Effects of polyphenolic-rich bark extracts of *Burkea africana* and *Syzygium cordatum* on oxidative stress

by

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"Only two things are infinite, the universe and human stupidity, and I'm not sure about the former"

Albert Einstein

Table of Contents

Declaration

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Subject of the work : Effects of polyphenolic-rich bark extracts of *Burkea africana* and *Syzygium cordatum* on oxidative stress

Declaration

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Abstract

Free radicals have been implicated in the progression of various diseases, such as cancers and cardiomyopathies. When the body is overburdened with free radicals and endogenous antioxidants become depleted, oxidative stress ensues with resultant damage to biomolecules. During oxidative stress high levels of reactive oxygen species are generated, cellular viability decreases, and apoptosis and lipid peroxidation are induced. Supplementation with exogenous supplements rich in antioxidants, such as herbal remedies containing polyphenols, could result in increased protection against oxidative stress. The aim of the study was to assess the effect of *Burkea africana* and *Syzygium cordatum* in a cellular oxidative stress model for the potential development of an antioxidant supplement.

Crude aqueous and methanolic extracts were prepared by solvent maceration, while a polyphenolic-rich extract was created through liquid-liquid extraction. Polyphenolic content and antioxidant activity was assessed in cell-free systems. Polyphenolic content was determined through the Folin-Ciocalteau and aluminium trichloride methods, while antioxidant activity was assessed by the Trolox Equivalence Antioxidant Capacity and 1,1-diphenyl-2-picrylhydrazyl radical assays. Identification of phytochemical classes was done through thin layer chromatography and biochemical reactions. Inherent cytotoxicity of samples was determined in four cell cultures (3T3-L1 pre-adipocytes, C2C12 myoblasts, normal human dermal fibroblasts and U937 macrophage-like cells) using the neutral red uptake assay. The effect on oxidative stress was assessed in 2,2`-azobis-(2-methylpropionamidine) dihydrochloride-exposed U937 macrophage-like cells with regards to reactive oxygen species generation, cytotoxicity, apoptosis, lipid peroxidation and GSH depletion.

Both *B. africana* and *S. cordatum* showed enrichment of polyphenols from the aqueous extract, to methanolic extract, to polyphenolic-rich extract. Antioxidant activity showed the same trend, which correlated well with the increased concentration of polyphenols, such as catechin, gallic acid and myricetin. Samples indicated toxicity in the 3T3-L1 and C2C12 cell lines, though no toxicity was noted in

the U937 cell line and normal human dermal fibroblast cultures. Free radical-induced generation of reactive oxygen species, cytotoxicity, lipid peroxidation and apoptosis was successfully reduced by crude extracts of *B. africana* and the polyphenolic-rich extracts of both plants between concentrations of 10 and 20 µg/ml. The crude extracts of *S. cordatum* were mostly ineffective in reducing these parameters, even though cell viability was increased. *B. africana* pre-treatment decreased reduced glutathione concentrations significantly in a dose-dependent manner, while the methanolic and polyphenolic-rich extract of *S. cordatum* increased concentrations moderately.

Polyphenolic-rich extracts of *B. africana* and *S. cordatum* had the most potent decrease in oxidative stress-related parameters in the present study, which could be attributed to the polyphenolic content and antioxidant activity. Limited cytotoxicity was apparent in two of the four cell lines tested; further isolation and purification needs to be carried out to assess the bioactive constituents which do not elicit a toxic response. Further investigation through the use of quantitative structure–activity relationship modeling could give more insight on conformational and chemical changes that need to be brought about to modify the bioactive phytochemicals for reduced cytotoxicity, but increased antioxidant activity.

Keywords: AAPH, antioxidant, apoptosis, *Burkea africana*, glutathione, lipid peroxidation, oxidative stress, polyphenols, reactive oxygen species, *Syzygium cordatum*

List of Figures

List of Tables

Abbreviations

A	Absorbance/Intensity
AAPH	2,2'-azobis-(2-methylpropionamidine) dihydrochloride
ABTS	2,2'-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid)
$ABTS^{\bullet+}$	ABTS radical
Ac-DEVD-AMC	Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin
AcOH	Acetic acid
$AlCl_3$	Aluminum trichloride
AMC	Amido-4-methylcoumarin
ANOVA	Analysis of variance
AP-1	Activator protein-1
Apaf-1	Apoptosis-protease-activating-factor-1
ARE	Antioxidant response element
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under the curve
Bcl-2	B-cell lymphoma 2 protein
BER	Base excision repair
$^{\circ}C$	Degrees centigrade
c	Concentration
Ca^{2+}	Calcium ions
CAT	Catalase
Cdk2	Cyclin dependent kinase 2
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
cm	Centimeter
cm^2	Centimeter squared
CO_2	Carbon dioxide
CRP	C-reactive protein
Cyt *c*	Cytochrome C
DCF	2',7'-dichlorofluorescein
DCFHDA	2',7'-dichlorodihydrofluorescein diacetate
DE	Diethyl ether

DF	Dilution factor
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EA	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin-3-gallate
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinase
EtOH	Ethanol
FCS	Foetal calf serum
g	Gram
g	Centrifugal force
G1	Gap 1 phase
G6PDH	Glucose-6-phosphate dehydrogenase
GAE	Gallic acid equivalents
GCLC	Glutamate-cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modifier subunit
γGCS	Gamma-glutamylcysteine synthetase
GPx	Glutathione peroxidase
GRx	Glutaredoxins
GS	Glutathione synthetase
GSH	Reduced glutathione
GSNO	S-nitrosoglutathione
G-SOH	Glutathione sulphenic
GSR	Glutathione reductase
GSSG	Oxidized glutathione
GST	Glutathione *S*-transferase
h	Hour
H_2O_2	Hydrogen peroxide

HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid
HO^-	Hydroxide anions
HO-1	Heme oxygenase-1
IAP	Inhibitor of apoptosis protein
IC_{50}	Concentration which inhibits 50% cell growth
IFN-γ	Interferon-gamma
IκB	Inhibitory protein kappa B
IL	Interleukin
INF-γ	Interferon-gamma
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KOH	Potassium hydroxide
λ_{ex}	Excitation wavelength
λ_{em}	Emission wavelength
L·	Lipid radicals
LC_{50}	Concentration that results in 50% lethality
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LOO·	Lipid peroxyl radicals
LOOH	Lipid hydroperoxides
m	Mass
M	Molarity
MDA	Malondialdehyde
MeOH	Methanol
min	Minutes
µg	Microgram
mg	Milligram
MKK4	Mitogen-activated protein kinase kinase 4
µl	Microlitre
ml	Milliliter
mM	Millimolar
µm	Micrometer

µM	Micromolar
MnSOD	Manganese superoxide dismutase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
N_2O_3	Dinitrogen trioxide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
$NaNO_2$	Sodium nitrate
NaOH	Sodium hydroxide
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa B
NHDF	Normal human dermal fibroblasts
NIOH	National Institute for Occupational Health
nM	Nanomolar
nm	Nanometer
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOS	Nitric oxide synthase
NQO-1	NADPH:quinone oxidoreductase-1
O_2^-	Superoxide anion
·OH	Hydroxyl radical
$ONOO^-$	Peroxynitrate anion
ONOOH	Peroxynitric acid
ORAC	Oxygen Radical Antioxidant Capacity
8-oxoGua	8-oxo-7,8-dihydroguanine
P	Phosphorus
PBS	Phosphate buffered saline
PC	Positive control
pH	Log hydronium ion concentration
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl fluoride
Pro-SH	Thiol-containing proteins

Prot-S	Thyil protein
Prot-SOH	Sulphenic protein
Prot-SNO	S-nitroso protein
QSAR	Quantitative structure–activity relationship
r	Correlation
RE	Rutin equivalents
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Rosswell Park Memorial Institution
RSH	Organic thiol species
RSO_2H	Sulphinic derivative
RSO_3H	Sulphonic derivative
RSSG	Radical mixed disulphide
SEM	Standard error of the mean
SOD	Superoxide dismutase
SRx	Sulphiredoxin
TBA	Thiobarbituric acid
TE	Trolox equivalents
TEAC	Trolox Equivalence Antioxidant Capacity
tert-BuOOH	*tert*-Butylhydroperoxide
TFC	Total flavonoid content
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor-alpha
TPC	Total phenolic content
TRx	Thioredoxins
U	Ubiquitin
UV	Ultraviolet
v	Volume
VND	Value not determinable
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Chapter 1
Literature Review

1. Introduction

Even with our modern pharmacopeia, there exists a wide variety of chronic illnesses that decrease quality of life and increase mortality. Diseases such as diabetes mellitus, neurodegeneration, cardiomyopathy and cancer result in the death of numerous people annually (Lesgards *et al.*, 2011). While each disease has its specific aetiology it has recently become apparent that increased oxidation in the body affects disease progression severely (Lesgards *et al.*, 2011). The latter is known as oxidative stress, where the body is exposed to a concentration of free radicals greater than which it can handle. Although free radicals are formed endogenously for various functions, overproduction or increased exposure to these radicals results in aggravated damage to healthy cellular morphology and function, which promotes disease progression (Pérez *et al.*, 2009; Zeraik *et al.*, 2011). Free radicals can initiate a sequential reaction of oxidation in cells, such as in the phospholipid membranes with subsequent formation of other free radicals (Gupta-Elera *et al.*, 2011).

Free radical-induced damage can be counteracted through the use of antioxidants. These molecules decrease the formation of free radicals and limit their harmful properties. Even though evolution has bestowed organisms with endogenous antioxidant systems, excessive free radical production during oxidative stress can result in saturation of the latter with failure to reduce oxidative damage (Irshad and Chaudhuri, 2002; Casanova *et al.*, 2008). During this overburdening of the endogenous antioxidant system, the use of exogenous sources can result in decreased oxidative damage with possible health benefits. Herbal therapies remain popular due to the presence of natural antioxidants and other highly active phytochemicals which could decrease oxidative stress and associated disease progression (Casanova *et al.*, 2008). The use of exogenous sources of antioxidants is justified, and while research has been done on various herbal remedies, very little information is present for that of African origin. Thus it is of importance to determine the possible use of African plants for antioxidant supplements.

2. *Free radicals*

Although free radicals have endogenous origins during normal physiological processes as well as in response to disease and toxicity (Pérez *et al.*, 2009; Zeraik *et al.*, 2011), they can also occur through exogenous sources, including ultraviolet (UV) radiation or exposure to oxidants (Irshad and Chaudhuri, 2002). They are highly reactive molecules or atoms that possess unpaired valence electrons in their outermost orbitals (Kotz and Treichel, 2003) and have short half-lives (Irshad and Chaudhuri, 2002). Due to their reactivity they are able to remove electrons from other molecules which can result in further production of free radicals (Farber, 1994). Reactive species appear to have hormetic properties depending on the concentration, as they are necessary for normal physiological functioning at low levels (Ristow and Schmeisser, 2011) and detrimental to macromolecules in abundance (Pérez *et al.*, 2009). At low concentrations they act as signaling molecules from mitochondria to other cellular compartments (Ristow and Schmeisser, 2011), regulate cell growth and assist in innate immunity (Vaziri, 2008). Pathophysiological effects of high free radical concentrations include the degradation of lipids, deoxyribonucleic acid (DNA) and proteins (Pérez *et al.*, 2009; Kedare and Singh, 2011). All these lead to large scale tissue damage, mutagenesis, carcinogenesis and aging (Pérez *et al.*, 2009; Kedare and Singh, 2011).

Free radicals are classified according to the molecular source of the unpaired electron, such as reactive oxygen (ROS) or nitrogen species (RNS) (Irshad and Chaudhuri, 2002). ROS are formed endogenously by, amongst others, the mitochondrial electron transport chain, phagocytic respiratory bursts, lipid oxidation and auto-oxidation of proteins (Irshad and Chaudhuri, 2002). A representation of ROS formation can be seen in Figure 1, where complex interactions between different species and enzymatic processes generate new radicals or detoxify them. Superoxide anion radical (O_2^-) is generated non-enzymatically in aerobic cells during the mitochondrial electron transport chain, but enzymatically in macrophages during inflammatory responses (Farber, 1994; Peterhans, 1997). Hydrogen peroxide (H_2O_2) is formed when oxygen spontaneously dimerizes, when O_2^- is enzymatically converted by superoxide dismutase (SOD) or when oxygen undergoes a two electron reduction (Farber, 1994; Irshad and Chaudhuri, 2002). H_2O_2 is relatively

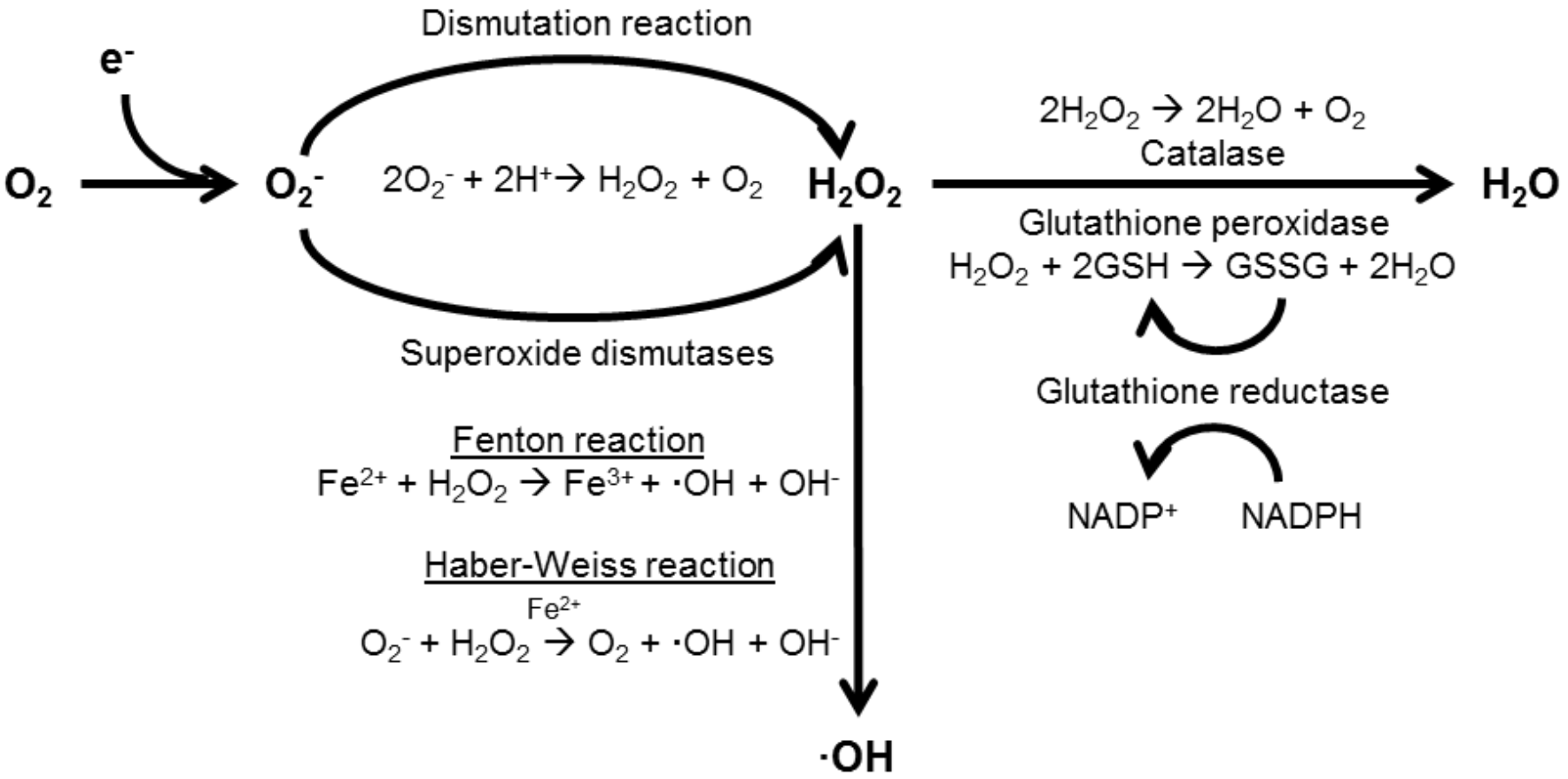


Figure 1: Commonly formed reactive oxygen species and their intracellular metabolism (modified from Goel and Khanduja, 1998)

weakly reactive, but does cross membranes easily (Irshad and Chaudhuri, 2002). Upon cleavage of the dioxygen bond in H_2O_2 through electron addition, hydroxide anions (HO^-) and hydroxyl radicals (·OH) are formed. The latter is a highly reactive molecule and a primary mediator of cellular toxicity (Farber, 1994). Additionally, through Fenton reactions with metal cations, H_2O_2 is broken down to ·OH (Farber, 1994). Other ROS include peroxyls, alkoxyls, hypochlorous acid, ozone and singlet oxygen (Irshad and Chaudhuri, 2002). Oxidative phosphorylation of fatty acids and amino acids generate energy sources such as adenosine triphosphate (ATP), which can promote ROS generation under increased metabolic rate. However, glucose metabolism can be independent of mitochondria and thus does not promote ROS, but is not as efficient in producing energy (Ristow and Schmeisser, 2011).

RNS include nitric oxide (NO), peroxynitrate anion ($ONOO^-$), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) (Irshad and Chaudhuri, 2002). NO, a physiological free radical, is formed enzymatically when L-arginine is converted to L-citrulline through nitric oxide synthase (NOS) (Sheikh *et al.*, 2010; Zhu *et al.*, 2011). Three NOS isoforms are present, classified according to their location and function – inducible (iNOS), neuronal (nNOS) and endothelial (eNOS) (Sheikh *et al.*, 2010). While necessary for normal physiological functioning such as smooth muscle relaxation (Irshad and Chaudhuri, 2002), high concentrations of NO interact with ROS to form toxic $ONOO^-$ and peroxynitric acid (ONOOH), which in turn aggravates oxidative damage to cellular constituents (Sheikh *et al.*, 2010; Zhu *et al.*, 2011). $ONOO^-$ is highly reactive, rapidly reacting with tyrosine, thiols, lipids, DNA and guanosine with their oxidation products resulting in cellular damage (Irshad and Chaudhuri, 2002). Overproduction of RNS results in a burden of NO_2 and N_2O_3, a state termed nitrosative stress (Irshad and Chaudhuri, 2002).

3. Oxidative stress

Although cells are equipped with their own endogenous antioxidant systems it can be overwhelmed during periods of severe free radical production. An imbalance arising from increased free radical production and/or decreased elimination through antioxidant activity results in oxidative stress (Farber, 1994; Pérez *et al.*, 2009). As oxidative stress is an imbalance in the redox homeostasis, it is of utmost importance that it is corrected (Goodman *et al.*, 2011). Once a cell is not able to defend against

the increased pro-oxidant (such as ROS and RNS) concentrations it will be left susceptible to attack by other oxidants and toxins (Pérez *et al.*, 2009; Slameňová *et al.*, 2011). Depending on the extent of stress, different pathways can be activated to counteract or halt progression of damage. If stress is too extensive, these adaptive processes will not be able to deter disease progression (Figure 2).

Oxidative stress has been implicated in up to sixty different ailments (with over 6000 publications highlighting the connection) (Lesgards *et al.*, 2011), including renal nephropathy, cardiopathy, myopathy, diabetes mellitus, Alzheimer's disease and Parkinson's disease (Barcelos *et al.*, 2011; Wang *et al.*, 2010; Chen *et al.*, 2011; Nirmala *et al.*, 2011). Oxidative stress markers have been found in various cancers (DNA lesions) (Kryston *et al.*, 2011) and inflammatory diseases (highly activated immune cells and oxidized low-density lipids [LDL]) (Barcelos *et al.*, 2011; Zeraik *et al.*, 2011). Oxidatively-induced damage to biomolecules results in cytotoxicity, which presents itself in various ways: apoptosis, lipid peroxidation, DNA degradation, protein modification and inflammation.

3.1. Apoptosis

Cells that are damaged can either follow a passive or active form of cell death, namely necrosis or apoptosis (programmed cell death) (Harwood *et al.*, 2005). The latter is a mechanism required not only for development or homeostasis, but also for defense against pathogens and removal of damaged cells (Adams and Cory, 1998). Depending on the cellular stimulus, apoptosis can occur through an extrinsic (death receptor) or intrinsic pathway (mitochondrial or stress), the latter being induced by, amongst others, ROS (Figure 3) (Harwood *et al.*, 2005; Cheng *et al.*, 2011).

The cysteine-aspartate protease class referred to as caspases is one of the main regulators of the apoptotic cascade. Once stimulated by an apoptosis response initiator, caspases proteolytically activate effector caspases. Extrinsic pathways result in caspase-8 and 10 activation, while caspase-2, 7 and 9 are involved in the intrinsic pathway. These eventually activate the effector caspases-3, 6 and 7 (Harwood *et al.*, 2005). Free radical-induced damage may result in caspase activation at low levels, while at high concentrations it could inactivate caspases, shifting death of cells to a necrotic pathway (Goel and Khanduja, 1998).

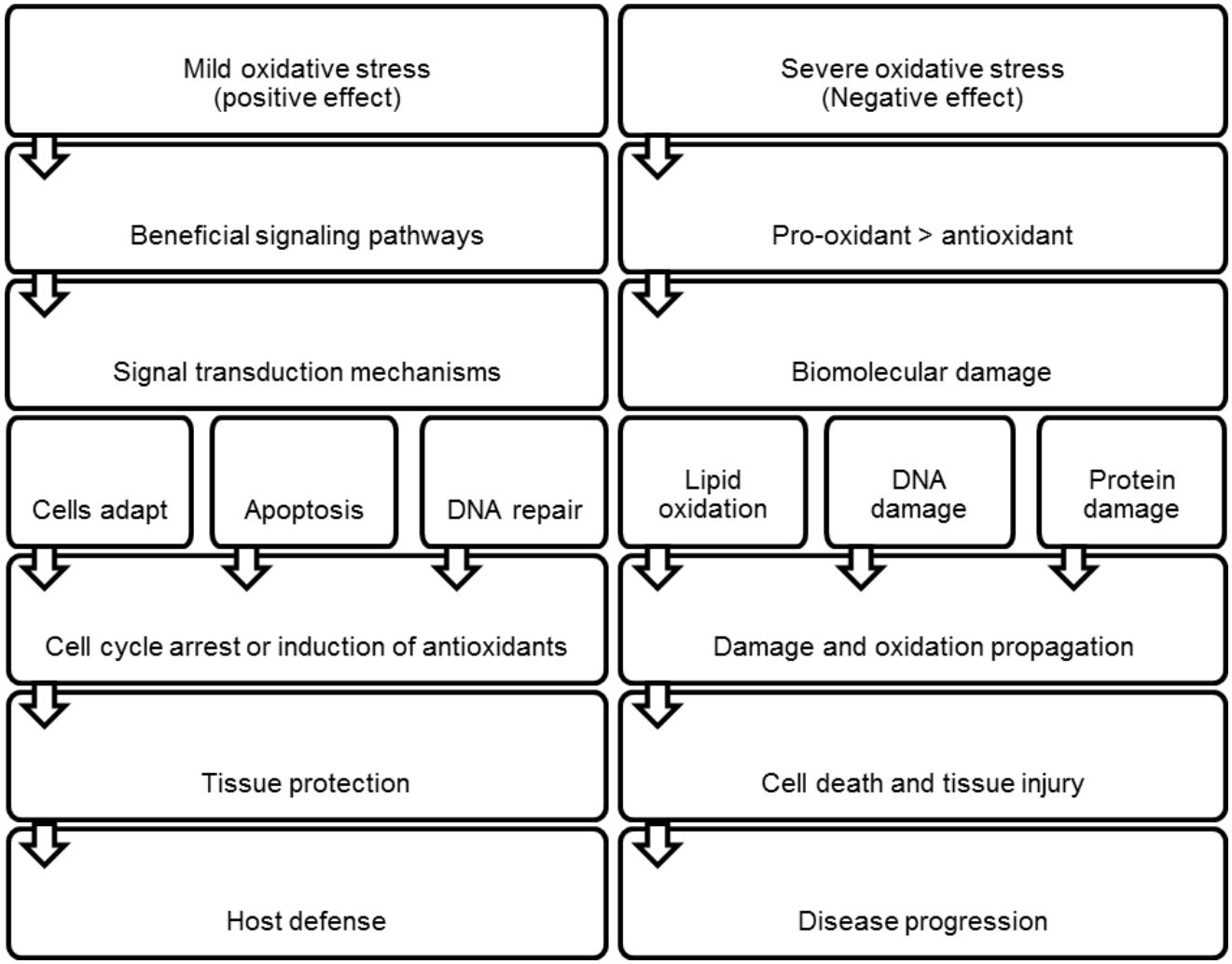


Figure 2: Responses to oxidative stress (modified from Irshad and Chaudhuri 2002)

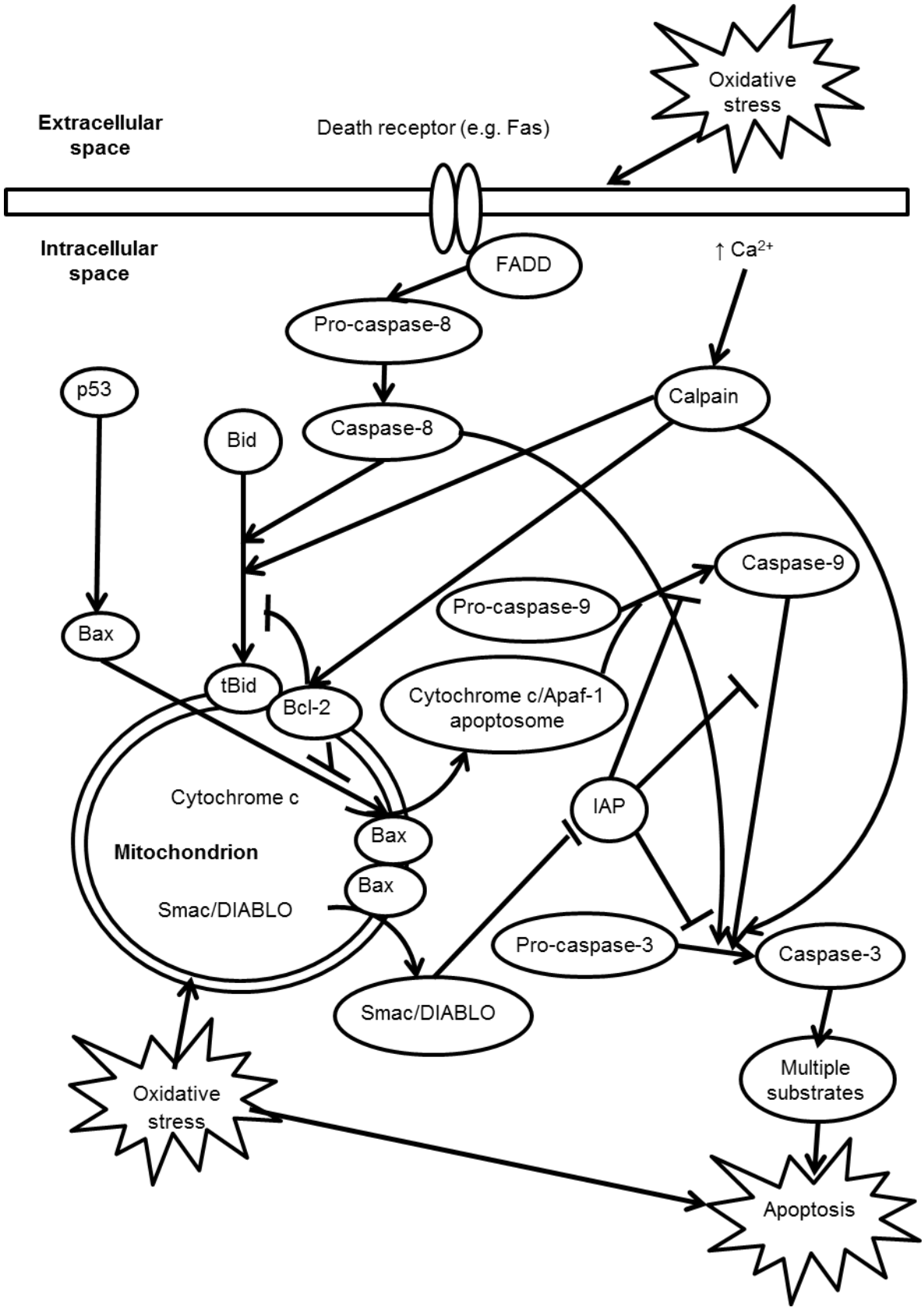


Figure 3: Mechanisms by which apoptosis can be induced; Ca^{2+} - calcium, IAP – inhibitor of apoptosis protein (modified from Goel and Khanduja, 1998; Harwood *et al.*, 2005)

Additionally, the B-cell lymphoma 2 (Bcl-2) family of apoptosis regulator proteins mediates apoptosis by either inhibiting (through the actions of Bcl-2, Bcl-xL, Bcl-w, etc.) or inducing apoptosis (through the actions of Bax, Bcl-xS, Bad, Bak, etc.) (Goel and Khanduja, 1998). Oxidative stress results in an increased Bax/Bcl-2 ratio (Zapolska-Downar *et al.*, 2008). Up-regulation of Bcl-2 protects against apoptosis, while increased Bax creates “pores” in the mitochondrial membrane (Harwood *et al.*, 2005; Du and Lou, 2008). Permeabilization depolarizes the mitochondrial membrane through decreased transmembrane potential and releases cytochrome *c* (Cyt *c*) into the cytosol (Du and Lou, 2008; Zapolska-Downar *et al.*, 2008; Cheng *et al.*, 2011). A complex is formed between released Cyt *c* and apoptosis-protease-activating-factor-1 (Apaf-1), which recruits and activates procaspase-9 (Tusi *et al.*, 2011). The activated complex (or apoptosome) then proteolytically activates procaspase-3 to caspase-3 (Harwood *et al.*, 2005; Zapolska-Downar *et al.*, 2008; Tusi *et al.*, 2011).

The activation of procaspase-3 to caspase 3 results in the formation of apoptotic bodies due to i) chromatin breakages as a result of dedicated deoxyribonuclease (DNase) release (Harwood *et al.*, 2005), ii) condensation of the cytoplasm and iii) membrane phospholipids alterations (Zapolska-Downar *et al.*, 2008). Literature suggests that caspase-9 is not always necessary to the intrinsic pathway, but rather functions as an enhancer of the cascade (Harwood *et al.*, 2005). Bcl-2 decreases apoptosis via a reduction of Cyt *c* efflux through stabilization of mitochondrial membrane potential. ROS-mediated apoptosis is indirectly decreased by Bcl-2 by reducing the damaging effects of the ROS on mitochondria and cellular organelles (Goel and Khanduja, 1998). Oxidative stress and other DNA damaging stimuli result in the up-regulation and expression of p53, a well-known mediator of apoptosis. Through the action of p53, cells that are DNA damaged udergo apoptosis, which limits the chances of cloning genetically damaged cells (Goel and Khanduja, 1998).

3.2. Lipid peroxidation

Lipid peroxidation results in the destruction of membrane constituents which occurs during periods of oxidative stress. Cellular membrane phospholipids are comprised of polyunsaturated fatty acids that contain weak carbon-hydrogen linkages due to neighboring double bonds (Farber, 1994). Lipid radicals (L·) form when potent radicals, such as ·OH remove hydrogen from the membrane, subsequently leaving

the carbon molecule with an unpaired electron. By reacting with molecular oxygen, L· can form highly oxidizing lipid peroxyl radicals (LOO·) (Farber, 1994). LOO· extract hydrogen atoms as well, and as such initiates sequential oxidation throughout the membrane with degradation of membrane phospholipids (Figure 4) (Farber, 1994). Lipid peroxidation products, such as F2-isoprostanes, malondialdehyde (MDA), 4-hydroxy-nonenal and acrolein further potentiate oxidation due to their high reactivity with biological macromolecules (Rahman and Adcock, 2006).

These reactive products are relatively stable and are able to cross membranes and attack targets distant from the oxidative origin (Rahman, 2003). Reactive aldehydes initiate inflammatory responses, are involved in abnormal regulation of cell growth and inhibition, as well as macrophage activation and depletion of endogenous antioxidants (such as reduced glutathione (GSH)) (Rahman, 2003; Rahman and Adcock, 2006). Acrolein depletes GSH through redox enzyme inhibition, increases transcription of NADPH:quinone oxidoreductase 1 (NQO1), messenger ribonucleic acid (mRNA) and may inhibit interleukin (IL)-8 synthesis (Rahman and Adcock, 2006). 4-Hydroxy-2-nonenal is able to complex with GSH, diminish endogenous stores and decrease its efficacy. At lower free radical concentrations, lipid peroxidation byproducts may alternatively induce glutamate cysteine ligase expression through an adaptive measure (Rahman and Adcock, 2006). 4-Hydroxy-nonenal has been implicated in the potentiation of mitochondrial dysfunction, induction of apoptotic signals, ROS generation and decreased cell viability (Raza and John, 2006). The movement of the normal inward-facing cell bilayer phospholipid, phosphatidylserine to be expressed on the outer layers of the plasma membrane during apoptosis – is thought to be mediated through lipid peroxidation mechanisms (Goel and Khanduja, 1998).

With loss of functional and structural stability comes an increase in membrane permeability, oxidation of intracellular constituents and ion leakage (Yilmaz and Parlak, 2011). Lipid peroxidation of monocytes and macrophages results in the formation of foam cells which are filled with oxidized lipids, characteristic of atherosclerosis disease progression (Kaliora *et al.*, 2006). It has been postulated that lipid peroxidation arises as an epiphenomenon in some cases, not being directly toxic but merely a side-effect of other oxidative toxicity mechanisms (Farber, 1994).

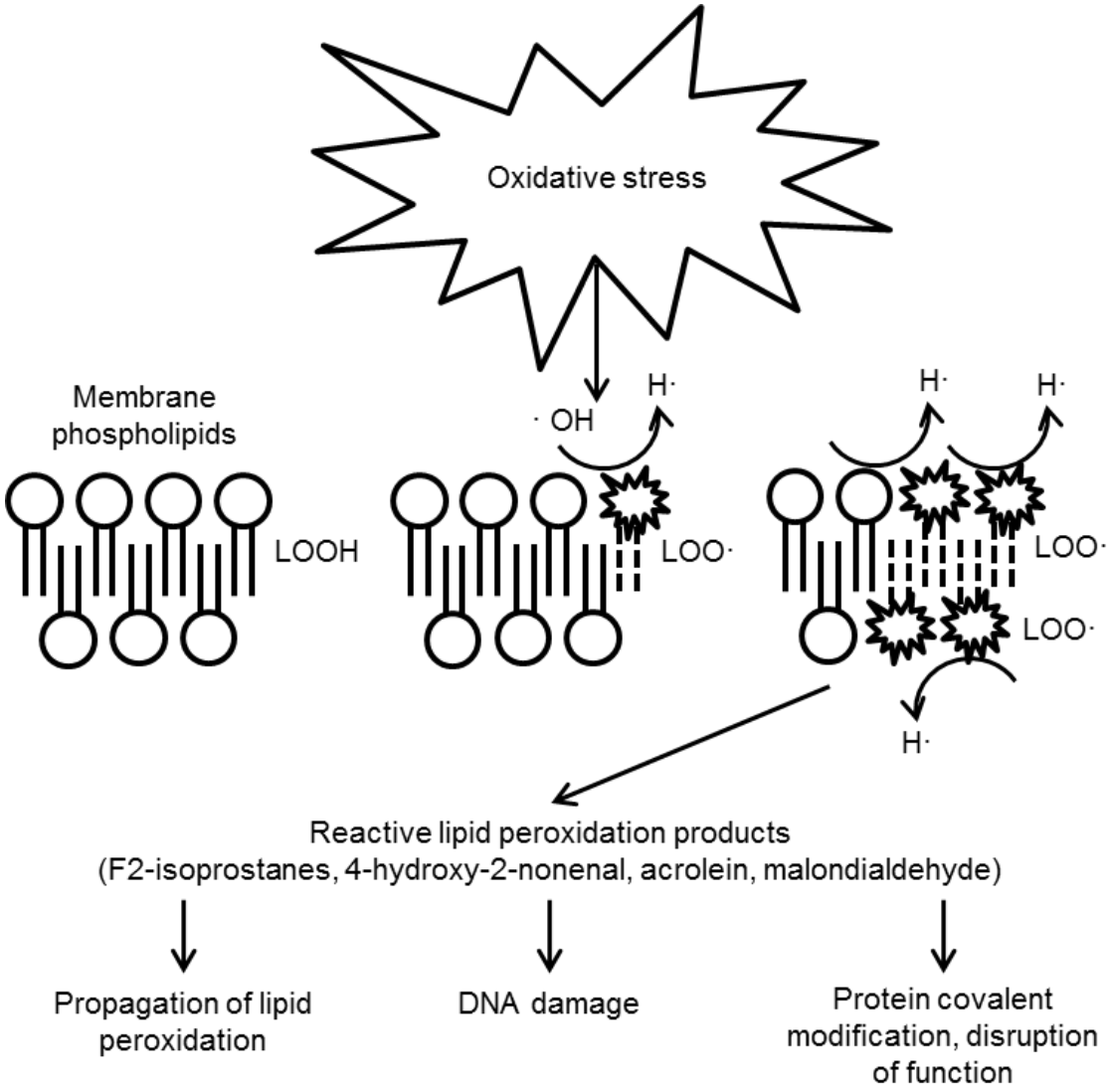


Figure 4: Mechanism of lipid peroxidation induced by oxidative stress (modified from Rahman and Adcock, 2006)

3.3. DNA degradation

Free radicals result in damage to DNA in the form of breakages, cross-linkages or base-modifications. Nucleic acids and chromatin, which can be fragmented and modified, are vulnerable to direct oxidation (Figure 5) (Goel and Khanduja, 1998; Broedbaek *et al.*, 2011). Typical products of damage include abasic DNA site damage, purine and pyrimidine oxidation, and single or double strand breakages (Kryston *et al.*, 2011). Hydrolysis of *N*-glycosidic nucleotide bonds results in the release of bases while the deoxy-ribose phosphodiester structure remains stable. Such an area is deemed an abasic site, which might not be lethal to cells but in high frequency can interefere with DNA polymerases and increase mutation frequency (Kryston *et al.*, 2011).

The most common oxidative base modifications include 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine, which originate from a C8-hydroxyl addition to form 8-hydroxy-7,8-dihydroguanil radicals (Broedbaek *et al.*, 2011; Kryston *et al.*, 2011). Pyrimidine-hydroxyl interactions at position 5 or 6 commonly form thymine glycol or cytosine glycol. These lesions are not considered highly cytotoxic, but they can result in an increased mutation rate which contributes to cellular damage, loss of function and cancer progression (Kryston *et al.*, 2011). Measurement of these lesions gives a good indication of the status of an individual. DNA lesions are mainly controlled through the action of base excision repair (BER) and nucleotide excision repair (NER) (Broedbaek *et al.*, 2011). Single strand breakages occur through hydrogen abstraction from 2-deoxyribose, sugar moieties, C3', C4' and C5' in DNA brought upon by free radicals such as •OH, LOO· and lipid peroxidation byproducts (Rahman and Adcock, 2006; Kryston *et al.*, 2011).

When damage is severe enough, two adjacent single strand breakages can form a double strand breakage (Kryston *et al.*, 2011). It is suggested that cells with higher proliferation capacity are more susceptible to such DNA damage (Kryston *et al.*, 2011). Furthermore, DNA repair can be affected by alteration or modification of DNA repair systems (Kryston *et al.*, 2011).

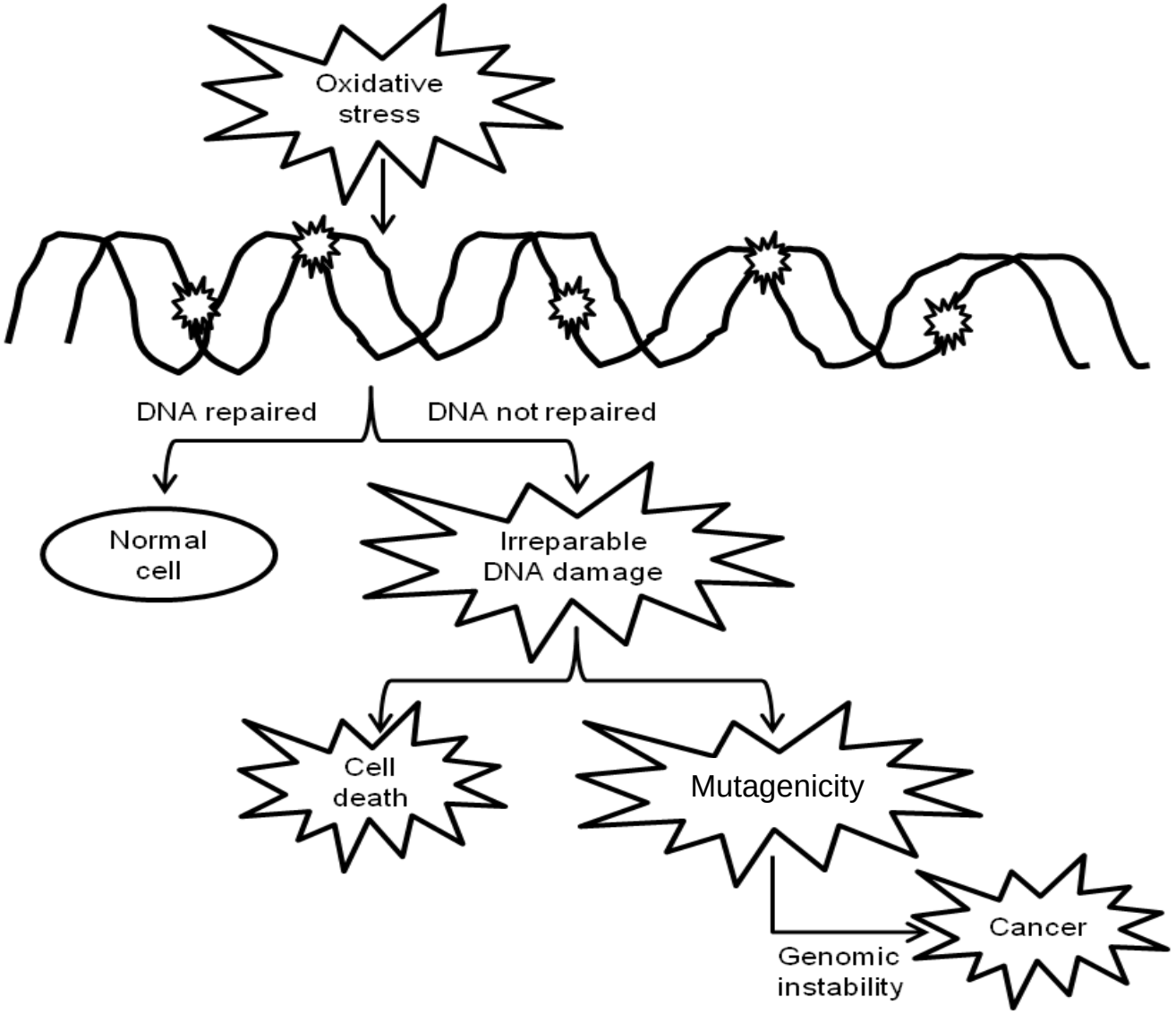


Figure 5: Induction of DNA damage during oxidative stress (modified from Kryston *et al.*, 2011)

3.4. Protein modification

Modification of proteins results in conformational changes and activity alterations. Amino acid constituents such as cysteine, methionine and tyrosine are easily oxidized and altered during oxidative stress (Rahman, 2003). Due to the susceptibility of sulfur atoms to oxidative stress-induced modification it can result in the sequential oxidation of cysteine. Subsequent sulphenic, sulphinic and sulphonic acids are formed – the latter two being better indicators of oxidative injury (Figure 6) (Wouters *et al.*, 2011). Sulfhydral modification in the membrane calcium transport system due to oxidative stress results in higher cytosolic calcium levels with induction of cellular toxicity (Goel and Khanduja, 1998). All these changes result in functional differences and potentiation of oxidative damage (Rahman and Adcock, 2006). These modifications require high levels of energy to reverse, but fortunately cellular mechanisms are in place to prevent their formation (Wouters *et al.*, 2011). Cysteine may be recycled in such instances between their reduced and oxidized forms (Rahman and Adcock, 2006).

Modification of the catalytic site of enzymes results in their inactivation, though *in vivo* results provide a more complex view. Acute free radical exposure may increase enzyme activity as an adaptative and protective response, whereas long-term exposure induces conformational inactivation and cytotoxicity (Guttmann and Ghoshal, 2011). Transcription factors (such as nuclear factor kappa B (NF-κB), p53 and others) containing critical cysteine regions are easily modified during high levels of oxidation, with subsequent diminished DNA-binding strength and weakened response. Many enzymes regulating expression of transcription factors are themselves also modified with altered efficacy (Goel and Khanduja, 1998). Damage to intracellular proteins can change their recognition by other molecules, such as where an increased vulnerability to nonlysosomal protease degradation is seen (Goel and Khanduja, 1998).

3.5. Inflammation

Oxidative stress results in inflammation due to damage to surrounding tissue and induction of inflammatory transcription factors such as NF-κB and activator protein 1

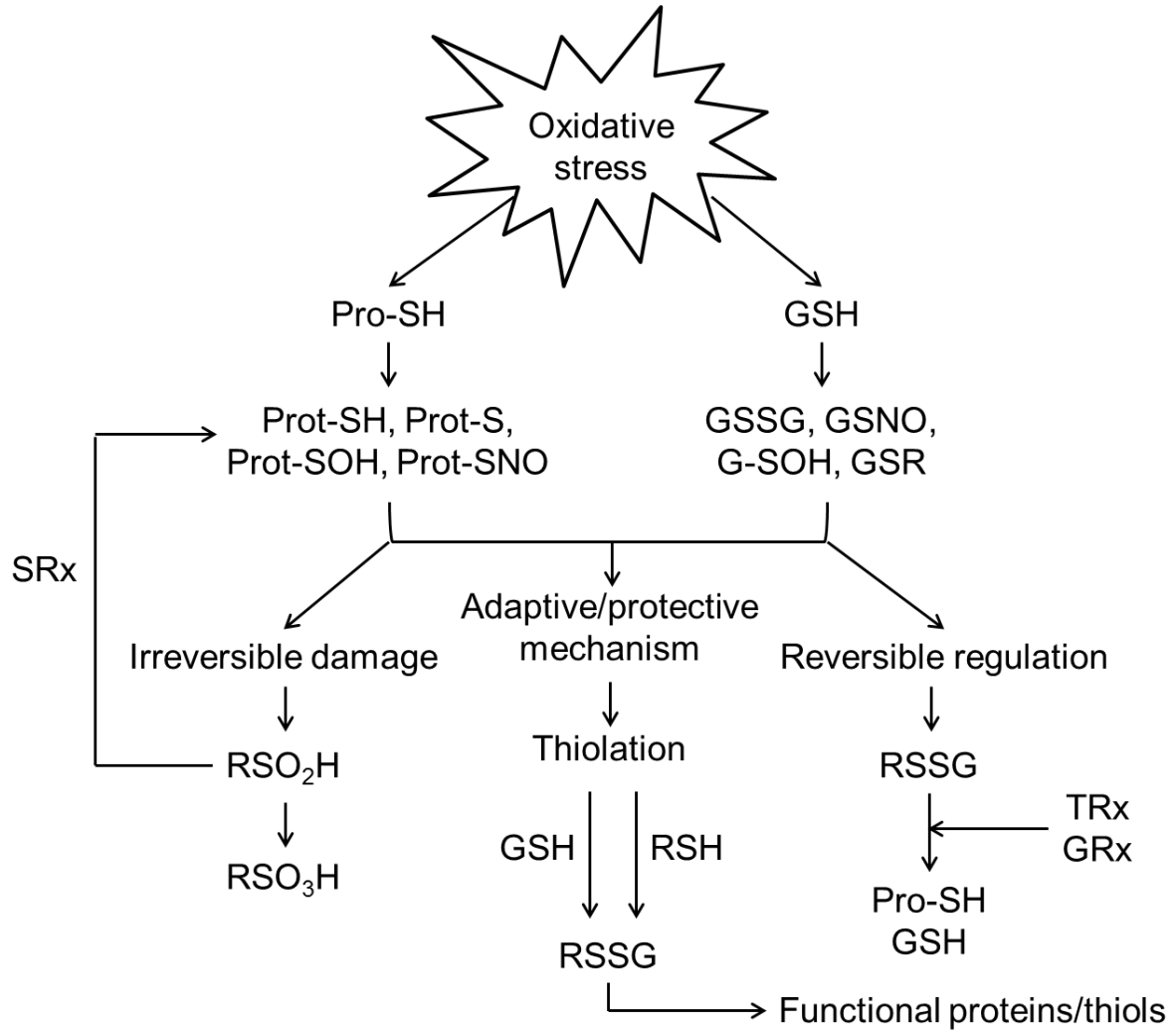


Figure 6: Protein modification as brought upon by oxidative stress and the involvement of glutathione. GSH – reduced glutathione, GSSG – oxidized glutathione, GSNO – S-nitrosoglutathione, G-SOH – glutathione sulphenic, GSR – glutathione reductase, GRx – glutathione reductase, RSH – other thiol species, RSSG – radical mixed disulphide , TRx - thioredoxins, GRx – glutaredoxins, Pro-SH – thiol-containing proteins, Prot-SH – protein thyil, Prot-SOH – sulphenic protein, Prot-SNO – S-nitroso protein, SRx – sulphiredoxin, RSO_2H – sulphinic derivative, RSO_3H – sulphonic derivative (modified from Rahman and Adcock, 2006)

(AP-1), as well as the release of pro-inflammatory cytokines and mediators (Figure 7) (Rahman and Adcock, 2006; Meja *et al.*, 2008; Vaziri, 2008). Activation of signal transduction pathways (such as c-Jun N-terminal kinase [JNK] and antioxidant-response element [ARE]) increases transcription of antioxidant genes, as well as promoting inflammatory responses. Increased activation of ARE results in the synthesis of i) antioxidant enzymes, such as glutamate-cysteine ligase catalytic subunit (GCLC) and heme oxygenase-1 (HO-1), and ii) detoxification enzymes, such as NQO-1 and glutathione *S*-transferase (GST) (Kokubo *et al.*, 2011). During oxidative stress the potency of the inflammatory response is higher (Rahman, 2003).

Elevated concentrations of IL-1β, IL-6, IL-8, interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) are considered pro-inflammatory and pro-oxidant. This is possibly through increased production of NO and $ONOO^-$ (Kaliora *et al.*, 2006; Sarir *et al.*, 2010). Elevated pro-inflammatory mediators, including C-reactive protein (CRP), are good indicators of chronic disease progression (Karlsen *et al.*, 2010). Activation of macrophages during inflammation results in further generation of O_2^- (with subsequent H_2O_2 and ·OH formation), leading to a self-perpetuating series of events (Rahman, 2003; Vaziri, 2008).

4. Antioxidants

To combat oxidation, pro-oxidant production, propogation and activity can be limited through the use of antioxidants. *In vitro* experimentation has been shown to reduce free radical-induced damage in oxidative stress models, such as those induced using 2,2`-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) as generator of •OH, alkyl radicals and peroxyl radicals through thermolysis (Figure 8) (Pinchuk and Lichtenberg, 2002; Pedriali *et al.*, 2008). This has further been seen in *in vivo* models where antioxidants reduce cellular damage, increase health and improve antioxidant status. This can ultimately reduce morbidity and development of chronic diseases such as cancer and heart failure (Casanova *et al.*, 2008; Meja *et al.*, 2008; Heo *et al.*, 2009; Su *et al.*, 2009; Hamed, 2011).

Antioxidants have the ability to scavenge and trap free radicals. These are generally either inhibitors of free radical chain reactions or preventative in nature (Figure 9),

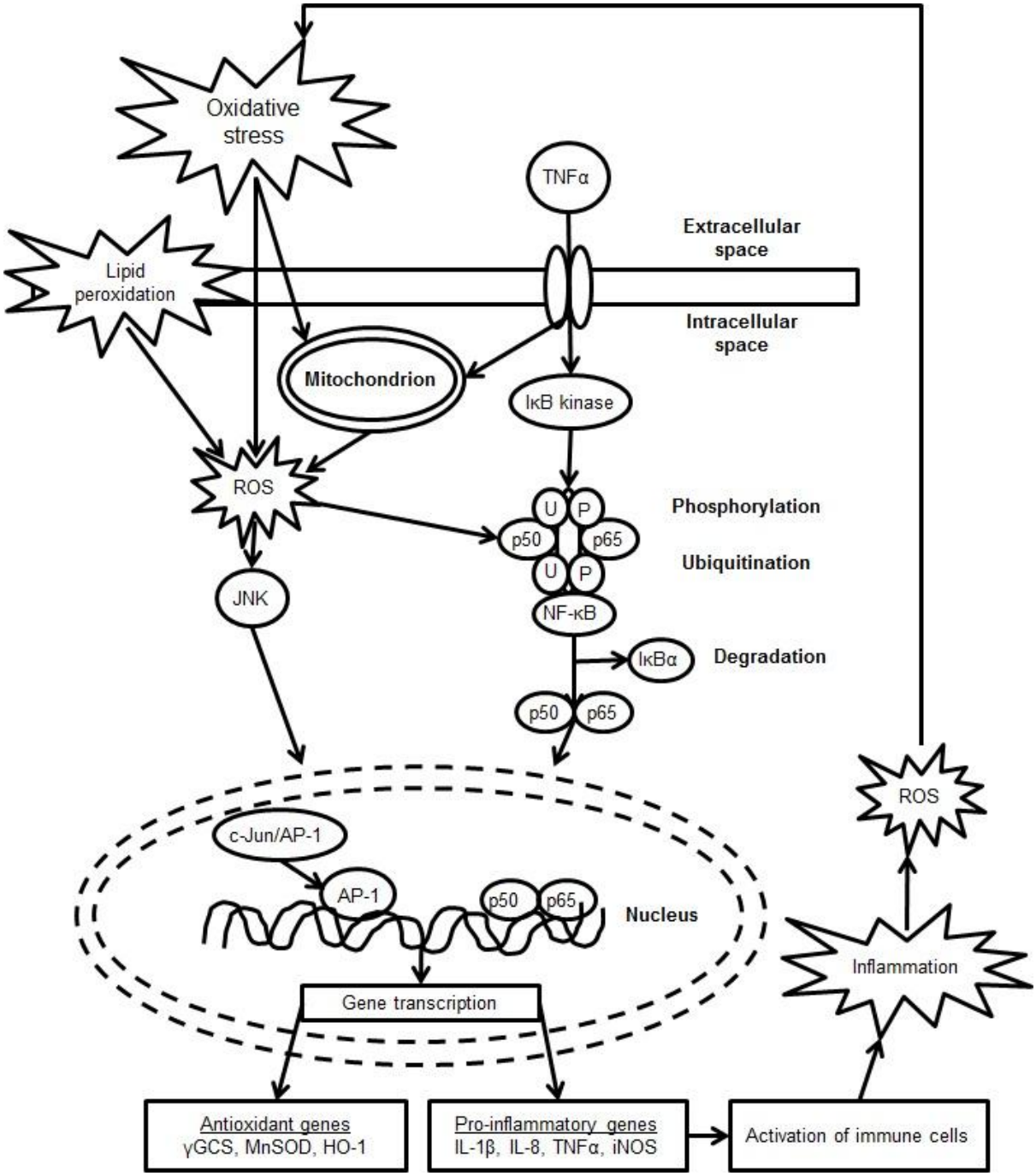


Figure 7: Inflammatory cycle induced by oxidative stress. TNF-α – tumour necrosis factor alpha, ROS – reactive oxygen species, IκB – inhibitory protein kappa B, U – ubiquitin, P – phosphorus, NF-κB – nuclear factor kappa B, γGCS – gamma-glutamylcysteine synthetase, MnSOD – manganese superoxide dismutase, HO-1 - heme oxygenase-1, IL – interleukin, iNOS - inducible nitric oxide synthase, JNK – c-JUN N-terminal kinase, AP-1 – activator protein 1 (modified from Rahman, 2003 and Vaziri, 2008)

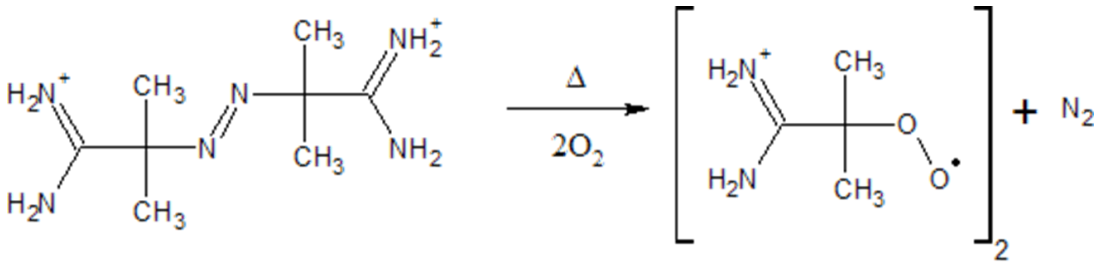


Figure 8: Generation of free radicals due to thermolysis of AAPH (modified from Pedriali *et al.*, 2008)

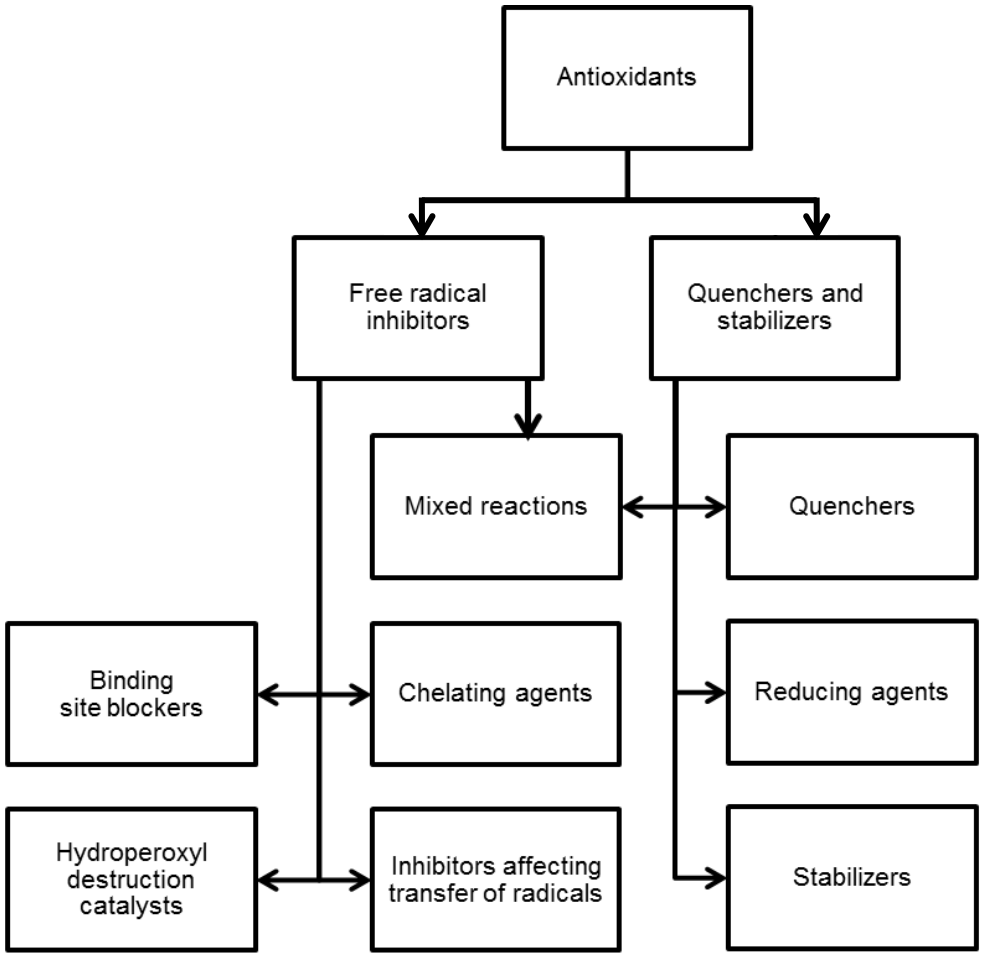


Figure 9: Mechanisms of free radical inhibition by antioxidants (modified from Pinchuk and Lichtenberg, 2002)

thus inhibiting free radicals to oxidize other compounds (de Beer *et al.*, 2002; Pinchuk and Lichtenberg, 2002; Souri *et al.*, 2008; Su *et al.*, 2009). A single electron or hydrogen atom is donated to a free radical which is reduced by neutralising its valence electron (Kotz and Treichel, 2003; Ruan *et al.*, 2008).

While humans do possess an endogenous antioxidant system, the burden of oxidative stress would deplete these stores leaving cells open to free radical-induced damage. This decrease is seen in oxidative stress-related diseases and exposure to free radical-generating agents, such as smoking (Rahman and Adcock, 2006). In studies concerning the antioxidant effects on chronic obstructive pulmonary disease sufferers, antioxidant usage (such as flavonoids, n-acetylcysteine and thiol compounds) increased quality of life and decreased disease parameters (such as acute exacerbation and lung function) (Rahman and Adcock, 2006). Thus, through exogenous supplementation with food, herbal remedies and pharmaceutical usage, antioxidant status could be increased with beneficial effects on oxidative stress.

4.1. Endogenous antioxidant systems

Endogenous defenses include various enzyme and non-enzyme molecules that aim to a) quench free radicals, b) diminish production of reactive species and c) chelate reactive metal ions (Irshad and Chaudhuri, 2002). Several enzymes, such as catalase (CAT), SOD, thioredoxin reductase, peroxiredoxin and glutathione peroxidase (GPx), play key roles in degrading ROS to less reactive compounds (Irshad and Chaudhuri, 2002; Pérez *et al.*, 2009; Kalim *et al.*, 2010; Ngo *et al.*, 2011). Metal ions such as selenium, manganese, copper and zinc are essential components to several enzymes involved in the reduction of free radicals (Irshad and Chaudhuri, 2002). Metal chelating agents can also have a level of antioxidant activity by reducing potential Fenton reactions and radical conversions (Kaliora *et al.*, 2006). The metalloprotein enzyme SOD is necessary for the quenching of O_2^- molecules, while the hemeprotein CAT in mitochondria degrades H_2O_2 to water and oxygen (Farber, 1994; Irshad and Chaudhuri, 2002; Nirmala *et al.*, 2011). One of the most important antioxidant systems present in the body, is the GSH (or γ-glutamylcysteinylglycine) system (Rahman and Adcock, 2006).

The tripeptide antioxidant GSH is one of the most abundant non-protein thiols present intracellularly in the nucleus, endoplasmic reticulum and mitochondria (Masella *et al.*, 2005). It also occurs in an oxidized form (GSSG). While GSH is present at high levels (1 to 10 mM intracellularly), GSSG comprises an insignificant portion (<1%) (Abbott *et al.*, 1986; Rahman and Adcock, 2006). Adaptive *de novo* synthesis of GSH during oxidation requires the amino acids glutamate, cysteine and glycine (Masella *et al.*, 2005). Cysteine is the rate limiting substrate for GSH production (Masella *et al.*, 2005; Rahman and Adcock, 2006). GSH formation is a two-step enzymatic process that involves glutamate cysteine ligase (the rate limiting enzyme) and glutathione synthetase (Masella *et al.*, 2005; Rahman and Adcock, 2006). Glutamyl transpeptidase, found in the plasma membrane, is necessary for the release of cysteine from cysteine-linked sources. GSH acts as regulator of glutamate cysteine ligase expression through feedback inhibition, and can be upregulated as an adaptive response during low levels of oxidation (Rahman and Adcock, 2006).

GPx is a heterogenous group of enzymes with a wide distribution, but all are functionally similar (Munz *et al.*, 1997). GPx enzymatically converts H_2O_2 and lipid hydroperoxides (LOOH) to water and less reactive hydroxy acids (Irshad and Chaudhuri, 2002) through reversible oxidization of GSH to GSSG (Figure 10) (Farber, 1994; Masella *et al.*, 2005). Glutathione reductase (GSR) catalyzes the reduction of GSSG to GSH for recycling purposes during which reduced nicotinamide adenine dinucleotide phosphate (NADPH) is oxidized to nicotinamide adenine dinucleotide phosphate ($NADP^+$). Thus by detoxifying H_2O_2 other cofactors are oxidized, and simultaneously there is an increase in glutathione-mixed disulfides from protein-bound thiols (Farber, 1994; Irshad and Chaudhuri, 2002). Overproduction of GSSG results in formation of mixed disulphides, depleting intracellular GSH levels (Masella *et al.*, 2005; Rahman and Adcock, 2006). Toxic xenobiotic electrophiles are detoxified by GST through conjugation to GSH. These conjugate products are transported to the extracellular space for removal (Masella *et al.*, 2005). These changes increase cytosolic calcium with subsequent risk of apoptosis, and potentiate oxidative stress (Farber, 1994; Harwood *et al.*, 2005). Homeostasis of the GSH/GSSG ratio is important for cellular function, and regulates the activity of the endogenous antioxidant system, including SOD and CAT (Rahman and Adcock, 2006). Disruptions in the intracellular GSH/GSSG ratio results in the

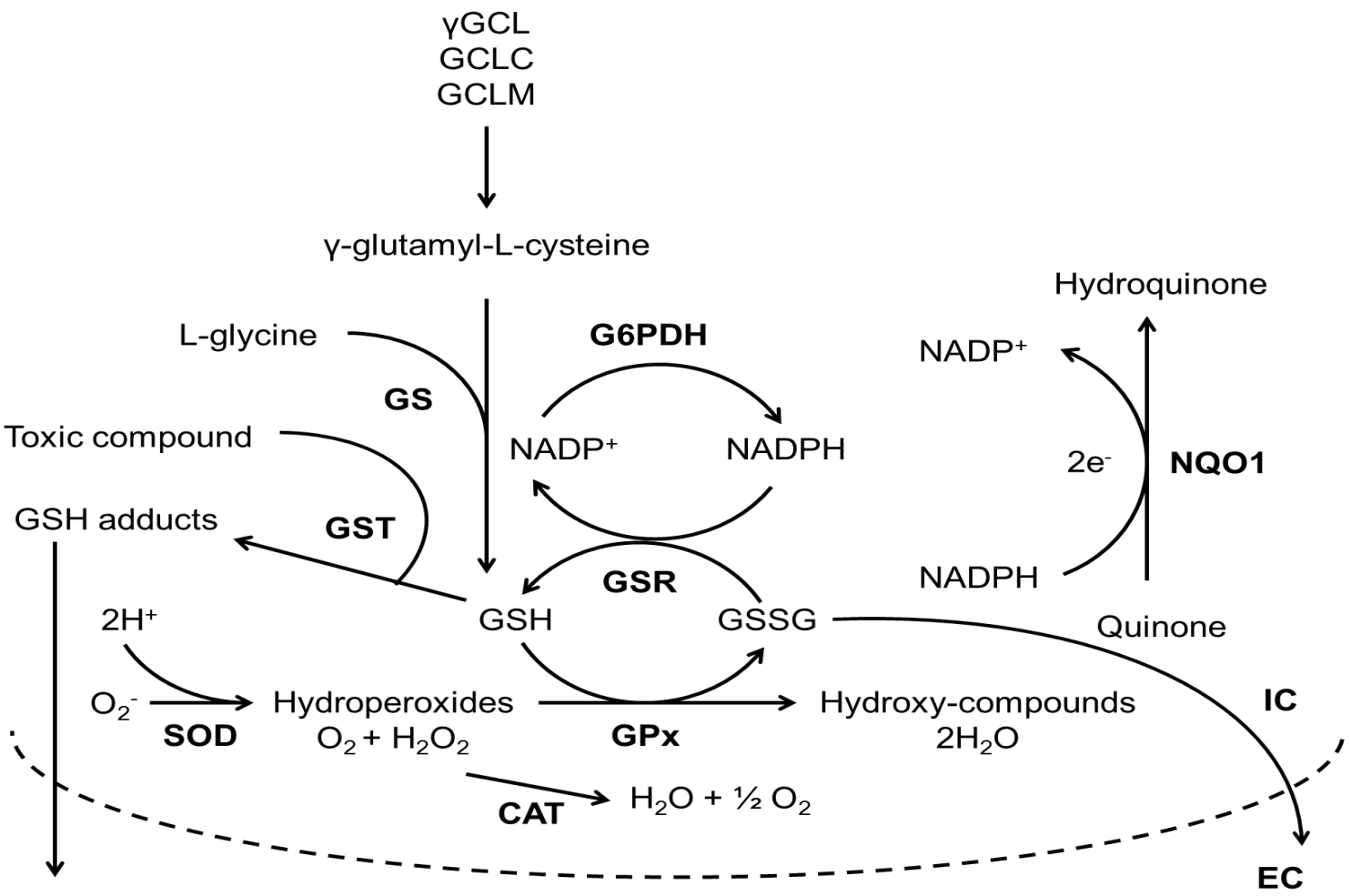


Figure 10: Glutathione pathway. GCLC – glutamate-cysteine ligase catalytic subunit, GCLM – glutamate-cysteine ligase modifier subunit, GS – glutathione synthetase, GSH – reduced glutathione, GSR – glutathione reductase, GPx – glutathione peroxidase, G6PDH – glucose-6-phosphate dehydrogenase, GSSG – oxidized glutathione, GST – glutathione S-transferase, H_2O_2 – hydrogen peroxide, SOD – superoxide dismutase, CAT – catalase, NQO1 – NADPH quinone oxidoreductase 1 (modified from Masella *et al.*, 2005 and Soyalan *et al.*, 2011)

induction of oxidant response transcriptional factors due to a thiol dependant redox shift, recycling of GSSG (through the sacrifice of NADPH) or increased excretion of GSSG into the extracellular space (which results in increased need for *de novo* synthesis of GSH) (Masella *et al.*, 2005; Rahman and Adcock, 2006). Thus, due to decreased endogenous stores, supplementation is needed to assist in removal of free radicals.

4.2. Exogenous antioxidant systems

Administration of exogenous antioxidants is beneficial as stated previously and acts as support to endogenous stores, which in periods of excessive oxidation is already at low concentrations (Nirmala *et al.*, 2011). Although supplementation can occur through food stuffs, herbal remedies remain an ever-popular form of treatment. Herbal remedies are commonplace and can be found at informal markets, health food stores or pharmacies (Rosecrans and Dohnal, 2009). The World Health Organization reports that approximately 80% of the African and Asian populace resort to traditional medicine such as herbal remedies to alleviate symptoms and progression of diseases (WHO, 2008). Various cultures and countries have formed their own pharmacopeia of locally grown plants which are popular due to the inexpensive procurement and abundance in the area (Rosencrans and Dohnal, 2009; Calixto, 2000). The use of plants as remedies for various ailments has formed the basis of our modern medicinal sciences, with many natural products appearing as pharmaceutical agents (Hutchings *et al.*, 1996). During 1981 and 2002 60% of cancer drugs were derived from natural sources, while 75% of new agents were targeted against infectious disease. Between 2001 and 2005, twenty three naturally-derived drugs were introduced as registered pharmaceuticals (Lam, 2007). Antioxidant plant sources are no different. Plants consist of a complex matrix of phytochemicals, which are bioactive constituents that are formed endogenously for, amongst other reasons, protection against oxidative stress.

Phytochemicals not only work in the plant, but also elicit antioxidant effects in humans (Srivastava and Shivanandappa, 2011). The unique carbon skeletons and conformations of phytochemicals create an immensely diverse range of chemical species which have been estimated to have a higher frequency of displaying bioactivity than man-made molecules (Kalim *et al.*, 2010; Lam, 2007). Although

synthetic antioxidant counterparts do exist, such as butylated hydroxytoluene and butylated hydroxyanisole, concerns have been expressed about potential toxicity and carcinogenicity (Mariod *et al.*, 2010), further justifying research into herbal therapies. This results in increased effort to elucidate possible natural lead molecules as alternatives.

Natural compounds with antioxidant activity, such as polyphenols, decrease oxidation-induced damage and have elevated free radical scavenging abilities due to their aromatic structures (Villaño *et al.*, 2007; Souri *et al.*, 2008). Polyphenolic compounds are omnipresent and stem as secondary metabolites of the shikimate and acetate-malonate pathways (Apak *et al.*, 2007; Su *et al.*, 2009). Many types of polyphenols exist and share the phenylpropanoid carbon skeleton (C_6 – C_3): phenolic acids, flavonoids, biflavonoids, hydroxycinnamic acids, stilbenes, simple phenols and proanthocyanidins (Figure 11) (Apak *et al.*, 2007).

Phenolic compounds are aromatic structures, generally hydrophilic and may occur as free phenols or complex glycosides when combined with sugars (Figure 12) (Harborne, 1973). More than 8 000 natural phenolic compounds have been characterized to date and range from simple to complex dimerizations (Prior and Cao, 2000), and are generally cinnamic or benzoic acid derivatives (de Beer *et al.*, 2002). Flavonoids form the largest class with various subdivisions (Figure 13) (de Beer *et al.*, 2002). Flavanoids have hydrogenated C2 and C3 chains, and include flavanones, flavanonols and dihydrochalcones (Corradini *et al.*, 2011). Flavan-3-ols are rarely glycosylated, while flavonols generally conjugate with glycosides such as glucose, galactose, rhamnose or xylose (de Beer *et al.*, 2002). Flavans are rare in nature (Corradini *et al.*, 2011). Proanthocyanidins are complex structures of flavan-3-ol subunits that yield anthocyanidins when cleaved (de Beer *et al.*, 2002).

Polyphenols exert a number of functions: anthocyanins act as flower pigments; flavonoids act as UV filters against radiation; flavanols act as growth and resistance factors; lignans add structural integrity of cell walls; tannins act as defensive, antimicrobial agents (Harborne, 1973; Apak *et al.*, 2007; Corradini *et al.*, 2011). Studies indicate that polyphenols elicit cytoprotective effects due to their anti-inflammatory, anti-oxidative, anti-tumor and DNA repairing activities (Figure 14).

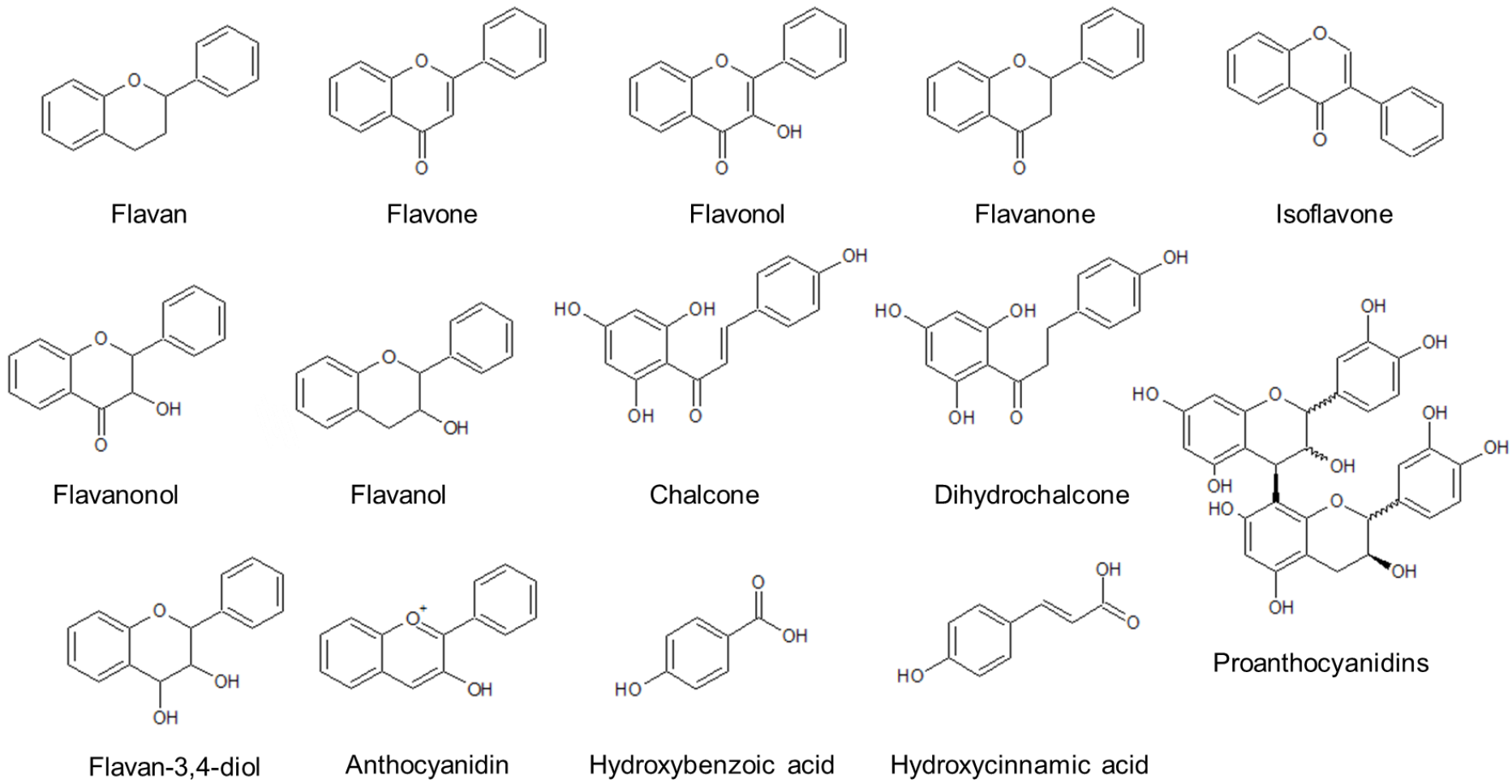


Figure 11: Backbones of different polyphenolic classes (modified from de Beer *et al.*, 2002 and Corradini *et al.*, 2011)

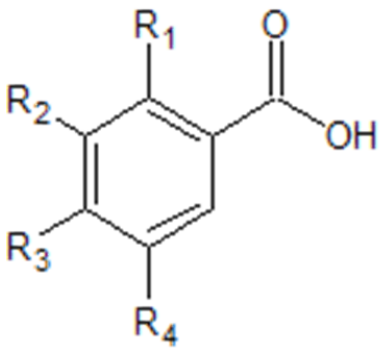
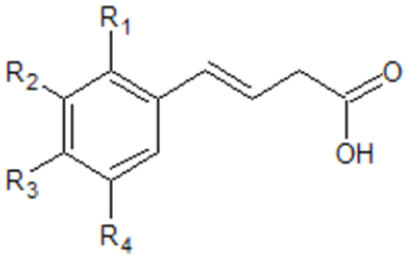
Type	Structure	Phenolic acid	R_1	R_2	R_3	R_4
Hydroxybenzoic acid		Salicylic acid	OH	H	H	H
		m-Hydroxybenzoic acid	H	OH	H	H
		p-Hydroxybenzoic acid	H	H	OH	H
		Protocatechuic acid	H	OH	OH	H
		Gallic acid	H	OH	OH	OH
		Vanillic caid	H	OCH_3	OH	H
		Syringic acid	H	OCH_3	OH	OCH_3
Hydroxycinnamic acid		*p*-Coumaric acid	H	H	OH	H
		Caffeic acid	H	O	OH	H
		Ferulic acid	H	OCH_3	OH	H
		Sinapic acid	H	OCH_3	OH	OCH_3

Figure 12: Various phenolic compounds found in nature (modified from Corradini *et al.*, 2011)

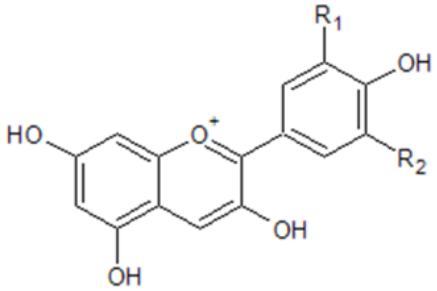
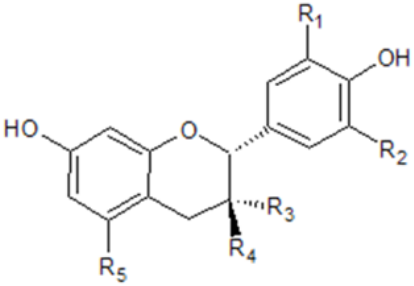
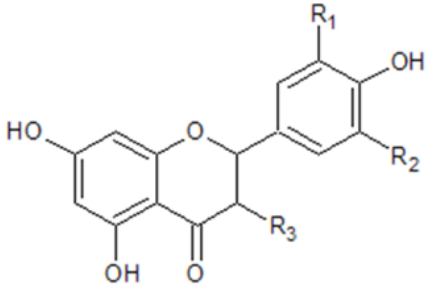
Type	Structure	Flavonoid	R_1	R_2	R_3	R_4	R_5
Anthocyanidin		Peonidin	OCH_3	H			
		Malvidin	OCH_3	OCH_3			
		Cyanidin	H	OH			
		Pelargonidin	H	H			
		Delphinidin	OH	OH			
		Petunidin	OCH_3	OH			
Flavan-3-ol		(+)-Catechin	H	OH	H	OH	OH
		(-)-Epigallocatechin	OH	OH	OH	H	OH
		(+)-Gallocatechin	OH	OH	H	OH	OH
		(-)-Epicatechin	H	OH	OH	H	OH
		(+)-Fisetinidol	H	OH	H	OH	H
		(-)-Epifisetinidol	H	OH	OH	H	H
		(+)-Afzelechin	H	H	H	OH	OH
		(-)-Epiafzelechin	H	H	OH	H	OH
		(+)-Robinetindol	OH	OH	H	OH	H
Flavonol		Quercetin	H	OH	OH		
		Dihydroquercetin	H	OH	OH, H		
		Myricetin	OH	OH	OH		
		Kaempferol	H	H	OH		
		Dihydrokaempferol	H	H	OH, H		
		Rutin	H	OH	Rutinose		

Figure 13: Various flavonoids found in nature (modified from de Beer *et al.*, 2003 and Corradini *et al.*, 2011)

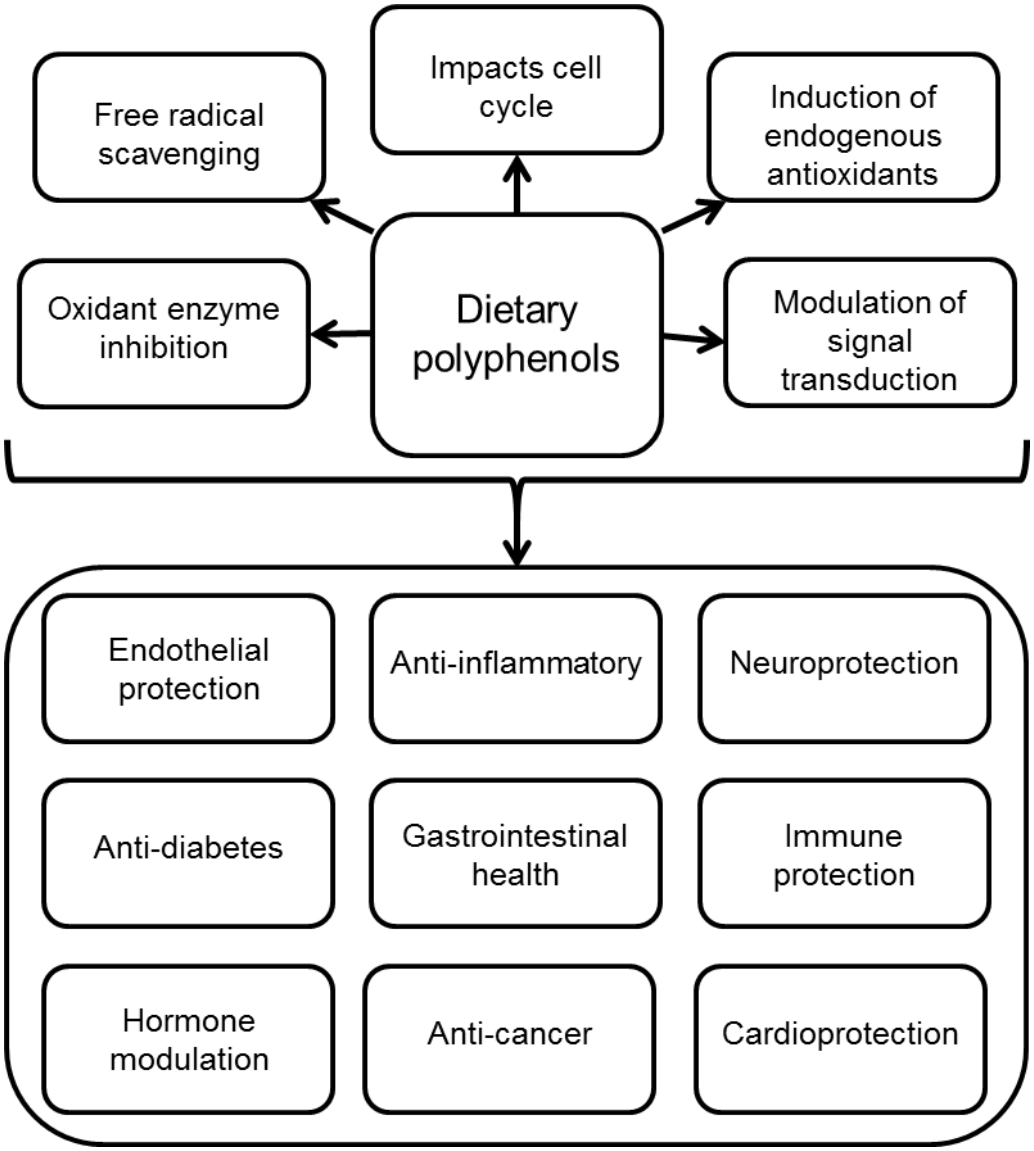


Figure 14: Examples of claimed beneficial effects of dietary polyphenol consumption (modified from Han *et al.*, 2007)

Many different types of plants rich in antioxidants are endemic to the African continent and yet literature concerning these plants in terms of their biological activity against oxidative stress is limited, and therefore warrants further investigation. Two plants of particular interest are *Burkea africana* Hook.F (Fabaceae) and *Syzygium cordatum* Hochst. ex C.Krauss (Myrtaceae).

B. africana, otherwise commonly known as the wild seringa (Figure 15), is found in sub-Saharan Africa. Ethnomedicinally, the bark is used to treat various conditions including heavy menstruation, abdominal pain, inflammation, pneumonia and as a wound dressing for topical ulcers (van Wyk and Gericke, 2000; Mathisen *et al.*, 2002). *S. cordatum*, otherwise known as the waterberry (Figure 16) is an evergreen tree which grows in moist environments (Hutchings *et al.*, 1996; van Wyk and Gericke, 2000). The bark is used ethnomedicinally as an emetic, treatment of diarrhea, stomach aches, chest complaints and wound healing (van Wyk and Gericke, 2000; Pallant and Steenkamp, 2008). Literature suggests their potential for cytoprotective functions against oxidation due to their high polyphenolic content (Mathisen *et al.*, 2002; van Wyk and Gericke, 2000), which could decrease the incidence of oxidative stress- related disorders (such as those found in inflammation and pulmonary diseases). Research concerning *B. africana* and *S. cordatum* is limited, thus justifying further investigation of these plants for the possible development of an antioxidant supplement. Further information regarding these plants are given in detail in the discussion.

Figure 15: *Burkea africana,* bark in left bottom corner (Wikipedia, 2008; Flora of Zimbabwe, 2012)

Figure 16: *Syzygium cordatum*, bark in left bottom corner (Venter and Venter, 1996; Wikimedia, 2008)

5. *Aims and objectives*

The aim of this study was to investigate the effects of various bark extracts of *B. africana* and *S. cordatum* on oxidative stress, with emphasis on the polyphenolic-rich extracts for potential future development.

The objectives of this study were to:

- Prepare crude aqueous and methanolic extracts of *B. africana* and *S. cordatum* bark, as well as polyphenolic-rich extracts
- Identify the major phytochemical constituents in the crude extracts and polyphenolic-rich extracts
- Determine the polyphenolic content of crude extracts and polyphenolic-rich extracts in terms of phenolics and flavonoids using the Folin-Ciocalteau and aluminium trichloride assays, respectively
- Determine the antioxidant activity of crude extracts and polyphenolic-rich extracts using the Trolox Equivalence Antioxidant Capacity (TEAC) and *1,1*-diphenyl-2-picrylhydrazyl (DPPH) assays
- Determine the *in vitro* cytotoxicity of crude extracts and polyphenolic-rich extracts in mammalian cell lines using the neutral red uptake assay
- Determine the *in vitro* effect of crude extracts and polyphenolic-rich extracts to attenuate oxidative stress in U937 cells induced by AAPH in terms of:
    - ROS generation
    - Cytotoxicity
    - Apoptosis
    - Lipid peroxidation
    - GSH depletion

Chapter 2
Materials and Methods

1. Preparation of plant extracts and polyphenolic-rich extracts

1.1. Collection of plant material

Bark of *B. africana* and *S. cordatum* was collected by Lawrence Tshikhudo and Dr N Hahn, respectively. No permission was needed for the collection of plant material. The identity was confirmed by a botanist and are catalogued at the Department of Toxicology (Onderstepoort Veterinary Institute, Pretoria) (*B. africana*, LT15) and the Soutpansbergensis Herbarium (*S. cordatum*, NH1880). Plant material was inspected for contamination by fungi and lichens, air-dried at room temperature for one week and ground to a fine powder (YellowLine Grinder, Merck).

1.2. Preparation of crude extracts

Powdered plant material (50 g) was extracted twice in 500 ml relevant solvent (distilled water or methanol, Appendix III), sonicated (Bransonic 52, Branson Cleaning Equipment Co.) for 30 min and kept at 4˚C for 24 h. Extracts were filtered through a vacuum-filtration system (pore size 0.22 µm) and concentrated either through lyophilization (Freezone® 6 Freeze Dry System, Labconco) or by means of *in vacuo* rotary-evaporation (Büchi Rotovapor R-200, Büchi) for aqueous and methanolic extracts, respectively. Methanolic extracts were reconstituted in absolute ethanol at a stock concentration of 100 mg/ml. Crude extracts were stored at -20˚C, and dissolved in phosphate buffered saline (PBS) to the desired concentrations (serial dilutions from 1 mg/ml) prior to use.

1.3. Preparation of polyphenolic-rich extracts

Fractionation of plant material to yield polyphenolic-rich extracts were carried out according to the methods of Jung *et al.* (2002), with modifications (Figure 17). Plant material (50 g) was defatted twice for 2 h with 80 ml hexane in a round bottom flask on a mechanical shaker (VRN-200 Shaker, Gemmy Industrial Corp.). Plant material was recovered by filtration through Whatman No. 1 filter paper and left under a fume hood to dry in order to remove residual hexane. Defatted plant material was

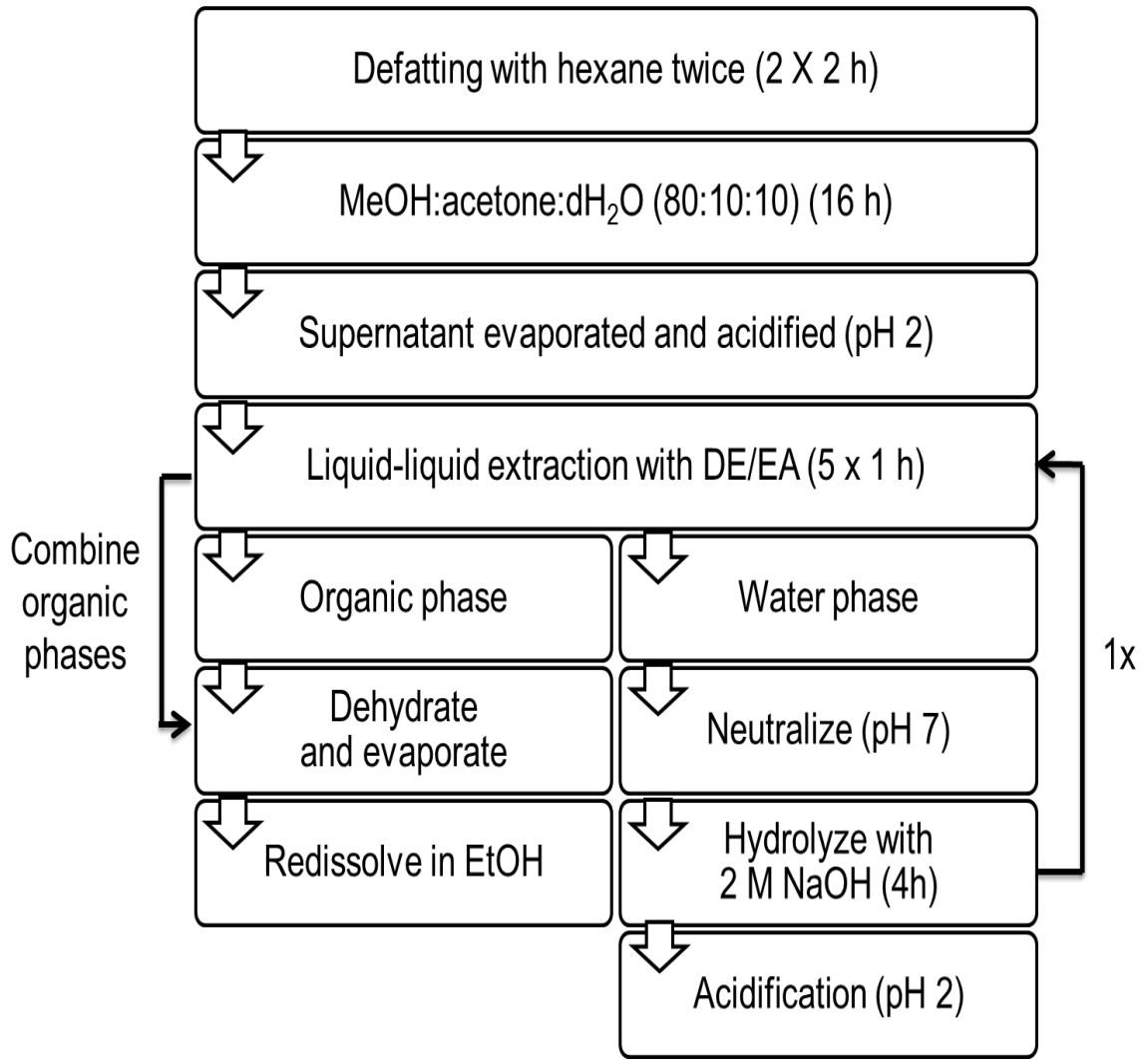


Figure 17: Fractionation procedure employed to create polyphenolic-rich extracts (adapted from Jung *et al.*, 2002); MeOH – methanol, dH_2O – distilled water, DE/EA – diethyl ether-ethyl acetate; EtOH – ethanol, NaOH – sodium hydroxide

macerated in 200 ml methanol:acetone:distilled water (80:15:5) at 4°C for 24 h. The mixture was filtered (pore size 0.22 µm) and concentrated to 10 ml *in vacuo* using a rotary-evaporator. The concentrated extract was diluted with 100 ml acidified water (pH 2, water phase) (Micro pH 2001, Crison) and partitioned five times with 100 ml diethyl ether-ethyl acetate (DE/EA, organic phase) for 1 h at a time. The organic phase was stored at -20°C until use. The water phase was neutralized to pH 7 with 2 M sodium hydroxide and lyophilized. Residue was hydrolyzed in 100 ml 2 M sodium hydroxide by shaking for 4 h on a mechanical shaker at room temperature. The solution was acidified to pH 2 with 6 M hydrochloric acid, and partitioned five times with 100 ml DE/EA for 1 h each. The water phase was discarded while all organic phases were combined, dehydrated with anhydrous sodium sulphate, vacuum-filtered (pore size 0.22 µm) and evaporated to dryness *in vacuo* to form the polyphenolic-rich extract. Residue was reconstituted in absolute ethanol to a stock concentration of 100 mg/ml. Polyphenolic-rich extracts were stored at -20˚C and diluted in PBS to the desired concentrations (serial dilutions from 1 mg/ml) prior to use.

2. Phytochemical screening

2.1. Thin layer chromatography (TLC)

Phytochemical screening of crude and polyphenolic-rich extracts for alkaloids, ascorbic acid, coumarins, flavonoids and phenolic acids were performed according to modified methods of Harborne (1973) (Table 1). C_{18} silica gel plates (10 x 10 cm) (Merck Chemicals, Darmstadt) were spotted with approximately 5 µg of crude and polyphenolic-rich extracts as well as standards, and developed using the specified mobile phases and sprayed with selective visualization agents.

2.2. Biochemical reactions

Crude and polyphenolic-rich extracts were screened for glycoside, saponin, terpenoid and steroid presence using chemical reactions as described by modified methods of Siddiqui and Ali (1997) (Figure 18). Screening for glycosides was done using a chemical reaction with sulphuric acid and ferric chloride. Crude and polyphenolic-rich extracts (300 µl, 3 mg/ml) were treated with 10 µl ferric chloride (5%) and 20 µl sulphuric acid. A red-brown reaction was an indiciation of glycoside presence.

Table 1: Development and selective spray reagents used to detect specific phytochemicals

Phytochemical	Mobile phase	UV visualization	Spray reagent
Alkaloids	MeOH:AcOH (200:3)	-	Dragendorff's reagent
Ascorbic acid	MeOH:AcOH (200:3)	254, 366 nm	Phosphomolybdinic acid
Coumarins	DE:toluene (1:1), 10% AcOH	254 nm	5% KOH
Flavonoids	chloroform:acetone:formic acid (75:15:10)	254, 366 nm	3% $NaNO_2$, 1% $AlCl_3$, 0.5 M NaOH
Apigenin			
Daidzein			
Epigallocatechin			
Genistein			
Hesperidin			
Kaempferol			
Myricetin			
Rutin			
Sinapic acid			
Vitexin			
Phenolic acids	MeOH:AcOH (200:3)	254, 366 nm	Folin-Ciocalteau
Benzoic acid			Phosphomolybdinic acid
Caffeic acid			
Catechin			
Cinnamic acid			
Ferulic acid			
Gallic acid			
p-Coumaric acid			
Quercetin			
Syringic acid			

MeOH – methanol ; AcOH – acetic acid ; DE – diethyl ether ; KOH – potassium hydroxide ; $NaNO_2$ – sodium nitrate ; $AlCl_3$ – aluminum trichloride ; NaOH – sodium hydroxide

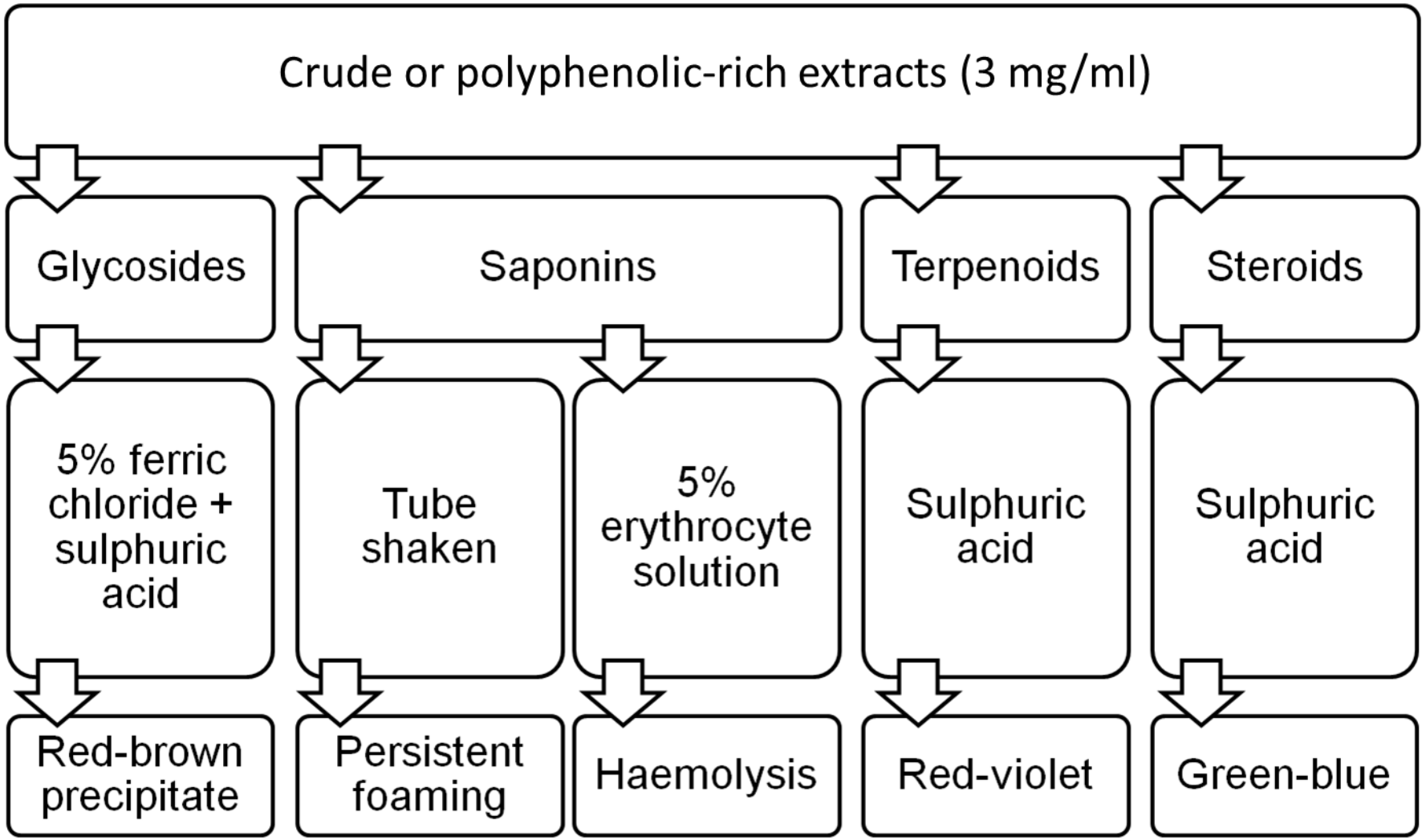


Figure 18: Procedure for detecting glycosides, saponins, terpenoids and steroids

The presence of saponins was determined through both a shaking and a haemolysis test. Extracts and polyphenolic-rich extracts (1 ml, 3 mg/ml) were shaken and persistent foam noted as positive for saponin presence. Additionally, heparinized blood (Department of Pharmacology Ethics Approval, Appendix I) was collected from a healthy volunteer, centrifuged at 200 *x g* for 5 min and the erythrocyte layer diluted in PBS to give a 5% suspension of red blood cells. Into 1.5 ml tubes was pipetted: 30 µl erythrocyte suspension and 170 µl crude extract, polyphenolic-rich extract or saponin standard (3 mg/ml). The contents were incubated for 1 h, after which tubes were centrifuged (5 min, 1000 *x g*) (Microfuge 16, Beckman Coulter) and the supernatant observed visually. A saponin-positive sample was identified if any visible haemolysis occured.

Terpenoid and steroid presence was determined via a chemical reaction with sulphuric acid. Extracts and polyphenolic-rich extracts (300 µl, 3 mg/ml) were treated with 20 µl sulphuric acid (98%). A red-violet and green-blue colour indicated the presence of terpenoids and steroids, respectively.

3. Determination of total polyphenolic content

3.1. Total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteu assay as described by Slinkard and Singleton (1977) with minor modifications. Gallic acid was used to prepare a standard curve. Into a tube was pipetted: 75 µl gallic acid standard (half serial dilutions of 1 mg/ml), crude or polyphenolic-rich extract, followed by 5925 µl distilled water, 375 µl Folin-Ciocalteu reagent (Merck, Darmstadt) and after 8 min incubation 1125 µl sodium carbonate solution (20%). The tubes were agitated and incubated in the dark for 2 h. Absorbance was measured at 765 nm (Lambda 25 UV/VIS Spectrophotometer, Perkin Elmer). Results are expressed as gallic acid equivalents (GAE mg/g extract ± standard error of the mean [SEM]) as calculated by the following equation:

$$GAE = \frac{c \times v \times DF}{m}$$

where, c = concentration obtained from standard curve (in mg/ml); v = volume obtained from initial extraction of plant material (in ml); DF = dilution factor of sample; and m = total weight of extract (in g).

3.2. Total flavonoid content (TFC)

TFC was determined using the aluminium trichloride assay as described by Dewanto *et al.* (2002) with minor modifications. Rutin hydrate was used to prepare a standard curve. Into individual wells of a 96-well plate was pipetted: 20 µl rutin hydrate (half serial dilutions of 1 mg/ml), crude or polyphenolic-rich extract, followed by 20 µl sodium nitrate solution (3%), 20 µl aluminum trichloride solution (1%) and 100 µl sodium hydroxide solution (0.5 M). Absorbance was measured at 570 nm (ELx800 Universal Microplate Reader, Bio-Tek Instruments, Inc.). Results are expressed as rutin equivalents (RE mg/g extract ± SEM) as calculated by the following equation:

$$RE = \frac{c \times v \times DF}{m}$$

where, c = concentration obtained from standard curve (in mg/ml); v = volume obtained from initial extraction of plant material (in ml); DF = dilution factor of sample; and m = total weight of extract (in g).

4. Determination of cell-free antioxidant activity

4.1. TEAC assay

The TEAC assay was used to determine the 2,2'-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid) radical ($ABTS^{\bullet+}$) scavenging ability of the crude and polyphenolic-rich extracts as described by Re *et al.* (1999). $ABTS^{\bullet+}$ (7.46 mM) was prepared in distilled water from 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and oxidized using 2.5 mM potassium peroxidisulfate at 4°C for 16 h. The oxidized solution was diluted with distilled water to an absorbance of 0.70 ± 0.02. Trolox was used to prepare a standard curve (half serial dilutions of 1 mg/ml) and samples were tested at four different concentrations (0.05 to 0.6 mg/ml). Into a cuvette was pipetted: 20 µl Trolox standard, crude extract, polyphenolic-rich extract or ascorbic acid (as antioxidant control) followed by 2 ml $ABTS^{\bullet+}$. Absorbance was measured at 734 nm after 1 min. Results are expressed as Trolox equivalents (TE ratio ± SEM) as determined by the following equation:

$$TE = \frac{slope(T)}{slope(S)}$$

where, slope(T) = slope of Trolox standards curve; slope(S) = slope of sample curve.

4.2. DPPH radical assay

The DPPH assay was used to determine the DPPH radical scavenging ability of the crude extracts and polyphenolic-rich extracts as described by Gyamfi *et al.* (1999) with minor modifications. Trolox was used to prepare a standard curve (half serial dilutions of 0.5 mg/ml) and samples were tested at four different concentrations (0.05 to 0.6 mg/ml). Into a 96-well plate was pipetted: 15 µl Trolox standard, crude extract, polyphenolic-rich extract or ascorbic acid followed by 185 µl DPPH solution (240 µM). Absorbance was measured after 15 min at 570 nm. Results are expressed as TE (ratio ± SEM) using the equation provided in Section 4.1.

5. Cytotoxicity

5.1. Primary cells and cell lines

Compounds can induce selective toxicity based on cellular type, thus it is of importance to determine inherent toxicity across a panel of cell types, including primary cells and cultures. All cell lines were cultured and maintained according to standard operating procedures of the Department of Pharmacology, University of Pretoria. Normal human dermal fibroblasts (NHDF) were purchased from Southern Medical, South Africa, while 3T3-L1 murine pre-adipocyte (#CL-173) and C2C12 murine myoblast (#CRL-1722) cell lines were purchased from the American Type Culture Collection (ATCC). Cultures of the above-mentioned cells were used to determine any cytotoxicity induced by supplementation with the crude and polyphenolic-rich extracts. Ethical clearance was obtained from the Ethics Committee of the University of Pretoria to carry out studies on commercial cell lines (Appendix II).

The U937 human pro-monocytic cell line was a gift from the National Institute of Occupational Health (NIOH), Johannesburg (South Africa). The U937 cell line is of human Caucasian origin which is commonly used as a model for inflammatory response. The cell line can be stimulated to differentiate into a macrophage-like state upon exposure to compounds such as phorbol-12-myristate-13-acetate (PMA) (Gingras and Margolin, 2000; Young *et al.*, 2008). Apart from inherent cytotoxicity, oxidative stress was induced in the U937 cell line as well.

5.2. Culture, maintenance and seeding of cells

Adherent NHDF, 3T3-L1 and C2C12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 µg/ml) in 25 cm^2 flasks at 37˚C and 5% CO_2. Once cells reached approximately 80% confluence, flasks were rinsed with PBS and cells detached using 3 ml Trypsin/Versene solution for 5 to 10 min. Cells were washed (200 *x g*, 5 min) (TJ-6 Centrifuge, Beckman), counted using the trypan blue exclusion assay (MicroStar 110, Reichert-Jung) with a haemocytometer, then diluted to 5 x 10^4 cells/ml in 2% FCS-supplemented DMEM. Trypan blue cannot cross intact cell membranes and will only stain dead cells (Fan *et al.*, 2009).

Non-adherent U937 cells were cultured in Roswell Park Memorial Insitition (RPMI)-1640 supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (100 µg/ml) in 25 cm^2 flasks at 37˚C and 5% CO_2. Cells were washed, centrifuged (200 *x g*, 5 min), counted using the trypan blue exclusion assay and diluted to 1 x 10^6 cells/ml in 10% FCS-supplemented RPMI-1640. Cells were stimulated to differentiate for 48 h with 32 nM PMA in 25 cm^2 flasks at 37˚C and 5% CO_2. Cells were recounted using the trypan blue exclusion assay and diluted to 1 x 10^6 cells/ml in 2% FCS-supplemented RPMI-1640.

Into a 96-well plate was placed: 100 µl cell suspension (5 x 10^3 cells/well and 1 x 10^5 cells/well for adherent and non-adherent cells, respectively) and 80 µl 2% FCS-supplemented medium. Plates were allowed to incubate at 37˚C and 5% CO_2 for 1 h or 24 h for non-adherent or adherent cell lines, respectively. Clear and white plates were used for spectrophotometrical and fluorescent assays, respectively.

5.3. Cytotoxicity of crude and polyphenolic-rich extracts

Cytotoxicity was determined using the neutral red uptake assay as described by Borenfreund *et al.* (1990) with minor modifications. Weakly cationic neutral red dye is accumulated in viable cells by binding to anionic sites within the lysosomal matrix and Golgi apparatus. This accumulation can be measured spectrophotometrically (Borenfreund *et al.*, 1990). The final concentration of ethanol for the methanolic and polyphenolic-rich extracts used in the cellular assays did not exceed 0.1%.

Inherent cytotoxicity of samples was determined in pre-seeded clear plates by addition of 20 µl medium (negative control), crude or polyphenolic-rich extracts (concentrations from 0.0078 mg/ml to 1 mg/ml) and incubation for 72 h at 37˚C and 5% CO_2. Medium was replaced with 100 µl neutral red medium (200 µg/ml) and incubated for 3 h. Plates were washed with PBS and centrifuged (200 *x g*, 5 min), left to dry and extracted using 100 µl neutral red eluent. Absorbance was measured at 540 nm (reference 630 nm) and the concentration which inhibits 50% cell growth (IC_{50}) was determined from the survival curves, using the GraphPad Prism 4 software package, when possible.

6. Attenuation of oxidative stress-induced parameters in U937 cells

6.1. Induction of AAPH-induced oxidative stress

Oxidative stress was induced in U937 cells through exposure to 1.5 mM AAPH for 48 h (except for ROS generation which was measured for 3 h), which allowed for extended generation of free radicals (Pietarinen *et al.*, 2006; Razinger *et al.*, 2010).

Into pre-seeded U937 plates was pipetted: 20 µl medium (negative control), positive control, 2.5, 5, 10 and 20 µg/ml crude or polyphenolic-rich extracts or 1 mM Trolox (as antioxidant comparison) and incubated for 1 h at 37˚C and 5% CO_2. For all experiments, except the ROS generation assay described in Section 6.3, plates were washed with RPMI-1640, centrifuged (200 *x g*, 5 min), treated with 1.5 mM AAPH (final reaction volume 200 µl) and incubated for 48 h (37˚C and 5% CO_2). White plates were used for all experiments to eliminate cross-fluorescence, except for the AAPH-induced cytotoxicity assay (Section 6.2) and the AAPH-induced lipid peroxidation assay (Section 6.5) which utilized clear plates. Positive controls used were 1% saponin (cell death control), 20 µM staurosprine (apoptosis control) and 20 mM H_2O_2 (lipid peroxidation and GSH depletion control).

6.2. Protection against AAPH-induced cytotoxicity

The ability of crude and polyphenolic-rich extracts to attenuate AAPH-induced cytotoxicity was measured using the neutral red uptake assay.

AAPH-exposed plates were assayed as described in Section 5.3, the values adjusted using the blank and expressed as the percentage viability relative to the negative control through the following equation:

$$Cell\ viability\ (\%) = \frac{A(sample)}{A(control)} \times 100$$

where, A(control) = average absorbance of triplicate negative control; A(sample) = triplicate absorbance of sample at a given concentration.

6.3. Protection against AAPH-induced ROS generation

The ability of crude and polyphenolic-rich extracts to attenuate AAPH-induced ROS generation was measured through the 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) method as described by Gutiérrez *et al.* (2007) with minor modifications. Acetate groups are cleaved intracellularly from non-fluorescent DCFHDA to trap the membrane impermeable dichlorodihydrofluorescein (DCF), which increases fluorescence almost 40-fold on oxidation to the DCF (Jakubowski and Bartosz, 2000; Gutiérrez *et al.* 2007).

Into pre-seeded U937 white plates was pipetted: 20 µl medium, crude extracts, polyphenolic-rich extracts (2.5, 5, 10 and 20 µg/ml) or 1 mM Trolox and 5 µM DCFHDA, which was incubated for 1 h at 37˚C and 5% CO_2. Plates were washed with PBS, centrifuged (200 *x g*, 5 min) and treated with 1.5 mM AAPH (final reaction volume 200 µl). Fluorescence was then measured over a period of 3 h at λ_{ex} = 485 nm and λ_{em} = 520 nm (FLUOstar Optima, BMG Labtech), and the values adjusted by subtracting the blank. The percentage inhibition of DCFHDA oxidation was determined through the following equation:

$$Inhibition\ (\%) = \frac{AUC(AAPH) - AUC(sample)}{AUC(AAPH)} \times 100$$

where, AUC(AAPH) = average area under curve of AAPH-exposed cells; AUC(sample) = average area under curve of sample-treated, AAPH-exposed cells.

6.4. Protection against AAPH-induced apoptosis

The ability of crude and polyphenolic-rich extracts to attenuate AAPH-induced apoptosis was measured through the caspase-3 activity assay as described by Banjerdpongchai *et al.*, (2010) with minor modifications. The probe, Acetyl-Asp-Glu-

Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), is cleaved by activated caspase-3 enzymatically to release the highly fluorescent amido-4-methylcoumarin (AMC) (Harwood *et al.*, 2005). Staurosporine, a serine/threonine kinase inhibitor and known apoptosis inducer (Giuliano *et al.*, 2004), was used as positive control.

AAPH-exposed plates were centrifuged at 200 *x g* for 5 min, medium was replaced with 25 µl cold lysis buffer and incubated for 15 min on ice. Thereafter, 100 µl caspase-3 substrate buffer contraining Ac-DEVD-AMC was added and plates incubated for 4 h at 37˚C. Fluorescence was then measured at λ_{ex} = 355 nm and λ_{em} = 460 nm, and the values adjusted by subtracting the blank. The percentage of apoptosis relative to the negative control was determined through the following equation:

$$Caspase\text{-}3\ activity\ (\%) = \frac{A}{A(control)} \times 100$$

where, A = fluorescent intensity of test sample; A(control) = fluorescent intensity of negative control.

6.5. Protection against AAPH-induced lipid peroxidation

The ability of crude and polyphenolic-rich extracts to attenuate AAPH-induced lipid peroxidation was measured through the thiobarbituric acid (TBA) assay as described by Stern *et al.*, (2010) with minor modifications. The lipid peroxidation product MDA forms a fluorescent adduct with TBA. H_2O_2 was used as positive control as it induces lipid peroxidation *in vitro* (Sheridan *et al.*, 1996).

From AAPH-exposed plates were taken aliquots (150 µl) of the supernatant which were mixed with 200 µl trichloroacetic acid (12.5%) and 400 µl TBA (1%) in plastic 5 ml tubes, vortex mixed and incubated for 20 min at 95˚C. 3-Methyl butan-1-ol (1 ml) was added to the mixture, vortex mixed and the organic layer left to separate from the aqueous. Into a white 96-well plate was transferred 100 µl of the 3-methyl butan-1-ol layer and the fluorescence measured at λ_{ex} = 544 nm and λ_{em} = 590 nm. Values were adjusted by subtracting the blank. The percentage of lipid peroxidation relative to the negative control was determined through the following equation:

$$Lipid\ peroxidation\ (\%) = \frac{A}{A(control)} \times 100$$

where, A = fluorescent intensity of test sample; A(control) = fluorescent intensity of the negative control.

6.6. Protection against AAPH-induced GSH depletion

The ability of crude and polyphenolic-rich extracts to attenuate AAPH-induced GSH depletion was measured through the monochlorobimane assay. Monochlorobimane and reduced glutathione form a fluorescent adduct through the action of GST (Fernandez-Checa and Kaplowitz, 1990).

Into AAPH-exposed plates was pipetted 50 µM monochlorobimane and plates were incubated for 1 h. Plates were washed twice with PBS, centrifuged (200 *x g*, 5 min) after which the fluorescence was measured at λ_{ex} = 355 nm and λ_{em} = 460 nm, and the values adjusted by subtracting the blank. The percentage of GSH depletion relative to the negative control was determined through the following equation:

$$GSH\ (\%) = \frac{A}{A(control)} \times 100$$

where, A = fluorescent intensity of test sample; A(control) = fluorescent intensity of the negative control.

7. Statistical analyses

All experiments were performed in triplicate on three separate days. Results are expressed as mean ± SEM as determined by GraphPad Prism 4. IC_{50}s were determined through use of non-linear regression (variable slope) where survival was below 50% of initial cell numbers. Results of attenuation of AAPH-induced parameters were compared with each other through use of one way analysis of variance (ANOVA) with a post-hoc Dunnett's test. Significance was noted as $p < 0.05$.

Chapter 3
Results

1. Extraction and phytochemical screening

Extraction yields are presented in Table 2, where *B. africana* yeilded a higher recovery for both the crude and polyphenolic-rich extracts than *S. cordatum*. Phytochemical screening results for these crude and polyphenolic-rich extracts are presented in Table 3. As expected, polyphenolic-rich extracts were enriched with polyphenolic phytochemicals due to the selective extraction sequence. Both plants tested positive for caffeic acid, cinnamic acid, epigallocatechin, gallic acid and sinapic acid – although *S. cordatum* required heating of the crude extracts for caffeic acid extraction. *B. africana* tested positive for catechin (only upon heating) and myricetin, while *S. cordatum* alone contained hesperidin. The polyphenolic-rich extracts of *B. africana* and *S. cordatum* also contained alkaloids, glycosides and terpenoids. Contradictory data was observed between the haemolysis and foam tests for the presence of saponins.

2. Total polyphenolic content and cell-free antioxidant activity

Polyphenolic content and antioxidant activity followed an expected trend with the relative polyphenolic concentration and antioxidant activity increasing from crude aqueous to crude methanolic to the polyphenolic-rich extract (Table 4). A 1.8-fold increase in polyphenol content from the crude aqueous to crude methanolic extracts in both plants was noted. Also a 1.4-fold and 2-fold increase in polyphenolic content from methanolic to polyphenolic-rich extract for *B. africana* and *S. cordatum*, respectively was found. *B. africana* extracts contained greater concentrations of polyphenols than *S. cordatum*.

Antioxidant activity was expressed as a ratio of the sample to Trolox, thus a value above 1 indicates superior activity in the test sample. The crude extracts of *B. africana* displayed greater antioxidant activity than those of *S. cordatum*. The polyphenolic-rich extract of *S. cordatum* however showed antioxidant activity greater

Table 2: Extraction yields for dry plant material

Plant	Extract/fraction	Extraction yield (%)
B. africana	Aqueous	11.79
	Methanolic	16.00
	Polyphenolic	11.00
S. cordatum	Aqueous	5.72
	Methanolic	11.00
	Polyphenolic	5.00

Table 3: Phytochemical screening of crude and polyphenolic-rich extracts

Phytochemical	*B. africana*			*S. cordatum*		
	A	M	P	A	M	P
Alkaloids	+*	+*	+#	+*	+*	+#
Ascorbic acid	-	-	-	-	-	-
Coumarins	-	-	-	-	-	+#
Flavonoids	+#	+#	+#	+#	+#	+#
Apigenin	-	-	-	-	-	-
Catechin	-	+*	+*	-	-	-
Daidzen	-	-	-	-	-	-
Genistin	-	-	-	-	-	-
Hesperidin	-	-	-	+#	+#	+#
Kaempferol	-	-	-	-	-	-
Myricetin	-	-	+#	-	-	-
Rutin	-	-	-	-	-	-
Sinapic acid	-	-	+#	+#	+#	+#
Vitexin	-	-	-	-	-	-
Glycosides	+#	+#	+#	+#	+#	+#
Phenolic acids	+#	+#	+#	+#	+#	+#
Benzoic acid	-	-	-	-	-	-
Caffeic acid	+#	+#	+#	+*	+*	+*
Cinnamic acid	-	+#	+#	+#	+#	+#
Epigallocatechin	-	+#	+#	-	-	+#
Ferulic acid	-	-	-	-	-	-
Gallic acid	-	+#	+#	-	-	+#
p-Coumaric acid	-	-	-	-	-	-
Quercetin	-	-	-	-	-	-
Syringic acid	-	-	-	-	-	-
Saponins (foam)	-	+#	+#	-	+#	+#
Saponins (haemolysis)	-	-	-	-	-	-
Steroids	-	-	-	-	-	-
Terpenoids	+#	+#	+#	+#	+#	+#

A – aqueous; M – methanolic; P – polyphenolic-rich; + positive indication for phytochemical; - negative indication for phytochemical; # immediate appearance; * appearance after heating (50°C)

Table 4: Total polyphenolic content and antioxidant activity of crude and polyphenolic-rich extracts of *B. africana* and *S. cordatum*

Plant	Extract	Total polyphenolic content (mg/g extract ± SEM)		Antioxidant activity (TE ± SEM)	
		TPC (GAE)	TFC (RE)	TEAC	DPPH
B. africana	Aqueous	260.07 ± 10.76^{a,b}	196.78 ± 7.33^{a,b}	1.15 ± 0.02$^{a.b,c}$	1.09 ± 0.02^{a,b}
	Methanolic	484.43 ± 13.70^{a}	334.31 ± 3.04^{a,c}	1.42 ± 0.02^{a,b}	1.46 ± 0.03^{a}
	Polyphenolic	699.07 ± 25.28^{a}	460.00 ± 18.85^{a}	2.03 ± 0.06^{a}	2.18 ± 0.03^{a}
S. cordatum	Aqueous	183.88 ± 5.57^{a}	130.56 ± 9.51^{a}	0.80 ± 0.01^{a}	0.74 ± 0.01^{a}
	Methanolic	260.80 ± 9.19^{a,b}	192.23 ± 8.87^{a,b}	1.09 ± 0.02^{a,c}	1.08 ± 0.02^{a,b}
	Polyphenolic	619.37 ± 11.27^{a}	334.00 ± 9.65$^{a.c}$	2.46 ± 0.10^{a}	2.98 ± 0.11^{a}
Ascorbic acid		-	-	2.80 ± 0.01^{a}	1.67 ± 0.01^{a}

GAE – gallic acid equivalents; TFC - Total flavonoid content; RE – rutin equivalents; TE – Trolox equivalents; ^{a} – values significantly different ($p < 0.05$); ^{b,c} – values not statistically different ($p > 0.05$)

than that of the polyphenolic-rich extract of *B. africana*, despite the lower concentrations of polyphenols. In the DPPH assay the polyphenolic-rich extracts of both plants presented with higher activity than ascorbic acid. The polyphenolic content and antioxidant activity of both plants was plotted against each other, and a linear correlation was observed (Figure 19), where phenolic content had a higher correlation ($r > 0.85$) than flavonoid ($r > 0.72$).

3. Inherent cytotoxicity of crude extracts and polyphenolic-rich extracts

Exposure of cultures of cell lines to crude and polyphenolic-rich extracts demonstrated a small dose response with a gradual decrease in cell viability as the test concentration increased (Figure 20). Pronounced toxicity was noted in C2C12 and 3T3-L1 cell lines, but not in the U937 and NHDF cells, where concentrations up to 100 µg/ml extract did not achieve an IC_{50} (Table 5). For all cells and cell lines tested, the greatest toxic effect was observed for the polyphenolic-rich extracts, with *B. africana* being slightly more toxic. For *B. africana* the methanolic crude extract and polyphenolic-rich extract were the most toxic, while the aqueous crude extract produced relatively less toxicity. The C2C12 cell line was most sensitive, while the 3T3-L1 cell line required approximately 2-fold the concentration for the same toxic effect. Crude extracts from *S. cordatum* were more toxic to the 3T3-L1 cell line than the C2C12, with the methanolic extract being less cytotoxic than the aqueous extract. The *S. cordatum* polyphenolic-rich extracts had a similar toxicity profile in both cell lines.

4. Attenuation of oxidative stress-induced parameters in U937 cells

4.1. Protection against AAPH-induced ROS generation

The effect of crude and polyphenolic-rich extracts on ROS formation was determined through DCF fluorescence. AAPH generated high levels of ROS over the course of 3 h, with a 16.94-fold increase in fluorescence relative to the negative control (Figure 21), while only a moderate 2.2-fold increase was noted between the blank to negative control. Trolox at 1 mM inhibited ROS formation by 95.73% (Figure 22). Inhibitory activity was similar to that seen in the cell-free antioxidant assays. Polyphenolic-rich extracts were the most effective at decreasing ROS generation, followed by methanolic extracts then aqueous extracts. While *B. africana* crude

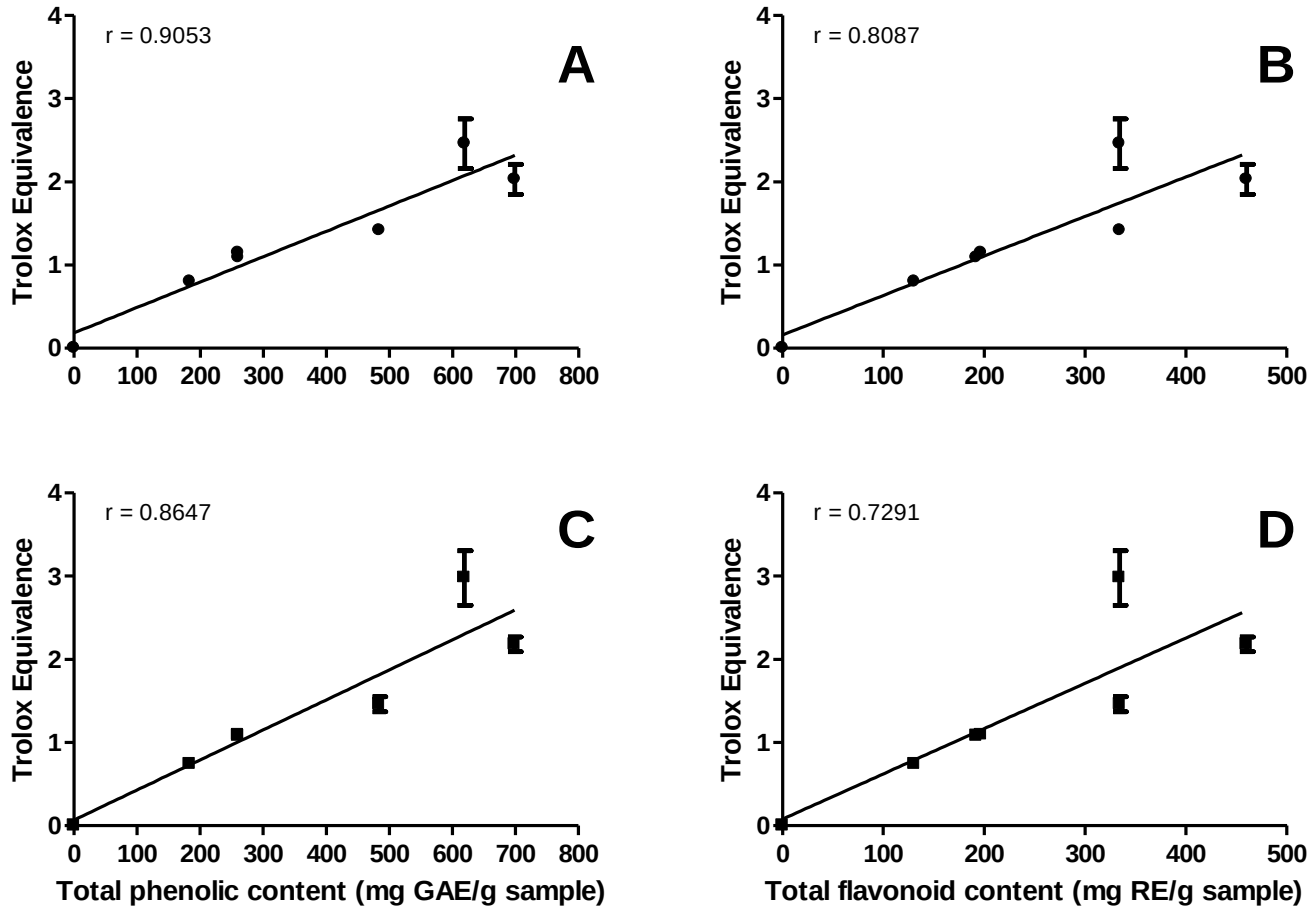


Figure 19: Correlation between polyphenolic content and antioxidant activity; A - phenolic content vs TEAC, B - flavonoid content vs TEAC, C - phenolic content vs DPPH, D - flavonoid content vs DPPH

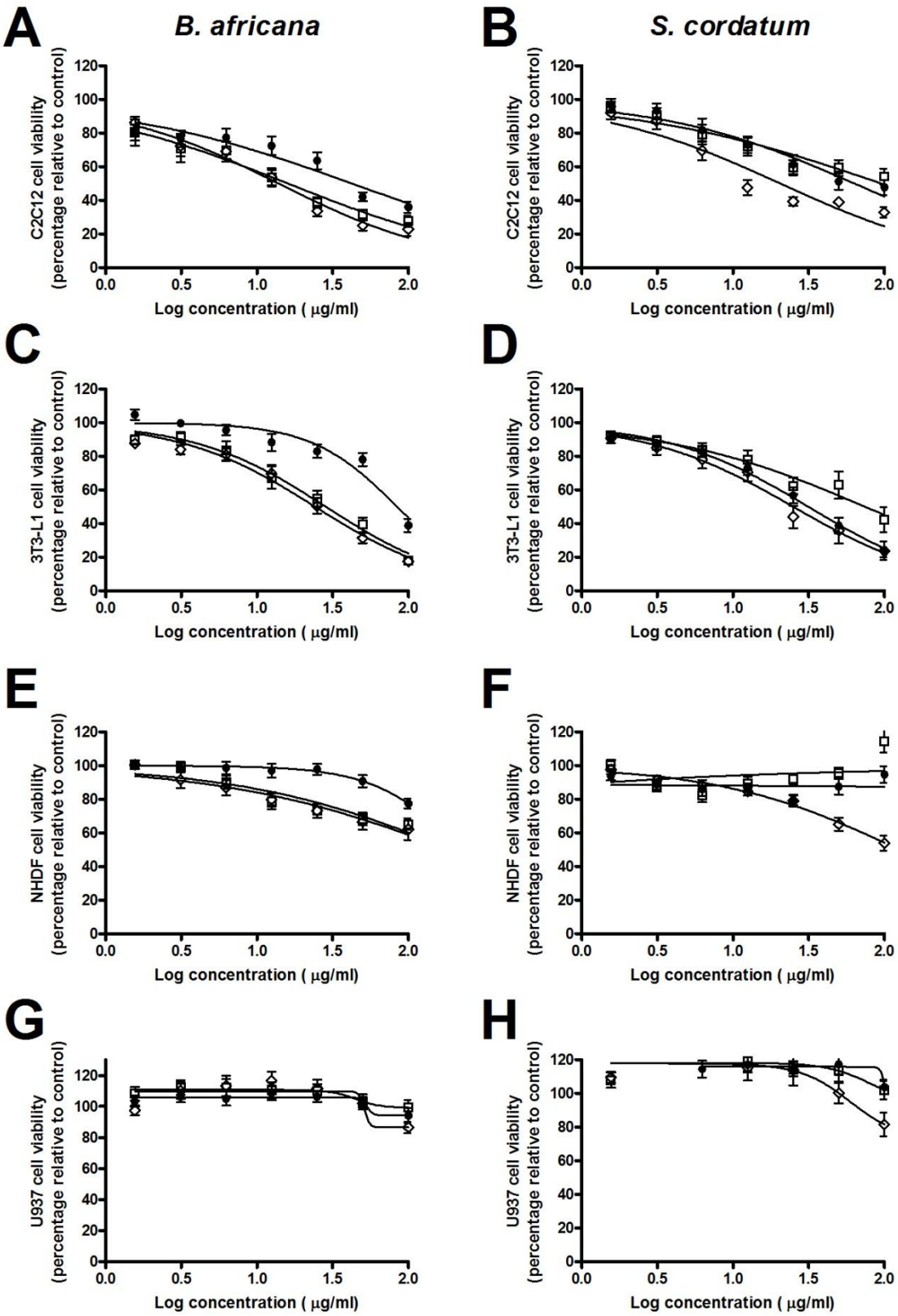


Figure 20: Cytotoxic effect of the crude (aqueous • / methanolic □) and polyphenolic-rich (◊) extracts from *B. africana* (A, C, E, G) and *S. cordatum* (B, D, F, H) in C2C12 myoblasts (A, B), 3T3-L1 pre-adipocytes (C, D), NHDF (E, F) and 48 h PMA-stimulated U937 monoblastic cells (G, H).

Table 5: Calculated IC_{50} values of crude and polyphenolic-rich extracts of *B. africana* and *S. cordatum* on different cell cultures

Plant	Extract	IC_{50} ± SEM (µg/ml)			
		C2C12	3T3-L1	NHDF	U937
B. africana	Aqueous	42.5 ± 1.1	84.5 ± 1.0	>100	>100
	Methanolic	16.0 ± 1.0	28.5 ± 1.0	>100	>100
	Polyphenolic	13.9 ± 1.0	24.3 ± 1.0	>100	>100
S. cordatum	Aqueous	63.4 ± 1.0	31.4 ± 1.0	>100	>100
	Methanolic	95.6 ± 1.1	74.6 ± 1.0	>100	>100
	Polyphenolic	20.5 ± 1.1	25.0 ± 1.0	>100	>100

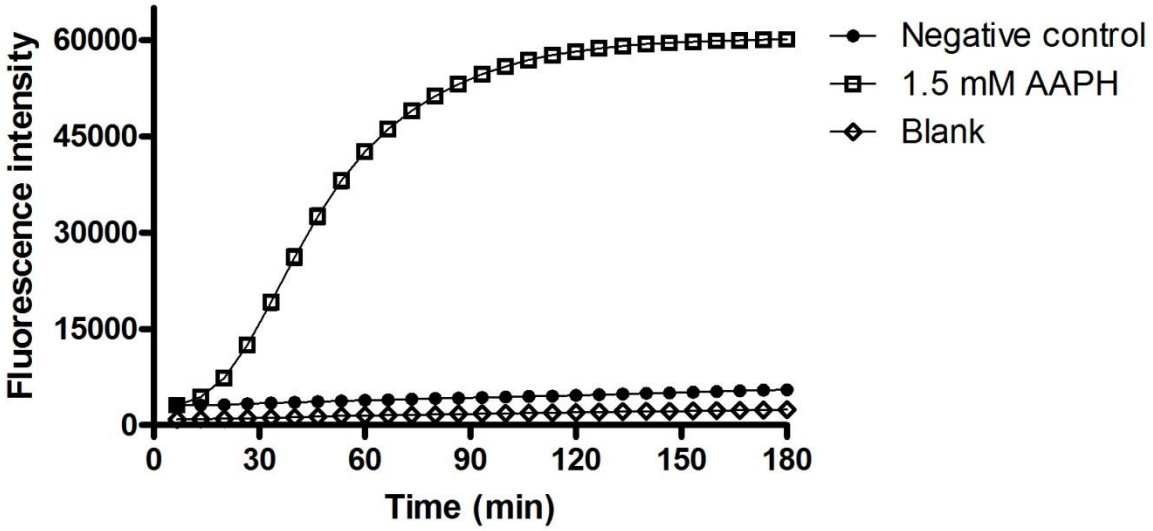


Figure 21: Generation of ROS by 1.5 mM AAPH over a 3 h period in U937 cells

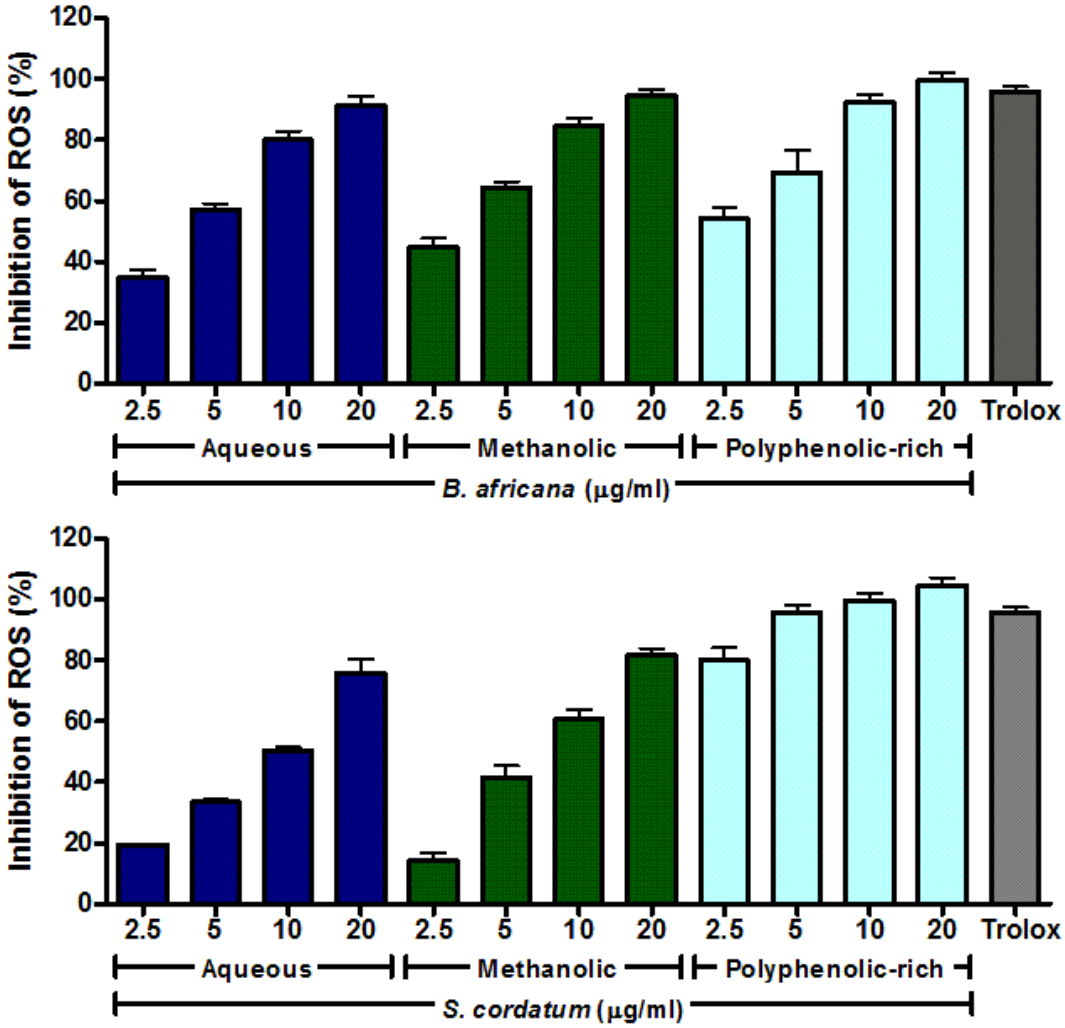


Figure 22: Inhibition of ROS generation in U937 cells pre-treated for 1 h with various concentrations of *B. africana* and *S. cordatum* extracts followed by 48 h exposure to AAPH

extracts had higher activity than *S. cordatum*'s crude extracts, the phenolic-rich fraction of *S. cordatum* had the greatest ROS attenuating effect, decreasing ROS generation by more than 80% at a concentration of only 2.5 µg/ml.

4.2. Protection against AAPH-induced cytotoxicity

Exposure to AAPH reduced cell viability to 73.68% after 48 h exposure (Figure 23). Concentrations of ≥10 µg/ml for all crude and polyphenolic-rich extracts were able to protect against this cytotoxicity in a dose-dependent manner after 1 h pre-treatment. Although not significant, the polyphenolic-rich extract of *S. cordatum* (96.57%) tended to provide higher protection than the polyphenolic-rich extract of *B. africana* (90.23%). Trolox was not able to induce significant protection against the AAPH-induced cytotoxicity.

4.3. Protection against AAPH-induced apoptosis

AAPH increased caspase-3 activity by 33.6% compared to the negative control, representing induction of apoptosis (Figure 24). Trolox decreased AAPH-induced apoptosis by 44.67%. *B. africana* decreased apoptosis in a dose-dependent manner with the highest activity seen for the polyphenolic-rich extract, followed by the methanolic then the aqueous extracts. Significant decreases ($p < 0.05$) in apoptosis were noted for 5, 10 and 20 µg/ml concentrations of *B. africana* crude extracts and the polyphenolic-rich extract. The ability to protect against apoptosis was less pronounced in *S. cordatum* where the crude extracts did not demonstrate a dose-dependent response, although there was a significant decrease ($p < 0.05$) noted in the following: aqueous extract at 5 and 20 µg/ml, and methanolic extract at 20 µg/ml. The polyphenolic-rich extract protected significantly ($p < 0.05$) at all concentrations tested. Overall, *B. africana* resulted in a higher level of protection against apoptosis, while only the polyphenolic-rich extract of *S. cordatum* was able to elicit a protective effect.

4.4. Protection against AAPH-induced lipid peroxidation

AAPH induced a significant increase ($p < 0.001$) in MDA formation (384.4%) after 48 h exposure, which was similar to that of the positive control, H_2O_2 (389.3%) (Figure 25). Trolox reduced AAPH-induced lipid peroxidation by up to 338.2%. *B. africana*

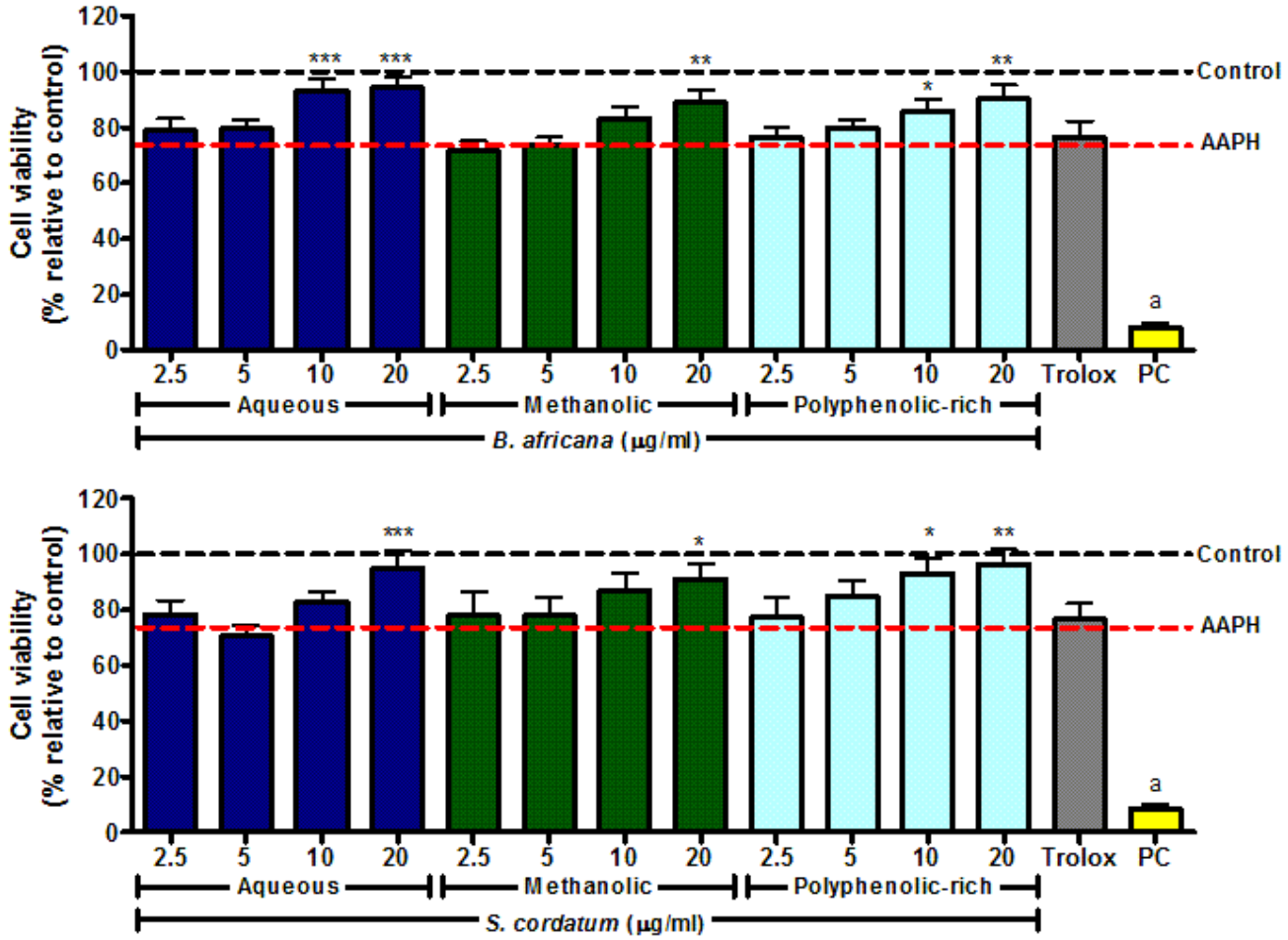


Figure 23: Protection against cytotoxicity in U937 cells pre-treated for 1 h with various concentrations of *B. africana* and *S. cordatum* extracts followed by 48 h exposure to AAPH; PC = Positive control (saponin); black and red lines represent negative control and AAPH-treated cells, respectively; control vs AAPH: a – $p < 0.001$; AAPH vs sample: *** – $p < 0.001$, ** – $p < 0.01$, * – $p < 0.05$

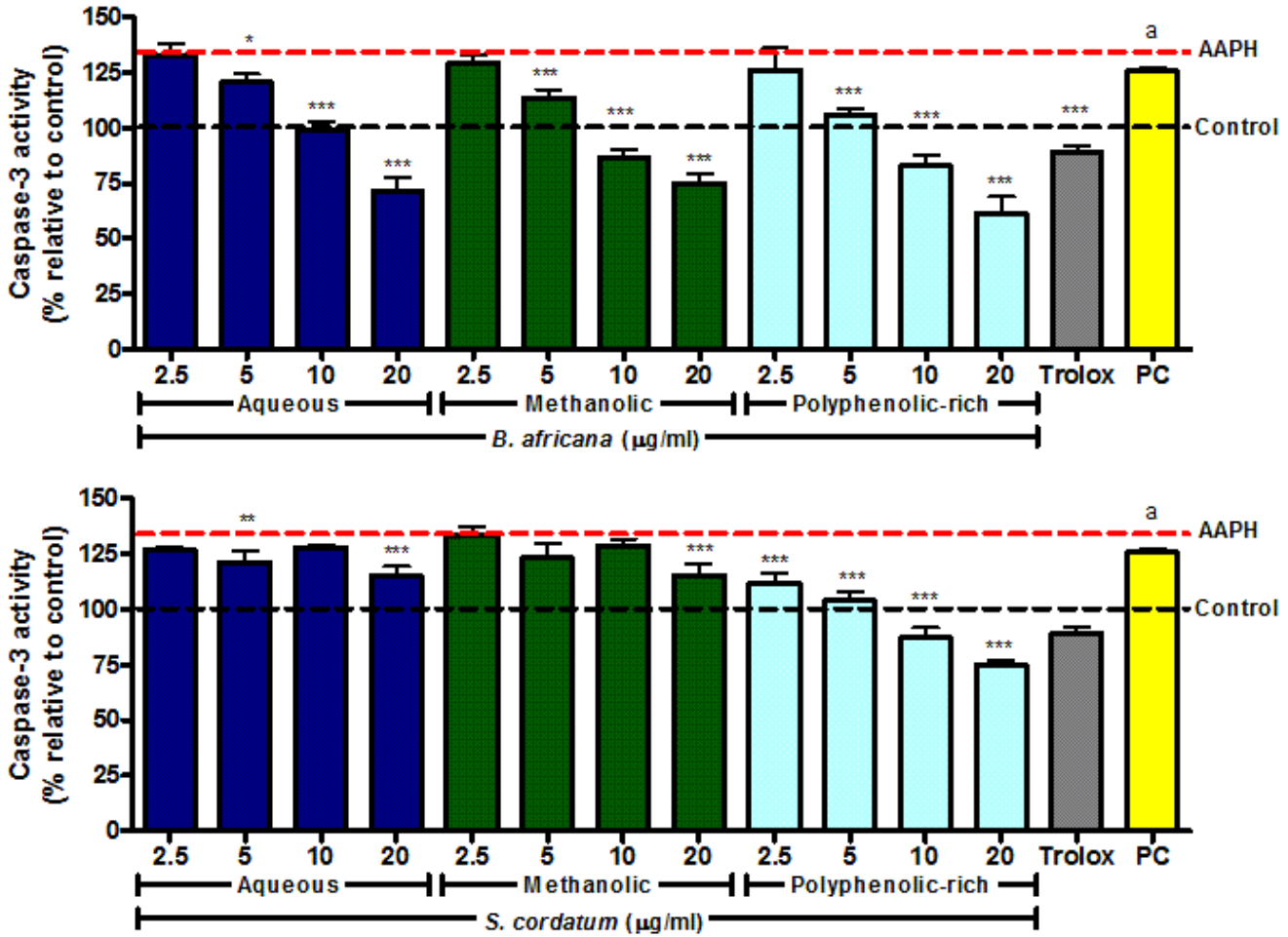


Figure 24: Inhibition of caspase-3 activation in U937 cells pre-treated for 1 h with various concentrations of *B. africana* and *S. cordatum* extracts followed by 48 h exposure to AAPH; PC = Positive control (staurosporine); black and red lines represent negative control and AAPH-treated cells, respectively; control vs AAPH: a – $p < 0.001$; AAPH vs sample: ******* – $p < 0.001$, ****** – $p < 0.01$, ***** – $p < 0.05$

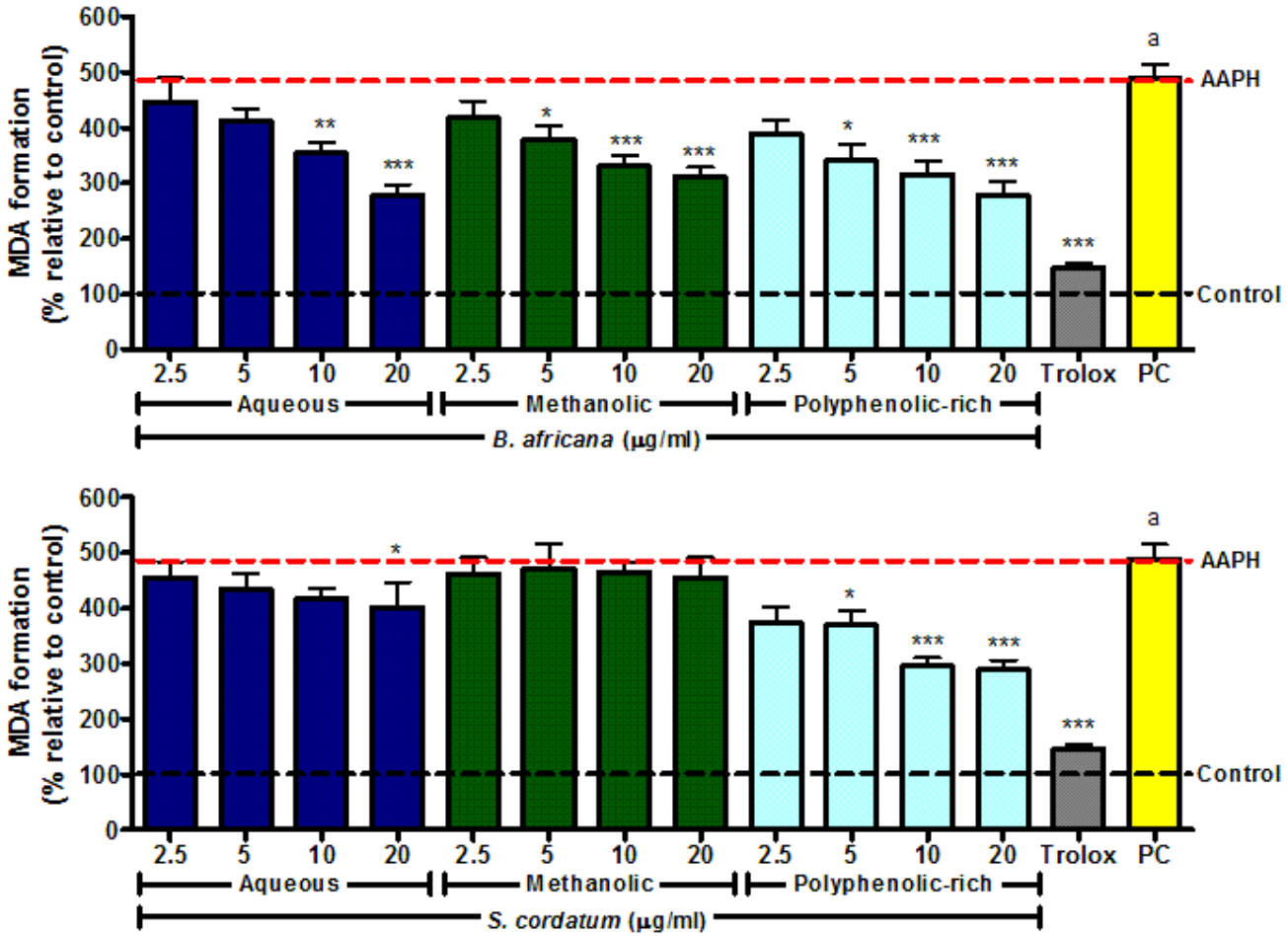


Figure 25: Inhibition of lipid peroxidation in U937 cells pre-treated for 1 h with various concentrations of *B. africana* and *S. cordatum* extracts followed by 48 h exposure to AAPH; PC = Positive control (H_2O_2); black and red lines represent negative control and AAPH-treated cells, respectively; control vs AAPH: a – $p < 0.001$; AAPH vs sample: *** – $p < 0.001$, ** – $p < 0.01$, * – $p < 0.05$

reduced lipid peroxidation in a dose-dependent manner. *S. cordatum*'s crude aqueous and methanolic extracts were not able to decrease lipid peroxidation significantly, although a slight trend was noted with the aqueous extract. However, the polyphenolic-rich extract did demonstrate inhibition of lipid peroxidation.

4.5. Protection against AAPH-induced GSH depletion

AAPH-induced oxidative stress resulted in a 10.24% reduction in GSH concentrations, while Trolox increased GSH concentrations by 11.8% from baseline (Figure 26). Pre-treatment of cells with extracts of *B. africana* prior to AAPH treatment showed a dose dependent decrease in GSH concentrations, which was greatest for methanolic crude extract. The aqueous crude extract of *S. cordatum* had a similar decrease at concentrations of greater than 10 µg/ml. The methanolic extract was the only extract to increase the GSH concentrations (as one would expect from antioxidant supplementation) with higher concentrations having the greater ($p < 0.05$) protective effect. The polyphenolic-rich extract of *S. cordatum* increased GSH concentrations in a dose-dependent manner, but a sharp decline was seen at 20 µg/ml.

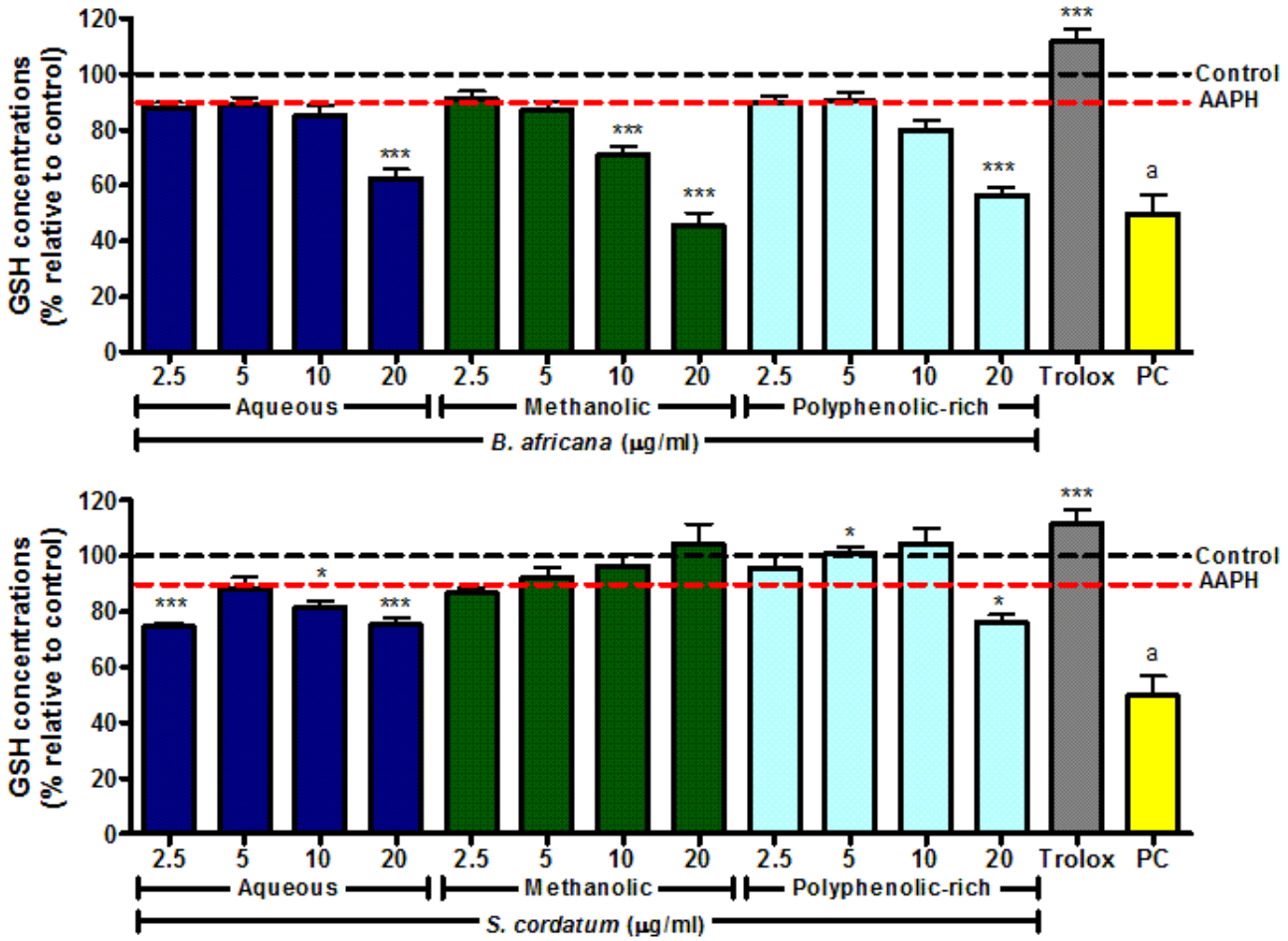


Figure 26: Effect on glutathione concentrations in U937 cells pre-treated for 1 h with various concentrations of *B. africana* and *S. cordatum* extracts followed by 48 h exposure to AAPH; PC = Positive control (H_2O_2); black and red lines represent negative control and AAPH-treated cells, respectively; control vs AAPH: a – $p < 0.001$; AAPH vs sample: ******* – $p < 0.001$, ***** – $p < 0.05$

Chapter 4
Discussion

1. Phytochemical composition, polyphenolic content and antioxidant activity

Selective fractionation increased the relative concentration of polyphenolic phytochemicals, however, the polyphenolic fractions were not still pure and interfering compounds (such as alkaloids and mixed glycosides) were still detected. Polyphenolic compounds are able to complex with other phytochemicals, such as alkaloids (Harborne, 1973), explaining their presence within the polyphenolic-rich extracts. Information regarding the phytochemical composition of *B. africana* and *S. cordatum* is scarce. *B. africana* has been reported to contain proanthocyanidins, catechin, epicatechin, fisetinidol and other phenolic acids contributing to its high antioxidant activity (Figure 27) (Diallo *et al.*, 2001; Mathisen *et al.*, 2002). In the current study, *B. africana* was also found to contain a variety of polyphenolic constituents: flavonoids such as catechin (supported by literature), epigallocatechin, myricetin and sinapic acid, and phenolic acids such as caffeic acid, cinnamic acid and gallic acid (Figure 28). Caffeic and sinapic acids are hydroxylated cinnamic acid derivatives found in various plant sources that present with antioxidant and anti-inflammatory activities (Pari and Jalaludeen, 2011; Prasad *et al.*, 2011; Terpinc *et al.*, 2011). Myricetin is a flavonol which also displays high antioxidant activity (de Beer *et al.*, 2002; Borde *et al.*, 2011). *B. africana* contained a higher concentration of polyphenols of the two plants tested.

S. cordatum has been reported to contain arjunolic acid, β-sitosterol, friedelin, epi-friedelinol, flavonoids, phenolic acids, glucose, proanthocyanidins, gallic acid, gallic-acid-ellagic acid complex, leucodelphinidin and leucocyanidin (Figure 29) (Candy *et al.*, 1968; Hutchings *et al*, 1996). Arjunolic acid, friedelin and epi-friedelinol are triterpenoids, while β-sitosterol is a phytosterol (Candy *et al.*, 1968). Polyphenolic constituents have been suggested to contribute to its antioxidant activity (Pallant and Steenkamp, 2008). The present study reveals that the polyphenolic-rich extract of *S. cordatum* contained the flavonoids hesperidin and sinapic acid, the phenolic acids caffeic acid, cinnamic acid, and gallic acid and epigaloocatechin (Figure 30). The

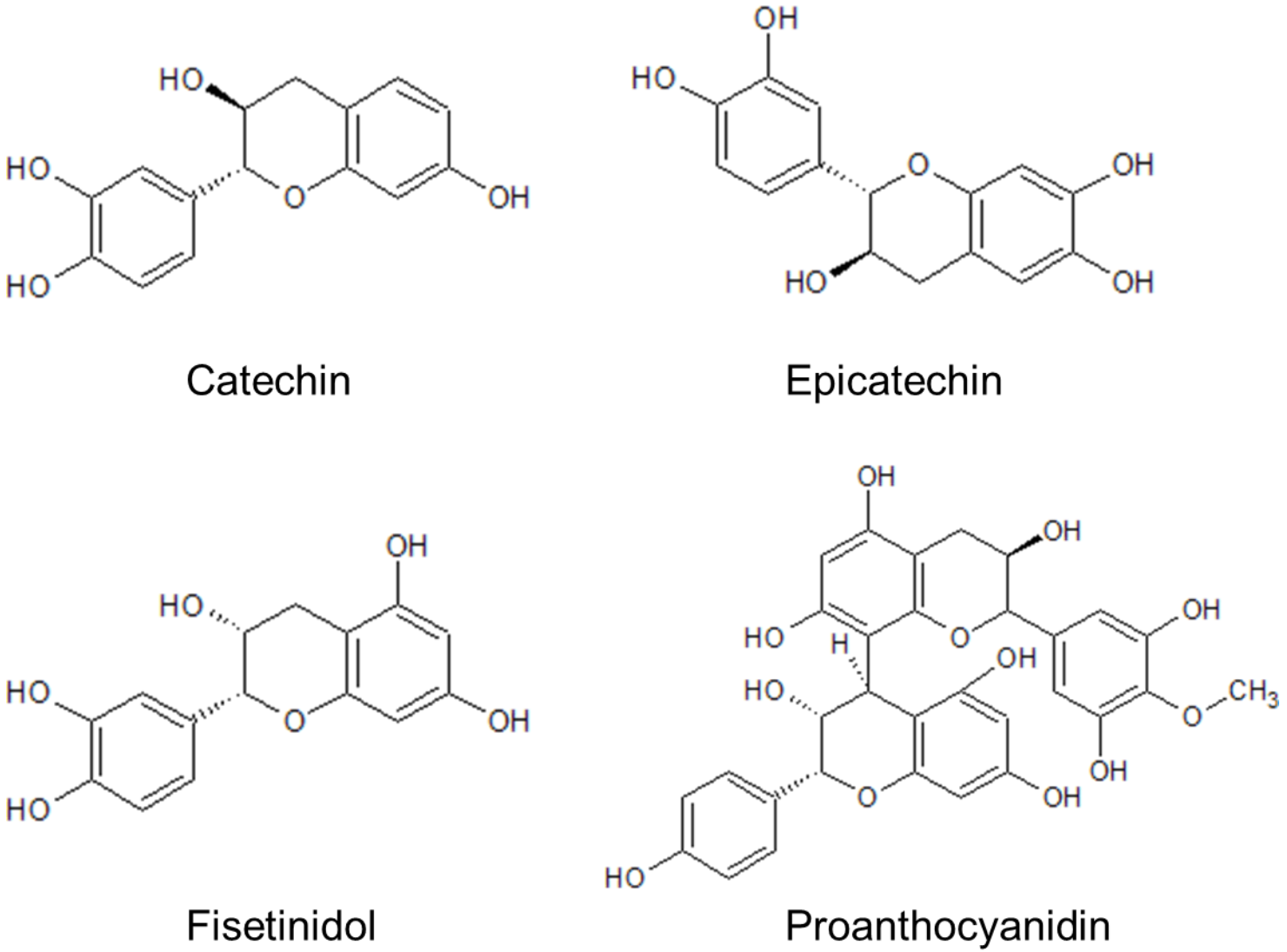
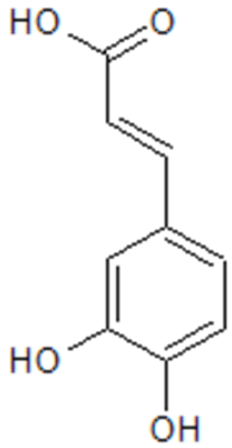
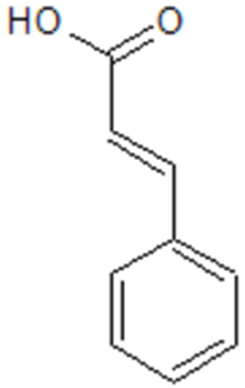


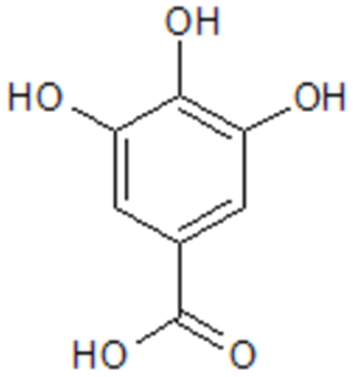
Figure 27: Phytochemicals previously isolated from *B. africana* (Source: Chemspider, 2012)



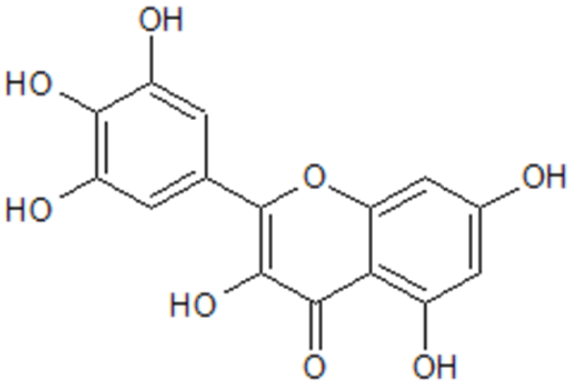
Caffeic acid



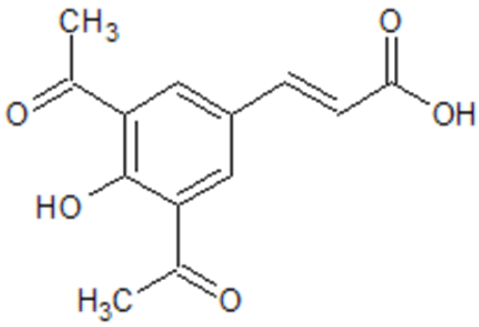
Cinnamic acid



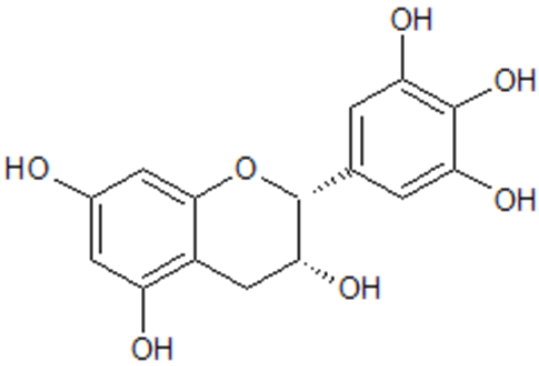
Gallic acid



Myricetin



Sinapic acid



Epigallocatechin

Figure 28: Polyphenols identified in *B. africana* during the present study not previously reported (Source: Chemspider, 2012)

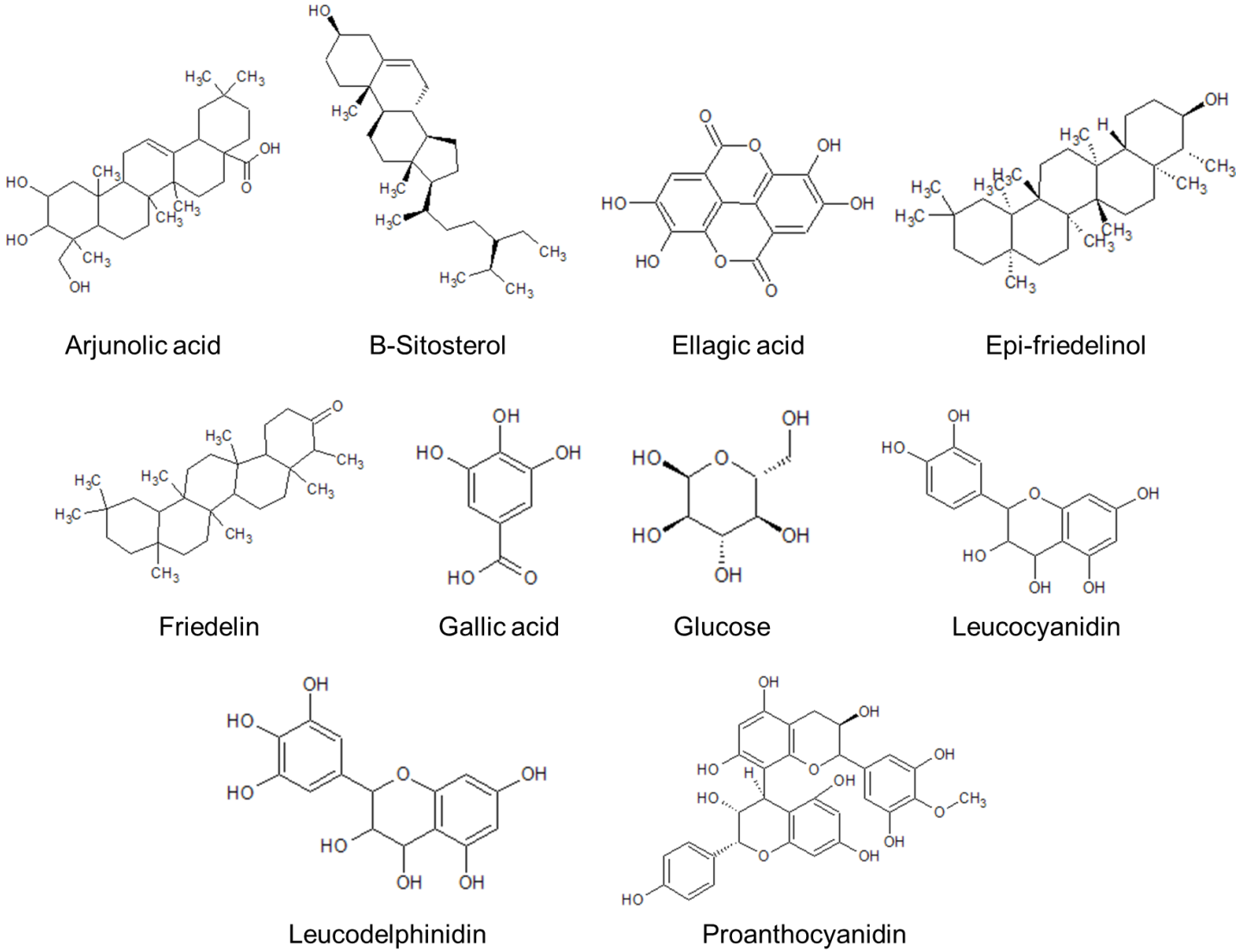


Figure 29: Phytochemicals previously isolated from *S. cordatum* (Source: Chemspider, 2012)

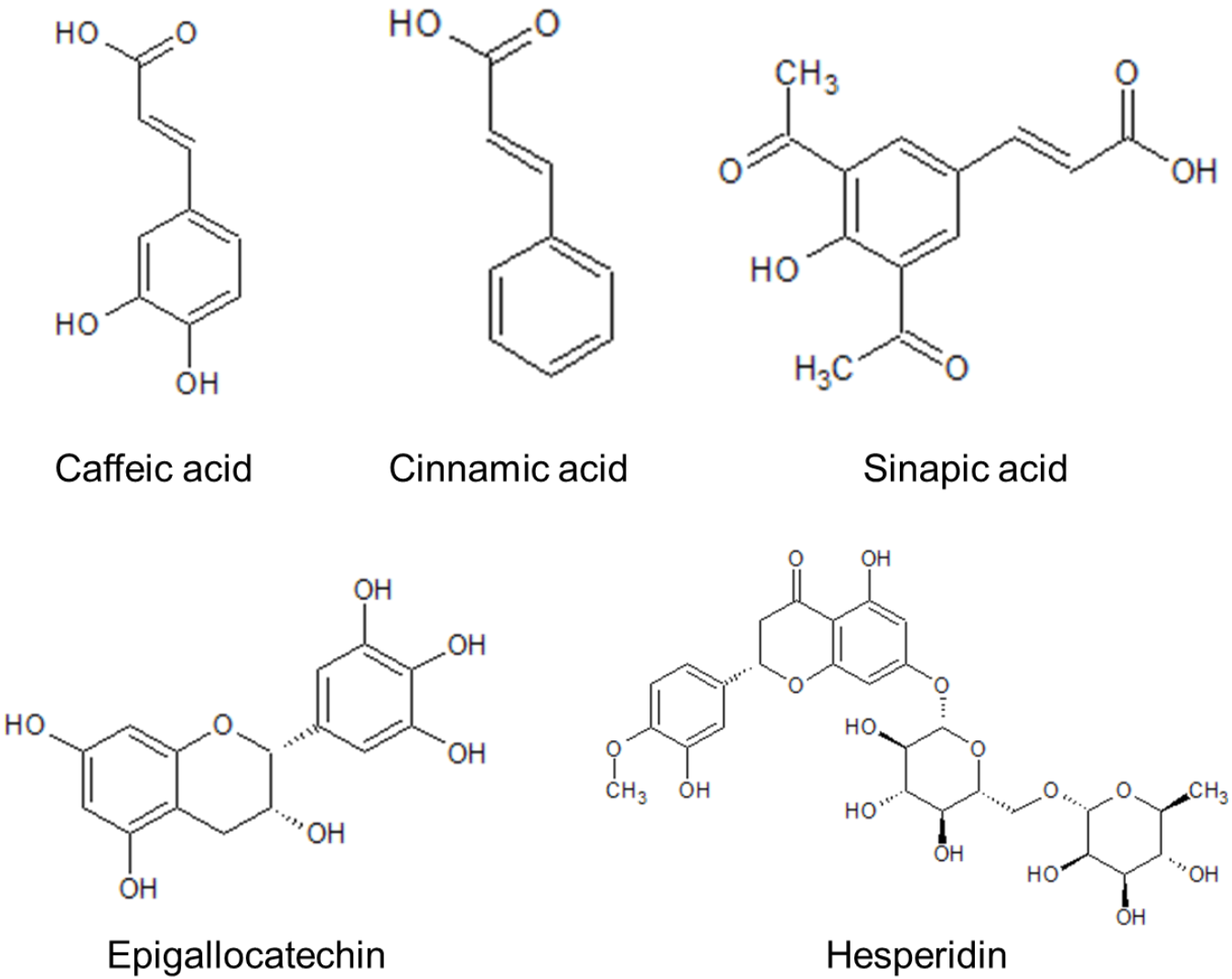


Figure 30: Polyphenols identified in *S. cordatum* during the present study not previously reported (Source: Chemspider, 2012)

presence of alkaloids, flavonoids, phenolic acids and terpenoids (Pallant and Steenkamp, 2008), as well as gallic acid (Candy *et al.*, 1968) is supported by literature. *S. cordatum* contained lower concentrations of polyphenols as shown by the polyphenolic quantitation assays.

Due to the existence of different free radical types and the conditions in which they are generated, it is imperative to analyze antioxidant potential of compounds using a variety of *in vitro* assays (Re *et al*, 1999; de Beer *et al.*, 2003). Antioxidants provide two primary methods of quenching free radical sources: single electron transfer and hydrogen atom transfer (Phipps *et al.*, 2007; Kedare and Singh, 2011). For this study TE was expressed as the ratio between the absorbance/concentration slope of the sample and the Trolox standard, resulting in a unitless ratio. This gives an indication of the antioxidant activity over a range of concentrations while ignoring the absolute concentration of the free radicals (which could differ between researchers and make comparison of results difficult). In the present study, the TEAC and DPPH assays produced similar results, indicating that the antioxidants present in the samples could quench free radicals by single electron transfer. Slightly higher activity was observed for the polyphenolic-rich extracts using the DPPH assay. Furthermore, the polyphenolic-rich extracts from both plants were able to produce better activity than ascorbic acid against the DPPH free radical, suggesting superiority in antioxidant activity. Various studies have reported positive correlations between antioxidant activity and polyphenolic content, which supports the findings observed in the present study (de Beer *et al.*, 2003*;* Sestili *et al.*, 2007; Guo *et al.*, 2008). In the present study, polyphenolic content was a better predictor of antioxidant activity than flavonoid content. Due to the correlation ($r > 0.72$) found between polyphenols and antioxidant activity, it can possibly be deduced that the high polyphenolic content results in the cell-free antioxidant activity. This is supported by literature (de Beer *et al.*, 2003; Apak *et al.*, 2007; Celli *et al.*, 2011).

B. africana crude extracts contained greater concentrations of polyphenols and antioxidant activity than that of *S. cordatum*. Previous studies support the antioxidant activity present in *B. africana*, with polyphenols being implied as the most likely contributers. Methanolic extracts of the bark of *B. africana* have been reported to scavenge free radicals in the DPPH and β-carotene assays (Diallo *et al.*, 2001).

Similarly, Mathisen *et al.* (2002) reported that the hydroethanol crude extract of *B. africana* was a good scavenger of the DPPH radical (IC_{50} = 8.5 µg/ml). Literature also supports the contribution that the polyphenolic constituents have with respect to antioxidant activity, including catechin, epicatechin, proanthocyanidins, gallic acid, myricetin (Rice Evans *et al.*, 1996), caffeic acid, epigallocatechin (de Beer *et al.*, 2003) and sinapic acid (Apak *et al.*, 2007).

No other studies concerning the *Burkea* genus could be found, making it necessary to compare with the family Fabaceae, specifically the subfamily Caesalpinioideae. Various plants in this subfamily have been shown to contain high antioxidant activity. Extracts of *Caesalpinia bonduc* (L.) Roxb. seeds (Gacche and Dhole, 2011), *Cassia auriculata* (L.) Roxb. (Juan-Badaturuge *et al.*, 2011), *Cassia fistula* L. fruits (Gacche and Dhole, 2011), *Ceratonia siliqua* L. germ flour (Custódio *et al.*, 2011a) and fruits (Custódio *et al.*, 2011b) have been reported to contain polyphenolic constituents with antioxidant activity comparable to *B. africana*. Additionally, it is known that plants in the Fabaceae family contain high levels of isoflavones, which could contribute to its antioxidant activity (Corradini *et al.*, 2011).

The Folin-Ciocalteau assay is known to react with interefering compounds, including sugars, aromatic amines, (Phipps *et al.*, 2007) peptides and other aromatic containing substances, which could explain why higher polyphenolic content was measured in the polyphenolic-rich extract of *B. africana*, yet the greater antioxidant activity was measured in the polyphenolic-rich extract of *S. cordatum.* Antioxidant activity of aqueous and methanolic crude extracts of *S. cordatum* bark has previously been described by Pallant and Steenkamp (2008). While the present study found higher activity in the methanolic (1.09) than aqueous (0.80) extract, Pallant and Steenkamp (2008) obtained higher activity in the latter (1.95 vs 0.80). Extraction and antioxidant methodology could possibly be attributable to the conflicting data. Antioxidant activity of the polyphenols found in *S. cordatum* is supported by literature for caffeic acid, epigallocatechin, leucocyanidin, leucodelphinin (de Beer *et al.*, 2003), gallic acid, hesperidin, proanthocyanidins (Rice-Evans *et al.*, 1996) as well as the triterpenoid saponin arjunolic acid (Candy *et al.*, 1968; Manna *et al.*, 2007; Sinha *et al.*, 2008; Hemalatha *et al.*, 2010).

Studies on other *Syzygium* species show comparable results to that of the present study. Extracts of *S. euphlebium* (Hayata) Mori leaves (Jiang *et al.*, 2006), *S. formosanum* (Hayata) Mori leaves (Jiang *et al.*, 2006), *S. polyanthum* (Wight) Walp. leaves (Kusuma *et al.*, 2011) and fruit and *S. cumini* (L.) Skeels (synonymn: *Eugenia* or *S. jambolanum*) seeds (Ruan *et al.*, 2008; Samadder *et al.*, 2011) and fruit (Afify *et al.*, 2011) displayed antioxidant activity attributable to polyphenols such as catechin, γ-sitosterol and derivatives of myricetin and cyanidin (Celli *et al.*, 2011).

Flavonoids and other polyphenols possess significant radical scavenging abilities and antioxidant potential (Table 6). The flavonoid-radical formed through neutralization is less reactive and can be eliminated faster than other free radicals (Corradini *et al.*, 2011). Three structural criteria have to be met for efficacious radical scavenging abilities in flavonoids: 1) the *o*-hydroxy structure of ring B which helps stabilize the radical and delocalizes electrons, 2) the 2,3 double bond and a 4-oxo function in the C ring, which assists in electron delocalization of ring B and 3) the 3-OH group of ring A and 5-OH group and 4-oxo function of ring C to increase radical scavenging efficacy (Figure 31) (Rice-Evans *et al.*, 1996; de Beer *et al.*, 2002). In the present study, many of the polyphenols, such as leucocyanidin, leucodelphinidin, catechin, epicatechin, myricetin and proanthocyanidins, comply with the aforementioned criteria which explain their antioxidant potential. Increased hydroxyl groups typically potentiate antioxidant activity (de Beer *et al.*, 2002), which is seen in the polyphenols identified in the present study. Metal chelating antioxidant properties of flavonoids rely on the benzopyran-4-one and *ortho*-dihydroxyphenyl moieties, while the 5-OH group does not add much activity (Souček *et al.*, 2011). It is thus evident that polyphenolic constituents increase the antioxidant potential of the crude and polyphenolic-rich extracts, supporting the results obtained during the cell-free antioxidant assays.

2. Inherent cytotoxicity of B. africana and S. cordatum

The viability of the four cell cultures used in this study was assessed through the neutral red uptake assay after exposure to the different crude and polyphenolic-rich extracts. Neutral red is taken up into viable lysosomes, from where it can be extracted by using an organic solvent and then measured spectrophotometrically.

Table 6: Reported antioxidant activity of polyphenols found to be present in *B. africana* and *S. cordatum*

Class	Polyphenols	$ABTS^{\cdot+}$ TE (mM)	TEAC value	ORAC TE (µM)	DPPH IC50 (µM)
Anthocyanidins	Cyanidin	4.40[a]	-	-	-
	Delphinidin	4.44[a]	-	-	-
Flavanols	Catechin	2.40[b]	3.14[c]	-	134 39[e]
	Epicatechin	2.50[b]	2.69[c]	28[d]	99.9 29[e]
	Epicatechin gallate	4.90[b]	3.51[c]	-	-
	Epigallocatechin	3.80[a]	-	-	-
	Epigallocatechin gallate	4.80[b]	3.15[c]	-	-
	Procyanidins	-	-	-	34[e]
Flavanones	Hesperidin	1.08[b]	1.40[c]	19[d]	-
Flavonols	Myricetin	3.10[a]		-	-
Phenolic acids	Caffeic acid	1.26[a]	1.39[c]	-	-
	Gallic acid	3.01[b]	3.48[c]	-	11.8 2[e]
	Sinapic acid	-	1.11[c]	-	-

ORAC – Oxygen Radical Antioxidant Capacity; TEAC – Trolox Equivalent Antioxidant Capacity; [a] de Beer *et al.*, 2002, [b] Rice-Evans *et al.*, 1996, [c] Apak *et al.*, 2007, [d] Weng *et al.*, 2011, [e] Martín *et al.*, 2011

(modified from Rice-Evans *et al.*, 1996; de Beer *et al.*, 2002, Apak *et al.*, 2007, Martín *et al.*, 2011 and Weng *et al.*, 2011)

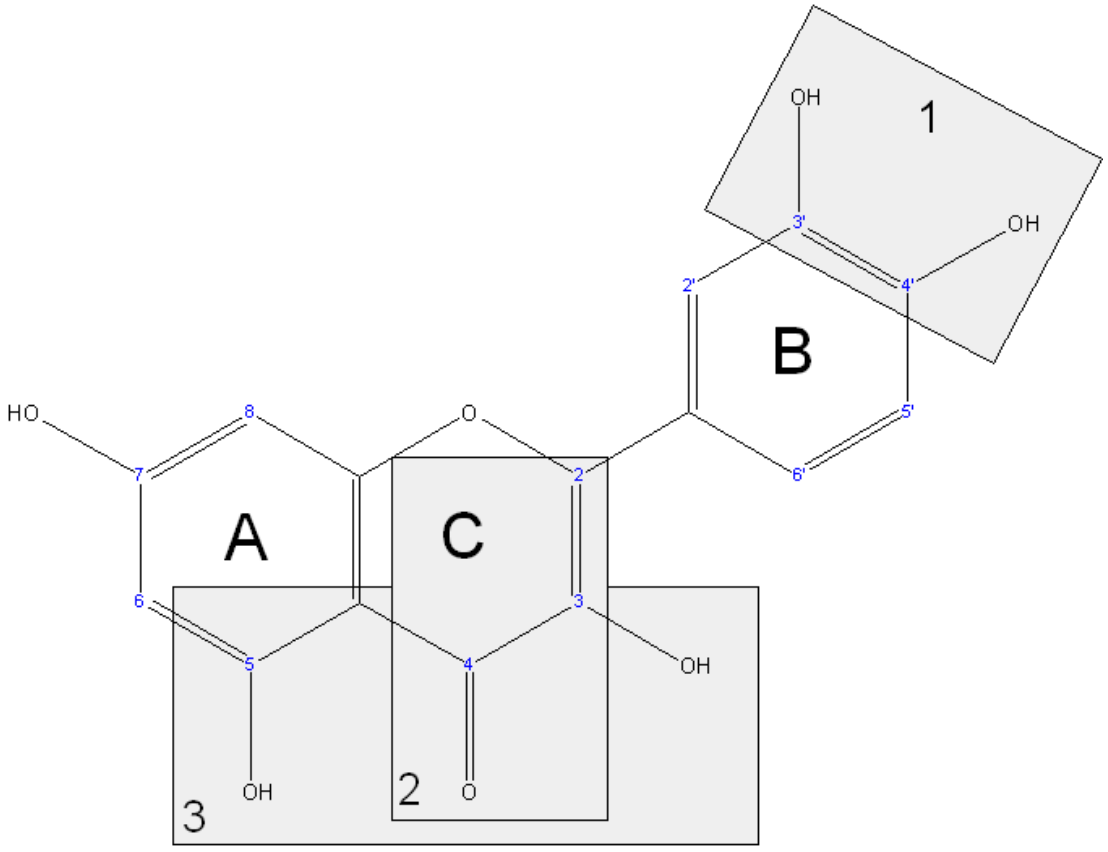


Figure 31: Chemical characteristics that allow for an increased antioxidant potential in flavonoids; A, B and C refer to the respective phenyl rings; 1, 2 and 3 refer to the respective criteria as referenced in text (modified from de Beer *et al.*, 2002)

As to date, this is the first study presenting cytotoxicity data for *B. africana* and *S. cordatum* in the four cell cultures tested. Cytotoxicity was only present in the 3T3-L1 and C2C12 cell lines, suggesting potential selective toxicity for cells of a muscle and fat origin as healthy dermal fibroblasts and immortalized macrophage-like cells did not present with any significant toxicity at the levels tested. Furthermore, the effects of a homogenous mixture cannot be excluded due to possible synergistic, additive or antagonistic effects that would not be seen when using a single compound.

B. africana ethanolic stem extracts induced brine shrimp toxicity with a concentration that resulted in 50% lethality (LC_{50}) of 87.24 µg/ml (Moshi *et al.*, 2006), which is comparable to the cytotoxicity observed in the present study in 3T3-L1 cells after exposure to the crude aqueous extract (84.5 µg/ml). Furthermore, cytotoxicity was also more pronounced in the C2C12 cell line than the 3T3-L1 cell line after exposure to the crude methanolic and polyphenolic-rich extract. This once again suggests potential selective toxicity for muscle and fat cells, though little toxicity for primary fibroblasts and macrophage-like cells. The mechanism through which toxicity is elicited is unknown. Although toxicity can be elicited by the polyphenols found in *B. africana*, including caffeic acid (Yoo *et al.*, 2005), gallic acid (Chen *et al.*, 2009) and sinapic acid (Fan *et al.*, 2009), these studies were performed on cancerous cell lines and thus may not have the same effect on the primary cultures. Isoflavone-containing LDL reduced cell viability in U937 cells (Tikkanen and Adlercreutz, 2000), however toxicity was not noted in the present study. This does not imply that the presence of isoflavones in Fabaceae (Corradini *et al.*, 2011) might have caused cell death in the 3T3-L1 and C2C12 cells.

It has been reported in literature that extracts from plants in the Fabaceae family exhibit toxicity in various cell lines. Dichloromethane and ethyl acetate extracts of *Intsia bijuga* (Colebr.) O. Ktze. leaves were cytotoxic (25 – 50 µg/ml) to various human cancerous cell lines (such as 786-0 renal carcinoma, A549 lung carcinoma, HT-29 colorectal adenocarcinoma, MCF-7 breast carcinoma, PC-3 prostate carcinoma and SNB-19 glioblastoma), while methanolic extracts showed no toxicity (>50 µg/ml) (Bradacs *et al.*, 2010). Methanolic extracts of *C. siliqua* fruit showed limited toxicity against Hela cells (IC_{50}s ranging between <2.5 and 15.9 mg/ml) (Custódio *et al.*, 2011b). Hexane and methanolic extracts of *Piliostigma reticulatum*

(DC.) Hochst. leaves were found to display toxicity in the brine shrimp toxicity assay with LC_{50} of 33.39 and 3.38 µg/ml, respectively. Polyphenolic constituents and derivatives were proposed to contribute to the toxicity (Babajide *et al.*, 2008).

The crude aqueous and polyphenolic-rich extracts of *S. cordatum* were more toxic than the methanolic extract, with IC_{50}s ranging from 20.5 to 63.4 µg/ml in the 3T3-L1 and C2C12 cell lines. *S. cordatum* bark extracts have been reported to show toxicity in human kidney epithelial cells (dichloromethane:methanol (1:1), IC_{50} = 26.80 µg/ml) (Sibandze *et al.*, 2010) and Vero green monkey kidney epithelial cells (methanolic and acetone extracts, IC_{50} = 212.00 µg/ml and 37.34 µg/ml, respectively) (Mativandlela *et al.*, 2008; Samie *et al.*, 2009). The large difference in toxicity in the latter two studies could be explained by the different solvents used for extraction, resulting in different phytochemical combinations being present in the extracts.

Other *Syzygium* species have been studied, with varying levels of cytotoxicity being found. Extracts of *S. polyanthum* (Wight) Walp. leaves (brine shrimp, >1000 µg/ml) (Kusuma *et al.*, 2011) and *S. malaccense* (I.) Merr. and L.M. Perry bark maceration (Neuro-2a neuroblastoma, >5 mg/ml) (Garrec *et al.*, 2005). No cytotoxicity was observed for the essential oil of *S. aromaticum* against bovine aortic endothelial cells (>134 µg/ml) (Machado *et al.*, 2011). *S. euphlebium* and *S. formosanum* at 100 µg/ml induced 63% and 21% cell death, respectively against primary melanocytes, respectively (Jiang *et al.*, 2006). *S. malaccense* leaves were cytotoxic (25 – 50 µg/ml) to 786-0, A549 and MCF-7 cancerous cell lines (Bradacs *et al.*, 2010). Ethanolic *S. cumini* extracts displayed anticancer activity by reducing leukaemic cell viability (up to 70.7% at 100 µg/ml) in the trypan blue exclusion assay, which was attributable to the antioxidants kaempferol 7 *o*-methylester and γ-sitosterol (Afify *et al.*, 2011). Although *S. cordatum* does not contain the γ-form, β-sitosterol has been reported, which could possibly contribute to cytotoxicity.

Toxicity data for the polyphenols; caffeic, gallic and sinapic acid; present in both plants is summarized in Table 7. Although polyphenols are powerful antioxidants, under certain conditions – such as high phytochemical concentrations, or in aqueous

Table 7: Reported cytotoxicty of various phytochemicals found to be present in *B. africana* and *S. cordatum* extracts that elicit *in vitro* cytotoxicity

Phytochemical	Cell line	Viability assessment	IC_{50} (µg/ml)	References
Caffeic acid	HT-1080	MTT	166.5	Prasad *et al.*, 2011
	HL-60	MTT	4.3	Yoo *et al.*, 2005
	HepG2	MTT	21.4	Yoo *et al.*, 2005
	3LL Lewis	MTT	16.1	Yoo *et al.*, 2005
	SNU-C5	MTT	23.3	Yoo *et al.*, 2005
Gallic acid	DU145	XTT	91.7	Chen *et al.*, 2009
	LNCaP	XTT	121.7	Chen *et al.*, 2009
	PC-3	XTT	94.6	Chen *et al.*, 2009
Sinapic acid	HL-60	Trypan blue	56.1	Fan *et al.*, 2009

Polyphenol concentrations standardized to µg/ml. MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, XTT – 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

media and cancerous cell culture systems – they can present with pro-oxidant activity (e.g. in aqueous media or high concentrations) (Fang *et al.*, 2005; Sestili *et al.*, 2007; Koleckar *et al.*, 2008). Even so, literature does not suggest this as the most possible cause for the inherent toxicity of the crude extracts and polyphenolic-rich extracts seen in the 3T3-L1 and C2C12 cell cultures. Polyphenolic compounds have been shown to induce toxicity in 3T3-L1 cells through cell cycle arrest and apoptosis, which could be explained by its anti-adipogenic activity *in vitro* (Chen *et al*., 2009; Sung *et al*., 2010). Epigallocatechin gallate (EGCG) at 22.9 µg/ml displayed toxicity in 3T3-L1 cells through diminished glucose uptake and ROS generation (Sung *et al.*, 2010). EGCG was shown to elicit cytotoxicity in both primary and cancerous cells (Chen *et al.*, 2009). EGCG was shown to result in apoptosis in 3T3-L1 cells through the cyclin dependent kinase 2 (Cdk2) pathway (45.8 µg/ml) (Wu *et al.*, 2005) and cell cycle arrest in the gap 1 (G1) phase (183.3 µg/ml) through the extracellular regulated kinase (ERK) and Cdk2 pathways (Hung *et al.*, 2005). Gallic acid induced apoptosis in 3T3-L1 cells at 22.9 µg/ml through both extrinsic and intrinsic pathways (Hsu *et al.*, 2007). From the information obtained it can thus be suggested that the toxicity inflicted on 3T3-L1 cells by *B. africana* and *S.cordatum* could be attributed to the inherent anti-adipogenic activity of certain polyphenols. Although the concentrations of specific polyphenols are not known, the combination thereof could result in higher anti-adipogenic activity with subsequent cell death. This however cannot be the only determinant as the crude aqueous extract of *S. cordatum* was more toxic than the methanolic extract, even though the latter contained a higher polyphenolic content. Furthermore, the polyphenolic-rich extracts of both plants had similar IC_{50}s (~25 µg/ml), though the polyphenolic content differed. This brings to light the importance of both the extraction methods and the extracted matrix. Toxicity could also have arisen from the increased concentration of interfering compounds, such as alkaloids, glycosides and terpenoids, though this needs to be examined further.

3. Induction of oxidative stress

In *in vitro* cellular systems induction of oxidative stress is commonly through exposure to H_2O_2 (Charoenteeraboon *et al.*, 2010), AAPH (Gupta-Elera *et al.*, 2011), *tert*-butylhydroperoxide (*tert*-BuOOH) (Sestili *et al.*, 2007) or iron sulphate (Martín *et al.*, 2011). Oxidative stress is assessed through the quantification of ROS

generation, as well as the damage it produces which includes apoptosis, DNA degradation, lipid peroxidation, mitochondrial membrane integrity, protein modification and inflammation (Sestili *et al.*, 2007; Martín *et al.*, 2011). During the present study, oxidative stress was induced in U937 cells by exposure to AAPH for 48 h. This procedure reduced cell viability, generated ROS, induced apoptosis and lipid peroxidation, as well as depleted intracellular GSH levels. Thus oxidative stress was successfully achieved *in vitro*.

4. Protection against oxidative stress-induced toxicity

4.1. ROS generation and subsequent cytotoxicity

The measurement of the fluorescent DCF probe over a period of 3 h yielded high levels of ROS in U937 cells after exposure to AAPH. This was however successfully inhibited in a dose-dependent manner through pre-treatment with the crude and polyphenolic-rich extracts of both *B. africana* and *S. cordatum*. The extent of inhibition was reflected by the antioxidant activity seen in the cell-free antioxidant assays, with polyphenolic-rich extracts being more effective than the crude extracts in protecting cells against ROS and cytotoxicity. Protection against cytotoxicity was increased while ROS generation was decreased in a dose-dependent manner with pre-treatment. As far as could be determined, this is the first study to assess the effects of *B. africana* and *S. cordatum* on a cellular oxidative stress model *in vitro*.

Pro-oxidant and cytotoxic activities have been described in cancerous cells (Rigano *et al.*, 2009; Custódio *et al.*, 2011a) by plants of the Fabaceae family, though this is not deemed as relevant to the present study. As stated earlier, high polyphenolic concentrations resulted in decreased ROS generation and decreased cytotoxicity when cells were pre-treated with crude or polyphenolic-rich extracts before exposure to oxidants.

A single study on another *Syzygium* species was found with regard to ROS generation. Samadder *et al.* (2011) investigated the effects of ethanolic seed extract of *S. cumini* on alloxan-induced oxidative stress in isolated pancreatic and hepatic cells from Swiss albino mice and found decreased ROS generation and subsequent higher cell viability.

Polyphenols are able to decrease ROS generation and cytotoxicity induced by various oxidants, as shown in Table 8 and Table 9, however this depends on the cell line or model used. In several cases, potentiation or direct damage is caused by polyphenols through oxidative stress, though this was only seen in some cell lines. The polyphenols listed in the tables are those that are present within the crude extracts and polyphenolic-rich extracts, which support the results obtained in the present study. Several studies have noted the reduction of oxidative stress in U937 cells through incubation with polyphenols, including catechin, epicatechin (Kanupriya *et al.*, 2006) and gallic acid (Charoenteeraboon *et al.*, 2010). Caffeic acid and epicatechin at concentrations of 0.1 µg/ml have been reported to decrease superoxide formation in U937 cells by approximately 40% (Froemming and O'Brien, 1997). These compounds could be responsible for the reduction in ROS generation. Zhang *et al.* (2011) reported that myricetin (3.2×10^{-4} µg/ml) reduced 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced ROS generation and cytotoxicity in MES23.5 rat dopaminergic cells by approximately 65% and 23%, respectively. Myricetin, present in *B. africana*, could thus contribute to the protective antioxidant activity. Myricetin has been shown to inhibit mitogen-activated protein kinase kinase 4 (MKK4) and JNK phosphorylation and scavenge free radicals, with subsequent reduced mitochondrial dysfunction, apoptosis and DNA damage (Zhang *et al.*, 2011). The concentrations listed in the latter two studies are also well below that found in the crude and polyphenolic-rich extracts used in the present study, which is a much more likely scenario for a homogenous plant extract. Anthocyanins (Youdim *et al.*, 2000), similar to leucocyanidin and leucodelphinidin, and arjunolic acid (Manna *et al.*, 2007; Pal *et al.*, 2011) have been reported to decrease oxidative stress *in vitro,* thus supporting the reduction caused by *S. cordatum* pre-treatment. It has been suggested that arjunolic acid works through antioxidant and metal-chelating activity (Manna *et al.*, 2007), which supports protection from ROS by non-polyphenolic antioxidants. Thus literature supports the possible protection against ROS by crude extracts and polyphenolic-rich extracts against oxidative stress.

4.2. Endogenous GSH content

Oxidative stress results in a decreased concentration of GSH (Nirmala *et al.*, 2011), such as seen in the present study, which ultimately increases the susceptibility of cells to free radical-induced damage. GSH can be measured through the formation

Table 8: Reported effect of phytochemicals found to be present in *B. africana* and *S. cordatum* extracts on ROS generation after exposure to oxidants

Sample tested		**ROS generator used**		**Generation assay**	**Cell line**	**Protection against ROS generation (%)**	**References**
Phytochemical	**Concentration (µg/ml)**	**Oxidant**	**Concentration**				
Arjunolic acid	97.7	Cadmium chloride	30 µM	DCFHDA	Isolated hepatocytes	VND	Pal *et al.*, 2011
Caffeic acid	0.1	H_2O_2	1 mM	DCFHDA	RGC-5	50	Nakajima *et al.*, 2009
Catechin	29.0	Doxorubicin	20 µM	DCFHDA	H9C2	27	Du and Lou, 2008
	72.6	Fenton reaction	0.5 mM:1.0 mM	DCFHDA	U373 MG	30	Martín *et al.*, 2011
	100.0	*tert*-BuOOH	200 µM	DCFHDA	U937	VND	Kanupriya *et al.*, 2006
	29.0	Xanthine oxidase-xanthine	100 mU/ml:0.5 mM	DCFHDA	H9C2	31	Du *et al.*, 2007
Ellagic acid	9.1	Cadmium chloride	30 µM	DCFHDA	Isolated astrocytes	36	Yang *et al.*, 2008
Epicatechin	72.6	Fenton reaction	0.5 mM:1.0 mM	DCFHDA	U373 MG	30	Martín *et al.*, 2011
	100.0	*tert*-BuOOH	200 µM	DCFHDA	U937	VND	Kanupriya *et al.*, 2006
Gallic acid	42.5	Fenton reaction	0.5 mM:1.0 mM	DCFHDA	U373 MG	32	Martín *et al.*, 2011
	4.5	H_2O_2	2 mM	DCFHDA	U937	50	Charoenteeraboon *et al.*, 2010

Hesperidin	30.5	H_2O_2	400 µM	DCFHDA	PC12	45	Hwang and Yen, 2008
	48.8	*tert*-BuOOH	150 µM	DCFHDA	LO2	93	Chen *et al.*, 2010
Myricetin	31.8	Doxorubicin	30 µM	NADPH-generating system	Minipig liver microsomes	45	Souček *et al.*, 2011
	3.2×10^{-4}	MPTP	200 µM	DCFHDA	MES23.5	65	Zhang *et al.*, 2011
Proanthocyanidin B4	57.9	Doxorubicin	20 µM	DCFHDA	H9C2	34	Du and Lou, 2008
	57.9	Xanthine oxidase-xanthine	100 mU/ml:0.5 mM	DCFHDA	H9C2	20	Du *et al.*, 2007

Polyphenol concentrations standardized to µg/ml. Values are calculated approximate averages from graphs and tables. VND – value not determinable, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Table 9: Reported effect of phytochemicals found to be present in *B. africana* and *S. cordatum* extracts on cell viability after exposure to oxidants

Sample tested		**Oxidant used**		**Cell line**	**Viability assay**	**Protection against cytotoxicity (%)**	**References**
Phytochemical	**Concentration (µg/ml)**	**Oxidant**	**Concentration**				
Arjunolic acid	97.7	Cadmium chloride	30 µM	Isolated hepatocytes	MTT	55	Pal *et al.*, 2011
	97.7	Cadmium chloride	30 µM	Isolated hepatocytes	LDH leakage	26	Pal *et al.*, 2011
Catechin	72.6	Fenton reaction	0.5 mM:1 mM	U373 MG	MTT	43	Martín *et al.*, 2011
	100.0	*tert*-BuOOH	200 µM	U937	Neutral red uptake	75	Kanupriya *et al.*, 2006
Cyanidin rutinoside	59.5	Amyloid-β	1 µM	PC12	MTT	22	Harvey *et al.*, 2011
	59.5	H_2O_2	200 µM	PC12	MTT	1	Harvey *et al.*, 2011
	59.5	*tert*-BuOOH	100 µM	PC12	MTT	10	Harvey *et al.*, 2011
Ellagic acid	9.1	Cadmium chloride	30 µM	Isolated astrocytes	MTT	>100	Yang *et al.*, 2008
Epicatechin	72.6	Fenton reaction	0.5 mM:1 mM	U373 MG	MTT	50	Martín *et al.*, 2011
	100.0	*tert*-BuOOH	200 µM	U937	Neutral red uptake	75	Kanupriya *et al.*, 2006

Epigallocatechin 3-gallate	45.8	Amyloid-β	1 µM	PC12	MTT	65	Harvey *et al.*, 2011
	45.8	H_2O_2	200 µM	PC12	MTT	30	Harvey *et al.*, 2011
	22.9	Lead	5 µM	SH-SY5Y	MTT	28	Suresh *et al.*, 2011
	45.8	*tert*-BuOOH	100 µM	PC12	MTT	5	Harvey *et al.*, 2011
Gallic acid	42.5	Fenton reaction	0.5 mM:1 mM	U373 MG	MTT	38	Martín *et al.*, 2011
Hesperidin	30.5	H_2O_2	400 µM	PC12	MTT	70	Hwang and Yen, 2008
	30.5	H_2O_2	400 µM	PC12	LDH leakage	>100	Hwang and Yen, 2008
	48.8	*tert*-BuOOH	150 µM	LO2	MTT	67	Chen *et al.*, 2010
	48.8	*tert*-BuOOH	150 µM	LO2	MTT	85	Chen *et al.*, 2010
Myricetin	3.2×10^{-4}	MPTP	200 µM	MES23.5	MTT	53	Zhang *et al.*, 2011
	3.2	Cumene hydroperoxide	100 µM	C6	MTT	9	Seibert *et al.*, 2011
	3.2	H_2O_2	500 µM	C6	MTT	24	Seibert *et al.*, 2011

Polyphenol concentrations standardized to µg/ml. Values are calculated approximate averages from graphs and tables. MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, LDH – lactate dehydrogenase

of GSH-monochlorobimane fluorescent adducts (catalyzed through GST) (Fernandez-Checa *et al.*, 1990). It would be expected that pre-treatment with antioxidants would increase the endogenous supply of GSH because of reversal of free radical production and retention of endogenous antioxidant stores. However, the inverse was seen with the crude and polyphenolic-rich extracts of *B. africana,* as GSH concentrations were significantly reduced after exposure to these extracts. The aqueous extract of *S. cordatum* had similar activity to that of *B. africana* in decreasing GSH activity, whereas the methanolic extract resulted in a dose-dependent increase in GSH at concentrations between 2.5 to 10 µg/ml. The highest concentration of the polyphenolic-rich extract (20 µg/ml) however depleted GSH. The change in trend at 20 µg/ml could possibly be interference between phytochemical constituents and the fluorescent adduct, though this is unlikely due to the washing steps involved in the assay.

While most studies support the increase in GSH concentrations brought on by plant extracts of the Fabaceae family (Yu *et al.*, 2005; Okada and Okada, 2007; Luqman *et al.*, 2009), by polyphenols such as isoflavonoids (Yu *et al.*, 2005) and by water soluble proteins (Okada and Okada, 2007), this was not observed in the present study for *B. africana*. No reports on the *in vitro* effects of *Syzygium* species on GSH could be found, but the increased antioxidant status, in terms of GSH, seen for the methanolic crude extract and polyphenolic-rich extract of *S. cordatum* could be attributable to the high polyphenolic content and antioxidant activity, as supported by literature. The reduction of ROS brought upon by pre-treatment with crude and polyphenolic-rich extracts would have reduced the amount of free radicals able to damage cells. This however does not fully explain the protection in the absence of GSH. Although the cells were essentially susceptable to oxidative attack due to decreased endogenous GSH, cell viability was not affected, thus stabilization of other parameters could be assumed, such as decreased apoptotic factors and protection against lipid peroxidation.

Although literature supports the increased antioxidant status *in vitro* by affecting the concentration of GSH and the linked enzymes (Table 10), the increased polyphenolic content mostly did not elicit this response. This effect, however, is supported by other literature. Zhang *et al.* (1997) described the ability of certain polyphenols, such

Table 10: Reported effect of phytochemicals found to be present in *B. africana* and *S. cordatum* on the endogenous GSH pathway after exposure to oxidants

Sample tested		**Oxidant used**		**Cell line**	**Protection against endogenous antioxidant depletion (%)**				**References**
Phytochemical	**Concentration (µg/ml)**	**Oxidant**	**Concentration**		**GSH**	**GPx**	**GR**	**GST**	
Arjunolic acid	100.0	Arsenic	1 mM	Isolated hepatocytes	63	54	77	43	Manna *et al.*, 2007
	97.9	Cadmium chloride	30 µM	Isolated hepatocytes	88	76	61	>100	Pal *et al.,* 2011
Caffeic acid	50	Caffeic acid	50 µg/ml	HT-1080	-80	-65	-	-	Prasad *et al.*, 2011
Catechin	2902.6	Catechin	10 mM	Isolated hepatocytes	-71	-	-		Galati *et al.*, 2002
	29.0	Doxorubicin	10 - 20 µM	H9C2	70	-	-	60	Du and Lou, 2008
	72.6	Fenton reaction	0.5 mM:1 mM	U373 MG	-	-226	>100	-	Martín *et al.*, 2011
Epicatechin	72.6	Fenton reaction	0.5 mM:1 mM	U373 MG	-	-8	>100	-	Martín *et al.*, 2011
Epigallocatechin 3-gallate	22.9	Lead	5 µM	SH-SY5Y	40	-	-	-	Suresh *et al.*, 2011
Gallic acid	42.5	Fenton reaction	0.5 mM:1 mM	U373 MG	-	>100	>100	-	Martín *et al.*, 2011
Hesperidin	30.5	H_2O_2	400 µM	PC12	-	>100	45	-	Hwang and Yen, 2009
Proanthocyanidin B4	57.9	Doxorubicin	10 - 20 µM	H9C2	78	-	-	67	Du and Lou, 2008

Polyphenol concentrations standardized to µg/ml. Values are calculated approximate averages from graphs and tables. Negative values represent increased depletion, while positive values indicate protection. – not assessed

as flavonoids and catechins, to decrease GSR activity, the enzyme responsible for recycling GSSG back to GSH. Phytochemicals with the ability to complex with GSH or alter enzyme activity could contribute to diminished GSH recycling, with a resultant increased GSSG/GSH ratio. Galati *et al.* (2002) ascribed the GSH depletion effects caused by dietary polyphenols due to conjugation with GSH and subsequent formation of *o*-quinone metabolites. Myricetin is known to auto-oxidize at physiological pH, which results in decreased glutathione stores due to removal of the radical species (Moskaug *et al.*, 2005). The latter could possibly describe the large GSH decrease seen after pre-treatment with the crude and polyphenolic-rich extract of *B. africana*. While GSH was decreased, it is possible that other endogenous antioxidants systems including SOD and CAT could have been increased and offered protection against oxidative stress. This however was not assessed in the present study. In the present study it would appear that the possibility exists that the extracts can reduce GSH by inhibiting the recycling enzymes or through conjugation. This GSH decrease however did not appear to influence cell viability.

4.3. Lipid peroxidation and apoptosis

Lipid peroxidation occurred at high levels (5-fold higher than control) after exposure of cells to AAPH, with subsequent formation of MDA. The MDA-TBA fluorescent adduct can be measured through fluorescence after extraction into a non-polar phase, such as butanol (Stern *et al.*, 2010). AAPH-induced apoptosis was evident by increased caspase-3 activity as determined by AMC fluorescence (Harwood *et al.*, 2005). Synthetic substrates used in caspase-3 activity assays are generally not highly specific, as they can be metabolized by other caspases (such as 6, 7, 8 and 10). It is noted that this non-specificity is unlikely to affect the experiment (Harwood *et al.*, 2005). Caspase activity can be diminished through oxidative deactivation, which would result in decreased fluorescent intensity of AMC due to reduced substrate cleavage. The addition of β-mercaptoethanol maintains a reduced cysteine state within the active sites of the caspase enzyme, which should reverse any possible oxidative modification (Harwood *et al.*, 2005).

While the crude and polyphenolic-rich extracts of *B. africana*, as well as the polyphenolic-rich extract of *S. cordatum*, was effective in reducing lipid peroxidation

and apoptosis, the crude extracts of *S. cordatum* could not prevent damage. This is most likely due to the polyphenolic content and antioxidant activity of the crude extracts of *S. cordatum*, being lower than the other extracts tested, which could be too low to prevent cytotoxicity. Furthermore, intracellular accumulation of polyphenols and antioxidants will allow for greater protection (Hwang and Yen, 2009), thus the phytochemicals present in *S. cordatum* may not be entering the cell sufficiently within the allocated 1 h pre-treatment time. Unexpectedly, the polyphenolic-rich extract of *S. cordatum*, being superior in antioxidant activity, did not surpass the anti-apoptotic ability of the polyphenolic-rich extract of *B. africana*. Other compounds in *B. africana*, apart from polyphenols, could thus be attributing to the activity seen. Lipid peroxidation by-products, such as MDA, are known to elicit apoptotic responses in cells (Raza & John, 2006), thus the reduced apoptosis seen in the present study could be related to the inhibition of lipid peroxidation caused by the crude and polyphenolic-rich extracts.

Mathisen *et al.* (2002) reported that *B. africana* hydroethanol extracts decreased bovine phospholipid damage (IC_{50} = 78 µg/ml). While no IC_{50} was determined for the lipid peroxidation assay, the crude and polyphenolic-rich extracts seemed to be more effective in the present study than that of Mathisen *et al.* (2002) with inhibition of approximately 43% for the polyphenolic-rich extract at 20 µg/ml. Various plants from Fabaceae have been shown to protect against lipid peroxidation (Sultana *et al.*, 2007; Xu *et al.*, 2007; Luqman *et al.*, 2009; Juan-Badaturuge *et al.*, 2011) which has been attributed to their antioxidant activity and polyphenolic content. The reported concentrations needed to elicit effective reduction in lipid peroxidation are comparable to those obtained in the present study.

Several species of *Syzygium* have been shown to decrease lipid peroxidation (Benherlal and Arumughan, 2007; Sultana *et al.*, 2007; Veigas *et al.*, 2007; Kumari *et al.*, 2010). This is similar to findings in the present study, where phenolic acids and anthocyanins have been identified as compounds responsible for this decrease (Benherlal and Arumughan, 2007; Veigas *et al.*, 2007). While anthocyaninin content was not determined, literature supports the presence of leucodelphinidin and leucocyanidin in *S. cordatum* which could contribute to the observed decreased lipid peroxidation in the present study.

Literature supports the ability of phytochemicals, including polyphenols, to reduce lipid peroxidation (Table 11) and apoptosis (Table 12) induced by oxidative stress, such as that observed in the present study. Phytochemicals such as arjunolic acid, catechin, epicatechin, gallic acid, hesperidin and myricetin reduced lipid peroxidation (Koleckar *et al*., 2008; Chen *et al*., 2010; Pal *et al*., 2011; Souček *et al*., 2011) and apoptosis (Du *et al*., 2007; Du and Lou, 2008; Hwang and Yen, 2008; Pal *et al*., 2011; Yu *et al*., 2011; Zhang *et al*., 2011) induced by oxidative stress. Furthermore, isoflavones which are present in the Fabaceae family, have been shown to decrease oxidative damage to lipids through incorporation into LDL particles (Tikkanen and Adlercreutz, 2000), which may contribute to the activity of the crude and polyphenolic-rich extract of *B. africana*. Verstraeten *et al.*, (2005) described the ability of procyanidins to decrease lipid peroxidation through hydrogen bonding to phospholipids, resulting in stabilization of membranes. Such activity ensures that ROS cannot enter the hydrophobic intracellular compartment and elicit further damage (Verstraeten *et al.*, 2005). Sinapic acid has been reported to be effective in reducing oxidative damage to sterols during heating (Kmiecik *et al.*, 2011). The most described mechanisms of reduced apoptosis were through the reduction in pro-apoptotic signals, upregulation of anti-apoptotic proteins and stabilization of the mitochondrial membrane. From the results obtained in the present study it is most likely that the polyphenolic content of the plant material is responsible for the anti-apoptotic effect. The higher anti-apoptotic activity present in *B. africana* extracts may suggest that unidentified non-antioxidant compounds are also decreasing apoptosis.

5. Potential for further development

As stated earlier, the potential for further development is hindered due to the cytotoxicity observed in two of the four cell lines tested. The concentrations necessary to elicit a protective effect in the U937 cell line (10 and 20 µg/ml) were also within the range of the IC_{50} for the two susceptible cell lines. Although protection against oxidative stress is evident, the polyphenolic-rich extracts will need to be purified further to determine the bioactive polyphenols or phytochemicals which can be developed for use as a supplement. The toxicity of the extracts is an unwanted variable in this development, but quantitative structure–activity relationship (QSAR) modeling (Kruhlak *et al*., 2012) could elucidate on possible structural modifications

Table 11: Reported effect of phytochemicals found to be present in *B. africana* and *S. cordatum* on lipid peroxidation after exposure to oxidants

Sample tested		**Oxidant used**		**Cell line**	**Protection against lipid peroxidation**		**References**
Phytochemical	**Concentration (µg/ml)**	**Oxidant**	**Concentration**		**MDA (%)**	**LDL oxidation (IC_{50})**	
Arjunolic acid	100.0	Arsenic	1 mM	Isolated hepatocytes	84	-	Manna *et al.*, 2007
	97.7	Cadmium chloride	30 µM	Isolated hepatocytes	90	-	Pal *et al.*, 2011
Caffeic acid	-	Cupric ions	N/S	Isolated LDL	-	0.24	Vinson *et al.*, 1995
	-	Iron	50 µM	Minipig microsomal lipids	-	4.5	Souček *et al.*, 2011
	-	NADPH generating system	2.5 mM	Minipig microsomal lipids	-	>30	Souček *et al.*, 2011
Catechin	-	Cupric ions	N/S	Isolated LDL	-	0.19	Vinson *et al.*, 1995
	-	Iron	50 µM	Minipig microsomal lipids	-	3	Souček *et al.*, 2011
	-	NADPH generating system	2.5 mM	Minipig microsomal lipids	-	22.2	Souček *et al.*, 2011
Cyanidin	-	Cupric ions	N/S	Isolated LDL	-	0.21	Vinson *et al.*, 1995
Ellagic acid	-	Cupric ions	N/S	Isolated LDL	-	2.50	Vinson *et al.*, 1995
Epicatechin	-	Iron	50 µM	Minipig microsomal lipids	-	1.6	Souček *et al.*, 2011
	-	NADPH generating system	2.5 mM	Minipig microsomal lipids	-	15.9	Souček *et al.*, 2011

Epicatechin 3-gallate	-	Cupric ions	N/S	Isolated LDL	-	0.14	Vinson *et al.*, 1995
	-	Cupric ions	N/S	Isolated LDL	-	0.14	Vinson *et al.*, 1995
Epigallocatechin	-	Cupric ions	N/S	Isolated LDL	-	0.10	Vinson *et al.*, 1995
Epigallocatechin 3-gallate	-	Cupric ions	N/S	Isolated LDL	-	0.08	Vinson *et al.*, 1995
Gallic acid	-	Cupric ions	N/S	Isolated LDL	-	1.25	Vinson *et al.*, 1995
	-	Iron	50 µM	Minipig microsomal lipids	-	6.7	Souček *et al.*, 2011
	-	NADPH generating system	2.5 mM	Minipig microsomal lipids	-	>40	Souček *et al.*, 2011
Hesperidin	-	Cupric ions	N/S	Isolated LDL	-	>16	Vinson *et al.*, 1995
	48.8	*tert*-BuOOH	150 µM	LO2	76	-	Chen *et al.*, 2010
Myricetin	15.9	Cumene hydroperoxide	1 mM	C6	12	-	Seibert *et al.*, 2011
	-	Cupric ions	N/S	Isolated LDL	-	0.48	Vinson *et al.*, 1995
	-	Iron	50 µM	Minipig microsomal lipids	-	4.2	Souček *et al.*, 2011
	-	NADPH generating system	2.5 mM	Minipig microsomal lipids	-	7.5	Souček *et al.*, 2011

Polyphenol concentrations standardized to µg/ml. Values are calculated approximate averages from graphs and tables. – not assessed

Table 12: Reported effect of phytochemicals found to be present in *B. africana* and *S. cordatum* on apoptosis after exposure to oxidants

Sample tested		**Oxidant used**		**Cell line**	**Protection against apoptosis (%)**		**References**
Phytochemical	**Concentration (µg/ml)**	**Oxidant**	**Concentration**		**Caspase-3 activity**	**Apoptotic cells**	
Arjunolic acid	97.7	Cadmiun chloride	30 µM	Isolated hepatocytes	75	43	Pal *et al.*, 2011
Caffeic acid	50.0	Caffeic acid	50 µg/ml	HT-1080	-	-85	Prasad *et al.*, 2011
Catechin	29.0	Doxorubicin	10 - 20 µM	H9C2	50	35	Du and Lou, 2008
	29.0	Xanthine oxidase-xanthine	100 mU/ml:0.5 mM	H9C2	-	31	Du *et al.*, 2007
Cyanidin	14.4	Taurodeoxycholic acid	400 µM	Huh7	100	-	Yu *et al.*, 2011
Epicatechin	29.0	Taurodeoxycholic acid	400 µM	Huh7	75	-	Yu *et al.*, 2011
Epigallocatechin 3-gallate	22.9	Lead	5 µM	SH-SY5Y	70	-	Suresh *et al.*, 2011
Hesperidin	30.5	H_2O_2	400 µM	PC12	87	-	Hwang and Yen, 2008
Myricetin	3.2×10^{-4}	MPTP	200 µM	MES23.5	80	-	Zhang *et al.*, 2011
Proanthocyanidin B4	57.9	Doxorubicin	10 - 20 µM	H9C2	65	20	Du and Lou, 2008
	57.9	Xanthine oxidase-xanthine	100 mU/ml:0.5 mM	H9C2	-	29	Du *et al.*, 2007

Polyphenol concentrations standardized to µg/ml. Values are calculated approximate averages from graphs and tables. – not assessed

that could be made to bioactive phytochemicals to remove toxicity while maintaining or improving cytoprotective activity.

Physiologically, extracts will have to reach concentration of at least 10 µg/ml to elicit a positive modulatory effect on oxidative stress parameters. The ability of polyphenolic-rich extracts to prevent or reduce oxidative stress will need to be determined on different cell lines, such as the NHDF cells to assess their potential beneficiality in other cellular systems. Furthermore, the pharmacokinetic parameters will need to be assessed to determine the phytochemical matrix present after supplementation has occurred. If one assumes that complete absorption will occur with supplementation (taking the extraction yields and average blood supply of 5 l into account), 0.5 g and 2 g of the polyphenolic-rich extracts of *B. africana and S. cordatum* would be sufficient to reach the concentrations needed to elicit a cytoprotective effect against oxidative stress. This would be a realistic dosage to administer. This also falls within the range of the lowest IC_{50}s obtained in the 3T3-L1 and C2C12 cells.

Chapter 5
Conclusion

The aim of this study was to assess the potential of polyphenolic-rich extracts of two plants, *B. africana* and *S. cordatum,* to reduce oxidative stress *in vitro*. Literature concerning both of these plants is scarce, with little information available on their *in vitro* oxidative stress-modulating capacity. This study assessed the *in vitro* oxidative stress-decreasing capacity of these plants to expand knowledge of the ethnomedicinal use and the potential of developing an antioxidant supplement from them. Further studies is needed to assess if antioxidant activity is correlated to ethnomedicinal usage.

Crude extracts of *B. africana* and *S. cordatum* displayed high levels of antioxidant acitivity in cell-free systems, which could be attributed to their phenolic and flavonoid content. Through fractionation, polyphenolic-rich extracts were obtained which demonstrated greater antioxidant activity than the crude extracts using both the TEAC and DPPH assays. Furthermore, this antioxidant activity was confirmed in an *in vitro* cellular system during an oxidative stress-model using U937 cells. Oxidative stress induced by AAPH decreased viability of cells due to generation of ROS and subsequent free radical-toxicity. Polyphenolic-rich extracts were not only successful in attenuating the intracellular ROS generation, but also physiological responses, such as reduced apoptosis and lipid peroxidation. These could possibly be through stabilization of phospholipid membranes, upregulation of anti-apoptotic factors, inhibition of caspase cascades or protection of the mitochondrial membrane. A decrease in GSH concentrations was however noted, but viability of cells was not affected.

Although protection was observed in U937 cells, the concentrations needed to elicit this effect was toxic in the 3T3-L1 and C2C12 cell lines. The mechanism of toxicity is not known, but could entail the presence of contaminating compounds such as alkaloids, glycosides and terpenoids, or the conversion of polyphenols to reactive byproducts. Furthermore, toxicity in 3T3-L1 cells could be due to the inherent anti-adipogenic effect of many polyphenols. Although cytotoxicity is an unwanted effect, isolation of the

bioactive components will allow further research into the potential for development of an antioxidant supplement. Furthermore, although *in vitro* data suggests the potential for beneficial use, confirmation would be required using *in vivo* studies to determine the effect of pharmacokinetic parameters on supplementation. Through the use of QSAR modeling, neccessary structural changes can be determined to decrease cytotoxicity.

The influence of free radicals on disease progression remains a problem, and therefore the development of new prophylactic measures is of importance. Supplementation with polyphenolic-rich extracts does result in the reduction of *in vitro* oxidative stress, which increases the viability of cells under oxidative stress. If development of an antioxidant supplement extracted from *B. africana* and *S. cordatum* were to be taken further, isolation, purification and potential QSAR modeled structural changes will need to be researched to allow for maximal cytoprotective effects, while eliminating risk of cytotoxicity. Further studies can be done to assess whether synergistic action will allow for greater activity between phytochemicals identified.

Chapter 6
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Appendix I

Ethical Approval for Collection of Blood

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 27/08/2009

Prof V Steenkamp
Department of Pharmacology
University of Pretoria

Best Prof Vanessa Steenkamp

RE.: Application for Blood Collection utilizing lymphocytes, macrophage, neutrophils and plasma.

Herewith acknowledgement that the above Application for blood collection has been received and tabled on 26/08/2009, and found to be acceptable by the Faculty of Health Sciences Research Ethics Committee.

With regards

DR R SOMMERS; MBChB, MMed (Int), MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

31 Bophelo Road ♦ H W Snyman Building (South) Level 2-34 ♦ P.O.BOX 667, Pretoria, South Africa, 0001 ♦ Tel:(0´2)3541330 ♦
♦ Fax: (012)3541367 / 0866515924 ♦ E-Mail: manda@med.up.ac.za ♦ Web: //www.healthethics-up.co.za ♦

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Appendix II

Ethical Approval for Use of Cell Cultures

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

DATE: 23/06/2011

TO:
Prof V Steenkamp
Dept of Pharmacology

Best Prof V Steenkamp

RE.: Commercial Lines: The use of Commercial lines ~ Mr W Cordier

During the meeting held on 22/06/2011, the use of Commercial Lines were discussed.

The Faculty of Health Science Ethics Committee approved the use of the cell lines for the various assays.

With regards

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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Appendix III
Reagents

1. Powder reagents

AAPH

AAPH was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 15 mM solution was prepared in RPMI-1640 prior to use.

$ABTS^{\cdot+}$

2,2'-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid) (ABTS) and potassium peroxidisulfate was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 7.46 mM ABTS/2.5 mM potassium peroxidisulfate solution was prepared by dissolving 19.2 mg ABTS and 3.31 mg potassium peroxidisulfate in 5 ml distilled water and incubating for 16 h at 4°C.

Aluminum trichloride

Aluminum trichloride was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 1% solution was prepared in distilled water and stored at room temperature.

Anhydrous sodium sulphate

Anhydrous sodium sulphate was obtained from Merck Chemicals (Darmstadt, Germany) in powder. A 10% w/v solution was used to dehydrate extracts.

Caspase-3 substrate buffer

Caspase-3 substrate buffer consisted of 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 5 µM Ac-DEVD-AMC obtained from Sigma-Aldrich (St Louis, USA) and 4.3 mM β-mercaptoethanol obtained from Merck Chemicals (Darmstadt, Germany). This was prepared by dissolving 476.6 mg HEPES and 58.4 mg EDTA in 100 ml distilled water. Caspase-3 substrate buffer was stored at 4°C. Thirty

minutes prior to experimentation 50 ul 100 mM PMSF, 3 ul 14.3 M β-mercaptoethanol and 10 µl 5 mM Ac-DEVD-AMC was added to 10 ml cold caspase-3 substrate buffer.

DCFHDA

DCFHDA was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 2 mM solution was prepared by dissolving 4.9 mg DCFHDA in 5 ml ethanol and storing at -2°C. A 50 µM working solution was prepared by diluting 100 µl stock solution with 3.9 ml PBS prior to use.

DPPH

DPPH was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A stock solution of 600 µM was prepared by dissolving 11.8 mg DPPH in 50 ml methanol and sonicating for 20 min. A working solution of 120 µM was prepared by diluting 20 ml stock solution with 80 ml methanol and sonicating for 20 min.

Dragendorff's reagent

Bismuth nitrate and potassium iodide were obtained from Merck Chemicals (Darmstadt, Germany) in powder form. Two solutions were prepared: A) 1.7% bismuth nitrate in 20% aqueous acetic acid, and B) 13% potassium iodide solution in 30% aqueous acetic acid. Solutions A and B were mixed in 4:1 ratio just prior to use.

DMEM

DMEM was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A solution of 1.04% was prepared in autoclaved, ultra-pure, pyrogen-free, deionized water and adjusted to pH 7.4 using sodium hydrogen carbonate obtained from Merck Chemicals (Darmstadt, Germany) in powder form. The solution was filtered *in vacuo* thrice (Sartorius, 0.22 µm), supplemented with 1% penicillin/streptomycin and stored at 4°C.

Iron (III) chloride

Iron (III) chloride was obtained from Merck Chemicals (Darmstadt, Germany) in powder form. A 5% solution was prepared in 32% hydrochloric acid and stored at room temperature.

Lysis buffer

Lysis buffer consisted of 10 mM HEPES, 2 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM EDTA, 0.5 mM PMSF obtained from Sigma-Aldrich (St Louis, USA) and 4.3 µM β-mercaptoethanol obtained from Merck Chemicals (Darmstadt, Germany). This was prepared by dissolving 71.5 mg HEPES, 36.9 mg CHAPS and 43.8 mg EDTA in 30 ml distilled water. Lysis buffer was stored at 4°C. Thirty minutes prior to experimentation 50 ul 100 mM PMSF and 3 ul 14.3 M β-mercaptoethanol was added to 10 ml cold caspase-3 substrate buffer.

Monochlorobimane

Monochlorobimane was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 25 mM stock solution was prepared by dissolving 5 mg in 880 µl dimethylsulfoxide (DMSO) and kept at -80°C in 20 µl aliquots. A 500 µM working solution was prepared by diluting two aliquots in 1.96 ml PBS prior to use.

Neutral red medium (200 µg/ml)

Neutral red medium consisted of 200 µg/ml neutral red reagent obtained from Sigma-Aldrich (St. Louis, USA) in cell culture medium. The solution was adjusted to pH 6.4 using 1 M potassium orthophosphoric acid obtained from Merck Chemicals (Darmstadt, Germany) in powder form. The solution was filter-sterilized (0.22 µm) and stored at 4°C.

PBS

BBL™ FTA hemagglutination buffer was obtained from BD (France) in powder form. A 0.9% solution was prepared in distilled water and stored at 4°C.

Phosphomolybdinic acid

Molybdophosphoric acid was obtained from Merck Chemicals (Gauteng, RSA) in powder form. A 20% solution was prepared in absolute ethanol and stored at room temperature.

Phytochemical standards

Apigenin, benzoic acid, caffeic acid, catechin, cinnamic acid, daidzein, epigallocatechin, ferulic acid, gallic acid, genistein, hesperidin, kaempferol, myricetin, *p*-coumaric acid, quercetin, rutin hydrate, saponin, sinapic acid, vitexin and warfarin were obtained from Sigma-Aldrich (St. Louis, USA) while ascorbic acid was purchased from Merck Chemicals (Darmstadt, Germany). All chemicals were in powder form. Standards were prepared to 1 mg/ml in methanol for TLC (all) or distilled water for antioxidant and polyphenolic assays (gallic acid, rutin hydrate and ascorbic acid) prior to use.

PMA

PMA was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A solution of 1 mg/ml was prepared in absolute ethanol and stored at -80°C in 500 µl aliquots. Prior to use a 1 µg/ml working solution was prepared by diluting 1.5 µl stock solution in 1.5 ml RPMI-1640 prior to use.

Potassium hydroxide

Potassium hydroxide was obtained from Merck Chemicals (Darmstadt, Germany) in powder form. A 5% solution was prepared in absolute methanol and stored at room temperature.

RPMI-1640

RPMI-1640 was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A solution of 1.04% was prepared in autoclaved, ultra-pure, pyrogen-free, deionized water and adjusted to pH 7.4 using sodium hydrogen carbonate obtained from Merck Chemicals (Darmstadt, Germany) in powder form. The solution was filtered *in vacuo*

thrice (Sartorius, 0.22 µm), supplemented with 1% penicillin/streptomycin and stored at 4°C.

Saponin

Saponin was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 1% solution was prepared in RPMI-1640 and stored at 4°C.

Sodium carbonate

Anhydrous sodium carbonate was obtained from Merck Chemicals (Darmstadt, Germany) in powder form. A 20% solution was prepared in distilled water and stored at room temperature.

Sodium hydroxide

Sodium hydroxide was obtained from Merck Chemicals (Gauteng, RSA) in powder form. Solutions of 0.5, 2 and 4 M were prepared by dissolving 2, 8 and 16 g in 100 ml distilled water and stored at room temperature.

Sodium nitrate

Sodium nitrate was obtained from Merck Chemicals (Darmstadt, Germany) in powder form. A 3% solution was prepared in distilled water and stored at room temperature.

Staurosporine

Staurosporine was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 2.14 mM stock solution was prepared by dissolving 0.5 mg in 500 µl DMSO and kept at -80°C in 10 µl aliquots. A 200 µM working solution was prepared by diluting aliquots with 100 µl RPMI-1640 prior to use.

TBA

2-TBA was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 1% solution was made in distilled water containing 0.1 M sodium hydroxide and 50 µM EDTA just prior to use. The solution was sonicated to assist in dissolution.

Trichloroacetic acid

Trichloroacetic acid was obtained from Merck Chemicals (Darmstadt, Germany) in crystaline form. A 12.5% solution was prepared in distilled water and kept at room temperature.

Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid)

Trolox was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 1 mg/ml solution was prepared in distilled water for the antioxidant assays, while a 10 mM solution was prepared by dissolving 5 mg Trolox in 2 ml RPMI-1640, respectively prior to use. Sonication was used to assist in dissolution.

Trypan blue counting fluid

Trypan blue was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 0.1% solution was prepared in distilled water and stored at room temperature.

2. *Liquid reagents*

Acids

Formic acid (85%), glacial acetic acid and hydrochloric acid (32%) were obtained from Merck Chemicals (Darmstadt, Germany) in liquid form and used undiluted.

FCS

FCS was obtained from The Scientific Group (Gauteng, RSA). Solutions were heat-inactivated through heating at 56°C for 45 min.

Folin-Ciocalteau reagent

Folin-Ciocalteau reagent (2 N) was obtained from Sigma-Aldrich (St. Louis, USA) in liquid form and used undiluted.

H_2O_2

H_2O_2 (30%) was obtained from Merck Chemicals (Darmstadt, Germany) in liquid form. A 200 mM solution was prepared by diluting 40 µl stock solution with 1.96 ml RPMI-1640 prior to use.

Neutral red eluent

Neutral red eluent consisted of absolute ethanol, distilled water and glacial acetic acid in liquid form (49:50:1). The solution was stored at 4°C.

Solvents

Acetone, chloroform, diethyl ether, DMSO, ethanol, ethyl acetate, hexane, methanol, 3-methyl butan-1-ol and toluene was obtained from Merck Chemicals (Darmstadt, Germany) in liquid form and used undiluted.

Trypsin/versene

Tyrpsin/versene solution was obtained from The Scientific Group (Gauteng, RSA) in liquid form and used undiluted.