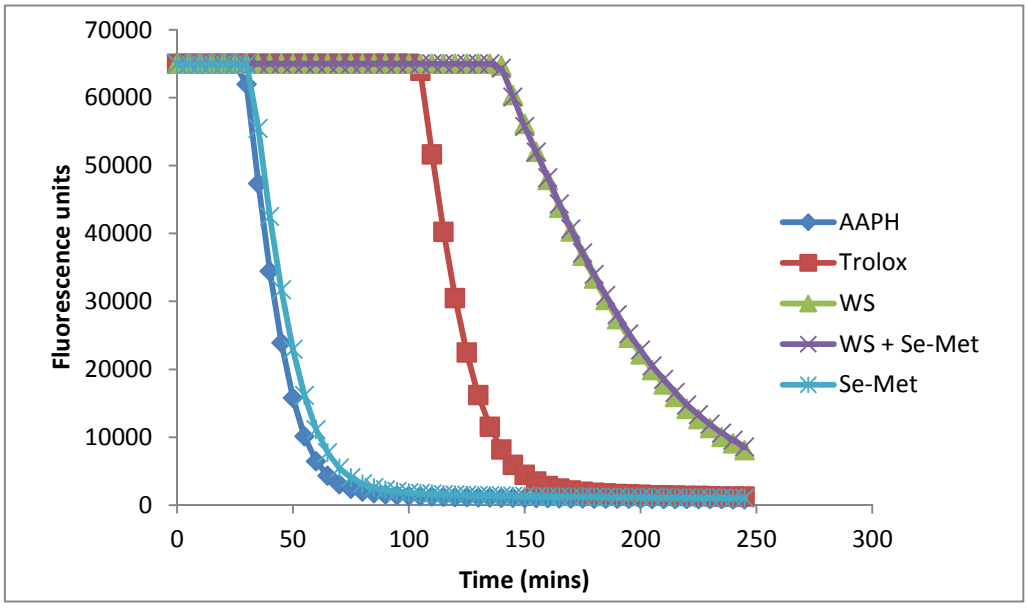


Figure 3.11: Representative quenching curves obtained in the study for fluorescein for the ORAC assay in the presence of AAPH, blank (water), Trolox 1000 µM, WS (100 mg/ml), WS + Se-Met (100 mg/ml WS+ 0.5 pg/ml Se-Met), and Se-Met (0.5pg/ml).

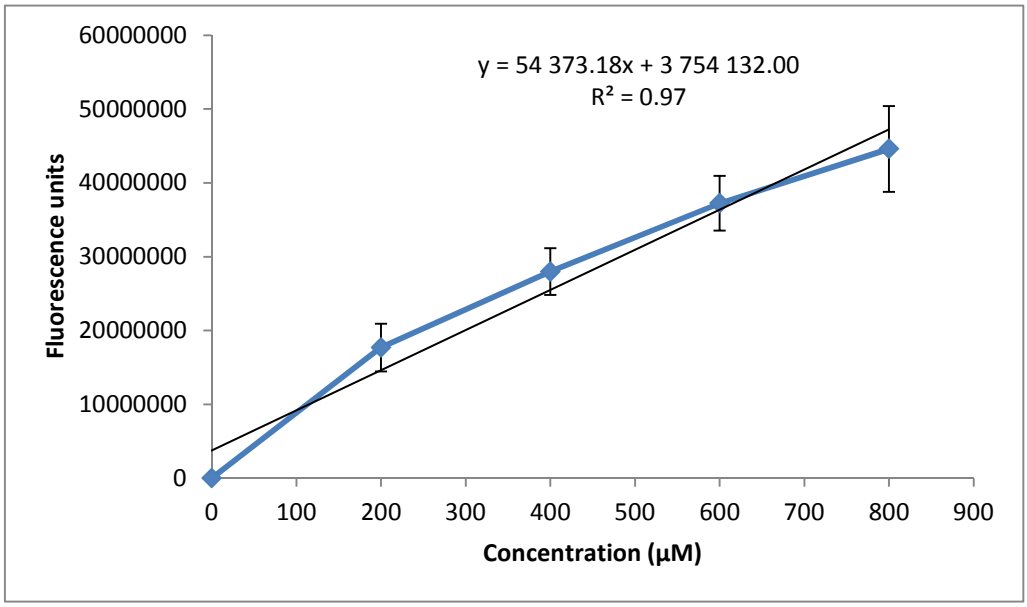


Figure 3.12: Trolox standard curve for ORAC assay. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM.

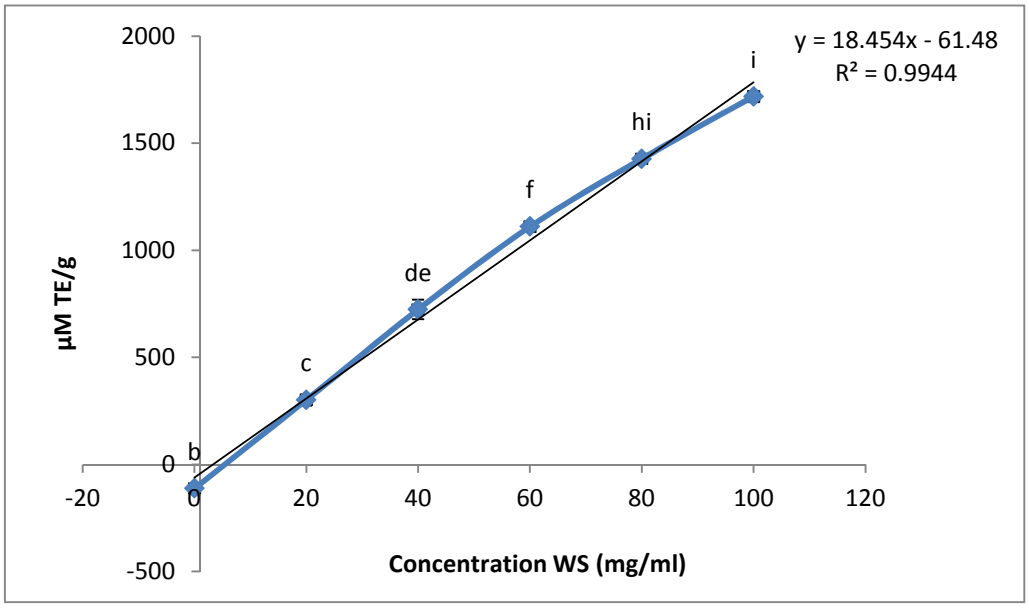


Figure 3.13: Antioxidant activity determined with the ORAC assay of WS (0–100 mg/ml), data expressed as µM TE/g. Data average of three experiments ± SEM. Means with different letters are significantly different, p≤0.001.

Table 3.4: Summary of the antioxidant activity of WS

Assay	TE	IC_{50}
DPPH	274.8 µmol/g WW	56.34 mg/ml
TEAC	22 µmol/g DW / 0.0084 mmol/g WW	60.30 mg/ml
ORAC	15.05 µM/g WW	

3.5 Summary

Water extracts of WS (3.13 – 100 mg/ml), as prepared for traditional medicinal purposes, contain polyphenolics and flavonoids which translate into antioxidant activity that can be accurately quantified using the TEAC and ORAC assays. In Chapter 4 and 5, it will be determined if this activity translates into cellular protection against oxidative damage in SC-1 fibroblast cells (Chapter 4) and chick embryo lung primary cells (Chapter 5), and if there is any interaction between WS and Se-Met.

Chapter 4: The effects of *Withania somnifera* and selenium, alone and in combination, on the SC-1 permanent cell line

4.1 Introduction

Medicinal plants contain many constituent molecules that possess antioxidant activity, and these include vitamins (e.g., vitamins C, E and carotenoids), flavonoids (e.g., flavones, isoflavones, anthocyanins and catechins), and polyphenols (e.g., epicatechin, quercetin-3-rhamnoside, gallic acid, rutin hydrate, ellagic acid and tannins) (Pal *et al.*, 2012). Many antioxidant molecules, for example polyphenols, show antioxidant activity at low concentrations, and may show anticancer effects i.e., cytotoxic effects at high concentrations (Mertens-Talcott *et al.*, 2005; Katiyar & Mukhtar, 1997; Roy *et al.*, 2003).

WS has been shown in the previous chapter of this study, as well as in other studies, to possess antioxidant compounds and activity (Russo *et al.*, 2001; Pal *et al.*, 2011). The antioxidant assays performed in the previous chapter, namely the TEAC and ORAC assays, are excellent indicators of the presence and activity of antioxidants, but fail to take into consideration the effect of absorption, distribution, metabolism and excretion (Girard-Lalancette *et al.*, 2009). When oxidative stress is measured within a cellular system, the results better reflect the effect of absorption, distribution, metabolism and excretion at a cellular level and, in turn, can serve as a basis for animal studies (Roy *et al.*, 2009). Furthermore, even though cellular antioxidant activity is found, it is necessary to determine if other components present in the extracts exert cellular toxicity.

The cell type that is used to evaluate antioxidant activity and/or toxicity is important as it should reflect the target cell type. To determine bio-safety, non-cancer derived fibroblast cell types, such as the L929 fibroblast cell line, can be used. Data obtained in such cell lines like can then be confirmed using primary cell cultures representative of the target tissue for which a medicinal plant is used.

Antioxidant research of late is more focused on interactions that occur naturally between antioxidants. Various types of interactions may result from the combination of two or more antioxidants, namely additive, synergistic and antagonistic. An additive effect refers to the sum of the effects of the individual components; a synergistic effect occurs when the effect is greater than the sum of the individual components; and an antagonistic effect is when the sum of the effects is less than the predicted sum of the individual components (Wang *et al.*,

2011). These effects can occur between the polyphenolics in the same extract i.e., for WS: epicatechin and gallic acid. Antioxidant effects could be enhanced if a synergistic effect between non-enzymatic and enzymatic antioxidants existed, such as polyphenolics and GPx. GPx activity is dependent on Se, a cofactor for this enzyme. By optimising Se levels, optimal GPx functioning occurs and increased synergism between GPx and polyphenolics is possible.

Standard antioxidant assays can be used to determine synergism between two polyphenolics, but not between non-enzymatic and enzymatic antioxidants. This can only be achieved using cellular models such as the SC-1 fibroblast cell line, which will be used in this chapter.

The main aim of this study is to determine the toxicity as well as the *in vitro* cellular and protective effects of WS and Se-Met, alone and in combination, using the SC-1 fibroblast cell line.

The specific objectives of this study are:

1. To determine if WS and Se-Met are cytotoxic using the combined NR and CV assays.
2. To evaluate the effects of WS and Se-Met, alone and in combination, on cellular morphology with light and phase contrast microscopy.
3. To determine the total and intracellular protection provided by WS and Se-Met, alone and in combination, for the SC-1 cell line against AAPH-induced oxidative damage using the DCFH-DA cell-based assay.
4. To determine the type of interaction (antagonistic, additive, synergistic) between WS and Se-Met in the DCFH-DA cell-based assay.

4.2 Materials

SC-1 fibroblasts

The embryonic *Mus musculis* (mouse) fibroblast (SC-1) cells were obtained from Highveld Biological Company, Johannesburg, SA.

Withania somnifera and seleno-methionine

The same WS extracts and Se-Met solutions as those used in Chapter 3 will be used in this study.

Media, supplements, reagents and plastic ware

Eagle's minimum essential medium (EMEM) powder, fetal calf serum (FCS) and antibiotic solution (containing, streptomycin, penicillin and fungicide) were obtained from Highveld Biological Company, Johannesburg, SA. Dulbecco's modified Eagle medium (DMEM), Neutral Red (NR), Crystal Violet (CV), dichlorofluorescein diacetate (DCFH-DA) and seleno-L-methionine (Se-Met) were obtained from Sigma-Aldrich, Atlasville, SA. Fixatives, acids, salts and organic solvents such as gluteraldehyde, acetic acid, formic acid and isopropanol were of analytic grade and together with ethylene diamine tetra acetate (EDTA) and dimethyl sulphoxide (DMSO) were obtained from Merck, SA. Trypsin was obtained from Life Technologies Laboratories, Johannesburg, SA. Sartorius cellulose acetate membrane 0.22 µm filters were obtained from National Separations, Johannesburg, SA. Water was double distilled and de-ionised (ddH_2O) with a continental water system and all medium, enzyme solutions and buffers were sterilized by filtration through a Millex 0.2 µm filter. Glassware was sterilized at 121℃ for 20 minutes in a Prestige Medical Autoclave (series 2100).

Disposable plastic ware used for cell culture was obtained from either Greiner Bio-One supplied by LASEC, Cape Town, SA or NUNCTM supplied by AEC-Amersham, Johannesburg, SA and included: 24 and 96 well plates, 25 cm^2 and 75cm^2 tissue culture flasks, 15 ml and 50 ml centrifuge tubes, 5 ml and 10ml pipettes and pipette tips (10, 25, 100, 200, and 1000 µl).

Laboratory facilities

All cell culture studies and microscopy were carried out in the cell culture laboratory of the Department of Anatomy, while fluorescence analysis was undertaken in the Department of Pharmacology, School of Medicine, Faculty of Health Sciences, University of Pretoria.

4.3 Methods

Cultivation, maintenance and preservation of the SC-1 fibroblast cell line

The SC-1 cell line was maintained in EMEM, supplemented with 10% FCS (EMEM/FCS) and a 1% antibiotic solution. An antibiotic stock solution was prepared by mixing 10 000 µg/ml penicillin G (sodium salt), 10 000 µg/ml streptomycin sulphate and 25 µg/ml Amphotericin B in 0.85% saline. A volume of 10 ml of the working solutions was added to 1 l of the prepared medium.

The cells were plated at 4x10^4 cells per ml in 25 cm^2 and 75 cm^2 cell culture flasks and were maintained until confluent at 37℃ at 5% CO_2. Once confluent, the SC-1 cells were

passaged by removing the medium from the confluent monolayer then adding 1 ml of a 5% trypsin solution prepared in PBS (0.2 M Na_2HPO_4, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl, pH=7.4). The flask was incubated at 37°C for 1 – 2 minutes. A 5 ml volume of medium was then added to the trypsin solution containing the detached cells, and transferred to a 15 ml centrifuge tube. The cells were collected by centrifugation at 1750 xg for 2 minutes. The medium was removed and the cells were re-suspended in 5 ml EMEM/FCS. The number of cells was determined by counting a 10 µl aliquot of cells using a haemocytometer.

The cell line was either used for the experiments described below, or stored for later use at -70°C. For storage, the cells were suspended in cell culture freezing medium at a concentration of $2x10^5$ cells per ml. The freezing medium was prepared by adding 10% DMSO and 80% FCS to EMEM or DMEM. A volume of 1.8 ml was transferred to the freezing vials and stored by slow freezing (first placed on ice, then in -20°C and finally in a -70°C freezer). The cells were stored for a maximum of six months with minimal loss of viability. For the following experiments, the vials containing the cells were thawed rapidly in warm water at 37°C. The cells were suspended in 5 ml medium supplemented with FCS, and collected by centrifugation. The supernatant was removed and the cells were suspended in fresh medium and plated in 25 cm^2 culture flasks.

Preparation of *Withania somnifera* extracts and seleno-methionine solutions

As described in Chapter 3, water extracts from the roots, leaves and stems of WS were prepared. A volume of 2.66 ml of an antibiotic stock solution was added to 66.6 ml WS extract (1:25) and the extract was filtered. The concentrations of WS used in the cytotoxicity assays are summarized in Table 4.1.

The RDA for selenium varies throughout the world, and is dependent on the various health regulation bodies, and selenium intake. The RDA in the USA is 55 µg/day; while in the UK it is 75 µg/day for adult males, and 60 µg/day for adult females (Papp *et al.*, 2007). For the purposes of this study, an average of 1 µg/kg/day for adults was chosen. According to Smit (2006), $1x10^9$ cells have a mass of 1 g. This data was extrapolated for the use of $2x10^4$ cells per 500 µl. The concentrations of Se-Met used in the cytotoxicity assays are presented in Table 4.1.

Table 4.1: Final concentrations of WS and Se-Met for NR and CV assays

WS	Se-Met		
mg/ml	pg/ml	nM	RDA
0.007	0.01	$5.1x10^{-5}$	½
0.042	0.02	$1.1x10^{-4}$	1
0.25,	0.10	$5.1x10^{-4}$	5
1.51	0.20	$1.1x10^{-3}$	10
9.09*	1.00	$5.1x10^{-3}$	50
	2.00*	$1.1x10^{-2}$*	100*

*Used to determine the cytotoxicity alone and in combination with phase contrast and light microscopy

The cellular effects of *Withania somnifera* and seleno-methionine on the SC-1 cell line

Cells were plated at a cell concentration of $2x10^4$ cells per 500 µl in 24-well flat bottom plates and incubated for 24 hours at 37°C and 5% CO_2 to allow the cells to attach to the tissue culture surface before conducting the following experiments.

To determine the cellular effects of WS and Se-Met alone, the SC-1 cells were exposed to 0 – 9.09 mg/ml WS and 0 – 2 pg/ml Se-Met. After 48 hours exposure for both WS and Se-Met, lysosomal membrane integrity and cell number were determined using the combined NR and CV assays respectively.

Neutral Red assay: lysosomal membrane integrity

After exposure, 15 µl of 0.15% NR prepared in a Dulbecco's phosphate buffered saline solution (DPBS) (A 10x DPBS stock solution was prepared by dissolving 2g/l KCl, 2g KH_2PO_4, 80g/l NaCl and Na_2HPO_4 in ddH_2O that was diluted 1:9 with ddH_2O prior to use) was added to the medium in each well and the plates were incubated for a further 90 minutes at 37°C in 5% CO_2. The medium was removed and the plates blotted dry. The cells were fixed for 10 minutes with 200 µl of a 1% acetic acid and 1% formaldehyde solution in ddH_2O. The fixative was removed and the wells were rinsed once again with the same solution to remove any excess NR dye. The NR was solubilised with 200 µl of a 1% acetic acid and 50% ethanol solution prepared in ddH_2O. The dissolved NR was transferred into a 96-flat well plate. Absorbance was measured at 570 nm using an ELx800 microplate reader. Data was expressed as percentage absorbance, with the control containing no sample, 100%.

Crystal Violet assay: cell number

Following the extraction of NR dye, the same plate was washed with DPBS and allowed to dry. The cells attached to the bottom of the plate were stained by adding a volume of 300 µl

of a 0.1% w/v CV solution prepared in 200 mM formic acid, pH 3.5, to each well. After staining for 60 minutes at room temperature, the plate was washed with ddH_2O and dried and the bound dye was extracted using a 10% acetic acid solution. Absorbance was measured at 570 nm and data was expressed as percentage absorbance, with the control containing no sample, 100%.

Phase contrast and light microscopy

In order to determine the short-term effects of WS and Se-Met, alone and in combination, on the SC-1 cell morphology, the highest concentrations of WS (9.09 mg/ml), Se-Met (2 pg/ml) and WS + Se-Met (9.09 mg/ml WS + 2 pg/ml Se-Met) used in the CV and NR assays were utilized. After exposure for 12 hours, cellular morphology was evaluated by phase contrast microscopy. The cells were then fixed in 2.5% gluteraldehyde in DPBS with a pH of 7.4 for one hour. Thereafter, the cells were stained with CV and cellular morphology was evaluated using light microscopy. For both phase contrast and light microscopy an Olympus IX71 microscope was used. Photographs were taken using a Zeiss Axiocam ERc5s camera and processed using Axiovision Vs40 v 4.8.2.0 software.

Oxidative/antioxidant effects of effect of *Withania somnifera* and seleno-methionine alone and in combination on the SC-1 cell line

The DCFH-DA cell-based assay measures the ability of WS and Se-Met, alone and in combination, to cause oxidative cellular damage, as well as protect the SC-1 cells from AAPH-induced oxidative damage. In terms of protection, two different parameters are measured i.e., total and intracellular protection. Total protection is a measure of the antioxidants that are in the extracellular medium and are able to exert an antioxidant effect before oxidative radicals reach the cell, and of the antioxidants that have crossed the cell membrane and are able to mediate protection from the intracellular compartment. Intracellular protection only measures the effect of antioxidants that have crossed the cell membrane.

For all experiments using DCFH-DA, SC-1 cells were plated at a concentration of $2x10^4$ cells per 100 µl medium in 96-well flat bottom plates and were kept at 37°C and 5% CO_2 to allow cells to attach to well surfaces. All experiments were conducted 24 hours later.

Total protective effects

For total protection, the SC-1 cells were incubated with DCFH-DA for one hour to allow the uptake of DCFH-DA into the cell. The medium was removed, and the samples (WS and Se-Met alone and in combination) were added together with AAPH. The change in fluorescence

was immediately measured over a period of one hour. All data was calculated as a percentage of the damage caused by AAPH (100%).

Withania somnifera

Volumes of 40 µl of a 20 µM DCFH-DA solution was added to each well and cell culture plates were placed at 37℃ and 5% CO_2 for 1 hour. The medium containing the DCFH-DA solution was then carefully removed. Cell culture plates were washed once with PBS, and plates were blotted dry.

To measure possible oxidative damage by WS, 20 µl of 2 mg/ml and 20 mg/ml WS was added without AAPH (PBS was added instead). For the measurement of total protection, 20 µl of a concentration range of 2 – 20 mg/ml WS was added to each well followed by 40 µl of a 15 mM AAPH solution (final concentration: 10 mM). The final concentrations of WS used are found in Table 4.2. Change in fluorescence was measured immediately over 60 minutes at 2 minute intervals.

Seleno-methionine

To measure the possible oxidative damage caused by Se-Met, 20 µl of 0.1 pg/ml and 16 pg/ml Se-Met was added without AAPH (PBS was added instead). For the measurement of total protection by Se-Met, 20 µl of a concentration range of 0.1 – 16 pg/ml was used, followed by the addition of 40 µl of a 15mM AAPH solution (final concentration: 10 mM). The final concentrations of Se-Met are summarized in Table 4.2. The change in fluorescence was measured immediately over 60 minutes, every 2 minutes.

***Withania somnifera* and seleno-methionine**

To determine the total protective effect of WS and Se-Met in combination, 20 µl of WS and Se-Met samples, followed by 40 µl of a 15 mM AAPH solution were added to each well. Final concentrations of WS and Se-Met used in combination are presented in Table 4.2.

Intracellular protective effects

Intracellular protection in the DCFH-DA cell-based assay represents the ability of the antioxidants to cross or bind the cell membrane. The SC-1 cells were incubated with DCFH-DA and WS and Se-Met, alone and in combination, for one hour. After incubation, the medium was removed, the wells were washed carefully and blotted dry to remove all extracellular DCFH-DA and WS and/or Se-Met. AAPH was then added and the plate was read immediately for changes in fluorescence over one hour. All sample data was calculated as a percentage of the damage caused by AAPH (100%).

Withania somnifera

The DCFH-DA was prepared as described above for total protection, and 40 µl of the DCFH-DA solution was added to each well together with 20 µl of a concentration range of 2 – 20 mg/ml WS. The plates were maintained for an additional hour at 37℃. The medium containing the DCFH-DA and the sample was then carefully removed, the plates were washed once with PBS, and blotted dry. Forty µl of 15 mM AAPH was added to each well and the change in fluorescence was read over 60 minutes. The final concentrations of samples were the same as for total protection (Table 4.2).

Seleno-methionine

The intracellular antioxidant protective effect of Se-Met was determined by adding 20 µl of a series of concentrations ranging from 0.1 – 16 pg/ml, together with 40 µl of DCFH-DA to each well. The final concentrations were the same as those for total protection as in Table 4.2. Following the addition of Se-Met, the cell culture plates were maintained for an additional hour at 37℃. The medium containing the DCFH-DA and Se-Met was then carefully removed. Cell culture plates were washed once with PBS and blotted dry. Forty µl of a 15 mM AAPH solution was added to each well and the change in fluorescence was measured over 60 minutes.

***Withania somnifera* and seleno-methionine**

The intracellular antioxidant effect of WS and Se-Met in combination was also determined. A volume of 20 µl WS and Se-Met samples were added to each well of the cell culture plates. The final concentrations are represented in Table 4.2. Cell culture plates were returned to the incubator for an additional hour and then the medium containing the DCFH-DA and WS + Se-Met was then carefully removed, the plates were then washed, dried and then 40 µl volume of a 15 mM AAPH solution was added to each well. Change in fluorescence was measured over 60 minutes.

Table 4.2: Final concentrations of WS and Se-Met alone and in combination for DCFH-DA assay

Alone		Combination		
WS (mg/ml)	Se-Met (pg/ml) (nM)	WS + Se-Met		Ratio
		WS (mg/ml)	Se-Met (pg/ml)	(mg/ml: pg/ml)
3.33	0.167 ($8.56x10^{-4}$)	2.50	0.125 ($6.37x10^{-4}$)	1:1
6.67	0.33 ($1.68x10^{-3}$)	5.00	1.25 ($6.37x10^{-3}$)	2:10
13.33	1.67 ($8.56x10^{-3}$)	10.00	12.50 ($6.37x10^{-2}$)	4:100
20.00	3.33 ($1.68x10^{-2}$)	15.00	0.25 ($1.27x10^{-3}$)	6:2
26.69	16.67 ($8.56x10^{-2}$)	20.00	2.50 ($1.27x10^{-2}$)	8:20
33.33	33.33 ($1.68x10^{-1}$)	25.00	25.00 ($1.27x10^{-1}$)	10:200

Interactions between *Withania somnifera* and seleno-methionine

The results obtained by WS and Se-Met alone were used to calculated the expected combinational effects of WS and Se-Met. A curve fit was applied to the graphs for the total and intracellular protective effects of WS and Se-Met alone. The y-values were calculated using the equations of the trendlines, and the values obtained for WS and Se-Met were added together and divided by two. The calculated result is the expected protection by WS and Se-Met in combination.

An interaction index (*II*) was calculated using the following equation:

$$II = {}^{EP}/_{OP}$$

EP represents the expected/calculated protection of the combination of WS and Se-Met and OP represents the actual/observed experimental protection of the combination of WS and Se-Met.

Where the *II* was greater than 1.1, a synergistic effect was described, and an *II* less than 0.9 represented an antagonistic effect. Where the *II* fell between 1.1 and 0.9, an additive effect was classified. In literature, a synergistic effect is typically expressed when $II > 1$, an additive effect when $II = 1$, and an antagonistic effect when $II < 1$ (Panya *et al.*, 2012; Yang & Lui, 2009). In order to further clarify the effect between WS + Se-Met, the classification system shown in Table 4.2 was chosen for this study.

Table 4.3: The classification of the type of interaction using the calculated interaction index

Synergistic effect	Additive effect	Antagonistic effect
$II > 1.1$	$1.1 > II < 0.9$	$II < 0.9$

Data management and statistical analysis

All data management and methods used for statistical analysis was the same as described in Chapter 3.

4.4 Results and discussion

Effect of *Withania somnifera* and seleno-methionine on the SC-1 cell line

To determine if WS and Se-Met, alone and in combination, protect cells against oxidative damage, it was first necessary to determine if WS and Se-Met are cytotoxic. For this purpose, the NR and CV assays were used.

Neutral Red and Crystal Violet assays

SC-1 cells were exposed to WS and Se-Met for 48 hours and the lysosomal membrane integrity and cell number were determined using the NR and CV assays respectively. NR, a red-coloured stain that colours the lysosomes of viable cells (Ishiyama *et al.*, 1996), is a sensitive indicator of cellular damage. It works on the principle that damage caused to the cells will alter the cell and lysosomal membranes, leading to lysosomal damage and a reduced uptake of NR (Chiba *et al.*, 1998). The NR assay is considered to be more sensitive than the CV assay in measuring cellular damage (Motsoane *et al.*, 2003).

The CV assay is a simple and easily reproducible cytotoxicity assay. CV is a positively charged dye which, at a neutral pH, stains structures such as DNA, RNA and protein. The amount of CV staining is correlated with the number of cells (Chiba *et al.*, 1998). The combined NR and CV assay determines lysosomal membrane integrity and cell number in a single cell population. This reduces the number of cells / plates required and eliminates variability due to plating concentration and media factors.

Cytotoxicity assays are widely used in *in vitro* studies (Fotakis & Timbrell, 2006). Molecules that cause cytotoxicity will target the cell membrane first, followed by organelles present in the cytoplasm such as the lysosomes and mitochondria, and then structural damage to the DNA will occur. The events that take place within the cell leading up to cell death are very complex. Cell death can occur via a number of processes, of which apoptosis and necrosis are two. Apoptosis is a process which is initiated by a signalling cascade that activates cysteine proteases, degrades the nuclear DNA of the cell and results in cell death (Raha & Robinson, 2001). It is characterised by very distinct morphological and biochemical events: cell shrinkage, blebbing of the plasma membrane, condensation of the cytoplasm and nucleus and formation of apoptotic bodies (Stoian *et al.*, 1996; Proskuryakov *et al.*, 2003).

Organelle integrity is maintained during apoptosis, and the organelles are enclosed within an intact plasma membrane (Elmore, 2007). Apoptosis will therefore not necessarily lead to a decrease in cell protein content and/or cell number, but functional changes will lead to decreased NR uptake. Changes in cell number will only occur once the cells have started to detach.

In contrast, during necrosis cells swell, causing damage to the cell membrane, and the cellular content leaks out of the cell (Proskuryakov *et al.*, 2003). Using the CV assay, this will translate into decreased CV staining i.e., decrease in cell number, although this is often not the case where cells are damaged and cell content has leaked out of the cell, but the cell is still attached to the cell culture surface.

For each assay, the sample data was expressed as a percentage of the control, DMEM containing 5% FCS, which was calculated as 100%. SC-1 cells were exposed to 0 – 9.09 mg/ml WS for 48 hours. A statistically significant decrease was observed in lysosomal membrane integrity at the highest concentration of 9.09 mg/ml (Figure 4.1A); however, this was not associated with a significant decrease in cell number (Figure 4.1B).

Pretorius and co-workers (2009) evaluated the cellular effects of a methanolic extract of WS on the MRC-5 (human embryonic lung-derived diploid fibroblast) cell line using NR, CV and MTT assays. The concentration range was much lower than that used in the present study (0.007 – 9.09 µg/ml WS). Low concentrations of methanolic extracts (up to 0.25 µg/ml WS) did not cause cell damage to the cell line, however higher levels had a negative effect on cell viability and numbers.

Russo *et al.* (2001) studied the effect of a methanolic extract of WS on the mitochondrial function and cell viability of human non-immortalized fibroblast cells using the MTT assay, another widely used cytotoxicity assay used to determine cell viability and the metabolic state of the cell. It is based on the conversion of tetrazolium salt, a soluble yellow dye, to an insoluble purple/blue formazan product by the enzyme mitochondrial succinate dehydrogenase (Chiba *et al.*, 1998; Ishiyama *et al.*, 1996). The formazan product is impermeable to cell membranes, and can therefore only accumulate in healthy cells (Fotakis & Timbrell, 2006). WS was found to be non-toxic at all the concentrations tested (12 – 25 µg/ml). The concentrations used by Russo *et al.* (2001) were much lower than those used in the present study. However, a methanolic extract would represent an enriched fraction of the bioactive component of WS. The MTT assay was not used in the present study, as studies in our laboratory have shown that SC-1 cells are poor MTT metabolisers. In this study only the

highest concentration of WS (9.09 mg/ml) produced a significant decrease in lysosomal membrane integrity using the NR assay, while there was no effect on cell number at any concentration of WS using the CV assay.

The SC-1 cells were exposed to a concentration range of 0 – 2 pg/ml ($5.1x10^{-5}$ – $1.1x10^{-2}$ nM) Se-Met, which physiologically translates to 0 – 100 x RDA of Se for adults. Using the NR assay to determine the effect of Se-Met on lysosomal membrane integrity, no statistical differences were observed at any of the concentrations of Se-Met when compared to the control, which contained no Se-Met. None of the concentrations exerted a cytotoxic effect whatsoever on the SC-1 cells. The CV assay indicated a slight mitogenic effect by Se-Met on the SC-1 cells, as almost all of the concentrations appeared to be of a higher value than the control, but this was not significantly different from the control.

In a study on the effect of different Se compounds, including Se-Met, on liver cells, Hoefig and co-workers (2011) examined the effect of Se-Met on three different cell lines: HepG2, Huh-7 and Hepa1-6. The authors measured the effect of a concentration range of 0.1 nM – 1 nM Se-Met after incubation periods of 24, 48 and 72 hours using the MTT assay. Se-Met and ebselen (a stable glutathione peroxidase mimetic) were the only two compounds found to have no toxicity at all concentrations (Hoefig *et al*., 2011). The present study confirms these results where for Se-Met (16 pM – 3.3 nM), no toxicity was found.

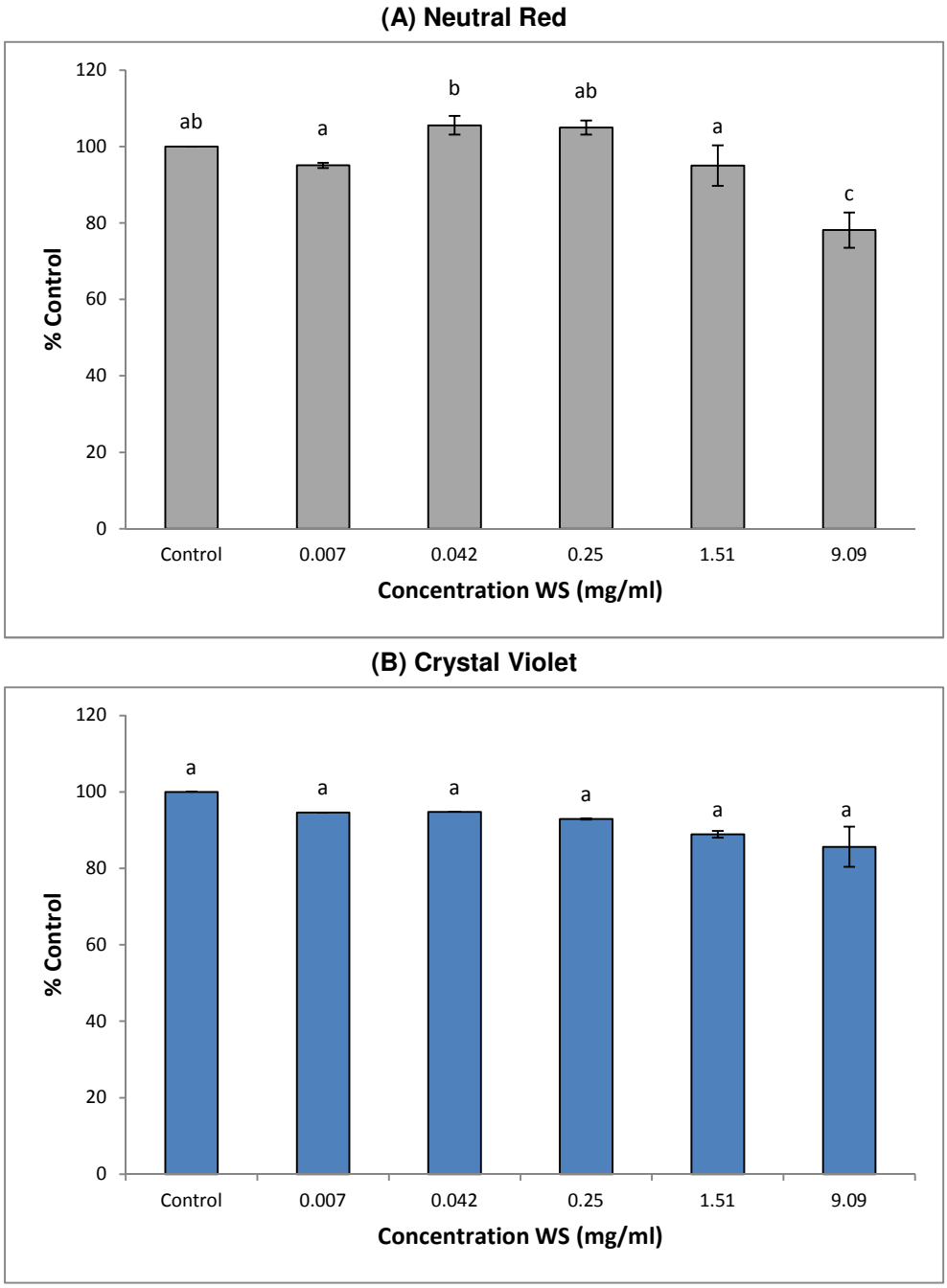


Figure 4.1: Effect of 24 hour exposure to WS on SC-1 lysosomal membrane integrity and cell number using the Neutral Red (A) and Crystal Violet (B) assays respectively. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001.

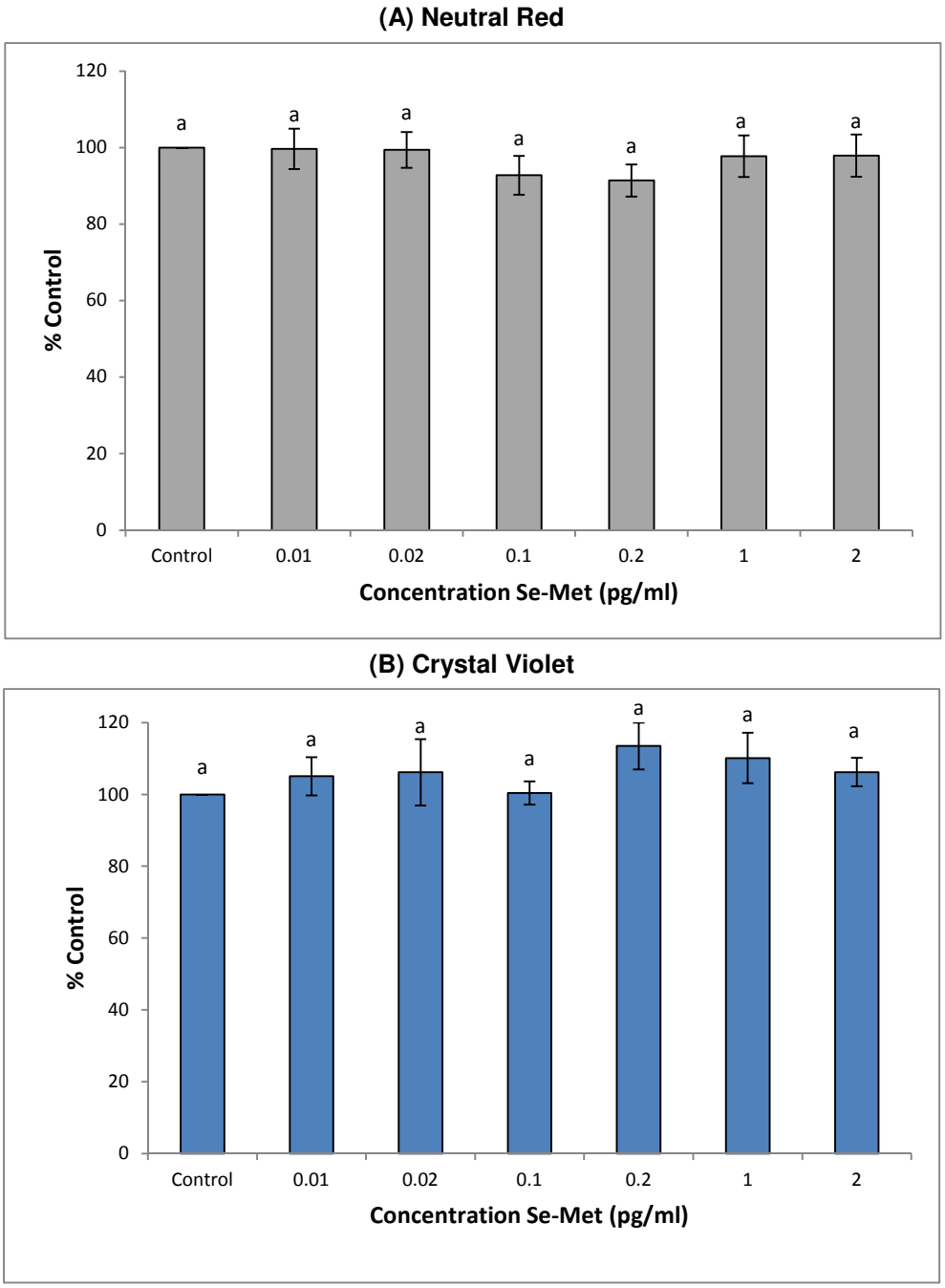


Figure 4.2: Effect of 24 hour exposure to Se-Met on SC-1 lysosomal membrane integrity and cell number using the Neutral Red (A) and Crystal Violet (B) assays respectively. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001.

Phase contrast and light microscopy

Bioassays are used to quantify the effects of complex mixtures such as plant extracts and molecules such as Se-Met. NR and CV assays may not give a clear indication of the effects occurring at a cellular level. With apoptosis, organelle structure remains intact and many cells remain attached to the cell culture surface (Elmore, 2007). In contrast, using microscopy, the effects of these compounds can be described. A limitation of morphological studies is that only a specific area or grouping of cells is evaluated.

The effect of the highest concentrations of WS, Se-Met and WS + Se-Met in combination on cell morphology was evaluated after 12 hours exposure. Cytotoxicity can cause cells to detach from a cell culture surface. For this reason, a 12 hour exposure time was chosen compared to 24 hours for the combined NR/CV assay. Effects on general cell morphology were evaluated using phase contrast microscopy and light microscopy with CV staining.

Controls, SC-1 cells not exposed to WS and/or Se-Met had typical, spindle-shaped fibroblast morphology (Figure 4.3A). The SC-1 cells were exposed to a concentration range of 0 – 9.09 mg/ml WS for 48 hours in the combined NR/CV assay. A statistically significant decrease was observed in lysosomal membrane integrity at the highest concentration of 9.09 mg/ml (Figure 4.1A). This, however, was not associated with a significant decrease in cell number (Figure 4.1B). Phase contrast and light microscopy with CV staining (Figure 4.3B) revealed, that after exposure to 9.09 mg/ml WS for just 12 hours, cells were rounded and starting to detach, and evidence of membrane blebbing and apoptotic bodies (arrows, Figure 4.3B) was observed. These changes are associated with apoptosis (Stoian *et al*., 1996; Proskuryakov *et al*., 2003).

The SC-1 cells were exposed to 0.01 pg/ml (0.5xRDA) – 2 pg/ml (100xRDA) Se-Met for 48 hours in the combined NR/CV assay, where no cytotoxicity was found (Figure 4.2A and 4.2B). Phase contrast and light microscopy with CV staining revealed that Se-Met was less toxic than WS, although some cells were rounded and starting to detach (arrows, Figure 4.3C) after exposure for 12 hours.

These observations, namely WS showing greater toxicity than Se-Met, were also seen when lysosomal membrane integrity was measured using the NR assay. In combination, increased toxicity was observed with phase contrast and light microscopy after 12 hours exposure, with almost all cells being rounded, clumped and detached (arrows, Figure 4.3D). Although indications are that these cells have undergone apoptosis, this can only be confirmed by apoptosis-specific assays, such as fluorescein-labelled annexin V, a phospholipid protein

with a high affinity for phosphatidylserine, which is translocated from the inner to the outer surface of the cell membrane for phagocytic recognition during apoptosis. Annexin V binds the phosphatidylserine and can be detected using flow cytometry (Vermes *et al.*, 1995).

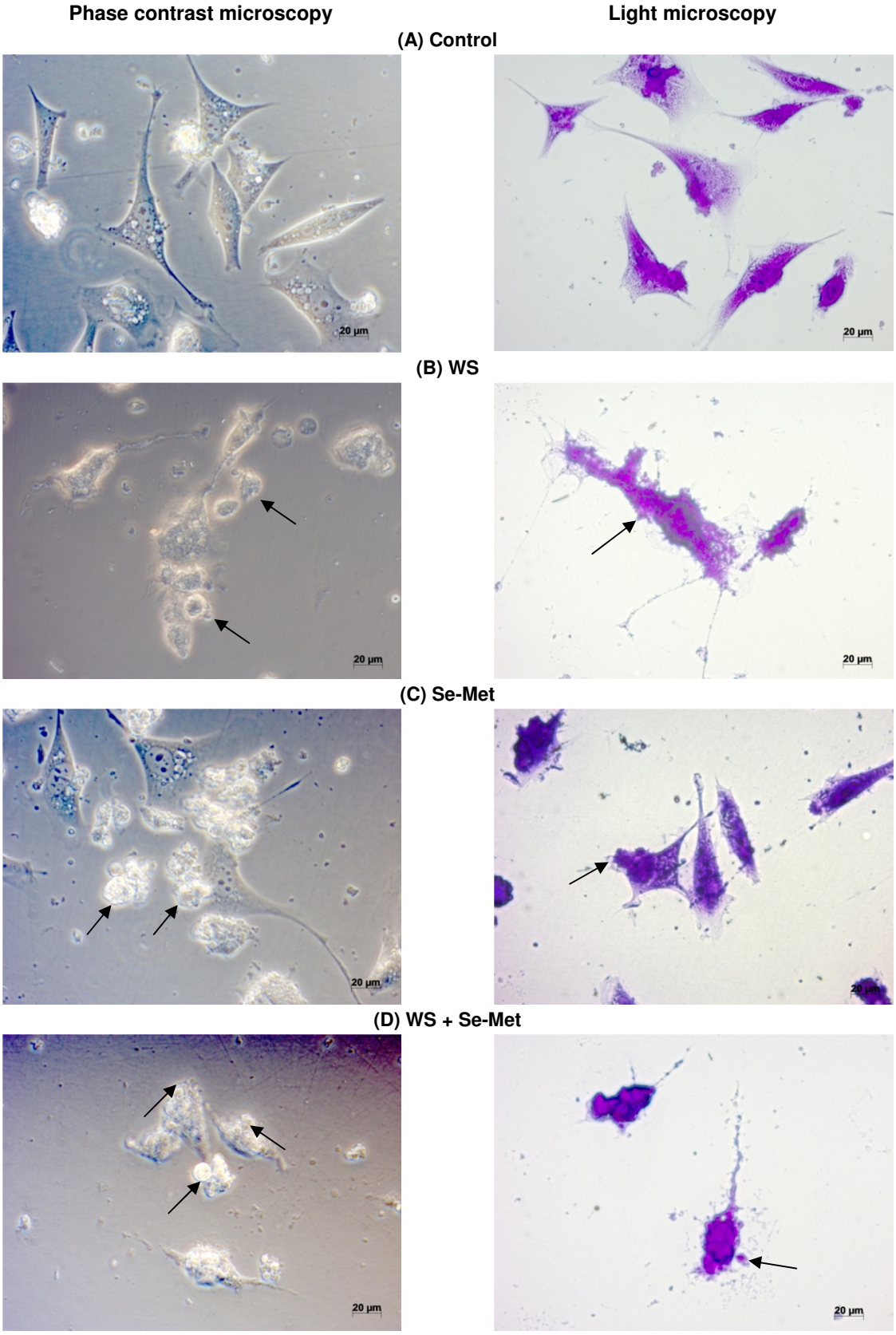


Figure 4.3: Phase contrast and light microscopy (using a CV stain) of SC-1 cells not exposed (A), and exposed to 9.09 mg/ml WS (B), 2 pg/ml Se-Met (C) and 9.09 mg/ml WS + 2 pg/ml Se-Met (D) for 12 hours. For phase contrast microscopy arrows indicate cells that are rounded and starting to detach as well as apoptotic bodies (B), rounded cells starting to detach (C), and most cells rounded, clumped and detached (D). For light microscopy arrows show evidence of membrane blebbing (B), rounded cells in (C) and most cells rounded and detached (D).

The cellular results are summarized below in Table 4.4, and in conclusion, toxicity was observed for WS after 12 – 48 hours exposure, while for Se-Met alone only slight toxicity was observed. However, in combination increased toxicity was found.

Table 4.4: Summary of cellular effect of WS and Se-Met alone and in combination on SC-1 cells

	WS (48 hours)				
	0.007 mg/ml	0.042 mg/ml	0.25 mg/ml	1.51 mg/ml	9.09 mg/ml
NR	ns	ns	ns	ns	↓ **(T)**
CV	ns	ns	ns	ns	ns
	Se-Met (48 hours)				
	0.01 pg/ml	0.02 pg/ml	0.1 pg/ml	0.2pg/ml	1 pg/ml	2pg/ml
NR	ns	ns	ns	ns	ns	ns
CV	ns	ns	ns	ns	ns	ns
	9.09 mg/ml WS (12 hours)					
Morphology: Rounded, detached cells, membrane blebbing						
	2 pg/ml Se-Met (12 hours)					
Morphology: Most cells are detached, some are rounded						
	9.09 mg/ml WS + 2 pg/ml Se-Met (12 hours)					
Morphology: Most cells rounded, detached, loss of cellular content						

ns = no significant differences compared to control, T = Toxic effect, ↓ = decrease in staining

Antioxidant studies using the traditional bioassays, such as the MTT, NR and CV assays, have not been successful. This has been attributed to several factors such as autoxidation, presence of Fe that catalyses the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow$ intermediate complexes $\rightarrow Fe^{3+} + OH + {}^{-}OH$ = pro-oxidant), and the antioxidant effects of added serum and vitamin C (Halliwell, 2006, Polyakov *et al.*, 2001). As a result, the DCFH-DA assay has become the method of choice when evaluating antioxidant activity. This assay is rapid and is undertaken in the absence of cell culture media.

Total protective effect of *Withania somnifera* and seleno-methionine, alone and in combination, against oxidative damage in the SC-1 cell line

Total protection in the DCFH-DA cell-based assay represents the ability of the antioxidants present in the extracellular environment, as well as those antioxidants that are taken up by the cells, to protect the cells from oxidative damage caused by AAPH. In the previous chapter, WS was shown to have antioxidant activity and this was investigated further using cell-based methodologies that are physiologically more relevant.

The ability of WS and Se-Met, alone and in combination, to protect the SC-1 cells against oxidative damage was assessed using the DCFH-DA cell-based assay. DCFH-DA is used to

detect the generation of reactive oxygen intermediates, and to assess the overall oxidative state of a cell (Afri *et al*., 2004). AAPH is used to generate peroxyl radicals, which oxidise DCFH-DA, converting it to the highly fluorescent dichlorofluoroscein (DCF) that has absorbance at 504nm (Ali SS *et al*., 2008, Girard-Lalancette *et al*., 2009).

For total protection, the SC-1 cells were incubated with DCFH-DA for one hour to allow the uptake of DCFH-DA into the cell. The medium was removed, and the samples (WS and Se-Met alone and in combination) were added together with AAPH. The change in fluorescence was immediately measured over one hour. All data was calculated as a percentage of the damage caused by AAPH (100%).

Intracellular protective effect of *Withania somnifera* and seleno-methionine, alone and in combination, against oxidative damage in the SC-1 cell line

Intracellular protection in the DCFH-DA cell-based assay represents the ability of the antioxidants to cross or bind the cell membrane. The SC-1 cells were incubated with DCFH-DA and WS and Se-Met, alone and in combination, for one hour. After incubation the medium was removed, the wells were washed carefully with water and blotted dry to remove all extracellular DCFH-DA and WS and/or Se-Met. AAPH was added and the plate was read immediately for changes in fluorescence over one hour. All sample data was calculated as a percentage of the damage caused by AAPH (100%).

The principles of total and intracellular protection in the DCFH-DA assay is illustrated schematically in Figure 4.4.

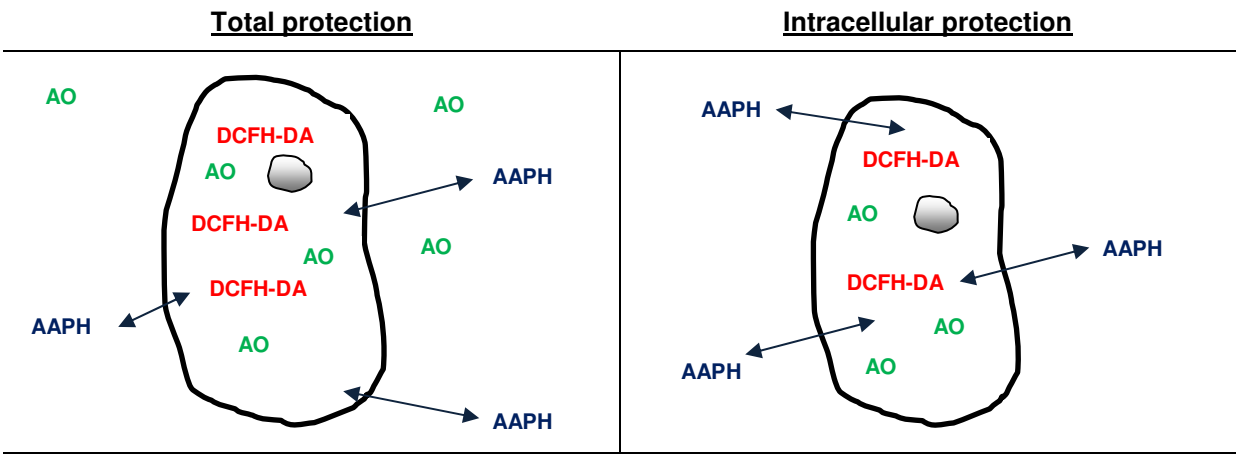


Figure 4.4: A schematic representation of total and intracellular protection methods using the dichlorofluorescein diacetate (DCFH-DA) assay. AO = antioxidant

Withania somnifera

(A) Total protection

The ability of WS to provide total protection for the SC-1 cells *in vitro* against oxidative damage caused by AAPH was tested using the DCFH-DA assay. In this study, WS shows cytotoxicity in the SC-1 cell line after 12 and 48 hours exposure, possibly via the process of apoptosis. Free radicals are able to induce apoptosis as a result of oxidative stress, and may also function as intermediates in destructive pathways triggered by other agents (Stoian *et al*., 1996, Drake, 2006). Therefore it was necessary to determine whether WS alone, in the absence of AAPH, causes cellular damage i.e., an increase in fluorescence following the loading of SC-1 cells using the DCFH-DA assay. For this purpose, the lowest and highest concentrations were used (3.33 and 33.33 mg/ml WS). Despite the increase in fluorescence to 17% and 8% for 3.33 and 33.33 mg/ml WS respectively, the levels were not significantly different from the control, where no WS was added (3.7%). The lack of toxicity suggests that the toxic effect observed following 12 and 48 hours exposure may be the result of a different mechanism, not related to radical damage, or that exposure times of 1 – 2 hours used in the DCFH-DA assay are not long enough to detect significant amounts of damage. The concentrations of WS used in the DCFH-DA assays were higher than those used in the NR and CV assays, because the exposure time was much shorter (1 – 2 hours as opposed to 48 hours).

Once it was determined that WS did not cause oxidative damage using the DCFH-DA assay, the antioxidant effect of WS was evaluated. AAPH alone without WS causes 100% damage, whereas the lowest concentration of WS with AAPH (3.33 mg/ml WS + AAPH) showed a significant increase in oxidation, 203% when compared to 100% caused by AAPH alone. No differences compared to AAPH alone were observed for 6.67 mg/ml WS + AAPH and 13.33 mg/ml WS + AAPH. The three highest concentrations of WS (20.00 mg/ml +AAPH – 33.33 mg/ml + AAPH) showed significant protection against the damage induced by AAPH (Figure 4.5A and Figure 4.6A). The increasing concentrations of WS showed a dose-dependent effect with R^2=0.92 (Figure 4.6A). The three lowest concentrations, which fell in the top left quadrant of the graph showed a pro-oxidant effect, while the three highest concentrations (higher than 12 mg/ml), falling in the bottom right quadrant, showed antioxidant properties (Figure 4.6A). In plant extracts, phenolics have shown a pro-oxidant effect under certain conditions, and this effect is dependent on the metal-reducing potential and chelating behaviour of the phenolics, the pH of the environment and the solubility characteristics (Sakihama *et al*., 2002). The effect observed in this study occurs at low concentrations and

may be related to the experimental conditions e.g., pH changes, increased radical formation by AAPH due to the interaction of AAPH with plant components.

(B) Intracellular protection

WS was tested for its ability to provide intracellular protection for the SC-1 cells *in vitro* against oxidative damage caused by AAPH, using the DCFH-DA assay. The same WS concentrations were used as for the total protection assay, ranging from 3.33 to 33.33 mg/ml. The SC-1 cells were exposed to the lowest (3.33 mg/ml) and highest (33.33 mg/ml) concentrations of WS in the absence of AAPH to rule out oxidative damage caused by WS. DCFH-DA alone caused 2% increase in fluorescence, and WS caused 4% and 11% increase in fluorescence for 3.33 mg/ml and 33.33 mg/ml respectively. These figures were not significant (Figure 4.5B). In the presence of AAPH, all concentrations of WS produced a significant decrease in AAPH-induced oxidative damage. Maximum protection was found at concentrations as low as 3.33 mg/ml. With increasing concentration, the percentage decrease remained constant, indicating that at 3.33 mg/ml a saturation point for protection had already been obtained (Figure 4.6B) and an average of 30% protection was maintained. No dose-dependent relationship was observed. The measured antioxidant cellular protective effects are a function of the number of cells plated and this may account for the absence of a dosage effect.

In summary, when observing the total protection of WS, WS alone does not cause oxidative damage, but when the lowest concentration of WS (3.33 mg/ml) is combined with AAPH, an increase in oxidative damage is observed, indicating that components of WS are interacting with AAPH to cause oxidative damage. In contrast, at the highest concentration (33.3 mg/ml), which also does not cause oxidative damage, causes a decrease in fluorescence in combination with AAPH, indicating an antioxidant effect. This implies that two processes may occur, namely pro-oxidant and antioxidant effects, and as the concentration of WS increases, it overrides the toxic effect. In contrast, when the intracellular effects are measured, no pro-oxidant effect is observed, indicating that the pro-oxidant effect occurs in the extracellular environment.

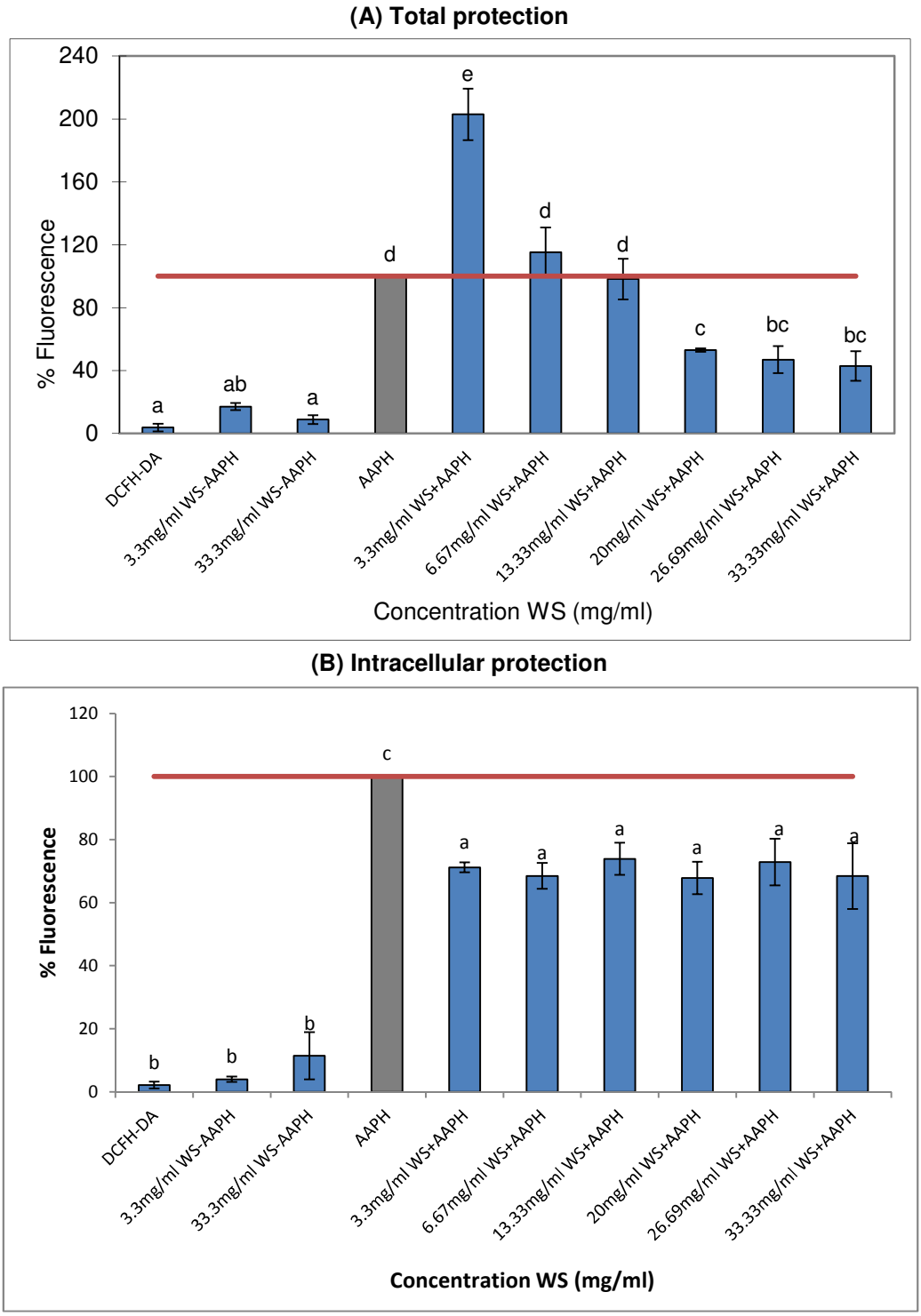


Figure 4.5: Total (A) and intracellular (B) effects of increasing concentrations of WS on AAPH-induced oxidative damage on SC-1 cells. Data is expressed as a percentage of the damage caused by AAPH + DCFH-DA, 100% damage. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001. Red line = 100% damage due to AAPH.

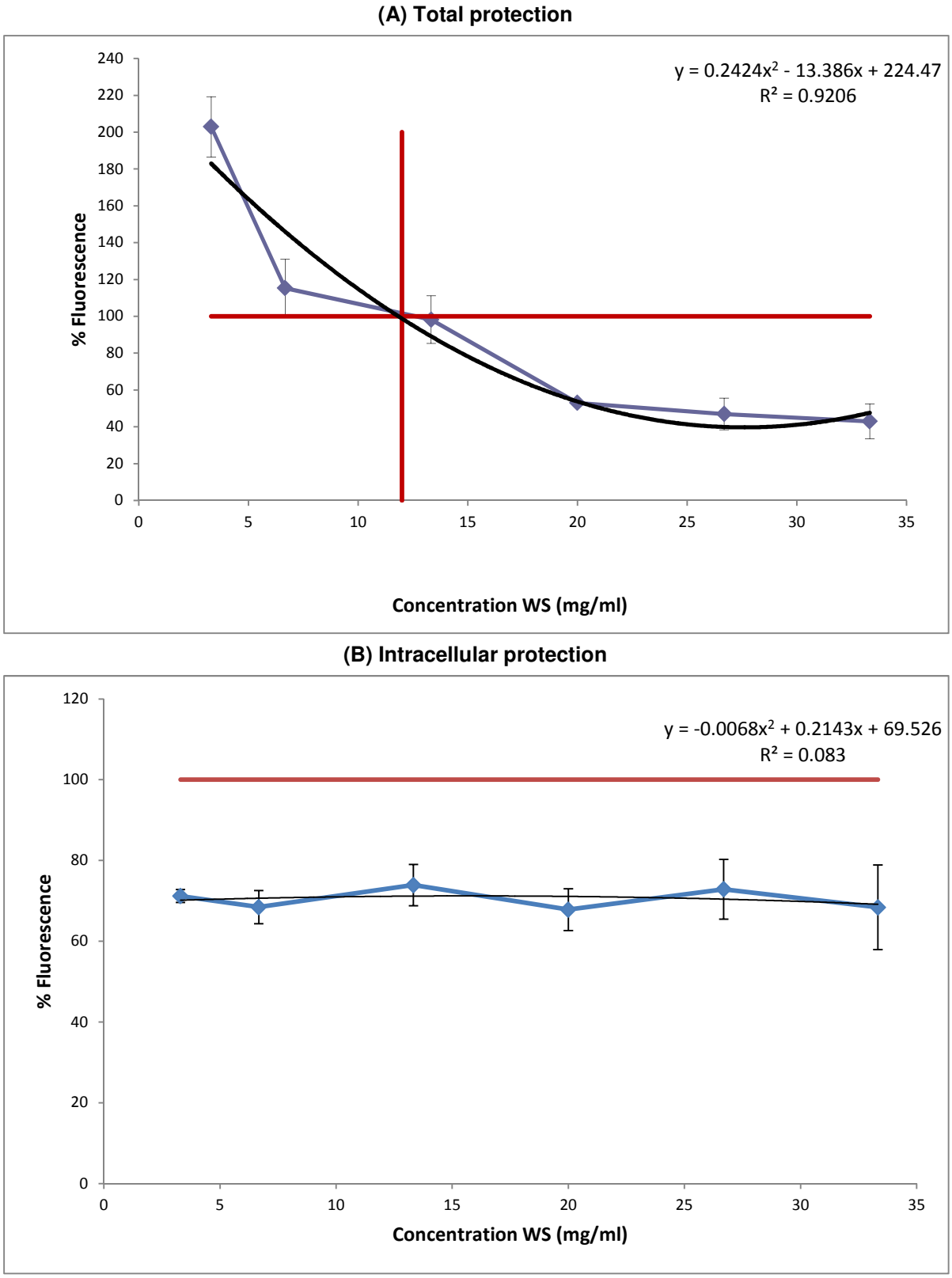


Figure 4.6: Total (A) and intracellular (B) effects of increasing concentrations of WS on AAPH-induced oxidative damage in SC-1 cells. Data is expressed as a percentage of the damage caused by AAPH + DCFH-DA, 100% damage. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001. Red line = 100% damage due to AAPH.

Seleno-methionine

(A) Total protection

The ability of Se-Met to provide total protection for the SC-1 cells *in vitro* against oxidative damage caused by AAPH was tested using the DCFH-DA assay. The lowest and highest concentrations of Se-Met without the presence of AAPH were used to elucidate any potential oxidative effects by Se-Met. Se-Met without AAPH produced results of 12% and -1% for 0.167 and 33.33 pg/ml respectively. These results were not significantly different to the effect of DCFH-DA alone (1.4%) (Figure 4.7A). These findings validate the results obtained in the NR and CV assays (12 – 48 hour exposure), where Se-Met was observed not to be cytotoxic at any concentrations.

The ability of Se-Met to provide total protection against oxidative damage induced by AAPH was measured at the concentrations indicated in Table 4.2. At all concentrations, 0.167 – 33.33 pg/ml, Se-Met in the presence of AAPH showed a significant pro-oxidant effect. This effect was the greatest at 0.33, 1.67 and 3.33 pg/ml, and less, although greater than AAPH, for 16.67 and 33.33 pg/ml (Figure 4.7A).

While Se is known to protect cells from endogenous and exogenous stressors, it is also capable of inhibiting cell proliferation and inducing cell injury. This is attributed to Se's chemical nature, and is dependent on the dose of Se, as well as the experimental model (Rudolf *et al*., 2008). Glutathione peroxidase-1 (GPx-1), a selenium-dependent antioxidant enzyme, exerts a dual role in reactive nitrogen species-related oxidative stress. Studies have shown that GPx-1 will promote peroxynitrite-induced cell death instead of protecting the cell from the oxidative damage (Maraldi *et al*., 2011). As for WS, increased pro-oxidant effects were found at lower concentrations. This effect may not be related to the compounds evaluated, but rather may be a function of experimental design or cellular effects.

(B) Intracellular protection

Se-Met plays a pivotal role in the cellular antioxidant defence system (Wojewoda *et al*., 2010). Se associates with selenoproteins within the cell, which with other Se-dependent mechanisms, protect cells against internal and external stressors by enhancing cellular antioxidant and DNA repair systems. Se-Met and GSH have been suggested to act as an antioxidant system, protecting cells against oxidants (Li *et al*., 2008; Zhou *et al*., 2009).
Se-Met without AAPH produced results of 2.1% and -1.3% for 0.167 and 33.33 pg/ml respectively. These results were not significantly different to the effect of DCFH-DA alone

(2.2%). No significant, pro-oxidant effect was observed (Figure 4.7B). Likewise for the NR and CV assays, following exposure for 12 and 48 hours no cytotoxicity was observed.

The antioxidant effect of Se-Met alone was evaluated in order to ascertain the intracellular protective abilities of Se-Met. In contrast to the results obtained for total protection (Figure 4.7A), Se-Met exhibited significant antioxidant effects at all concentrations in the presence of AAPH, except for 1.67 pg/ml Se-Met + AAPH. There was a high standard error of mean at this concentration, and this may be the reason for the observation. Similar to the results obtained for intracellular protection of WS alone, there was no significant difference between any of the concentrations, and no dose-dependent trend was observed, suggesting a saturation of protection (approximately 40%) in the intracellular compartment.

The intracellular protective effects of 0.5, 1 and 1.5 µM Se-Met were measured with the DCFH-DA assay using SKNBE cells by Maraldi *et al.*, 2011. After just 1 hour, 0.5 µM Se-Met gave rise to a significant increase in ROS (150%, where the control is 100%). After 6 hours, a strong decline in ROS was observed (75%), suggesting that Se-Met was acting in combination with GPx to exert an antioxidant effect. After 18 hours, ROS production increased again to approximately 150% in a dose-dependent manner. After 18 hours, 1 µM Se-Met was at approximately 180%, and 1.5 µM at 200%. This may indicate that the activity of GPx, eventually increased by Se incubation, was not sufficient to scavenge the oxidative stress. These findings suggest that apoptosis is a function of redox unbalance, and that Se-Met is able to induce free radical damage, essentially acting as a pro-oxidant, at low concentrations (8 – 80 µg/l) (Maraldi *et al.*, 2011).

The findings by Maraldi *et al.* (2011) do not reflect the findings in this study on intracellular protection by Se-Met, where at all concentrations a significant intracellular effect was observed. A difference in cell type, as well as the GPx content of the cells, may contribute to the results obtained.

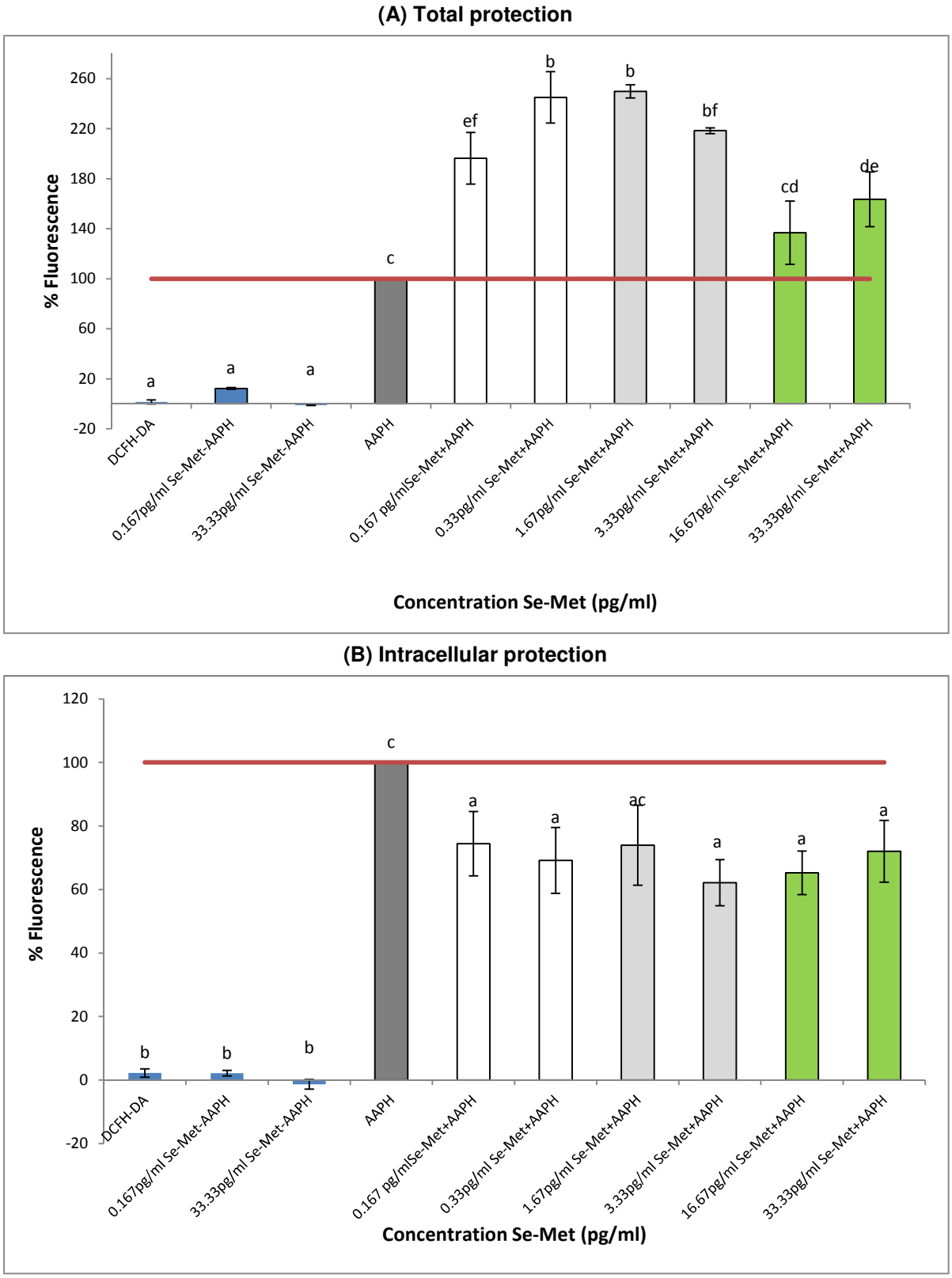


Figure 4.7: Total (A) and intracellular (B) effects of increasing concentrations of Se-Met on AAPH-induced oxidative damage in SC-1 cells. Data is expressed as a percentage of the damage caused by AAPH + DCFH-DA, 100% damage. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Red line = 100% damage due to AAPH.

***Withania somnifera* and seleno-methionine in combination**

The antioxidant properties of WS and Se-Met in combination were evaluated at the following concentration combinations: 2.5 mg/ml WS + 0.125 pg/ml Se-Met, 5.0 mg/ml WS + 1.25 pg/ml Se-Met, 10 mg/ml WS + 12.5 pg/ml Se-Met, 15 mg/ml WS + 0.25 pg/ml Se-Met, 20 mg/ml WS + 2.5 pg/ml Se-Met and 25 mg/ml WS + 25 pg/ml Se-Met (Table 4.2).

(A) Total protection

Interaction between compounds is a function of the ratio of the two compounds relative to one another. To identify the best ratio combination, different concentrations of WS were combined with different concentrations of Se-Met. For this reason ratios of 1:1, 2:2:, 4:10, 6:20, 8:100 and 10:200 were used (Table 4.2). Antioxidant effects would be the result of concentration and the ratio of WS and Se-Met relative to each other.

The lowest (2.5 mg/ml WS+0.125 pg/ml Se-Met) (12.8%) and highest (25 mg/ml WS + 25 pg/ml Se-Met) (14.4%) in the absence of AAPH showed no significant pro-oxidant effects compared to DCFH-DA alone (3.5%).

In the presence of AAPH, with the exception of 2.5 mg/ml + 0.125 pg/ml Se-Met (pro-oxidant effect), 5.0 mg/ml WS + 0.25 pg/ml Se-Met (pro-oxidant effect) and 10 mg/ml WS + 12.5 pg/ml Se-Met (not significant antioxidant effect), all the other combinations showed significant antioxidant effects (Figure 4.8A). With all of the combinations, WS + Se-Met had a greater antioxidant effect than similar concentrations of WS alone. 2.5 mg/ml WS + 0.125 pg/ml Se-Met in the presence of AAPH produced 142% increase in fluorescence, while the 5 mg/ml WS + 0.25 pg/ml Se-Met produced 148% increase in fluorescence. Both of these readings are much lower than the 203% increase in fluorescence that 3.33 mg/ml of WS alone produced in the presence of AAPH (Figure 4.5A). Ten mg/ml WS + 1.25 pg/ml Se-Met in the presence of AAPH reduced the oxidative damage caused by AAPH by 20%, and 15 mg/ml WS + 2.5 pg/ml Se-Met in the presence of AAPH reduced the damage caused by AAPH by 29%. Both of these combinations are lower than the 2% protection offered by 13.33 mg/ml WS alone, in the presence of AAPH. Twenty mg/ml WS + 12.5 pg/ml Se-Met combination with AAPH offered 63% protection as opposed to the 47% that 20 mg/ml WS alone with AAPH offered. 25 mg/ml WS + 25 pg/ml Se-Met in the presence of AAPH reduced the damage caused by AAPH by 61%, while 20 mg/ml WS alone, in the presence of AAPH, only reduced the AAPH-induced damage by 53%. Se-Met alone showed only significant pro-oxidant effects in the presence of AAPH at all concentrations (Figure 4.7A).

Se-Met seems to add to the protective effect offered by WS, suggesting an interaction of antioxidants. These results represent both the extra- and intracellular protective effects of antioxidants as the portion that is absorbed provides physiologically relevant intracellular protective effects, it is necessary to determine this effect.

(B) Intracellular protection

The lowest (2.5 mg/ml WS + 0.125 pg/ml Se-Met) (4.9%) and highest (25 mg/ml WS + 25 pg/ml Se-Met) (0.2%) of these concentrations were used without AAPH to test for any possible oxidative effects. There was no significant difference between these results and DCFH-DA alone (2.2%) (Figure 4.8B).

A wide concentration range and several different combinations of WS and Se-Met were used to identify those concentration combinations that show a significant intracellular antioxidant effect. The combinations without AAPH showed no cytotoxic effects, there was no statistical significance between the results obtained, and DCFH-DA alone. All the combinations of WS and Se-Met, except 5 mg/ml WS + 0.25 pg/ml Se-Met (antioxidant effect, not significant), showed significant intracellular protection similar to that observed for WS intracellularly (Figure 4.5B) and Se-Met intracellularly (Figure 4.7B). In conclusion, for WS and Se-Met, alone and in combination, intracellular antioxidant effects were the predominantly observed effect.

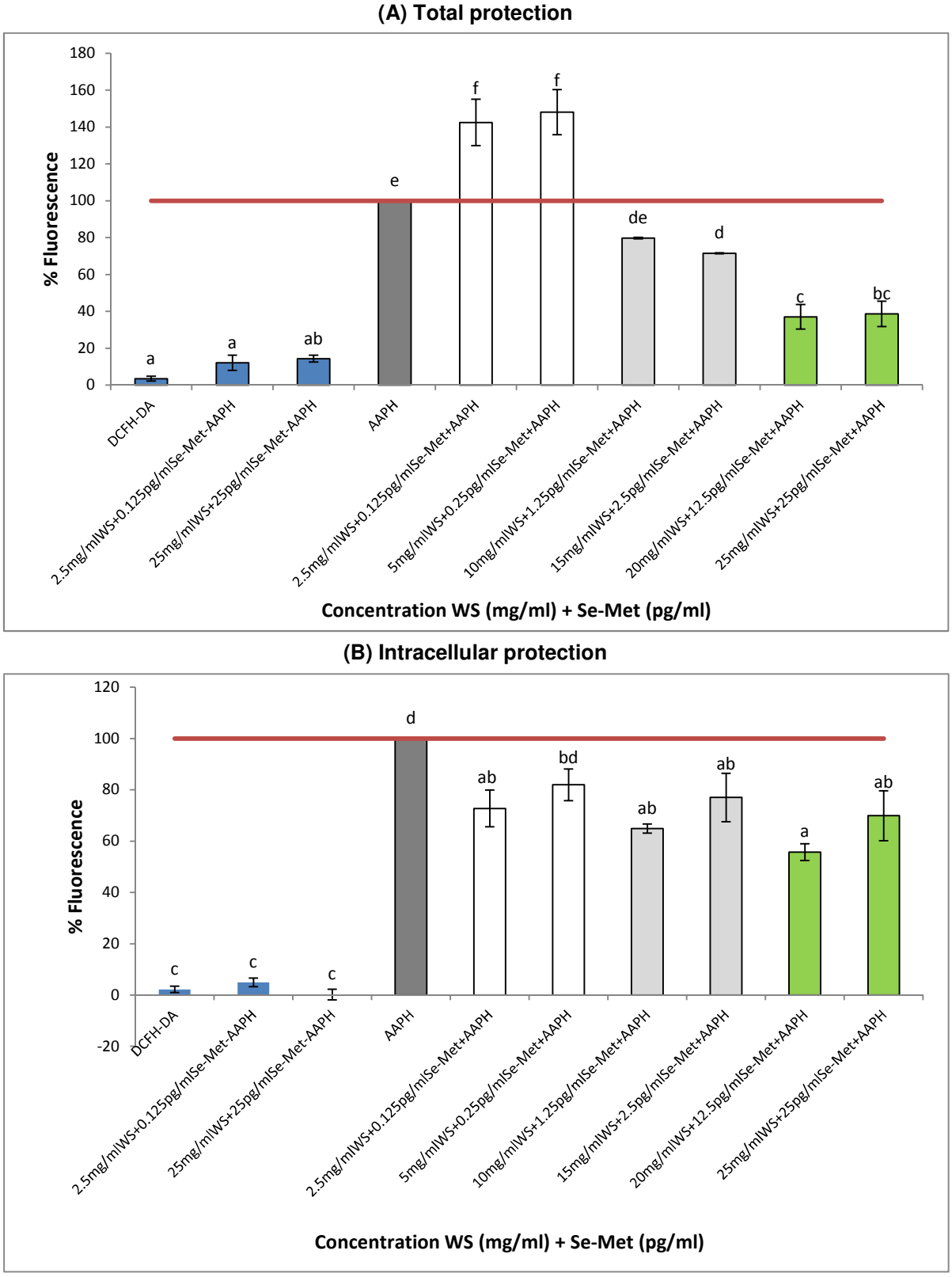


Figure 4.8: Total (A) and intracellular (B) effects of increasing concentrations of WS and Se-Met on AAPH-induced oxidative damage in SC-1 cells. Data is expressed as a percentage of the damage caused by AAPH + DCFH-DA, 100% damage. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001. Red line = 100% damage due to AAPH.

A summary of the ability of WS, and Se-Met, alone and in combination, to provide intracellular protection against AAPH-induced is shown in Table 4.5. All significant increases and decreases are shown, as compared to the damage caused by AAPH.

Table 4.5: Summary of total and intracellular protective effects of WS and Se-Met, alone and in combination, on SC-1 cells

WS (mg/ml)	3.33	6.67	13.33	20.00	26.69	33.33
Total	↑ (PO)	ns	ns	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**
Intracellular	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**

Se-Met (pg/ml)	0.167	0.33	1.67	3.33	16.7	33.3
Total	↑ (PO)	↑ (PO)	↑ (PO)	↑ (PO)	ns	↑ (PO)
Intracellular	↓ **(AO)**	↓ **(AO)**	ns	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**

WS + Se-Met (mg/ml + pg/ml)	2.5+0.125	5.0+1.25	10+12.5	15+0.25	20+2.5	25+25
Total	↑ (PO)	↑ (PO)	ns	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**
Intracellular	↓ **(AO)**	ns	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**

↓decrease in fluorescence, ↑increase in fluorescence, AO = antioxidant, PO = pro-oxidant, ns = not significant

Interactions between *Withania somnifera* and seleno-methionine

(A) Total protection

The results obtained for the total protective effects of WS and Se-Met alone were used to calculate the expected combinational effects between WS and Se-Met. A curve fit was applied to both graphs, and using the equations of these trend lines, expected values were calculated. The equation of the polynomial trend line obtained using the WS values (Figure 4.6A) was $y=0.2424x^2–13.386x+224.47$, $R^2=0.92$. The equation of the polynomial trend line obtained using the Se-Met data (Figure 4.8A) was $y=0.198x^2–8.8823x+237.44$, $R^2=0.75$.

Using these equations, the y-values were calculated for both WS and Se-Met, which were then added together and divided by two. The calculated result represents the expected total protection by WS and Se-Met in combination. The interaction index (*II*) was calculated using the following equation:

$$II = {}^{EP}/_{OP}$$

EP represents the expected/calculated protection of the combination of WS and Se-Met and OP represents the actual/observed protection of the combination of WS and Se-Met (Figure 4.9A).

The results obtained are illustrated in Table 4.6. Synergistic effects were observed at all concentration combinations of WS and Se-Met, with increased synergism occurring with the increase in concentration of WS and Se-Met.

A synergistic, antioxidant effect was observed between WS and Se-Met for the four highest concentration combinations (13.33 mg/ml WS + 1.67 pg/ml Se-Met – 33.33 mg/ml WS + 33.33 pg/ml Se-Met), while a synergistic pro-oxidant effect was observed for the two lowest concentration combinations (3.33 mg/ml WS + 0.167 pg/ml Se-Met – 6.67 mg/ml WS + 0.33 pg/ml Se-Met). These results indicate that concentration combinations higher than 13.33 mg/ml WS + 1.67 pg/ml Se-Met are ideal for achieving antioxidant synergism between WS and Se-Met, and the optimal WS:Se-Met ratios have been identified for total protection in the SC-1 cell line. These findings are noteworthy and emphasize the importance of antioxidant synergy. The protection offered by the combinations of WS and Se-Met against oxidative damage is increased compared to WS and Se-Met evaluated individually. Future research should focus on the mechanism of interaction as well as factors contributing to this effect such as concentration and exposure time.

Table 4.6: TOTAL EFFECT: Expected and observed combinational effects of WS and Se-Met, SC-1 cells

Combinations		% Damage		Interactions	
WS (mg/ml)	**Se-Met (pg/ml) (nM)**	**Expected**	**Observed**	***II***	**Effect**
3.33	0.167 ($8.56x10^{-4}$)	210.93	142.45±12.57	1.48	Synergistic
6.67	0.33 ($1.68x10^{-3}$)	193.17	148.06±12.18	1.30	Synergistic
13.33	1.67 ($8.56x10^{-3}$)	170.95	79.71±0.42	2.14	**Synergistic**
20.00	3.33 ($1.68x10^{-2}$)	161.43	71.53±0.31	2.26	**Synergistic**
26.69	16.67 ($8.56x10^{-2}$)	240.25	37.05±6.64	6.49	**Synergistic**
33.33	33.33 ($1.68x10^{-1}$)	400.77	38.66±6.82	10.37	**Synergistic**

(B) Intracellular protection

The results of the intracellular protection of WS and Se-Met alone were used to calculate the expected interaction between WS and Se-Met in combination. The equation of the linear trend line obtained using the WS values (Figure 4.6B) was $y = -0.0298x + 70.961$, $R^2=0.018$. The R^2 value is low due to the lack of a clear dosage effect. The equation of the polynomial trend line obtained using the Se-Met data (Figure 4.8B) was $y = -0.0046x^3 + 0.2586x^2 – 3.5601x + 73.979$, $R^2=0.60$.

The y-values were calculated using these equations, and the values obtained for both WS and Se-Met were added together and divided by two, providing the expected intracellular

protection by WS + Se-Met. The interaction index (*II*) was calculated using the observed values (Figure 4.9B) and the results are summarized in Table 4.7.

An additive effect was observed at the three lowest, as well as at the highest combination of WS and Se-Met. At 20 mg/ml WS + 3.33 pg/ml Se-Met, a very weak antagonistic effect occurred, while at 26.69 mg/ml WS + 16.67 pg/ml Se-Met, a synergistic, antioxidant effect was observed.

Table 4.7: INTRACELLULAR EFFECTS: Expected and observed combinational effects of WS and Se-Met, SC-1 cells

Combinations		% Damage		Interactions	
WS (mg/ml)	**Se-Met (pg/ml) (nM)**	**Expected**	**Observed**	***II***	**Effect**
3.33	0.167 ($8.56x10^{-4}$)	72.13	72.76±7.18	0.99	Additive
6.67	0.33 ($1.68x10^{-3}$)	71.79	81.96±6.14	0.88	Additive
13.33	1.67 ($8.56x10^{-3}$)	69.65	64.89±1.76	1.07	Additive
20.00	3.33 ($1.68x10^{-2}$)	67.59	77.02±9.43	0.88	Antagonistic
26.69	16.67 ($8.56x10^{-2}$)	67.59	55.74±3.28	1.22	**Synergistic**
33.33	33.33 ($1.68x10^{-1}$)	70.79	69.90±9.74	1.01	Additive

Antioxidant synergism has been reported with between various compounds such as vitamins E and C, vitamin E and β-carotene, catechin and malvidin 3-glucoside, flavonoids and urate, and tea polyphenols and vitamin E. Yang & Liu (2009) investigate the interaction between apple extracts and quercetin-3-β-D-glucoside using the MCF-7 (human breast cancer) cell line, and reported synergistic effects on MCF-7 cell proliferation. Jia and co-workers (1999) investigated the ability of the main components of green tea polyphenols to protect against linoleic acid peroxidation, and reported synergistic antioxidant effects in combination with α-tocopherol.

4.5 Summary

Forty-eight hours exposure of SC-1 cells to WS and Se-Met showed limited toxicity, and this was only for WS at 9.09 mg/ml with the NR assay. In contrast, at the highest concentration of WS, microscopy revealed toxicity for WS alone and in combination with Se-Met. Total protection studies revealed pro-oxidant effects at low concentrations of WS and Se-Met, alone and in combination. At higher concentrations an antioxidant effect was observed for WS alone and in combination with Se-Met. In contrast, intracellular antioxidant effects were observed at all concentrations for WS and Se-Met, alone and in combination. Synergism between WS and Se-Met was observed at all concentrations in the total protection assay, with mostly additive effects occurring intracellularly.

In conclusion, some toxicity was observed for WS alone and in combination with Se-Met at high concentrations. Antioxidant studies revealed significant antioxidant effects specifically related to intracellular effects, and these effects were predominantly additive.

Chapter 5: The effect of *Withania somnifera* and selenium, alone and in combination, on chick embryo lung primary cultures

5.1 Introduction

The level of airborne pollution, especially in heavily populated areas and developing countries, is escalating. Tobacco smoke, traffic-related and photochemical pollution, particulate matter and other contaminants have all been associated with inflammatory conditions like allergies, asthma, and emphysema (Alessandrini *et al*., 2010, Taur & Patil, 2011). Several of these conditions are coupled with changes in the levels of eosinophils, mast cells, lymphocytes, cytokines and other inflammatory cells. It has been hypothesized that an increase in ROS generation in the respiratory system has adverse effects on lung growth and pulmonary function, resulting in associated inflammatory diseases (Dworski, 2000, Wichmann *et al.,* 2009, Girard-Lalancette *et al.,* 2009).

A healthy cell, which maintains equilibrium between ROS generation and elimination, has the ability to protect itself against ROS with integral antioxidant enzymatic systems. However, if an unbalance occurs due to a higher ROS production than antioxidant defence capacity, a cellular system will enter a pathological state caused by oxidative stress. Certain foods containing antioxidant compounds can assist in preventing pathologies (Girard-Lalancette *et al.,* 2009). Likewise, medicinal plants with antioxidant properties will also be able to protect against oxidative damage. Antioxidants that have the ability to improve the antioxidant status of cells lining the respiratory system can lower the risk of oxidative damage and possibly prevent diseases of the respiratory tract.

In the previous chapters, water extracts of WS were found to have antioxidant activity which translated into significant levels of cellular protection against oxidative damage in the SC-1 cell line. In this chapter, the effects of WS, alone and in combination with Se-Met, will be investigated a more physiologically relevant model. For this purpose, primary cell cultures derived from chick embryo lungs (CELC), will be established. A cell culture system can elucidate the interactions between a drug/chemical and tissue at the cellular, subcellular or molecular level, and it allows for the examination of organ-specific toxicity effects of compounds. Established cell lines, such as the SC-1 fibroblasts, have increased viability compared to primary cultures, and are easier to maintain. However, these cells have a tendency to lose many of their differentiated functions associated with the tissue *in vivo*. Cell

lines have been shown to present characteristics of cancer or transformed cells (Acosta *et al.*, 1985).

The main aim of this study is to determine the toxicity, as well as the *in vitro* cellular and protective effects, of a water extract of WS and Se-Met, alone and in combination, using established CELC.

The specific objectives of this study are:

1. To establish and optimise methodologies used to isolate lungs from chick embryos and establish primary cultures of these CELC.
2. To determine if WS and Se-Met are cytotoxic to the CELC using the combined NR and CV assays.
3. To evaluate with light and phase contrast microscopy the effect of WS and Se-Met, alone and in combination, on CELC morphology.
4. To determine if WS and Se-Met, alone and in combination, protect the CELC against AAPH-induced oxidative damage using the DCFH-DA cell-based assay.
5. To determine the type of interaction, if any, between WS and Se-Met using the DCFH-DA cell-based assay.

5.2 Materials

Chick embryo lung primary cultures

One-day-old hatching eggs were obtained from Eagle's Pride Hatchery in Pretoria, South Africa, and incubated for 13 days at 37°C and 5% CO_2.

Withania somnifera and seleno-methionine

The WS extracts and Se-Met solutions described in Chapter 4 will be used in this study.

Media, supplements, reagents and plastic ware

The same media, reagents, supplements and plastic ware used for the cultivation for SC-1 cells were used in this study. In addition, Hank's buffered saline solution (HBSS) was obtained from Highveld Biological Company, Johannesburg, SA.

Laboratory facilities

All cell culture and microscopy studies were undertaken in the cell culture laboratory of the Department of Anatomy, while fluorescence analysis was carried out in the Department of Pharmacology, School of Medicine, Faculty of Health Sciences, University of Pretoria.

5.3 Methods

Establishment of primary chick embryo lung cell cultures

One-day-old hatching eggs were obtained from Eagle's Pride Hatchery in Pretoria, SA, and incubated at 37ºC and 5% CO_2 content for 13 days. The embryos were removed from the eggs and terminated immediately by decapitation. The lungs were dissected from the embryo. The tissue was cut into small fragments and washed three times with HBSS. Single cell suspensions were prepared by enzymatic digestion with trypsin. Enzymatic action was inhibited by DMEM containing 7% FCS. The cell suspension was plated in 24-well plates to allow for the attachment of the CELC. The primary cultures were maintained at 37ºC and 5% CO_2 content for 24 hours to allow for attachment and differentiation of cells. Phase contrast microscopy was used for the evaluation of cell types and to determine the effect of WS and Se-Met, alone and in combination, on cell morphology.

The cellular effects of *Withania somnifera* and seleno-methionine on the CELC

WS extracts and Se-Met solutions were prepared as described in Chapter 4 and exposure to WS and Se-Met was as described for the SC-1 cells in Chapter 4 (Table 5.1). After exposure for 48 hours, lysosomal membrane integrity and cell number was determined as described in Chapter 4 for the SC-1 cells.

Table 5.1: Final concentrations of WS and Se-Met for NR and CV assays

WS	Se-Met		
mg/ml	**pg/ml**	**nM**	**RDA**
0.007	0.01	$5.1x10^{-5}$	½
0.042	0.02	$1.1x10^{-4}$	1
0.25,	0.10	$5.1x10^{-4}$	5
1.51	0.20	$1.1x10^{-3}$	10
9.09*	1.00	$5.1x10^{-3}$	50
	2.00*	$1.1x10^{-2}$*	100

*Used to determine the cytotoxicity alone and in combination with phase contrast and light microscopy

The short-term effects of WS and Se-Met, alone and in combination, on the CELC morphology was evaluated by phase contrast microscopy after exposure for 12 hours. The cells were then fixed and stained with CV, and cellular morphology was evaluated using light microscopy as described in Chapter 4 for the SC-1 cells.

Oxidative/antioxidant effects of *Withania somnifera* and seleno-methionine, alone and in combination, on the CELC

Likewise, the total and intracellular effects of WS and Se-Met, alone and in combination, were determined at the same concentrations and under the same conditions as described in Chapter 4. The concentrations used are summarized in Table 5.2.

Table 5.2: Final concentrations of WS and Se-Met alone and in combination for DCFH-DA assay

Alone		Combination		
WS (mg/ml)	Se-Met (pg/ml) (nM)	WS + Se-Met		Ratio
		WS (mg/ml)	Se-Met (pg/ml) (nM)	(mg/ml: pg/ml)
3.33	0.167 ($8.56x10^{-4}$)	2.50	0.125 ($6.37x10^{-4}$)	1:1
6.67	0.33 ($1.68x10^{-3}$)	5.00	1.25 ($6.37x10^{-3}$)	2:10
13.33	1.67 ($8.56x10^{-3}$)	10.00	12.50 ($6.37x10^{-2}$)	4:100
20.00	3.33 ($1.68x10^{-2}$)	15.00	0.25 ($1.27x10^{-3}$)	6:2
26.69	16.67 ($8.56x10^{-2}$)	20.00	2.50 ($1.27x10^{-2}$)	8:20
33.33	33.33 ($1.68x10^{-1}$)	25.00	25.00 ($1.27x10^{-1}$)	10:200

Interactions between *Withania somnifera* and seleno-methionine

The interactions between WS and Se-Met were calculated and expressed as described in Chapter 4.

Data management and statistical analysis

Data management and methods used for statistical analysis were as described in Chapter 3.

5.4 Results and discussion

As a developing country, South Africa is subject to many sources of air pollution. The burning of coal, oil and natural gas in industrial processes, power generation and vehicles, the burning of wood and kerosene, cigarette smoke, insecticides and household materials all contribute to this problem. Some of the more common air pollutants that can have a major impact on health are sulphur dioxide, oxides of nitrogen, particulate matter, lead, ozone, carbon monoxide and volatile organic compounds, e.g., benzene. Chronic exposure to these pollutants can lead to associated inflammatory respiratory conditions such as wheeze, shortness of breath, sinusitis, rhinitis, bronchitis, pneumonia (Matooane *et al.*, 2004). A poor nutritional status will exacerbate these conditions, e.g., low nutritional intake of Se will result in reduced enzymatic antioxidant activity and an increased risk of oxidative damage.

The respiratory system and the lungs in particular, are a specific target for toxicity, as this system is directly exposed to the environment (Cross *et al.,* 1998). All eukaryotic cells have

the ability to utilize energy produced from the reduction of oxygen to water. With each reduction, a small number of oxygen intermediates, such as $O_2^{\bullet-}$, H_2O_2, HO^- and $^1O_2$ are produced, which are highly unstable and reactive. The cells of the lungs are exposed to the highest concentration of oxygen of any cells of the body and are subsequently a specific target for oxidant injury (Martin *et al.,* 1981).

Lung cell lines used in toxicological studies include the A549 human lung epithelial cell line, the H1299 lung lymph node tissue (www.atcc.org), and the squamous lung cancer cell line, EBC-1 (www.bioinfoweb.com). These specific cell lines do not take the cellular complexity of the lungs into account. Mammalian lung tissue consists of type I and type II pneumocytes and alveolar macrophages. Type I pneumocytes are thin, elongated cells with flattened nuclei and sparse mitochondria and organelles. These cells cover the alveolar basement membrane, and their shape and diminished cytoplasm contribute to the efficacy of the air-blood barrier. Type II pneumocytes are rounded cells with dark nuclei and cytoplasm rich in mitochondria and smooth and rough endoplasmic reticulum. Type II pneumocytes produce and secrete surfactant. The third cell type are the alveolar macrophages, which have a defence function in the lungs, and are present in the alveolar air spaces and phagocytose debris. Type II pneumocytes are the precursors to type I pneumocytes, and when alveolar epithelium is exposed to toxic agents and the sensitive type I pneumocytes are destroyed, type II pneumocytes increase in size and number (Stevens & Lowe, 2005).

The development of the chick lung, especially with regard to morphology and biochemistry, greatly resembles that of the mammalian lung. Therefore many conclusions can be drawn from studies done on the chick embryo lung. Researchers have described cells closely resembling type I and type II pneumocytes in both adult and embryonic chick lung cells (Compton *et al.,* 1981).

Birds have a much higher metabolic rate then mammals and other vertebrates, and therefore have some unique modifications to their respiratory system, which allow air to pass through their lungs faster. Bird lungs lack alveoli, as well as the anastomosis of the branches of the bronchi, resulting in a sponge-like structure. The lungs are not elastic and therefore cannot expand, but have air sacs extending posteriorly, which are able to expand and contract and force air through the lungs where gaseous exchange takes place. The abdominal muscles are responsible for this action (Bellairs & Osmond, 2005).

The embryonic development of the chick lung can be divided into three periods, namely the glandular period (3-12 days of incubation), vascular period (12-16 days of incubation) and finally the alveolar period (16 days to hatching). The developmental process concludes in the formation of the air-blood barrier. The histological events that take place during the development of the chick lung are similar to those occurring in mammalian lung development. In this study, the lungs were removed during the vascular period (day 13), and this period is analogous to the canalicular stage of development in mammalian lungs (17-27 weeks) (Chen *et al.,* 1986). As lungs develop, the number of undifferentiated mesenchymal cells decreases, while the number and size of the developing alveolar epithelial cells and capillary endothelial cells increases and the alveolar walls are formed. The differences between and similarities of the stage of development in the chick and mammalian lung are expounded in Table 5.3.

Table 5.3: Comparison of the stages of lung development in the chick and the mammal

Chick lung		**Mammalian lung**	
Period	**Characteristic events** (Chen *et al.,* 1986)	**Period**	**Characteristic events** (Joshi *et al.,* 2007)
Vascular 12-16 days	Atrium buds off from parabronchial epithelium Mesenchyme condenses Network of capillaries invade mesenchyme next to atrium Differentiation of epithelial cells Smooth muscle cells grow into crisscross formation lining openings of atria and parabronchi	**Canalicular** 17-27 weeks	Differentiation of type I and type II pneumocytes Formation alveolar capillary barrier

The development of the chick embryo is dependent on aerobic metabolism, and approximately halfway through the 21-day process, mitochondrial respiration and oxygen uptake are increased dramatically to provide energy for important functions like tissue growth, transport of nutrients and maintenance of the heart beat. These increases in metabolic rates can lead to the production of ROS and other free radicals. Three antioxidant enzymes found within the chick embryo act together to protect it against potential oxidative damage and these are SOD, CAT and GPx. SOD converts the $O_2^{\bullet -}$ radical formed from electron leakage from the electron transport chain to H_2O_2, and CAT and GPx both have the ability to convert H_2O_2 to H_2O (Surai, 1999). The presence of these antioxidant enzymes protects the embryonic tissues during sensitive times of development, and prepares for any potential oxidative hazards associated with hatchability, which is the percentage of eggs set to hatch that do in fact hatch (Blood *et al*., 2007). At day 13, the activity of SOD, CAT and GPx are low, and continue to decrease until day 19 of development, after which the enzyme

levels start to increase (Surai, 1999). The low levels of antioxidant enzymes may be of significance when investigating the role of Se-Met in the protection of the CELC.

Establishment of the chick embryo lung primary culture

Primary cultures were established from the lungs of chick embryos at day 13 of development. The chick embryo is in the vascular stage of development and the alveolar epithelial cells have started differentiating, the capillary endothelial cells have increased and the alveolar walls have formed (Chen *et al.,* 1986). Following immediate decapitation, the lungs were dissected from the embryo and cut into very small fragments. These were washed with HBSS and single cell suspensions were prepared by enzymatic digestion with trypsin. The cell suspension was plated and the primary cultures were maintained at 37ºC and 5% CO_2 content for 24 hours to allow for attachment and differentiation of cells before any experiments were undertaken. Arrows show a cluster of CELC tissue in (B), rounded cells in (C), similar in morphology to type II pneumocytes found in human lung tissue, and thin, elongated cells in (D), resembling type I pneumocytes found in human lung tissue. While the lung cells in the CELC may differ from those found in human lung tissue, cells with similar morphology, and thus similar functions, are present.

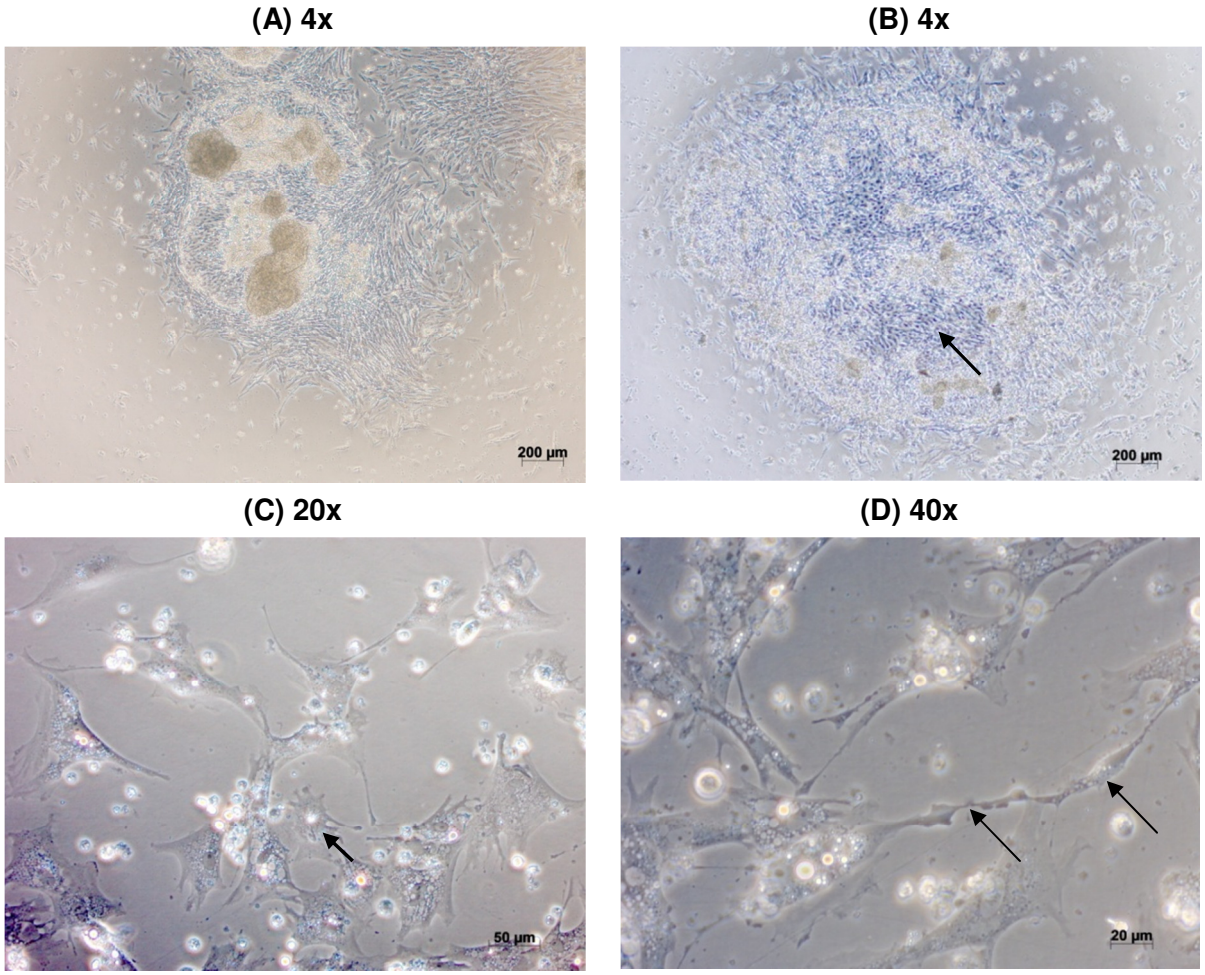


Figure 5.1: Morphology of chick embryo lung primary cultures after 24 hours *in vitro*. (A) and (B) 4x, (C) 20x and (D) 40x magnification. Arrows show a cluster of CELC tissue in (B), rounded cells in (C), similar in morphology to type II pneumocytes found in human lung tissue, and thin, elongated cells in (D), resembling type I pneumocytes similar to those found in human lung tissue.

The cellular effects of *Withania somnifera* and seleno-methionine on the CELC

Neutral Red and Crystal Violet assays

The CELC were exposed to WS and Se-Met, alone and in combination, for 48 hours. The lysosomal membrane integrity and cell number were subsequently determined using the combined NR/CV assay. For each assay, the sample data was expressed as a percentage of the control (100%). The combined assay was chosen because lysosomal membrane integrity and cell number could be determined in the same sample, thereby using fewer samples. This is an advantage particularly when using primary cultures, as there is usually a high degree of variability due to the heterogeneity of the cell population, i.e., cell type, growth rates and degree of differentiation of cell types.

A concentration range of 0.007 – 9.09 mg/ml WS was used in this study, and a significant decrease in lysosomal membrane integrity was observed at 0.25 mg/ml and 9.09 mg/ml (Figure 5.2A). At a concentration of 9.09 mg/ml WS, the SC-1 cells (Chapter 4) appeared to be more sensitive to the effects of WS than the CELC, as at this concentration the lysosomal membrane integrity decreased to 78% compared to 90% for the CELC. Cell number was determined using the CV assay (Figure 5.2B). A decrease in cell number was observed at all concentrations except for 1.51 mg/ml WS in contrast to the SC-1 cell line (Chapter 4), where no effect was observed at any concentration.

To investigate the effect of Se-Met on CELC, a concentration range of 0.01 pg/ml (½ of the RDA) to 2 pg/ml (100 x RDA), was added to the CELC for 48 hours, and the cells were then assayed using the combined NR/CV assay. At a concentration of 0.01 pg/ml and 0.02 pg/ml, a significant increase in lysosomal membrane integrity was observed (Figure 5.3A) and this translated into a significant increase in cell number at 0.02 pg/ml (Figure 5.3B). No loss of cellular function was observed at any other concentration.

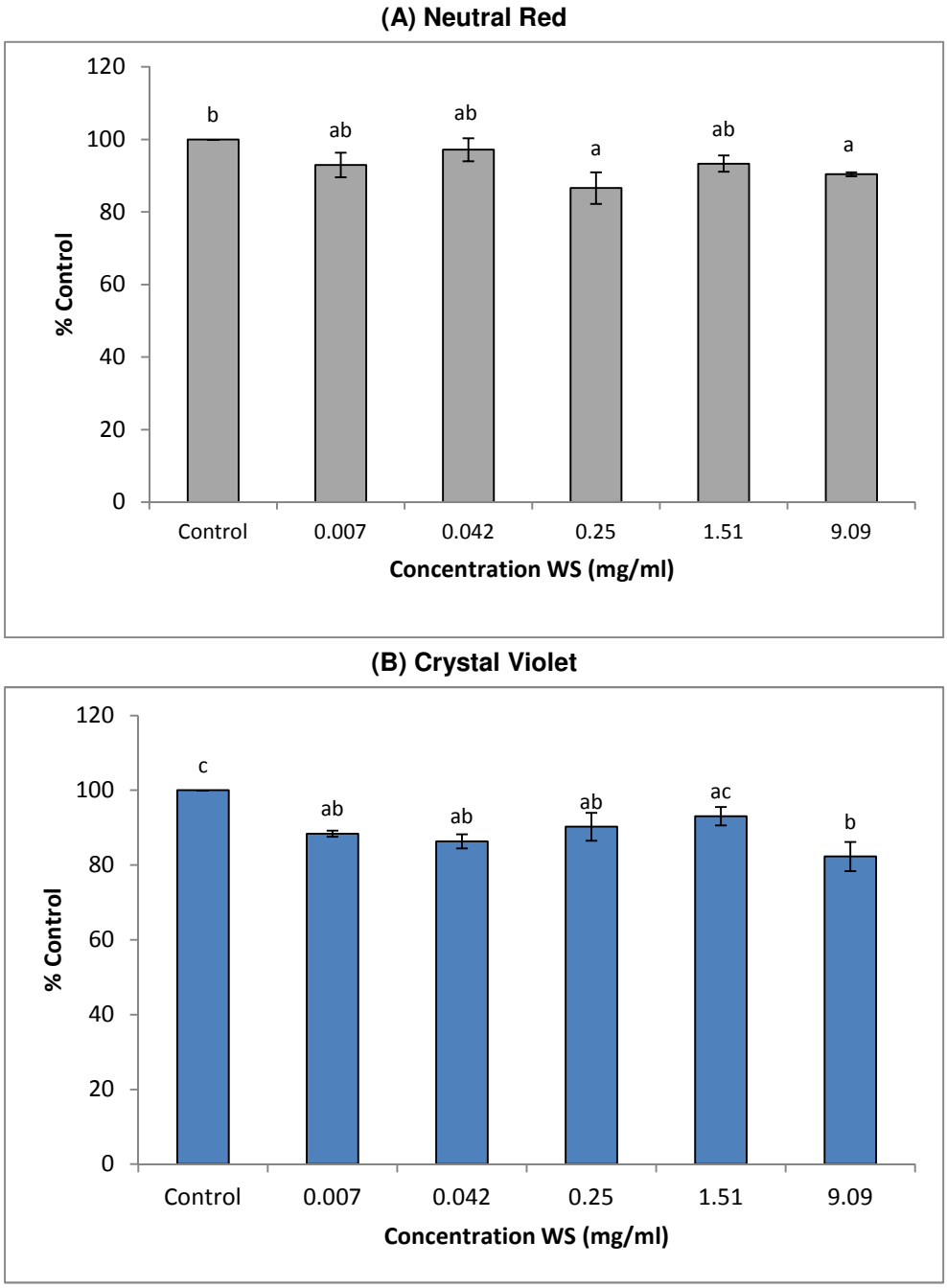


Figure 5.2: Effect of 24 hour exposure to WS on CELC lysosomal membrane integrity and cell number using the Neutral Red (A) and Crystal Violet (B) assays respectively. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001.

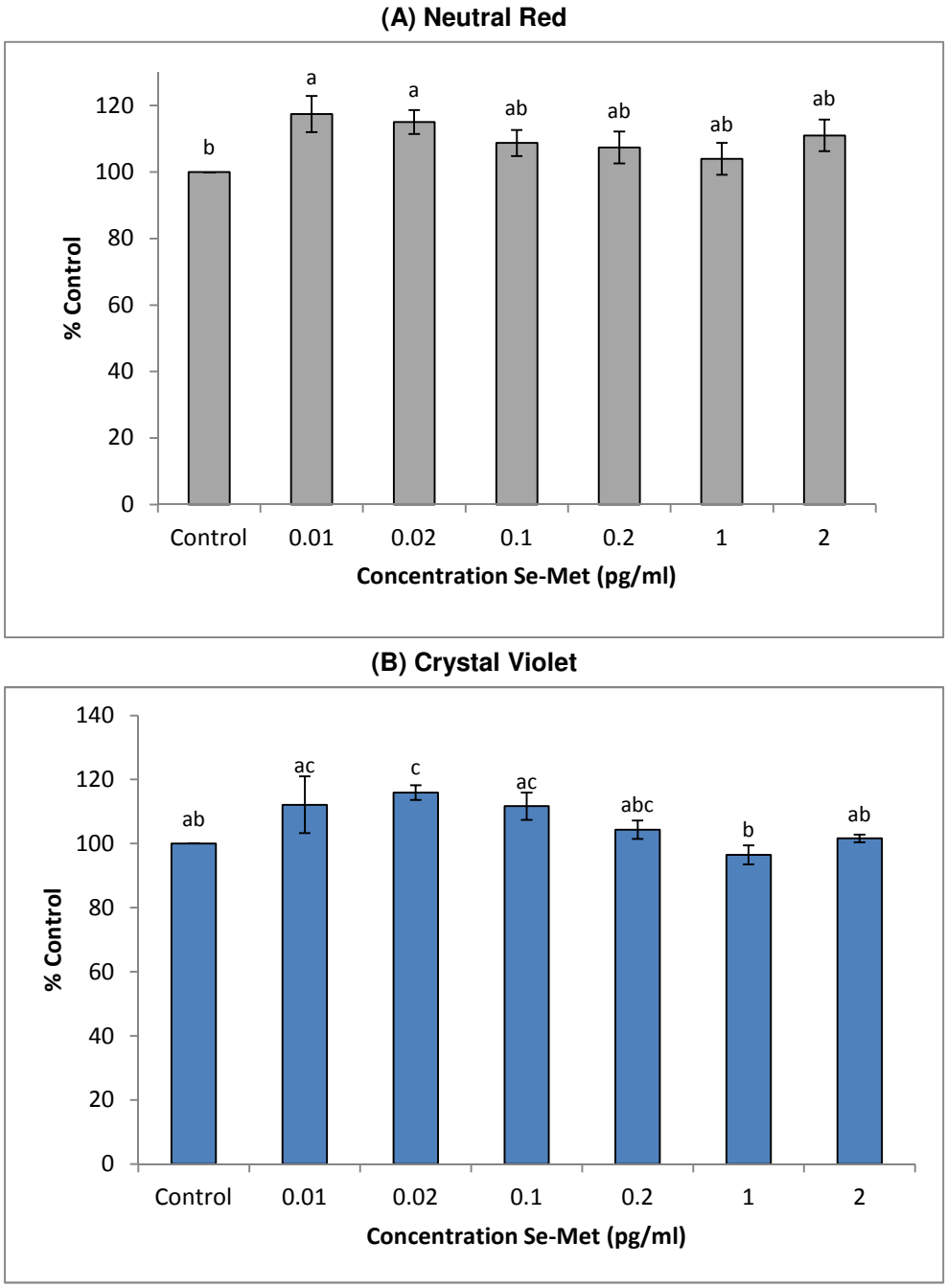


Figure 5.3: Effect of 24 hour exposure to Se-Met on CELC lysosomal membrane integrity and cell number using the Neutral Red (A) and Crystal Violet (B) assays respectively. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001.

Phase contrast and light microscopy

At 9.09 mg/ml WS (Figure 5.4B), after 12 hours of exposure, phase contrast microscopy revealed an increase in the number of vacuoles present in the cytoplasm and the CELC were rounded and starting to detach. Vacuolation can be a natural process associated with the secretion and digestion of cellular products, and occasionally vacuoles are permanent features of cells. However, the presence of vacuoles can also be an adaptive response to injury. In cases where the cell is not able to adapt and limit the damage, cell death will result (Henics & Wheatley, 1999).

At 2 pg/ml Se-Met, phase contrast microscopy revealed normal cellular morphology, compared to the control. (Figure 5.4C). CELC exposure to the highest concentrations of WS and Se-Met (9.09 mg/ml WS + 2 pg/ml Se-Met) in combination revealed a decrease in the degree of vacuolation compared to WS alone (9.09 mg/ml) (Figure 5.4D), although rounded cells and membrane blebbing were observed. Selenium is an essential component of the antioxidant enzymes GPx, TRx and selenoprotein P. A Se deficiency has been proven to decrease the activities of these enzymes, increase lipid peroxidation and cause cell death (Saito *et al*., 2003; Drake, 2006). Indications are that Se-Met supplementation protects the CELC against the toxicity of WS at high concentrations in contrast to observed effects in SC-1 cells.

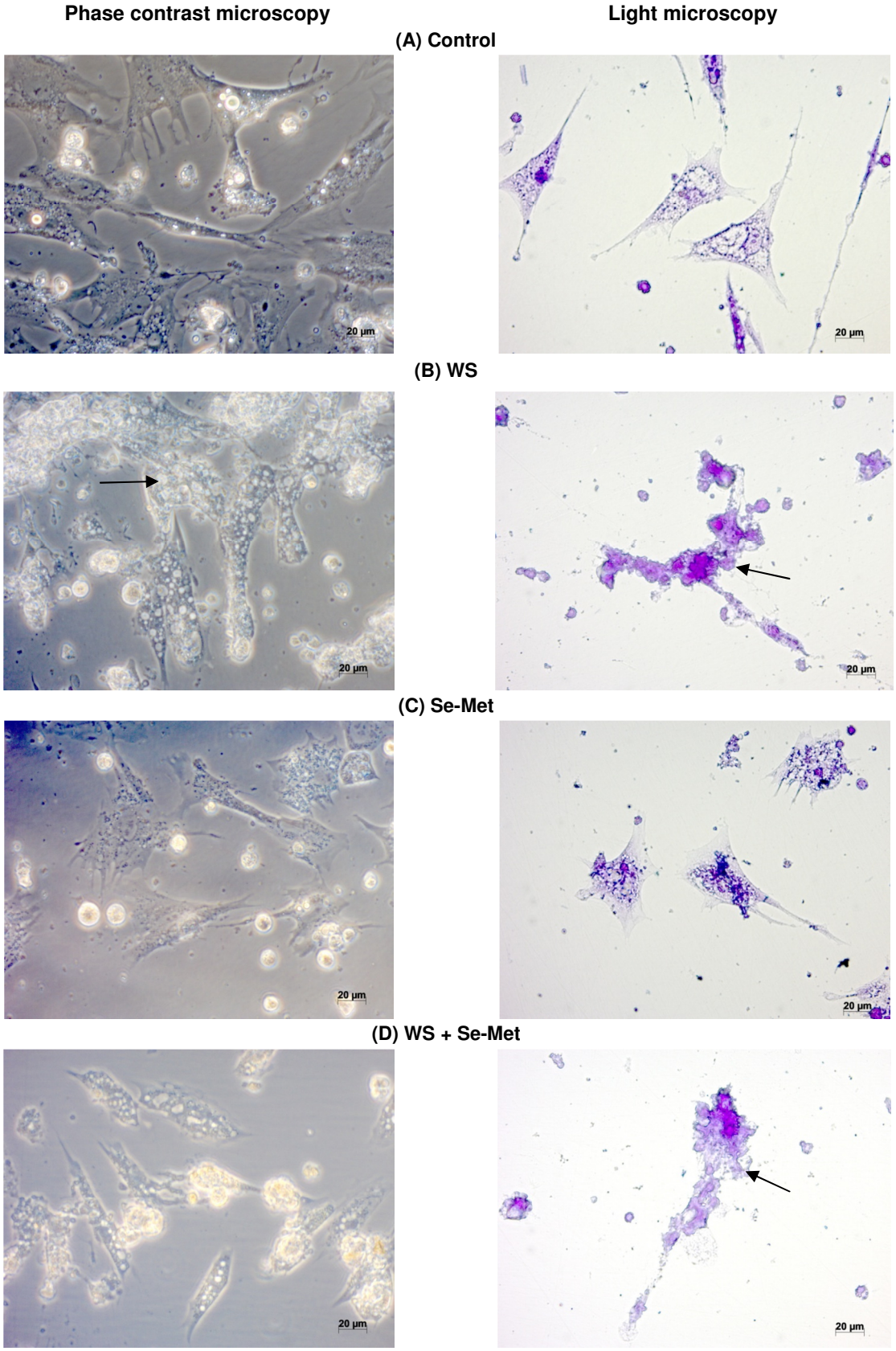


Figure 5.4: Phase contrast and light microscopy (using a CV stain) of CELC not exposed (A), exposed to 9.09 mg/ml WS (B), 2 pg/ml Se-Met (C), and a combination of 9.09 mg/ml WS and 2 pg/ml Se-Met for 12 hours (D). For phase contrast microscopy: arrows showing vacuolation in (B). For light microscopy: arrows show evidence of rounded and blunted cells, membrane blebbing and loss of cellular content in (B) and (D).

Table 5.4: Summary of the cellular effect of WS and Se-Met alone and in combination on CELC

	WS (48 hours)				
	0.007 mg/ml	**0.042 mg/ml**	**0.25 mg/ml**	**1.51 mg/ml**	**9.09 mg/ml**
NR	ns	ns	↓ **(T)**	ns	↓ **(T)**
CV	↓ **(T)**	↓ **(T)**	↓ **(T)**	ns	↓ **(T)**

	Se-Met (48 hours)					
	0.01 pg/ml	**0.02 pg/ml**	**0.1 pg/ml**	**0.2pg/ml**	**1 pg/ml**	**2pg/ml**
NR	↑**(M)**	↑**(M)**	ns	Ns	ns	ns
CV	ns	↑**(M)**	ns	Ns	ns	ns

9.09 mg/ml WS (12 hours)
Morphology: Rounded, blunted, more vacuoles, few detached cells
2 pg/ml Se-Met (12 hours)
Morphology: Similar to control
9.09 mg/ml WS + 2 pg/ml Se-Met (12 hours)
Morphology: Most cells rounded, detached, loss of cellular content. Decrease in degree of vacuolation

ns = no significant differences compared to control, M = Mitogenic effect, T = Toxic effect, ↑ = increase in staining, ↓ = decrease in staining

Protective effects of *Withania somnifera* and seleno-methionine, alone and in combination, against oxidative damage in the CELC

Total protection represents the ability of the antioxidants present in the extracellular environment, as well as those taken up by the cells, to protect the cells from oxidative damage. Intracellular protection signifies the ability of the antioxidants to cross or bind the cell membrane.

Withania somnifera

(A) Total protection

The ability of WS to provide total protection to the CELC *in vitro* against oxidative damage caused by AAPH was tested using the DCFH-DA assay. The concentrations of WS used are summarized in Table 5.2. The CELC were exposed to the lowest (3.33 mg/ml) and highest (33.33 mg/ml) concentrations of WS in the absence of AAPH to measure the potential oxidative effect of WS. Although low levels of cytotoxicity were observed, (12% for 3.33 mg/ml WS and 16% for 33.33 mg/ml WS), there was no significant difference between the two WS samples and the control of DCFH-DA alone (11%) (Figure 5.5A). The concentrations of WS used in the DCFH-DA assays were higher than those used in the NR and CV assays, because the exposure time was much shorter (1-2 hours as opposed to 48 hours).

In the presence of AAPH, all concentrations except for one, 6.67 mg/ml WS, showed significant total protective effects when compared to the 100% of AAPH. At this concentration (6.67 mg/ml WS + AAPH), however, there was a fairly high standard error of mean, and this could be the reason for it not showing a statistical difference, as it was well below the reading obtained for AAPH (Figure 5.5A). The increasing concentrations of WS showed a dose-dependent effect (Figure 5.6A). All the other concentrations of WS showed an antioxidant, protective effect in the presence of AAPH. The highest concentration of 33.33 mg/ml WS had a reading of 53% fluorescence, which can be interpreted as 47% protection. The ability of WS to provide total protection to the CELC against AAPH-induced oxidative damage is much better than for SC-1 cells (Chapter 4) at lower concentrations, but similar at higher concentrations.

(B) Intracellular protection

WS was tested for its ability to provide intracellular protection to the CELC *in vitro* against oxidative damage caused by AAPH, using the DCFH-DA assay. The same concentrations of WS were used as for the total protection assay, ranging from 3.33 mg/ml to 33.33 mg/ml (Table 5.2). The CELC were exposed to the lowest (3.33 mg/ml) and highest (33.33 mg/ml) concentrations of WS in the absence of AAPH in order to measure the potential oxidative effect of WS. Once again, low levels of cytotoxicity were observed (13% for 3.33 mg/ml WS and 19% for 33.33 mg/ml WS), but there was no significant difference between DCFH-DA alone (12%) and the two WS controls (Figure 5.5B). Only the highest concentration of WS in the presence of AAPH (33.33 mg/ml WS + AAPH) showed a significant decrease when compared to AAPH alone (Figure 5.5B). Similar, but statistically better results were obtained in Chapter 4, for the intracellular protection of the SC-1 cells by WS.

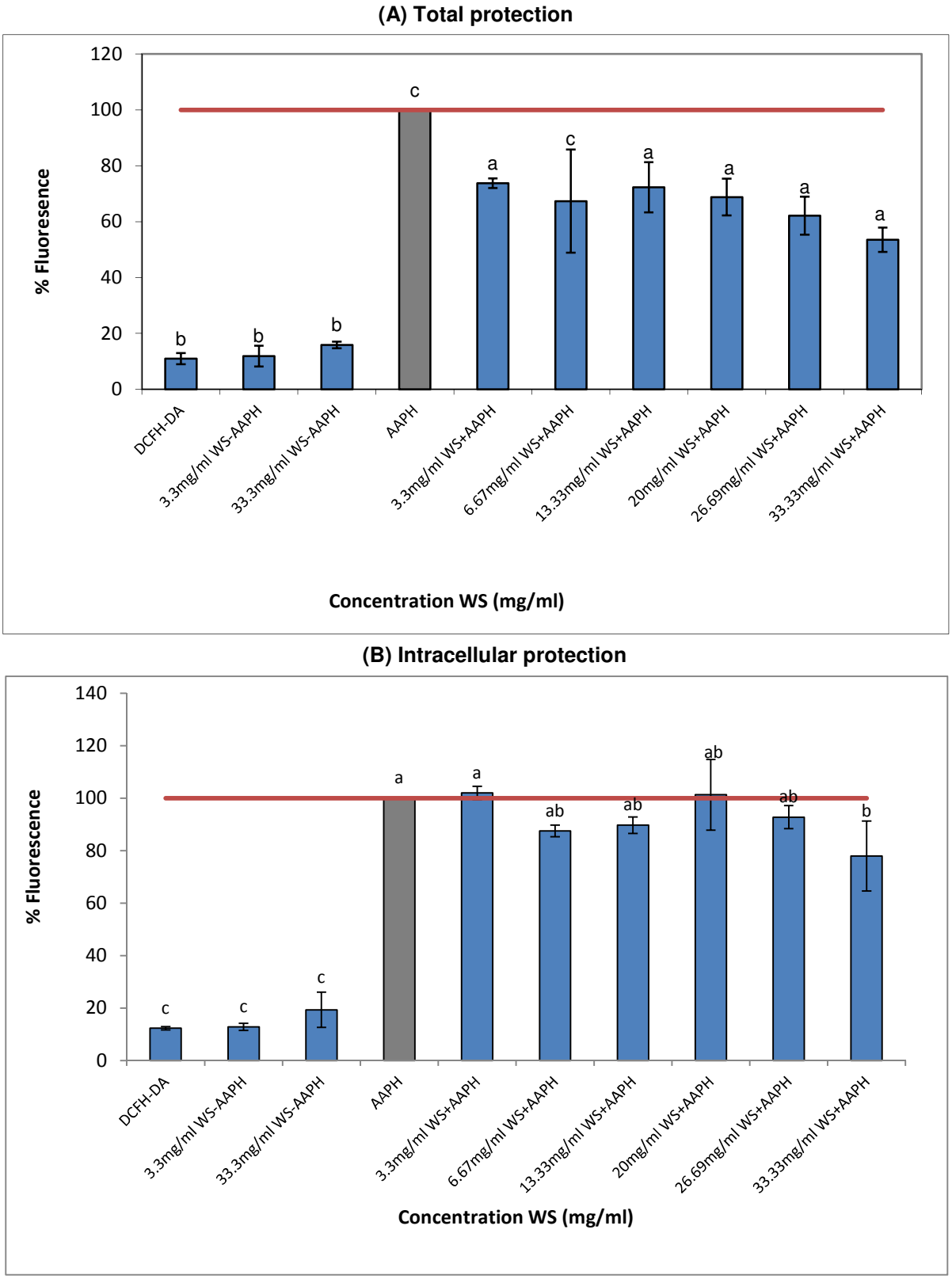
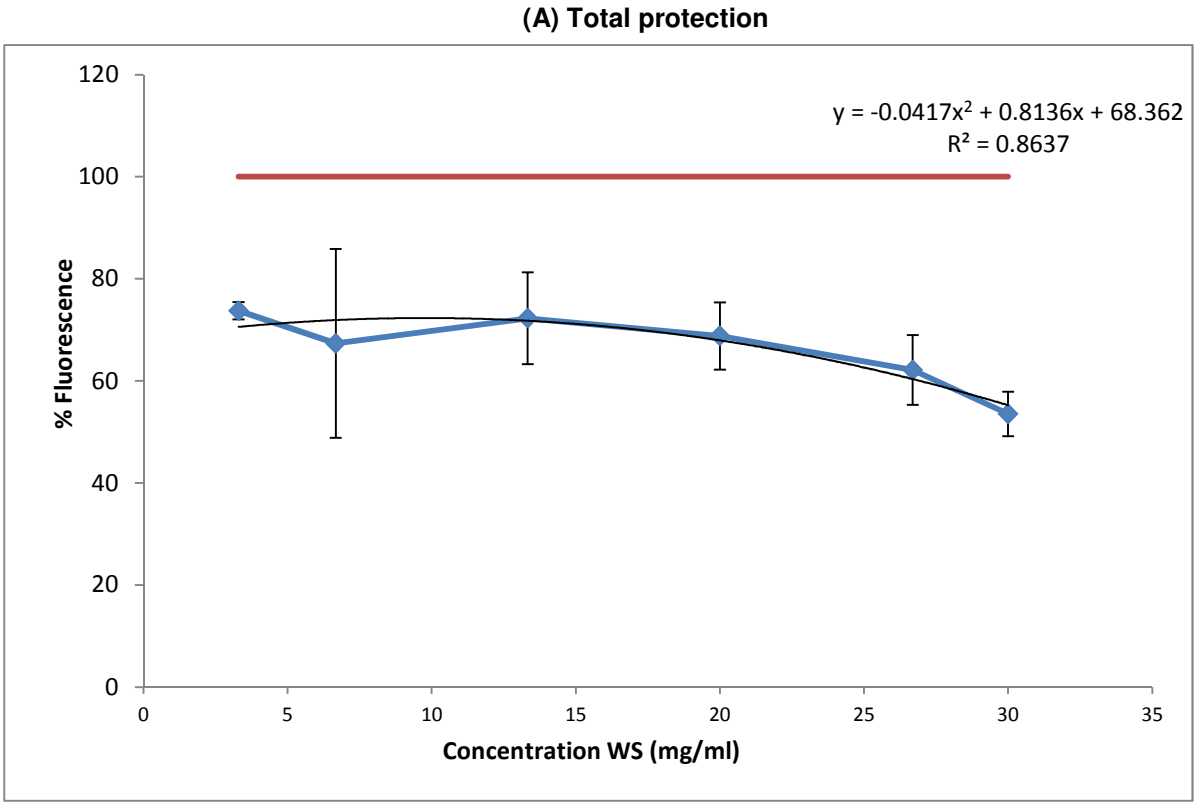


Figure 5.5: Total (A) and intracellular (B) effects of increasing concentrations of WS on AAPH-induced oxidative damage in CELC. Data is expressed as a percentage of the damage caused by AAPH + DCFH-DA, 100% damage. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001. Red line = 100% damage due to AAPH.



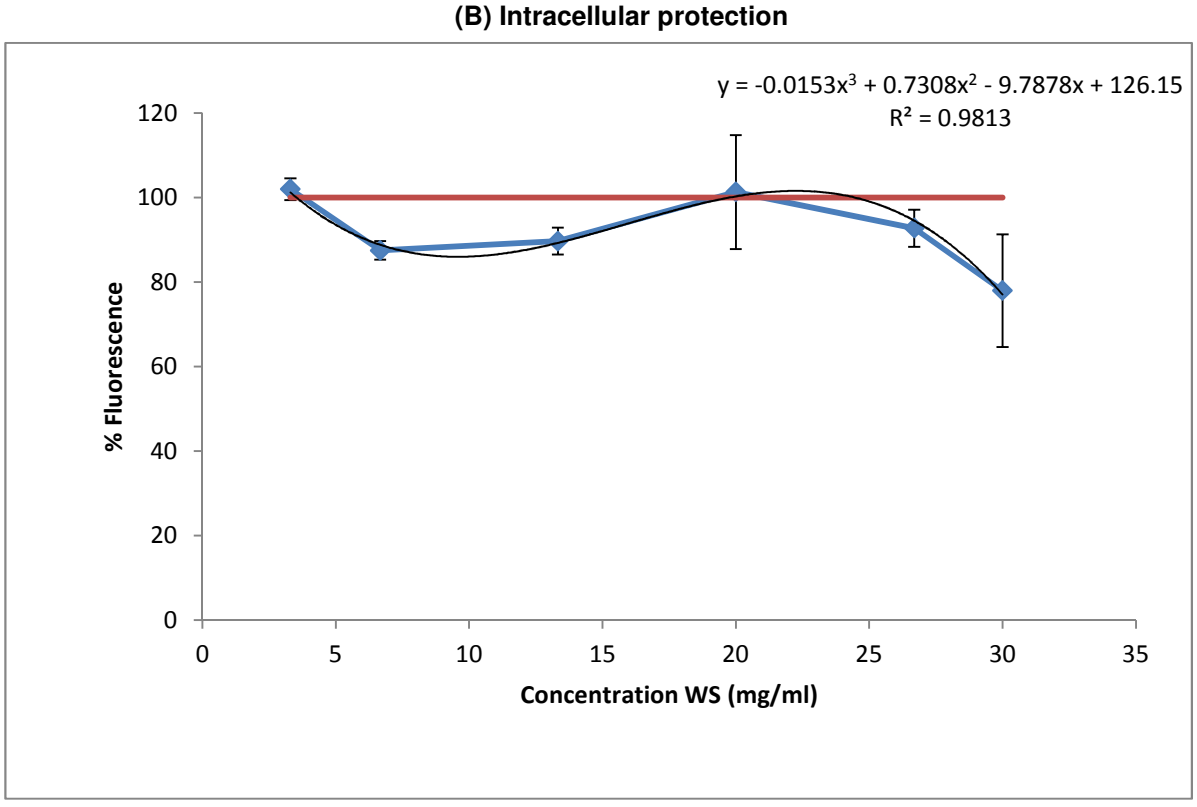


Figure 5.6: Total (A) and intracellular (B) effects of increasing concentrations of WS on AAPH-induced oxidative damage in CELC. Data is expressed as a percentage of the damage caused by AAPH + DCFH-DA, 100% damage. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Red line = 100% damage due to AAPH.

A major criticism of *in vitro* studies is that these studies do not account for absorption, distribution, metabolism and excretion, and although this effect occurs *in vitro*, it may not occur *in vivo*.

The ability of WS to protect rat testis against cadmium (Cd)-induced oxidative damage was investigated by Prithiviraj and co-workers in 2013. Cd (2.5 mg/kg body weight), a toxic metal and industrial hazard, was administered as cadmium chloride to rats intraperitoneally in a single dose. Whole root of WS, dissolved in 2% gum acacia, was administered daily at a dose of 1000 mg/kg body weight for 30 days. The generation of ROS was evaluated thereafter with the DCFH-DA assay using fresh rat testis homogenate. WS significantly reduced the total ROS level compared to Cd alone, and it also increased the levels of antioxidant enzymes SOD, CAT, GSH, GPx, glutathione-*S*-tranferase (GST), α-tocopherol and ascorbic acid in the testes after Cd ingestion (Prithiviraj *et al*., 2013). This indicates that WS is absorbed and shows antioxidant protective effects at peripheral sites, such as the prostate, and could have similar effects in the respiratory system.

The protective effect of WS has been evaluated in a clinical trial for infertile male patients. The ability of WS root to improve semen quality by protecting sperm from oxidative damage caused by excess ROS generation was investigated. The testicular environment is metabolically very active, resulting in ROS generation, which is necessary for sperm function. Ideally, just enough ROS would be generated by the Sertoli cells for spermiogenesis, capacitation and acrosome reaction. Excessive production of ROS, however, may result in oxidative damage, leading to lipid peroxidation of plasma membranes, and a change in sperm function and fertilizing capacity. For three months, the participants were prescribed 5 g/day of WS root powder purchased from an authorised dealer in India. The roots were dried in the shade and ground into a fine powder. The powder was taken with milk. ROS concentration in the sperm cells was measured before and after treatment with WS using the DCFH-DA assay. WS offered protection against excess ROS production, as it significantly lowered the ROS concentration in all the test groups. Apoptosis was also measured before and after treatment using annexin –V binding with flow cytometry. WS treatment significantly reduced the incidence of apoptosis in almost all the groups. WS was also able to improve semen quality, which may have a positive effect on infertility (Shukla *et al*., 2011). These results are reflected in the present study, where WS provided significant total protection to the CELC against the oxidative damage induced by AAPH and indications are that the antioxidant components are absorbed from the gastrointestinal tract and, as for sperm, may also protect the lung tissue from oxidative damage.

Seleno-methionine

(A) Total protection

The total protective effect of Se-Met on the CELC against the oxidative damage induced by AAPH was investigated. The concentrations of Se-Met are summarized in Table 5.2. The cells were exposed to the lowest (0.167 pg/ml) and highest (33.33 pg/ml) concentrations of Se-Met in the absence of AAPH in order to measure the potential oxidative effect of Se-Met. A low level of cytotoxicity was observed, but there was no statistically significant difference between DCFH-DA alone (6%) and the two Se-Met controls (14% for 0.167 pg/ml and 7% for 33.33 pg/ml) (Figure 5.7A). The concentrations of Se-Met used in the DCFH-DA assays were higher than those used in the NR and CV assays, because the exposure time was shorter.

The concentrations of Se-Met used with AAPH showed significant decreases at 0.33 pg/ml Se-Met + AAPH, 3.33 pg/ml Se-Met + AAPH, 16.67 pg/ml Se-Met + AAPH and 33.33 pg/ml Se-Met + AAPH, compared to the control of AAPH (100% damage). All the readings showed a protective trend, but were not necessarily statistically significant. No dose-dependent effect was observed. The results obtained for total protection in the CELC were much better than those obtained for the SC-1 cells (Chapter 4), where Se-Met was shown to act as a pro-oxidant. The higher protection in the CELC may be attributed to the presence, although low, of SOD, CAT and GPx in the chick embryo.

(B) Intracellular protection

The intracellular protective effect of Se-Met against the oxidative damage induced by AAPH was measured using the same concentrations of Se-Met as for the total protection assay – a range from 0.167 pg/ml to 33.33 pg/ml. The CELC were exposed to the lowest (0.167 pg/ml) and highest (33.33 pg/ml) concentrations of Se-Met without AAPH in order to measure the potential oxidative effect of Se-Met. There was no statistically significant difference between DCFH-DA alone (12%) and the two Se-Met controls (13% for 0.167 pg/ml and 18% for 33.33 pg/ml) (Figure 5.7B). Once again, the concentrations of Se-Met used in the intracellular DCFH-DA assay were higher than those used in the NR and CV assays, because the exposure time was much less.

Most of the concentrations of Se-Met used in the presence of AAPH showed statistically significant decreases when compared to the control of AAPH (100%), indicating protection against the oxidative damage induced by AAPH. The three highest concentrations of Se-Met (3.33 pg/ml Se-Met + AAPH – 33.33 pg/ml Se-Met + AAPH) showed antioxidant properties in

the intracellular protection assay, while the lowest concentration (0.167 pg/ml Se-Met + AAPH) exhibited a slight pro-oxidant effect on the cells.

The ability of Se-Met to protect primary hippocampal and cortical neurons against β-Amyloid (Aβ) and iron/hydrogen peroxide (Fe^{2+}/H_2O_2)-mediated cell death was investigated by Xiong *et al*. in 2007. Both Aβ and Fe^{2+}/H_2O_2 play a central role in Alzheimer's disease. Cell survival was quantified by counting the number of undamaged neurons in a pre-marked microscopic field before (0 hours) and during treatment (3, 6, 9, and 16 hours), and by measuring the release of lactate dehydrogenase (LDH) into the medium. Se-Met at 1, 2 and 5 μM in combination with Fe^{2+}/H_2O_2 significantly increased neuron survival compared to Fe^{2+}/H_2O_2 treatment alone, and 1 and 2 μM Se-Met provided significant dose-dependent protection against Aβ, as observed under the microscope. Measuring LDH release, a sign of tissue breakdown, 1 μM Se-Met was shown to significantly protect the neurons after 9 hours. Xiong *et al*. (2007) also measured the effect of Se-Met on GPx activity. In neuron cultures pre-treated with Se-Met, GPx activity was significantly increased when compared to cultures treated with Fe^{2+}/H_2O_2 and Aβ alone. The authors concluded that Se-Met may be suitable as an antioxidant therapeutic agent in Alzheimer's disease.

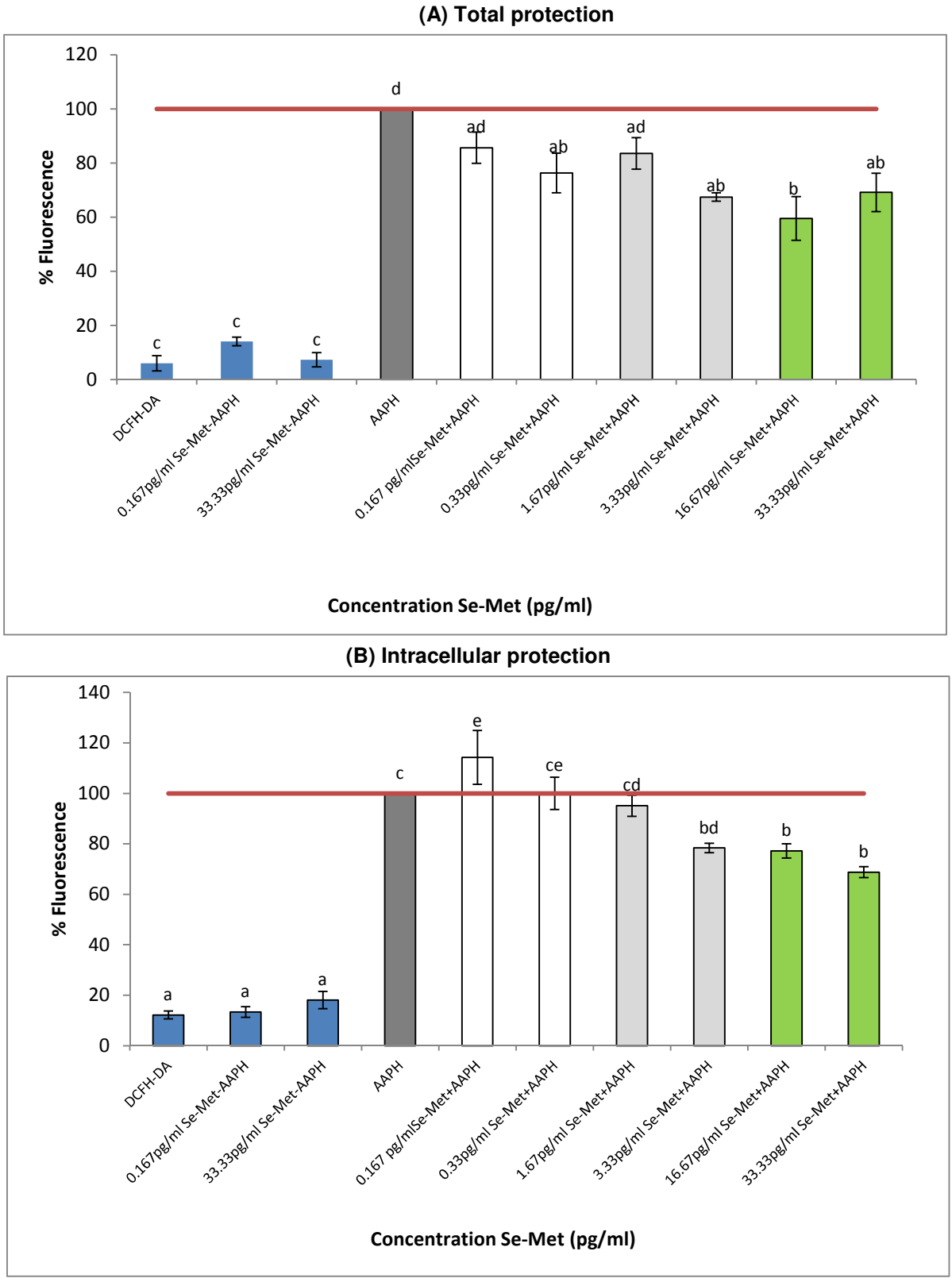


Figure 5.7: Total (A) and intracellular (B) effects of increasing concentrations of Se-Met on AAPH-induced oxidative damage in CELC. Data is expressed as a percentage of the damage caused by AAPH + DCFH-DA, 100% damage. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001. Red line = 100% damage due to AAPH.

***Withania somnifera* and seleno-methionine in combination**

(A) Total protection

WS and Se-Met were combined in order to test their ability to provide total protection for the CELC against oxidative damage induced by AAPH, and to investigate the potential for interaction between antioxidants. The concentrations used in this experiment are summarized in Table 5.2. The lowest (2.5 mg/ml WS + 0.125 pg/ml Se-Met) and highest (25 mg/ml WS + 25 pg/ml Se-Met) of these concentrations were used without AAPH to test for any possible oxidative effect of the products. The combinations without AAPH showed very low cytotoxicity, and there was no statistical significance between these results obtained (9% for 2.5 mg/ml WS + 0.125 pg/ml Se-Met and 17% for 25 mg/ml WS + 25 pg/ml Se-Met), and the results for DCFH-DA alone (11%). This validates results obtained in the cytotoxicity study. The lowest combination of WS and Se-Met (2.5 mg/ml WS + 0.125 pg/ml Se-Met) with AAPH showed a pro-oxidant effect, producing a result much higher than obtained for AAPH alone. This result was also much higher than the result obtained for 3.33 mg/ml WS in the presence of AAPH, 74% (Figure 5.5A), as well as for 0.167 and 0.33 pg/ml Se-Met with AAPH, 86% and 76% respectively (Figure 5.7A). This indicates a possible antagonistic interaction between WS and Se-Met. All the other concentration combinations of WS and Se-Met in the presence of AAPH produced a decrease when compared to AAPH alone, but only the highest concentration combination, 25 mg/ml WS + 25 pg/ml Se-Met, offered statistically significant protection, a 45% decrease in fluorescence compared to AAPH alone. With the exception of the lowest combination concentration, similar results to those seen with WS and Se-Met alone were obtained (Figure 5.8A).

(B) Intracellular protection

WS and Se-Met were also combined in order to test their ability to provide intracellular protection to the CELC against oxidative damage induced by AAPH. The concentrations of WS and Se-Met combined were the same as those used in the total protection assay (Table 5.2). The lowest (2.5 mg/ml WS + 0.125 pg/ml Se-Met) and highest (25 mg/ml WS + 25 pg/ml Se-Met) of these concentrations were used without AAPH to test for any possible oxidative effect. The combinations without AAPH showed low cytotoxic effects (10% for 2.5 mg/ml WS + 0.125 pg/ml Se-Met and 26% for 25 mg/ml WS + 25 pg/ml Se-Met), and, when compared to the control of DCFH-DA alone (14%), there was no statistical significance found. While all the combinations of WS and Se-Met showed a general protective effect, only the highest concentration (25 mg/ml WS + 25 pg/ml Se-Met) showed a statistically significant decrease when compared to AAPH alone (Figure 5.8B).

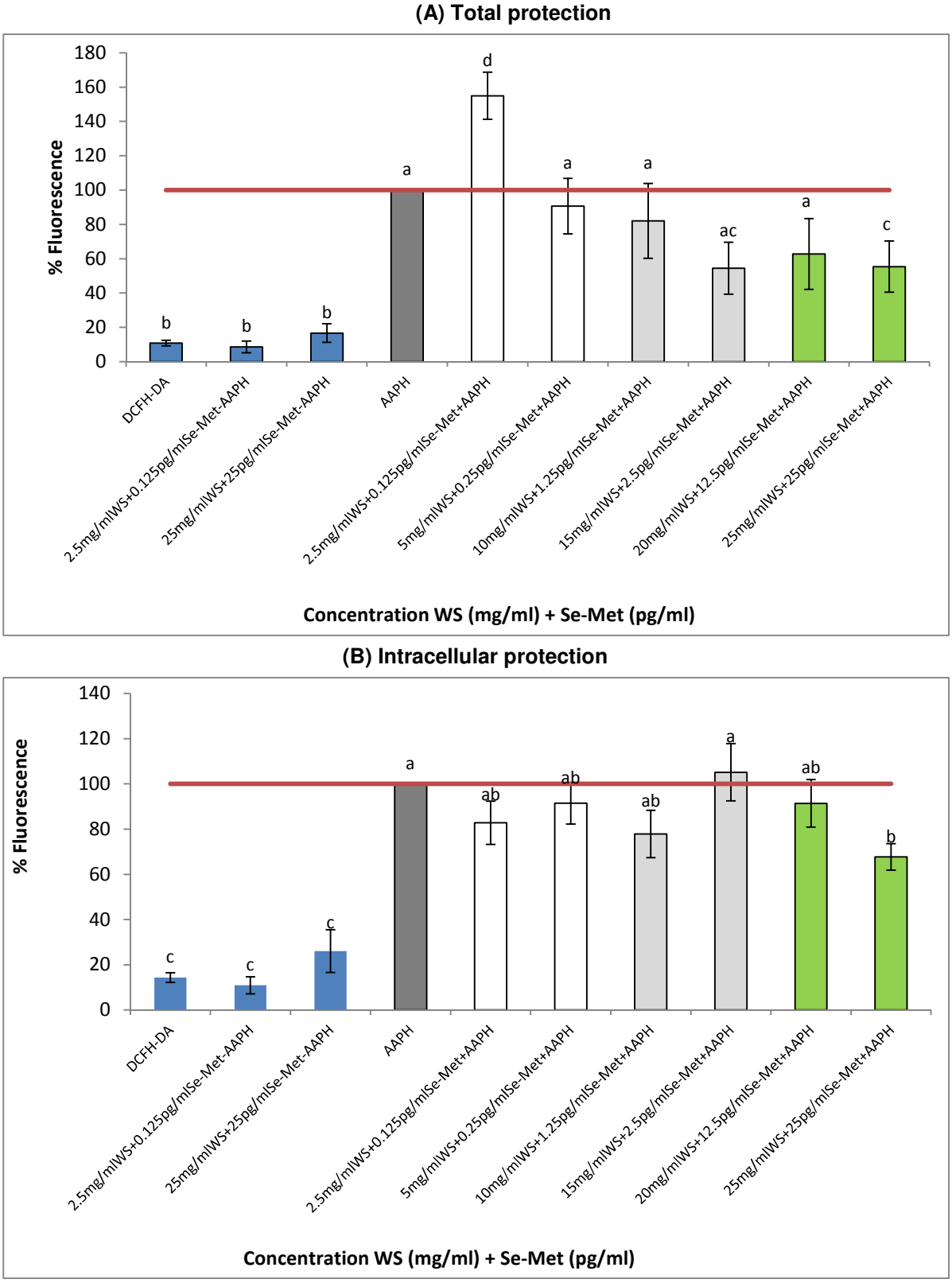


Figure 5.8: Total (A) and intracellular (B) effects of increasing concentrations of WS and Se-Met on AAPH-induced oxidative damage in CELC. Data is expressed as a percentage of the damage caused by AAPH + DCFH-DA, 100% damage. Average of 3 independent experiments, each assay point in quadruple. Data expressed as mean ± SEM. Means with different letters are significantly different, p≤0.001. Red line = 100% damage due to AAPH.

A summary of the ability of WS and Se-Met alone and in combination, to provide total and intracellular protection against AAPH-induced oxidative damage in CELC is shown in Table 5.5. All significant increases and decreases are shown, as compared to the damage caused by AAPH.

Table 5.5: Summary of total and intracellular protective effects of WS and Se-Met, alone and in combination, on CELC

WS (mg/ml)	3.33	6.67	13.33	20.00	26.69	33.33
Total	↓ **(AO)**	ns	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**
Intracellular	ns	ns	ns	ns	ns	↓ **(AO)**

Se-Met (pg/ml)	0.167	0.33	1.67	3.33	16.7	33.3
Total	ns	↓ **(AO)**	ns	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**
Intracellular	↑(PO)	ns	ns	↓ **(AO)**	↓ **(AO)**	↓ **(AO)**

WS + Se-Met	2.5+0.125	5.0+1.25	10+12.5	15+0.25	20+2.5	25+25
Total	↑ (PO)	ns	ns	ns	ns	↓ **(AO)**
Intracellular	ns	ns	ns	ns	ns	↓ **(AO)**

When comparing the results obtained for the SC-1 cells (Chapter 4) and the CELC, similar antioxidant effects are observed in the total protection assay for WS alone at higher concentrations; in the intracellular protection assay for Se-Met alone at higher concentrations; and in both the total and intracellular protection assays at the highest concentration ratio for WS and Se-Met in combination.

Interactions between *Withania somnifera* and seleno-methionine

(A) Total protection

The results obtained for the total protective effects of WS and Se-Met alone on the CELC were used to calculate the expected combinational effects between WS and Se-Met. A curve fit was applied to both graphs, and using the equations of these trend lines, expected values were calculated. The equation of the polynomial trend line obtained using the WS values (Figure 5.6A) was $y=-0.029x^2+0.5106x+69.609$, $R^2=0.90$. The equation of the polynomial trendline obtained using the Se-Met data (Figure 5.8A) was $y=0.0629x^2–2.4625x+81.678$, $R^2=0.76$.

Using these equations, the y-values were calculated for both WS and Se-Met, which were then added together and divided by two. The calculated result represents the expected total

protection by WS and Se-Met in combination. The interaction index (*II*) was calculated using the following equation:

$$II = {}^{EP}/_{OP}$$

EP represents the expected/calculated protection of the combination of WS and Se-Met and OP represents the actual/observed protection of the combination of WS and Se-Met (Figure 5.9A).

The results obtained are illustrated in Table 5.6. Antagonistic effects were observed at the two lowest combinations of WS and Se-Met concentrations, weak synergistic effects observed at the middle two concentration combinations, at all concentration combinations of WS and Se-Met, with increased synergism occurring with the increase in concentration of WS and Se-Met.

Synergistic antioxidant effects are observed at 13.33 mg/ml WS + 1.67 pg/ml Se-Met, 20 mg/ml WS + Se-Met + 3.33 pg/ml Se-Met and 33 mg/ml WS + 33.33 mg/ml Se-Met. These results are similar to those seen for total protection in the SC-1 cells (Chapter 4), except for the antagonistic effect seen at 26.69 mg/ml WS + 16.67 pg/ml Se-Met. This result may be due to a plating effect, or experimental error.

Table 5.6: TOTAL EFFECT: Expected and observed combinational effects of WS and Se-Met, CELC

Combination		% Damage		Interactions	
WS (mg/ml)	**Se-Met (pg/ml) (nM)**	**Expected**	**Observed**	***II***	**Effect**
3.33	0.167 ($8.56x10^{-4}$)	76.12	154.95±13.70	0.49	Antagonistic
6.67	0.33 ($1.68x10^{-3}$)	76.28	90.61±16.15	0.84	Antagonistic
13.33	1.67 ($8.56x10^{-3}$)	74.46	82.08±21.84	0.91	**Synergistic**
20.00	3.33 ($1.68x10^{-2}$)	71.10	54.47±15.10	1.31	**Synergistic**
26.69	16.67 ($8.56x10^{-2}$)	60.18	62.76±20.62	0.96	Antagonistic
33.33	33.33 ($1.68x10^{-1}$)	61.70	55.37±14.88	1.11	**Synergistic**

(B) Intracellular protection

The results of the intracellular protection of WS and Se-Met alone were used to calculate the expected interaction between WS and Se-Met in combination. The equation of the polynomial trend line obtained using the WS values (Figure 5.5B) was $y=0.0082x^3+0.4228x^2–6.1054x+115.57$, $R^2=0.85$. The equation of the polynomial trend line obtained using the Se-Met data (Figure 5.7B) was $y=0.0503x^2–2.619x+101.43$, $R^2=0.70$.

Using the equations above, the y-values were calculated, and the values obtained for both WS and Se-Met were added together and divided by two, providing the expected intracellular protection by WS + Se-Met. The interaction index (*II*) was calculated using the observed values (Figure 5.8B) and the results are summarized in Table 5.7.

Synergistic antioxidant effects were observed at the three lowest concentration combinations, an additive effect at 20.00 mg/ml WS + 3.33 pg/ml Se-Met, and antagonistic effects at the two highest combinations of WS and Se-Met. At the highest concentration combination 33.33 mg/ml WS + 33.33 pg/ml Se-Met the interaction was extremely antagonistic.

Table 5.7: INTRACELLULAR EFFECT: Expected and observed combinational effects of WS and Se-Met, CELC

Combination		% Damage		Interactions	
WS (mg/ml)	**Se-Met (pg/ml) (nM)**	**Expected**	**Observed**	***II***	**Effect**
3.33	0.167 ($8.56x10^{-4}$)	102.37	82.80±9.55	1.24	**Synergistic**
6.67	0.33 ($1.68x10^{-3}$)	102.87	91.48±9.20	1.12	**Synergistic**
13.33	1.67 ($8.56x10^{-3}$)	111.68	77.85±10.45	1.43	**Synergistic**
20.00	3.33 ($1.68x10^{-2}$)	114.08	105.12±12.62	1.09	Additive
26.69	16.67 ($8.56x10^{-2}$)	79.50	91.40±10.52	0.87	Antagonistic
33.33	33.33 ($1.68x10^{-1}$)	5.30	67.77±5.86	0.08	Antagonistic

Comparison between interactions in SC-1 cells and CELC

The interactions between WS and Se-Met in the SC-1 fibroblast cell line and the CELC are compared in Table 5.8. Similar interactions are observed for the total protection assay at 13.33 mg/ml WS + 1.67 pg/ml Se-Met, 20 mg/ml WS + 3.33 pg/ml Se-Met and 33.33 mg/ml WS + 33.33 pg/ml Se-Met.

Table 5.8: Comparison of total and intracellular interactions in SC-1 and CELC

Combination		SC-1		CELC	
WS (mg/ml)	**Se-Met (pg/ml) (nM)**	**Total**	**Intracellular**	**Total**	**Intracellular**
3.33	0.167 ($8.56x10^{-4}$)	**Synergistic**	Additive	Antagonistic	**Synergistic**
6.67	0.33 ($1.68x10^{-3}$)	**Synergistic**	Additive	Antagonistic	**Synergistic**
13.33	1.67 ($8.56x10^{-3}$)	**Synergistic**	Additive	**Synergistic**	**Synergistic**
20.00	3.33 ($1.68x10^{-2}$)	**Synergistic**	Antagonistic	**Synergistic**	Additive
26.69	16.67 ($8.56x10^{-2}$)	**Synergistic**	**Synergistic**	Antagonistic	Antagonistic
33.33	33.33 ($1.68x10^{-1}$)	**Synergistic**	Additive	**Synergistic**	Antagonistic

BOLD = beneficial antioxidant effects, RED = similar effects between SC-1 and CELC

5.5 Summary

CELC, a physiologically relevant cell culture model was established to investigate the cytotoxic and antioxidant effects of WS and Se-Met alone and in combination. WS was cytotoxic at 0.25 mg/ml (NR assay) and 9.09 mg/ml (NR and CV assays). Se-Met was not cytotoxic at any concentrations evaluated, but a mitogenic effect was observed at 0.01pg/ml (NR assay) and 0.02 pg/ml (NR and CV assays). Microscopy revealed cell vacuolation following exposure to WS. Se-Met showed no toxicity and vacuolation of CELC was reduced when exposed to WS in combination with Se-Met.

Significant antioxidant protective effects against AAPH-induced oxidative damage were observed for WS and Se-Met, alone and in combination, in the CELC. Dosage-related total antioxidant effects were observed for WS (13.33 – 33.33 mg/ml). For Se-Met, an intracellular dosage-related effect was observed from 3.33 – 33.33 pg/ml / $8.56x10^{-4}$ - $1.68x10^{-1}$ nM. At a combination of 25 mg/ml WS and 25 pg/ml Se-Met both total and intracellular antioxidant effects were observed. Antagonism between WS and Se-Met was observed at low concentrations in the total protection assay, with weak synergism and antagonism occurring at higher concentrations. Synergism between WS and Se-Met was observed at low concentrations intracellularly, with antagonistic interactions occurring at higher concentrations.

Chapter 6: Concluding discussion

Rationale for the study

Oxidative damage is associated with several lifestyle diseases and related to the respiratory system, a primary target of oxidative damage, causing inflammatory conditions like allergies, asthma and emphysema. The ability of the body to counteract oxidative elements is dependent on the oxidative status of the individual. A deficiency of Se will result in decreased antioxidant enzymes levels, e.g., GPx, and a poor response to oxidative damage.

Medicinal plants are often used to treat respiratory disorders often associated with poor antioxidant status, and such plants include WS. WS is reported to possess antioxidant, anti-inflammatory, antitumour, antistress, immunostimulatory, haemopoetic and rejuvenating properties. It is reported to have a positive effect on the endocrine, cardiopulmonary and central nervous systems.

Various strategies are used to evaluate the properties of medicinal plants. The isolation and characterisation of the major compounds provide very little information specifically related to the activity, efficacy and possible toxicity of the extracts as traditionally used. In this study, a water infusion or decoction of WS from the roots, stems and leaves was used. Interactions between antioxidants can occur between polyphenolics in a medicinal plant extract and with other antioxidants, for example vitamins and/or enzymatic antioxidants, glutathione peroxidase (GPx) (containing Se-Met). Three types of interactions can occur and are additive, synergistic and antagonistic.

Summary of results

The cellular effects of WS and Se-Met, alone and in combination, on the SC-1 cell line and CELC were investigated. Little or no cytotoxicity was observed using the NR and CV assays, and therefore the antioxidant content and properties of WS and Se-Met, alone and in combination, was evaluated.

Water extracts of WS (3.13 – 100 mg/ml), as prepared for traditional medicinal purposes, were found to contain polyphenolics and flavonoids, which translated into antioxidant activity that was quantified using the TEAC and ORAC assays. As expected, Se-Met did not show antioxidant activity using the abovementioned assays, and was used as a negative control.

The ability of WS and Se-Met, alone and in combination, to protect the SC-1 cells and the CELC against AAPH-induced oxidative damage was investigated using the DCFH-DA assay, a more physiologically relevant assay.

In the SC-1 cell line, WS provided total protection at high concentrations and intracellular protection at all concentrations. Se-Met provided only intracellular protection to the SC-1 cells, and exerted pro-oxidant effects in the total protection assay. For the combination of WS and Se-Met, similar results were observed as for WS alone. Synergism between WS and Se-Met was observed at all concentrations in the total protection assay, with mostly additive effects occurring intracellularly.

Using the CELC to investigate the ability of WS and Se-Met, alone and in combination, to provide protection against oxidative damage, WS provide total protection at all concentrations, in contrast to the results obtained with the SC-1 cells, and showed intracellular antioxidant activity at higher concentrations. Se-Met protected the SC-1 cells at low concentrations in the total protection assay, but showed pro-oxidant effects at higher concentrations. Intracellularly, Se-Met exhibited pro-oxidant effects at low concentrations, and antioxidant effects at higher concentrations. The combination of WS and Se-Met provided total and intracellular protection to the CELC at the highest concentration combination. Antagonism between WS and Se-Met was observed at low concentrations in the total protection assay, with weak synergism and antagonism occurring at higher concentrations. Synergism between WS and Se-Met was observed at low concentrations intracellularly, with antagonistic interactions occurring at higher concentrations.

The results demonstrate that protection is cell specific, and dependent on the concentration of the individual components, as well as the ratio at which the combinations are used.

Hypothesis 1:

Water extracts of WS (3.13 – 100 mg/ml), as prepared for traditional medicinal purposes, were shown to contain polyphenolics and flavonoids, which translated into significant antioxidant activity that was accurately quantified using the TEAC and ORAC assays. The DPPH assay provided tentative information on antioxidant activity in this study, because the µM TE/g and IC_{50} were calculated from data where decolouration was not complete.

Hypothesis 2:

WS and Se-Met, alone and in combination at non-cytotoxic concentrations, displayed significant cellular protection against oxidative damage and this was a function of cell line, concentration and ratios between WS and Se-Met.

Hypothesis 3:

For the SC-1 cells, synergistic antioxidant effects were observed in the total protection assay at higher WS:Se-Met ratios. Intracellularly, a synergistic antioxidant effect was only observed at 26.69 mg/ml WS + 16.67 pg/ml Se-Met (8 WS:20 Se-Met). For the CELC, similar synergistic antioxidant total effects were observed as for the SC-1 cells. Intracellularly, synergistic antioxidant effects were observed at lower WS:Se-Met ratios.

Limitations of the study

Cell-based studies provide an indication of effects but do not take absorption, distribution, metabolism and excretion into account, which may have an effect on the antioxidant potential of the plant extract in the lungs.

Although polyphenolics of WS have been identified, factors such as the geographical region where samples are collected and the season (summer vs. winter, dry vs. wet) will have an impact on the polyphenolic content of WS. The Se content of the soil will also have an effect on WS, as plants that have been enriched in Se by fertilization with selenite have exhibited higher antioxidant activity. WS with a higher Se status will influence the results obtained in the combination assays. It is important to identify and quantify the specific polyphenolic content of WS that was sourced in Gauteng, South Africa.

Cytotoxicity was evaluated for WS and Se-Met alone only, and not in combination. Antagonistic effects may occur as was observed with the microscopic evaluation of SC-1 and CELC cultures. This is a major limitation related to the cytotoxicity studies.

Firstly, the limitations identified above should be addressed, namely identification of the bioactive polyphenolics with HPLC-MS. Measurement of Se-Met-associated enzyme activity and lastly the further, detailed evaluation of toxicity.

For the evaluation of antioxidant activity, chick embryo lung primary cultures were used from the vascular period of development, which corresponds to the canalicular period of mammalian development (17 – 27 weeks). However, this stage does not represent fully differentiated phenotypes. The cellular content of Se-Met-dependent enzymes is unknown,

and this information would have assisted with the interpretation of the observed cellular antioxidant effects in both the SC-1 and CELC cultures.

The use of a positive control, for example, N-acetyl cysteine a physiologically relevant antioxidant, in the antioxidant assays, namely DPPH, TEAC and ORAC would have provided a better basis for comparison of results.

Cell death was evaluated solely by the observation of morphological changes using phase contrast and light microscopy. The use of fluorescence-activated cell sorting (FACS) using propidium iodide or Annexin V markers, or even Hoechst staining of the nucleic acids to be evaluated by fluorescence microscopy or flow cytometry would have provided a better understanding of the method of cell death.

Future directions

The effects of digestion on WS can be evaluated using a simulated *in vitro* digestion model, where antioxidant activity is evaluated following each stage of digestion. Antioxidant activity can be assessed in cell lines representing the stomach, liver and colon. Once the molecule/s responsible for antioxidant effects have been identified using HPLC-MS (high performance liquid chromatography mass spectrometry), animal studies can be undertaken where levels of these antioxidants can be measured in the liver, lungs and blood. Using an animal model, e.g., BALB/c mice (often used in basic, non-clinical research) on a selenium-deficient diet, it would be possible to evaluate the health benefits of WS alone and in combination with Se-Met in greater detail.

Oxidative damage is not limited to the respiratory system, and can affect other organ systems such as the central nervous system and gastrointestinal tract. The antioxidant effect of WS and Se-Met, alone and in combination, on other cell lines should be considered. For example, the HS683 cell line (human brain fibroblasts) could be used to test for cytotoxicity in the central nervous system, and Caco-2 (heterogeneous human epithelial colorectal adenocarcinoma cells) or HT29 (human colon colorectal adenocarcinoma cells) permanent cell lines could be used for the gastrointestinal tract. The identification of specific antioxidants within WS will allow for retesting in the cell systems.

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