In vitro evaluation of antioxidant properties of
Rosa roxburghii plant extract

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Acknowledgements

Abstract

Rosa roxburghii, also known as "Burr Rose" or "Chestnut Rose", originated in southwest China and was introduced to the botanic garden in Calcutta around 1824. It was named after William Roxburgh who was the superintendent. The extract of fruit of the *Rosa roxburghii* plant is the base ingredient of a range of products that is commercially sold under the *Cili Bao®* label. The extract is composed of a wide range of substances of nutritional value, in particular a relatively high amount of antioxidants such as ascorbate and plant phenols. It has been reported before that supplementation with the fruit extract resulted in increased red blood cell superoxide dismutase, catalase and the reduced form of glutathione. An enhancement of the antioxidant status could contribute to the protection against several diseases where oxidative stress is a major factor in the pathology, such as atherosclerosis, cancer and immunity stress. Several anecdotal reports with little (published) scientific support claim that human supplementation of the *Rosa roxburghii* extract to the diet has a protective effect against several diseases, including the above mentioned. Medicinal and herbal plants are used in large sections in developing countries for primary care and there is now also an increase in the use of natural therapies in developed countries. However, plant extracts can also consist of anti-nutritional and possible toxic components, such as oxalic acid and nitrates, which could express cytotoxic and genotoxic activities. Therefore, understanding the health benefits but also the potential toxicity of these plants is important. The objective of this study was to investigate the beneficial properties of *Rosa roxburghii* extract from an antioxidant potential perspective and in particular to investigate the safety of the product for human consumption. For this purpose *in vitro* evaluation of the cellular toxicity, mutagenicity and genotoxicity was performed. In addition, specific biochemical parameters relating to the antioxidant status of the product, i.e. antioxidant capacity, oxidative stress prevention and glutathione redox state profiles were investigated *in vitro* as well as *in vivo*.

The results indicated that *Rosa Roxburghii* fruit extract was not mutagenic when tested with *Salmonella typhimurium* strains TA 98, TA 100 and TA 102 in the Ames test. The results, however, pointed towards an antimutagenic effect of the extract in these strains against metabolic activated mutagens 2-acetylaminoflurorene (2-AAF) and aflatoxin B_1, and the direct-acting mutagen, methanesulfonate (MMS). In primary rat hepatocyte, *Rosa roxburghii* extract did not elicit double or single strand DNA damage and cell viability loss using the single cell gel electrophoresis (Comet assay), lactate dehydrogenase leakage test or the mitochondrial conversion test of MTT to formazan (MTT test). Again the opposite effect was observed: pre-treatment of hepatocytes with *Rosa roxburghii* extract significantly reduced the effect of oxidative stress-induced cellular- and genotoxicity. These results point to a protective effect against oxidative stress which is reflected in an increase of the antioxidant capacity and glutathione redox state (GSH/GSSG) *in vitro* (lymphoblasts) and *in vivo* (humans) reported in this study. This study underlines the previously suggested potential of this plant extract as a natural and safe antioxidant supplement.

Keywords: Rosa Roxburghii, antioxidant capacity, mutagenicity, genotoxicity, cytotoxicity

Opsomming

Rosa roxburghii, wat ook bekend staan as "Burr Rose" of "Chestnut Rose" het sy oorsprong in Suidwes China en is vir die eerste keer bekend gestel aan die botaniese tuine in Calcutta in 1842. Dit is vernoem na William Roxburgh wat in dié tyd die opsigter by die tuine was. Die ekstrak van *Rosa roxburghii* is die basis bestanddeel van die *Cili Bao*® reeks produkte. Dit bestaan uit 'n wye reeks komponente met voedingswaarde en in die besonder 'n relatiewe hoë hoeveelheid antioksidante soos askorbiensuur en plantfenole. Daar is alreeds voorheen aangetoon dat aanvullings met die plant ekstrak lei tot 'n verhoging in superoksied dismutase, katalase en die gereduseerde vorm van glutatioon. 'n Verhoging van die antioksidantstatus in mense kan bydra tot verskeie siektes waar oksidatiewe stres 'n primêre faktor is in die patologie, byvoorbeeld arterosklerosis, kanker en stres van die immuniteit. Verskeie nie-wetenskaplike en ongepubliseerde gegewens stel dat supplentering van die dieet met *Rosa roxburghii* 'n voordelinge gevolg het teen verskeie siektes, insluitende bogenoemde. Medisinale plante en kruie word oor die algemeen gebruik in ontwikkelende lande vir primêre sorg, maar daar is nou ook 'n toename in die gebruik van natuurlike terapieë in ontwikkelde lande. Plant ekstrakte kan egter ook uit toksiese stowwe soos oksaalsuur en nitrate bestaan, wat sitotoksiese en genotoksiese aktiwiteite in die liggaam kan veroorsaak. Dit is dus belangrik om nie net die gesondheidsvoordele nie, maar ook die moontlike toksiese gevolge van die plante te verstaan. Die doel van die studie is om die moontlike voordelige eienskappe van *Rosa roxburghii* ekstrak te ondersoek vanuit 'n antioksidant oogpunt en om die veiligheid van die produk vir menslike gebruik te toets. Vir hierdie doel is (*in vitro*) evaluering van sellulêre toksisiteit, mutagenisiteit en genotoksisiteit gedoen. Afgesien daarvan is verskeie biochemiese parameters wat in verband staan met veranderinge in die antioksidant status, byvoorbeeld die antioksidant kapasiteit, oksidatiewe stres en glutatioon redoksstatus, *in vitro* sowel as *in vivo* ondersoek.

Die resultate het aangetoon dat *Rosa roxburghii* ekstrak nie mutagenies is soos getoets deur die *Salmonella typhimurium* genotipes TA 98, TA 100 en TA 102 in die Ames toets. Inteendeel, die ekstrak was anti-mutagenies vir *Salmonella typhimurium,* wat vooraf met metabolies geaktiveerde 2-asetielaminofluroreen (2-AAF), aflatoksien B_1 en die direkte mutageen, metaansulfonaat (MMS) onderskeidelik behandel was. In primêre rot hepatosiete het die *Rosa roxburghii* ekstrak geen dubbel- of enkelstring DNA-skade tot gevolg gehad nie, en daar was geen lewensvatbaarheidsverlies soos gemeet met die enkelsel-gelektroforese (komeet-analise), laktaatdehidrogenase vrystellingstoets en die omskakeling van tetrazoliumsout (MTT) na formazaan (MTT-toets). Weereens is die teendeel aangetoon: voorafbehandeling van hepatosiete met *Rosa roxburghii* ekstrak het ook die effek van oksidatiewe stres-geïnduseerde sellulêre- en genotoksisiteit aansienlik verlaag. Hierdie resultate dui op die beskermende effek van *Rosa roxburghii* teen oksidatiewe stres, wat deur 'n verhoging in die antioksidantkapasiteit en glutatioon reduksstatus (GSH/GSSG) *in vitro* (limfoblaste) en *in vivo* (mense) gereflekteer word. Hierdie studie ondersteun die voorgestelde potensiaal van hierdie plant ekstrak as 'n natuurlike en veilige antioksidant supplement.

Sleutelwoorde: *Rosa roxburghii, antioksidantkapasiteit, mutagenisiteit, genotoksisiteit, sitotoksisiteit.*

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Abbreviations

γ-GCH	L-γ-glutamyl-L-cysteine synthetase
2-AAF	2-acetylaminofluorene
8-OH-dG	8-hydroxy-deoxyguanosine
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
AFB_1	Aflatoxin B_1
ATP	Adenosine 5' triphosphate
BCA	Bicinchoninic acid
BMI	Body mass index
COMET	Single cell gel electrophoresis assay
CHE	Christian Higher Education
DMSO	Dimethylsulfoxide
DTNB	5,5'dithiobis-(2-nitrobenzoic acid)
FCS	Fetal calf serum
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2ethanesulfonic acid)]
INT	2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
MMS	Methyl methanesulfonate
MRC	Medical Research Council
MT-2 cells	Murine lymphoblast cells
MTT	(3-(4,5-dimethylthiazol)-2-yl)-2,5-diphenyl tetrazolium bromide
M2VP	1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate
NAD^+	Nicotinamide adenine dinucleotide
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STD	Standard deviation
t-BHP	Tert-butyl hydroperoxide
TE	Trolox Equivalents
WHO	World Health Organisation

LIST OF ADDENDA

ADDENDUM A   Authors' guide to *Food and Chemical Toxicology*

ADDENDUM B   Authors' guide to *Journal of Nutrition and Biochemistry*

PREFACE

I.   Background and Motivation

Oxidative stress plays a central role in the mechanism and pathogenesis of cardiovascular related diseases such as atherosclerosis, a variety of cancers and other chronic diseases, as well as the undesirable effect of the ageing process and age related neurodegenerative diseases such as Alzheimer disease (Gilgun-Sherki, 2000, and Cheng, 2001). Atherosclerosis and related cardiovascular diseases and cancer remains some of the leading causes of death in the western world (Klausner, 1999 and Lee, 1998). The deeper understanding of the mechanisms involved in mitochondria-related diseases, such as the role of reactive oxygen species, emphasize the role of antioxidants (Chinnery & Turnbull, 2001). Although our body consist of an endogenous antioxidant defence system, it is not always so effective especially since exposure to damaging environmental factors is increasing (Gilgun-Sherki, 2001). The exogenous intake of antioxidants could be very effective in diminishing the effect of oxidative stress, as shown in studies (Cheng *et al.*, 2000, Urbanavicius, 2003, Kruidenier and Verspaget, 2002).

Although secondary prevention is the most cost-effective means of management in some diseases, it seems unethical to wait until the patient experiences these diseases (Fuster, 2000). A variety of individuals are nowadays more intrested in taking personal control over their health, not only in prevention but also in the treatment of these diseases, which is particularly true for a variety of chronic diseases such as cancer, diabetus and athritis (Kincheloe, 1997). The interest in the use of natural medicinale plants is expanding not only in developing countries but also in the industrial countries (WHO, 1998). However, it is important not only to evaluate the medicinal claims of these plants but also safety prospects (Lewis, 2001).

As a dietician, but also because of the interest of our division in the investigation and diagnosis of mitochondrial diseases for which antioxidants are the main therapeutic approach, it is of interest to investigate antioxidants. The "natural antioxidant product", *Rosa roxburghii* extract came, to our attention via the interest displayed by Mr. G. Joubert from Cili Health, SA.

II.   Structure of this dissertation

This dissertation is in an article format. The empirical work consists of two studies. The first study was a methodological investigation of the cytotoxic, genotoxic, mutagenic and antioxidant properties of the *Rosa roxburghii* plant extract *in vitro*. The second study was a randomised, paired, placebo-controlled, single-blind, and parallel feeding control study.

Following this preface, Chapter 1 provides background information necessary for the interpretaion of the data in the article. An overview is given of the principles behind the formation of free radicals and reactive oxygen species, resulting in oxidative stress. Primary and secondary consequences of oxidative stress and their possible relationship in pathogenesis of diseases are discussed. This is followed by the concept of the antioxidant defence systems, endogenous and exogenous, and special attention is given to the role of dietary antioxidants. Furthermore, the known characteristics of the natural plant extract and antioxidant, *Rosa roxburghii* extract, are provided. Chapter 2 consists of the submitted paper on the cytotoxic, genotoxic, mutagenic and antioxidant characteristics of *Rosa roxburghii* extract (submitted for publication in to the journal *Food and Chemical Toxicology*). Chapter 3 is the submitted paper on the results of the randomised, paired, placebo-controlled, single-blind, and parallel feeding control study (submitted for publication in to *Journal of Nutritional Biochemistry*). In chapter 4 a general discussion and summary of all the results

are provided, recommendations are made and conclusions are drawn. The relevant references of Chapters 2 and 3 are provided at the end of each chapter according to the authors' instructions of the specific journals in which the articles will be published. The references used in the unpublished Chapters (Preface and Chapter 1) are provided according to the mandatory style stipulated by the Potchefstroom University for Christian Higher Education (CHE) at the end of the dissertation.

III.   Authors' contribution
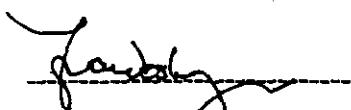
The study reported in this dissertation was planned and executed by a team of researchers. The contribution of each of the researchers is depicted in the table below.   Also included in this section is a statement from the co-authors confirming their individual roles in the study and giving their permission that the article may form part of this dissertation.

NAME	ROLE IN THE STUDY
Ms. C.S. Janse van Rensburg B.Sc. Hons. (Biochemistry), B.Sc (Dietitian)	Design, planning, execution of *in vitro* study, laboratory analysis, and compilation of data. Compilation and writing of *in vivo* study.
Dr. F.H. van der Westhuizen Ph.D. (Biochemistry)	Project co-ordinator and scientist; responsible for all aspects of the study. Study leader of C.S. Janse van Rensburg
Mr. E. Erasmus M.Sc. (Biochemistry)	Sub co-ordinator of the study design and co-study leader of C.S. Janse van Rensburg.
Prof. P.J. Pretorius Ph.D. (Biochemistry)	Contribution toward planning of the *in* vitro study (especially the Comet assay) and writing of paper.
Ms. J. Marnewick Ph.D. (Biochemistry)	Planning and co-ordinating of the AMES test and providing the necessary facilities.
Mr. G.S. Rautenbach B.Sc Hons. (Biochemistry)	Laboratory analysis and assistant.
Prof. W. Oosthuizen Ph.D. (Nutritionist)	Design and planning of *in vivo* study.
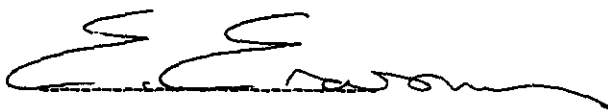
Prof. J.S. Jerling Ph.D (Nutritionist)	Design and planning of *in vivo* study.
Ms C. Huysamen B.Sc	Laboratory assistant (Comet analysis)
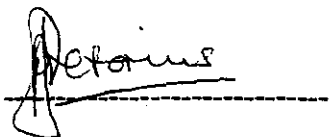
I declare that I have approved the above mentioned article and that my role in the study as indicated above is representative of my actual contribution and that I hereby give my consent that it may be published as part of the M.Sc. dissertation of C.S. Janse van Rensburg
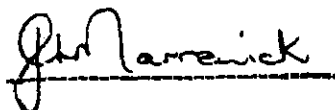
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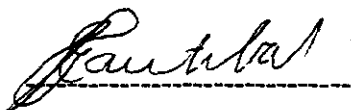
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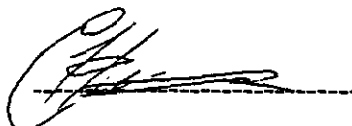
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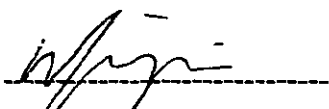
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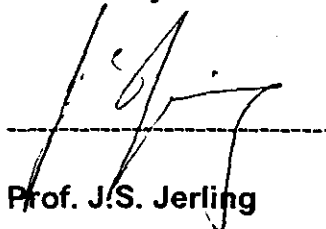
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CHAPTER 1   LITERATURE OVERVIEW AND OBJECTIVES

1.1   Introduction

This chapter presents a concise review of the literature that is necessary for the understanding and interpretation of the two articles presented in this dissertation. The first article is a methodological article. There are still unanswered fundamental questions regarding the toxicology and beneficial properties of the traditionally used, medicinal plant extract, *Rosa roxburghii*. One of these methodological questions is whether the high antioxidant content of the plant extract could at least partially contribute to the beneficial properties of *Rosa roxburghii* extract. However, it is not only important to investigate the beneficial properties but also the safety of this plant extract for human consumption and treatment in certain diseases. Once the effects were known, we were able to incorporate those results into an *in vivo* study.

1.2   Free radicals and reactive oxygen species

A free radical may be defined as any molecular species, which contain one or more unpaired electron. The presence of these unpaired electrons increases the molecules' chemical reactivity (Halliwell, 1978). Because of the need to pair its single electron, a free radical must attract a second electron from a neighbouring molecule, causing the formation of yet another free radical and self-propagating a chain reaction. This loss of electrons is called oxidation, and free radicals are referred to as oxidising agents because they tend to cause other molecules to donate their electrons. This broad definition includes the hydrogen atom, most transition metals and the oxygen molecule (Halliwell and Gutteridge, 1984). Therefore, not surprisingly, oxygen is a common reactant in free radical reactions either directly or indirectly, forming reactive oxygen species (ROS) such as singlet oxygen, superoxide and hydrogen peroxide (Urbananvicius, 2003).

Although oxygen could be harmful to most aerobic organisms, it is essential for the production of energy. Oxidising of macronutrients producing electrons accepted by electron carriers, such and nicotinamide adenine dinucleotide (NAD^+). The resulting reduced electron carriers are reoxidised by oxygen in the mitochondria, producing adenine trinucleotipe phosphate (ATP) (Gautheron, 1984). Most of the oxidative processes in cells generally result in a transfer of electrons to oxygen to form water without release of intermediates; however a small number of oxygen radicals are inevitably formed due to leakage in electron transfer reactions (Sauer *et al.*, 2001).

1.2.1. Different types of free radicals and reactive oxygen species

Molecular oxygen is, in fact, a bi-radical possessing two unpaired electrons of parallel spin in different antibonding orbitals. One way to increase the reactivity of oxygen is to move one of the unpaired electrons in a way that alleviates the spin restriction. However the singlet O_2 in biological systems, has no unpaired electrons and is therefore not a free radical but a *ROS* (Halliwell and Gutteridge, 1984 and Ebadi *et al.*, 2001). If a single electron is accepted by the ground-state oxygen molecule, it must enter one of the antibonding orbitals forming the free radical, superoxide (O_2^-). Superoxide is the primary oxygen free radical produced by mitochondria. Ubisemiquinone generated in the course of electron transport reactions in the respiratory chain donate electrons to oxygen and thereby provides a constant source of superoxide (Raha and Robinson, 2000).

A two-electron reduction of oxygen radical results in the formation of the peroxide ion (O_2^{2-}), which in the physiological pH will protonate to form hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 1984). Superoxide and hydrogen peroxide are *ROS* but are not normally toxic. However, in the presence of transition metals such as iron and copper, the Haber-Weiss reaction is catalysed and the highly reactive hydroxyl radical is formed (Tabatabaei *et al.*, 1998).

1.2.2. Endogenous and exogenous origin of free radicals

Normal cell metabolism, for example the incomplete reduction of molecular oxygen by the mitochondrial respiratory chain, results in a continuous generation of *ROS*, including superoxide radicals and hydrogen peroxide (Skaper *et al.*, 1997 and Rasilainen *et al.*, 2002). Further sources of ROS originates at leukocyte activation during inflammation (Rasilainen *et al.*, 2002) as well as enzymes such as xanthine oxidase, aldehyde oxidase and hyperoxidase (Dinçer *et al.*, 2002). Free radicals can also arise from exogenous sources such as fatty food, smoking, alcohol, environmental pollutants, radiation, ozone, toxins and ultraviolet light (Urbanavicius, 2003). In Table 1 the biological relevant free radicals are summarised.

Table 1: Biologically Relevant Free Radicals (Nowak et al., 1994).

Free radicals	Comments
ROS Superoxide $O_2^{\cdot}$ $O_2 \rightarrow O_2^{\cdot}$ *(Oxidase)*	Generated either (1) directly during auto-oxidation in mitochondria or (2) by enzymes in the cytoplasm, such as xanthine oxidase or cytochrome P450; once produced, it can be inactivated spontaneously or more rapidly by the enzyme superoxide dismutase (SOD) $O_2^{\cdot} + O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$
Hydrogen peroxide (H_2O_2) $O_2^{\cdot} + O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$ (SOD) *or* Oxidases present in peroxisomes $O_2 \text{ peroxisome } O_2^{\cdot} \rightarrow H_2O_2$ (SOD)	Generated by the enzyme superoxide dismutase (SOD) or directly by oxidases in intracellular peroxisomes.

Hydroxyl radicals ($OH^{\cdot}$) $H_2O \rightarrow H^{\cdot} + OH^{\cdot}$ *or* $Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^{\cdot} + OH^{-}$ *or* $H_2O_2 + O_2^{\cdot} \rightarrow OH^{\cdot} + OH^{-} + O_2$	Generated by the hydrolysis of water caused by ionizing radiation or by interaction with metals, especially iron (Fe) and copper (Cu). Iron is important in toxic oxygen injury because it is required for maximal oxidative cell damage.
$NO^{\cdot} + O_2^{\cdot} \rightarrow ONOO^{-} + H^{+}$ $\uparrow\downarrow$ $OH^{\cdot} + NO_2 \leftrightarrow ONOOH \rightarrow NO_3^{-}$	NO by itself is an important mediator that can act as a free radical; it can be converted to another radical – peroxnitrite anion ($ONOO^{-}$), as well as NO_2^{-} and NO_3^{-}.

1.3 Functions of free radicals and ROS in biological systems

Oxidation-reduction based regulation of gene expression appears to be a fundamental regulatory mechanism in cell biology. Therefore it is important to notice that free radicals play an important role in processes such as the immune function and processes such as vasculogenesis during exercise (Sen, 2001). Endogenous ROS is seen as "life signals" which is involved in the modulation of general signalling cascades during embryogenesis, proliferation and unfortunately also in proliferating cancer cells (Sauer *et al.*, 2001). ROS act in concert with intracellular Ca^{2+} in signalling pathways which regulate the balance of cell proliferation versus cell cycle arrest and cell death (Ermak and Davies, 2001).

1.4. Oxidative stress

Oxidative stress is characterised as a shift of the cellular redox status to a more oxidising state, due to the over generation of ROS (Ermak, 2001). Mammalian cells usually have effective endogenous antioxidant defence systems to cope with the toxic ROS generated in the course of aerobic life metabolism (Guaiquil et al., 2001). In some circumstances the continuous exposure to pro-oxidants may lead to an increase in reactive species beyond the body defence system, and cause irreversible oxidative damage (Kashimato, 1999 and Tseng, 1997). Oxidative stress is imposed on cells as a result of one of three factors: 1) an increase in oxidant generation, 2) a decrease in antioxidant protection, or 3) a failure to repair oxidative damage (Fiers et al., 1999).

1.5. Consequences of oxidative stress

ROS can oxidise important macromolecules leading to metabolic and structural modification that can lead to cell death. These modifications include lipid peroxidation, protein denaturation and cross-linking, enzyme inhibition, DNA strand scission, base modification and mutations, and changes in cell surface receptor and cell permeability (Blain et al., 1997). The three major consequences of free radical damages are (1) alterations of DNA, (2) lipid peroxidation and lysosome damage, (3) and alterations of proteins. The oxidative damage to various molecules and cells may result not only in the toxicity of xenobiotics but is also likely to be involved in numerous pathological events, including metabolic disorders, diabetes mellitus, cellular aging, and reperfusion damage after myocardial infarction (Langseth, 1995 and Urbanaviscius et al., 2003).

1.5.1 Alterations of DNA

In humans, the number of oxidative hits to DNA per cell per day have been estimated to be as high as 10 000. Oxidative lesions of DNA accumulate with

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age (Sen, 2001). Free radicals react with DNA and RNA leading to somatic mutations and to disturbances of transcription and translation (Ebadi *et al.*, 2001). ROS can react with DNA either at the sugar-phosphate backbone that leads to strand fragmentation or at a base that results in a chemically modified base (Ebadi *et al.*, 2001). The modification of DNA bases can result in the formation of DNA adducts which, during the course of attempted repair or replication, can lead to DNA mutations, and in the end development of neoplastic cells (Lampe, 2003). Oxidative DNA damage plays an important role in the pathophysiology of cancer, however, lately there is also strong experimental evidence for a "mutation theory of atherosclerosis", which underlines the similarity between atherosclerotic and carcinogenic processes (Andreassi and Botta, 2003).

Mitochondrial DNA is also highly susceptible to oxidative damage. Mitochondrial DNA is located close to the inner mitochondrial membrane where the ROS are generated and is not protected by histone proteins as in the case of nuclear DNA. All these factors increase the susceptibility of mitochondrial DNA to oxidative damage (Van Remmen & Richardson, 2001). There is indirect evidence that oxidation of DNA, especially mitochondrial DNA damage, may be a major cause of aging and age-associated degenerative diseases. This supports the mitochondrial theory of aging (Ebadi *et al.*, 2001).

1.5.2 Lipid peroxidation and lysosomal damage

Oxidative damage to lipids can occur directly by peroxidation initiated by ROS or indirectly through the production of highly reactive aldehydes (Van Remmem & Richardson, 2001). Lipid peroxidation is the destruction of unsaturated fatty acids. Fatty acids of lipids in membranes possess double bonds between some of the carbon atoms. Such bonds are vulnerable to attack by oxygen-derived free radicals for example OH$^-$. The lipid-radical interactions themselves yield peroxides, that set off a chain reaction resulting in membrane, organelle and

cellular destruction. Lipid peroxidation may alter fluidity and permeability, and compromise the integrity of the barrier (Sen, 2001). The damaged membranes inhibit oxygen, nutrient and water transport to cells and increase lysosome enzymes leaking, which digest the cell itself and surrounding cells, eventually leading to the lowering in the immune system (Urbanavicius, 2003).

Lipid peroxidation also plays a key role in the development of cardiovascular disease, such as atherosclerosis. The damage or dysfunction of the vascular endothelial layer followed by the migration of low density lipoproteins (LDL) in the sub-endothelial space, are the initial steps in the process of atherosclerosis (Andreassi and Botto, 2003, Klausner, 1999). Oxidised LDL also effects the expression of several proteins involved in the initiation and progression of atherosclerosis (Steinberg & Chait, 1998).

Cardiolipin, a major lipid component of the inner mitochondrial membrane, is especially sensitive to oxidative stress because of its high degree of unsaturation. Oxidation of cardiolipin can be particularly detrimental to the mitochondria because this lipid plays a critical role in the functioning of key mitochondria proteins such as cytochrome C oxidase (Van Remmem & Richardson, 2001).

1.5.3.   Protein alterations

Oxidative protein damage is widespread within the body at rest. At rest, 0.9% of the total oxygen consumed by a cell contributes to protein oxidation. Most of the damage done by protein oxidation is irreparable. Proteins that have been damaged by ROS are highly susceptible to proteolytic degradation (Sen, 2001). Certain components of proteins such as tyrosine, methionine, tryptophan and sulfhydryl residues are highly susceptible to oxidative damage (Sen, 2001). Free radicals react with proteins leading to enzyme inactivation and disruption of cellular function (Ebadi et al., 2001). Oxidative damage can be introduced to

proteins by reaction with aldehydes, formed during lipid peroxidation, e.g. malondialdehyde that reacts with the amino group (Csallany *et al.*, 1984)

Due to the close physical association of lipids and proteins in membranes, oxidative damage of mitochondrial proteins as a direct result of oxidative stress or as a consequence of lipid peroxidation can result in protein cross-linking, degradation of proteins and loss of function. Several mitochondrial electron transport chain enzymes, e.g. ATPase are sensitive to inactivation of oxidative stress. Therefore, inactivation of these proteins could lead to impaired mitochondrial function (Van Remmem & Richardson, 2001).

1.6. Antioxidant protection

Antioxidants could help prevent oxidative stress via various antioxidant protecting mechanisms, such as scavenging of reactive oxygen/nitrogen species or their precursors, inhibition of ROS formation by metal-chelating and activation of detoxifying/defensive proteins of endogenous enzymes (Roig *et al.*, 2002 and Gilgun-Sherki, 2000).

1.6.1. Endogenous antioxidant system

Mammalian cells usually have effected endogenous antioxidant defence systems to cope with the toxic ROS generated in the course of aerobic life metabolism (Guaiquil *et al.*, 2001), as summarised in Figure 1. It consists of antioxidative enzymes, such as superoxide dismutase, catalase, GSH peroxidase and of small antioxidant molecules, such as GSH and Vitamins E and C (Rasilainen *et al.*, 2002).
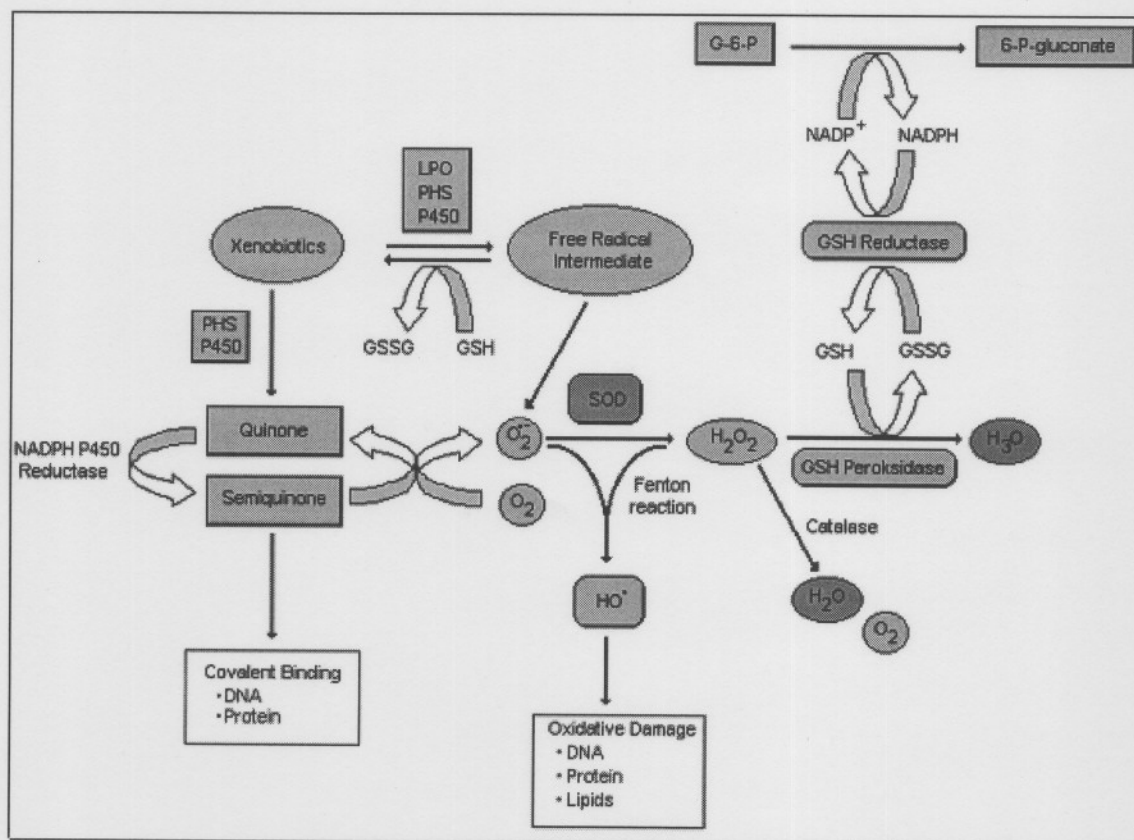
Figure 1. *Endogenous ROS production and antioxidant defence system.*

The major intracellular source of ROS is the mitochondrial electron transport chain, where the transfer of one electron to oxygen occurs from the stable semiquinone produced during reduction of ubiquinone by complexes I and II. Another source of electron transport is the endoplasmic reticulum where electrons leak from the NADPH cytochrome P450 reductase. ROS are also produced by peroxisomal β-oxidation of fatty acids, cytochrome P450 metabolism of xenobiotics and tissue-specific enzymes. Under normal conditions, ROS are cleared from the cells by the endogenous antioxidant system, consisting of superoxide dismutase (SOD), catalase, or GSH peroxidase. The imbalance between cellular production of ROS and the natural antioxidant defence system results in oxidative damage of lipids, proteins and DNA (Hayes *et al.*, 1999). GSH= reduced glutathione, GSSG = oxidised glutathione

1.6.1.1. Enzymatic antioxidant system

There are several ways to defend against free radicals. The antioxidant enzyme system is one of the most important defense mechanisms. Superoxide dismutase, GSH peroxidase and catalase are the most significant enzymes in antioxidant defense mechanism (Gilgun-Sherki *et al.*, 2001), as illustrated in Figure 1. SOD is the first line antioxidant and neutralises superoxide radical anions into H_2O_2, while catalase is specific for the removal of H_2O_2. GSH peroxidase, which uses GSH to remove hydrogen peroxide, can also reduce organic peroxidase into their corresponding alcohol and is therefor essential for humans in the antioxidant system (Sauer *et al.*, 2001).

1.6.1.2. Small molecular antioxidants (GSH and antioxidant vitamins)

Non-enzymatic components of the antioxidant defense system interrupt the free radical-initiated chain reaction of oxidation or scavenge and disable free radicals before they react with cellular components (Lampe, 1999). GSH is the main intracellular low molecular mass thiol. It is implicated in numerous antioxidant mechanisms and is also the co-substrate of enzymatic detoxification processes GSH peroxidase (see Figure 1) (Diez *et al.*, 2001 and Hayes *et al.*, 1999). GSH reacts rapidly and non-enzymatically with free radicals, cytotoxic Fenton reaction products (hydroxyl radicals), N_2O_3 and peroxynitrite (cytotoxic products formed by the reaction of nitric oxide with O_2 and superoxide, respectively. GSH also participates in the reductive detoxification of hydrogen peroxide and lipid peroxides. These reactions lead directly or indirectly to the formation of oxidised glutathione (GSSG) (Griffith, 1999). GSH also combines with several xenobiotics and their electrophillic metabolites, enhancing their elimination in bile and urine. This protects cells by preventing binding of these labile intermediaries with vital molecules (Estevez *et al.*, 1994). Another role of GSH is to maintain thiol-dependant enzymes and vitamins C and E in their active forms (Rasilainen *et al.*, 2002).

Other thiols, such as cysteine, are also able to directly react with free radicals, such as H_2O_2 and thus reduce oxidative stress. Cysteine also supports GSH synthesis (Rasilainen et al., 2002).

1.6.1.3. CoQ_{10} (ubiquinol)

Ubiquinol (CoQ_{10}) is a fat-soluble antioxidant produced in the human body, which also regenerates vitamin E (Langseth, 1995). CoQ_{10}, an electron carrier in the inner mitochondrial membrane, stabilises the respiratory chain components and acts as an antioxidant. CoQ_{10} might play an important role in the treatment of mitochondrial oxidative stress of Parkinson's disease (Ebadi et al., 2001).

1.6.2 Exogenous antioxidant system

Since the endogenous antioxidant defences are not always completely effective, especially in certain pathophysiology events such as Complex I mitochondrial defects (Chinnery and Turnbull, 2001) and because the exposure to damaging exogenous factors are increasing, it seems reasonable to state that exogenous antioxidants could play a very effective role in preventing oxidative related diseases (Cheng et al., 2001). The majority of antioxidants such as vitamin C, β-Carotene and polyphenols are diet depended (Gilgun-Sherki et al., 2001). Antioxidants studies also indicate that some B vitamins may also play important roles in the cellular antioxidant defence systems (Hu et al., 1995). The dietary intake of trace elements, such as Fe, Cu, Mn and Zn plays an important role in antioxidant metallo-enzyme functioning. For example, mitochondrial superoxide dismutase is a manganese-containing enzyme and GSH peroxidase is selenium-dependent (Lampe, 2003).

1.6.2.1. Ascorbic acid (Vitamin C)

Vitamin C is a major aqueous-phase antioxidant, which acts as the first line of defence against free radicals and which spares or regenerates vitamin E (Steinberg & Chait, 1998). Vitamin C and vitamin E quench free radicals by providing hydrogen atoms that can pair up with unpaired electrons of free radicals (R + H = RH) and become self-oxidised in the process (Jacob et al., 1999). Studies have shown interaction between vitamin C and GSH. The redundancy of GSSG by vitamin C may help to ensure important cellular antioxidant protection as well as to maintain GSH levels (Jacob et al., 1995). However, ascorbic acid can also act as a pro-oxidant in the Fenton reaction with iron (Gruss-Ficher and Fabian, 2002).

1.6.2.2.   Vitamin E (α-Tocopherol)

The term "vitamin E", is a collective name for numerous different tocopherols and tocotrienols, which share the same biological activity (Langseth, 1995). Vitamin E is incorporated into the hydrophobic core of lipoproteins where it plays a role in the termination of free radical-mediated reactions, by scavenging the intermediate peroxyl radicals thereby preventing the chain reaction of lipid peroxidation (Timberlake, 2002). In addition to the vitamin E free radical scavenging effect, it also influence the enzymatic antioxidants, by increasing catalase, GSH peroxidase and GSH reductase activities (Cheng et al., 2001).

1.6.2.3   Carotenoids

Carotenoids are the pigments that are responsible for the bright colours of fruits and vegetables. β-carotene may work synergistically with vitamin E in scavenging radicals and inhibiting lipid peroxidation (Timberlake, 2002). Primary prevention trials using β-carotene have shown either no effect on cardiovascular disease endpoints or an insignificant increase in mortality (Steinberg & Chait, 1998). Dietary carotenoids include β-carotene, α-carotene, lycopene, luitein, zeaxanthin and β-cryptoxanthin (Langseth, 1995). Lycopene is mostly associated with a decreased risk of prostate cancer (Lee, 1999). Fawzi et al.

(2000) also reported the increased in immune system after the consumption of tomatoes (high in lycopene) in malnourished children. β-carotene differs in many ways from the other antioxidants and generalisations between studies are discouraged (Timberlake, 2002). The Beta-Carotene and Retinal efficacy trial (CARET trial) involved men and women at high risk of lung cancer due to cigarette smoking. A combined treament of beta-carotene (30 mg daily) and retinol (25,000 IU daily) was evaluated. However supplementation has unexpectedly appeared to increase cancer risk among smokers, and was stopped. In the same study there was a non-statistically significant trend towrd increaced cardiovascular disease (Omenn *et al.*, 1996). This increased in the incidence of lung cancer was observerd in the finish alpha tocopherol beta-arotene (ATBC) cancer prevention trial as well. There was no effect of antioxidant on incidence of fatal or nonfatal myocardial infarction (Virtamo *et al.*, 1998; Rapola *et al.*, 1997). However, in the Physician's health study, there was no differences in the incidence of cancer, cardiovascular disease, myocardial infarction, stroke or overall mortality attributable to carotene (Hennekens *et al.*, 1996). It has been hypothesized that cigarette smake carcinogens such as benzo(a)pyrene and metabolites, may directly react with beta-carotene (Kleinjans *et al.*, 2004).

1.6.2.4 *Flavonoids and polyphenole (tannins)*

Flavonoids and tannins are plant-based polyphenoles that cannot be synthesized by animal organisms. They are widely distributed in plants as protective substances against harmful external influences. As a result, animals and humans depend exclusively on an exogenous dietary supply of flavonoids (Hässig *et al.*, 1999). The main classes of polyphenols are phenolic acids (such and flavonoids. Phenolic acids mainly consist Cinnamic acids, Benzoic acids, Ellagitannins (Scalbert *et al., 2004*). Caffeic acid is the major representative of hydroxycinnamic acid and mainly occurs in foods as an ester with quinic acid called chlorogenic acid (5-caffeoylquinic acid) found in coffee. Chlorogenic acid

and caffeic acid are antioxidants in vitro and they migh inhibit the formation of mutagenic and carcinogenic *N*-nitroso compounds and inhibit DNA damage in vitro (Tapiero *et al.*, 2002).

The flavonoids include two major groups of related compounds, namely the anthoxanthins (such as various flavonols, flavones, Isoflavones, Flavanones) and anthocyanins (various Flavanols such as catechin and epicatechin) (Anon, 1994; Scalbert *et al.*, 2002). Flavonols have been reported to have a range of antioxidant characteristics, including scavenging of free radicals, chelating of metals and the activation of detoxifying defensive proteins (Wang *et al.*, 2000 and Lodivici *et al.*, 2001). However, the ability of flavonoids to provide health benefits does not stop at their antioxidant properties. They have an ability to interact with cell-signaling cascades, therefore influencing the cell at a transcriptional level, and to down-regulate pathways leading to cell death (Roig *et al.*, 2002), e.g. the binding of proteins such as proteases and suppression of their enzymatic activity (Hässig *et al.*, 1999). The antioxidant properties of flavonols against coronary heart diseases are well demonstrated, for example the paradoxical protection of red wine, a rich dietary source of flavonoids, against atherosclerosis and cardiovascular disease (Hertog, 1993). Flavonoids inhibit LDL oxidation and cyclo-oxygenase, preventing aggregation of platelets (Anon, 1994 and Hässig *et al.*, *1999*).

Polyphenols show a structural diversity which largerly influenced their bioavialability. Caffeic acids are easily absorbed through the gut barrier, whereas large molecular weight polyphenols such as proathocyanidins are poorly obsorbed (Scalbert *et al.*, 2002). It is therefore, essential to know the different factors controlling variouse polyphenols bioavailability in different diets (Tapiero *et al.*, 2002). Quercetin and rutin (a glycoside of quercetin), bioavialability is low, while theres a high bioavailability for catechins in green tea, isoflavones in soy, flavanones in citrus fruits or athocyanidins in red wine. Interindividual variations have also been observerd (Tapiero *et al.*, 2002)

1.6.2.4   Antioxidant cocktails

Numerous studies have shown that a single vitamin or mineral supplementation has a beneficial effect on the antioxidant defence system, such as vitamin E in the prevention of coronary heart disease (Gey *et al.*, 1991) and vitamin C in cancer (Gruss-Fisher, 2002). However, the various antioxidant mechanisms are complementary to one another because they act on different oxidants or in different cellular compartments (Langseth, 1995). The chemical nature of an antioxidant determines the location of cell defence, for example: vitamin E, the fat-soluble antioxidant, is located in cell membranes and functions in the protection against lipid peroxidation (Sen, 2001).

Furthermore, antioxidants also interact in synergistic ways and have "sparing effects" in which one antioxidant protects another against oxidative destruction (Hässig *et al.*, 1999). A classical example of this is between vitamin C and E, which become oxidised in the process of quenching free radicals. However, the active reduced forms of the vitamins are believed to be regenerated by reduction with GSH and/or other reductants such as ascorbate and flavonoids (Jacob *et al.*, 1995). The sparing effect of these antioxidants is illustrated in Figure 2.
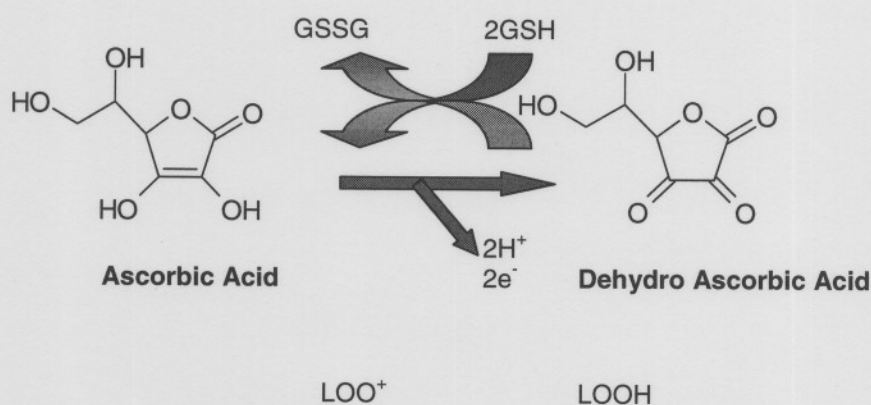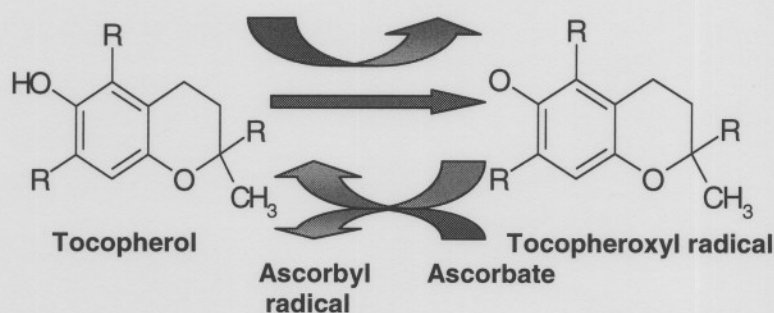
Figure 2: Recycling of antioxidants, vitamins C and E (Jacob et al., 1995). LOO·= lipid radical; LOOH= lipid hydroperoxide

The overall combined effect of a dietary supplement upon anti- and pro-oxidant balance in the human body becomes more meaningful to investigate than the individual effect of different components (Cheng *et al.*, 2001 and Roig *et al.*, 2002). However, the extent in which the many *in vitro* interactions and synergisms, , actually occur *in vivo* remains yet to be determined (Jacob *et al.*, 1995). Cheng *et al.* (2001) indicates that the overall combined effect of a multi-supplement increased the antioxidant defence system (chemical and enzymatic indicators) in healthy persons, and the chemical indicators (plasma vitamin C, vitamin E and β-carotene, total GSH and selenium) responded faster and to a larger extent than the enzymatic analyses (catalase; glutathione peroxidase and superoxide dismutase activities).

1.7   The *Rosa roxburghii* product.

The *Rosa roxburghii* product, which is commercially available under the trade mark, *Cili Bao®* from the company, Cili Health SA, is refined from natural, wild *Rosa roxburghii* fruit, found in a certain area in Southwest China. The Rose plant (also known as 'Burr Rose', or 'Chestnut Rose') was introduced to the Botanic Garden at Calcutta around 1824. It was named after William Roxburgh who was the superintendent (Austin, 1990). *Rosa Roxburghii* extract is composed of various antioxidants such as SOD, vitamin C and E. The supplementation with *Rosa roxburghii* extract increased the SOD, catalase and GSH levels in

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erythrocytes in old healthy participants (age of 50-57 years) (Ma *et al.*, 1997). The high content of antioxidants may contribute to the protective role it play as a traditional medicine in atherosclerosis (HU *et al.*, 1994), cancer and immunity stress (Zhang *et al.*, 2001). In a controlled dietary study done in China, supplementation of fruit juice, which included *Rosa roxburghii*, reduced urinary N-nitrosoproline, a noncarcinogenic N-nitroso compound (Lampe, 2003). Nitrosation in humans can be estimated by monitoring excretion of N-nitroso proline. Table 2 gives a summary of the nutrient content of *Rosa roxburghii*.

Table 2: The chemical composition of *Rosa roxburghii* fruit extract. Adopted by Zhang *et al.* (2001).

Index	*Fruit	Juice
Ascorbate (mg/ml)	3.48	1.3
Vitamin E (ug/ml)	2.16	0.81
Vitamin B1 (mg/ml)	1.67	0.64
Total SOD (U/ml)	5911	5174
Zn (mol/l)	17	15.5
Ca (mol/l)	2703.1	865.3
As (mg/kg)	0.5	ND**
Pb (mg/kg)	1	ND**

* Mass wasn't indicated by authors. ** Not detected

1.8   Use of plant extracts for medicinal purposes

Increased consumption of fruits and vegetables has been well known for their protection against various diseases (Cao *et al.*, 1998). The high content of phytochemicals has complementary or overlapping mechanisms such as antioxidative, antimutagenic and anticarcinogenic, which protects the body against diseases such as atherosclerosis and cancer (Lampe, 1999). Medicinal and herbal plants are used by large sections in developing countries for primary health care, however, there is also an increase in the use of natural therapies in industrial countries, due to the perception that natural remedies are somehow safer and more effective than pharmaceutically remedies (Elvin-Lewis, 2000). Medicinal plants are important for pharmacological research and drug

development, not only as a direct therapeutic agents, but also as starting material for the synthesis of drugs or as models for pharmacological active compounds (WHO, 1998). However, plant extracts also consist of antinutritional and possible toxic components, such as oxalic acid, nitrate and erucic acid, which could express cytotoxic and genotoxic activities. Therefore, understanding the health benefits but also the potential toxicity of these plants is important (Yen *et al.*, 2001).

1.9   Aims and Objectives

Aims and Objectives
The aims and objectives of this dissertation were:

Aim
Investigating the safety of *Rosa roxburghii* plant extract for human comsumption *in vitro*.

Objectives

- Determine the possible mutagenic-, cytotoxic- and genotoxic properties of the plant extract.

- In addition to this, investigate the possible beneficial properties; anti-mutangenicity, DNA repair capability and the antioxidant capacity.

Aim
Investigate the antioxidant capacity of *Rosa roxburghii* plant extract *in vivo* in a controlled feeding system.

Objectives

- Determine if *Rosa roxburghii* extract increases the base line

antioxidant capacity in healthy humans subjects.

- Determine if the plant extract has any influence on the GSH/GSSG redox status in healthy human subjects.

- Determine whether changes in antioxidant capacity are associated with superoxide dismutase and GSH synthetase enzyme activities.

1.10   Strategy

Several methods exist to determine the toxicity of a compound on various levels in cellular function. A commonly used assay is the MTT-test which tests the conversion of the colourless substrate, tetrazolium, to the coloured formazan product by cells (via mitochondrial succinate dehydrogenase) and serves as an indirect measurement of cell viability (Mossmann, 1983). Another commonly used cytotoxicity assay is the LDH-release assay which tests the release of lactate dehydrogenase from the cell due to necrosis and as a result of a toxic compound (Tseng et al., 1997). Mutagenicity of a compound can be determined by the Salmonella mutagenicity assay (Ames assay) - a commonly used assay to monitor the mutagenicity potential of natural products (Maron and Ames, 1982). Some plant extracts also consist of nonmutagens and antimutagens compounds or antioxidants that complement DNA repair systems (Gozález-Avila, 2002). Genotoxicity was determined by the single cell gel electrophoresis assay, which is a rapid and sensitive method for measuring DNA strand breaks (Singh et al., 1988). This is an effective method for the genotoxic or antigenotoxic investigation of plants extracts in primary rat hepatocytes (Yen et al., 2001). GSH is involved in several fundamental biological functions, including free radical scavenging, detoxification of xenobiotics and carcinogens, as well as redox reactions. Therefore measuring the ratio between GSH and GSSG will be an effective indicator of an antioxidant scavenging ability of an antioxidant (Asensi et

al., 1999 and Tietze, 1969). Another method to measure the effectiveness of an antioxidant, is determining the overall antioxidant capacity of the serum (Prior *et al.*, 2003 and Ou *et al.*, 2001).

CHAPTER 2

Protective effect of *Rosa roxburghii* against mutagenicity, genotoxicity, cytotoxicity and oxidative stress in primary rat hepatocytes

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Abstract

A few reports exist on the beneficial medicinal properties of the fruit of the *Rosa roxburghii* plant, which has been used for therapeutic purposes such as atherosclerosis and cancer in South-western China. Despite its use as a natural antioxidant supplement, little is known about the possible toxic effects of this plant extract. We evaluated the toxic-, mutagenic-, and genotoxic effects of *Rosa roxburghii* in primary rat hepatocytes *in vitro*. The antioxidant potential of *Rosa roxburghii in vitro* was also investigated. Using the MTT and LDH-leakage assays, no toxicity of the extract could be was observed; in fact cellular viability appeared to increase dose-dependently and oxidative stress induced *tert*-butyl hydroperoxide (*t*-BHP) toxicity reduced significantly using these assays. Similarly, no mutagenicity was detected using the Ames assay, but *Rosa roxburghii* showed an antimutagenic effect against several known mutagens (2-acetylaminofluorene, aflatoxin B_1 and methyl methanesulfonate) using the *Salmonella typhimurium* strains TA 98, TA 100 and TA 102. In addition, a decrease of baseline level as well as oxidative stress (*t*-BHP) induced DNA damage occured *in vitro* with co-incubation with the *Rosa roxburghii* extract as measured by single cell gel electrophoresis/Comet assay. This protection against oxidative stress was reflected in the increase of antioxidant capacity (ORAC assay) and the increase of GSH/GSSG ratio. These results underline the potential of this fruit extract as an antioxidant supplement with beneficial properties against oxidative stress-related damage.

Keywords

Abbreviations

Tert-butyl hydroperoxide (t-BHP), reduced glutathione/oxidised glutathione (GSH/GSSG), World Health Organization (WHO), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 5,5'dithiobis-(2-nitrobenzoic acid) (DTNB), Dimethylsulfoxide (DMSO), 2-acetylaminofluorene (2-AAF), aflatoxin B_1 (AFB_1), methyl methanesulfonate (MMS), Christian Higher Education (CHE), N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid] (HEPES), Fetal calf serum (FCS), lactate dehydrogenase (LDH), (3-(4,5-dimethylthiazol)-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate-buffered saline (PBS), MT-2 cells (murine lymphoblast), Bicinchoninic acid sollution (BCA), oxygen radical absorbance capacity (ORAC), standard deviation (STDEV), 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium (INT), Single cell gel electrophoresis (Comet assay), M2VP (1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate) .

1. Introduction

Although modern medicine is well developed in most countries of the world, large sections of the populations in developing countries still rely on medicinal plants and herbal medicines for primary health care. Moreover, recognition of herbal medicine's clinical, pharmaceutical and economic value in natural therapies has increased greatly in industrialised countries (Elwin-Lewis, 2000). Evaluation of these products and ensuring their safety and effectivity present important challenges (WHO, 1998). The dietary supplementation of the extract of the fruit of the *Rosa roxburghii* extract plant is one such plant that has been previously described to hold putative beneficial medicinal potential for diseases such as arthrosclerosis, cancer and immunity stress (Zhang *et al.*, 2001). It has also been suggested to limit the undesirable effects of ageing (Ma *et al.*, 1997) and is marketed as a natural antioxidant supplement. Limited scientific information, however, is available on the possible side effects of this supplement for use in humans. In light of the existence of several non-nutritional substances in plants that expresses cytotoxic and genotoxic activities (Ames, 1983), an investigation into these aspects is necessary to evaluate the potential of this product for human consumption.

The possible mutagenic, toxic and genotoxic properties of an extract of the *Rosa roxburghii* extract *in vitro* was *investigated*. The liver is the major target organ of oxidative stress induced by xenobiotics. Therefor primary cultures of hepatocytes, that is well described in pharmacological and toxicological studies (Lautraite *et al.*, 2002; Richert *et al.*, 2001) were used for this investigation. *Tert*-butyl hydroperoxide (*t*-BHP) were utilised as an oxidative damage inducer. *t*-BHP is a short chain analogue of lipid hydroperoxide and is metabolised into free radical intermediates by the cytochrome P450 system in hepatocytes. This in turn, initiates lipid peroxidation, glutathione depletion and cell damage (Wang *et al., 1999;* Yau *et al.,* 2002; Tseng *et al.,* 1997). In addition, selected biochemical

properties of the extract *in vitro* were evaluate for its putative antioxidant potential were also investigated.

2. Materials and Methods

2.1 Materials

2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), William's E Medium, Hank's balanced salt solution, type VII collagen, type IV collagenase, *t*-BHP, GSH, glutathione reductase, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 2-acetylaminofluorene (2-AAF), aflatoxin B_1 (AFB_1), methylmethane sulfonate (MMS), dimethylsulfoxide (DMSO), L-glutahione, and 5,5'dithiobis-(2-nitrobenzoic acid) (DTNB), 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP) and fluorescein sodium salt were purchased from Sigma Chemical Co. (Johannesburg, South Africa). Diaphorase, 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium (INT), pyruvate were purchased from Roche (Johannesburg, South Africa). Oxoid nutrient broth no2 was purchased from Rob Dyer Surgical (Johannesburg, South Africa), fetal calf serum and antibiotics from Laboratory specialised service (Johannesburg, South Africa) other chemicals used were from Merck (Johannesburg, South Africa) and of the highest grade available commercially. An extract, of which the processing is kept confidential, of the *Rosa roxburghii* extract fruit was received from Cili Health, South Africa. The extract was neutralised with KOH and filtered (0.2µm) before dilutions were made.

2.2 Mutagenicity and antimutagenicity (Ames) tests

The *S. typhimurium* bacterial reverse mutation assay was conducted by using the standard plate incorporation procedure of Maron and Ames (1983). The following dilutions of the evaporated extract of the received *Rosa roxburghii* solution were used in the assay: 1:2 (50%), 1:5(20%), 1:10(10%) and 1:50(2%)

(v/v), diluted in water (H_2O) or DMSO. *S. typhimurium* strains TA 98, TA 100, and TA 102 were provided by Dr. B.N. Ames (Berkeley, CA). For the mutagenicity assay, 0.1 ml of the various *Rosa roxburghii* dilutions, 0.5 ml of S9 activation mixture and 0.1 ml of an overnight bacterial culture were carefully mixed with 2 ml of molten top agar containing 0.05 mM biotin-histidine. This mixture was dispersed onto minimal glucose agar plates. The His$^+$ revertant colonies were counted after incubation at 37°C for 48hrs using a Quebec Colony Counter (American Optical Corp., Buffalo, New York). Each sample was assayed using five plates per treatment.

Antimutagenicity of *Rosa roxburghii* extract dilutions against different mutagens were assessed using the standard plate incorporation assay as described above, except that 0.1ml of each mutagen, 2-acetylaminofluorene (2-AAF, 10 µg per plate), aflatoxin B_1 (AFB$_1$, 20 and 50 ng per plate) and methyl methanesulfonate (MMS, 20 mM) was added as well (Mortelmans and Zeiger, 2000). A liver S9 homogenate (0.72 nmol cytochrome P450/mg protein) was prepared by inducing male Fischer rats (200 g) with aroclor-1245 as described by Maron and Ames (1983). The S9 homogenate was incorporated into the S9-mixture at 2 mg protein per ml. The same mutagen used as positive controls in the various strains during the mutagenicity assays were used. The effect of the metabolic activated mutagens was monitored against TA 98 and TA 100 in the presence of S9 mixture, respectively. The direct acting mutagen MMS was monitored using TA 102 in the absence of S9 mixture. Control plates containing only top agar, the various *S. typhimurium strains* and solvent (H_2O or DMSO) were used to obtain the background estimations of spontaneous revertant counts.

2.3 Preparation of primary rat hepatocytes

Male Sprague Dawley rats (body weight $\approx$ 300g) were obtained from the Laboratory Animal Centre of the Potchefstroom University for CHE. Hepatocytes were isolated by collagenase liver perfusion *in situ* as described by Wang and

Lautt (1997). Only preparations with cell viability greater than 90% as determined by trypan blue exclusion test were used for subsequent (as described in the SIGMA, catalog 2004) *in vitro* experiments. Primary hepatocytes were cultured in William's E Medium supplemented with 10 mM HEPES, 10% fetal calf serum (FCS), 0.1 U/ml insulin, 2 mM L-gluthamine, and antibiotics (penicillin 250 U/ml and streptomycin 250 µg/ml) at 37°C under 95% humidity and 5% CO_2. The cells were either seeded onto collagen coated 96-well plates (Nunc) at 4×10^4 cells/well for cytotoxicity studies, or 6-well plates (Nunc) at 1×10^6 cells/well for genotoxicity studies (Comet assay). The medium was changed after 2 hrs of incubation with William's E Medium, supplemented with 0.5% serum, 0.1 U/ml insulin, 2 mM L-gluthamine, 2 mM proline, 10 mM pyruvate and antibiotics. All subsequent assays were performed in this medium containing the various treatments.

2.4 Cytotoxicity assays

The potential toxicity was assessed in two ways: through measurement of lactate dehydrogenase (LDH) released into the culture medium by cells dying after exposure to the *Rosa roxburghii* extracts and through formazan (MTT) reduction by cells surviving in culture. Primary rat hepatocytes were incubated for 3 hours or 24 hours with dilutions of *Rosa roxburghii* extract ranging from 0-5% (v/v) in final reaction mixture. Cells were incubated for a further additional 2 hours with or without the addition of 0.8 mM *t*-BHP. Triton X-100 (0.1%) was used as positive control that denotes 100% cytotoxicity. The MTT assay were performed essentially as described by Alley *et al.* (1988), and the LDH-leakage assay as described before by Korzeniewski and Callewaert (1983).

2.5 Single cell gel electrophoresis (Comet assay)

Measurement of DNA single and double strand breaks was performed by the comet assay (single cell gel electrophoresis) essentially as described by Singh *et*

al. (1988). Primary hepatocytes were plated onto collagen-coated 6-well plates at a density of 1 x 10^6 cells/well. Cells were incubated for 14 hours with *Rosa roxburghii* using the same final concentration as described before for the cytotoxicity assay, with or without addition of 0.8 mM *t*-BHP for an additional 2 hours. To evaluate the effect of *Rosa roxburghii* extract on DNA repair, cells were either used immediately after this incubation period or the medium was replaced with new medium and the cells incubated for a third incubation period of 15 or 30 minutes. After the incubation periods cells were washed twice with phosphate-buffered saline (PBS) and detached by incubation with collagenase. Cells were collected by centrifugation, washed once more and resuspended in PBS. The cells were dissolved in low melting agarose and spread on a precoated agarose microscopic slide. After solidification, slides were immersed in the lysing solution (5 M NaCl, 0.4 M EDTA, 10 ml Triton X-100 and 10% DMSO) overnight (4°C). Slides were then washed and transferred to an electrophoresis chamber and treated with alkaline electrophoresis buffer (0.6 M NaOH and 0.05 M EDTA, pH 13) for 25 min. Electrophoresis were performed for another 25 minutes (30V). After neutralisation with Tris buffer (0.5 M Tris.Cl, pH 7.5) nuclei were stained with ethidium bromide (20 µg/ml), and the %DNA in the tail measured using an Olympus X70 fluorescence microscope and the CASP software program.

2.6 Glutathione redox status

Primary rat hepatocytes were prepared as described before. Chang cells (hepatocellular carcinoma) were cultured in Dulbecco's Modified Eagle's Medium, 10% FCS and antibiotics (penicillin 250 U/ml and streptomycin 250 µg/ml). MT-2 cells (murine lymphoblast), which was a kind gift from Dr. A Puren from the NICD, South Africa, were cultured in RPMI 1640 medium supplemented with 10% inactivated FCS and antibiotics. All cell lines were incubated at 37°C in a humidified incubator with 5% CO_2. The cells were seeded at a density of approximately 5 x 10^6 cells per 75 cm^2 flask and incubated for 24 hr with the

various antioxidant dilutions as described before and with or without the addition of 0.8 mM t-BHP.

The cellular content of the reduced glutathione (GSH) and oxidised glutathione (GSSG) was determined essentially using the recycling assay described by Tietze (1969). Briefly, the cells were washed twice with PBS, scraped from the bottom, suspended in PBS and divided into two separate fractions for the (total) GSHt and GSSG determinations, respectively. M2VP was used as GSH scavenger. Sample preparation and analyses were subsequently performed as described before (Asensi et al., 1999). Protein content was determined in the fractions prior to acid precipitation using the BCA method (Smith et al., 1985).

2.7   Antioxidant capacity analysis

The total antioxidant capacity in the samples prepared for glutathione redox state analyses was measured using the adapted oxygen radical scavenging capacity (ORAC) method as described by Ou et al. (2001). The extraction conditions described by Prior et al. (2003), to determine both lipophilic and hydrophilic antioxidant capacity in biological samples, were used to determine the antioxidant capacity of the *Rosa roxburghii* extract directly. In the final assay mixture with a volume of 200 µl, fluorescein (56 nM) was used as a target for free radical attack by AAPH (240 mM) as a peroxyl radical generator. A BioTEK fluorescence plate reader was used and the decay of fluorescence of fluorescein (excitation 485 nm, emission 520 nm) measured every 5 min for 2 hours. Trolox was used as standard and antioxidant capacity expressed as µM trolox units.

2.8   Data Analysis

Results are expressed as the average ± STD. Version 6 of the statistica program was used and statistical analyses were performed using Student's t-test with significance differences considered when $p < 0.05$.

3.   Results

3.1   Mutagenicity

Table 1 summarizes the results of the mutagenicity and antimutagenicity assays. For the dilutions used, no mutagenicity was found towards any of the three strains used. The concentrated form of *Rosa roxburghii* did not increase the number of revertants significantly, indicative of a non-mutagenic effect. In fact, the presence of *Rosa roxburghii* had a significant protective effect against mutagenicity induced by the various chemical mutagens and carcinogens used in the plate incorporation assay. The positive control plates showed an increase of 23-fold for TA 98, 4-fold for TA 102 and a 37-fold increase for TA 100 strain when comparing with spontaneous revertant plates.

Mutagenicity of AFB_1, which requires metabolic activation, was significantly ($p < 0.05$) lowered at all *Rosa roxburghii* extract dilutions. At 20 ng and 50 ng (result not shown) AFB_1, all three dilutions showed a protective effect against AFB_1-induced mutagenicity. Even at a 10% concentration *Rosa roxburghii* decreased the number of Histidine revertants more than three-fold. 2-Acetylaminofluorene (2-AAF), a mutagen used against TA 98, showed a clear dose response for the various *Rosa roxburghii* dilutions. Even the 2% concentration was still effective in reducing the number of His^+ revertants by nearly half. At the 50% concentration complete protection against 2-AAF was achieved.

MMS, which is a direct acting mutagen against TA 102 showed only a slight reduction in His^+ revertants of a approximately 20% reduction at the 50% *Rosa roxburghii* extract concentration.

3.2 Cytotoxicity

Both the cytotoxicity assays (MTT and LDH-release assays) showed that *Rosa roxburghii* extract up to 5%(v/v) concentration exhibited no toxic effect to the cells, even after 24hr incubation (Figure 1). The cellular viability in fact increased dose-dependantly and significantly at most dilutions compared to the baseline levels. Although the background contribution of the extract was at least in part normalised by the controls used, it is not clear if these controls were sufficient. Oxidative stress induced by *t*-BHP effectively reduced cell viability in both MTT and LDH-release assays comparable to the positive control (Triton X-100). Co-incubation with *Rosa roxburghii* extract significantly protected cell viability against t-BHP in a dose-dependant manner at from dilution 0.04% and higher. At the highest *Rosa roxburghii* extract concentrations a complete protection against the effect of t-BHP occurred after 3 or 24 hours pre-incubation with *Rosa roxburghii* extract.

3.3   Single cell gel electrophoresis (Comet assay)

Primary rat hepatocytes showed a baseline DNA damage with approximately 18% of cells falling into in classes 4-5, which indicates major DNA damage to exist (Figure 2A).   The co-incubation of *Rosa roxburghii* extract reduced the percentage of cells in these two classes from 11% at 0.008% (v/v) *Rosa roxburghii* extract to almost non-existent at higher concentrations.   With the inclusion of t-BHP (Figure 2C) the extent of DNA damage is clearly higher compared to the baseline with 15% of cells falling into class 5 and a notable decrease in class I (undamaged cells). With co-incubation of 0.008% (v/v) *Rosa roxburghii* extract the percentage of class 5 cells were much less and at higher concentrations the effect of t-BHP-induced damage were not detectable. From Figure 2B and figure 2D an indication of time-dependant DNA repair can be clearly noted. With the inclusion of t-BHP the DNA tail% decreased notably over the 30 min repair period (Figure 2B). With the inclusion of 0.2% *Rosa roxburghii*

extract the number of damaged cells clearly were less over this time period which indicates an enhancement of DNA repair (Figure 2D).

3.4   *Antioxidant status and GSH/GSSG ratio*

The total hydrophilic antioxidant capacity of the *Rosa roxburghii* extract used is higher (115.3 mM trolox equivalents) than the lipophilic antioxidants (1.351 mM) as measured by the ORAC method. The total antioxidant capacity of the *Rosa roxburghii* extract that was used for dilutions in this study is 1154.1 mM Trolox equivalents (water soluble ORAC analysis). Cellular antioxidant capacity also increased dose-dependent when incubated with *Rosa roxburghii* extract dilutions up to 0.08% in primary rat hepatocytes (Table 2). The 0.32% concentration however was lower than the 0.08% concentration which may indicate a maximum antioxidant capacity is reached at approximately 0.08%.

It was surprising to note that the glutathione ratio decreased dose-dependently from 27 to 9 with an increase in *Rosa roxburghii* extract content in the media. This is contrary to what was found in the two other cell lines that was also investigated previously. In Chang liver cells (hepatocellular carcinoma) the ratio increased dramatically from 108 to 1149 when incubated with *Rosa roxburghii* extract at dilutions ranging from 0.04 to 2 % (v/v) (Table 3). Similarly, a 0.8% *Rosa roxburghii* extract also increases the GSH/GSSG ratio in MT-2 lymphoblast from a baseline ratio of 62 to 440 (results not shown). Total glutathione levels remains constant in all of these analyses suggesting only changes in GSSG state levels to occur.

4.   Discussion

This study made use of the primary cultures of a hepatocyte system, which is widely used in the literature to assess the metabolism of xenobiotics (Mckay *et al.*, 2001). This *in vitro* system is mainly used to explore possible toxic effects of

a *Rosa roxburghii* plant extract that is commercially available. This extract contains various natural occurring antioxidants, notably ascorbic acid, which is reflected in its relatively high antioxidant capacity (in the concentrated form that we used) of 1154mM trolox equivalents as compared to other commonly known fruit juices which has capacities of between 3 and 30mM trolox equivalents (Prior, 2003). The putative protective effect of *Rosa roxburghii* extract against oxidative stress induced cytoxicity was investigated as well.

Mutagenicity was monitored using the Ames test. Results obtained with TA 98 for frameshift mutations, and TA 100 for base-pair substituents, show that *Rosa roxburghii* extract is not mutagenic for this two strains as well as for *Salmonella* TA 102 strain. In general *Rosa roxburghii* extract showed a dose response protection against metabolically activated carcinogens, 2-AAF and AFB_1. In strain TA 102 the *hisG428* mutation is an ochre mutation, TAA, in the *hisG* gene can be reverted by both transitions and transversions and by mutagens that cause oxidative damage (Mortelmans and Zeiger, 2000). In the present study protection against the direct acting mutagen MMS was found at 1:2 dillution. Similar weak protection against direct acting mutagens such as MMS was also found in herbal products high in polyphenols, such as herbal teas (Marnewick *et al.*, 2000; Yamade and Tomita, 1994). The lack of in vivo antioxidative enzymes, necessary for the protection against oxidation could explain these effects Marnewick *et al.* (2000), also reported that some of the polyphenols are relatively polar, which will associate with the polar constituents in the medium whilst the non-polar mutagen MMS, is more likely to associate with the bacterial cellular matrix. This could partially explain the less effective action of *Rosa roxburghii* extract at low concentration against the MMS, while in at higher concentration of *Rosa roxburghii* extract the possibility of interaction increased.

Rosa roxburghii extract did not show any cytotoxicity or genotoxicity even at levels far higher than is needed for effective scavenging of free radicals in. The results also indicate that components in the extract are not metabolised to toxic

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metabolites in hepatocytes. If the background controls in these assays are sufficient to eliminate any contribution of the extract to the results, cell viability of primary hepatocytes increases substantially in a dose dependant manner. This putative increase in cell viability may be explained partially by protection against the cellular stress due to re-perfusion, including oxidative stress, occurring after isolation (Ferraris et al., 2002 and Richert et al., 2001). It may also be due to an enhanced cellular metabolic activity or defence mechanism/s. Similar results were found by us when Chang liver cells (hepatocellular carcinoma) were investigated in the same way (results not shown). Genotoxicity experiments also clearly indicated that Rosa roxburghii extract protects primary hepatocyte against cellular DNA damage and t-BHP induced oxidative DNA damage. This phenomenon has previously been described for several natural plant products with diverse nutritional content (Mayne, 2003; Duthie et al., 1996; Pool-Zobel et al., 1997).

The antioxidant potential of Rosa roxburghii extract was further underlined with the significant increase in total antioxidant capacity in primary hepatocytes after pre-treatment with Rosa roxburghii extract, and the increased GSH/GSSG ratio in Chang cells and MT-2 lymphoblasts. Although the same increase in GSH/GSSG ratio was not found in primary hepatocytes, the enzymology of primary hepatocytes, including glutathione reductase (Richert et al., 2001), catalase (Wang et al., 1998) and cytochrome P450 (Richert et al., 2001; Fahrig et al., 1998) are significantly affected during and after perfusion and isolation. This suggests that primary hepatocytes are not an ideal cellular system to investigate the contribution of a putative modulator to GSH metabolism and redox fluxes in vitro. However, an increase in GSH/GSSG ratio was recently also observed in a human controlled-feeding supplement study using Rosa roxburghii extract (presented for publication). Modulation of Glutathione redox state is known to occur in elevated antioxidant status (Griffith, 1999) which is probably due to a protective effect of the reduced form of glutathione during oxidative stress rather than an increase in the GSH pool.

This study indicates that the use of *Rosa roxburghii* fruit extract is anti-mutagenic, anti-cytotoxic and anti-genotoxic in liver cells and supports previously described beneficial properties to cellular oxidative stress. Limited information is still available on certain components in this extract, i.e. flavonoids, which may direct future studies. The question remains if these beneficial properties are due to a selection of components in the extract or a unique and effective combination of components, including antioxidants, that work synergistically.

7. Acknowledgements

8. References

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Tables and Figures

Table 1   Mutagenicity and antimutagenicity (Ames) assay

Histidine revertants per plate

Strain	TA98		TA100		TA102	
Mutagen	none	+2-AAF (10ng)	none	+AFB (20ng)	none	+MMS (20mM)
Control (-)	25 ± 7	27 ± 4	91 ± 7	123 ± 5	154 ± 10	189 ± 9
Control (+)	Nd	626 ± 128	Nd	333 ± 42	Nd	750 ± 16
RR (2%)	29 ± 7	382 ± 27*	Nd	Nd	Nd	Nd
RR (10%)	34 ± 10	108 ± 32*	86 ± 12	101± 24*	169 ± 8	740 ± 76
RR (20%)	34 ± 4	80 ± 21*	88 ± 9	68 ± 16*	193 ± 14	732 ± 32
RR (50%)**	42 ± 9	27 ± 7*	81 ± 12	54 ± 28*	176 ± 5	608 ± 48*

Nd= Not determine; RR= Rosa Roxburghii
*Indicates significant differences between positive mutagenic control and RR dilution tested ($p <$ 0.05). n = 3. **Dillution of orginal extract 2:1 (v/v) with H_2O.

Table 2   Glutathione redox state and antioxidant capacity analysis in primary rat hepatocytes

| Rosa Roxburghii (%, v/v)	GSHt (nmol/mg)	GSH (nmol/mg)	GSSG (nmol/mg)	GSH/GSSG	Antioxidant capacity (µM)
0	0.0172	0.016	0.0006	26.67	1081
0.005	0.0142	0.013	0.0006	21.67	1454
0.02	0.0204	0.018	0.0012	15.00	1759
0.08	0.013	0.012	0.0009	13.33	2354
0.32	0.011	0.009	0.0010	9.00	1385

n = 2, std < 10% of mean

## Table 3   Glutathione redox state analysis in Chang liver cells

Rosa Roxburghii (%, v/v)	GSHt (nmol/mg)	GSH (nmol/mg)	GSSG (nmol/mg)	GSH/GSSG
0	7.5074	7.371	0.0682	108
0.04	6.9284	6.833	0.0477	143
0.08	6.9972	6.939	0.0291	238
1.2	6.3304	6.291	0.0197	319
2.0	6.5584	6.547	0.0057	1149

n = 2, std < 10% of mean
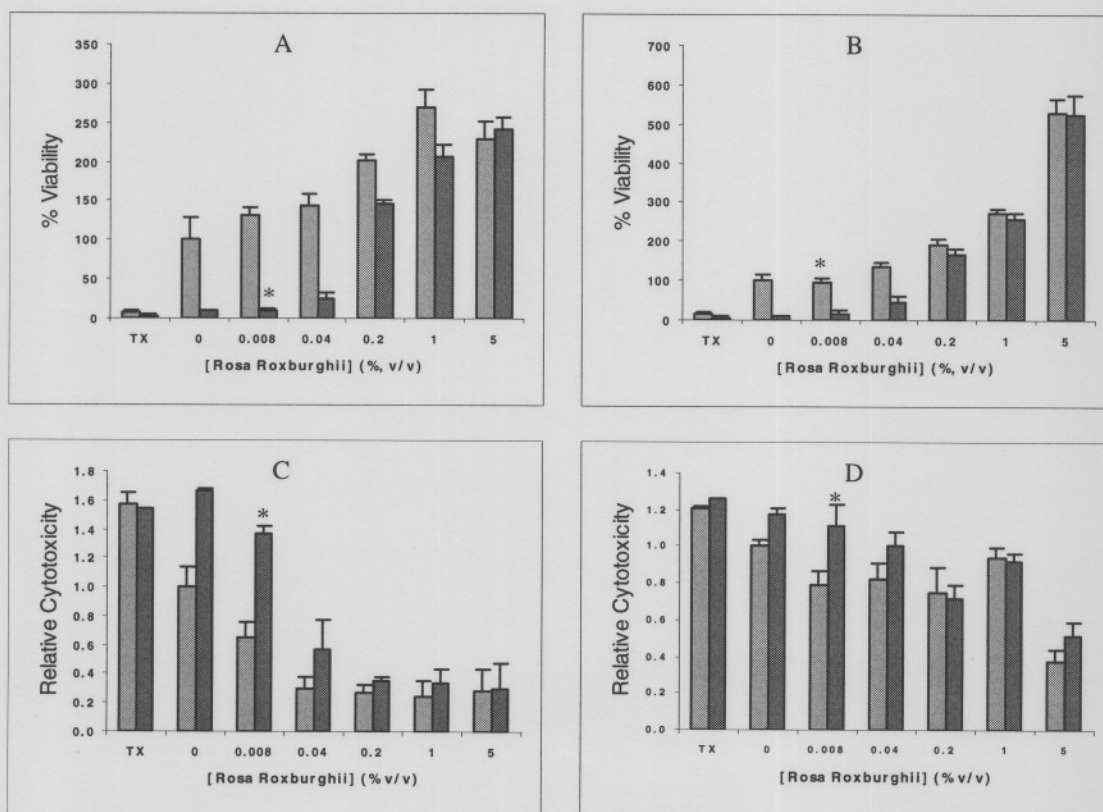
Figure 1   Cytotoxicity analyses

MTT (A and B) and LDH-release (C and D) assays were performed after 3 hours (A and C) or 24 hours (B and D) incubation with different RR dilutions. Analyses were performed in triplicate and the error bars indicate standard deviation of the mean. The dark bars indicate results where an additional two-hour incubation with 0.8 mM t-BHP was included. Triton X-100 (TX) was used as the positive control. Changes from baseline values (0 %) were all significant (p < 0.05) except as indicated by the asterisk. T= *t*-BHP
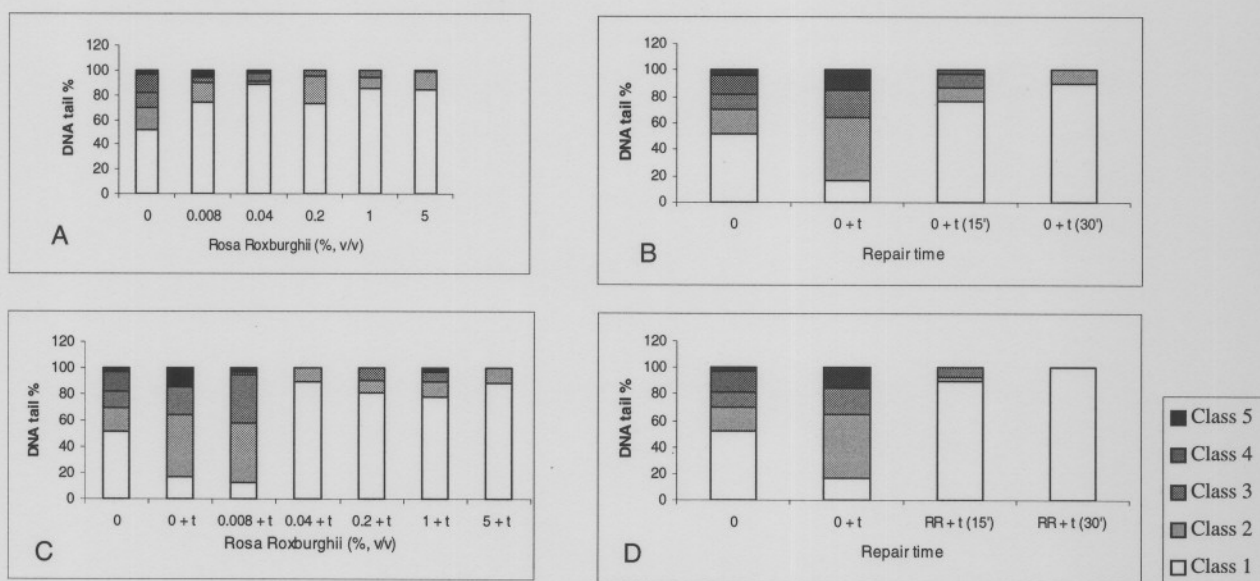
Figure 2 Single cell gel electrophoresis (Comet assay)

Single and double strand DNA breaks induced by different concentrations of RR was estimated in the absence (A) or presence (C) of 0.8 mM t-BHP. The repair time without RR after t-BHP-induced DNA damage was also compared over 15 and 30 minutes (B) and the repair time in the presence of 0.2% RR (V/v) over 15 and 30 minutes (D). The extent of DNA damage was classified as the DNA tail % damage in the following way: class 1, <6%; class 2, 6-17%; class 3, 17.1-34%; class 4, 34.1-60%, class 5 > 60%

CHAPTER 3

Increases in human plasma antioxidant capacity after supplementation with *Rosa roxburghii* in a controlled feeding study

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Abstract

Rosa roxburghii is a wild plant that grows in South-Western China and has been used for centuries in this region as a medicinal remedy against various diseases. The high amount of antioxidant components and antioxidant potential may contribute to the beneficial effects of this plant. The objective of this study was to investigate whether supplementation with *Rosa roxburghii* extract would significantly alter the antioxidant capacity of treated subjects. Thirty-six young, healthy, non-smoking individuals of ages between 21-27 years were recruited from the university campus area to participate in this randomised placebo-controlled single-blind trial. All participants resided at their own homes but received all meals and beverages on a specific location throughout the five-week study period. During the first two weeks of the study no supplements were given. This period was followed by an intervention period of 21 days where the subjects continued to receive the same three daily meals and 4 capsules with every meal that contained either evaporated *Rosa roxburghii* extract or the placebo. The change in plasma antioxidant capacity of the intervention significantly increased after *Rosa roxburghii* supplementation compared to the placebo group (p=0.02). Glutathione redox status ratio (GSH/GSSG) of the intervention group increased significantly compared to the placebo group (p=0.03). No significant changes in superoxide dismutase activity were found. The results obtained suggest that the

high content of antioxidants in *Rosa roxburghii* extract can increase the plasma antioxidant capacity in humans.

Introduction

Rosa roxburghii is a wild plant of the *Rosa* genus that grows in the mountain plateau areas of South-western China. Extract from its fruit have traditionally been used for medicinal purposes and are believed to have a protective effect against various diseases, including arteriosclerosis, cancer, and immunity stress [1]. The fruit extract is commercially available under the trademark, Cili Bao® at Cilli health SA. The beneficial effects of this plant extract have been associated with antioxidant components and a subsequent increase in the antioxidant status in erythrocytes of healthy old participants [2,3]. Depending on the process of extraction an extract of the fruit contains, amongst other, vitamins C (1.3 mg/ml), E (0.81 µg/ml), B1 (0.64mg/ml), superoxide dismutase (SOD) (5174 U), zinc (15.5 mol/l) and calcium (865.3 mol/l) [1]. It has also been suggested that its relatively high SOD content contributes highly towards its beneficial properties [4,5].

A putative beneficial effect of *Rosa roxburghii* extract on the prevention and treatment of diseases may not be due exclusively to the action of single antioxidants, such as vitamin C. It may also result from the action of lesser known compounds or from a concerted action of a combination of different antioxidants present in the juice. The synergistic effect of antioxidants is well known, therefore, combinations of antioxidants may be more effective than larger quantities of a single antioxidant [6].

Although previous investigations indicated that diet supplementation with *Rosa roxburghii* leads to an increase of superoxide dismutase, catalase and reduced glutathione in blood [3]. These investigations were not carried out under controlled feeding conditions and similar studies done on Western populations

could not be found. As antioxidants are commonly found in the diet it is essential to support these claims in a controlled feeding study. We investigated the effect of supplementation with *Rosa roxburghii* extract on selected markers for antioxidant status on presumed healthy individuals in a randomised placebo-controlled, controlled-feeding study. Although oxidative stress-related parameters are not expected to vary markedly in healthy individuals, it has been shown that an increase of antioxidant status can be expected with a higher consumption of fruit and vegetables [7,8].

Methods and materials

Study design and subjects

The study was a randomised, paired, placebo-controlled, single-blind parallel study. The study was conducted only after informed written consent from participants and ethical approval from the Ethics Committee of the Potchefstroom University for CHE (project 02M06) were obtained. Thirty-six volunteers aged between 21-27 years were recruited from the university campus area. Equal numbers of men and women were recruited. All study participants were in good health as determined by a medical history questionnaire and physical examination. The exclusion criteria were the following: (1) a history of cardiovascular, hepatic, gastrointestinal or renal disease, (2) frequent alcohol use, (3) use of antibiotics or any dietary supplements within 4 weeks prior to the start of the study, and (4) smokers or excessively physically active volunteers. Women were specifically excluded from the study if they were pregnant, lactating, using exogenous hormones, or had a history of menstrual irregularities.

Diet control

All participants resided at their own homes but received all meals at a specific location throughout the five-week study. The meals consisted of a typical combination of food that the participants usually eat and were recorded for nutritional value. For the first two weeks, participants followed the prescribed diet but no supplements were given. Based on age, gender, and body mass index (BMI) subjects were paired and then randomly allocated to either an intervention or a placebo group. The BMI values were 22.7 ± 2.7 kg/m^2 and 22.8 ± 3.0 kg/m^2 for the placebo and intervention groups, respectively. The first two weeks was followed by an intervention period of 21 days where the subjects continued to receive the same three daily meals and 4 capsules with every meal that contained either evaporated *Rosa roxburghii* extract or the placebo. The subjects had to take the capsules in the presence of the researchers ensuring 100% compliance. The *Rosa roxburghii* capsules represented a total of 24 ml of the unevaporated supplement that has an antioxidant capacity of 120 mM Trolox equivalents.

The subjects were restricted from using antioxidant supplements and alcoholic beverages during the intervention period. When averaged over a 3 day diet, the percentages of energy from protein, fat, and carbohydrate were 15%, 39%, and 45%, respectively (see Table 1). Participants maintained their usual daily activities and recorded any additional food intake or unusual physical activity, as well as illnesses and medication inevitably used during the study. Dietary intakes throughout the intervention period were estimated by calculating the nutrient content according to the menus served to the participants, by using Food Finder (MRC, MedTech, Cape Town, South Africa) dietary analysis software. Compliance to the diet was monitored by record keeping of meals attended, medicine taken, and food eaten in between meals.

Blood and urine samples

Fasting blood and urine samples were taken before and after the 21-day intervention period. Venous blood samples were collected with minimal stasis between 07:00 and 10:00 to avoid the effect of diurnal variation. Fasting urine samples was taken and stored at -20°C until analysis.

Antioxidant capacity

Plasma antioxidant capacity was determined by the oxygen radical absorbance capacity (ORAC) assay using plasma prepared from heparinised blood [9]. Plasma samples were de-proteinised with 0.5 M perchloric acid (1:1, v/v) when samples were taken and stored at –80°C until analysis. The ORAC assay was carried out on a BioTEK fluorescence plate reader, with excitation wavelength at 530nm and emission wavelength at 590nm as described previously [9]. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 240 mM) was used as a peroxyl radical generator in the reaction. Trolox was used as a reference standard and the total plasma antioxidant capacity was expressed as µM trolox equivalents.

Blood glutathione and SOD

The redox state of glutathione was measured in whole blood using the spectrophotometric GSH/GSSG ratio assay (GSH/GSSG-412™) from OXIS Research (Portland, Oregon). Erythrocyte SOD activity was determined by monitoring the auto-oxidation of pyrogallol as described before [10]. Enzyme activity was expressed as units per milligram of haemoglobin.

Blood histology

Total blood counts in EDTA-blood were measured using a Coulter counter (Beckman, A^c.T^{TM} 5diff Cap Pierce Hematology Analyzer) according to manufacturers' instructions.

8-hydroxy-deoxyguanosine (8-OH-dG)

DNA oxidation was estimated by measurement of 8-OH-dG in fasting urine samples using the immunological detection assay (8-OHdG-EIA™) from OXIS Research.

Statistical analysis

Data before and after the 21-day intervention period was analysed using the Statistica version 6 program. Data were checked for outliers and normality assessed using the Shapiro Wilk's W test. To evaluate changes before and after the intervention period the Wilcoxon matched pair tests (non-parametric) were used as indicated on the graphs. For evaluating the net changes from before to after the intervention period between the placebo and intervention groups, the Mann Whitney U test (non-parametric) were used. In both cases $p < 0.05$ was considered to be statistically significant.

Results

Diet and compliance

The macronutrient and average antioxidant intake by study participants during the study period are shown in Table 1.

Table 1: Daily consumption of macronutrients and antioxidants.

Macro/micronutrients	Mean	Maximum	Minimum
Total energy (kJ)	11546 ±1446	13109	8168
Proteins (g)	103.8 ± 21.5	131.5	73.6
Fats (g)	118.6 ± 23.3	151.5	72.8
Carbohydrates (g)	307.0 ± 47.0	410.4	242.9
Vitamin A (RE)	3487.4 ± 2367	6726	610
Vitamin C (mg)	140.5 ± 114	347	41
Vitamin E (TE)	14 ± 5	23.39	4.26

The compliance of the two groups was similar with respect to adhering to the prescribed diet. Many of the participants developed flu during the study and took medication, albeit for a short period. The medications used contained substances such as paracetamol, aspirin and vitamin C that may have effected the antioxidant status participant. Of these participants, 9 were in the placebo group and 5 in the intervention group. however these participants were not excluded from the study. Wine, fruits, vegetables and fruit juices were consumed by certain subjects between meals, although it was infrequent and in small quantities. For example, red wine consumption for the placebo group was 0.7 portions/person/week and for the intervention group 0.73 portions/person/week. The consumption of fruits, vegetables and fruit juices for the placebo group was 0.6 portions/person/week and for the intervention group 0.53 portions/person/week. Three subjects, two from the placebo and one from the intervention group were excluded from the results due to compliance concerns.

Antioxidant capacity analysis

The average baseline fasting deproteinated plasma antioxidant capacity were 1164 µM trolox units for the placebo group and 978 µM trolox units for the intervention group which is only slightly lower than values previously reported for healthy individuals (1200–1300 µM) using the same method [11]. The value for the control group remained similar after 21 days of the intervention at 1146 µM

but the value for the intervention group increased significantly (p = 0.036) to an average of 1091 μM trolox units after day 21 (see Figure 1A). This change was also significant when compared to the net change in the placebo group over the three week intervention period (p = 0.02).

Glutathione redox state and SOD

Reduced glutathione (GSH) levels in the placebo group remained relatively constant over the intervention period with an average of $1108 \pm 234\mu M$ to $1062 \pm 237\mu M$. In the intervention group a slight, albeit significant compared to the placebo group ($p = 0.03$, Mann-Whitney U test), increase of 1038 ± 215 μM to $1066 \pm 222\mu M$ was observed (Figure 1B). This was accompanied by a slight decrease of the oxidised form (GSSG) in the intervention group with a change of $2.3 \pm 1.0\mu M$ to $1.6 \pm 0.7\mu M$ over the intervention period. The resulting glutathione redox ratio (GSH/GSSG) in die intervention group changed significantly compared to the placebo group from 525 ± 232 to 712 ± 263 ($p = 0.027$, Mann-Whitney U test). In the placebo group this shift was not observed with GSSG levels of 2.7 ± 1.1 μM to 2.4 ± 0.9 μM and GSH/GSSG ratios of 483 ± 280 and 492 ± 185, respectively.

No significant changes were detected in erythrocyte SOD activity over the intervention period (Figure 1C). In the placebo group average activities were 1.18 ± 0.11 and 1.16 ± 0.1 U/mg Hg before and after intervention and in the intervention group the activities were 1.19 ± 0.12 and 1.18 ± 0.1 U/mg Hg over the same period.

Blood histology

No significant changes in any of the blood cell parameters occurred at any time during the study (Results not shown).

8-OH-dG

8-OH-dG levels in urine varied greatly in both groups. It is unclear why these values varied so much. No significant changes could, be detected and no correlation could be made with any biochemical of physical parameter.

Discussion

Aerobic life is characterized by a steady formation of pro-oxidants balanced by antioxidants. To maintain this balance, continuous supply of antioxidants is required, and if this is not met, oxidative damage occurs which contribute to the pathophysiology of various major pathological diseases [9,12]. However, cells have an extensive endogenous defence system against oxidative stress that consists of antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase and other small antioxidants, such as GSH, vitamin C and E [13]. Although cells are normally equipped with enough of these endogenous scavenging systems, a large amount of oxygen free radicals are generated under certain circumstances, for example cancer, which can overwhelm the endogenous antioxidant supply [14]. It is therefore commonly believed that exogenous consumption of antioxidants contributes to the cellular defence against oxidative stress and related diseases.

The fruit extract of the *Rosa roxburghii* plant is a product that has been used in certain areas in China for various diseases as a putative antioxidant contributor. The reason for this is the relatively high content vitamin C in the *Rosa roxburghii* extract which is the main contributor to its high antioxidant capacity (in the concentrated form that we used) of 1154 mM trolox equivalents as compared to other commonly known fruit juices which has capacities of between 3 and 30mM trolox equivalents (11). It also contains high levels of superoxide dismutase, although it is unlikely that this has any nutritional significance due to digestion and flavonoids [1]. All of these antioxidants are well known for their effective antioxidant characteristics [12,15,16]. However, the combination of these antioxidants may be more effective than that of individual antioxidants would

have been, due to the possible synergistic working of these compounds [15,17]. We have some evidence that this plant extract is not toxic and mutagenic *in vitro* (results presented for publication elsewhere). In a pilot study as well as this study no apparent clinical and biochemical side effects were observed. We have conducted this supplement study that provides evidence that, in a controlled feeding design, *Rosa roxburghii* extract indeed enhances antioxidant status in healthy individuals.

The increase in the GSH/GSSG ratio of the participants in the intervention group, and particularly the increase in GSH, indicates that the consumption of *Rosa roxburghii* extract may have contributed to this increase in the enzymatic antioxidant capacity of these participants. According to Griffith (1999), GSH synthesis is regulated by at least three factors: level of γ-GCH (L-γ-glutamyl-L-cysteine synthetase), the availability of its substrates such as L-cysteine and, feedback inhibition of GSH on γ-GCH. In a previous study done in vitro (unpublished data), an increased in γ-GCS was not observed. GSSG is further reduced to GSH by GSSG reductase in a NADPH-dependent reaction. However, GSSG reductase normally maintains the total glutatione pool in a predominant reduced state, thus, redox cycling between GSSG and GSH does not usually have a major influence on cellular GSH levels, except in extreme levels of oxidative stress [18]. *Rosa roxburghii* extract a poor source of amino acids, has negligible levels of L-cysteine (0.01417 mg/ml). Another possible explanation is that *Rosa roxburghii* extract could have a sparing effect on amino acids, and contribute towards energy production. In addition to its constituent amino acids, GSH synthesis requires ATP, and thus could also have a limiting effect [19]. The increase in the overall enzymatic antioxidant status after the consumption of *Rosa roxburghii* extract may decrease the net loss of GSH through oxidation.

All the participants in the placebo and intervention group followed the same diet during the test period, although there were minor deviations from the prescribed diet as discussed earlier. The increase in antioxidant capacity of the intervention

group can be attributed to the supplementation of *Rosa roxburghii* extract as plasma samples were deproteinated and uric acid levels, at least in urine, were decreased and is unlikely to contribute to the increased antioxidant capacity.

In conclusion, this study underlines the previously suggested potential of this plant extract as a natural and safe antioxidant supplement. However, the exact antioxidant mechanisms of *Rosa roxburghii* extract still needs to be defined.

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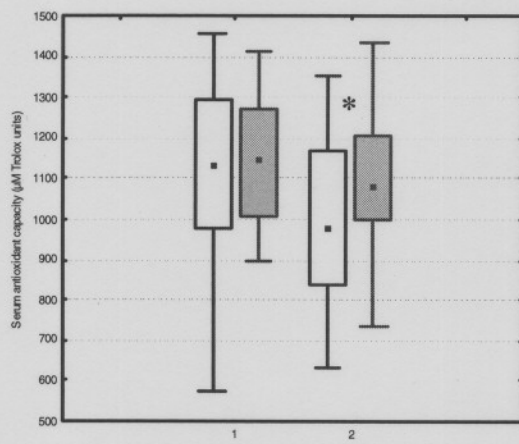
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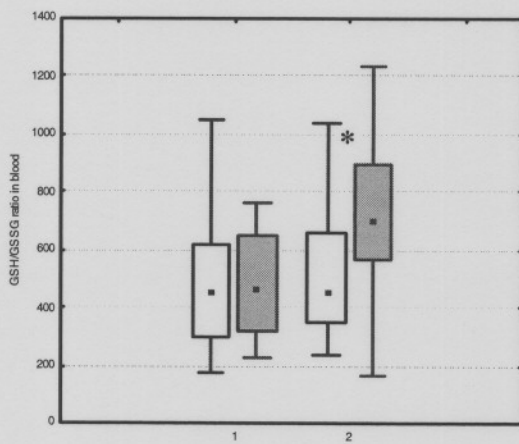
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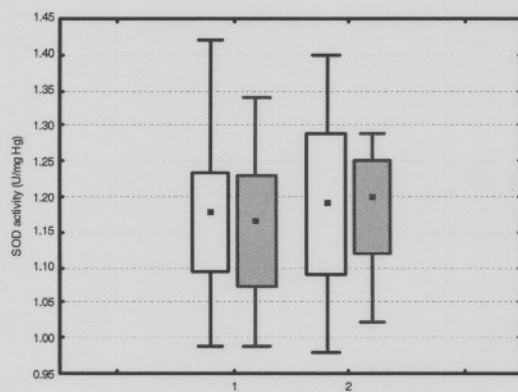
Figure 1   Antioxidant status parameters in blood

Antioxidant capacity (A), glutathione redox state (B), and SOD activity (C) were measured in serum, whole blood and erythrocytes, respectively, of placebo (1) and test (2) groups at the start (open boxes) and end (striped boxes) of the 21 day supplement period. Boxes indicate 25 – 75 percentiles with points indicating median values. Whiskers indicate minimum and maximum outliers. The asterisk indicates statistical significant change between day 1 and day 21 (p = 0.0036, Wilcoxon matched pairs test).

CHAPTER 4   GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1   Introduction

In recent years there has been a remarkable increase in scientific publications dealing with oxidative stress which include: the metabolism of free radicals, reactive oxygen species and nitrogen species (Urbanavicius, 2003; Halliwell and Gutteridge, 1984; Cheeseman and Slater, 1993). Evidence linking chronic diseases and oxidative stress (Guyton and Kensler, 1993; Kruidenier and Verspaget, 2002; Singal et al, 2000). Supplementation of single vitamins or minerals to increase the antioxidant defence system (Cheng, 2001; Gaziono, 1999; Kritharides and Stocker, 2002); the putative high beneficial effects of diets containing fruit, and vegetables which could at least partially explained by their high   antioxidant content (Cao et al., 1998 and Pérez et al., 2002). The perception that natural remedies are somehow safer and more efficacious than pharmaceutically remedies increase the interest of the public in natural therapies (Elvin-Lewis, 2000). The need to make health care affordable for all emphasises the necessity to evaluate these products ensuring their safety and efficacy, as well as the characterising of the active parts for further pharmaceutical research and drug development (WHO, 1998).

The liver is the major target organ of oxidative stress induced by xenobiotics and is widely used in pharmacological, toxicology and metabolic studies (Berry et al., 1991 and Mckay et al., 2001). Therefore the use of primary rat hepatocytes were the most appropriate in vitro system to not only evaluate the toxicity but also the antioxidant potential of Rosa roxburghii extract.

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4.2. Motivation for study

Antioxidants play a part in the therapy of various diseases such as diabetus (Atalay & Laaksonen, 2002), bowel diseases (Kruidenier & Verspaget, 2002) and HIV (Jaruga *et al.*, 2002) and also in the possible prevention of diseases such as cancer (Lee, 1999), cardiovascular disease (Gaziano, 1999) and neurodegenerative diseases (Sauer *et al.*, 2001). The deeper understanding into the mechanisms involved in mitochondria-related diseases, such as the role of reactive oxygen species, even more emphasise the role of antioxidants (Chinnery & Turnbull, 2001). Our division has been investigating and assisting the diagnosis of mitochondrial disease, i.e. disorders of mitochondrial bio-energetics. The main therapeutic approach to this complex group of diseases has been the enhancement of the defence against radical-induced damage and related processes such as cellular necrosis and apoptosis. This underlies our interest in antioxidants, including "natural antioxidant products" such as *Rosa roxburghii* extract, which came to our attention via the interest displayed by Mr G Joubert from Cili Health, SA.

Rosa roxburghii is a plant extract from the *Rose* genus which is used as a traditional medicine against diseases such as atherosclerosis, cancer and immunity stress (Zhang *et al.*, 2001). The high content of antioxidants *in Rosa roxburghii* extract could be partially contributed towards the health benefits of *Rosa roxburghii*. The objective of this study was firstly, to investigate the toxicity of *Rosa roxburghii* extract *in vitro*, in primary rat hepatocyte cultures as well as its mutagenicity as assessed by the Ames test, and secondly, to evaluate the antioxidant potential of *Rosa roxburghii* extract *in vitro* and *in vivo*. Similar studies may have been performed before by researchers in China, but since no data exists in published English scientific literature these objectives, in our view, needed to be revisited.

4.3.   Summary of the main findings and recommendations

4.3.1. The antimutagenicity and -genotoxicity of *Rosa roxburghii* extract

The Ames *Salmonella/*microsome mutagenicity assay (Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that lead to gene mutations, for example TA 98 detects frameshift mutations, TA 100 detects base-pair substitution mutations and TA 102 detects transition/transversion mutations. This strain also detects mutagens that cause oxidative damage (Mortelmans and Zeiger,   2000). Mutagenic chemicals are also capable of inducing cancer. Antimutagens and antioxidants (phenolic compounds, catechin, ascorbic acid and β-carotene), frequently found in plants, protect against mutagens and complement DNA repair systems (González-Avila *et al.*, 2002). However, some plants have toxic activities that express cytotoxic and genotoxic activities (Yen *et al.*, 2001). It is therefor necessary to test medicinal plants for potential toxicity. *Rosa roxburghii* extract showed no mutagenicity in the *Salmonella* (AMES) test against TA 98, TA 100 and TA 102. Our results indicated, in fact, that *Rosa roxburghii* extract has an antimutagenic effect against the metabolic activating mutagene 2-AAF and AFB_1. Protection against the direct acting mutagen MMS was found at 50% concentration, however similar protection was not found at lower concentrations. The lack of *in vivo* enzymes that contribute towards antioxidant defence systems against oxidative mutagenes, may lower the effect of *Rosa roxburghii* extract against MMS. It is also possible that the protective role of *Rosa roxburghii* extract does not exclusively relies on the antioxidants present in the extract. Further experiments are needed to evaluate the minimum *Rosa roxburghii* extract concentration necessary for antimutagenic protection against AFB_1, as a 10% concentration of *Rosa roxburghii* extract was still effective against this mutagene. For the further evaluation of the antimutagenicity effect of *Rosa roxburghii* extract other mutagens could be included such as the oxidative mutagens, cumolhydroperoxide (CHP) and hydrogen peroxide (H_2O_2). The use of double layer technique described by Glatt and Oesch (1977), with respect to the

mutagens that required metabolic activation could provide more insight into the possible mechanisms involved in the antimutagenic effects against these mutagens.

4.3.2. Genotoxicity

Although there is a strong correlation between mutagenicity and carcinogenicity, some substances found to be non-mutagenic are carcinogenic, such as polychlorinated pesticides (Maron and Ames, 1982; Mortelmans and Zeiger, 2000). To further evaluate the genotoxicity of *Rosa roxburghii* extract, the single gel electrophoresis assay (Comet assay) was performed. From the results, it was possible to deduce that *Rosa roxburghii* extract is not genotoxic and even increase the oxidative stress (*t*-BHP)-induced DNA damage repair in a time dependent manner. *Rosa roxburghii* extract also prevented DNA damage due to oxidative stress or "baseline" damage induced by the processing of the cells during perfusion and cultivation. Although the protective effect of *Rosa roxburghii* extract could take place extracellular, by preventing *t*-BHP entering the cells, *t*-BHP is metabolised into free radical intermediates by cytochrome P-450 in hepatocytes. It is this more likely that the protection takes place intracellular. To support this, further studies could be done where the *Rosa roxburghii* extract is removed after pre-incubation and before oxidative stress is induced with *t*-BHP. Evaluation of the specific repair mechanism could be extended, for example by using specific endonucleases in the Comet assay to give an indication of the type of damage repaired.

4.3.3. The cytotoxicity of *Rosa roxburghii* extract

The cytotoxicity analysis with the MTT test and LDH-leakage assays supports the antitoxicity and protective role of *Rosa roxburghii* extract against oxidative cell damage, as induced by *t*-BHP, over a three-hour and 24 hour period. This supports the mutagenicity test in the presence of S9 and also that components in

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the extract are not metabolised to toxic compounds. An unexpected finding was that cell viability of primary hepatocytes actually increased substantially in a dose dependant manner. This increase in the measured cell viability% may be explained partially by protection against the cellular stress, including oxidative stress occurring after isolation of the primary hepatocytes due to reperfusion (Ferraris et al., 2002 and Richert et al., 2001).

We mainly used the primary culture hepatocyte in vitro system to explore possible toxic effects of a Rosa roxburghii fruit extract that is commercially available. In comparative studies with other antioxidants the concentrated fruit extract of Rosa roxburghii extract contains a remarkably high antioxidant capacity at approximately 1154 mM TE, compared to commonly used fruit juices which ranges between 32.7 mM (blueberry) and 4.3 mM (apple) TE (Prior et al., 2003). It was therefore decided to investigate, in parallel, its protective effects against oxidative stress induced toxicity. The present study showed that Rosa roxburghii extract had a highly effective protective effect in primary hepatocytes against t-BHP.

4.3.4 Antioxidant capacity

The antioxidant potential of Rosa roxburghii extract was further underlined with the significant increase in total antioxidant capacity in primary hepatocytes after pre-treatment with Rosa roxburghii extract, and the increased GSH/GSSG ratio in Chang cells and MT-2 lymphoblasts. The same increase in GSH/GSSG ratio was however not found in primary hepatocytes. Literature indicates that the enzymology of primary hepatocytes which includes glutathione reductase (Richert et al., 2001), catalase (Wang et al., 1998) and cytochrome P450 (Richert et al., 2001; Fahrig et al., 1997) are significantly changed during and after perfusion. This suggests that primary hepatocytes are not an ideal cellular system to investigate GSH metabolism and redox fluxes in vitro.

4.3.5 Enhancement of antioxidant status in the supplementation study

Previous investigations (Ma *et al.*, 1997) indicated that diet supplementation with *Rosa roxburghii* extract leads to an increase of antioxidant capacity in blood. However, these investigations were not carried out under controlled-feeding conditions. Antioxidant enzymes act through complementary mechanisms, such as scavenging of reactive oxygen/nitrogen species or their precursors, inhibition of ROS formation by metal-chelating and activation of detoxifying/defensive proteins of endogenous enzymes (Roig *et al.*, 2002 and Gilgun-Sherki, 2000). These mechanisms and antioxidants commonly found in the diet protect the cell against oxidative stress and it is essential to support these claims in a controlled-feeding study *in vivo*. We investigated the effect of supplementation with *Rosa roxburghii* extract on selected markers for antioxidant status on presumed healthy individuals in a placebo-controlled, controlled-diet study. Although oxidative stress-related parameters are not expected to vary markedly in healthy individuals, it has been shown that an increase of antioxidant status can be expected with a higher consumption of fruit and vegetables (Lampe *et al.*, 1999 and Coa *et al.*, 1998). The *in vivo* increase in the antioxidant capacity and GSH/GSSG ratio of the intervention group compared to the placebo group over a 21-day intervention period, support the antioxidant protection found *in vitro*. The increase in GSH/GSSG ratio likely results from the overall antioxidant protection and not from the increase in GSH synthesis. There is strong epidemiological evidence regarding other small molecular antioxidants in prevention of diseases (Lee, 1999, Gilgun-Sherki *et* al., 2001 and Jacob *et al.*, 1995). *Rosa roxburghii* extract contains large amounts of these antioxidants, especially ascorbic acid, which we believe is the major contributor in *Rosa roxburghii* extract to prevention of oxidative stress, especially considering the *in vivo* and *in vitro* glutathione redox data, which ascorbic acid is known to modulate. However, the combination of these antioxidants may be more effective than that of individual antioxidants would have been, due to the synergistic working of these compounds (Steinbert and Chait; 1998 Hässig *et al.*, 1999). Furthermore, the chemical nature of an

antioxidant determines the location of cell defence, for example: Vitamin E, the fat-soluble antioxidant, is located in cell membranes and functions in the protection against lipid peroxidation (Sen, 2001). The overall combined effect of multi-nutrients in this cocktail seems to increase the antioxidant defence system and therefore contribute towards the protective role against reported diseases. Further studies, especially in the modulation of antioxidant enzymes and DNA repair enzymes are needed to clarify the specific protective role of *Rosa roxburghii* extract. Little information is still available on the breakdown of key components, i.e. phenols in this fruit extract, which may direct future studies.

4.3.6. Future studies and recommendations

- The studies showed that *Rosa roxburghii* extract contains potent antioxidant activities in both *in vitro* and *in vivo* systems. Further studies on isolating, identifying and characterising the active constituent(s) may provide useful leads in understanding the pharmacological effects of *Rosa roxburghii* extract.

- The Isolation, identifying and characterisation of possible polyphenols in *Rosa roxburghii* extract is necessary to understand the antioxidant working mechanism.

- Although studies showed effective protection against the oxidative stress inducer *t*-BHP, in both cytotoxic and genotoxic assays, further studies are needed to investigate the protective mechanism, e.g. the use of enzyme analysis.

- Investigation regarding the perfusion and cultivation effect on GSH status in primary rat hepatocytes is necessary to be preformed, to understand the decrease in the GSH/GSSG ratio in the presence of *Rosa roxburghii* extract.

- Further comparative studies with other antioxidants and antioxidant extracts are necessary.

- We experienced problems with Cili Health South Africa in providing us with crucial information regarding the processing of the plant extract and the exact micro- and macronutrient content of the plant extract we used in the studies. Above mentioned information is needed for publication purposes.

- Identification of micro- and macronutrient content of *Rosa roxburghii* extract.

- The characteristics of *Rosa roxburghii* extract make it a promising candidate for further studies designed to obtain more evidence on its protective effect against previously claimed health benefits such as atherosclerosis as well as mitochondrial related diseases.

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ADDENDUM A

The Journal of Nutritional Biochemistry

Guide for Authors

The editors of *The Journal of Nutritional Biochemistry* welcome the submission of original manuscripts on experimental and clinical nutrition as it interfaces with biochemistry, molecular biology, physiology, pharmacology and toxicology. The scope of the journal will encompass the broad area of *in vitro* and *in vivo* studies of mechanistic aspects of nutritional sciences. The chief criteria for acceptance of papers submitted for publication are originality, quality and clarity of the work reported. All contributions must be based on original, unpublished research and will be peer reviewed. All authors bear responsibility for ensuring the integrity and quality of their reported research. It is the author's responsibility to secure permission to use figures or tables that have been reported elsewhere.

Contributions may be classified as regular research articles, reviews, rapid communications or methodological papers. Most review articles will be by invitation from the editor. Authors interested in submitting a review should first contact the editorial office. Rapid publication of original manuscripts is a goal of the journal. Manuscripts must be written in English. Each manuscript is considered for publication with the understanding that it is not being submitted to any other journal. Upon acceptance for publication, papers are subject to editorial amendment.

Submission Guidelines

Provide a cover letter indicating the name, mailing address, telephone and fax numbers, as well as the e-mail address of the individual to whom correspondence and questions should be directed. The cover letter also must state that all listed authors have contributed to this work and have agreed to

submit the manuscript to this Journal, that no part of the work has been published before, except in abstract form, and that all human and animal studies have been reviewed by the appropriate ethics committees. The editor reserves the right to reject manuscripts that do not comply with the above-mentioned requirements.

- Submit an original and three copies of a complete manuscript, including references, tables, figures and figure legends. Electronic submission of the complete manuscript in Microsoft Word or Rich Text Format (PC or Mac format) must be included on a diskette with the initial submission.
- On the cover letter, provide names, mailing and e-mail addresses, telephone and fax numbers of three to five potential referees who would be suitable reviewers of your manuscript.
- Arrange the manuscript in the following order: title page, abstract, text, acknowledgements, references, footnotes, tables, figure legends and figures.
- Double-space the manuscript and type it on 8.5 X 11 inch (21.5 X 28 cm) white bond paper with 1-inch margins on all four sides.

Manuscript Format

Title page

- Give the first name, middle initial, and the last name of all authors.
- Provide the name and address of the corresponding author to whom reprint requests should be sent.
- List each author's institutional affiliation(s).
- Provide a running title of up to 50 characters.
- Acknowledge grants, sponsors, and funding sources.
- List up to six key words.

Abstract

- Provide an abstract of a single paragraph with up to 250 words summarizing the hypothesis tested, experimental design, results, and conclusions. Do not cite references and avoid abbreviations.

Text

- Start the text on a new page. Arrange the text into four parts: Introduction, Methods and Materials, Results, Discussion.

SI Units

- All laboratory data should be presented in SI units. See Young, DS. Implementation of SI units for clinical laboratory data. J Nutr Biochem 1990; 1: 599-633.

Illustrations

- It is the author's responsibility to provide original, camera-ready artwork for all illustrations.
- For line art submit original drawings or glossy black and white prints (5" X 7"). All labels should be professionally lettered in Helvetica or a comparable typeface. Typewritten labels will not be accepted.
- Figure legends should be typed, double spaced, and should appear at the end of the manuscript.
- To properly submit digital artwork, please see "Artwork Instructions" on http://authors.elsevier.com for details on image formats, sizing, naming conventions, preparation, and file delivery of your digital artwork. Digital artwork that does not conform to these instructions will be rejected and the hardcopy.

References

- References should be numbered sequentially in the order of their citation in the text, e.g., [1,2], and appear at the end of the main text. Style references as follows:

For journal articles:

Brown M, Evans M, McIntosh M. Linoleic acid partially restores the triglyceride content of conjugated linoleic acid-treated cultures of 3T3-L1 preadipocytes. J Nutr Biochem 2001,12:381-7.

- Letters to the Editor will be considered for publication at the discretion of the editor. Submission of a letter constitutes permission for publication. Letters are subject to editing and abridgement.

ADDENDUM B

FOOD AND CHEMICAL TOXICOLOGY

General Information

The Journal publishes papers that fulfil the views as laid down in the Aims and Scope section. The Journal's main purpose will be the publication of papers reporting and interpreting original unpublished toxicological research, particularly studies promoting an understanding of the mechanisms underlying toxic effects or improvements in methods for predicting adverse effects. Papers reporting the toxicological examination of specific chemicals or consumer products will be published, irrespective of the positive or negative nature of the results, provided the tests and reporting meet current standards of adequacy.

Manuscript Format

Manuscripts should be written in clear and concise English; incomprehensible manuscripts will be returned to the authors for rewriting. If submitting hard copies, manuscripts should be printed on *one side of the paper* only and *double spaced* throughout, with at least 2.5-cm margins. All pages must be numbered, including the *title page*, which should carry the title of the paper, the surnames and initials of the authors, the names and addresses of the institutions where the work was done (with the affiliation of each author clearly indicated), named corresponding author, and keywords. Titles consisting of declarative or interrogative sentences are not acceptable. In order to raise the standard of papers, and inherently of the Journal, scientific quality needs to be at a high level. Authors are encouraged to use electronic submission, providing contact details of at least three relevant putative reviewers. The Editors will not be bound to choose from these proposed peers. A signed covering letter should be sent to the Editor at the time of submission.

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Abstract -a self-contained summary of the objectives, results and significance of the study, not exceeding 200 words. Uninformative sentences such as "the significance of the results is discussed" are not acceptable. *Introduction* -a concise and clear statement on the background,purposes and significance of the work.

Materials and Methods -a detailed description of the experimental design and of any new or improved methods. Well established methods and techniques may be identified by reference only.

Results -presented concisely, with the aid of tables or figures where appropriate. Duplication between this section and the Discussion must be avoided.

Discussion -a succinct interpretation of the data. Extensive literature reviews and highly speculative comment are discouraged.

Acknowledgements -providing recognition of sources of funding and donations of materials, and including any thanks the authors may wish to accord for advisory, technical or other assistance, since authorship should be limited to those who have made a major contribution to the study and to the preparation of the paper. Authors are advised to obtain approval for the wording of any acknowledgement from those whose help is noted.

References and Footnotes -For *Food and Chemical Toxicology* the Harvard style system is to be used: author's names (no initials)and dates (and specific pages, only in the case of quotations) are given in the main body of the text,

e.g.(Philips, 1990, p. 40). References are listed alphabetically at the end of the paper, double spaced and conform to current journal style.

For Journals: Macintyre, S., MacIver, S., Sooman, A., 1993. Area, class and health: should we be focusing on places or people? Journal of Social Policy 22, 213-234.

Other publications: Where there is doubt include all bibliographical details.

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References may be listed if they are 'in press', but the journal that has *accepted* the paper for publication must be identified. Submitted papers or papers in preparation should not be listed; such work should be mentioned in the text only, as 'unpublished data' or 'personal communication' (with surnames, initials and year). These sections should be followed by the tables in numerical order, each on a separate sheet, and finally by a typed list of the legends to the figures (see below).

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These must be intelligible without reference to the text and should be planned to fit the page size of the Journal. Tables and figures should be numbered, in arabic numerals, in the sequence in which they are mentioned in the text. The same data may not be reproduced in both a table and a figure. Each table must have a title and on each column there should be a heading that clearly identifies the data therein. Illustrations and diagrams should be kept to a minimum; the figure number must appear only on the reverse side, together with the author's name and an arrow marking the top. Figures can be submitted on disk, labelled to include the software program used, but the originals must also be supplied. Three copies of any half-tones (unmounted and on glossy paper)are required; for line drawings, an original (in black drawing ink on good-quality paper or computer generated figures of laser quality) or a glossy print is required for reproduction,

together with two clear photocopies for the referees. No information that can be included in the legend should appear on the figure and the following standard symbols are preferred for line drawings: (closed triangle), (open triangle), (closed circle), (open circle), (circle with dot), (closed box), □ (open box), (x), (diamond). The legends for photomicrographs must state the staining method and magnification. Authors should bear in mind that the figures they submit may be subjected to photographic reduction. The cost for illustrations in colour will be the responsibility of the contributor(s)(an estimate may be obtained from the Editorial Office at TNO BIBRA International Ltd). If figures that have already been published under copyright are to be reproduced in the Journal (e.g. in reviews), copies of letters from the first publisher and the original author giving permission for such reproduction must always accompany the submitted manuscript. For more information about submitting graphics and artwork please visit the following website: http://www.elsevier.nl/locate/authorartwork

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These should include the definitions of any abbreviations used in the text and any author's current address, if different from that given below the title. Other footnotes, as distinct from literature references, should be avoided in the text as far as possible. When they are essential, they should be identified by superscript numbers. All the footnotes except those for tables should be printed on a separate sheet, with an indication of the manuscript page to which the footnote refers.

Nomenclature

The metric system is the standard for all measurements. Test chemicals and enzymes must be clearly identified, wherever possible with the aid of CAS Registry and EC numbers.

Abbreviations

These should be used sparingly; they should be defined when first used n the paper but also listed in alphabetical order under *Abbreviations* as a footnote to the title page (see abo