THE EFFECTS OF *TULBAGHIA VIOLACEA* (WILD GARLIC) LEAF AND BULB EXTRACTS ON AN OESOPHAGEAL CANCER CELL LINE (SNO)

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ABSTRACT

***Ethnopharmacological relevance*:** Indigenous plants such as *Tulbaghia violacea*(TV) and *Allium sativum* (garlic) are traditionally used as natural remedies to treat a variety of ailments, including cancer. This study investigated the effects of TV leaf and bulb extracts and garlic extract on a cancerous oesophageal cell line (SNO).

***Materials and methods*:** The methylthiazoltetrazolium (MTT) assay was used to determine the IC_{50} of TV leaf (TVL) (250µg/ml) and TV bulb extracts (TVB) (25µg/ml) and garlic (500µg/ml). Extracts were treated individually and in combination for a period of 24 hours. Oxidative damage and intracellular glutathione levels were assessed using the Thiobarbituric Acid Reactive Substances (TBARS) Assay and GSH-Glo™ Luminometry Assay, respectively. The CellTiter-Glo® Luminescent Cell Viability Assay was used to assess ATP activity. Induction of apoptosis and mitochondrial membrane potential were determined via the Caspase-Glo® 3/7 Assay, Caspase-Glo® 8 Assay, Caspase-Glo® 9 Assay and JC-1 Mitoscreen Assay, respectively. Morphological apoptotic changes were determined using the Hoechst 33342 stain. Expressions of p53, PARP and NF_KB activities were determined by western blotting.

***Results*:** Bulb and leaf extracts of TV increased lipid peroxidation compared to the control (p >0.05), whilst garlic and combination of TV leaf and bulb (TVB + TVL) extracts significantly decreased lipid peroxidation relative to the control (p< 0.05). Endogenous glutathione levels significantly decreased in all TV treatments compared to the control (p<0.05).However, garlic was accompanied by insignificantly increased intracellular glutathione levels compared to the control (p> 0.05). The percentages of depolarised mitochondria in all treated cells were significantly decreased compared to untreated cells (p< 0.05).ATP levels increased significantly in garlic and combination (TVB + TVL) treated cells as compared to the control (p< 0.05), yet no significant

differences were noted in TVL and TVB treatments (p> 0.05). Caspase8 and caspase 9 activitiessignificantly increased in garlic and combination treated cells relative to the control (p< 0.05). A similar trend was noted for caspase 3/7 activity in garlic and combination treatments (p< 0.05). However, initiator and executioner activities in TVL (p> 0.05) and TVB (p> 0.05) treatments did not significantly differ from the control (p> 0.05). All treatments (including garlic) resulted in increased DNA fragmentation and condensation. All treatments decreased p53 expression (p< 0.05), PARP expression (p< 0.05) and NF_KB expression (p>0.05) compared to the control.

***Conclusions*:** All TV extracts and garlic induces apoptosis in the oesophageal cancerous SNO cell line through changes in oxidative stress, antioxidant systems, and nuclear chromatin condensation, as well as through induction of nuclear genes and signalling pathways. Since inhibition of apoptosis is a principal alteration in cancer, induction of apoptosis would result in a decrease in cancer cell growth. Thus, TV could be exploited as a potential anti-cancer agent.

KEYWORDS

T.violacea; SNO cells; garlic; lipid peroxidation; apoptosis

DECLARATION

I hereby declare that this dissertation is entirely my own work and no part of this dissertation has been submitted to any other institution.

The research described was carried out in the Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, College of Health Science, University of KwaZulu-Natal, Durban under the supervision of Ms RB Myburg and Ms MR Serumula.

SIGNATURE: _____

Ms S Moonsamy

DATE: _____

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LIST OF ABBREVIATIONS

ABBREVIATIONS	COMPLETE NAMES
%:	Percentage
°C:	Degrees Celsius
ACE:	Angiotensin-converting enzyme
AGE:	Aged Garlic Extracts
Apaf-1	Apoptotic-Inducing Factor 1
ATP	Adenosine Triphosphate
BCA:	Bicinchoninic Acid Assay
BSA:	Bovine Serum Albumin
CCM:	Complete Culture Medium
CDK	Cyclin-dependent Kinase
$-CH_2-$	Methylene Group
Cu^{+}:	Monovalent Copper Ion
Cu^{2+}:	Divalent Copper Ion
$CuSO_4$:	Copper Sulphate
cm^3:	Cubic Centimeters
DADS:	Diallyldisulphide
DAS:	Diallylsulphide
DMSO:	Dimethyl Sulphoxide
ECL:	Chemiluminescent Substrate
EDTA:	Ethylenediaminetetraacetic Acid
EMEM:	Eagles Minimum Essential Medium
ELISA:	Enzyme-linked Immunosorbent Assay
FACS:	Fluorescence-Activated Cell Sorting
FADD	Fas-Associated Death Domain
GPx	Glutathione Peroxidase
GSH:	Glutathione
HRP:	Horse-radish Peroxide
hr (s):	Hour(s)

H_2	Hydrogen Atom
H_20	Water
H_2O_2	Hydrogen Peroxide
H_3PO_4:	Phosphoric Acid
IC_{50}:	Half Maximal Inhibitory Concentration
kDa:	Kilodaltons
KZN:	KwaZulu-Natal
LMPA:	Low Melting Point Agarose
LOO^{--}	Lipid Peroxyl Radical
M:	Molar
MDA:	Malonaldehyde
mg:	Milligrams
ml:	Milliliters
mg/ml:	Milligrams per Milliliter
µg/ml:	Micrograms per Milliliter
µl:	Microlitre
µM:	Micromolar
mM:	Millimolar
mins:	Minutes
m.s.:	Millisecond
MTT:	Methylthiazoltetrazolium
NF_KB:	Nuclear Factor Kappa B
n:	Number of Observations
nm:	Nanometers
NaCl:	Sodium chloride
Na_2EDTA:	DiSodiumEthylenediaminetetraacetate
NaOH:	Sodium Hydroxide
OC:	Oesophageal Cancer
O_2	Oxygen
O_2-	Superxide anion
p</>:	Probability less than/greater than
PARP:	Poly (ADP-ribose) Polymerase
PBS:	Phosphate Buffered Saline Solution

PFA:	Paraformaldehyde
PI:	Protease Inhibitor
RLU:	Relative Light Units
RNS	Reactive Nitrogen Species
ROS:	Reactive Oxygen Species
rpm:	Reserves per Minute
SA	South Africa
SAC:	S-allyl-L-Cysteine
SDS-PAGE:	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
secs:	Seconds
SH:	Sulfhydryl
SNO:	Human Oesophageal Cancerous Cell Line
SOD	Superoxide Dismutase
TBARS Assay	Thiobarbituric Acid Reactive Substances Assay
TBA/BHT:	Thiobarbituric Acid (1%, w/v) / 0.1mM Butylatedhydroxytoluene
TNF-α	Tumour Necrosis Factor- α
TRIS:	Tris(hydroxymethyl)aminomethane
TTBS:	Tween 20-Tris Buffered Saline
TV*:*	*Tulbaghiaviolacea*
TVB:	*Tulbaghiaviolacea*bulb
TVL:	*Tulbaghiaviolacea*leaf
USA:	United States of America
V:	Volts/voltage

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CHAPTER 1

INTRODUCTION

Oesophageal cancer (OC) is a commonly occurring malignancy on the eastern seaboard of South Africa (SA) that is associated with high mortality and morbidity rates (Aldina *et al*., 2004). Globally, OC is eighth with 316 000 new cases diagnosed annually and with an associated mortality of being ranked sixth (Aldina *et al*., 2004). There are geographical differences that exist for OC, where Asia and Sub-Saharan Africa have the highest incidence of OC (Jemal *et al*., 2010). The incident rate of OC in Black South African males is associated with dietary exposure to *Fumonisin* B_1-containing foods (Mqoco *et al*., 2010). Additional risk factors include excessive smoking and alcohol consumption (Myburg *et al*., 2002). The incident rate is higher in males than in females for OC (Jemal *et al*., 2010).

Conventional treatment refers to a common form of therapy that is widely used by patients worldwide (Style, 2007). This form of treatment has been differed away from due to the number of side effects that are associated with this form of therapy. The observed side effects such as vomiting, diarrhoea, headaches, abdominal cramps etc. are worsened by this form of therapy. Several studies have reported that cancer patients themselves have declined the use of conventional therapy and instead opted for complementary and alternative medicine (CAM) (Verhoef *et al*., 2008). Declining conventional therapy is not only based on medical factors, but to a greater extent emphasis is based upon personal factors (Verhoef *et al*., 2008). The global burden associated with cancer demonstrates a need for safer alternative treatment strategies (Sharma *et al*., 2010). In addition, a trend has developed where cancer patients prefer novel treatments such as traditional medicine or traditional healers (Galabuzi *et al*.,

2010). These medicinal herbs used by traditional healers possess an array of functions, such as anti-bacterial, anti-fungal, anti-platelet, hypoglycaemic, anti-atherosclerotic, anti-cancer and chemoprotective properties (Amagase *et al.*, 2001; Amagase, 2006; Khanum *et al*., 2004; Maeola, 2005; Omar and Al-Wabel, 2010; Ncube *et al*., 2011).

For decades, *Allium sativum* commonly known as garlic, has been used as both flavouring in food preparations and exploited for its potential health benefits in both *in vivo* and *in vitro* studies (Shukla and Kalra, 2007; Omar and Al-Wabel, 2010).Numerous studies have demonstrated that garlic and its constituents actively inhibit tumour growth both *in vivo* and *in vitro* (Bungu *et al*., 2006; Shukla and Kalra, 2007; Omar and Al-Wabel, 2010).Another medicinal herb belonging to the *Alliaceae* family, viz. *Tulbaghia violacea*(TV), has been exploited by the Zulu as a tonic that is effective against a myriad of common ailments such as influenza and fevers, tuberculosis, infections, asthma and eradicate intestinal problems such as bloating or intestinal worms (Bungu *et al*., 2006; 2008, Street, 2008). It been hypothesised that TV has similar biological activities and compounds as domesticated garlic (Bungu *et al*., 2006). Organosulphurous compounds of a thiosulfate nature found in both TV and garlic extracts are responsible for most of the conferred medicinal properties of these plant extracts (Maeola, 2005; Bungu *et al*., 2006; Omar and Al-Wabel, 2010; Van Huyssteen *et al*., 2011). Specifically, the anti-proliferative effects conferred by garlic and TV extracts may be attributed to organosulphurous compounds such as ajoene, allicin, diallyl disulfide (DADS) and diallyl sulfide (DAS) (Bungu *et al*., 2006; Omar and Al-Wabel, 2010).

It is thought that garlic exerts its anti-carcinogenic effect through various mechanisms, such as the induction of apoptosis, increasing glutathione (GSH) levels to detoxify free-radicals

and effective DNA repair mechanisms (Khanum *et al*., 2004).Apoptosis is the principle mechanism used by several anti-cancer agents (Bungu *et al*., 2006; Shukla and Kalra, 2007; Omar and Al-Wabel, 2010). Allicin has been shown to induce apoptosis in various cancerous cell lines by caspase-dependent (Omar and Al-Wabel, 2010) and caspase-independent (Park *et al*., 2004) mechanisms. Other hallmarks of apoptosis occurring in TV-treated cancer cell lines included condensation of chromatin and fragmented DNA (Bungu *et al*., 2004; 2006).

Several *in vivo* and *in vitro* studies have reported that garlic and its constituents are actively anti-proliferative (Shukla and Kalra, 2007; Omar and Al-Wabel, 2010). Indeed, TV-extracts have been used by the ethnic population as a remedy against cancer (Bungu *et al*., 2006). The present study differs from other studies in terms of the parameters investigated. The SNO cell line and both TV leaf and bulb in crude extracts were used. However, the molecular mechanisms remain unknown. Therefore, this study investigated the effects of garlic, TV leaf and TV bulb extracts individually and in combination on an oesophageal cancer cell line (SNO). The SNO cell line is a well-differentiated squamous cell carcinoma originally derived from a 62-year-old patient, Mr. SN (Myburg *et al*., 2002).

Objectives

- To determine the cytotoxicity and IC_{50} (half maximal inhibitory concentration) of garlic and TV leaf and bulb extracts on SNO cells using the methylthiazoltetrazolium (MTT) assay.

- To investigate the effect of TV extracts and garlic on mitochondrial membrane potential in the SNO cells using the JC-1 mitoscreen assay.

- To evaluate whether TV extracts and garlic alters the metabolic active state of the SNO cells in terms of cell viability using the CellTitre-Glo® cell viability assay.

- To determine the effect of TV and garlic extracts on lipid peroxidation in SNO cells as indicated by the levels of malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) assay.

- To determine whether TV leaf and bulb extracts and garlic alters intracellular glutathione (GSH) levels in SNO cells using the GSH-Glo™ luminometry assay.

- To assess the effect of TV extracts and garlic on initiator caspase 8 and caspase 9 activities in SNO cells using the Caspase-Glo® 8 assay and Caspase-Glo® 9 assay, respectively.

- To examine the effect of TV leaf and bulb extracts and garlic on executioner caspase 3/7 activity in SNO cells using the Caspase-Glo® 3/7 assay.

- To evaluate whether TV extracts and garlic alters protein expression levels such as p53, poly (ADP-ribose) polymerase (PARP) and nuclear factor kappa B (NF_KB) protein expressions in SNO cells by using western blotting.

- To investigate the effect of garlic and TV extracts on the integrity of DNA in SNO cells using the Hoechst 33342 stain.

CHAPTER 2

LITERATURE REVIEW

2.1. Medicinal plants

2.1.1. Traditional medicinal plants

Traditional medicine is defined as "health practices, beliefs, approaches and knowledge that incorporates plant, animal, and mineral based medicines, spiritual based theories, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being" (Galabuzi *et al*., 2010). Many developed and developing countries including Africa use traditional medicine as the primary basis for providing healthcare (Galabuzi *et al*., 2010). For instance, atleast 80% of African populations are inclined to utilise traditional medicine for healthcare requirements (Kassaye *et al*., 2006).

In both modern and traditional medicine, medicinal plants are considered as healing agents in helping to cure various ailments (Galabuzi *et al*., 2010). Medicinal plants are used readily by the poorly developed communities in rural areas, because the individuals in these areas lack access to proper health care facilities. This may be attributed to the fact that the communities are very impoverished, transportation is not readily available or expensive and sometimes the nearest clinic might be many miles away (Elubjoba *et al*., 2005). Therefore, these communities rely heavily on traditional medicine as their primary source of health care, since the availability of traditional healers and a variety of medicinal plants are found at their

disposal. To these inhabitants, these plants offer low cost maintenance and are readily available (Elubjoba *et al*., 2005). For example, 80% of the population in Ethiopia rely on traditional medicine, because of the numerous traditional healers that can cultivate “miracle cures” from the medicinal plants at their disposal (Kassaye *et al*., 2006).

Medicinal plants have been imperative to the field of pharmacology and drug development, because the natural compounds in medicinal plants have been used in developing drugs or used as possible models in the research (Kassaye *et al*., 2006; Galabuzi *et al*., 2010).

2.1.2.Somecommonly used medicinal plants in South Africa

In South Africa, some of the commonly used medicinal plants include *Allium sativum, Tulbaghia violacea (TV), Sutherlandia frutescens and Moringa oleifera. Sutherlandia frutescens* has been used to treat various ailments including internal cancers (Phulukdaree *et al*., 2010). The phytochemicals are found in the leaves of *S.frutescens* and these include L-canavanine and D-pinitol (Phulukdaree *et al*., 2010). The anti-diabetic and anti-cancer properties of *S.frutescens* may be attributed to the presence of D-pinitol (Phulukdaree *et al*., 2010). *Moringa oleifera*, known as “drumstick herbs”, is considered a healthy food-based therapeutic herb amongst humans (Fahey, 2005; Cajuday*et al*., 2010). The potent antioxidant properties conferred by *M.oleifera* are attributable to antioxidant pigments such as carotenoids, beta-carotene and xanthine (Fahey, 2005; Cajuday*et al*., 2010). In addition, kampherol and quercetin may also contribute to strong antioxidant effects of *M.oleifera* (Fahey, 2005; Cajuday*et al*., 2010).

2.2. Allium sativum

Allium sativum, commonly known as garlic (Figure 1), has been used for years in the preparations of food and has been studied extensively for its potential health benefits in both *vivo and in vitro* studies (Shukla and Kalra, 2007; Omar and Al-Wabel, 2010). This medicinal herb possesses an array of functions, such as anti-bacterial, anti-fungal, anti-platelet, hypoglycaemic, anti-atherosclerotic, anti-cancer, antioxidant and chemoprotective properties (Amagase *et al*., 2001; Amagase, 2006; Khanum *et al*., 2004; Maeola, 2005; Omar and Al-Wabel, 2010; Ncube *et al*., 2011). Experimental studies have revealed that of the variety of garlic preparations, aged garlic extracts (AGE) possess neuroprotective, hepatoprotective and antioxidant functions, adding to the vastness of garlic's biological activities (Amagase *et al*., 2001).

Figure 1: *Allium sativum* in its purist form (Girilal, 2006).

In ancient times, Egyptians consumed excessive amounts of garlic to enhance strength (Lanzotti, 2006), whereas Greeks and Romans used garlic as a form of therapy for skin diseases, breathing problems and intestinal infections (Amagase *et al*., 2001; Lanzotti, 2006).

To date, garlic has gained popularity as an excellent “disease-preventive food” source (Amagase *et al*., 2001). Clinical studies have reported that various garlic preparations have been used to treat respiratory diseases, enhance immune and antioxidant defences, and reduce cardiovascular and hypertension(Amagase *et al*., 2001; Kemper, 2008).It has long been realised that subjecting a particular food source to extraction aids in eliminating undesirable characteristics and thus enhancing its effectiveness (Amagase, 2006).

2.3. Chemistry

Garlic has a complex and diverse chemical background (Kemper, 2008). This structural complexity of garlic possibly developed as a self-defence mechanism used to eradicate intoxicating and non-intoxicating insults (Amagase, 2006). Garlic in its purest form(Figure 1) is comprised of two non-volatile sulphur-containing compounds called gamma (¥)-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (Omar and Al-Wabel, 2010). Alliin (Figure 2),chemically known as S-methyl-L-cysteine (Khanum *et al*., 2004),is derived from S-allyl-L-cysteine sulfoxides and this compound is stored garlic bulbs (Amagase *et al*., 2001). Alliin is a sulfoxide, which is situated on the amino acid cysteine and contains four stereoisomers, but only one of these is present in garlic (Omar and Al-Wabel, 2010).

2.3.1. Organosulphurous thiosulfinate composition in garlic

When garlic is crushed or cut, sulfoxides are converted into thiosulfinates (Amagase *et al*., 2001; Lanzotti, 2006). Alliin undergoes enzymatic transformation (Figure 2) into thiosulfinates such as organosulphurous compounds (OSCs) and other alkyl alkane-

thiosulfinates in a reaction catalysed by alliinase (Amagase *et al*., 2001; Khanum *et al*., 2004; Lanzotti, 2006). Alliinase is an enzyme that functions at an optimum pH of 6.5 (Amagase, 2006). The formation of thiosufinates occurs in a pH dependent manner between pH 2 to 10, when garlic bulbs are immersed in aqueous solutions (Amagase, 2006). Thiosulfinates are not generated in the stomach, because of the absolute acidic conditions, hence pH 3.6 and below leads to an inhibition of alliinase activity (Amagase, 2006). The activity of alliinase is stimulated by two co-factors namely SAC and pyridoxal phosphate (Amagase, 2006).

2.3.1.1. Allicin: An example of an organosulphur compound found in garlic

In 1944, allicin (Figure 2), known scientifically as diallyl thiosulfinate and chemically as 2-propene-1-sulfinothioc acid S-2-propenylester was discovered by Callavito and Bailey(Omar and Al-Wabel, 2010).This is an example of an organosulphurous compound from a thiosulfinate background that is typically responsible garlic's characteristic odour that is released upon being bruised, crushed or cut (Khanum *et al*., 2004; Omar and Al-Wabel, 2010). Allicin is formed when the garlic bulb is crushed or cut, which occurs when the odourless compound alliin is converted by alliinase into allicin (Figure 2) (Khanum *et al*., 2004).

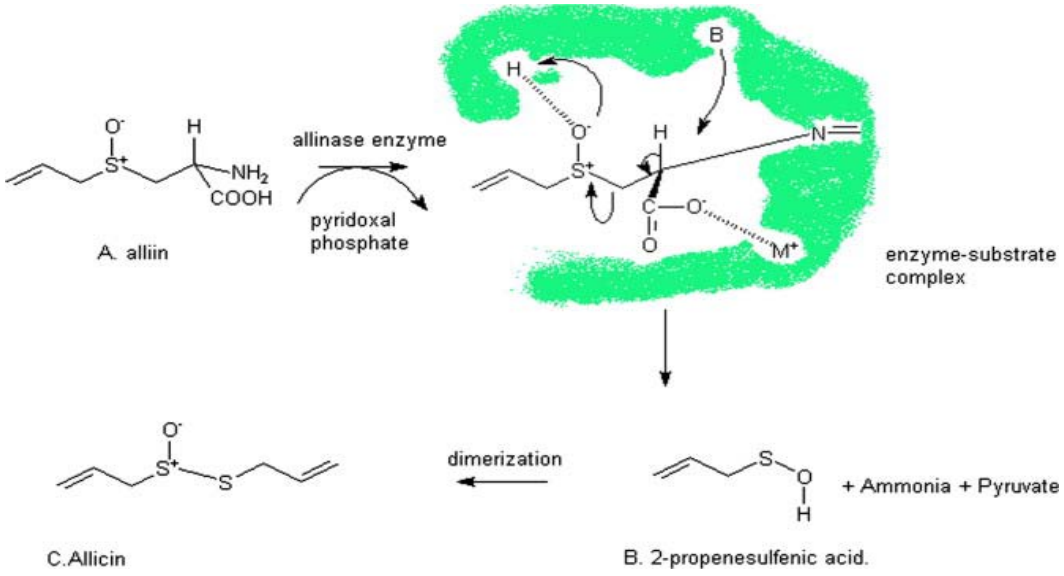


Figure 2: Anabolism pathway of Allicin (Omar and Al-Wabel, 2010).

The medicinal properties of garlic are attributed to its organosulphurous compounds. Allicin is said to be responsible for the conferred medicinal properties of garlic (Maeola, 2005; Lanzotti, 2006; Kemper, 2008; Omar and Al-Wabel, 2010; Van Huyssteen*et al*., 2011).Oommen and his colleagues(2004), concluded from their experiments that allicin has anti-cancer properties (Oommen *et al*., 2004). When murine and human cancer cell lines were treated with allicin, it prompted an inhibitory effect on cancer cell growth (Oommen *et al*., 2004). Furthermore, they also reported that allicin induced apoptosis by inducing caspase and poly-ADP-polymerase (PARP) activation (Oommen *et al*., 2004).On the other hand, Park and his colleagues (2005), reported that allicin induced apoptosis in gastric epithelial cancer cells independent of caspase activation(Park *et al*., 2005).

2.3.1.2. Degradation of allicin found in garlic

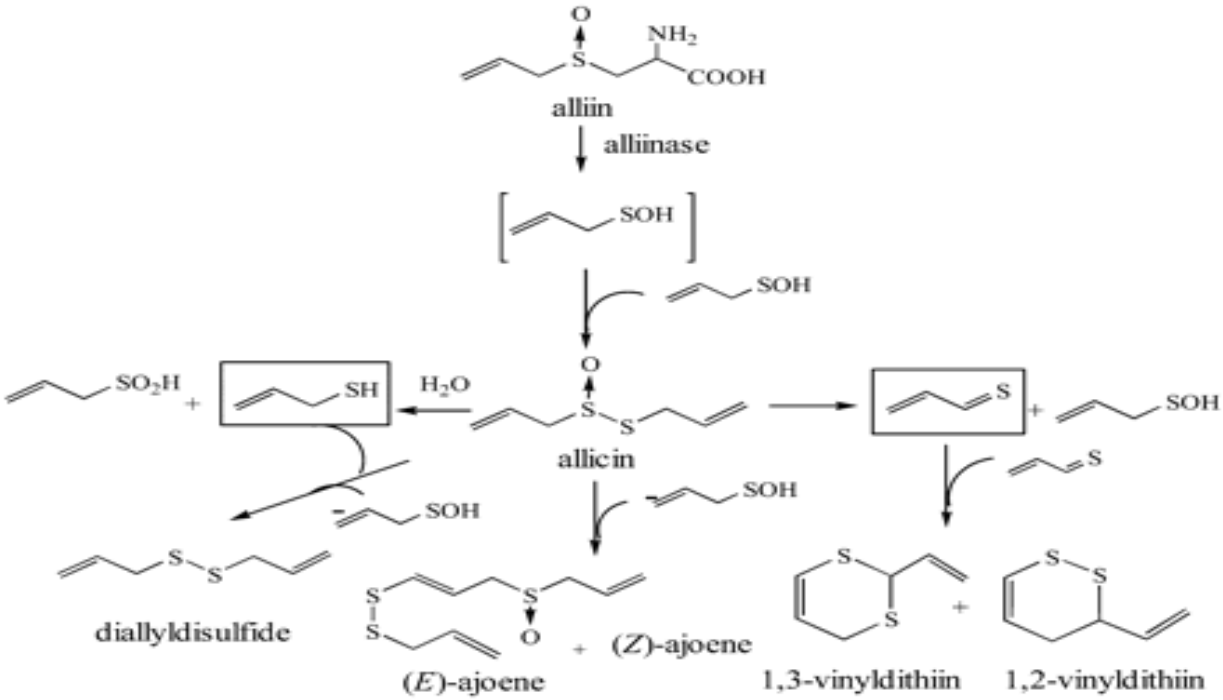


Figure 3: Catabolism of Allicin into other reactive intermediates(Amagase, 2006).

In Figure 3, it can be observed that allicin undergoes further enzymatic degradation by producing additional bioactive compounds found in garlic. In the first pathway (Figure 3), three molecules of allicin combine to form two molecules of ajoene and water (Khanum *et al*., 2004).Ajoene contributes to the anti-carcinogenic properties of garlic (Omar and Al-Wabel, 2010). Ajoene also inhibits platelet aggregation, controls blood lipid levels, is anti-thrombotic, hence prevents blood clotting; and reduces cholesterol levels by inhibiting the activity of angiotensin-converting enzyme (ACE) liberated from the kidneys, and therefore garlic may exert potent cardiovascular effects (Hassan, 2004).

In the second pathway (Figure 3), allicin is conjugated with diethyl ester to yield numerous organosulphurous volatile compounds found in garlic (Khanum *et al*., 2004, Lanzotti, 2006). Some of the bioactive volatile compounds include diallyldisulfide (DADS), diallylsulfide (DAS), and diallyltrisulfide (Lanzotti, 2006).

Another organosulphurous volatile compound found in garlic is vinyldithiins (Figure 3). Vinyldithiins are formed when allicin undergoes beta-elimination, thus allowing for thioacrolein to become dimerised by a Diels-Ader mechanism. Experiments performed on perfused rat liver tissue revealed that vinyldithiins produced no reactive intermediates, whereas other experimental studies have shown that DAS biotransformed into sulfates in the stomach and some is reabsorbed in the cytosol (Amagase, 2006).

2.3.1.3. Water-soluble organosulphurous compounds found in garlic

S-allyl-cysteine (SAC) (Figure 4) is a compound that is formed when ¥-glutamyl-S-allyl-L-cysteine is oxidised by gamma-glutamyl transpeptidase (Amagase, 2006) and this occurs when garlic is soaked in alcohol and aqueous solutions (Amagase *et al*., 2001; Lanzotti, 2006). Scientific evidence has shown that S-allyl-L-cysteine (SAC) is a stable, odourless and water-soluble compound with hepatoprotective, antioxidant, and anti-cancer properties (Amagase *et al*., 2001). Presently in experimental studies that evaluate garlic and its effects, SAC is considered as the “only reliable human compliance marker” (Amagase *et al*., 2001).

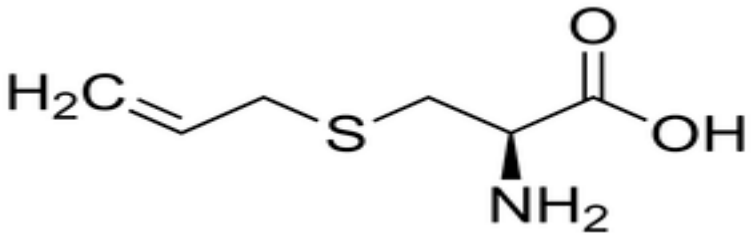


Figure 4:S-allyl-L-cysteine (SAC) found in garlic (http://www.sigmaaldrich.com/catalog/product/SIGMA/SML0337).

2.3.2. Saponins found in garlic

Besides the dominant organosulphurous compounds found in garlic extracts and various garlic preparations, studies have concluded that garlic extracts possess non-organosulphurous compounds (Amagase, 2006; Lanzotti, 2006). Steroidal saponins (Figure 5),have been detected by thin-layer chromatography (TLC) (Lanzotti, 2006). Eruboside-B is an example of a steroidal compound that has been shown to elicit potent antifungal activity against the fungus, *Candida albicans*, inhibit the cancer process and is anti-thrombotic (Amagase, 2006).

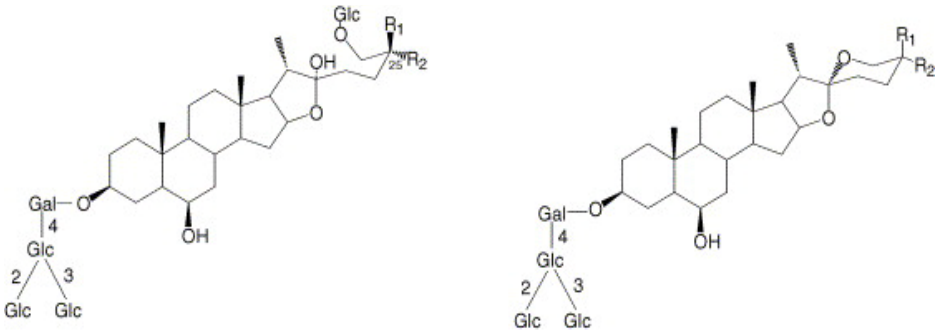


Figure 5: Steroidal saponins found in garlic (Lanzotti, 2006).

2.3.3. Phenolic compounds found in garlic

Phenolics are a class of compounds that contain a benzene ring that is bonded to a hydroxyl ion and are concentrated in high quantities in garlic bulbs (Lanzotti, 2006). Flavonoids (Figure 6),a commonly occurring phenolic, are said to be responsible for the potent antioxidant activity that is elicited by garlic (Lanzotti, 2006). Flavonoids are further divided into N-feruloytyrosine and N-feruloytyramine (Figure 6) compounds in garlic bulbs which exhibit anti-fungal properties (Lanzotti, 2006).

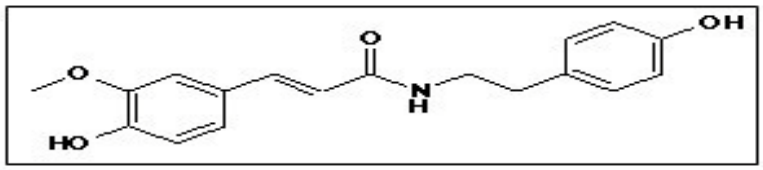


Figure 6: N-feruloytyramine an example of a flavonoid found in garlic (http://www.chemfaces.com/natural/N-trans-Feruloyltyramine-CFN97135.html).

2.4. Tulbaghia violacea (TV)

Figure 7: *Tulbaghia violacea* (TV), an indigenous plant to KwaZulu-Natal (KZN), Eastern Cape, and Limpopo Provinces with health benefits(http://www.bbc.co.uk/gardening/plants/plant_finder/images/large_db_pics/large/*tulbaghia_violacea*.jpg).

Known commonly as “wild garlic, wilde knoffel (Afrikaans), *isihaqa* (Zulu) or itswele lomlambo (Xhosa)”, *Tulbaghia violacea* (TV) (Figure 7) is a plant indigenous to KwaZulu-Natal (KZN), Eastern Cape, Limpopo, and Zimbabwe (Davison; Maeola, 2005; Belewa *et al*., 2010). This proliferative bulbous plant (Figure 7) with elongated, constricted and strapped-like leaves and pinkish tubular flowers grows up to 30cm in length (Maeola, 2005; Belewa *et al*., 2010). It has been exploited for decades by the ethnic population as a natural remedy to cure several ailments such as influenza and fevers, tuberculosis, skin infections, asthma and eradicate intestinal problems such as bloating or intestinal worms (Maeola, 2005; Bungu *et al*., 2006; Street, 2008). Furthermore, the leaves and roots of TV are used as snake repellents and in food dishes as vegetables or decorations (Street, 2008).

2.4.1. Biological activities of *T.violacea*

Recently, papers have reported that TV has anti-fungal and anti-microbial properties. Belewa *et al.*, (2011), concluded that aqueous extracts of TV has effective anti-fungal activity against the plant pathogen, *Aspergillus flavus*. They also revealed that the extracts were potent in decreasing aflatoxin production (Belewa *et al*., 2010). Nteso and Pretorius (2006), reported that crude extracts of TV are very effective at eliciting anti-microbial activities against a broad spectrum of bacterial species, such as *Botrytis cinerea* and *Rhizoctonia solani* (Nteso and Pretorius, 2006). Methanol leaf extracts of TV reduced hypertension and heart rate in the Wistar rats (Raji *et al*., 2012). Bungu *et al*., (2008), have shown that the bulb and leaf extracts of TV have anti-thrombotic and anti-coagulant properties (Bungu *et al*., 2008). Therefore, TV could be considered as an alternative to garlic, but further verification needs to be conducted on TV. Bungu and his colleagues (2006), also concluded from *in vitro* experiments that TV

had anti-cancer properties. But further research is needed to fully elucidate this property of TV (Bungu *et al*., 2006).

An *in vitro* investigation to determine the chemical composition of TV using hydro-distillation methods and extracting essential oils from the rhizomes of TV was performed by Olorunnisola *et al*., (2011). They also investigated the antioxidant and toxic potential of TV (Olorunnisola *et al*., 2011). Olorunnisola and his colleagues (2011) showed that TV consisted of dimethyl disulfide, dimethyl trisulfide, and methyl, 2, 4-dithiapethane to name a few (Olorunnisola *et al*., 2011). The essential oils displayed some positive antioxidant activity (Olorunnisola *et al*., 2011).

Researchers have hypothesised that TV has similar biological activities and compounds as domesticated garlic, since both belong to the same *Alliaceae* family (Bungu *et al*., 2006). Therefore, the biological effects of TV may be attributed to the secondary metabolites that are found in garlic.

2.5. Cancer

Cancer is the leading cause of disease associated with global incidence and high mortality rates. Cancer is not confined to any culture, ethnic group or country. In 2008, 12.7 million new cases of cancers were diagnosed versus 7.6 million cancer deaths worldwide (Jemal *et al*., 2011). The most prominent types of cancers diagnosed and associated with mortality

included lung, stomach, breast, liver and colorectal cancers and these accounted for greater than 40% of new cases been diagnosed in 2008 (Jemal *et al*., 2011).

The World Cancer Factsheet13 (August, 2012), depicts all the statistics with regards to global cancer burden in terms of incidence, deaths and prevalence and also the worldwide cancer trends and future projections (Jemal *et al*., 2011).Global cancer burden places deleterious effects on economies and governments of countries worldwide. Cancer burdens economic growth and health care facilities, and also affects many poorly and underdeveloped communities. Because of the lack of investments and emphasis that are not placed on cancer research and treatment regimens, it continues to add to the rising global cancer burden (Jemal *et al*., 2011).Furthermore, many countries such as developing nations are faced with financial constraints and as a result have problems investing in establishing health care infrastructures and education. Many nations have begun to collaborate and form organisations that address the economic burden that is associated with cancer and possibly these solutions would be the stepping stone to reducing rising global burden associated with cancer (Jemal *et al*., 2011).

Currently, cancer is treated with conventional therapies, which are cancer treatments that are commonly used worldwide (Styles, 2007). These include chemotherapy, radiation and even surgery (Styles, 2007). Although, in the short-term this form of therapy provides suitable treatment, however, the observed side effects associated with conventional therapy such as nausea, diarrhoea, headaches, fatigue and dizziness are worsened in long-term usage. In some cases using conventional therapy over a long period of time may cause immense or even irreversible damage to the human body and could even accelerate the rate of death. For the mentioned above reasons, therefore, any “natural” therapy that is less harmless on the body

and better than consuming hundreds or even thousands of drugs is welcomed in cancer research. A trend has developed for where cancer patients prefer using alternative treatments such as traditional medicine or traditional healers (Galabuzi *et al*., 2010).

2.5.1. Oesophageal cancer

Globally, oesophageal cancer (OC) (Figure 8) is ranked as the eight cause of cancer diagnosed with an associated mortality rate of being ranked sixth (Jemal *et al*., 2010). Differences exist in geographical distribution of OC (Park and Moon, 2008). For example, Asia and Sub-Saharan Africa have the highest incident rates of OC (Jemal *et al*., 2010). Squamous epithelial oesophageal cancer is the most common type of OC diagnosed. The incident rate of OC is higher in males than females and increases with age (Jemal *et al*., 2010). There is poor prognosis for OC and literature cites survival rate to between 3-5 years (Jemal *et al*., 2010).

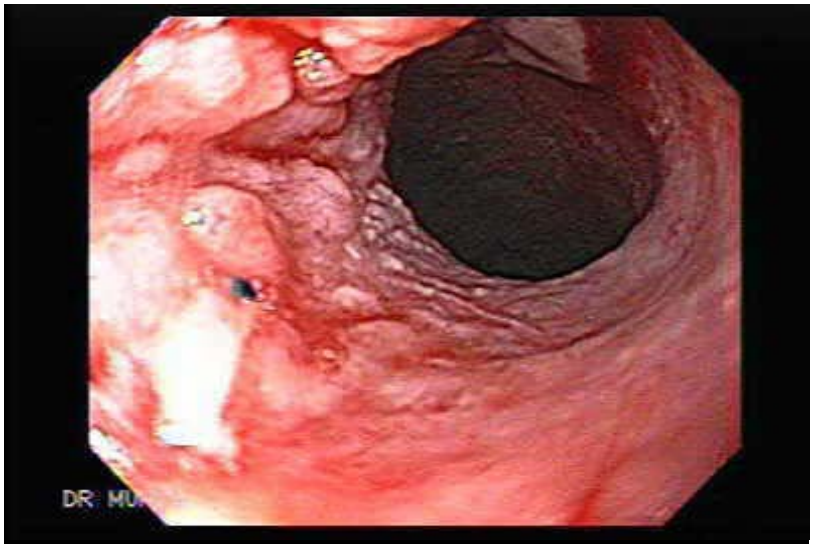


Figure 8: Squamous epithelial oesophageal carcinoma(https://cbwcancerproject.wikispaces.com/Ed+Sullivan).

Oesophageal cancer (OC) is a considered as a commonly occurring malignancy on the Eastern Sea Board Coast of South Africa (SA) that is connected with high mortality and morbidity rates (Alidina *et al*., 2004). In South Africa, OC is characterised by a high frequency and incident rate particularly in black South African males (Myburg *et al*., 2002). Excessive smoking and alcohol consumption are the main risk factors for OC (Jemal *et al*., 2010). Other risk factors include mycotoxin exposure, poor nutrition, sex and age, all of which lead to the progression of OC (Jemal *et al*., 2010).

2.6. *Allium sativum's* anti-cancer properties

Research over the years has reported on *Allium sativum* having anti-carcinogenic properties both *in vivo* and *in vitro* (Bungu *et al.,* 2006; Shukla and Kalra, 2007; Omar and Al-Wabel, 2010). Chinese researchers using gastric cell lines have shown that fresh garlic extracts and diallyltrisulfide were more potent in killing these cancer cells (Khanum *et al.*, 2004). Studies have shown that organosulphurous compounds such as ajoene, allicin, DADS and DAS found in garlic are responsible for garlic's anti-cancer properties. These compounds either inhibit cancer cell growth or induce cell death (Bungu *et al*., 2006; Omar and Al-Wabel, 2010). An experimental study has reported that ajoene inhibits skin cancer in mice and that garlic oil produced the same effect(Khanum *et al*., 2004; Powolny and Singh, 2008).In another study, DAS that was administered to rats inhibited oesophageal tumour formation, particularly during the initiation phase of the cell cycle (Khanum *et al*., 2004; Powolny and Singh, 2008).

To date, research has reported on the correlation of organosulphur compounds in garlic

eliciting their effects via the induction of apoptosis. For example, the earliest study revealed that human colon cancer cells treated with DADs showed apoptotic characteristics such as fragmented DNA and condensed chromatin (Powolny and Singh, 2008). Leukemic cells that were treated with ajoene induced apoptosis as their mechanism of cellular death(Omar and Al-Wabel, 2010).Other publications have stated that organosulphurous compound activity in inducing cell death appeared to be related to a class of mitochondrial proteins called the Bcl-2 family of apoptotic proteins (Powolny and Singh, 2008). For instance, apoptosis induced in SH-SY5Y neuroblastoma and lung cancer cell lines when treated with DAS and DADS corresponded to an increase in the Bax/Bcl-2 ratio (Powolny and Singh, 2008).

It is thought that garlic exerts its anti-carcinogenic effect through its organosulphurous compounds via. a number of mechanisms. These mechanisms include the scavenging of free-radicals, apoptosis and inhibition of mutagenesis, by modulating cancer cell metabolism, halting the progression of the cell cycle, monitoring histone formation, increasing glutathione (GSH) levels, increasing the activity of enzymes such as glutathione-S-transferase, catalase, and DNA repair mechanisms (Khanum *et al*., 2004).

2.6.1. Mechanisms of anti-cancer functions

2.6.1.1. Free-radical scavenging and antioxidant properties

Oxygen and its reactive intermediates are considered detrimental to organisms including humans (Ott *et al*., 2007). This prompted organisms to develop defence mechanisms against these reactive intermediates. Molecular oxygen (O_2) is the derivative of numerous reactive

compounds and free-radicals that are termed reactive oxygen species (ROS) (Figure 9) (Turrens, 2003). Most of the ROS are generated during adenosine triphosphate (ATP)production (Figure 9), when a leakage of electrons occurs in complex I and III of the mammalian mitochondrial electron transport chain (ETC) (Halliwell and Chirico, 1993). Examples of ROS include superoxide anions(O_2^-), hydrogen peroxide (H_20_2) and oxides(Figure 9) (Ott *et al*., 2007). The superoxide anion is formed when O_2 loses one electron and is the anion responsible for setting off the ROS cascade (Figure 9) (Turrens, 2003). In return, O_2^-becomes dismutated by superoxide dismutase to formH_20_2, which undergoes reduction to form water and hydroxyl (OH^-) and other anions (Turrens, 2003).

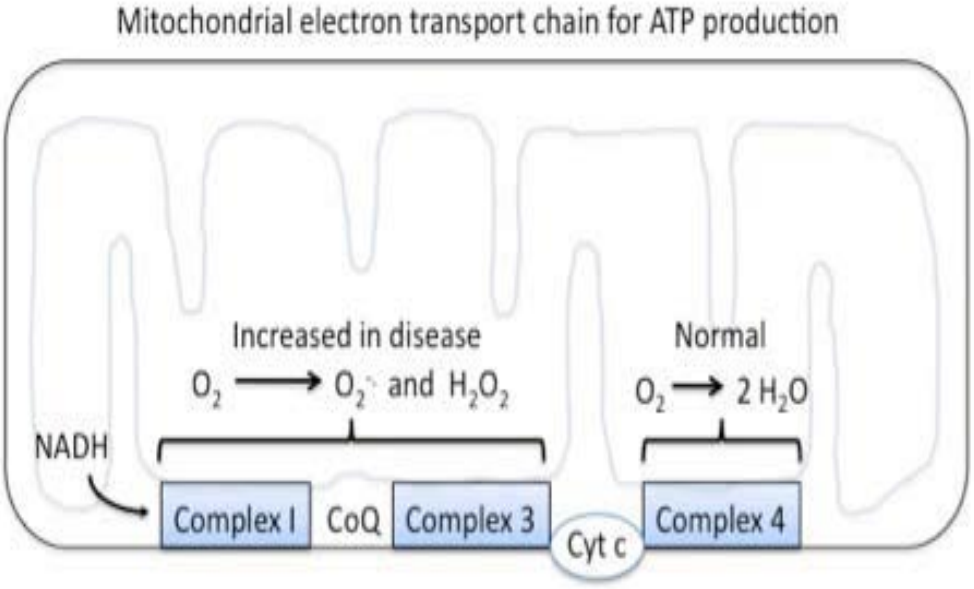


Figure 9: Mitochondrial respiratory chain used for the formation of ATP consequently allowing for the formation of ROS such as superoxide, hydrogen peroxide and oxides (Jones, 2011).

Oxidative stress is defined as tipping the balance in the favour of excessive ROS production over antioxidant defence mechanisms (Halliwell and Chirico, 1993; Ott *et al*., 2007). In humans, oxidative stress is considered as a mechanism in causing many diseases including cancer, chronic inflammatory illnesses and cardiovascular diseases (Ott *et al*., 2007). Antioxidants are molecules that are employed to scavenge free-radicals and destroy these

anions, hence offering protection against the development of oxidative stress (Ramoutar and Brumaghim, 2007). Some of the well-known endogenous antioxidant molecules include glutathione (GSH), glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Halliwell and Chirico, 1993; Ott *et al*., 2007). Superoxide dismutase (Figure 10) hydrolysesO_2^- into H_20_2using magnesium as a co-factor; in return $H_20_2$is further degraded to water (H_20) by GPx where it is eliminated from the mitochondria into the cytosol (Figure 10) (Ott *et al*., 2007). The most prominent mitochondrial antioxidant enzyme is GSH (Ott *et al*., 2007).

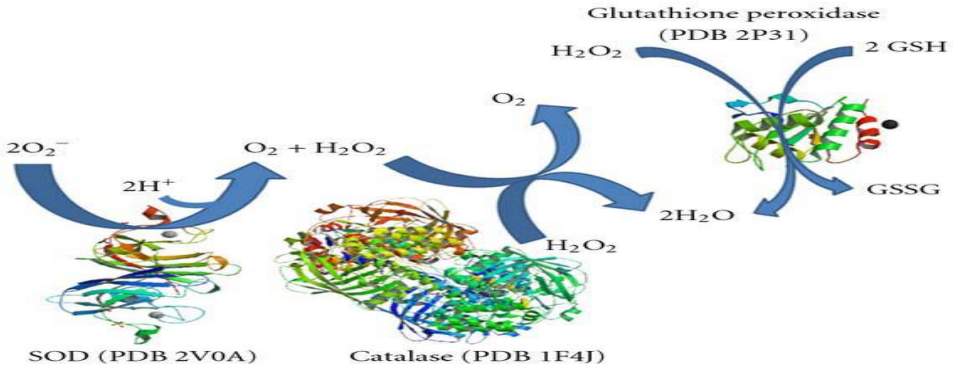


Figure 10: Antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) that are responsible for detoxifying ROS. Superoxide dismutase hydrolyses O_2^- to H_20_2, in turn GPx reduces $H_20_2$to form H_20 (Melo *et al*., 2011).

Glutathione (GSH) (Figure 11) is a tripeptide that contains three amino acids such as cysteine, glutamate and glycine (Jones, 2011). The structural conformation of GSH is such that it can be anabolised and catabolised independent of the body's protein, as a result of containing these amino acids. Also, the structure of GSH ensures that the sulphur atom found in cysteine is responsible for GSH's function (Jones, 2011). Glutathione reacts with a variety of reactive radicals, where electrons donated by GSH are accepted by these oxygen radicals and in turn, two molecules of GSH are reduced to form an oxidised GSH called glutathione disulfide (GSSG) (Figure 11) (Jones, 2011). When oxidants accept the electrons, this

interrupts the normal oxidative process and thus GSH neutralises the oxygen radicals and prevents oxidative damage to cellular components (Jones, 2011). Therefore, the redox potential of a cell is maintained.

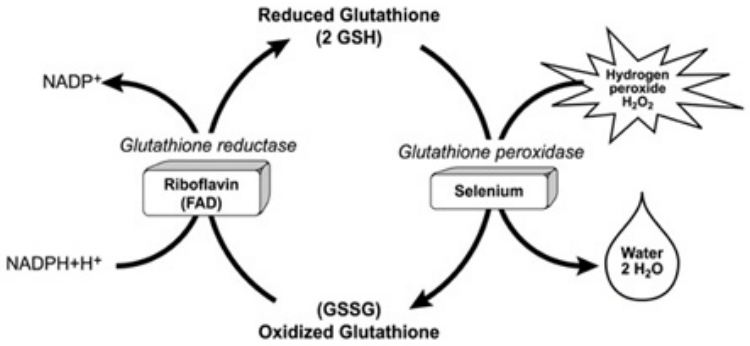


Figure 11: Glutathione (GSH), a tripeptide antioxidant molecule responsible for detoxifying ROS. Glutathione reductase catalyses the reduction of GSH to GSSG, an oxidised form of GSH with NADPH oxidised to $NADP^+$(http://lpi.oregonstate.edu/infocenter/vitamins/riboflavin/gsh.html).

This antioxidant is distributed in a variety of body fluids and tissues in organisms. Literature has suggested that a strong correlation exists between GSH and diseases (Jones, 2011). Reduced GSH levels and associated risk factors that include age, diet and lifestyle have been implicated in diseases such as cardiovascular disease, respiratory and infectious diseases (Jones, 2011). Therefore, both low GSH and risk factors can dampen the GSH defenses that are elicited by the body in order for essential health and functioning of the body (Jones, 2011).

Antioxidant defense mechanisms in organisms also consist of exogenous antioxidants that are obtained from diets. Some of the well-known exogenous antioxidants include vitamin C and E, flavanols (kaempferol) and carotenoids (β-carotene) (Bouayed and Bohn, 2010). These exogenous antioxidants work synergistically with endogenous antioxidants to produce optimum antioxidant defenses (Bouayed and Bohn, 2010). Specifically, the exogenous antioxidants are important to maintain the balance between oxidants and antioxidants in organisms (Bouayed and Bohn, 2010). For instance, GSH regenerates vitamin E and vitamin C offers protection against oxidative damage to lipid membranes, thereby preventing oxidative stress in living organisms (Bouayed and Bohn, 2010).

To date, numerous literature suggests that medicinal plants possess exceptional free radical scavenging properties owing to their bioactive compounds, and so are efficient in aiding defense against oxidative stress in cancerous cells (Madhuri and Pandey, 2005; Ncube *et al*., 2011). For instance, Auroma *et al*., (2006),showed in a set of experiments where high levels of tissue malondialdehyde (MDA), hydroperoxide and diene conjugates were formed by feeding alcohol to rats for 45 days, the administration of water-soluble proteins from garlic reduced the peroxidative changes by increasing GSH levels and other antioxidant enzyme activities (Auroma *et al*., 2006). This an example amongst other studies that have reported on the antioxidant property of garlic extracts. Not much research has been conducted on TV extracts to date. However, Chung (2006), concluded that allicin inhibits the activity of xanthine oxidase, thereby resulting in the formation of O_2^- and ultimately contributes to lipid peroxidation (Chung, 2006). Furthermore, allyl cysteine and alliin are water-soluble in nature whilst allyl disulfide is lipid-soluble in nature and found in membranes (Chung, 2006); therefore these compounds are possibly responsible for interfering with the redox potential of a cancer cell.

Reactive oxygen intermediates have been found to bind irreversibly to macromolecules causing damage to organelles and altering cellular functions; hence this leads to impacting cellular viability and potentiates cell death in a cell (Ott *et al*., 2007). Oxidative damage can occur to DNA, proteins and lipid molecules (Ramoutar and Brumaghim, 2007). Oxidative DNA damage causes alterations to nitrogenous bases i.e. purines and pyrimidine bases, single and double stranded DNA strands (Ott *et al*., 2007). In the energy production process ROS targets mitochondrial DNA, which encodes major proteins that are involved in the respiratory chain (Halliwell and Chirico, 1993; Ott *et al*., 2007). So if mitochondrial DNA is mutated in its function, this affects protein formation and location and indirectly affects electron transport, leading to altered ATP production and cellular activity (Halliwell and Chirico, 1993; Ott *et al*., 2007). As a result of energy deprivation, an organism is pushed towards programmed cell death.

All membranes consist of lipids in the form of polyunsaturated fatty acids and are thus susceptible to oxidative damage, thus allowing for lipid peroxyl radical formation (Halliwell and Chirico, 1993). Lipid peroxyl radical formation is divided into three stages: initiation, propagation and termination (Catala, 2012). The initiation phase begins when a highly reactive molecule (OH^{-}) abstracts a hydrogen (H_2) atom from methylene group ($-CH_2-$) forming simple free-radicals (Catala, 2012). In turn, the carbon radical is transformed into a conjugated diene, which in return binds with $O_2$to form a lipid peroxyl radical (LOO^{--}) (Catala, 2012).The propagation phase occurs when the lipid peroxyl radical abstracts $H_2$from an adjacent fatty acid molecule, thus producing a lipid hydroperoxide or peroxide (Catala, 2012). Finally, the termination phases is characterised by the production of a hydroperoxide (Catala, 2012).

Therefore, if free-radicals do not conjugate with antioxidant molecules such as GSH, this leads to induction of lipid peroxidation and subsequently MDA, a by-product of lipid peroxidation is produced. The increased lipid peroxidation levels targets the cell membranes causing cell surface receptors such as death domain receptors to be expressed and bind to initiator molecules such as caspase 8, which converges onto caspase 3 causing activation of the apoptotic pathways (Chowdhury *et al*., 2008). That is how oxidative damage is linked to apoptosis.

2.6.1.2. Apoptosis

Apoptosis is defined as programmed cell death and forms part of the evolutionary and homeostatic balance in maintaining an organism's well-being (Chowdhury *et al*., 2008). Cell death is a physiological process that is needed for cellular homeostasis and is crucial in the overall development and functioning of multicellular organisms (Hajra and Liu, 2004).

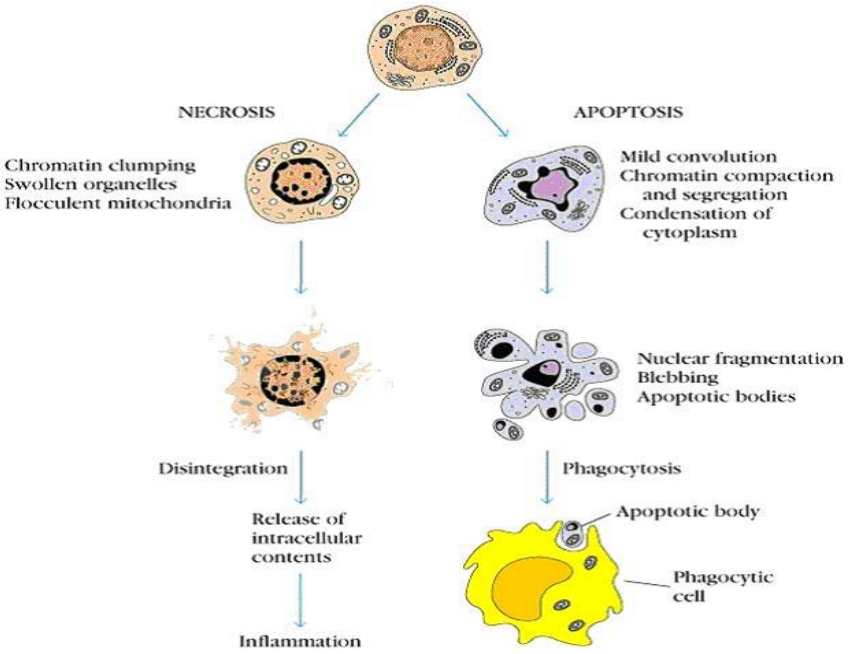


Figure 12: Cell death classified as apoptosis vs. necrosis(http://www.aibnsus.org/AcdamicEmblem.html).

Cell death can be classified into apoptosis and necrosis (Figure 12). Apoptosis is considered as a controlled type of cell death with no inflammation and occurs in response to mild cellular injury, whereas necrosis occurs in response to serious injuries and has associated inflammation (Kanduc *et al*., 2002). The main morphological characteristics of apoptosis are cell shrinking, vacuole swelling and an intact membrane, whereas there is cellular blebbing, vacuole shrinking and leakage of membranes (Figure 12) (Kanduc *et al*., 2002).

Programmed cell death is carefully controlled by cysteine aspartate molecules termed caspases (Hajra and Liu, 2004; Chowdhury *et al*., 2008). Caspases comprise of initiator proteins (e.g. caspase 8 and caspase 9) which initiate cellular death and in return these proteins activate downstream executioner proteins (e.g. caspase 3) which carry out the further processing of apoptosis (Chowdhury *et al*., 2008).

There are three types of apoptotic pathways, namely the intrinsic and extrinsic apoptotic pathways. The intrinsic apoptotic pathway can be divided into two sub-domain pathways, the mitochondrial apoptotic pathway and the endoplasmic reticulum (ER) pathway (Rao *et al*., 2004). The mitochondrial apoptotic pathway is initiated by intracellular apoptotic molecules such as stress, DNA damage, growth factors and oxidative stress (Hajra and Liu, 2004; Chowdhury *et al*., 2008). This then facilitates release of the cytochrome c from the mitochondria into cytosol, where it becomes conjugated to apoptotic-inducing factor-1(apaf-1) and pro-caspase 9 forming an apoptosome. Thus, procaspase 9 is activated initiator protein called caspase 9 (Hajra and Liu, 2004; Chowdhury *et al*., 2008). Caspase 9 then activates the

executioner protein, caspase 3 that is responsible for carrying out cell death (Hajra and Liu, 2004; Chowdhury *et al*., 2008). The ER pathway is triggered by ER stress, when there is an accumulation of calcium ions and accumulation of misfolded proteins (Rao *et al*., 2004). Like the mitochondrial instrinsic pathway, the ER pathway uses caspase 9 and caspase 12 to execute apoptosis, but by an unknown mechanism (Rao *et al*., 2004). Programmed cell death is not only executed by apoptosis, but also by autophagy. Autophagy is an intracellular catabolic process, that facilitates the uptake of cellular constituents that are degraded via lysosomal degradation (Salminen and Kaarniranta, 2009). Many signalling proteins such as p53 and nuclear factor kappa B (NF_KB) are involved in the regulation of the autophagic pathway (Salminen and Kaarniranta, 2009).

The extrinsic or cell-mediated death receptor apoptotic pathway is executed by death ligands that include tumour necrosis factor-alpha (TNF-α) and Fas molecules (Hajra and Liu, 2004; Chowdhury *et al*., 2008). These ligands become bound to death surface receptors leading to receptor dimerisation and forming complexes such as Fas-associated death domain (FADD) complex, which binds and activates initiator caspase 8 and in return caspase 3 becomes activated to execute apoptosis (Hajra and Liu, 2004; Chowdhury *et al*., 2008).

2.6.1.2.1. The role of p53 in apoptosis

The most important role of p53 as a tumour suppressor gene is a likely adaptation from organisms to enable them to survive the continuous mutative changes that they are exposed to and enable their existence. This transcription factor is activated in response to cellular stress

signals such as DNA damage, hypoxia and oxidative stress in cells. This protein functions to scan the genome, detect DNA damage and initiate repair mechanisms (Haupt*et al*., 2003). p53 functions as a growth inhibitor in preventing tumour formation by inhibiting cellular proliferation through cell cycle arrest and senescence and promoting apoptosis of damaged or infected cells with DNA damage (Haupt*et al*., 2003). p53 is a component of the death-receptor apoptotic pathway (Haupt *et al*., 2003).Also, p53 is a key player in cellular processes such as angiogenesis (new blood vessel formation), DNA repair and cell differentiation (Haupt*et al*., 2003).

Normally, when DNA damage or cell cycle abnormalities are observed, p53 becomes activated and binds to a transcription factor p21, which in turn becomes activated and binds to a G1-S/cyclin-dependent kinase 2 (CDK2) complex and inhibits cell cycle progression. This allows for enough time for DNA repair to occur and if DNA damage is beyond being repaired, damaged cells then undergo apoptosis, thus allowing for genomic stability. When p53 becomes mutated, it allows a cancer cell to divide, proliferate and invade a particular organ and overcome the above mentioned protective mechanisms and thus promoting for tumour progression (Haupt*et al*., 2003).

The p53-dependent apoptotic pathway regulates many downstream transcriptional genes that contain p53 responsive elements, such as FAD, Apaf-1, Noxa etc. which are involved in apoptosis (Benchimol, 2001; Haupt *et al*., 2003). As already stated, apoptosis is regulated by one of two apoptotic pathways, either the extrinsic or intrinsic pathways. p53 influences the death receptor apoptotic pathway by regulating the transcription of FAD, a death receptor protein (Benchimol, 2001). This protein contains a death domain and provides a link between

DNA damage mediated p53 activation and caspase activation (Benchimol, 2001). Thus, a scenario for extrinsic apoptosis occurs in the following manner: when DNA damage is sensed, it activates p53 and this leads to transcription and activation of FAD. Once, FAD is activated, it binds to FADD and results in the recruitment and activation of initiator caspase 8 that in turn through which caspase 3 becomes activated and executes apoptosis (Benchimol, 2001).

On the other hand, Bax, a pro-apoptotic protein that is located in the mitochondria has a p53-binding site and thus is regulated by p53 activation (Benchimol, 2001). So, the intrinsic apoptosis scenario is activated, when in the mitochondria Bax is activated by p53, Bax allows for the release of cytochrome c from mitochondria into the cytoplasm, and then it binds to Apaf-1, calcium ions and pro-caspase 9 forming an apoptosome through which activation of this initiator protein caspase 9 occurs, subsequently causing the activation of downstream effector caspase 3/7 responsible for apoptosis (Benchimol, 2001).

2.6.1.2.2. The role of Poly (ADP-ribose) polymerase (PARP) in apoptosis

Poly(ADP-ribose)polymerase (PARP) is a DNA-binding protein that influences DNA repair, DNA replication, modulation of chromatin structure and apoptosis (Bressenot*et al*., 2009). Poly (ADP-ribose) polymerase (PARP) recognises DNA strand interruptions and is cleaved into two fragments, namely the C-terminal cleavage product: 89kDa segment and the N-terminal cleavage product: 24kDa segment, respectively (Bressenot*et al*., 2009).

2.6.1.2.3. The role of nuclear factor kappa B (NF_KB) in apoptosis

Inflammation is defined as the infiltration of immune cells and cytokines amplifying their responses in response to tissue injury or invasion. For decades, scientists have tried to provide evidence of how inflammation is linked to cancer progression (Hoffnung, 2011). In 2000, the answer was discovered when researchers suggested that nuclear factor kappa B(NF_KB), was the "master-control complex" between linking inflammation to the growth and metastasizing of the breast cancer cells (Hoffnung, 2011). It has been said that NF_KB enhances angiogenesis, as well as the synthesis of genes that regulate pro-inflammatory cytokine synthesis (e.g. chemokines and interleukins) and amplification of their response, thus allowing for progressive tumour proliferation and cancer (Karin 2008; Hoffnung, 2011). Thus, NF_KB is a crucial factor regulating DNA transcription and incorrect coding of this protein has been linked to the development of cancer (Karin, 2008). The transcription factor NF_KB functions as a switch from anti-apoptotic to pro-apoptotic functioning (Vousden, 2009).The anti-apoptotic functions of NF_KB are elicited by Bcl 2 family and the pro-apoptotic role of NF_KB is regulated by the p53 response (Vousden, 2009).

Since cancer is regarded as a disease of the cell cycle, apoptosis is the principle mechanism used by several anti-cancer agents (Bungu *et al*., 2006; Shukla and Kalra, 2007; Omar and Al-Wabel, 2010). Balasenthi *et al*., (2002), investigated the apoptotic potential of garlic in preventing Syrian mouse carcinomas and concluded that garlic's anti-carcinogenic effects are because of induction of apoptosis by garlic as well as suppression of anti-apoptotic proteins such as Bcl-2 (Balasenthi *et al*., 2002).

CHAPTER3

MATERIALS AND METHODS

3.1. Materials

The SNO cell line was purchased from Highveld Biologicals (Johannesburg, South Africa). All tissue culture reagents were purchased from Whitehead Scientific (Johannesburg, South Africa). The primary antibody for β-actin (Mouse anti-β-actin),GSH-Glo™ gluathione assay, CellTitre-Glo® cell viability assay, Caspase-Glo® 3/7 assay, the Caspase-Glo® 8 assay and Caspase-Glo® 9 assay were purchased from Promega (South Africa). The JC-1 dye was obtained from BD Biosciences (South Africa) and cOmplete, Mini Cocktail Protease Inhibitors (Cat no. 04 693 124 001) were purchased from, Roche Applied Sciences (South Africa). Methylthiazoltetrazolium (MTT) salt, primary antibodies for p53 (rabbit anti-p53), Poly (ADP-ribose) polymerase (PARP) (goat anti-PARP), and Nuclear factor kappa beta (NF_KB) (rabbit anti-NF_K B) were purchased from Sigma (Germany). Horse-radish peroxide (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG and rabbit anti-goat IgG) were purchased from Abcam (South Africa) and rabbit anti-goat IgG secondary antibody was purchased from Bio-Rad (USA). Unless otherwise stated, all other consumables were purchased from Merck (South Africa).

3.2. Preparation of TV leaf (TVL) and TV bulb (TVB) and garlic bulb methanol extracts

Fresh rhizomes of *T.violacea* and *Allium sativum* (garlic) and leaves of *T.violacea* were collected and authenticated at the University of KwaZulu-Natal, Durban, South Africa, by botanist, Prof. H. Baijnath. The rhizomes and leaves were washed with water to remove any impurities and allowed to air dry at room temperature for 72hrs. Subsequently, a Waring blender (Waring Products Division, Dynamics Corporation of America, New Hartford, Connecticut) was used to crush the roots and leaves until finely ground. The blended samples were soaked in methanol for 48hrs, after which the pulp was filtered and the filtrate concentrated in a rotary evaporator (Genevac LTD, Ipswich, England) for 48hrs. Thereafter, the filtrate (3ml) was aliquoted into little vials and allowed to freeze dry in a freeze drier (SP Scientific Warminster, PA) at -40^{0}C.Once, freeze-dried, the powdered extracts were stored at -20^{0}C until needed. When required, stock solutions (1mg/ml) were prepared and diluted accordingly. The stock solution for each plant extract was prepared as follows: 10mg of garlic and TV-extract powders were weighed out and dissolved in 10ml of 5% complete culture medium (CCM) (Refer to Appendix A), yielding a plant stock solution of 1mg/ml(Refer to Appendix A). For the MTT assay, for each plant extract 9 serial dilutions (0-500µg/ml) were prepared from the various stock solutions (Refer to Appendix A).Controls were incubated in 5% CCM (1ml) only.

3.3. Cell culture maintenance

The SNO cancer cell line, an adherent cell line, was cultured in 75cm³ culture vessels in 5% CCM (Refer to Appendix A): Eagles minimum essential medium (EMEM) augmented with 25ml (5%) foetal calf serum, 1% Penstrep fungizone, and 1% L-glutamine, and incubated at 37°C. Cell growth was monitored every 48hrs using a phase-contrast microscope. Once confluency (80%) was attained, cells were trypsinised, reconstituted in CCM and counted using the trypan blue exclusion assay to determine cell viability. The counted cells were then either subcultured or cryopreserved.

The process of trypsinisation, is used to remove adherent cells that are attached to a culture vessel into suspension nature. Briefly, cells were washed thrice with 0.1M phosphate buffered saline (PBS, pH7.4) (Refer to Appendix B) (2ml) and the supernatant was discarded between each wash. Trypsin (5ml) was added to the culture vessel and incubated in a 37°C incubator with occasional monitoring every 30secs until cells were detached from each other and well rounded. Once trypsinised, the trypsin was discarded, the culture vessel was sealed and cells dislodged with moderate banging against the palms of the hands. Thereafter, 5ml of CCM was added into the culture vessel and the cell suspension transferred into a 15ml sterlin tube. To determine cell viability and cell number the trypan blue method was used. Briefly, a cell suspension containing 150µl CCM, 50µl cell suspension and 50µl 0.4M trypan blue was prepared, pipetted (10µl) onto a haemocytometer and covered with a coverslip. Cells were counted in five quadrants and the average number of cells was taken and calculated as follows:

$$Cell\ number\ =\ Average\ cell\ numbers \times dilution factor\ (5) \times 10^4\ cells$$

The cell suspension was diluted to $1x10^6$ cells/ml and transferred into new 75cm³ culture vessels. Usually, a 1:3 split was used when subculturing.

Cryopreservation of cells occurred in a similar manner as described above for the subculturing of cells. Following counting, the cells were pelleted by centrifugation the pellet was re-suspended in freezing medium (10%CCM containing 10% DMSO) to obtain a dilution of $1x10^6$ cells/ml. The cell suspension was transferred to cryovials (2ml) that were subsequently placed into a Nalgene cooler and stored at –80°C.

3.4. Methylthiazoltetrazolium (MTT) assay to measure cytotoxicity and obtain an IC_{50} in SNO cells

Cell viability is often used as a measure for detection of cell proliferation and cytotoxicity in cultured cells. The MTT assay (Figure 13) measures how viable a living cell is when MTT salt, a yellow tetrazolium compound is metabolically reduced to a purple formazan compound as $NAD^+$is reduced to NADH in glycolysis (Mosmann, 1983). Therefore, MTT reduction forms a linkage to cellular metabolism and indicates changes in mitochondrial activity (Mosmann, 1983). Mosmann (1983), said that mitochondrial dehydrogenases are responsible, but found that cytosolic reduction takes place. Red blood cells can also reduce MTT to formazan.

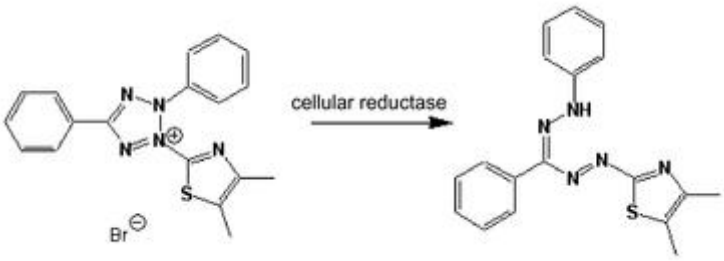


Figure 13: The reduction of MTT salt, a yellow tetrazolium to a purple formazan compound cataslysed by cellular reductase (http://en.wikipedia.org/wiki/MTT_assay).

This assay determined the cytotoxicity and IC_{50} (being the half maximal inhibitory concentration) of garlic and TV leaf and bulb extracts on SNO cells. Cells ($1x10^4$ cells; 200µl) were plated in a 96-well plate and allowed to attach overnight. Once cells were attached, media was removed and replaced with the test concentrations (0-500µg/ml) (Refer to Appendix A) of each extract in triplicates (CCM served as the control) and incubated for 24hrs. Thereafter, media was removed and replaced with 10µl MTT salt solution (5mg/ml in PBS)(Refer to Appendix C) and 100µl CCM. After 4hrs incubation, the supernatant was discarded and replaced with 100µl dimethyl sulphoxide (DMSO) and incubated for 1hr. Optical densities were measured with Bio-Tek µQuant spectrophotometer at 567nm with a reference wavelength of 690nm. The average absorbance was used to determine average cell viability:

$$\% \text{ cell viability} = \frac{\text{Average absorbance of sample}}{\text{Average absorbance of control}} \text{ x } 100$$

Thereafter, the IC_{50} for these extracts was extrapolated using GraphPad Prism v5.0 (GraphPad Software Inc., La Jolla, USA) and these IC_{50}values were used in subsequent assays.

For all subsequent experiments,SNO cells were grown to confluency in 75cm^3 culture vessels and treated with CCM only, 250µg/ml TVL, 25µg/ml TVB, 500µg/ml garlic and combination of TVB + TVL (250µg/ml and 25µg/ml). The flasks were trypsinised and the cells counted for subsequent assays as described above (Refer to section 3.3).

3.5. Determination of mitochondrial membrane potential (ΔΨm) in SNO cells

In order to assess mitochondrial potential membrane permeable lipophilic cationic flourochromes are used as probes. These probes penetrate the cells and emit fluorescence. The J-aggregate cationic dye-1 (JC-1) is an example of such a fluorochrome. The fluorescence emitted following JC-1 penetration of a cellis proportional to its concentration and gives an indication of mitochondrial state (Perry *et al*., 2011). The JC-1 dye exists as monomers at low dye concentrations (i.e. JC-1 emits green fluorescence and is measured in the green channel in the flow cytometer at wavelengths 527nm and 590nm);and aggregates at high concentrations (JC-1 taken up by mitochondria emits red fluorescence in the red channel in the flow cytometer (Perry *et al*., 2011).The type of fluorescence emitted gives an indication of whether mitochondria are polarised (green fluorescence) or depolarised (red fluorescence) (Perry *et al*., 2011).

The mitochondrial membrane potential (ΔΨm) of the SNO cells was determined using the fluorescence-activated cell sorting (FACS) and JC-1 mitoscreen assay (BD Biosciences) according to the manufacturer's instructions. Treated cells (1x10^5 cells, 100µl PBS) were dispensed into polystyrene cytometry tubes. The cells were then stained with the JC-1 dye

(150µl) (Refer to Appendix D) and incubated for 10mins at 37°C. Subsequently the cells were washed with JC-1 wash buffer (400µl), centrifuged and resuspended in 300µl of sheath fluid. Data from fluorescent labeled cells (15000 events) were acquired using a FACSCalibur (BD Biosciences) benchtop cytometer and analysed using CellQuest PRO v4.02 software (BDBiosciences).

3.6. Evaluation of the metabolic activity of SNO cells exposed to garlic and TV extracts using the CellTitre-Glo® cell viability assay

The CellTitre-Glo® cell viability assay is used for quantifying the number of viable cells and is expressed in terms of ATP concentrations. The principle of this assay entails beetle luciferin becoming mono-oxidised to oxyluciferin catalysed by luciferase and co-factors such as O_2, magnesium and ATP (Figure 14) (Hannah *et al*., 2001). The luminescent signal is directly proportional to ATP levels.

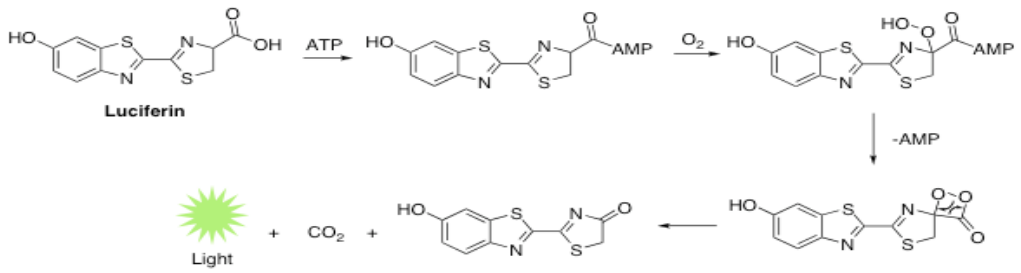


Figure 14: Luciferase catalsyes the mono-oxygenation of luciferin (http://smellslikescience.com/a-language-of-light-from-a-tiny-molecule/).

The number of viable cells that were metabolically active was determined by using the CellTitre-Glo® cell viability assay (Promega) according to the manufacturer's instructions. Cells ($1x10^6$cells, 100µl PBS) were plated in a 96-well opaque luminometer plate and exposed to extracts (Refer to section 3.4.) for 24hrs. Thereafter, the supernatant was discarded and replaced with 100µl per well of ATP Glo® working reagent (CellTitre-Glo® substrate to the CellTitre-Glo® buffer) (Refer to Appendix D). The plate was then placed on a shaker to allow for the mixing of contents for 2mins. Thereafter, the plate was incubated for 10mins at room temperature in the dark, which allowed for stabilisation of the luminescent signal. Luminescence was recorded as relative light units (RLU) and analysed using the Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA).

3.7. Determination of lipid peroxidation in SNO cells

The thiobarbituric acid reactive substances (TBARS) assay was performed to determine the effect of TV and garlic extracts on lipid peroxidation as indicated by the levels of malondialdehyde (MDA). Mitochondria are considered principle sites for ROS production (Ott *et al*., 2007). During ATP production the pumping of protons from the inner mitochondrial matrix into the electron transport chain (ETC) facilitates the leakage of electrons, which irreversibly binds to oxygen molecules forming free radicals such as superoxides and hydrogen peroxides that are responsible for causing oxidative damage to mitochondrial membranes, lipids, proteins and DNA (Ott *et al*., 2007). Since all membranes are composed of lipids in the form of polyunsaturated fatty acids, they are susceptible to oxidative damage and allows for lipid peroxyl radical formation (Halliwell and Chirico, 1993). If these free-radicals are not conjugated to antioxidant molecules such as GSH, lipid

peroxidation is induced and MDA, a by-product of lipid peroxidation is produced (Halliwell and Chirico, 1993).

Treated cells ($1x10^5$cells) were pelleted and homogenised in 0.2% H_3PO_4 (500µl), and the homogenate (400ul) was transferred to glass tubes. Each glass tube subsequently received: 2% H_3PO_4 (200µl), 7% H_3PO_4 (400µl) and thiobarbituric acid (1%, w/v)/0.1mM butylatedhydroxytoluene solution (TBA/BHT) (400µl)(Refer to Appendix E).A positive control and sample blank were also prepared. The glass tube for the positive control contained MDA (1µl) in addition to the above reagents, whereas the sample blank received CCM (400µl) in place of TBA/BHT. Following adjustment of the pH to 1.5 using 1M HCl, the tubes were boiled for 15mins at 100°C and allowed to cool to room temperature. Thereafter, butanol (1 ml) was pipetted into each tube and vortexed for approximately 60secs each. The tubes were allowed to stand until a butanol phase had developed. The butanol phase (top) was removed from each tube, added into an eppendorf tube and centrifuged at 13 000rpm for 5mins. Each sample was aliquoted (200µl) into a microtitre plate in duplicate. The optical densities were measured on the BioTek µQuant spectrophotometer at dual wavelengths of 532nm and 600nm. The averaged absorbance values were used to calculate the MDA concentrations ([MDA]) using the following equation:

$$[\text{MDA}]\ (\mu\text{M}) = \frac{\text{Average absorbance value of sample}}{156\text{mM}} \times 1000$$

3.8. Determination of antioxidant (GSH) activity in SNO cells

Intracellular glutathione (GSH) levels were assessed using the GSH-Glo™ luminometry assay (Promega) according to the manufacturer's directions. Glutathione is an antioxidant molecule

that principally functions to scavenge free-radicals such as superoxide, hydrogen peroxide and hydroxyl radicals, thus offering protection against oxidative damage in cells (Halliwell and Chirico, 1993). In other words, GSH is crucial in maintaining the redox potential of cells. Once this redox potential is altered, it sets off a cascade of downstream events in a cell such as oxidative damage, apoptosis etc. (Halliwell and Chirico, 1993).

The principle of this assay is governed by the reduction of a luciferin derivative to luciferin in the presence of GSH, which is catalysed by glutathione-S-transferase(Held, 2010).The GSH-Glo™ luminometry assay was used to determine whether TV extracts and garlic altered intracellular GSH levels in SNO cells.

Following the 24hr incubation, treated cell pellets were re-suspended in PBS and 50µl aliquots containing $1x10^4$cells were pipetted into an opaque microtitre plate in duplicates. A 5µM stock solution was used to prepare the GSH standards (0-5µM). Each standard (50µl) was pipetted into duplicate wells of the microtitre plate. A 2x GSH-Glo™ working solution (Refer to Appendix E) was added to the respective wells (50µl/well) and incubated at 25°C for 30mins in the dark. The luciferin detection reagent (50µl) (refer to Appendix E) was subsequently pipetted into all sample wells. Luminescence was then measured using the Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The GSH concentration in each sample was determined by extrapolation from the GSH standard curve (Refer to AppendixJ).

3.9. Assessment of caspase activities indicative of apoptosis in SNO cells

Apoptosis or programmed cell death is a physiological process that is needed by all organisms for maintenance of cell homeostasis (Hajra and Liu, 2004; Chowdhury *et al*., 2008). Cell death is coordinated by cysteine-aspartases called caspases (Hajra and Liu, 2004; Chowdhury *et al*., 2008). Caspase 8 is an initiator molecule that is a component of the intrinsic/mitochondrial apoptotic pathway, whereas caspase 9 is the key initiator molecule of the extrinsic/death receptor apoptotic pathway (Hajra and Liu, 2004; Chowdhury *et al*., 2008). Both of these apoptotic pathways eventually converge at an executioner protein called caspase 3/7, which is responsible for carrying out apoptosis(Hajra and Liu., 2004; Chowdhury *et al*., 2008).

Luminometry was used to assess initiator and executioner caspase activities. Assessment of initiator caspases 8 and 9 and executioner 3/7 activities were carried out by using the Caspase-Glo®assays (Promega) according to the manufacturer's instructions. Caspase-Glo® substrates and caspase buffers were mixed to produce Caspase-Glo® reagents, respectively (Refer to Appendix F). Control and treated cell suspensions ($1x10^4$cells, 50µl) were added into duplicate wells of a 96-well opaque microtitre plate. The Caspase-Glo® reagents (10µl) were pipetted into each of the sample containing wells. The plate was then agitated on a shaker for 10secs at 500rpm. The plate was then incubated for 30mins in the dark at room temperature and RLU were assessed on the Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA).

3.10. Immunodetection of protein expressions namely p53, poly(ADP-ribose)polymerase (PARP), and nuclear factor kappa B (NF_KB) in SNO cells

The mechanism of apoptosis in SNO cells was evaluated by the protein expression levels of p53, PARP and NF_KB, respectively. Cells were cultured in a 6-well ELISA plate and treated as above. Following the 24hr incubation, attached cells were washed (500µl PBS). Cells were then lysed in cell lysis buffer (Sigma, Germany) (Refer to Appendix G) and placed on ice for 10mins.In a circular motion cells were scrapped, lysates (200µl) transferred to eppendorf tubes and centrifuged (Eppendorf 5804R, Hamberg, Germany) at 10 000rpm for 10mins at 4° C. The resulting supernatants were transferred to clean labelled eppendorf tubes and placed on ice.

The protein concentration of each sample was determined using the bicinchoninic acid (BCA) assay (Refer to section 3.10.1. below) and standardised samples were subjected to SDS-PAG E (Refer to section 3.10.2. below).

3.10.1. The bicinchoninic acid (BCA) assay

The BCA assay is based upon the central component bicinchoninic acid, which is a water-soluble compound that reduces Cu^{2+} ions to Cu^{+} ions, thus forming a purple compound. This assay is rapid and easy to perform and has a high sensitivity to varying protein concentrations.

From a bovine serum albumin (BSA) stock (1mg/ml), in six eppendorf tubes BSA standards were prepared (0, 0.2, 0.4, 0.6, 0.8 and 1mg/ml) (Refer to Appendix G). The samples and standards (25µl) were aliquoted into the 96-well plate in duplicates. The BCA reagent (200µl) (Refer to Appendix G) was added to each well and incubated at 37ºC for 30mins. Absorbance was measured at 562nm using the Bio-Tek µQuant spectrophotometer and absorbance values were used to construct a standard curve and determine protein concentration of each sample (Refer to Appendix J). Samples were standardised to the lowest protein concentration 1mg/ml, which were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3.10.2. Samples subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis for detection of p53, PARP and NF_KB protein expression by western blotting

Western blotting (Figure 15) is a process used to identify a particular protein of interest amongst numerous proteins within samples that is bound to a specific antibody and hence a specific antibody-antibody reaction (Kurien *et al*., 2011).The size and expression level of a particular protein can be determined (Kurien *et al*., 2011).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)(Figure 15) is a technique that is used for separating proteins according to their size in cell culture samples and tissues (Chevalier, 2010).The procedure begins with loading of samples into wells on a gel and then proteins are coated with SDS. Sodium dodecyl sulfate (SDS) is a detergent that

dentures proteins so that the proteins are in linear form and it coats the proteins with a negative charge, thus when an electric field is applied the negatively charged proteins will migrate through the gel towards the positive side (Chevalier, 2010).Also, having proteins linearised allows for faster migration through the gel and proteins can be separated by their respective sizes. The smaller proteins migrate fastest through the gel and are found at the bottom of the gel, whereas larger proteins are found at the top of the gel (Chevalier, 2010).

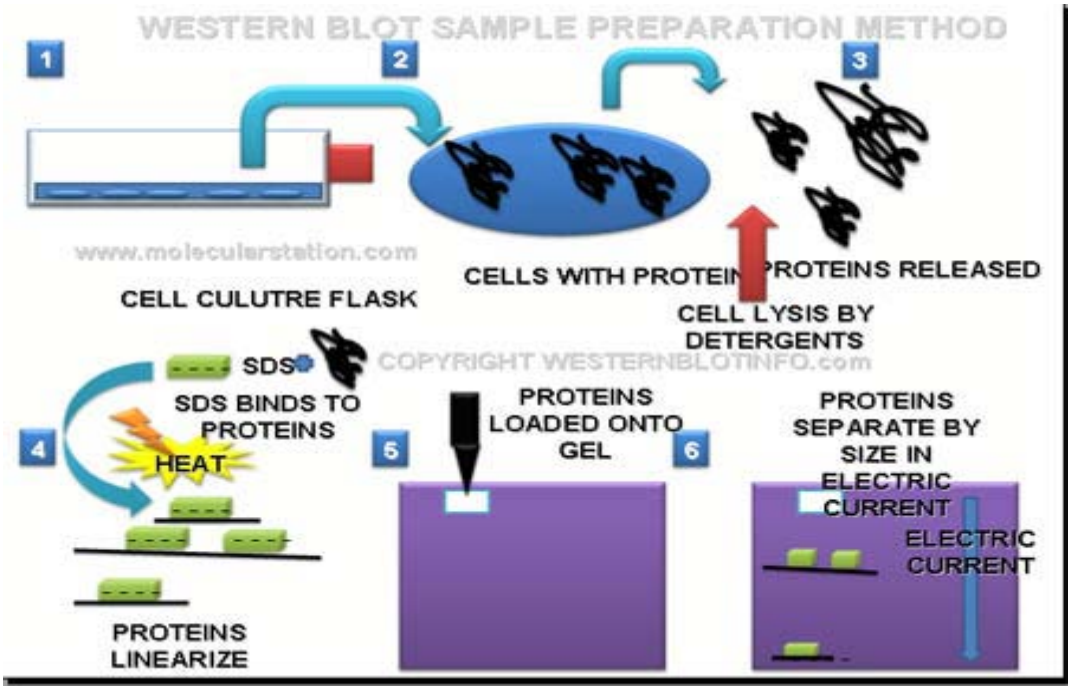


Figure 15: Western blotting procedure for a detection of protein expression levels (http://www.molecularstation.com/protein/western-blot/).

Standardised samples (1mg/ml) were then prepared or SDS-PAGE analysis; where in five eppendorfs added standardised protein (100µl) and 1x laemmli sample buffer (20µl) (Refer to Appendix G). Samples were denatured by addition of 1x laemmli buffer and heated at 100°C for 5mins, so that β-mercaptoethanol was activated and SDS could allow for protein

unfolding. Equal amounts of proteins (25µl) and molecular weight markers were loaded into each well on a 7.5% resolving gel (Refer to Appendix G) unless otherwise stated. Proteins were then electrophoresed at 150V for 1hr (Power Pac Universal, Bio-Rad, USA) submerged in 1x Tank Running Buffer (1L) (Refer to Appendix G). Separated proteins were transferred to nitrocellulose membranes and allowed to transfer at 400mA for 45mins.Transfer of proteins was as follows: fibre pad, nitrocellulose membrane, SDS-PAGE gel, fibre pad with all components submerged in little transfer buffer (Refer to Appendix G) just covering the transfer sandwich. The membranes were washed with 1x Tween 20 tris buffered saline (TTBS) (Refer to Annexure G) and blocked with 2% BSA in TTBS overnight (Refer to Appendix G). The blots were incubated with primary antibodies (rabbit anti-p53, goat anti-PARP and rabbit anti-NF_KB) (Sigma, Germany) diluted 1:5000in 2% BSA in TBBS, on a shaker at room temperature for 1hr. Thereafter, membranes were washed thrice with TTBS wash buffer for 10mins each and incubated for 1hr with HRP-conjugated goat anti-rabbit IgG (Abcam) or rabbit anti-goat IgG (Bio-Rad, USA) diluted 1:2000 in 2% BSA in TBBS.Protein bands were detected using the chemiluminescent substrate (ECL Western Blotting detection reagents, Gaithersburg, USA) using the Chemi-doc XRS gel documentation system. Protein bands were scanned and quantitative estimates developed using Quantity-one™ image analysis software (Bio-Rad, USA). Data was expressed in arbitrary values as individual sample optical density/β-actin ratio.

3.10.3. Detection of β-actin protein expression

Membranes have to be stripped before probing for β-actin protein expression. The respective membranes were removed from the refrigerator and placed on the shaker and allowed to

equilibrate to room temperature for 1hr. The TTBS wash buffer (Refer to AppendixG) was discarded and 5ml H_20_2 was added to each membrane and incubated at 37ºC for 30mins. The membranes were washed once with TTBS wash buffer for 10mins on the shaker. Monoclonal anti-β-actin antibody diluted in 1% BSA in TTBS (5ml) (1:5000, Promega, South Africa) was used as a standard control marker. The diluted antibody was added to each membrane and incubated for 1hr at room temperature. The membranes were then rinsed four times with TTBS wash buffer for 10mins each and then rinsed in dH_2O. Protein bands were detected using the chemiluminescent substrate (ECL Western Blotting detection reagents, Gaithersburg, USA) by usage of the Chemi-doc XRS gel documentation system. Protein bands were scanned and quantitative estimates developed using Quantity-one™ image analysis software (Bio-Rad, USA). Data was expressed in arbitrary values as individual sample optical density/β-actin ratio.

3.11. Detection of DNA fragmentation and nuclear condensation in SNO cells

Fragmented DNA and condensed chromatin are morphological changes indicative of cells undergoing apoptosis (Bungu *et al*., 2006).Hoechst 33342 dye is an effective method for detection of such morphological changes in a cell undergoing cell death (Smith *et al*., 1991). The Hoechst stain recognises and binds effectively to Adenine-Tyrosine (A-T) base pairs in nucleotides (Smith *et al*., 1991). Hoechst 33342 dye stains all DNA blue, however when chromatin condensation occurs, the nuclei are brightly fluorescent (Smith *et al*., 1991).Since the Hoechst 33342 dye is blue in colour, apoptotic cells are brightly fluorescent and this

denotes nuclear changes, whereas non-apoptotic cells are not brightly fluorescent and remain intact (Bungu *et al*., 2006).

The Hoechst 33342 stain was utilised to investigate the effect of garlic and TV extracts on the integrity of DNA. Cells were grown to confluency in a 24-well ELISA plate. Treated cells were washed thrice with PBS (37°C), and then fixed in 10% paraformaldehyde (PFA) (Refer to Appendix H) (2ml) at 37°C for 5mins. The fixative was then discarded and cells washed three times with PBS. Hoechst 33342 working solution (Refer to Appendix H)(100µl) was added into each well and incubated for 15mins at 37°C. The cells were again washed with PBS. The cells were visualised and images captured using the Olympus inverted microscope and six fields of views were captured at a magnification of 200x. Exposure time was kept constant for all treatments at 15m.s.

3.12. Statistical analysis

GraphPad Prism v5.0 (GraphPad Software Inc., La Jolla, USA) was used to analyse data statistically. The results were expressed as mean ± standard deviations (SD). One-way ANOVA was employed to calculate the statistical significances for all of the assays and for the MTT Assay samples and IC_{50} values. Post-test analysis was employed either as Tukey Kramer Multiple Comparisons Test or Kruskal-Wallis test with Dunn's Multi-Comparison test characterised for non-parametric data. Level of statistical significance was set at $p< 0.05$.

CHAPTER 4

RESULTS

4.1. Methylthiazoltetrazolium (MTT) cytotoxicity and IC_{50} values of garlic and TV extracts

The cytotoxic effects of garlic and TV bulb and leaf extracts on the SNO cells was determined by exposing the cancer cells to a series of the plant extract dilutions (0µg/ml-500µg/ml) for 24hrs. The IC_{50} determined for garlic (Figure 16A), TVB (Figure 16B) and TVL (Figure 16C) were 500µg/ml, 25µg/ml and 250µg/ml, respectively. A representative graph for each extract is shown in Figure 16A-C.

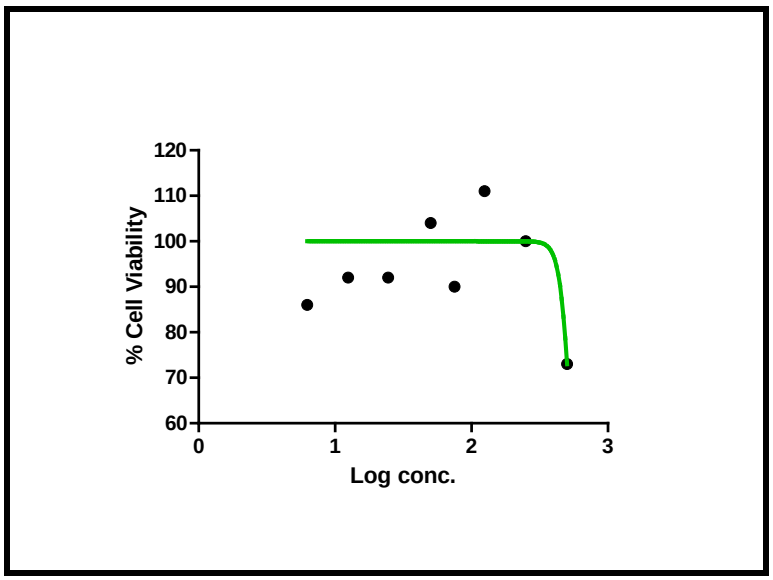


Figure 16A: Cell viability determined by MTT assay of garlic-treated SNO cells for 24hrs used to determine the IC_{50} for garlic extracts.

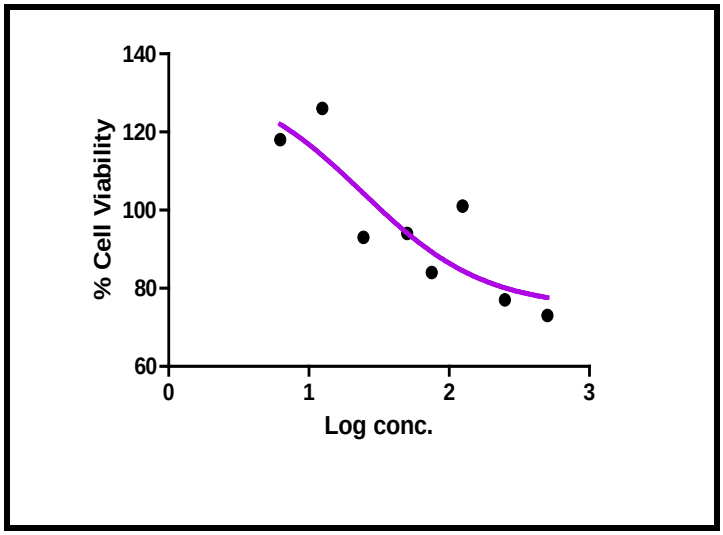


Figure 16B: Cell viability determined by MTT assay of TVB-treated SNO cells for 24hrs used to determine the IC_{50} for TVB extracts.

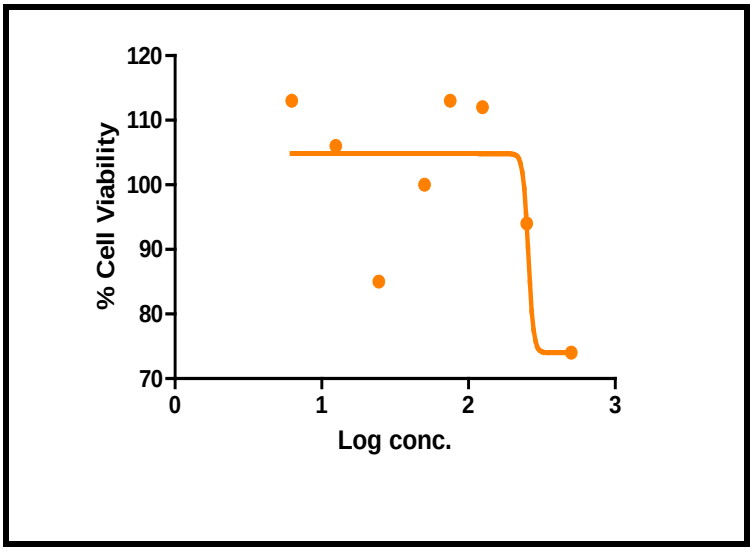


Figure 16C: Cell viability determined by MTT Assay of TVL-treated SNO cells for 24hrs used to determine the IC_{50} for TVL extracts.

4.2. Mitochondrial membrane potential in SNO cells

The JC-1 stain was utilised to determine changes in ΔΨm following treatment with the various extracts and results are represented as % depolarisation that gave an indication of mitochondrial integrity. Decreased mitochondrial membrane potential is associated with decreased polarisation. Untreated SNO cells contained the highest percentage of depolarised mitochondria as compared to all treated cells (Figure 17). Mitochondrial depolarisation was significantly decreased following exposure to all the extracts (***p= 0.0003). The % of depolarised mitochondria was more than halved, decreasing from 28±2% in the control to 12±2% following exposure to the garlic extract,15±2% following exposure to TVB, 11±2% following exposure to TVL and 7%±2% following exposure to combination treatment (p< 0.05) (Figure 17). Combination treatment and TVL displayed the lowest % depolarisation.

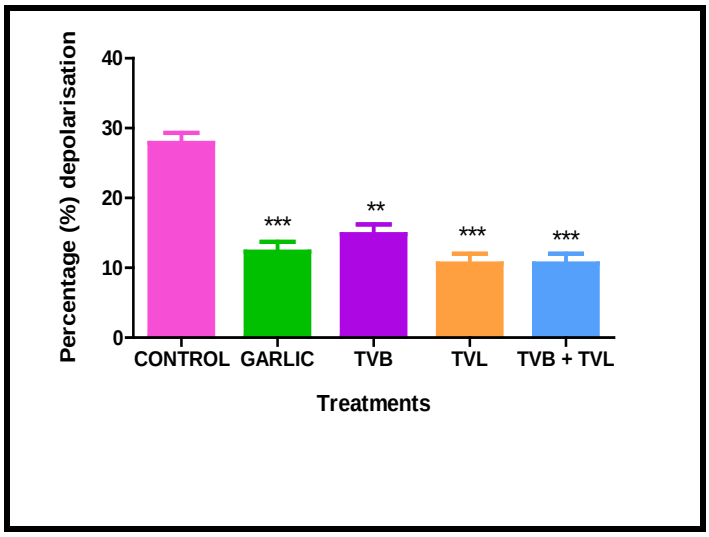


Figure 17: Mitochondrial depolarisation decreased significantly in all treated cells as compared to the control (p< 0.05). (**p< 0.001 and ***p< 0.0001).

4.3. The effect of garlic and TV extracts on the metabolic activity of SNO cells

Metabolic activity of SNO cells was assessed by using CellTitre-Glo® cell viability assay. The ATP production increased from 4617000 ± 190300RLU in the control to 8597000 ± 14480 in garlic treated cells, representing an approximately 2-fold significant increase($p < 0.05$)(Figure 18).Similarly, ATP levels were significantly increased in combination treatment($p < 0.05$). No significant differences were noted in TVL and TVB treatments ($p > 0.05$) (Figure 18).

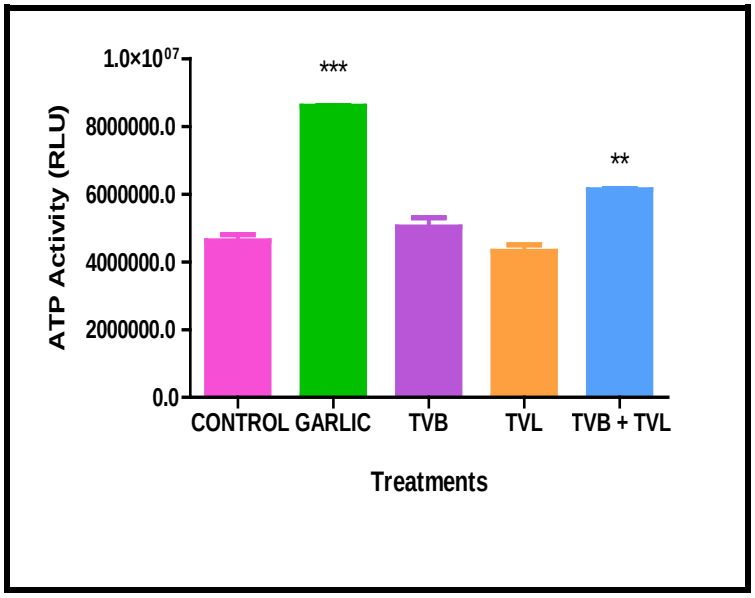


Figure 18: The effect of TV and garlic on ATP levels in treated SNO cells. ATP levels increased significantly in garlic and combination (TVB + TVL) treated cells as compared to the control ($p < 0.05$), but were non-significantly decreased in TVL-treated cells as compared to untreated cells ($p > 0.05$). (**$p < 0.001$ and ***$p < 0.0001$).

4.4. The effect of TVL, TVB and garlic extracts on lipid peroxidation in SNO cells

Lipid peroxidation was assessed using the TBARS assay and reported as [MDA] (Figure 19). One-way ANOVA showed that the data was significantly different from each other (**p= 0.0011). However, the only significant increase in [MDA] was displayed by TVL (0.212µM) (p< 0.05). The [MDA] in the control was 0.165µM, which represents basal levels of MDA in the cell that occurs as a normal part of metabolism (Figure 19). Garlic caused [MDA] to decrease to 0.139µM (Figure 19). Treatment with TVB did not alter [MDA] relative to the control (p> 0.05), but the combination decreased [MDA] as compared to the control (p> 0.05) (Figure 16).The decreased [MDA] (Figure 19) in the garlic and combined treatments corresponds to increased ATP production (Figure 18) in the samples.

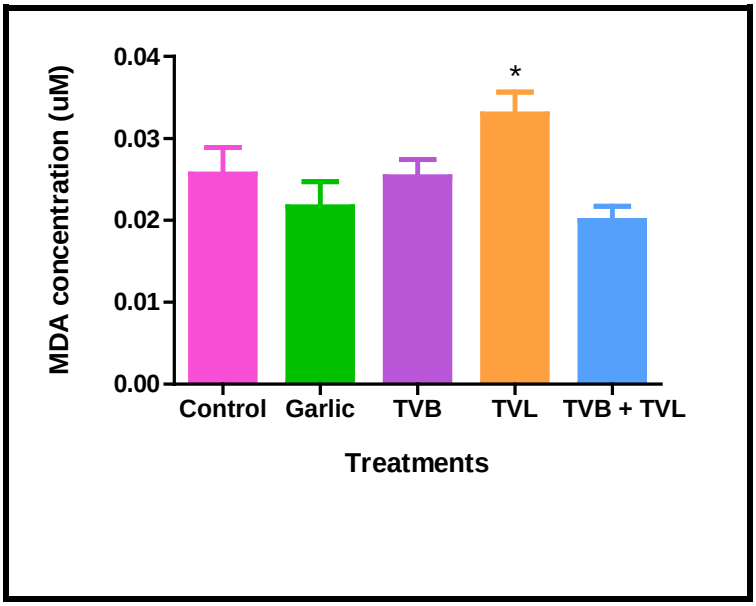


Figure 19: Lipid peroxidation represented as [MDA] in TV and garlic treated SNO cells. The TVL extract showed the significantly highest level of lipid peroxidation (p< 0.05), while garlic and combination treatments decreased lipid peroxidation (p< 0.05) compared to the control. (*p< 0.05).

4.5. The antioxidant (GSH) activity conferred by garlic and TV extracts in SNO cells

Intracellular GSH concentrations were measured in TV and garlic `treated SNO cells. Basal GSH levels were recorded as 20.8µMin the control. Garlic caused a significant increase in GSH concentration as compared to the control ($p< 0.05$) (Figure 20). In contrast, all TV extracts caused a significant decrease in GSH concentrations compared to the control ($p< 0.05$), with GSH concentration decreasing to 12.5µM, 11.3µM and 10.4µM in TVB, TVL and combined treatments respectively (Figure 20).

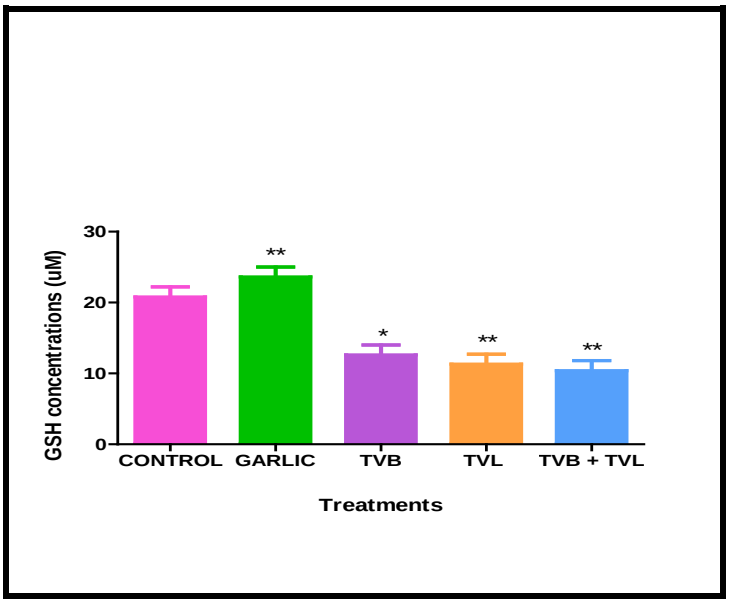


Figure 20: Intracellular GSH concentrations were significantly increased in garlic treated SNO cells ($p< 0.05$). However, a notably significant decrease in GSH concentrations occurred for all TV treatments ($p< 0.05$). (*$p< 0.05$ and **$p< 0.001$).

4.6. Garlic and TV extracts effects on initiator caspase 8 and 9 and effector caspase 3/7 activities in SNO cells

Effector and initiator activities were assessed in TV and garlic treated SNO cells. Caspase 8 activity increased significantly in garlic ($p< 0.05$) and combination treatments ($p< 0.05$) compared to the control (Figure 21A). A similar trend was observed for caspase 9 activity, where garlic extracts caused a greater than 2-fold increase ($p< 0.05$) in caspase 9 activity and combination treatment almost doubled ($p< 0.05$) caspase 9 activity (Figure 21B). In response to initiator activation, executioner caspase 3/7 activity also significantly increased in garlic ($p< 0.05$) and combination-treated cells ($p< 0.05$) (Figure 21C). Initiator and executioner activities in TVL ($p> 0.05$) and TVB ($p> 0.05$) treatments did not significantly differ from the control ($p> 0.05$) (Figure 21A-C).

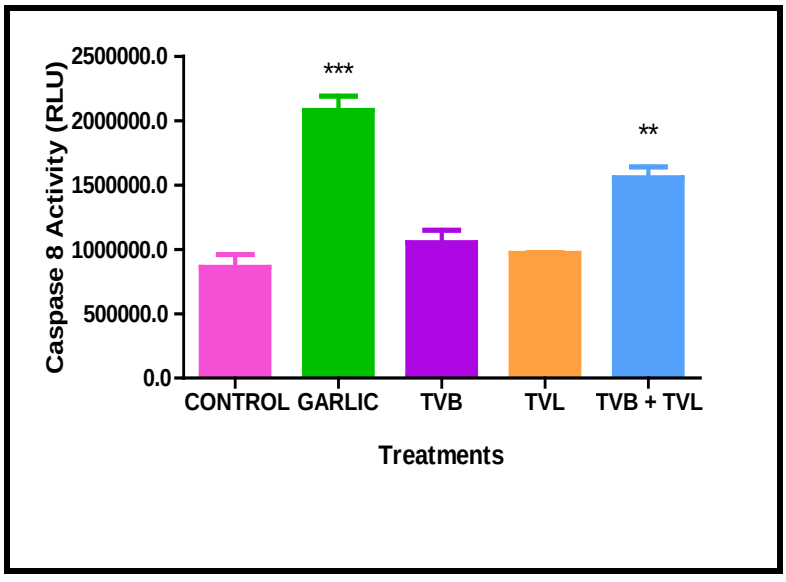


Figure 21A: Caspase 8 activity in treated cells was compared to the control. Caspase 8 activity was increased significantly in garlic ($p< 0.05$) and combination ($p< 0.05$) treated cells. Activity was not significantly altered in TVB and TVL treated cells ($p > 0.05$). (**$p< 0.001$ and ***$p< 0.0001$).

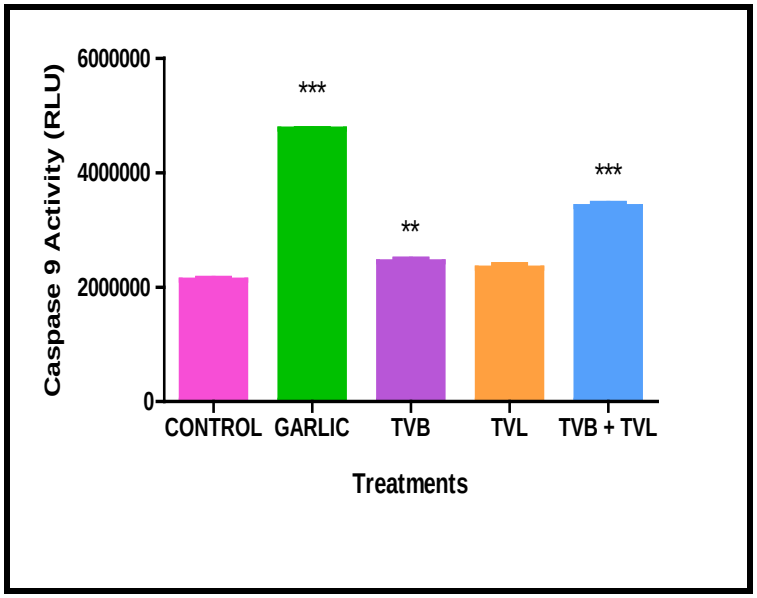


Figure 21B: Caspase 9 activity in treated cells was compared to the control. Caspase 9 activity was increased significantly in garlic ($p< 0.05$) and combination ($p< 0.05$) treated cells. Activity was not significantly altered in TVB and TVL treated cells ($p > 0.05$). (**$p< 0.001$ and ***$p< 0.0001$).

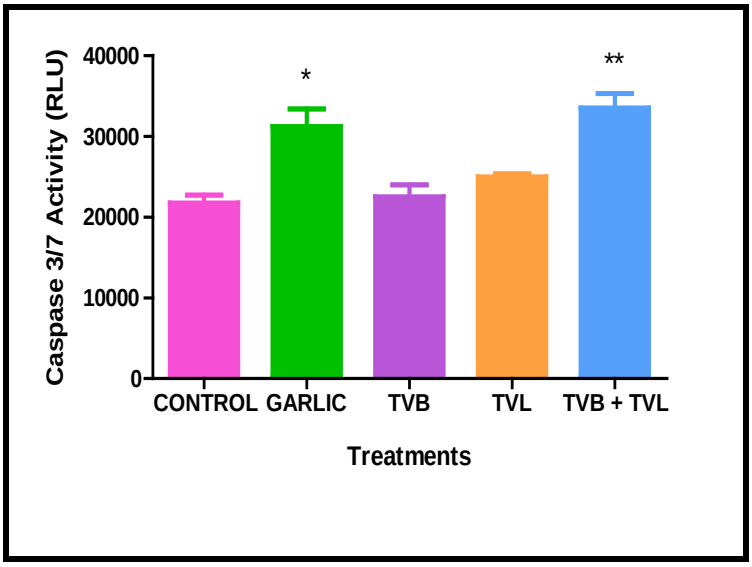
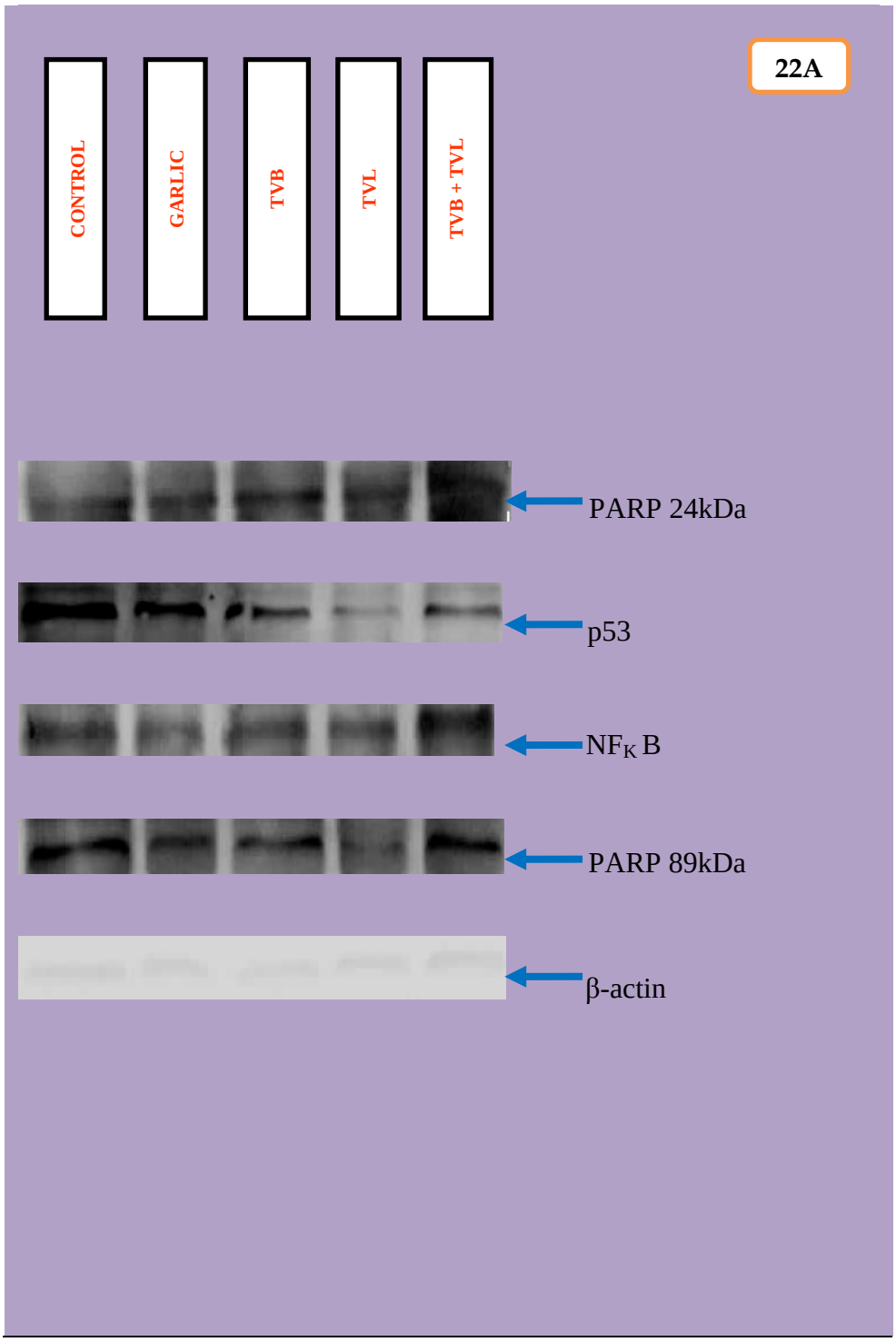


Figure 21C: Caspase 3/7 activity in treated cells was compared to the control. Caspase 3/7 activity was increased significantly in garlic ($p< 0.05$) and combination ($p< 0.05$) treated cells. Activity was not significantly altered in TVB and TVL treated cells ($p > 0.05$). (*$p< 0.05$ and **$p< 0.001$).

4.7. Detection of protein expression in SNO cells

To elucidate the precise apoptotic signalling pathway by which TV executes apoptosis in SNO cells, western blotting was employed to determine the protein expressions of three regulatory proteins in treated cells. The three proteins investigated were p53, PARP and NF_KB. Following treatment with the various extracts, p53 expression was decreased in all treatments (Figure 22A). Band intensity was slightly decreased following treatment with garlic ($p > 0.05$), whereas p53 expression was significantly downregulated in all TV treatments ($p < 0.05$), with the most significant decrease occurring for TVL treatments (Figure 22B). A slight decrease occurred in expression of NF_KB following exposure to the various extracts, however this was not significant ($p > 0.05$) (Figure 22A and 22B). The PARP segment was activated into 89kDa fragments and 24kDa fragments in both treated and untreated SNO cells. Garlic and TVL extracts caused a significant decrease in PARP 89kDa fragment ($p < 0.05$) (Figure 22A and 22B); however a slight but non-significant decrease in PARP 24kDa fragment ($p > 0.05$) was observed for all treatments (Figure 22A and 22B). Similarly, PARP 89kDa expression was non-significantly downregulated following exposure to TVB and combination treatments ($p < 0.05$) (Figure 22A and 22B).


22A
CONTROL
GARLIC
TVB
TVL
TVB + TVL
PARP 24kDa
p53
NF$_K$ B
PARP 89kDa
β-actin

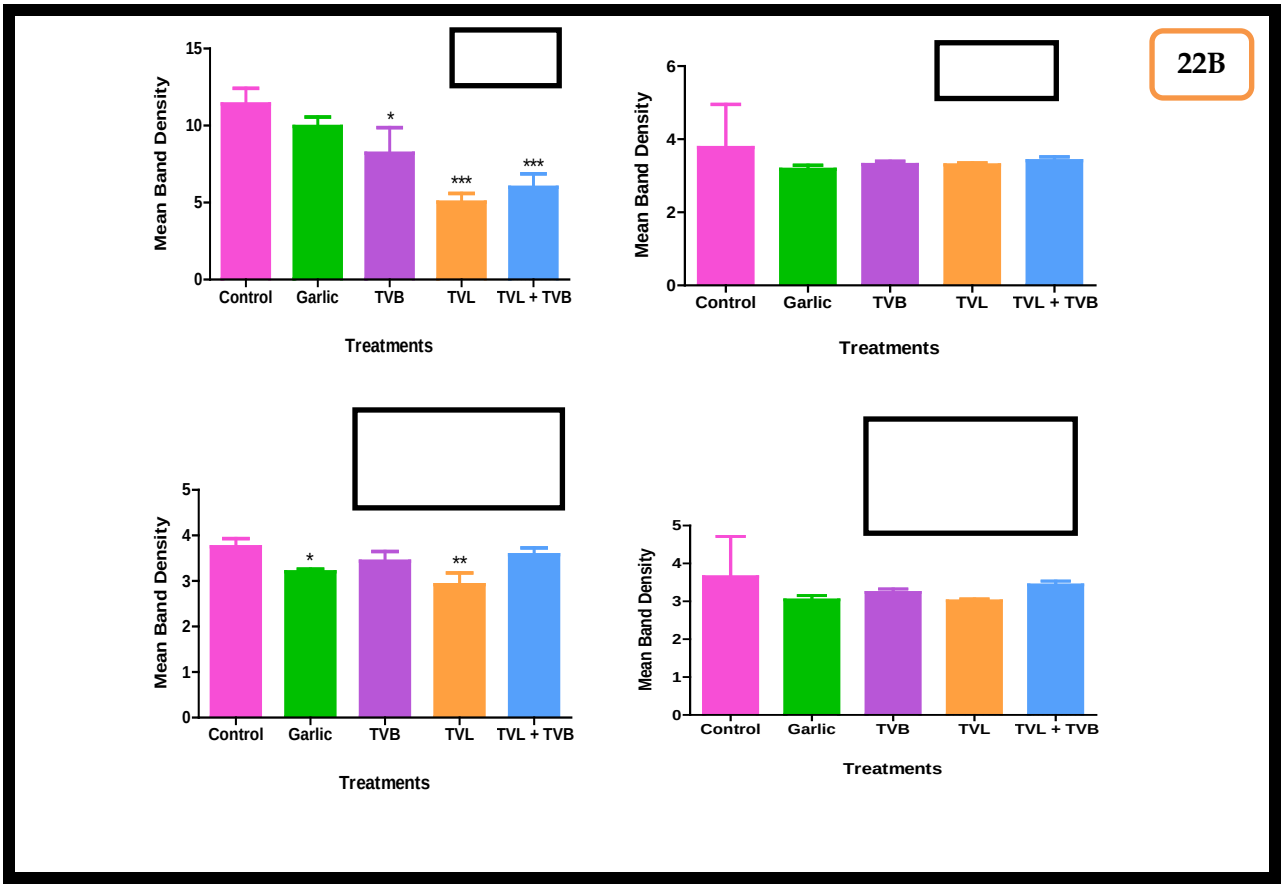
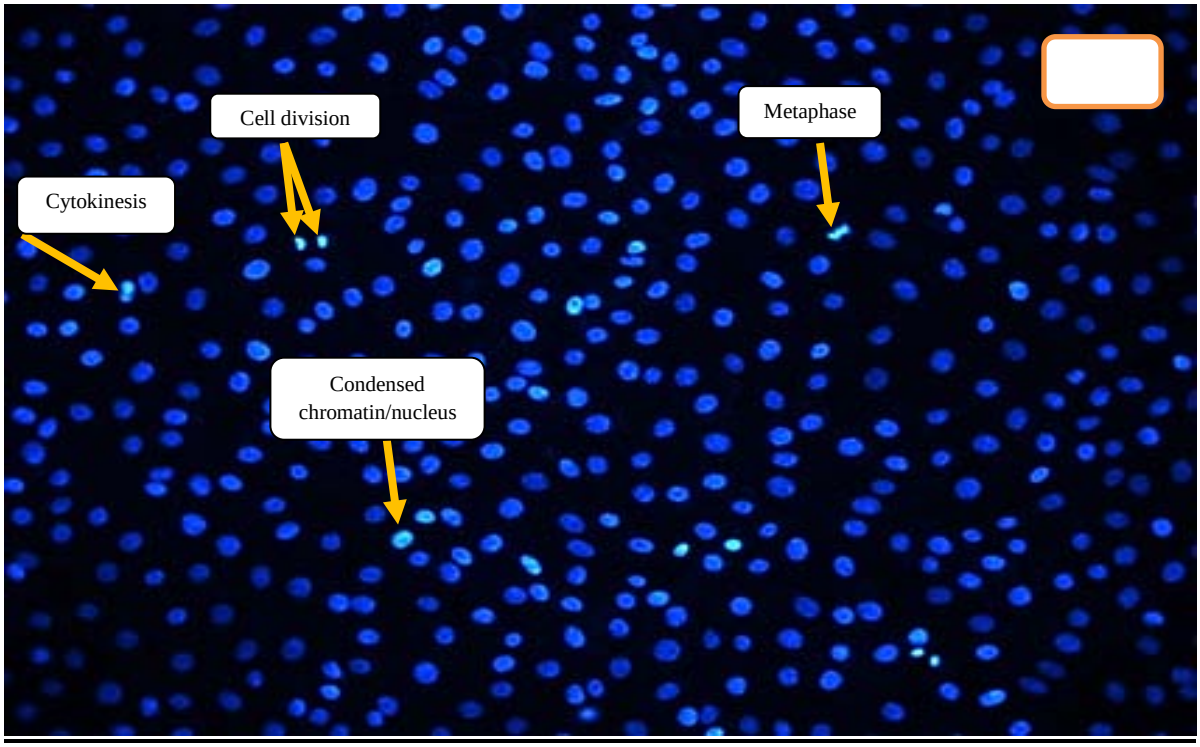


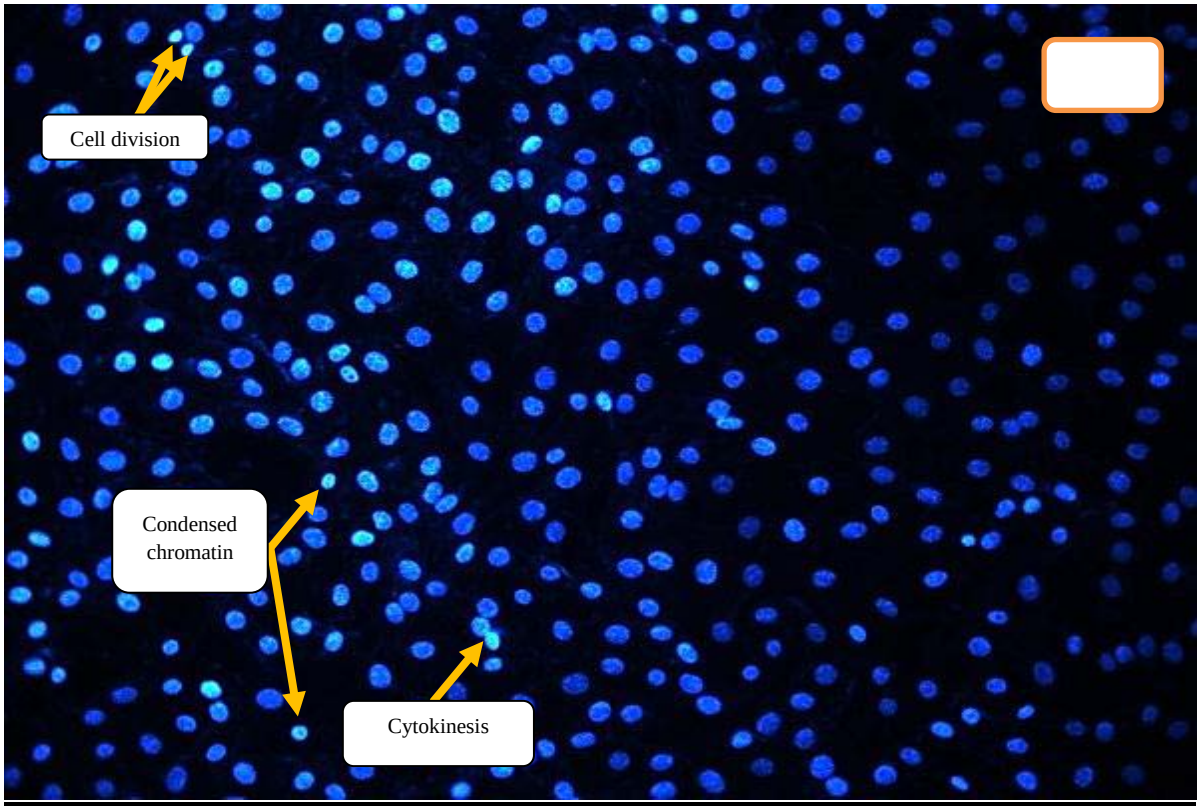
Figure 22: Detection of p53, PARP and NF_KB expression in SNO cells following treatment with garlic and TV extracts for 24hrs. **A:** Representative of western blot analysis of p53, NF_K B, PARP 24kDa and PARP 89kDa fragments with β-actin as a reference point. **B:** Band intensities obtained for all treatments show that protein expression was unaltered or slightly downregulated. (*p< 0.05, **p< 0.001and ***p< 0.0001).

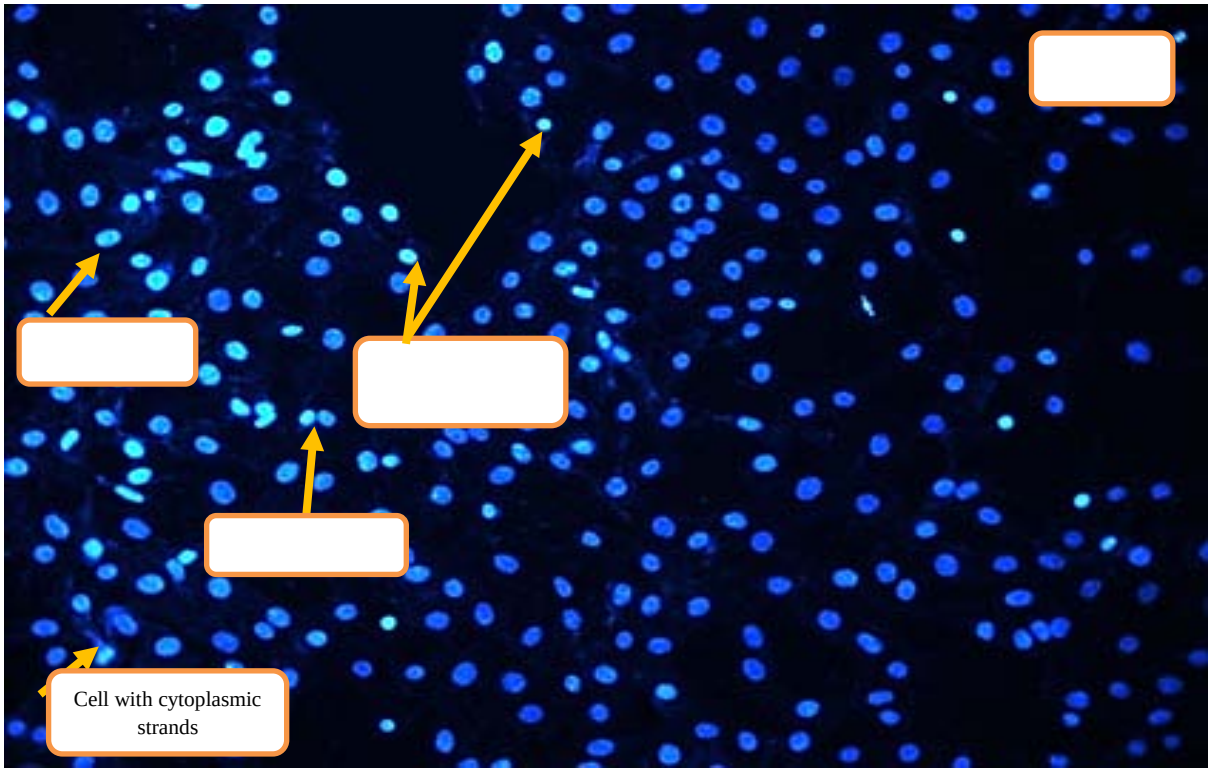
4.8. Fragmented nuclear condensation in SNO cells

Hoechst 33342 stain was used to analyse chromatin condensation, a hallmark of apoptosis, when SNO cells were treated with TV and garlic extracts. In the various images, SNO cells exposed to the various extracts showed cells observed at different stages of the cell cycle, such as cytoplasm condensed, cell division and cytokinesis or cells in metaphase (Figure

23A-E). Although nuclei were uniformly stained in the control cells, there was evidence of cell division and separation, as well as cells were observed in the metaphase of the cell cycle (Figure 23A). SNO cells following exposure to the garlic extract displayed a greater amount of cells/nuclei that showed chromatin condensation. Similarly, cell division and splitting was also noted in Figure 23B and 23C. However, in Figure 23C, it showed many TVL-treated cells/nuclei that were most brightly fluorescent indicating condensation of chromatin. Also seen in Figure 23C, cells were observed in metaphase and some cells were attached to cytoplasmic strands. The number of cells treated with the TVB extract did not differ from the control (Figure 23D). Combination treated cells showed few cells with brightly fluorescent nuclei, which gave an indication of condensed chromatin (Figure 23E).




Cell division
Condensed chromatin
Cytokinesis


Cell with cytoplasmic strands

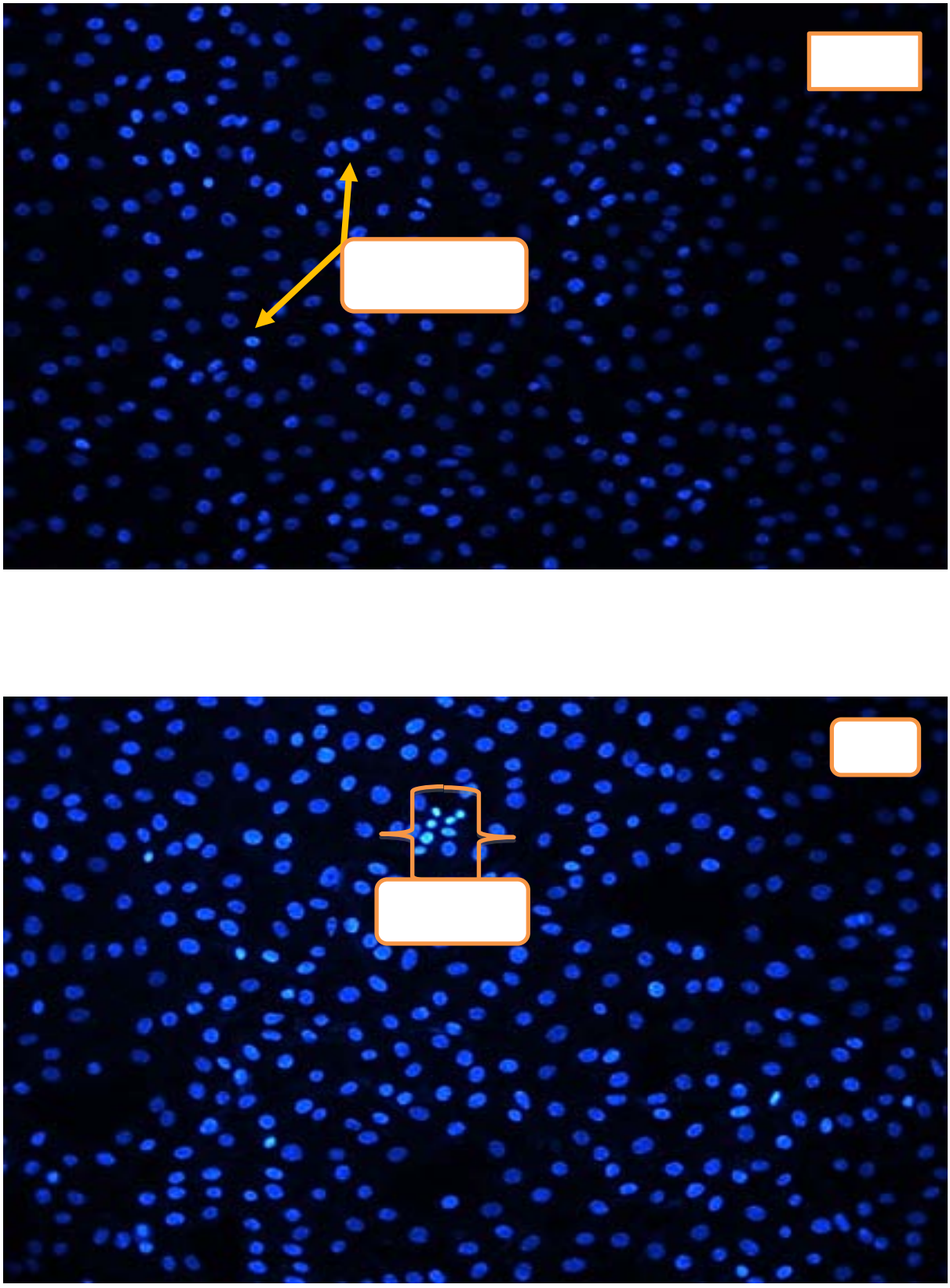


Figure 23: SNO cells stained with Hoechst 33342 dye showing SNO cells at different stages of mitosis (200X).Untreated cells **(A)**, 500µg/ml garlic-treated cells **(B)**, 125µg/ml TVL-treated cells **(C)**, 25µg/ml TVB-treated cells **(D)**, and combination (TVB + TVL)-treated cells **(E)** for a 24hr incubation.

CHAPTER 5

DISCUSSION

Numerous studies have reported that garlic and its constituents are actively anti-proliferative and inhibit tumour growth both *in vivo* and *in vitro* (Shukla and Kalra, 2007; Omar and Al-Wabel, 2010). Indeed, TV extracts have been used by the ethnic population as a remedy against cancer (Bungu *et al*., 2006).However, molecular mechanisms remain unknown; therefore this study investigated the molecular effects of garlic and TV extracts on cancerous SNO cells.

In this study, the MTT assay was used as an indicator of cell viability and cytotoxicity. Generally, the MTT assay measures intracellular levels of NADH, as reduced NADH levels is often correlated to decreased cell viability (Mosmann, 1983). However, in the present study, none of the % cell viability values were below 50% for any given concentration (Appendix I, Tables 2-4), with an average viability for the bulb treatment ranging from 73%-128% and that of the leaf extract ranging from 73%-113%. Although a true IC_{50} concentration could not be obtained, the values obtained from Graphpad analysis (25µg/ml and 125µg/ml) because they concurred with documented values used in other studies (Bungu *et al*., 2006). The MTT assay results were confirmed by measuring intracellular ATP levels, which served as an alternative indicator of cell viability. Using the above mentioned IC_{50} concentrations and the Cell-Titre-Glo® method, increased ATP production was shown in the combination-treated cells. In addition, ATP levels for TVB and TVL exposed cells did not

differ from the control (Figure 18). This implies that these IC_{50} concentrations had no profound effect on cell viability.

The results of this investigation have shown that SNO cells are susceptible to the effects of garlic and TV extracts *in vitro*. This study showed that exposure to TV and garlic extracts caused a significant decrease in depolarised mitochondria (Figure 17) and maintenance and/or increased ATP production (Figure 18). Depolarisation is associated with disruption of the electron transport chain gradient (ETC) and dissipation of the mitochondrial membrane potential (Chowdhury*et al*., 2008). The increase in polarised mitochondria that occurred following exposure to the extracts is associated with an intact ETC and mitochondrial membrane potential conducive to the production of ATP (Chowdhury*et al*., 2008). Indeed, garlic and combination treatments caused an increase in ATP concentrations. Increased levels of glucose uptake were observed following treatment of Chang liver cells and C2C12 muscle cells with aqueous and ethanol root TV extracts (Van Huyssteen *et al*., 2011). Therefore, the extracts might have increased the rate of glucose uptake and as a result increased the rate of glycolysis, leading to an enhanced rate of the functioning of the Krebs cycle and consequently a production of reducing equivalents such as NADH and $FADH_2$. These reducing equivalents are the main substrates that create electrochemical gradients across the mitochondrial membranes when fed into the ETCat complex I and II, resulting in increased ATP production (Figure 18). Therefore, TV and garlic extracts exert a positive effect on metabolism and thus contributes to the increasing levels of cellular ATP.

Mitochondria are the principle sites for ROS production (Ott *et al*., 2007). During electron transfer that occurs in the process of energy production, some electrons react with oxygen to

form free-radicals that may cause oxidative damage to cellular macromolecules including lipids and DNA (Ott *et al*., 2007). This would account for residual amounts of MDA found in the control cells (Figure 19).Polyunsaturated fatty acids found in membrane lipids are susceptible to oxidative damage, thus allowing for lipid peroxyl radical formation (Halliwell and Chirico, 1993). However, cells have endogenous antioxidant reserves that neutralise free-radicals and counteract their damaging effects. When these protective mechanisms are overwhelmed oxidative stress occurs (Halliwell and Chirico, 1993). Exposure to TVL extracts caused significant increase in MDA levels compared to other treatments and the control (Figure 19), while these levels did not vary significantly from the control in other treatments. All TV treatments significantly reduced intracellular GSH concentrations, but only garlic caused a significant increase in GSH concentrations (Figure 20).

Literature suggests that medicinal plants possess excellent free radical scavenging properties owing to their phytochemicals, and as a result offers protection against oxidative stress in cancer cells (Madhuri and Pandey, 2005; Ncube *et al*., 2011).The increased GSH and decreased ROS following garlic exposure may be attributed to the presence of SAC which would replenish the oxidised GSH (Yedjou and Tchounwou, 2012). Additionally, the sulfhydryl (SH) group on SAC may be responsible for the detoxification of ROS. In this study, decreased endogenous GSH concentrations in TV extracts may be viewed as an attempt to neutralise ROS that has been produced and may be viewed as successful in TVB (Figure 20). However, whether the increased ROS in TVL may be attributed to the low concentration of phenolic compounds in the leaf compared to garlic and TVB remains undetermined because no studies have compared the concentrations of the antioxidants in the different plant components. Additionally, TV leaf extracts may possibly contain low

concentrations of SH-containing compounds which may have facilitated the ROS detoxification.

Olorunnisola *et al.* (2011), have demonstrated that methanol root extracts of TV possess excessive amounts of flavonoids, tannins and phenolic compounds and they also reported that antioxidant potentials occurred in a time-dependent manner (Olorunnisola *et al*., 2011).Likewise, other authors have revealed the high phenolic content of garlic (Amagase, 2006).Even though TVB may have the highest concentration of allicin, which would contribute to increase ROS, this was counteracted by the high levels of endogenous and phenolic compounds. However, it may be that lower levels of phenolic and SH compounds are present in the leaves that are insufficient to counteract the effects of allicin. Phenolic compounds have been reported to have crucial implications as the key molecules responsible for the antioxidant properties of garlic and TV (Amagase *et al*., 2001; Amagase, 2006; Olorunnisola *et al*., 2011; 2012a; 2012b).

The increased ROS targets the cell membranes causing recruitment of death domain receptors involved in apoptosis (Ott *et al*., 2007, Chowdhury *et al*., 2008). Apoptosis is the principle mechanism used by severalanti-cancer agents including TV (Hajra and Liu, 2004; Bungu *et al*., 2006; Shukla and Kalra, 2007; Chowdhury et al., 2008; Omar and Al-Wabel, 2010). The levels of initiator caspases 8 and 9 and executioner caspase 3/7 were measured in order to validate whether the mode of cell death induced by TV extracts and garlic were caspase-dependent. In this study, the garlic and combined treatments significantly increased the levels of caspase 8 (Figure 21A), caspase 9 (Figure 21B) and caspase 3/7 (Figure 21C), respectively. Interestingly the individual TV extracts did not induce significant differences in

all caspases tested (Figure 21A-C).Therefore, the garlic extract and combination treatments were effective in inducing apoptosis in the treated SNO cells. This implies that garlic and combination treatments are causing apoptosis by extrinsic and intrinsic pathways and are caspase-dependent.

Balasenthi *et al*. (2002), concluded that garlic's anti-cancer effects are because of induction of apoptosis (Balasenthi *et al*., 2002). Organosulphurous compounds of garlic are responsible for the induction of cell death (Bungu *et al*., 2006; Omar and Al-Wabel, 2010).An experimental study demonstrated that allicin induced apoptosis in four cancer cell lines *viz.* HeLa, SiHa, SW-480 and L-929 cancer cells via caspase dependent mechanisms (Bungu *et al*., 2006), whereas Park *et al*. (2004), revealed from their studies on an epithelial cancer cell line, that allicin caused cell death via caspase independent mechanisms (Park *et al*., 2004).It has been hypothesised that TV has similar biological activities and compounds as domesticated garlic, since both belong to the same *Alliaecea* family (Bungu *et al*., 2006).Thus, the activation of caspases observed in the combined treatment confirms the induction of apoptosis in these cells. The *Tulbaghia* extracts could possibly cause apoptosis via the intrinsic and extrinsic apoptotic pathways in SNO cells and is therefore anti-proliferative.

Studies have reported that caspase activation is linked to PARP cleavage, where caspase 3/7 is responsible for cleaving the 113kDa PARP fragment into two segments, a larger 89kDa fragment and a smaller 24kDa fragment (Bressenot*et al*., 2009). This is evident in the present study, when cells were exposed to the various extracts, the 113kDa PARP segment cleaved into its 89kDa and 24kDa segments, respectively (Figure 22A and 22B). Also, PARP

cleavage is an indication that apoptosis has been induced, because the high of levels of active caspase 3/7 activity (Figure 21C) and the morphological changes observed in the Hoechst 33342 dye staining (Figure 23A-E) correlates to the time-dependent PARP cleavage (Figure 22A and 22B). Therefore, it may be deduced that the extracts are partial potent inducers of PARP cleavage and apoptosis in these oesophageal cancerous cells. However, this effect is minimal as demonstrated by the significantly decreased PARP cleavage levels of both the 89kDa and 24kDa fragments following exposure to the extracts (Figure 22A and 22B). A possible mechanism may be a blockage that occurs between caspase 3/7 and the onset of PARP cleavage. An experimental study has reported that ajoene is responsible for the induction of PARP cleavage in cancer cells *in vitro* (Bungu *et al*., 2006). Hence, TV extracts may be inducing a caspase 3/7 independent apoptotic pathway in SNO cells.

Poly(ADP-ribose)polymerase cleavage has been shown to have an effect on downstream effectors such as p53 (Yelamos *et al*., 2011). The transcription of various genes is regulated by the binding of p53 to the DNA in sequence-specific manner (Benchimol, 2001). Mutation of p53 alleles results in the aberrant transcription of genes or defective products that do not bind to DNA, thus favouring tumorigenesis (Benchimol, 2001).An experimental study has shown that oesophageal cancer cells express p53 genes (Rigberg *et al*., 1999). In this study, p53 expression was decreased in all treatments (Figure 22A and 22B). The possible mechanism may be as a result of mutated p53 allele functioning. The decreased p53 levels may be due to the decreased ROS levels and reduced oxidative stress, as evident in garlic andcombination treatments (Figure 19). As oxidative stress functions as one of the endogenous signals for p53 activation(Benchimol, 2001), thus decreased lipid peroxidation, leads to reduced oxidative damage and hence contributed to significantly reduced p53 levels in all TV treatments.

In the present study, a slight decrease occurred in expression of NF_KB following exposure to the various extracts (Figure 22A and 22B). Studies have stated that garlic extracts have potent antioxidant abilities that indirectly depress NF_KB activities and hence play a role in preventing tumour proliferation; whereas DAS and thiacremonone found in garlic directly inhibit NF_KB activity (Hoffnung, 2011). Therefore, the presence of DAS and thiacremonone compounds in the garlic and TV extracts may be responsible for the downregulation of NF_KB. Another possible mechanism may be due to the potent linking of antioxidants such as GSH to NF_KB activity (Hoffnung, 2011).Therefore, TV extracts directly downregulates NF_KB activity in SNO cells through its constituents and prevents SNO cell proliferation; whereas garlic induces a direct and indirect effect on NF_KB activity in SNO cells through its antioxidants and bioactive compounds preventing cancer progression.

T.violacea extracts and garlic induce cell death in the oesophageal cancerous cells, as demonstrated by the nuclear condensed chromatin in the control, TV-treated and garlic-treated cells stained with Hoechst 33342 dyes, which are hallmarks of apoptosis (Figure 23A-E). These results are in accordance with other research. For instance, Bungu and colleagues (2006), concluded from experiments on four TV-treated cancer cell lines that cells stained with Hoechst 33342 dye displayed nuclear condensed chromatin and fragmented DNA and so concluded that the extract induced apoptosis in the four cancer cell lines (Bungu *et al*., 2006). Therefore TVcauses cell death in cancerous cells exposed to this plant extract and is anti-proliferative in nature. Thus, these cancerous cells use apoptosis as a mode of cell fate instead of necrosis.

CHAPTER 6

CONCLUSION

This study investigated the effects of garlic, TV leaf and TV bulb individually and in combination on oesophageal cancer cell line (SNO). All the TV extracts and garlic induced apoptosis in the oesophageal cancerous SNO cell line. The production of ROS potentiates lipid peroxidation and in return this causes changes in the ETC or plasma membranes, which trigger activation of initiator caspases and these, activate effector caspases and then effector caspases have further downstream effects by causing PARP cleavage. Thus, apoptosis is being induced in the SNO cells. Furthermore, DNA damage induced by all the extracts was observed by changes in nuclear chromatin condensation and thus DNA damage lead to activation of further downstream signalling proteins or genes that included activation of p53 and NF_KB.

The results of this investigation have shown that SNO cells are susceptible to the effects of garlic and TV extracts *in vitro*. This study showed that exposure of the TV and garlic extracts to SNO cells resulted in a decrease of the percentage of depolarised cells and maintenance and/or increased ATP production. Exposure of TVL extracts significantly induced lipid peroxidation in the SNO cells, whilst exposure to TVB and garlic extracts induced minimal amounts of lipid peroxidation in the SNO cells. Interestingly, the exposure of the TV extracts to SNO cells significantly decreased intracellular GSH concentrations, whereas exposure of the garlic extract to the SNO cells significantly increased GSH concentrations. Exposure of the TV extracts to the SNO cells induced apoptosis, but more interestingly, the combination

extracts induced significant pro-apoptotic effects in the SNO cells. The TV extracts downregulate the protein expression levels of apoptosis-related proteins in SNO cells.

Since inhibition of apoptosis is a principal alteration in cancer, induction of apoptosis would result in the removal of cells through the cell death process and may be the mechanism by which TV and garlic exerts its anti-proliferative effects. Thus apoptosis is being induced, making these plant extracts potentially effective as therapeutic agents and may be exploited as potential anti-cancer agents.

In this study, SNO cells had only exposure to a 24hr treatment regime before subsequent assays were conducted. Possibly these cells should be exposed for 48hrs and 72hrs to determine chronic effects. Also, in the present study methanol extraction was conducted, possibly a water extraction vs. a methanol extraction to compare the effects of both extractions on the effects it produces on the SNO cells and to determine which extraction method is more effective.

As not much research has been conducted on TV and its safety and efficacy as a possibly therapeutic agent, a comparison should be conducted of a cancerous cell line to a normal cell line. Also, as DNA fragmentation is a morphological change of apoptosis, the comet and annexin assays should be conducted to fully elucidate apoptosis as the principle mechanism responsible for TV and garlic’s anti-cancer properties.

As ROS are not the only free-radicals produced in cells, a detection of other radicals such as reactive nitrogen species (RNS) using the nitrates assay should be conducted and a comparison of both ROS and RNS to determine which free-radical species causes a higher vs. a lower degree of oxidative stress in cancerous cells or in both normal vs. cancerous cells. Furthermore, maybe a detection of changes in heat shock protein expression as this is important in relation to ROS formation.

As TV belongs to the same plant family as garlic, therefore studies have postulated that TV may contain the same bioactive compounds that are found in garlic and hence similar functions as that of garlic. Therefore, a comparison to purified bioactive components of the crude extracts should be conducted to determine what actual biological effects these extracts share.

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APPENDIX

AppendixA: Reagent lists-Preparation of plant extracts section

5% Complete culture media (CCM):

Add these reagents to 500ml Eagles minimum essential medium (EMEM):

25ml (5%) foetal calf serum

5ml(1%) Penstrepfungizone

5ml (1%) L-glutamine

Swirl and tilt the bottle to allow mixing of components.

Store 5% CCM at -4ºC if not used and if 5% CCM is to be used, place in a 37ºC incubator for 30mins for warming up.

Plant stock solution for each extract (1mg/ml):

Weigh out 10mg of each plant extract powder (i.e. TVB, TVL, garlic) and dissolve in 10ml of 5% CCM to yield a stock solution of 1mg/ml.

Used stock solution to prepare serial dilutions as follows: $C_1V_1 = C_2V_2$, where C_1 = initial stock solution (1mg/ml), V_1 = x (initial concentration of extract solution, µl), C_2 = range of final concentration values chosen (µg) and V_2 =final chosen stock concentration (µl).

Table1: Serial dilutions prepared for each plant extract for the 24hr MTT assay.

Concentration Range (µg/ml)	**Extract Solution (µg/ml)**	**5% CCM (µl)**
0	0	2000
6.25	12.5	1987.5
12.5	25	1975
25	50	1950
50	100	1900
75	150	1850
125	250	1750
250	500	1500
500	1000	1000

Appendix B: Reagent lists-Cell culture maintenance section

0.1M Phosphate buffered saline solution (1L PBS):

Stock A: 0.2M Sodium dihydrogen orthophosphate (Dissolve 6.24g in 200ml dH_2O)

Stock B: 0.2M disodium hydrogen orthophosphate (Dissolve 11.32g in 400ml dH_2O)

Stock C: 1M Sodium chloride (Dissolve 4.25g in 500ml dH_2O)

Then add 160ml of Stock A and 340ml of Stock B to 500ml of Stock C and pH must be adjusted to pH 7.4 and PBS must be autoclaved twice before usage.

Appendix C: Reagent lists-Methylthiazoltetrazolium section

Methylthiazoltetrazolium (MTT) salt preparation:

Add these reagents to yield 5mg/ml MTT reagent:

10mg MTT salt

2ml PBS

Dissolve MTT salt (10mg) in Phosphate Buffered Saline Solution (PBS) (2ml), to yield a concentration of 5mg/ml in a 15mlsterlin tube that is then wrapped in aluminum foil and stored in a dark cupboard at 25°C.

Appendix D: Reagent lists-Mitochondrial membrane potential and Cell viability/ATP section

JC-1 dye preparation:

Take one vial of JC-1 obtained in the reagent kit and reconstitute in 125µl DMSO. Then seal the vial and invert several times to allow for mixing of contents and yield a JC-1 dye stock solution.

CellTitre-Glo® working reagent:

Remove both the CellTitre-Glo® buffer and CellTitre-Glo® substrate from storage and allow equilibrating to room temperature for 48hrs. Once equilibrated to room temperature, transfer the CellTitre-Glo® buffer into a bottle that contains the CellTitre-Glo® substrate and allow for reconstitution of the CellTitre-Glo® substrate. Recap the bottle and gently invert the bottle to allow for mixing of components to yield a CellTitre-Glo® working reagent solution.

AppendixE: Reagent lists-Lipid peroxidation and antioxidant (GSH) section

Thiobarbituric acid reactive substances (TBARS) reagents:

2% H_3PO_4 in dH_2O

Dilute 2% phosphoric acid to 50ml with dH_2O.

7% H_3PO_4 in dH_2O

Dilute 7% phosphoric acid to 50ml with dH_2O.

Thiobarbituric acid (TBA)/Butylated hydroxytoluene Solution (50ml):

0.1g NaOH

0.5g TBA

250µl BHT from 20mM BHT in ethanol stock solution

Dissolve all reagents in 40ml with dH_2O.

Then top up to 50ml with dH_2O.

A 2x GSH-Glo™ working solution:

Make up 5ml of 2x GSH-Glo™ Reagent according to the manufacturer's instructions as follows:

To 5ml GSH-Glo™ Reaction Buffer must add 100µl luciferin-NT substrate and 100µl glutathione-S-transferase. Seal the bottle and allow contents to mix by inverting the bottle. This is made up immediately when required.

Luciferin detection reagent:

A bottle of reconstitution buffer that contains esterase is added to the amber bottle of luciferin detection reagent and the bottle is recapped and contents are allowed to mix.

Appendix F: Reagent lists-Assessment of caspase activities section

Caspase-Glo® working reagent solutions:

Caspase-Glo® working reagent	Caspase-Glo® buffer	Caspase-Glo® substrate
Caspase-Glo® 8 working reagent	Caspase-Glo®8 buffer	Caspase-Glo®8 substrate
Caspase-Glo® 9 working reagent	Caspase-Glo®9 buffer	Caspase-Glo®9 substrate
Caspase-Glo® 3/7 working reagent	Caspase-Glo®3/7 buffer	Caspase-Glo®3/7 substrate

As depicted in the above table, the different Caspase-Glo® working reagent solutions are made up by adding the respective Caspase-Glo® buffers to the amber bottle of the corresponding Caspase-Glo® substrates. Thereafter, the bottle is sealed and inverted several times to allow for mixing of the components.

AppendixG: Reagent lists-Immunodetection of protein expression using western blotting section

Working solution of BCA (Used in protein standardisation only):

198µl BCA (198µl x 22 wells = 4356µl)

4µl $CuSO_4$(4µl x 22 wells = 88µl)

Bovine serum albumin (BSA) standards (Used in protein standardization only):

From 1mg/ml BSA stock (weigh 1mg BSA and add 1 ml dH2O, vortex to dilute),

BSA Standards are made up as follows:

0: 100µl dH_2O

0.2: 80µl dH_2O; 20µl BSA

0.4: 60µl dH_2O; 40µl BSA

0.6: 40µl dH_2O; 60µl BSA

0.8: 20µl dH_2O; 80µl BSA

1: 0µl dH_2O; 100µl BSA

Laemmli sample buffer (5x, 37.75ml) (Used in SDS-PAGE sample preparing only):

4.5ml Tris (1M, pH 6.8)

3g SDS

1ml Bromophenolblue (1%)

15ml Glycerol

7.5ml β-mercaptoethanol

3.75ml dH_2O

Vortex all components to become diluted.

Tank running buffer (1x, 1L) (Used in SDS-PAGE only):

Made up 1L of 1x Tank Running Buffer as follows:

Took 100ml of 10x SDS running buffer and topped up with 900ml of dH_2O and placed bottle in the refrigerator until used.

Wash buffer (1x, 1L) (Used in western blotting only):

Made up 1L of 1x TTBS Wash Buffer as follows:

Took 100ml of 10x TTBS wash buffer and added 900ml of dH_2O.

Then, adjusted pH to 7.4 and topped up to 1000ml with dH_2O.

Thereafter, add 500µl of Tween 20 and allow contents to mix well and place in the refrigerator.

2% BSA in TTBS:

Weigh out 1mg of BSA and dissolve in 50ml TTBS. Thereafter, allow contents to mix and place in refrigerator until required.

Cell lysis buffer:

Contains 200µl per well, cytobuster, 4x protease inhibitor, 4x phosphatase inhibitor.

Gels used in electrophoresis:

Components	**7.5% Resolving gel**	**4% Stacking gel**
dH_2O	4.85ml	6ml
Tris	1.25M, 2.5ml	0.5M, 2.5ml
10% SDS	100µl	100µl
Bis/Acrylamide (fridge)	2.5ml	1.33ml
10% APS	50µl	100µl
TEMED (fridge)	5µl	10µl
Total	**10ml**	**10ml**

Appendix H: Reagent lists-DNA fragmentation and nuclear condensation section

10% Paraformaldehyde (PFA) solution:

Weigh out 2g of PFA and dissolve it in 18ml of PBS.

Mix the compounds together by vortexing.

Then heat the mixture in water bath at a temperature of 60ºC to obtain a clear solution.

The pH must be adjusted to pH 7.4 and then topped up to 20ml with PBS.

Hoechst 33342 stock solution:

5µg/ml Hoechst 33342 working solution as follows:

Add 5µl of Hoechst 33342 stock solution (10mg/ml) to a sterlin tube and top up to 10ml with PBS.

Appendix I: Tables

MTT analysis

Table 2: Cell viability determined by MTT Assay for Garlic-treated SNO cells for 24hrs

SAMPLES (µg/ml)	AVERAGE	% CELL VIABILITY	LOG CONC.
0	0.416	100	
6.25	0.358	86	0.795
12.5	0.385	92	1.096
25	0.382	92	1.39
50	0.434	104	1.7
75	0.376	90	1.875
125	0.464	111	2.096
250	0.416	100	2.397
500	0.302	73	2.7

Table 3: Cell viability determined by MTT Assay for TVB-treated SNO cells for 24hrs

SAMPLES (µg/ml)	AVERAGE	% CELL VIABILITY	LOG CONC.
0	0.416	100	
6.25	0.491	118	0.795
12.5	0.524	126	1.096
25	0.385	93	1.39
50	0.391	94	1.7
75	0.348	84	1.875
125	0.419	101	2.096
250	0.322	77	2.397
500	0.302	73	2.7

Table 4: Cell viability determined by MTT Assay for TVL-treated SNO cells for 24hrs

SAMPLES (µg/ml)	AVERAGE	% CELL VIABILITY	LOG CONC.
0	0.416	100	
6.25	0.468	113	0.795
12.5	0.442	106	1.096
25	0.352	85	1.39
50	0.417	100	1.7
75	0.473	113	1.875
125	0.466	112	2.096
250	0.391	94	2.397
500	0.309	73	2.7

Appendix J: Standard curves

Glutathione (GSH) analysis section

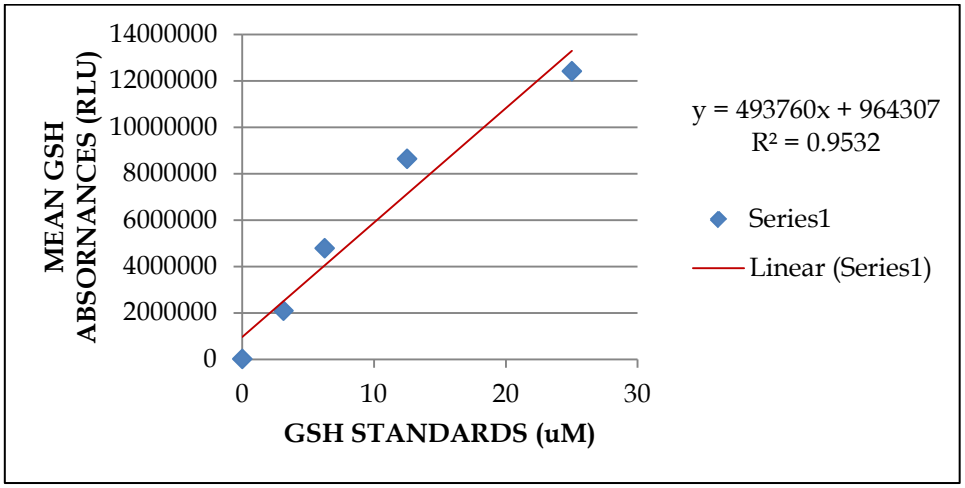

Figure 24: Glutathione standard curve with mean points from duplicates(n=2). (R^2 = 0.9532).

Protein extraction and western blot analysis section

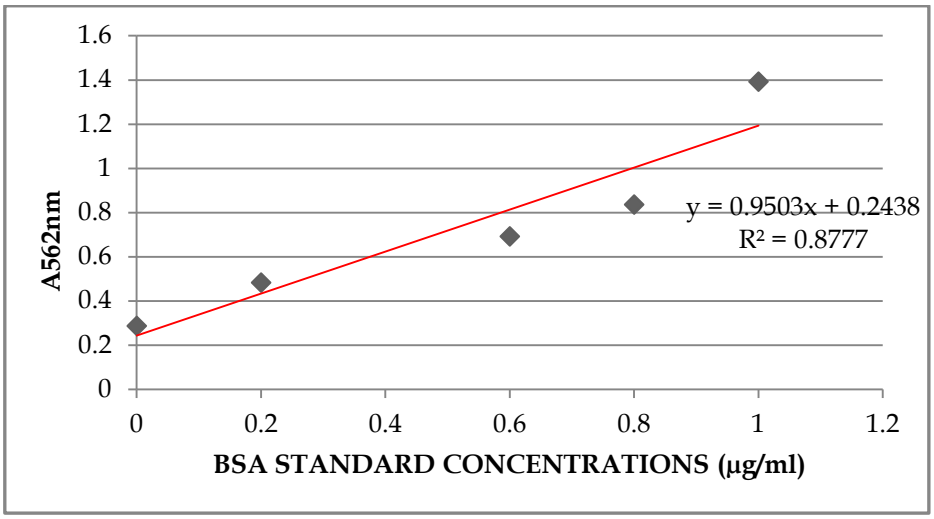

Figure 25: BSA Standard concentrations at the different absorbance values in duplicates (n = 2). (R^2 = 0.8777).