Investigations into the cancer modulating properties of *Aspalathus linearis* (Rooibos), *Cyclopia intermedia* (Honeybush) and *Sutherlandia frutescens* (Cancer bush) in oesophageal carcinogenesis

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Thesis presented for the Degree of Magister Scientiae in the Department of Medical Bioscience, Faculty of Natural Science at the University of the Western Cape, Bellville

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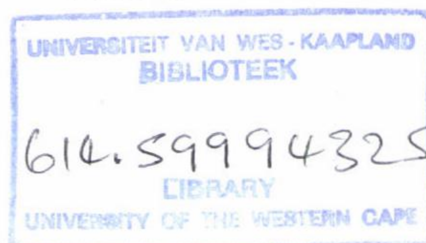

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DECLARATION

I declare that the work contained in this thesis is my own, and has not previously in its entirety, or in part, been submitted for any degree or examination at any other university. All sources I have used or quoted have been indicated and acknowledged by complete references.

L.G. Sissing 28.02.2008

Linda G Sissing **Date**

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DEDICATION

This thesis is dedicated to my daughter, Tiffany Joy

Everything I do, I do it for you.

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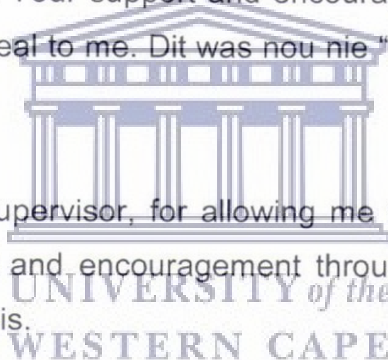

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ABSTRACT

This thesis contains scientific evidence on the anticancer properties of two unique South African herbal teas, rooibos and honeybush, and the herbal plant *Sutherlandia frutescens* (cancer bush) using an *in vivo* oesophageal cancer model in rats and a human cancer cell line *in vitro*. Green and black teas (*Camellia sinensis*), known to modulate oesophageal cancer in rats were included as references. The unfermented herbal teas reduced tumour multiplicity (number and size) by significantly ($P<0.05$) decreasing the total mean number and the mean sizes of papillomas. Green and black teas significantly ($P<0.05$) reduced the total mean number of papillomas, while the mean size was markedly reduced. The *Sutherlandia frutescens* infusion markedly reduced the total mean number and mean size of papillomas. Modulation of the growth kinetics of the early neoplastic oesophageal lesions by the teas, presumably the polyphenolic constituents, is likely to reduce their proliferative activity. A total polyphenol intake threshold of 7 mg/100 g body weight is proposed for the herbal teas to effectively modulate oesophageal papilloma development in rats. Specific polyphenol subgroup ratios also appear to be important, as "fermentation" chemically alters polyphenols and reduces the water soluble polyphenol content of rooibos and honeybush and therefore reduced their chemopreventive properties. The cytotoxicity and anti-proliferative properties of the different teas were also evaluated in a human oesophageal cancer cell line. Although the cytotoxicity could not be measured accurately when using the lactate dehydrogenase (LDH) release and tetrazolium bromide (MTT) colorimetric assays as a result of colour interference of the teas, satisfactory results were obtained when employing flow cytometry, CellTiter Glo® Luminescence and BrdU incorporation cell proliferation assays. Plant matrix and media interactions effected by *Sutherlandia frutescens* also prevented the determination of cytotoxic parameters in the cancer cells. When utilising flow cytometry and cell viability assays, unfermented rooibos tea displayed properties comparable to the *Camellia sinensis* teas, while honeybush exhibited weaker effects. Unfermented rooibos, however, exhibited a more potent inhibitory effect on cell proliferation when compared to green and unfermented honeybush teas. Impairment of the proliferative capacity of the papillomas and the human cancer cell line could be important in utilizing the herbal teas as chemopreventive agents against oesophageal cancer.

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ABBREVIATIONS

2-AAF	2 acetylaminofluorine
AFB_1	Aflatoxin B_1
B[*a*]P	benzo[*a*]pyrene
CCL_4	Carbon tetrachloride
DNA	Deoxyribonucleic acid
DMBA	Dimethylbenz[a]anthrocene
DMEM	Dulbecco's modified eagle's medium
DPPH	a,a-diphenyl-ß-picrylhydrazyl
ELISA	Enzyme-linked immunosorbant assay
EGCG	Epigallocatechin-3-gallate
E/A	Ethyl acetate
FBS	Fetal bovine serum
FDE	Freeze-dried extract
FB_1	Fumonisin B_1
GABA	Gamma-aminobutyric acid
HBSS	Hank's balanced salt solution
HCAs	Heterocyclic amines
HAA	Heterocyclic aromatic amines
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
LDH	Lactate dehydrogenase
L-glut	L-glutamine
MBN	Methylbenzylnitrosamine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMBA	N-Nitosomethylbenzylamine
ORAC	Oxygen radical absorbance capacity
PhIP	2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine
PSF	Penicillin, Streptomycin, Fungizole
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
TPP	Total polyphenols

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IC_{50} A concentration of FDE/ml that exerts an inhibitory action (cytotoxicity, cell viability or anti-proliferation) of 50%.

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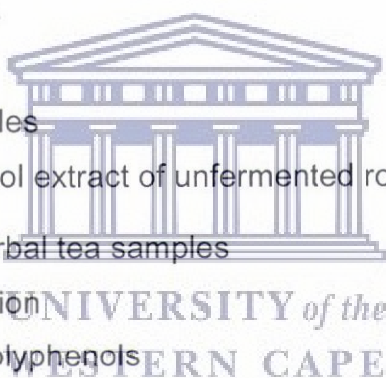
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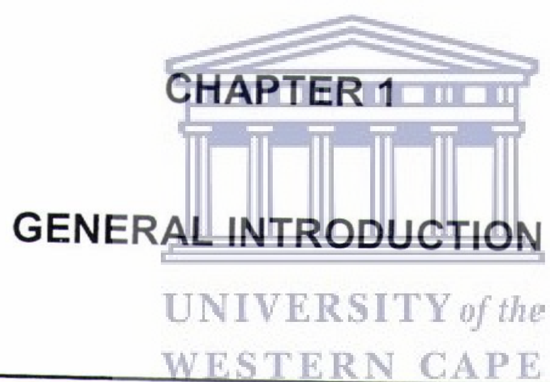
CHAPTER 1

GENERAL INTRODUCTION



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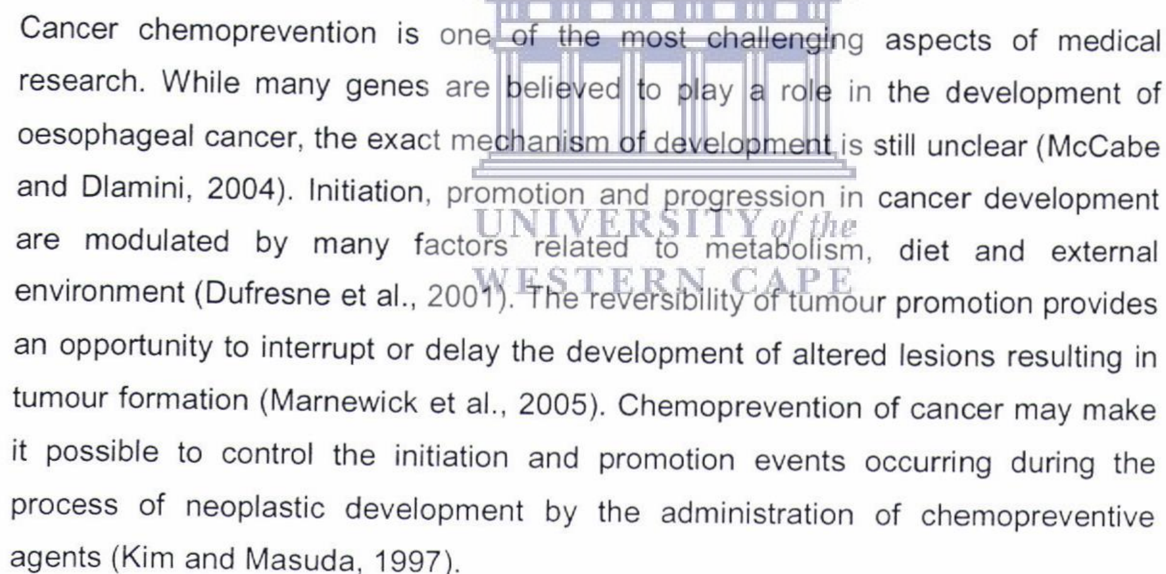
GENERAL INTRODUCTION

Cancer is the largest single cause of death in both men and women in most countries (Parkin *et al.*, 1999), with oesophageal cancer (OC) being one of the fastest growing, ranking as the ninth most common malignancy in the world. It is particularly common in South Africa, specifically among the population of the former Transkei region in the Eastern Cape (Jaskiewicz *et al.*, 1987; Somdyala *et al.*, 2003a & b). A similar cluster of people who have an unusually high incidence live in China, Japan, the Russian Republics, Iran, South America and parts of France (Bolger *et al.*, 2001; Parkin *et al.*, 2005). However, treatment of OC in a developing country is extremely challenging and it is the socio-economics of South Africa alone that presents a significant hurdle in the combat against cancer. As with many other types of cancer, the outcome of OC depends on how advanced it is when it is diagnosed, but overall, OC has a very poor prognosis (Johnson, 2004).



Cancer chemoprevention is one of the most challenging aspects of medical research. While many genes are believed to play a role in the development of oesophageal cancer, the exact mechanism of development is still unclear (McCabe and Dlamini, 2004). Initiation, promotion and progression in cancer development are modulated by many factors related to metabolism, diet and external environment (Dufresne et al., 2001). The reversibility of tumour promotion provides an opportunity to interrupt or delay the development of altered lesions resulting in tumour formation (Marnewick et al., 2005). Chemoprevention of cancer may make it possible to control the initiation and promotion events occurring during the process of neoplastic development by the administration of chemopreventive agents (Kim and Masuda, 1997).

There is a great need, worldwide, to identify compounds with the ability to prevent cancer. In South Africa it is not only imperative to identify compounds because of the high incidence of OC, but also to ensure that the average inhabitant can benefit from the findings in a cost effective manner. Several studies in animals and humans support the hypothesis that green tea and tea polyphenols are

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chemopreventive agents for oesophageal cancer (Gao *et al.*, 1994; Sun *et al.*, 2002; Wang *et al.*, 1995). Rooibos (*Aspalathus linearis)* and honeybush (*Cyclopia intermedia)* are herbal teas indigenous to South Africa, and have a history of anecdotal health benefits. Recent studies show that rooibos and honeybush teas display significant antimutagenic, antioxidant and antitumour activity, and contain components that possess significant chemopreventive properties (Marnewick *et al.*, 2005; Snijman *et al.*, 2007; van der Merwe *et al.*, 2006), which may be attributed to either a single and/or a combination of polyphenols. The biological characterization of herbal tea fractions with respect to their antioxidant, anti-proliferative, and pro-apoptotic properties could be important initial steps in developing chemopreventive products against OC development.

The present thesis reviews different aspects of oesophageal cancer, chemical carcinogenesis, the chemopreventive properties of plants and detailed aspects regarding the South African herbal plants, *Aspalathus linearis*, *Cyclopia intermedia* and *Sutherlandia frutescens* (Chapter 2). Black and green teas (*Camellia sinensis*) were used as experimental controls, since numerous studies have been conducted on the anticancer properties of these popular teas (Han and Xu, 1990; Hirose *et al.*, 1993; Katiyar and Mukhtar, 1996; Steele *et al.*, 2000; Yang and Wang, 1993). Differences in the polyphenolic constituents and/or known compounds produced by these herbal plants are highlighted. As rooibos, honeybush and *Sutherlandia* exhibited chemopreventive properties in different *in vitro* studies (Chinkwo, 2005; Marnewick *et al.*, 2004, 2005; Southon, 1994; Swaffar *et al.*, 1994; Tai *et al.*, 2004; van der Merwe *et al.*, 2006), and the extracts of rooibos and honeybush inhibited mouse skin cancer development, the present study was conducted to investigate the preventive properties in an oesophageal cancer model utilising methylbenzylnitrosamine as carcinogen (Chapter 3). The major emphasis of the study was on the inhibition of papilloma development, which signifies the effect that the herbal tea constituents have on the cancer-promoting phase of chemical carcinogenesis. Detailed tea intake profiles of the tea polyphenolic composition and known flavonoid and xanthone constituents were monitored.

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Studies in human cancer cell lines have made an important contribution to investigate possible mechanisms involved in the chemopreventive properties of compounds and/or extracts from plant origins. The underlying mechanisms involved in the regulation of different cell survival parameters have been used to predict possible outcomes and provide information on the cytotoxic effects of chemopreventive agents (Roy *et al.*, 2001; Stewart *et al.*, 2003). A human oesophageal cancer line (WHCO5) was selected to investigate the selective cytotoxic and anti-proliferative properties of the herbal plants. The possible effects of the known herbal tea polyphenols and/or other constituents are discussed and their activities debated.

The main findings of the study are integrated and summarised in Chapter 5 while supportive experimental data are summarised in the Addendum section.

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CHAPTER 2

LITERATURE REVIEW

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2.1 Introduction

Cancer is the largest single cause of death in both men and women in most countries (Parkin *et al.*, 1999). It was estimated that there are 1.9 million new cases, 6.7 million deaths, and 24.6 million persons alive with cancer per annum (Parkin *et al.*, 2005). The most commonly diagnosed cancers are lung (1.35 million), breast (1.15 million), and colorectal cancer (1 million), while the most common causes of cancer death are lung (1.18 million deaths), stomach (700 000 deaths), and liver (598 000 deaths) (Parkin *et al.*, 2005).

There has been striking variations in the risk of different cancers by geographic area. Most of the international variation may be due to exposure to known or suspected risk factors related to lifestyle or the environment, and provide a clear challenge to prevention (Parkin *et al.*, 2005). Cancer is a major health problem and the effects thereof ultimately extend into the community (WHO, 1992). For many cancers, curative treatment is generally not possible and this may be exacerbated by poverty, health inequalities, and inappropriate facilities (Somdyala, 2002). Approaches for cancer prevention in relation to diet are important to reduce the incidence, particularly in areas where dietary deficiencies contribute to risk factors in cancer development. The focus should be on cost-effective health care strategies of which dietary changes are one (ICMR Bulletin, 2001).

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Epidemiological data suggested that the major cause of cancer is largely due to environmental factors as the risk of developing this disease varies greatly between populations, independent of genetic factors (Johnson, 2004).

2.2 Environmental Carcinogens

A carcinogen is a substance known to cause cancer in experimental animals and/or in humans, and is contracted by inhalation, ingestion and percutane absorption. Exposure to chemical carcinogens may be work-related, and may include products of the combustion of petroleum, processing of tobacco or industrial processes, which generate aromatic amines. Three principal classes of food-related mutagens that may be implicated in alimentary carcinogenesis have

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been identified. These include heterocyclic aromatic amines, polycyclic aromatic amines (Goldman and Shields, 2003) and the nitrosamines that may be formed during food processing and also by the colonic flora acting on digestive residues of meat proteins (Bingham, 1999). It has been shown that cooking certain meats at high temperatures creates chemicals that are not present in uncooked meats (NCI, 2004). Heterocyclic amines (HCAs) are formed during the cooking of muscle from beef, pork, fowl and fish. 2-amino-3-methylimidazo [4,5-*f*]quinoline (IQ) is an example of an HCA derived from amino acids during high temperature cooking. According to the National Cancer Institute (2004) 17 different HCAs resulting from the cooking of meat, that may increase the risk of human cancer, have been identified (NCI, 2004). Naturally occurring carcinogens include mycotoxins such as aflatoxin B_1 and fumonisin B_1, which are produced by fungi that contaminate human food sources (Miller and Marasas, 2002; Shephard et al., 1996, 2005). Many substances in the environment such as sunlight and pollutants cause DNA mutations and are thus considered as environmental carcinogens (Belpomme *et al*., 2007; Clapp *et al*., 2007; Irigaray *et al*., 2007).

It is important to note that many carcinogens are converted to reactive mutagenic compounds after metabolic activation. These include most environmental carcinogens such as aflatoxins, polycyclic aromatic hydrocarbons, and nitrosamines, which are pro-carcinogens that have to be metabolically activated to electrophilic reactive species that interact with cellular constituents such as the DNA, protein, etc. (Lin *et al*., 1999). Several deactivating enzymes exist, which play a role in the metabolic conversion of these reactive species into water-soluble conjugates that are excreted from the body (Fig. 1).

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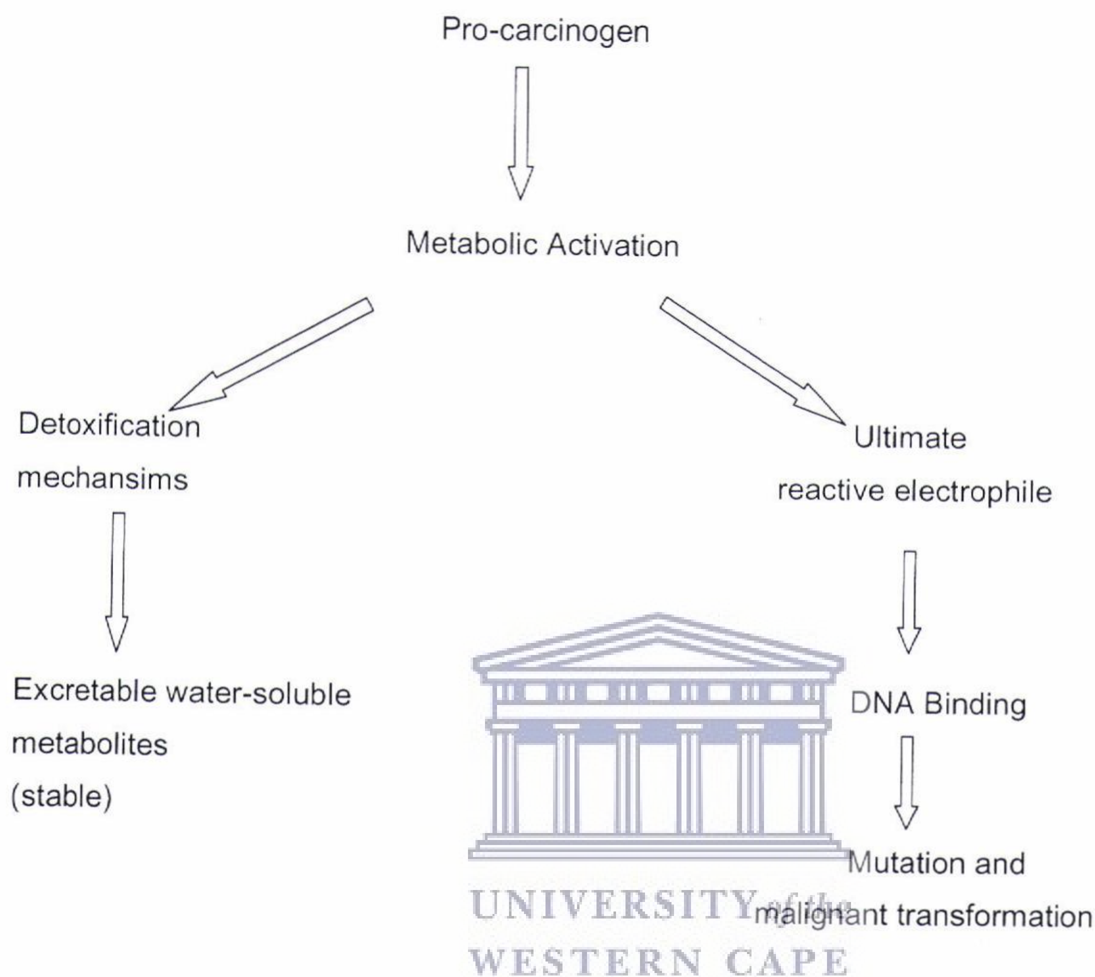

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Fig. 1 Pro-carcinogens are converted to DNA reactive electrophiles, or detoxified to suitable stable products and excreted (Adapted from Lin *et al.*, 1999).

2.3 Carcinogenesis

Tumourigenesis or carcinogenesis is a multistep process including cellular mutation and transformation, progression to hyper-proliferative lesions that culminate in the acquisition of invasive potential, angiogenic properties and establishment of metastatic lesions (Lin *et al.*, 1999). This multistep process consists of three phases: (i) tumor **initiation**, (ii) **promotion** and (iii) **progression**, which are subject to multistep levels of control (Butterworth and Goldsworthy, 1991) (Fig. 2).

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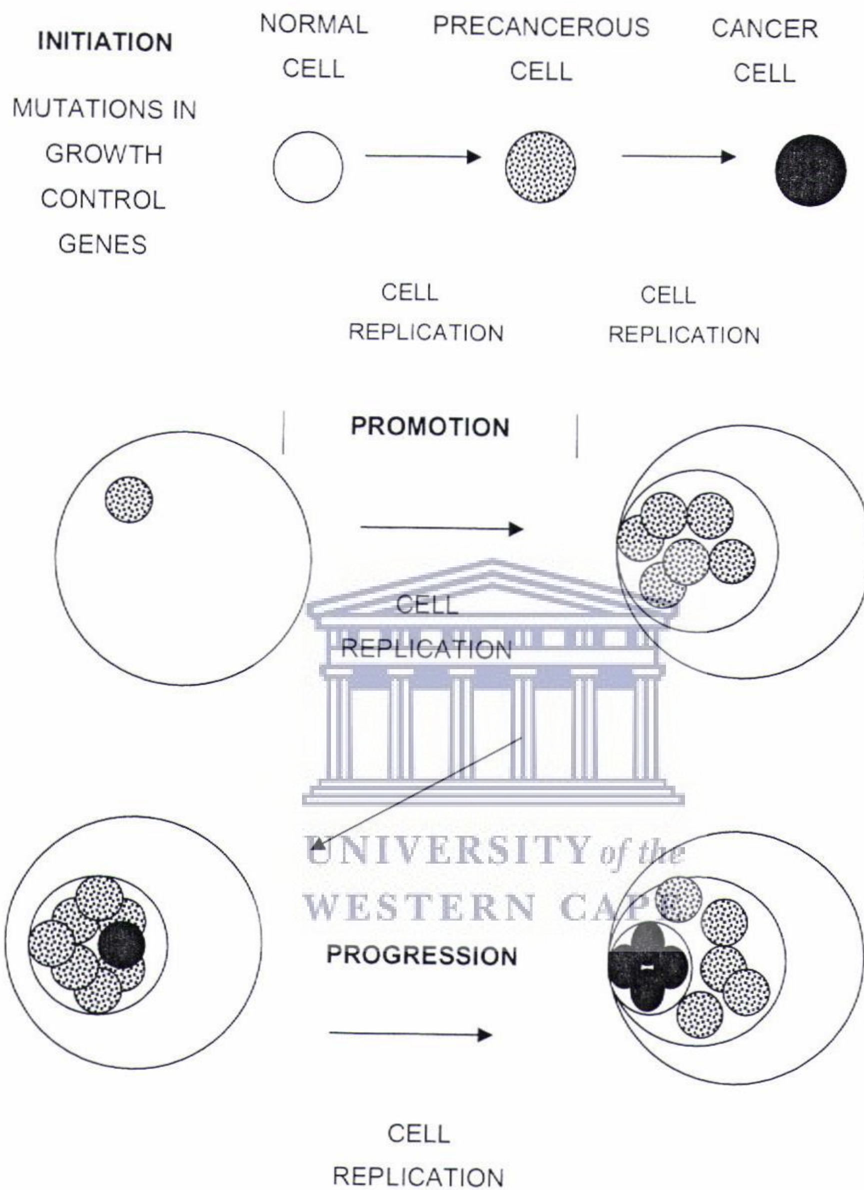

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Fig. 2 Schematic representation of the multistep process of carcinogenesis (adapted from Butterworth and Goldsworthy, 1991).

(i) **Initiation** occurs when a carcinogen reacts with deoxyribonucleic acid (DNA) to produce a mutation. This initial process involves carcinogen metabolism, DNA repair and cell proliferation. Initiation is irreversible,

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inheritable and confers a growth advantage as a consequence of a new biochemical phenotype, which make the cell resistant to the adverse effects of carcinogens and/or cancer promoters (Faber, 1996; Klaunig *et al.*, 1998). The initiated cell, however, is and does not necessarily become a cancer cell.

(ii) **Promotion** occurs when tumour promoters contribute to carcinogenesis, mainly through non-genotoxic mechanisms. They influence the proliferation of initiated cells to form benign lesions that may regress, or acquire additional mutations to become a malignant neoplasm. The process whereby the initiated cell population is expanded is called differential inhibition and/or stimulation (Faber, 1996; Schulte-Hermann *et al.*, 1993, 2000).

(iii) Tumour **progression** describes the process during which benign lesions are transformed into cancer and, under certain instances, develop the ability to invade and establish distant metastasis. This process is characterized by chromosome instability and mutations in oncogenes and tumor suppressor genes. The mutations may reflect selections of cells suited for neoplastic growth that may further increase chromosome instability. The dysfunction of gene products is important in the development of the cancer phenotype.

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2.4 Characteristics of tumours

Tumours are diverse and heterogeneous, but all share the ability to proliferate beyond the constraints limiting growth in normal tissue. Deregulated cell proliferation, together with suppressed apoptosis, constitutes the minimal common platform upon which all neoplastic evolution occurs (Evan and Vousden, 2001). Cancer cells differ from non-cancer cells in four fundamental ways (Kastan, 1997; Evan and Vousden, 2001):

(i) **Uncontrolled proliferation**: Proliferation occurs in non-cancer as well as in cancer cells, but non-cancer cells stop dividing after the cells are fully differentiated in contrast to cancer cells.

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(ii) **Loss of contact inhibition**: Non-cancer cells stop growing when they come into direct contact with other cells. This is a form of communication called "contact inhibition". Cancer cells seemed to have lost this contact inhibition, thus having a greater ability to grow and extend.

(iii) **Lack of adhesion**: Non-cancer cells adhere to one another if they are of the same kind via a process called adhesion. In cancer cells there is a lack of adhesion that gives them the ability to detach from the original clone and migrate to other areas.

(iv) **Inability to differentiate**: Differentiation is the specialization of cells and tissues from immaturity to maturity in order to perform specific cellular functions. During this process the cells lose their ability to proliferate. An undifferentiated cell maintains its proliferative capability and does not take on a normal cellular function. Cancer cells tend to be less differentiated and maintain their proliferative capability (Kastan, 1997; Evan and Vousden, 2001).

2.5 Regulation of cellular growth

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Cellular homeostasis in tissues is maintained by three mechanisms namely, proliferation, differentiation and cell death (apoptosis) (Lockshin and Zakeri, 2001). Apoptotic cell death is a physiological mechanism that eliminates unwanted cells by triggering the cell's suicide program (Kerr *et al.*, 1972). Numerous morphological changes occur when apoptosis is induced. These include membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation, followed by rapid engulfment of cell debris by neighbouring cells (Christop, 2003). Impairment of this mechanism may generate pathological conditions that may include developmental defects such as autoimmune diseases, neurodegeneration or cancerous neoplasia (Reed *et al.*, 2001). Apoptosis therefore plays a crucial role in developing and maintaining normal cellular homeostasis whereby old and/or damaged cells are removed.

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During cell proliferation each cell progresses through the cell cycle where various protein complexes regulate the events during each phase (Fig. 3).

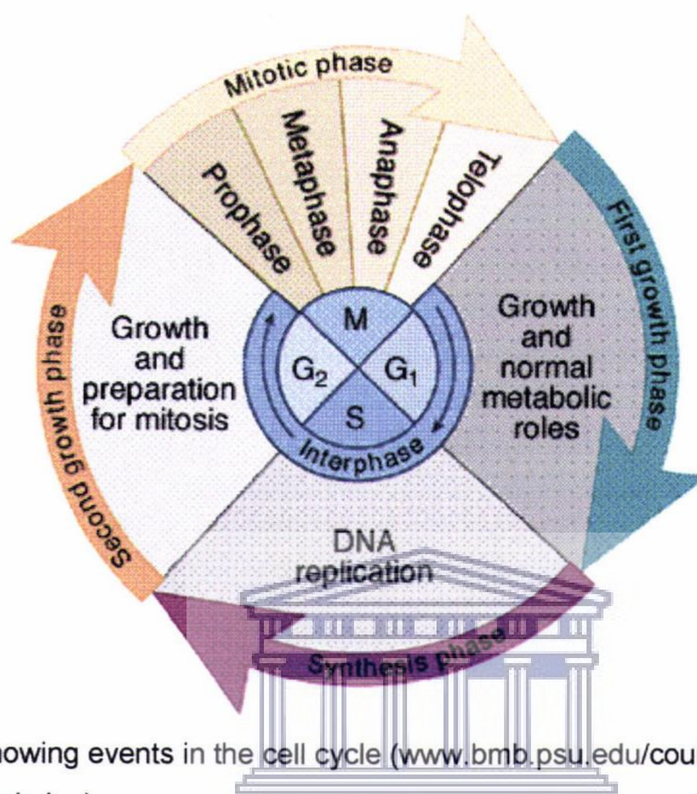

Fig. 3 Diagram showing events in the cell cycle (www.bmb.psu.edu/courses/ biotc489/notes/cycle.jpg)

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Completion of the cell cycle requires the co-ordination and regulation of a variety of macromolecular synthesis, assemblies and movements. The chromosomes replicate, condense, segregate and de-condense, and the spindle poles duplicate, separate and migrate to opposite ends of the nucleus during mitosis (Hartwell and Kastan, 1994). In mammals the spindle checkpoint ensures equal chromosome segregation at almost every cell division.

2.5.1 Checkpoints and restriction sites

All cells undergo cell cycle regulation during which an essential assessment mechanism exists to determine the integrity of the cell. This will determine whether the cell may proceed to the next stage or whether it should "commit suicide" via the process of apoptosis (McCabe and Dlamini, 2004). These regulatory events,

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known as checkpoint signaling pathways, ensure the proper order and timing of events (Hartwell and Weinert, 1989; Murray, 1992). Checkpoints also ensure that the events of the cell cycle occur in a well-defined sequence to maintain the integrity of the cell and its genetic material. Three major checkpoints exist, namely:

(i) Gap I phase, which recognizes damaged DNA and is mediated by p53

(ii) Gap II phase, which senses damaged and/or unreplicated DNA; and

(iii) Mitosis (metaphase), which checks the spindle assembly and ensures that all the chromosomes are attached to the mitotic spindle by a kinetochore (Vermeulen *et al*., 2003).

Many signal transduction systems, including checkpoint control, undergo adaptation whereby the restriction diminishes with time in the presence of a constant stimulus. As a result, the cell may proceed through the cell cycle, even though the original defect in the checkpoint control has not been removed or cannot be repaired (Sandell and Zakhian, 1993). A defect in any of the checkpoints can generate a signal that stops progression in the others, even if they are not physically dependent. If the genome is damaged cell cycle progression is delayed, predominantly in G1 or G2, until the damage is repaired (McGowan, 2003).

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Mutations affecting the checkpoint proteins are frequent in all types of cancer. The tumour suppressor protein, p53, is a sequence-specific DNA-binding protein that is able to induce either cell cycle arrest or apoptosis (Vermeulen *et al*., 2003). DNA damage and incomplete replication of DNA that prevents chromosome segregation, in turn prevents the G2-M transition. Improper functioning of the mitotic spindle at metaphase can also arrest cell cycle progression (Vermeulen *et al*., 2003).

2.5.2 Cell cycle control and carcinogenesis

Potentially oncogenic proliferative signals are coupled to a variety of growth-inhibitory processes, such as the induction of apoptosis, differentiation of senescence, each of which restricts subsequent clonal expansion and neoplastic

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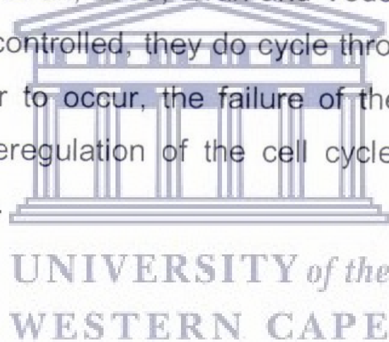
evolution. Tumour progression occurs only in the very rare instances where these growth-inhibitory mechanisms are thwarted by compensatory mutation. Defects in surveillance of each of these components result in forms of genetic instability that characterize precancerous and cancerous cells. These defects are all common during cancer cell evolution (Hartwell and Kastan, 1994). Cellular damage normally leads to cell cycle arrest, which could lead to genetic instability if the cell cycle checkpoint is impaired. Experimental evidence supports the view that loss of the G1-S checkpoint and apoptotic signals may contribute to genomic instability and tumourigenesis by loss of a mechanism for eliminating cells with genetic damage. This could occur early in cancer progression, leading to the inappropriate survival of genetically damaged cells. Alternatively, it could occur later in tumourigenesis and contribute directly to survival of cells in appropriate physiological conditions (Hartwell and Kastan, 1994; Lin *et al.*, 1999; Evan and Vousden, 2001). Although the growth of cancer cells is uncontrolled, they do cycle through the same phases as non-cancer cells. For cancer to occur, the failure of the apoptotic safeguard mechanisms, as well as the deregulation of the cell cycle is required (Kastan, 1997; Evan and Vousden, 2001).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2.6 The oesophagus

2.6.1 Structure and function

The oesophagus is a muscular tube that connects the mouth to the stomach (Fig. 4a) and carries food into the stomach. The wall of the oesophagus has several layers (Fig.4b), of which the mucosa, the innermost, is where cancer usually originates. This layer is composed of thin, flat non-keratinized epithelial squamous cells, and a thin layer of connective tissue, the lamina propria, followed by a thin layer of smooth muscle tissue, the muscularis mucosae. The submucosa, which may contain secretory glands, is found beneath this layer. The next layer is the muscularis externa, which is made up of an inner band of circular and an outer band of longitudinal muscle. The last layer, the adventitia, is formed by connective tissue (http://courseweb.edteched.uottawa.ca/Medicine-

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histology/English/Gastrointestinal/Gastro_Esophagus.htm). Carcinoma of the oesophagus originates in the epithelial cells in the mucosa and infiltrates the underlying layers.

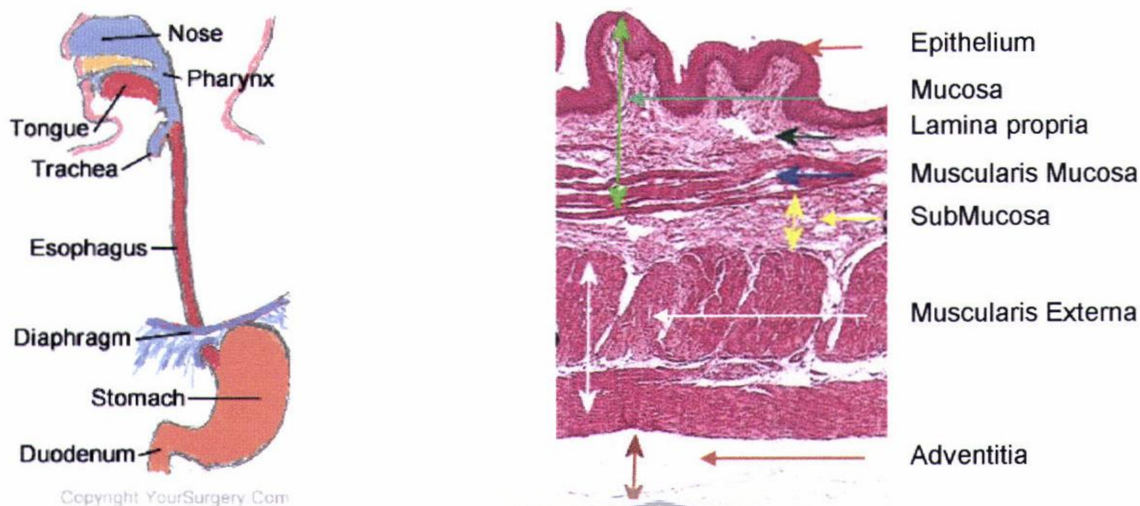

Fig. 4(a) Fig. 4(b)

Fig. 4 Diagrams illustrating the position of the oesophagus (4a) and the layers of the oesophagus (4b) (http://courseweb.edteched.uottawa.ca/Medicine-histology/Englsih/Gastrointestinal/Gastro_Esophagus.htm).

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2.6.2 Oesophageal cancer

Cancers of the alimentary tract are, collectively, amongst the major causes of morbidity and mortality from cancer across the world today (Johnson, 2004). Most cancers of the alimentary tract are carcinomas derived from the rapidly dividing population of stem cells that renew the epithelium forming the barrier between the lumen and the interior milieu. As with many other types of cancer, the outcome of oesophageal cancer depends on how advanced it is when initially diagnosed, but overall, oesophageal cancer has a poor prognosis. Symptoms would include weight loss, difficulty or pain when swallowing, difficulty in swallowing solids and, with further progression of the cancer, even liquids become difficult to swallow. At this stage the disease has usually transgressed beyond that of curable, with approximately 60 percent of the circumference of the oesophagus being infiltrated (Johnson, 2004).

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There are two main types of cancer of the oesophagus, namely, squamous cell carcinoma (SCC), which is common at the upper end, and adenocarcinoma that is more common at the lower end (Johnson, 2004). SCC represents the most common oesophageal malignancy and demonstrates wide regional variations in incidence and causal associations.

2.6.3 *Incidence*

Oesophageal cancer ranks the ninth most common malignancy in the world and recent evidence has shown that its incidence is increasing (McCabe and Dlamini, 2004). The incidence of oesophageal cancer varies considerably with geographic location and also, to some extent, among ethnic groups within a common area. Information on the incidence of cancer in South Africa has become available with the establishment of a National Cancer Registry (NCR) in 1986 (Sitas and Isaacston, 1989). In South Africa and Zimbabwe the incidence of oesophageal cancer was shown to be higher in the black population than among whites, whereas persons of mixed or Asian/Indian background, in South Africa, have rates that varied between those of blacks and whites. Oesophageal cancer in South Africa was shown to be the second most common cancer among all South African men, and the most common cancer in black males (Rose and McGlashan, 1975). According to the National Cancer Registry (1993-1995), oesophageal cancer incidence in the former Transkei has been much higher when compared with the oesophageal cancer rates in South Africa. Cancer registry in this region dated back to the 1950's (Rose, 1965; Burrell, 1969). The highest rate has been recorded in the southwestern districts of Transkei, Centani and Butterworth (Rose, 1975; Jaskiewicz *et al.*, 1987; Makuala *et al.*, 1996; Somdyala, 2002; Somdyala *et al.*, 2003). According to a recent report (Somdyala, 2002) there is a steady increase of oesophageal cancer in the northeastern districts of Lusikisiki and Bizana.

High incidence areas in other countries include China (21 per 100 000), Iran (43.4 per 100 000 for males and 36.3 per 100 000 for females) (Semnani *et al.*, 2006); South America (13 per 100 000), Western Europe (11 per 100 000), Japan (9 per

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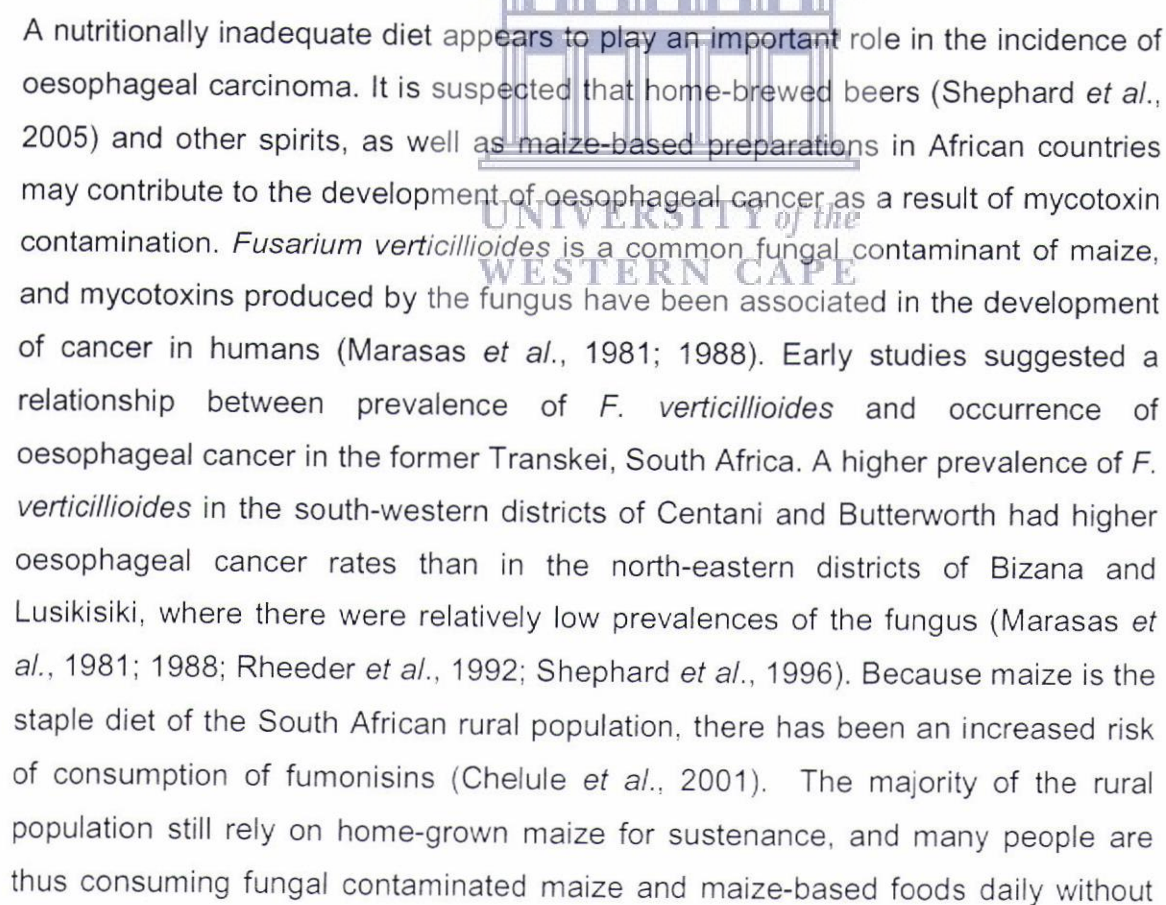
100 000); and the former Soviet Union (8 per 100 000). Oesophageal cancer was ranked the highest among Black Americans (Parkin *et al.*, 1997), and was reported to be higher among males than females (Safdar and Khan, 2003).

2.6.4 Diet and other risk factors

Human oesophageal cancer has been associated with tobacco smoking (Rose, 1975), alcohol consumption (Tuyns *et al.*, 1979; Walker *et al.*, 1979), and nutritional deficiencies (Van Rensburg, 1985; Jaskiewicz, 1987; 1988). Associations with the ingestion of certain foods that are contaminated with N-nitroso compounds and fungal toxins (Somdyala, 2002; Parkin *et al.*, 2005), all of which are dependent on geographical area, have been correlated with oesophageal cancer. Oesophageal cancer is therefore a multifactorial disease with no single causative agent thus far identified.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A nutritionally inadequate diet appears to play an important role in the incidence of oesophageal carcinoma. It is suspected that home-brewed beers (Shephard *et al.*, 2005) and other spirits, as well as maize-based preparations in African countries may contribute to the development of oesophageal cancer as a result of mycotoxin contamination. *Fusarium verticillioides* is a common fungal contaminant of maize, and mycotoxins produced by the fungus have been associated in the development of cancer in humans (Marasas *et al.*, 1981; 1988). Early studies suggested a relationship between prevalence of *F. verticillioides* and occurrence of oesophageal cancer in the former Transkei, South Africa. A higher prevalence of *F. verticillioides* in the south-western districts of Centani and Butterworth had higher oesophageal cancer rates than in the north-eastern districts of Bizana and Lusikisiki, where there were relatively low prevalences of the fungus (Marasas *et al.*, 1981; 1988; Rheeder *et al.*, 1992; Shephard *et al.*, 1996). Because maize is the staple diet of the South African rural population, there has been an increased risk of consumption of fumonisins (Chelule *et al.*, 2001). The majority of the rural population still rely on home-grown maize for sustenance, and many people are thus consuming fungal contaminated maize and maize-based foods daily without

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being aware of the possible cancer risk (Fandohan *et al.*, 2003). Oesophageal cancer has therefore become a disease that urgently need a consistent tool for early diagnosis, as well as an effective therapeutic strategy that ensures non-recurrence, best quality of life, and an increased life span (McCabe and Dlamini, 2004).

Epidemiological data suggested that more than 80% of cancers are attributable to lifestyle, of which one third is diet-related (Bushman, 1998), the latter of which play an important role in both the development and prevention of cancer. Cancers most commonly associated with diet include oesophageal, stomach, colon, liver and the prostate (Farombi, 2004). The consumption of meat, fatty foods of animal origin and salted foods increases the incidence of cancer, whereas the intake of foods from plant origin is believed to reduce the chance of cancer incidence (Safdar and Khan, 2003).

2.7 Chemoprevention

We are all exposed to and affected by environmental carcinogens from diverse sources. It is therefore imperative to find a way to neutralize these carcinogens or protect against their adverse effects (Surh, 1999). Chemoprevention is gaining more attention as this approach aims to decrease overall cancer morbidity and mortality by using substances that are capable of preventing different phases of cancer development. Since deregulated proliferation and inhibition of apoptosis plays a critical role during cancer development, they present two obvious targets for therapeutic intervention in cancer. The success of reinstating the apoptotic response in tumour cells depends on the extent to which such therapies confine death to the cancer cells, and allow survival of normal, healthy tissue (Evan and Vousden, 2001).

Chemoprevention can be defined as the prevention of cancer by the administration of one or more chemical entities, either as individual drugs or as natural-occurring constituents in the diet (Lippman *et al.*, 1994). Chemopreventive agents can be

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characterized as inhibitors of carcinogen formation, "blocking" agents and "suppressing" agents (Wattenburg, 1985), and is considered as a strategy to block, reverse or retard the process of carcinogenesis from the very early stages (Lippman *et al.*, 1994; Surh, 2003). Blocking agents are the anti-carcinogens that are responsible for the prevention of initiation, while suppressing agents inhibit the development of tumours from the initiated cells (Johnson, 2004). A limiting factor in human cancer therapy is the level of toxicity the agent has on normal tissue (Hartwell and Kastan, 1994). Since the goal in cancer therapy is to target cancer cells while sparing healthy cells, the ideal chemopreventive agent should have little or no toxic effect; a high efficacy; preferably administered orally; and cost-effective (Morse and Stoner, 1993).

Although the reduced risk of cancer and cardiovascular disease has been associated with daily intake of fresh fruits and vegetables, phenolic constituents of dietary origin and medicinal plants may also play a role in reducing the risk of human cancer (Heim *et al.*, 2002). Recently, natural herbs and pure compounds that are present in the human diet have attracted attention as chemopreventive agents (Kohlmeier *et al.*, 1997; Surh, 1999). Antioxidant-bearing foods are believed to reduce the risk of cancers and other chronic diseases by eliminating free radicals, which are highly reactive molecules thought to contribute to cancer development by damaging DNA (Lin *et al.*, 1999). Broadly, the chemopreventive agents may act through detoxification mechanisms or by antimutagenic processes at both the initiation and promotion steps of carcinogenesis.

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Alternative natural remedies are gaining interest among the public and health care workers in the protection and/or prevention against various diseases (Astin, 1998). Most plants and/or plant products used at present have a long history of use and have served a particular purpose in maintaining health and quality of life (Bast *et al.*, 2002). The use of medicinal plants in traditional healing in most developing countries as a normative basis for the maintenance of good health has been widely observed (UNESCO, 1996). In Africa up to 80% of the population uses traditional

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medicine for primary health care, and 25% of modern medicines are made from plants first used traditionally (WHO, 2003).

2.8 Tea as a possible tool for chemoprevention

Several compounds of plant origin, of which tea is one, have been evaluated as chemopreventive agents. Tea has been shown to be the most popular beverage next to water, and is consumed by more than two-thirds of the world's population (Katiyar and Mukhtar, 1996; Ahmad *et al.*, 1998).

The variety of processing methods and tea drinking differ in different parts of the world, with the three most popular types being green, black and oolong tea. Black tea constitutes about 80% of the tea manufactured in the world, and is mainly consumed in Western and some Asian countries, while green tea constitutes about 20% and is mainly consumed in Asia, some countries in North Africa and the Middle East. Oolong tea (2%) is consumed in some parts of China and Taiwan (Katiyar and Mukhtar, 1996; Ahmad *et al.*, 1998). While tea is a popular beverage, the consumption of green tea has been associated with a lower risk of several types of cancer including stomach, oesophagus and lung (Lin *et al.*, 1999). Worldwide interest in green tea as a preventive agent for human cancer has increased because it has been shown to have beneficial effects in a various organ systems (Suganuma *et al.*, 1999). The widespread consumption of tea throughout the world has, as such, aroused interest in the possibility of its use in chemoprevention of carcinogenesis and mutagenesis (Gupta *et al.*, 2002).

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In South Africa, herbal teas derived from two endemic plants, *Aspalathus linearis* (rooibos tea) and *Cyclopia spp.* (honeybush tea), are also consumed regularly. Both rooibos and honeybush teas have been shown to contain a complex mixture of polyphenolic compounds (Keoppen and Roux, 1965, 1966; Rabe et al., 1994; Ferreira *et al.*, 1998). Their polyphenolic constituents differ, not only from each other, but also from green and black teas (Von Gadow *et al.*, 1997a; Kamara *et al.*, 2003). Herbal teas are not regarded as true teas since they are not derived from

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the original tea plant (Trevisanato and Young-In, 2000), but they are ingested in the same manner as green and black teas (Bushman, 1998). In addition to being safe, enjoyable and inexpensive, the herbal teas provide a natural source of compounds that may protect against various human diseases (Joubert and Ferreirra, 1996; Standley *et al.*, 2001).

2.9 *Camellia sinensis* (Green and black teas)

2.9.1 History and processing

The plant was originally discovered and grown in Southeast Asia thousands of years ago and, according to Chinese mythology, the emperor, Shen Nung, discovered tea for the first time in 2737 B.C. (Harbowy and Balentine, 1997). The tea plant (*Camellia sinensis*) is a tree species with leathery, dark and slightly serrated evergreen leaves, which is cultivated in large plantations and/or estates (Fig. 5).

Fig. 5 Tea pickers in *Camellia sinensis* plantations
(ww.teafountain.com/.../pictures/pick_tea250.jpg)

Green tea is obtained from the leaves of *Camellia sinensis* var. *sinensis*, while black tea is obtained from the leaves of *Camellia sinensis* var. *assamica*, and differences in tea processing determines the final product.

During the production of green tea the leaves are rapidly subjected to heat treatment after harvest to prevent any oxidative fermentation. Thereafter, they are

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withered, rolled and dried (Lin *et al.*, 1996; Wiseman *et al.*, 1997). During the manufacturing of black tea the leaves are withered, macerated, fermented and dried at high temperature (Tomlins and Mashingaidze, 1997; Wang *et al.*, 2000). In the preparation of black tea the catechins are oxidized into theaflavins and thearubigins, yielding the typical dark colour and flavour (Fig. 6).

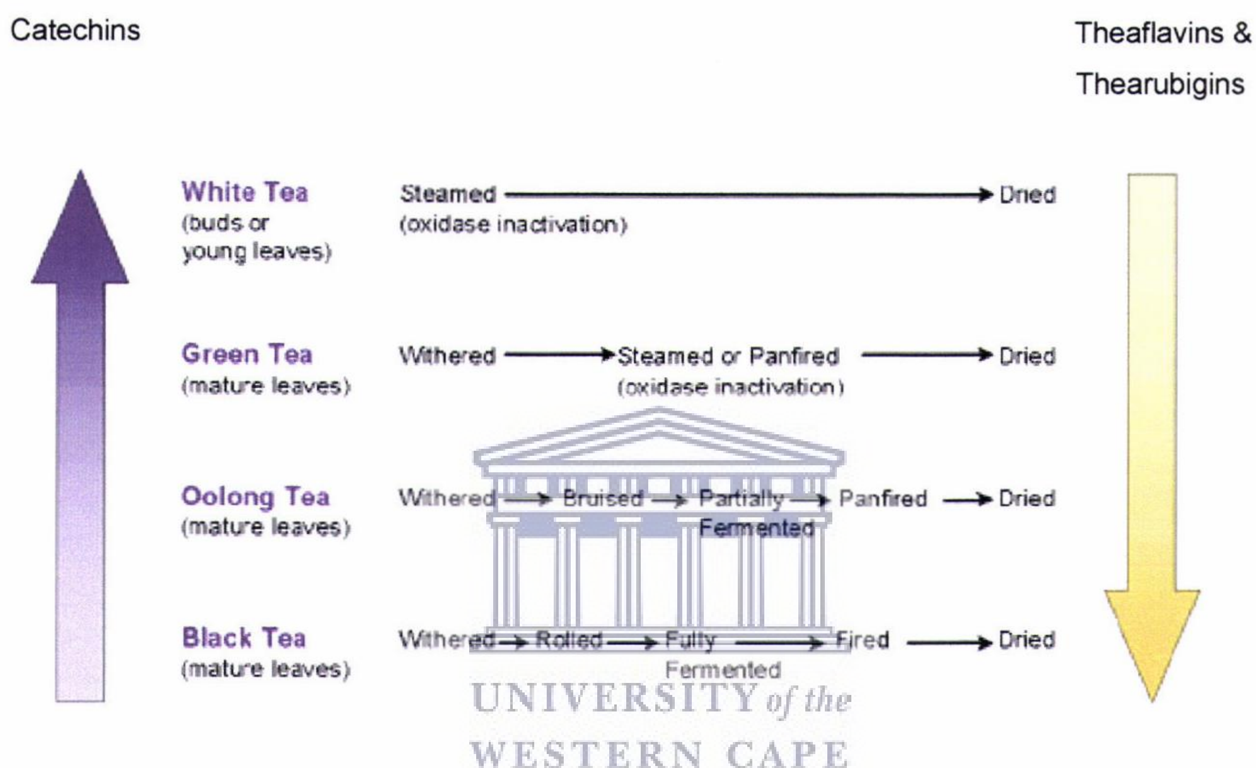

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Fig 6 Diagram showing tea processing steps and the effects on tea polyphenol content. (http://lpi.oregonstate.edu/f-w02/tea.html).

2.9.2 Major phenolic constituents

2.9.2.1 Green tea

The major polyphenolic fraction in green tea is the group of flavonoids made up of flavanols, which are the catechins (Graham, 1992; Wiseman *et al.*, 1997). The principal catechins in fresh tea leaves and green tea are (-)-epigallocatechin-3-gallate (EGCG); (-)-epigallocatechin (EGC); (-)-epicatechin-3-gallate (ECG); (-)-epicatechin (EC); (+)-gallocatechin (GC); and (+)-catechin (C). EGCG is the most abundant catechin and accounts for 6-16% on a dry weight basis (Zhu *et al.*, 1999). The catechins are colourless, water-soluble compounds that lend bitterness

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and astringency to green tea infusions, while the specific taste, colour and aroma of manufactured tea are associated with modifications to the catechins (Wang *et al*., 2000) (Fig. 7).

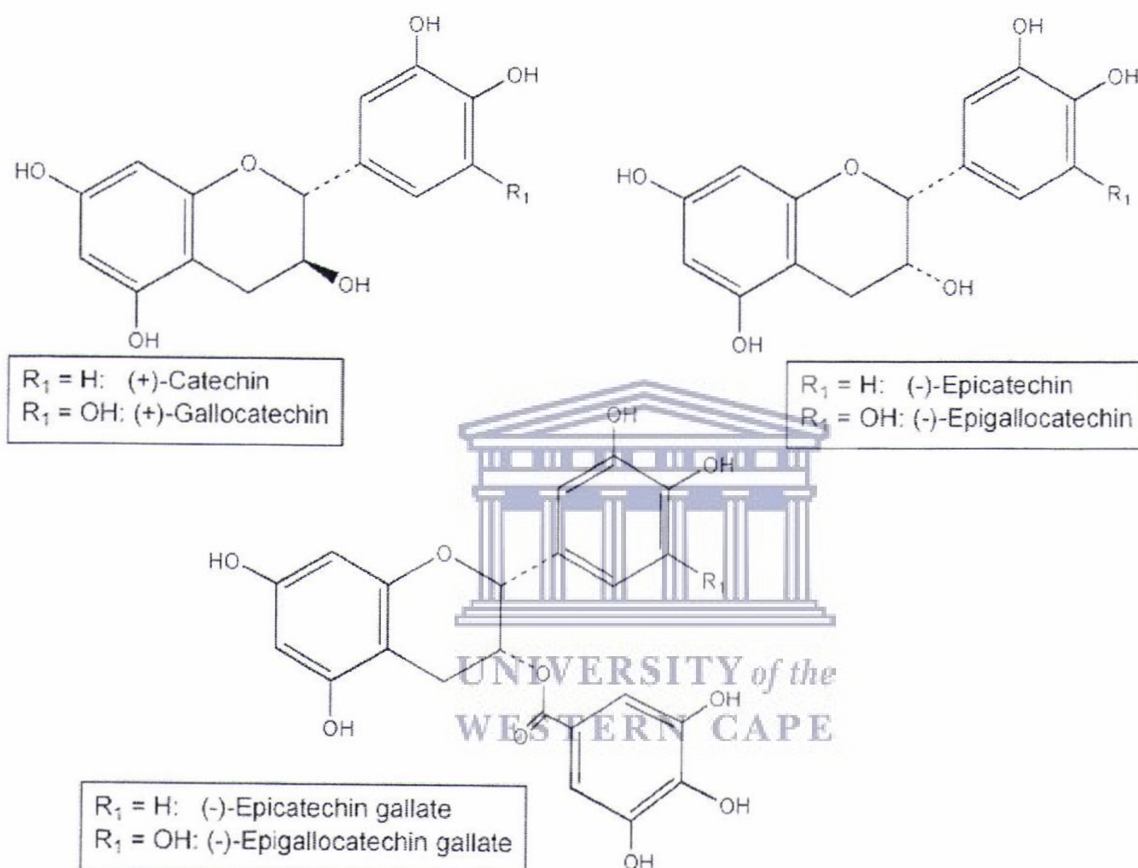

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Fig. 7 Chemical structure of the major green tea catechins (lpi.oregonstate.edu/.../flavcatechins.jpg)

2.9.2.2 Black tea

The pigments of black tea have been divided into orange-red coloured theaflavins (Fig. 8) and brownish thearubigins. Theaflavins are formed from the polymerization of catechins during fermentation (Leung *et al*., 2001). There are four main theaflavins: theaflavin, theaflavin-3-gallate, theaflavin-3'3-gallate, and theaflavin 3,3'-digallate. The thearubigins are a heterogeneous group of phenolic pigments

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with a relatively higher molecular mass and are the principal contributors to the black tea polyphenols (Yang *et al*., 2000).

A typically brewed black tea beverage contains approximately 3-10% (w/w) catechins, 2-6% theaflavins and >20% (w/w) thearubigins of the tea soluble solids per dry weight (Balentine *et al*., 1997; Wiseman *et al*., 1997; Lambert and Yang, 2003). Theaflavins are extracted into an organic solvent, while thearubigins remain in the aqueous phase. Theafulvins are generally isolated from thearubigins and eluted by 50% acetone in water (Dhawan *et al*., 2002).

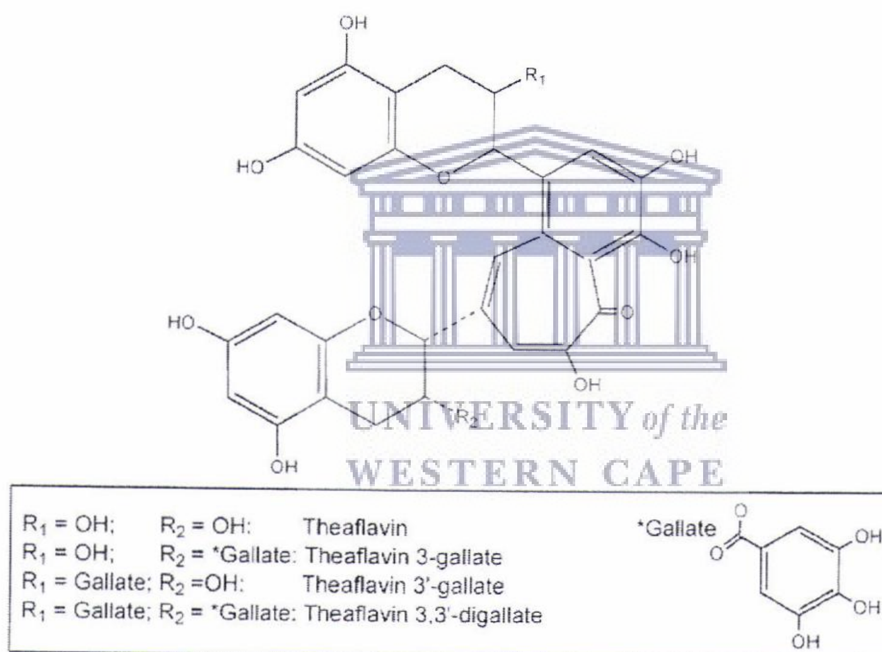

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Fig. 8 Chemical structure of major black tea theaflavins
(lpi.oregonstate.edu/.../tea/flavthea.jpg)

2.9.2.3 Health benefits of green and black teas

In traditional Chinese and Indian medicine, green tea has been used as a stimulant, a diuretic (to promote the excretion of urine), an astringent (to control bleeding and help heal wounds), and to improve heart health (Liao *et al*., 2001; McKay and Blumberg, 2002). Other traditional uses of green tea include treating flatulence, regulating body temperature and blood sugar, promoting digestion and

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improving mental processes. Green tea and/or green tea extracts have also been used to combat cardiovascular disease, cancer, oral health, bone health, thermogenesis, cognitive function, iron status, kidney stones, diabetes, immune responses, antibacterial and antiviral activity, dermatological conditions and various others (Liao *et al.*, 2001; McKay and Blumberg, 2002). Many of these benefits are ascribed to the antioxidant properties and free radical scavenging ability of green and black teas.

Both green and black teas have been shown to contain similar amounts of flavonoids, but differ in chemical structure (Balentine *et al.*, 1997; Wiseman *et al.*, 1997; Yang *et al.*, 2000; Leung *et al.*, 2001), yet they both have been shown to possess possible health benefits.

2.9.3 Biological activity of tea polyphenols

The possible health benefits of green tea are attributed to the catechins. These green tea catechins have antioxidant activity, and may also have anticarcinogenic, anti-inflammatory, anti-atherogenic, thermogenic and antimicrobial activities (Yang and Wang, 1993; Katiyar and Mukhtar, 1997; Gupta *et al.*, 2002). Green tea catechins have also been found to up regulate the level of some hepatic phase II enzymes that are involved in the detoxification of some xenobiotics, including chemical carcinogens (Conney *et al.*, 1999). The tea flavanols interact with cellular signal pathways controlling proliferation, differentiation and apoptosis of various tumour cells (Gee and Johnson, 2001).

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2.9.3.1 Antioxidant activity

The role of antioxidants has attracted much interest with respect to their protective role against free radical damage that may be the cause of many diseases, including cancer (Bradford, 1976). The catechins interact with numerous structurally unrelated molecules and may alter cellular mechanisms, cell membranes, proteins, lipids, nucleic acids as well as cellular factors important for normal and abnormal cell growth that are the key to cancer development and

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metastasis. Some of these interactions may be responsible for the anticarcinogenic and antimutagenic properties of green and black teas (Wang *et al.*, 1988; Wang *et al.*, 1989). A number of *in vitro* studies using different assays have reported on the antioxidant activity of green, black and oolong teas, as well as their respective phenolic constituents (Rice-Evans, 1999). Studies conducted showed that certain constituents of green and black teas are responsible for its antioxidant activity (Vinson and Dabbagh 1998; Azam *et al.*, 2004) and that the pure catechins and phenolic acids were found to exert a greater effect than vitamin C, E and *ß*-carotene in an *in vitro* lipoprotein oxidation model. The antioxidant activity of the catechins using the Rancimat method varies in the order EGCG > EGC > ECG > EC, with EGCG being the single strongest antioxidant as well as the major catechin of green tea (Ho *et al.*, 1992).

Serafini *et al.* (1994) were of the very first groups to show, in an *in vivo* study, that both green and black teas increased the plasma antioxidant capacity of healthy adults. The *in vitro* antioxidant capacity of the various teas and their constituents is dependent on the type of assay used and does not reflect factors such as bioavailability and metabolism. The fact that catechins are metabolized (Lee *et al.*, 2002; Crespy and Williamson, 2004) could play an important role in the *in vivo* antioxidant assay.

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2.9.3.2 Antimutagenicity

An investigation by Muto et al. (2001) demonstrated that green tea constituents, especially epicatechin gallate (ECG) and epigallocatechin gallate, inhibited the metabolic activation of benzo[*a*]pyrene (B[*a*]P), 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) and aflatoxin B_1 (AFB_1) by human cytochrome P450s (CYPs) in the *Salmonella typhimurium* mutagenesis assays. In another study the antimutagenic effect of tea extracts was investigated using the *Salmonella typhimurium* strains TA98 and TA100 against five indirect mutagens, aflatoxin B_1 (AFB_1), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-b)indole (Trp-P-1), 2-amino-6-methyldipurido[1,2-*a*:3',2'-d]imidazole (Glu-P-1), benzo[*a*]pyrene (B[*a*]P), and 2-

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amino-3-methylimidazo[4,5-f]quinoline (IQ) (Yen and Chen, 1995). In general, the semi-fermented teas (oolong and pouching) displayed a greater antimutagenic effect than that of the other teas for some mutagens. Green tea exhibited the strongest inhibitory effect on IQ and Glu-P-1 toward both strains. Concerning the strains TA98 and TA100, black tea exerted the weakest inhibitory activity against the five mutagens studied. Green tea extract, as well as (+)-catechin and (-)-epicatechin were investigated for their antioxidant activity, and was also tested against mutagenesis using the tester strain *Salmonella typhimurium* TA102. Although all three agents possess antimutagenic activity, (-)-epicatechin was shown to exert greater activity when compared with the other two agents (Geetha *et al.*, 2004). Eight brands of teas derived from *Camellia sinensis* examined for inhibitory or potentiating effects on the mutagenicity of heterocyclic aromatic amines (HAA) utilizing the *Salmonella typhimurium* assay (Starvric *et al.*, 1994) displayed potent antimutagenic effects against most HAA.

The antimutagenic activity of theafulvins, isolated from black tea, demonstrated a dose-dependent inhibition of most of the food mutagens tested, presumably by inhibiting the cytochrome P450-dependent bioactivation (Catterall *et al.*, 1998). A more recent study showed that theafulvins display a biphasic response in the Ames test against aflatoxin B_1 (Catterall *et al.*, 2003). It was shown that theafulvins either inhibited the microsomal activation of AFB_1 or potentiated the mutagenic activity of AFB_1 in a dose-dependent manner in the presence of the S-9 activation system. The latter was ascribed to the inhibition of the phase II enzymes by theafulvins resulting in the incomplete deactivation of the mutagenic intermediate, aflatoxin B_1 8,9-oxide.

2.9.4 Carcinogenesis studies using *Camellia sinensis*

Many *in vivo* studies in rodents were conducted with aqueous extracts or phenolic fractions of green and black teas to investigate the possible effects of tea against cancer development. These studies showed that tea protected against many types of cancers in skin, lung, oesophagus, stomach, liver, small intestine, pancreas,

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colon, bladder, prostate and mammary gland (Han and Xu, 1990; Hirose *et al.*, 1993; Katiyar and Mukhtar, 1996; Yang and Wang, 1993).

2.9.4.1 Animal studies

Tea and its polyphenolic constituents inhibit chemically induced carcinogenesis in various animal models. Many studies demonstrated that the topical application or oral feeding of phenolic extracts and individual catechin derivatives of green and black teas have anticarcinogenic activities on skin (Katiyar and Mukhtar, 1996; Lu *et al.*, 1997; Liu *et al.*, 1998). In addition to the preventive effects against skin carcinogenesis, green and black tea polyphenols were effective against chemically induced carcinogenesis of various other organs. In particular, green tea inhibited N-nitromethylbenzylamine (NMBA)-induced rat oesophageal tumourigenesis (Wang *et al.*, 1995). Whole extracts of black tea were also shown to be inhibitory in this model system when given either during or after NMBA administration (Xu and Han, 1990; Wang *et al.*, 1995; Morse *et al.*, 1997). A study conducted by Morse *et al.* (1997) reported that theaflavins significantly reduced NMBA-induced oesophageal tumour formation. Other studies showed that high doses of black or green tea polyphenols and/or theaflavins and EGCG reduced the tumour multiplicity (number and size of papillomas in each rat), but not the oesophageal cancer incidence when orally administered to rats (Yang and Wang, 1993; Steele *et al.*, 2000).

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2.9.4.2 Studies in humans

Several human epidemiological studies indicated that green tea may possess anti-carcinogenic properties (Katiyar and Mukhtar, 1996), though studies on the relation between tea consumption and altered cancer risk have been inconsistent, making a general conclusion regarding the relationship between tea consumption and cancer prevention difficult (IARC, 1991; Yang and Wang, 1993). The inconsistency in findings may be due to the lack of proper study design, difference in lifestyle habits, type of tea and duration of tea consumption and/or other potential confounding factors (Blot *et al.*, 1994; Hakim *et al.*, 2000). For example, in most

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parts of Europe and parts of the United States black tea is more commonly consumed, while in some Asian countries, Japan and China, mostly green tea is consumed.

Although the mechanisms involved in the biological effects of green and black teas are unclear and far from complete, their exact mechanism of action at cellular level needs to be understood, as they are important in the design of strategies for the prevention and treatment of cancer.

2.10 *Aspalathus linearis* (rooibos tea)

2.10.1 Origin and processing

Aspalathus linearis is endemic to the Cedarberg region of the Western Cape, about 250 kilometers north of Cape Town, South Africa (Fig. 9), and is totally unrelated to the tea plant, *Camellia sinensis*.

Fig. 9 Map of the Cedarberg region (red) of the Western Cape, South Africa. (Rooibos Ltd, Clanwilliam, South Africa).

Indigenous inhabitants of the mountainous regions of the Western Cape in South Africa were the first to collect wild rooibos and use it to make a beverage almost 300 years ago (Joubert and Schulz, 2006). These people discovered that they could brew a sweet, tasty infusion from rooibos leaves and stems that they cut, bruised with wooden hammers, fermented in heaps, and then sun-dried.

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Aspalathus linearis has a single basal stem that divides just above the ground surface into multiple thin branches that carry bright green needle-like leaves, and produces small yellow flowers in spring through early summer (Fig. 10) (http://www.rooibosltd.co.za). From here it is processed, packaged and dispatched worldwide.

Fig. 10 Rooibos, a fynbos species endemic to the Clanwilliam/Cedarberg area. (www.clanwilliam.info/ info/images/rooibos1.jpg)

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At present, two types of rooibos tea can be produced depending on the processing methods. During the fermentation of rooibos tea, the leaves are oxidized, lending to the distinctive red colour, aroma and flavour of the tea. The cut leaves and stems are wetted, bruised between rollers to induce chemical oxidation or "fermentation" and left in heaps. After the oxidation process, which lasts between 12-24 hours depending on the tea itself and the temperature of the heap, the cuttings are thinly spread out in the hot sunlight to dry. Further processing is carried out at the factory. This includes sieving, grading, steam pasteurization and drying over a hot airbed dryer. The end product is subject to strict microbiological tests before it is made commercially available. The rooibos is then weighed, packed, either in tea bags or in loose-leaf form, and marketed under various brand names (http://www.rooibosltd.co.za).

The second method of processing this herbal beverage yields the unfermented or "green" form. This is manufactured by immediately drying the harvested plant material in an oven before cutting it into pieces and sieving it, followed by factory processing. This method prevents the chemical oxidation that takes place during

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the fermenting process. A minimally oxidized product, known as "green" rooibos has recently come onto the local and international markets (Joubert and Schulz, 2006).

2.10.2 Anecdotal health claims

In 1968 a young South African mother, whose baby was allergic to milk and suffered with colic, fed her baby rooibos tea. The baby gained weight, and was cured of the chronic restlessness, vomiting and stomach cramps. She published all her observations in a book: Babies, Allergies and Rooibos Tea in 1974 and communicated the findings through the press and other public forums (Morton, 1983). The antispasmodic activity of the tea was later suggested to be ascribed to two components isolated from processed rooibos tea, quercetin and luteolin (Snykers en Salemi, 1974).

Non-phenolics such as D-pinitol have been said to help lower blood-sugar levels and may be used as an expectorant, while alpha-hydroxy acids, ingredients in beauty preparations and dermatological creams are also found in rooibos (Shindo and Kato, 1991). Rooibos tea has also been claimed to relieve insomnia and calm nervous tension (Ferreira *et al.*, 1997), possibly due to the fact that it does not contain caffeine (Morton, 1983; van Wyk *et al.*, 2002).

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2.10.3 Constituents of rooibos tea

Rooibos tea is known to be a rich source of flavonoids and phenolic acids (von Gadow *et al.*, 1997a; Bramati *et al.*, 2002). The phenolic constituents of rooibos tea differ from that of green and black teas, and are unique in that rooibos tea contains the dihydrochalcones, aspalathin (Fig. 11) (Koeppen and Roux, 1966), and nothofagin (Fig. 11), which is similar in structure to aspalathin. Other flavonols present in rooibos tea include quercetin and the quercetin glycosides, hyperoside and rutin/isoquercitrin (Fig. 12) (Rabe *et al.*, 1994). Rooibos tea also contains flavones that comprise orientin, iso-orientin (Koeppen et al., 1962; Koeppen and Roux, 1965), vitexin, isovitexin (Fig. 13), chrysoeriol (Rabe *et al.*, 1994) and

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luteolin (Fig. 14) (Snyckers & Salemi, 1974). During the fermentation process of rooibos tea, aspalathin is oxidized to dihydro-iso-orientin (Joubert, 1996). The flavanols, (+)-catechin is present, which together with (-)-epicatechin, form the chain-extending units of the rooibos procyanidin type tannin (Joubert and Ferreira, 1996). Phenolic acids present in rooibos include caffeic acid, ferulic acid, p-hydroxybenzoic acid, p-coumaric acid, vanillic acid, protocatechuic acid, and syringic acid (Rabe *et al.*, 1994). The dihydrochalcones aspalathin and nothofagin have been identified as the major polyphenols in unfermented rooibos tea (Joubert, 1996).

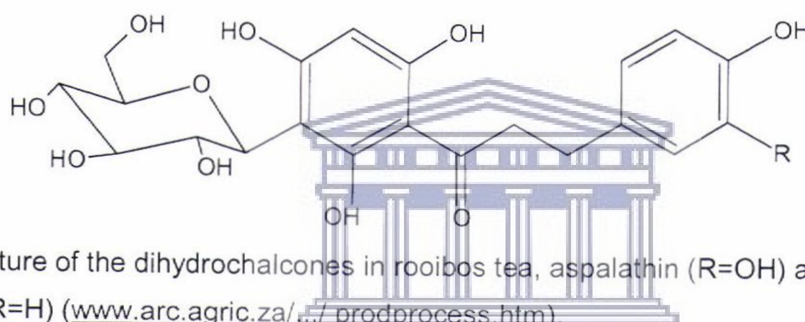

Fig. 11 Structure of the dihydrochalcones in rooibos tea, aspalathin (R=OH) and nothofagin (R=H) (www.arc.agric.za/.../ prodprocess.htm).

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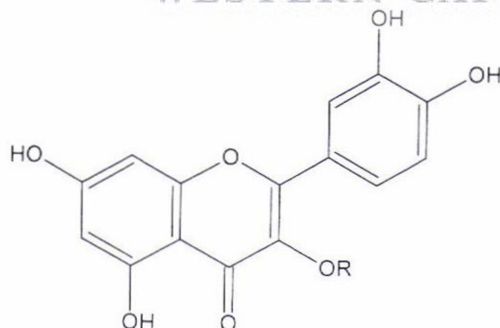

Fig. 12 Structure of quercetin (R=H), isoquercitrin (R= β-D-glucopyranosyl and rutin (R= rutinosyl).

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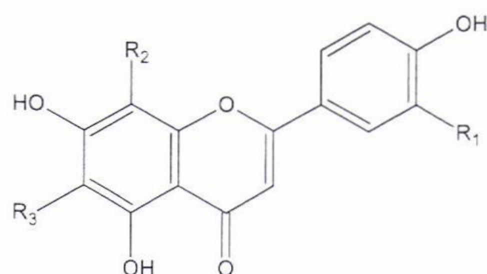

Fig. 13 Structure of iso-orientin [R_1=OH, R_3= glycopyranosyl (glucose)]; isovitexin [R_3= glycopyranosyl (glucose)]; orientin [R_1= OH, R_2=glycopyranosyl (glucose)] and vitexin [R_2=glycopyranosyl (glucose)].

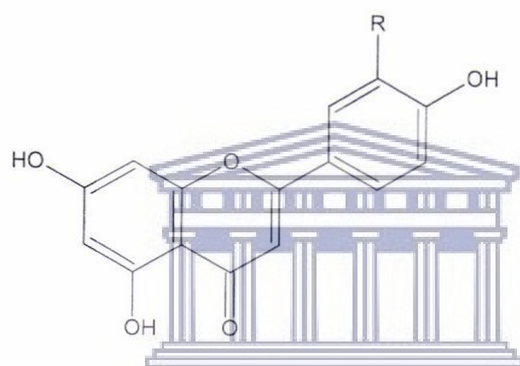

Fig. 14 Structure of luteolin (R=OH), and chrysoeriol (R=OCH_3).

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2.10.4 Biological properties of rooibos tea

2.10.4.1 Antioxidant activity

The antioxidant activity of aqueous extracts in rooibos tea was compared to that of green, oolong and black teas (Von Gadow *et al.*, 1997a) on a water soluble solids basis, utilizing the *a,a*-diphenyl-ß-picrylhydrazyl (DPPH) radical scavenging and ß–carotene bleaching methods. In the DPPH radical scavenging method, antioxidant activity of their water soluble solids decreased in the order: green > unfermented rooibos > fermented rooibos > semi-fermented rooibos > black > oolong, while in the ß–carotene bleaching method, in the order: green > black > oolong > fermented rooibos > unfermented rooibos > semi-fermented rooibos.

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The effect of extraction time on the antioxidant potency of aqueous extracts was monitored utilizing the DPPH radical scavenging and ß–carotene bleaching methods (Von Gadow *et al.*, 1997b). An increased antioxidant activity was noticed as a function of extraction time when measured by the Rancimat method. No effect was noticed when using the ß–carotene bleaching method. The antioxidant activity, when using the Rancimat method, was significantly increased when heating the aqueous extract over a period of 30 minutes. Increasing extraction time resulted in increasing induction time for a fixed ratio of soluble solids to lard. The increasing antioxidative effect can be attributed to compositional changes of the TWSS since a fixed ratio of TWSS to lard was used (Von Gadow *et al.*, 1997c). It was also noted that an ethyl acetate soluble fraction of an aqueous extract displayed a higher antioxidant activity than the resultant water-soluble polymerized polyphenol when using the β-carotene bleaching and Rancimatt methods.

The antioxidant potential of aqueous extracts of rooibos collected at different processing stages was evaluated using the hydrogen donating, as well as the superoxide anion radical scavenging abilities (Standley *et al.*, 2001). The fermentation process reduced the total polyphenolic content, as well as the antioxidant potential of aqueous extracts. Since fermentation lowers the antioxidant levels of rooibos, the development of green rooibos and extracts enriched in aspalathin, with higher antioxidant potency has commenced and is being introduced on the markets (Joubert *et al.*, 2006).

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Joubert *et al.* (2004) compared the antioxidant activity of unfermented rooibos with that of fermented rooibos including an aqueous extract, an ethyl acetate soluble fraction, and a crude polymeric fraction of the infusions by quantifying the hydrogen donating, as well as the superoxide anion radical scavenging capacities. The unfermented extracts and crude fractions were found to be more effective scavengers of DPPH than their fermented counterparts. Fermentation also reduced the superoxide anion scavenging ability of the aqueous extract and ethyl acetate soluble fraction, but not the crude polymeric fraction (Joubert *et al.*, 2004). The

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antioxidant and pro-oxidant activities of aqueous extracts and crude polyphenolic fractions of rooibos was evaluated using a linoleic acid-Tween-buffer emulsion for lipid peroxidation and the deoxyribose degradation assay based on a Fenton reaction model system (Joubert *et al.*, 2005). The inhibition of lipid peroxidation by the samples, with the exception of the ethyl acetate fraction, which had the highest total polyphenol (TPP) content, correlated directly with their TPP content. The pro-oxidant activity of the aqueous extracts and their crude polymeric fractions showed a direct correlation with respect to their dihydrochalcone and flavonoid content when using the deoxyribose degradation assay. The dihydrochalcone content of rooibos has been shown to decrease during fermentation (Joubert *et al.*, 2004; Joubert, 1996), and this contributed to a decrease in the pro-oxidant activity of the aqueous extracts (Joubert *et al.*, 2005).

The antioxidant activity of aspalathin was compared with rooibos flavonoids and phenolic acids as well as the synthetic antioxidants, a-tocopherol, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Von Gadow *et al.*, 1997c). Aspalathin exhibited stronger antioxidant activity than all the phenolic acids when utilizing the ß-carotene bleaching method, but was less effective than a-tocopherol, BHT, and BHA. When using the DPPH radical scavenging method, aspalathin was found to have radical scavenging abilities comparable to a-tocopherol, BHT, and BHA and the phenolic acids, with the exception of caffeic acid. When comparing the antioxidant activity according to the Rancimat method, both BHT and a-tocopherol were poor antioxidants in terms of induction time. Aspalathin displayed a higher antioxidant activity than the phenolic acids, with the exception of caffeic acid. Aspalathin and the other polyphenols present in rooibos tea displayed antioxidant activity comparable to the reference compounds, a-tocopherol, BHT and BHA (Von Gadow *et al.*, 1997c).

The antioxidant activity and protective effect of rooibos tea on DNA strand scission were investigated utilizing various antioxidant assay systems and DNA strand nicking. The 75% ethanol extract displayed higher hydrogen donating capacity and

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scavenging activity of hydrogen peroxide than the water extract, with the exception of the rate constant with hydroxyl radical. While the peroxyl radical-induced DNA strand scission was prevented by both the ethanol and aqueous extracts, the hydroxyl radical-induced DNA strand scission was not (Lee and Jang, 2004).

In an *in vivo* study in rats by Marnewick *et al.* (2003), the antioxidant capacity was evaluated utilizing the oxygen radical absorbance capacity (ORAC). While the hepatic ORAC levels were marginally to significantly reduced by the Chinese green and black teas, no effect was noticed when using rooibos tea. The GSH redox potential was altered as rooibos significantly decreased the GSSG level and markedly increased the concentration of reduced glutathione (GSH) in the liver, resulting in an increased in the GSH/GSSG ratio. These changes were suggested to be indicative of a reduced oxidative status stress in the liver that will protect against free radical damage (Marnewick *et al.*, 2003). In another study Ulicna et al. (2006) showed that the antioxidant constituents of aqueous rooibos tea partially prevented oxidative stress in streptozotocin-induced diabetic rats and can be used as supportive therapy in cases where free radicals are involved.

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The effect of rooibos tea on liver antioxidant status and oxidative stress was investigated in a rat model of carbon tetrachloride (CCl_4)-induced liver damage (Kucharská *et al.*, 2004). After rooibos tea and N-acetyl-L-cysteine (NAC), a synthetic antioxidant, was administered to CCl_4–damaged rats, the concentrations of coenzyme Q_9 (CoQ_9H_2 and CoQ_9) and a–tocopherol were restored in the liver, and the formation of malondialdehyde (MDA) inhibited. The improved regeneration of CoQ_9 may explain the beneficial effects of rooibos, and was suggested to be a safe and effective hepatoprotector in patients with liver disease.

2.10.4.2 Antimutagenic properties

The antimutagenic activity against various mutagens by tea extracts and polyphenols has been demonstrated in microbial systems (*Salmonella typhimurium* and *Escherichia coli*), mammalian cell systems and *in vivo* animal tests (Kuroda

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and Hara, 1999). Research regarding the antimutagenicity and chemopreventive properties of tea has focused mainly on green and black teas, and these studies indicated that polyphenol preparations and/or water extracts from these teas decreased the mutagenicity of various genotoxic carcinogens (Gupta *et al.*, 2002). Aqueous extracts of fermented and unfermented rooibos tea was reported to possess antimutagenic activity against 2-acetylaminofluorene (2-AAF)-induced mutagenesis using strain TA98 in the *Salmonella* mutagenicity assay (Marnewick *et al.*, 2000; Standley, 2001). However, these extracts offered no or only weak protection against the direct-acting mutagens, methylmethane sulfonate (MMS), cumolhydroperoxide (CHP), and hydrogen peroxide (H_2O_2) (Marnewick *et al.*, 2000). A study by van der Merwe *et al.* (2006) showed that, against 2-AAF, fermented rooibos showed similar protective properties than oolong and green teas with a weaker protection when compared to black tea. When using aflatoxin B_1 as a mutagen, fermented rooibos exhibited weaker protection than the *C. sinensis* teas. Unfermented rooibos exhibited weaker protection than fermented rooibos and the *C. sinensis* teas. It should be noted that differences in the antimutagenicity exists with regards to the effect of the tea preparation, the mutagen selected, changes and variations in the phenolic content due to natural plant variation, and differences in the geographical area (van der Merwe *et al.*, 2006).

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Aqueous extracts of unfermented and fermented rooibos significantly enhanced the activities of the hepatic phase II metabolizing enzyme, glutathione-S transferase (Marnewick, *et al.*, 2003). Unfermented rooibos also significantly increased the activity of UDP-glucuronosyl transferase. *Ex vivo* studies showed that the mutagenic response of aflatoxin B_1 (AFB_1) against *Salmonella* strain TA 100 was significantly inhibited by the cytosolic fraction from rats that were treated with fermented and unfermented rooibos teas. Although there was a reduction in the activation of AFB_1 in the microsomal fractions prepared from the livers of rats treated with rooibos tea, no protection was observed against 2-AAF–induced mutagenesis.

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A recent study by Snijman *et al.* (2007) showed that luteolin and chrysoeriol, minor flavonoid constituents of unfermented rooibos exhibited comparable antimutagenic properties against 2-AAF and AFB_1 to the potent green tea flavonoid epigallocatechin gallate. The major rooibos flavonoids, aspalathin and nothofagin and their structural analoques only exhibited moderate protective effects, with an apparent threshold effect over a specific dose range. Other flavonoid mutagen interactions included mutagenic, comutagenic and promutagenic effects.

2.10.4.3 Anti-tumour properties

The inhibition of tumor promotion in mouse skin by an ethanol/acetone (E/A) soluble fraction of a methanol extract of rooibos was studied using 7,12-dimethylbenz[a]anthracene (DMBA) in 12-o-tetradecanoylphorbol-13-acetate (TPA)-induced mice (Marnewick *et al.*, 2005). The rooibos tea fractions have been shown to decrease the tumour volume and the mean number of tumours, as well as causing a delay in the development of tumours (Marnewick *et al.*, 2005). The green tea E/A fraction was more effective than the rooibos extract in suppressing skin tumourigenesis. The green tea E/A fraction also exhibited the highest protective activity against hepatic microsomal lipid peroxidation. Even though evidence has shown that aqueous extracts of rooibos influences the development and size in tumour formation (Marnewick *et al.*, 2005), subsequent studies need to be conducted to elucidate the exact mechanisms involved.

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2.10.4.4 Anti-aging properties

Age-induced deterioration of the central nervous system (CNS) is partly due to the cytotoxic effect of reactive oxygen species (ROS) generated in the brain. Rooibos tea has been shown to exhibit potent antioxidant activity by scavenging oxygen, hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (Yoshikawa *et al.*, 1990; Von Gadow, 1997). The results of a study by Inanami *et al.* (1995) suggested that rooibos flavonoids suppressed the accumulation of lipid peroxides in the brain associated with the aging process, and chronic administration of

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rooibos tea prevented the accumulation of lipid peroxides, associated with age, in several regions of rat brain.

2.10.4.5 Antihemolytic effect

An investigation was conducted in Japanese quails to determine whether rooibos tea supplement and/or rooibos extracts protected erythrocytes against cell lysis (Simon *et al.*, 2000). Long-term consumption of rooibos tea had no effect on peroxide or hypotonia-induced hemolysis. However, there was a decrease in peroxide-induced hemolysis of erythrocytes incubated with rooibos and black tea, and no hemolysis induced by hypotonic sodium chloride solution. A hot water extract of rooibos tea exhibited stronger inhibition of hemolysis than a powdered rooibos tea supplemented food.

2.11 *Cyclopia intermedia* (Honeybush tea)

2.11.1 Origin and processing

Honeybush tea (*Cyclopia intermedia*) is a traditional indigenous herbal beverage from South Africa, with the plant belonging to the Cape fynbos biome, growing only in the coastal districts of the Western and Eastern Cape Provinces from Darling to Port Elizabeth (Fig. 15).

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Fig. 15 Map of Western Cape in South Africa, illustrating the regions where honeybush is grown (http://www.itmonline.org/arts/honeybush.htm).

Honeybush, like rooibos is totally unrelated to the tea plant, *Camellia sinensis*.

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The earliest mention of the honeybush plant in botanical literature was in 1705 (Kies, 1951). Dutch and British colonists in the Cape recognized honeybush as a suitable substitute for black tea, probably based on native practices for the treatment of coughs and other upper respiratory symptoms. Honeybush tea is prepared from the leaves, stems and flowers of several species of *Cyclopia* including *C. sessiliflora*, *C. genistoides*, *C. subternata* and *C.* (Du Toit *et al.*, 1998) (Fig. 16).

Fig. 16 Photograph taken in a honeybush plantation (www.rooibostea.co.za/ images/asnapp-logo.jpg).

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As with rooibos tea, honeybush tea is manufactured in both the fermented and unfermented forms. The manufacture of the fermented tea includes four main steps: harvesting, shredding, oxidation (fermentation) and drying. The shredding disrupts the cellular integrity to facilitate the oxidation process (http://www.rooibos.ch/honeybush.html).

Oven oxidation is the preferred method, since it yields a product of greater consistency and better quality. A preheated oven is used and the oxidation process is somewhat shorter (24-36 hours). The final product is then sieved to remove the bigger pieces, steam pasteurized and bulk-packed for local and international markets. Honeybush tea is traditionally a very coarse product, with the finer tea material used for teabags and the coarser material for brewing loose tea (http://www.rooibos.ch/honeybush.html).

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The unfermented from of honeybush tea is obtained by immediate drying of the raw cut plant material in a drying tunnel of approximately 40°C for twelve hours, followed by sieving and pulverization (http://www.rooibos.ch/honeybush.html).

2.11.2 Anecdotal health claims

Like rooibos, honeybush tea also has very low tannin content and contains no caffeine (Greenish, 1881; Terblanche, 1982). Traditionally, honeybush tea has been used as a diuretic, appetite stimulant, treatment for colic babies, a cough syrup, a stimulator of milk production in lactating women, and for prevention of stomach ulcers (Du Toit *et al.*, 1998). High levels of (+)-pinitol, which is useful as an expectorant (Beecher *et al*, 2003), have been shown to be present in honeybush (Ferreira *et al.*, 1998; Kamara *et al.*, 2004). Phytoestrogenic activity has been associated with isoflavonoids, coumestans as well as the flavonoids groups, flavones and flavanones (Narayyanan *et al.*, 1987; Miksicek, 1995), all of which have been shown to be present in *C. intermedia*. Phytoestrogens are used in the treatment of menopausal symptoms in women (Chiechi, 1999).

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2.11.3 Phenolic constituents of Cyclopia

Honeybush tea differs from rooibos in the major classes of phenolic compounds present, some of which include mangiferin, isomangiferin (Fig. 17), hesperetin, naringenin, hesperidin, narirutin, eriodictyol, and eriocitrin (Fig. 18). De Nysschen *et al.*, (1996) have shown that mangiferin, hesperidin and isosakuranetin are the major compounds present in most of the *Cyclopia* species. Mangiferin and hesperidin have recently been quantified and identified as the major compounds from unfermented *C. intermedia*, *C. sessiliflora* and *C. genistoides* (Joubert *et al.*, 2002). The phenolic compositions of the *Cyclopia* species are influenced by a wide range of factors including different samples of the same species, selected from various regions (Joubert *et al.*, 2008).

A methanol extract from the leaves and stems of *Cyclopia intermedia* was shown to contain a phenolic acid, isoflavones, flavanones, coumestans and xanthones

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(Ferreira *et al.*, 1998; Kamara *et al.*, 2004). The flavones, luteolin and (+)-pinitol were also identified to be present in *C. intermedia* (Ferreira *et al.*, 1998). Analysis of acetone and methanol extracts prepared from unfermented *C. subternata* revealed the presence of compounds also found in *C. intermedia* namely (+)-pinitol, hesperidin, luteolin and mangiferin (Kamara *et al.*, 2004).

Although there are differences in phenolic composisition, different *Cyclopia* species may have distinctive biological properties (Richards, 2003).

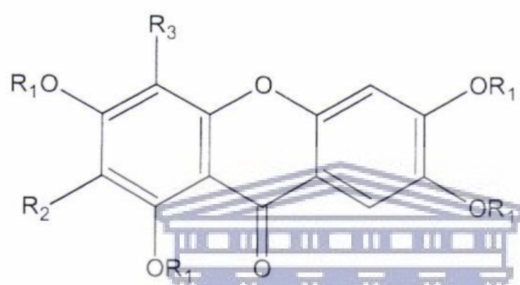

Fig. 17 Structure of mangiferin (mangiferin R_1=R_3=H, R_2=2-β-D-glucopyranosyl) and isomangiferin (R_1=R_2=H, R_3= 2-β-D-glucopyranosyl) (www.wilshiretechnologies.com/ anti.htm)

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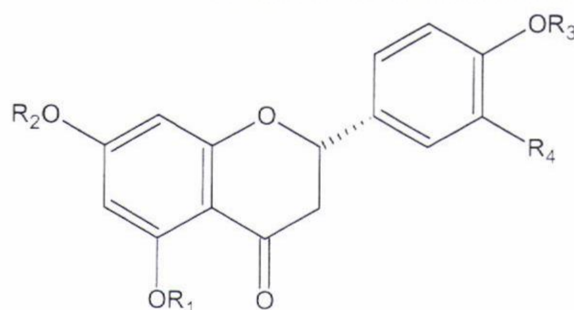

Fig. 18 Structure of hesperetin (R_1=R_2=H, R_3=Me, R_4=OH); hesperidin (R_1=H, R_2 = rutinosyl, R_3=Me, R_4=OH); naringenin (R_1=R_2=R_3=R_4=H); eriodictyol (R_1=R_2=R_3=H, R_4=OH); narirutin (R_1= R_3= R_4, R_2 = rutinosyl); eriocitrin (R_1= R_3= H, R_2 = rutinosyl, R_4=OH) (www.axxora.com/free_ radical_scavengers-LKT-H1)

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2.11.4 Biological activities of *Cyclopia* species

2.11.4.1 Antioxidant properties

Despite the limited scientific data on the biological activities of honeybush tea (Richards, 2003), it has been reported (Hubbe and Joubert, 2000a; Hubbe and Joubert, 2000b) that *C. intermedia*, *C. sessiliflora*, *C. genistoides* and *C. subternata* all has superoxide anion radial ($O_2^{\cdot-}$) scavenging activity, as well as hydrogen donating ability. Aqueous extract of unfermented *C. sessiliflora,* with the highest total polyphenol content, was the most effective scavenger of both the DPPH radical as well as the superoxide anion radicals. The oxidation process during the manufacturing of the tea has been shown to decrease the antioxidant potential of honeybush tea (Hubbe and Joubert, 2000; Richards, 2003). Generally, unfermented *C. intermedia* and *C. sessiliflora* showed the highest antioxidant activity, while *C. genistoides* showed a greater antioxidant activity in the fermented honeybush plant material (Richards, 2003).

In an *in vivo* study, fermented and unfermented *C. intermedia* aqueous extracts has been shown to reduce oxidative stress and enhance the antioxidant capacity of rat liver after chronic exposure (Marnewick *et al.*, 2003). Honeybush tea significantly decreased the level of GSSH in the liver, resulting in an increase of the GSH/GSSH ratio, presumably by stabilizing GSH. The increase in the GSH/GSSH ratio in the liver of the herbal tea-treated rats may be a result of a reduced oxidative stress or an increased antioxidant capacity in the cell, making it less susceptible to oxidative damage (Marnewick *et al.*, 2003). When using the Oxygen Radical Absorbance Capacity (ORAC) assay, no effect was noticed in the liver of rats treated with the honeybush teas (Marnewick *et al.*, 2003).

2.11.4.2 Antimutagenic properties

Aqueous extracts of unfermented *C. intermedia* tea was shown to exhibit antimutagenic activity against two metabolically activated carcinogens, 2-AAF and AFB_1, and also against the direct-acting mutagen, CHP, but no protective effect was found against two other direct-acting mutagens, H_2O_2 and MMS (Marnewick *et*

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al., 2000). Anti-mutagenic activity of aqueous extracts of honeybush tea was compared to that of rooibos, green, oolong and black teas in the *Salmonella* mutagenicity assay using AFB_1 and 2-AAF as mutagens (van der Merwe *et al.*, 2006). *Cyclopia subternata*, *C. genistoides* and *C. sessiliflora* demonstrated protective activity against both mutagens with the exception of unfermented *C. genistoides* against 2-AAF, which had either protective or enhanced mutagenesis depending on the concentration used. Unfermented *C. intermedia* and *C. subternata* showed similar antimutagenic activity against AFB_1 as fermented rooibos, while the latter showed similar protective properties as that of unfermented *C. intermedia* and *C. sessiliflora* when using 2-AAF as mutagen (van der Merwe *et al.*, 2006). Fermented *C. sessiliflora* and *C. subternata* exhibited a higher protective effect than unfermented rooibos when using AFB_1 and 2-AAF as mutagens, respectively. The antimutagenic activity of *Cyclopia* spp. was significantly lower than black tea, while unfermented *C.intermedia* and *C.subternata* exhibited a similar protective effect as green tea against AFB_1-induced mutagenesis. When using 2-AAF, black tea also exhibited a higher protective effect than the honeybush teas, while unfermented *C. intermedia* and *C. sessiliflora* exhibited similar effects as green tea. Of the *Cyclopia* spp., fermented *C. intermedia* and *C. genistoides* exhibited the lowest antimutagenic effect against 2-AAF, while fermented *C. intermedia* showed the lowest protection against AFB_1–induced mutagenesis. Unfermented *C. genistoides* displayed weak antimutagenic activity at a low concentration, while it enhanced the mutagenesis of 2-AAF at higher concentrations. There was no mutagenic response by itself in the absence or presence of metabolic activation when using tester strain TA98 (van der Merwe *et al.*, 2006).

Hesperidin, eriodictyol and narirutin, constituents of *Cyclopia* species, enhanced the mutagenicity of 2-AAF, which could explain the pro-mutagenic effect of aqueous extracts of unfermented *C. genistoides* on 2-AAF mutagenesis. The anti-mutagenic activity of the herbal teas was shown to be mutagen-specific, and proposed to be affected by fermentation, plant type, plant age, time harvested and

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the geographical location, presumably as a result of changes and/or variation in phenolic composition (van der Merwe, 2006).

Similar to rooibos, aqueous extracts of fermented and unfermented honeybush also enhanced the activity of hepatic glutathione-S transferase and marginally increased the activity of the UDP-glucuronosyl transferase (Marnewick, *et al.*, 2003). *Ex vivo* studies showed that liver cytosolic fractions obtained from rats exposed to aqueous extracts of fermented and unfermented *C. intermedia* inhibited the mutagenic response of aflatoxin B_1 (AFB_1) against *Salmonella* strain TA 100. Liver microsomal preparations of rats treated with unfermented honeybush tea also have a reduced ability to activate AFB_1 to its active mutagenic metabolite (Marnewick *et al.*, 2004).

2.11.4.3 Inhibition of tumuor promotion

In a recent study, topical application of an ethanol/acetone (E/A) soluble fraction of a methanol extract of honeybush tea was shown to protect against TPA-induced tumour promotion in mouse skin (Marnewick et al., 2005). The extract decreased the tumour volume, the mean number of tumours, as well as delaying the onset of tumour development. Similar to the rooibos extract, honeybush exhibited a weaker protection against skin tumour formation than the green tea extract. However, the honeybush teas exhibited a better protection than the rooibos teas.

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2.12 *Sutherlandia frutescens* subspecies *microphylla*

2.12.1 Origin

Sutherlandia frutescens subspecies *microphylla* (unwele in Zulu; kankerbos in Afrikaans; cancer bush in English) is a perennial, woody shrub that is native to South Africa (Tai *et al.*, 2004) (Figure 19). *Sutherlandia frutescens* occurs naturally throughout the dry parts of southern Africa, in Western Cape and up the west coast as far north as Namibia and into Botswana, and in the western Karoo to Eastern Cape. The genus *Sutherlandia* includes six species that can be reduced to two, namely *Sutherlandia frutescens* and *Sutherlandia tomentosa*. *Sutherlandia*

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frutescens is further divided into three subspecies, namely subsp. *frutescens,* subsp. *microphylla,* and subsp. *speciosa*.

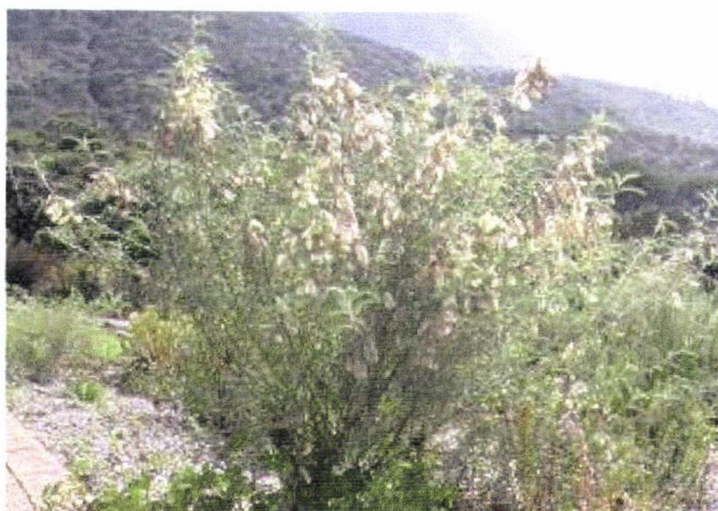

Fig. 19 Sutherlandia bush with dry pods (www.plantzafrica.com/.../ lessertbush.jpg).

2.12.2 Anecdotal health claims

The medicinal use of the plant may have originated with the Khoi and Nama people, who used decoctions, externally to wash wounds, and internally for fevers and a variety of ailments. The leaves are mainly used for medicinal purposes, but the stems are also included when making the infusion. *Sutherlandia frutescens* has been used as an old Cape remedy for stomach problems and internal cancers (van Wyk *et al*., 1997; van Wyk and Gericke, 2000). Currently, *Sutherlandia frutescens* is available in tablet or pressed leaf powder form. *Sutherlandia frutescens* has been reported to have diverse medicinal uses that include the treatment of inflammation and viral diseases (Rood, 1994), but there has been no scientific documentation on the pharmacology, biochemistry and mechanism of action of this plant (Fernandes *et al*., 2004). Although scientific evidence supporting the anecdotal health claims regarding *Sutherlandia frutescens* is both limited and conflicting, studies are ongoing to establish affirmative data.

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2.12.3 Constituents of *Sutherlandia frutescens*

The main constituents of *Sutherlandia frutescens* thought to be active include L-canavanine (Fig. 20), a non-protein amino acid, D-pinitol (Fig. 21), an inositol found

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in many types of legumes, L-arginine, an antagonist of L-canavanine (Fig. 22), and gamma-amino butyric acid (GABA) (Fig. 23) an amino acid analogue, (Gericke *et al.*, 2001).

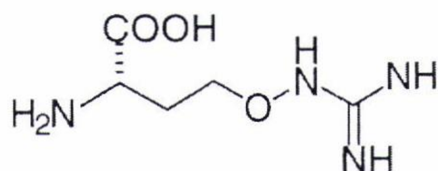

Fig. 20 Structure of the L-canavanine molecule (http://aem.asm.org/cgi/content/full/64/12/4683).

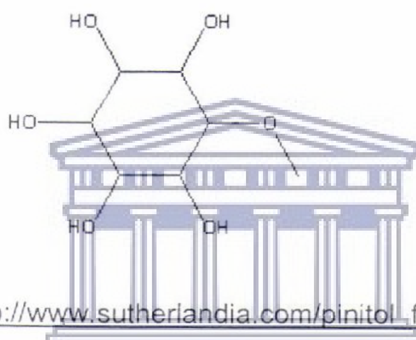

Fig. 21 Structure of D-pinitol (http://www.sutherlandia.com/pinitol_frame.htm).

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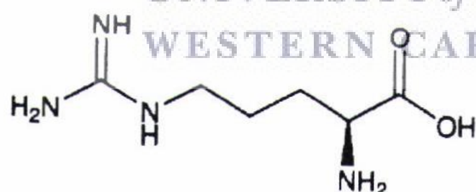

Fig. 22 Structure of L-arginine (http://morelife.org/figures/arginine.jpg)

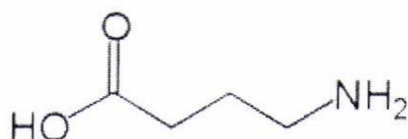

Fig. 23 Structure of gamma-amino butyric acid (http://en.wikipedia.org/wiki/GABA)

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Other minor bioactive compounds include asparagines and secondary plant metabolites such as saponin, methyl and propyl parabens, a preservative combination with anti-microbial abilities that prevent the formation of bacteria, hexadecanoic acid, gamma sitosterol, sigmast-4- en -3-one and several long chain alcohols (Tai *et al.*, 2004).

2.12.4 Biological properties

Aqueous extracts of *Sutherlandia frutescens* have been reported to have anti-proliferative effects on cancer cells (van Wyk *et al.*, 2002; Tai *et al.*, 2004). Chinkwo (2005) have shown that *Sutherlandia frutescens* extracts induced apoptosis in cancer cells when compared to staurosporine and ceramide. L-canavanine is believed to have antibacterial, antifungal, and antiviral activity (Rosenthal, 1997), as well as anti-tumour properties, (Southon, 1994; Swaffar *et al.*, 1994). L-arginine is an antagonist of L-canavanine regarding the anti-proliferating properties of the latter. L-arginine exhibits antiviral properties by contributing to nitric oxide (NO) synthesis and enhanced oxidative injury. GABA exhibits neurotransmitter inhibitory action, while D-pinitol has antiinflamatory properties (Sia, 2004; Tai *et al.*, 2004).

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2.12.4.1 Antioxidant properties

Utilizing a cell free system, *S. frutescens* was found to possess superoxide and hydrogen peroxide scavenging activities, which could account for the anti-inflammatory properties (see section 2.12.4.3) (Fernandes *et al.*, 2004).

Katerere and Eloff (2005) investigated the antioxidant activity of *S. frutescens* using the DPPH assay on thin layer chromatography. Different extracts of various solvents, and two different extraction methods were prepared. The strongest free radical scavenging activity was obtained with extracts prepared when using more polar solvents such as ethanol, acetone and water.

Sutherlandia demonstrated antioxidant activity in the ABTS hydroxyl radical cation scavenging assay, using Trolox as antioxidant reference. The ethanolic extract had

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radical scavenging activity when compared to Trolox. In murine macrophages/monocytes (Tai et al., 2004), the ethanolic extract of *Sutherlandia*, pinitol and canavanine inhibited lipopolysaccharide (LPS)-activated NO production. Although the ethanolic extract did not significantly stimulate NO production in the cells not treated with LPS, Tai *et al*. (2004) observed a significant increase in NO production in the cells treated with 1 µg/ml of LPS.

2.12.4.2 Anti-mutagenic properties

Reid et al. (2006) investigated the mutagenic and antimutagenic effects of *S. frutescens* were in the *Salmonella typhimurium* assay. The study was conducted in the presence of a liver metabolizing system (S-9) using 2-aminoanthracene for both TA98 and TA100. Sodium azide and 4-nitroquiniline -1-oxide were used against TA100 and TA98 in the absence of S-9, respectively. Dichloromethane extracts of *S. frutescens* exhibited antimutagenic properties against TA98 and TA100 in the presence of S9, while a 90% methanol extract showed no activity against 2-aminoanthracene-induced mutagenesis towards TA98 and TA100.

2.12.4.3 Anti-inflammatory properties

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Numerous anti-inflammatory agents have been shown to exert chemopreventive activity by targeting cycloxygenase (COX)-2, a key enzyme involved in inflammation. A methanol extract of *S. frutescens* inhibited 12-O-tetradecanoylphorbos-13-acetate (TPA)-induced COX-2 expression in human breast epithelial cells and in mouse skin *in vivo* (Na *et al*., 2004). The methanol extract also inhibited the DNA binding of NF-κB, a transcription factor regulating COX-2 expression by TPA, in a dose-dependent manner. A study by Kundu et al. (2005) showed that topical application of a methanol extract of *S. frutescens* inhibited TPA-induced COX-2 expression in mouse skin. The anti-inflammatory effects of a hot water extract of *S. frutescens* were demonstrated against the formation of reactive oxygen species in neutrophils affected by L-formyl-L_methionyl-L-leucyl-L-phenylalanine (Fernandes *et al*., 2004). An aqueous *Sutherlandia* shoot extract inhibited the albumin-induced acute inflammation in

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mice. Secondary metabolites and/or other chemical constituents are suggested to account for the anti-inflammatory effect (Na *et al.*, 2004).

It must be noted that different preparations of *S. frutescens* (fresh, whole plant extracts; single compound extracts; or tablets containing raw herb powder) have been used in investigations using *S. frutescens*, and these variations in chemical composition may have contributed to variations in results (Sia, 2004). It is possible that the individual compounds found in *S. frutescens* may be acting synergistically in the human physiological system, thereby mediating greater clinical benefit than is obtained by single compounds.

Summary

Frequently used plants in traditional medicine are assumed to be safe due to their long-term use (Elgorashi *et al.*, 2002) and are considered to have no side effects because they are "natural" (Popat *et al.*, 2001). The medicinal uses of many of these plants have been passed down from generation to generation, and are based mostly on anecdotal evidence, thus having no scientific substantiation.

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Natural antioxidants are ideal ingredients for inclusion into chemopreventive agents since they play a role in reducing oxidative stress associated with DNA damage and degenerative diseases such as atherosclerosis, coronary heart disease, cancer and acceleration of the ageing process (Richards, 2003).

Tea contains high amounts of flavonoids such as flavanols, flavonols, theaflavins and thearubigins (Balentine *et al.*, 1997), and a number of epidemiological studies have linked tea consumption, mostly green tea, to a reduced risk of cancer in humans. The effect of tea on carcinogenesis in animal models is unique, as no other agent tested for possible chemopreventive effects has shown such efficacy at the concentrations usually consumed by humans (Dreosti *et al.*, 1997).

Although no epidemiological studies have been conducted on rooibos, honeybush and *Sutherlandia*, the physiological effect demonstrated by these herbal infusions

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in some *in vitro* and *in vivo* systems are convincing. To establish the mechanisms of action of the infusions and/or active constituents, sound scientific evidence is necessary in order to substantiate anecdotal claims with the aim of developing and implementing successful chemopreventive strategies. In addition to being indigenous to South Africa these plants and/or their extracts provide a natural source of compounds that may protect against various diseases.

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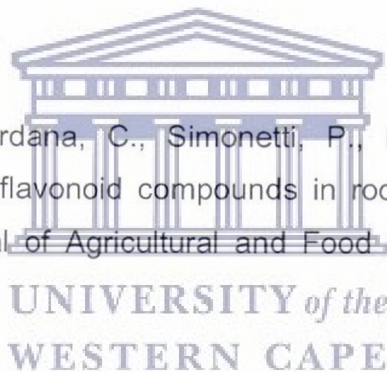
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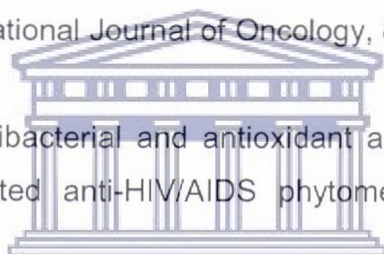

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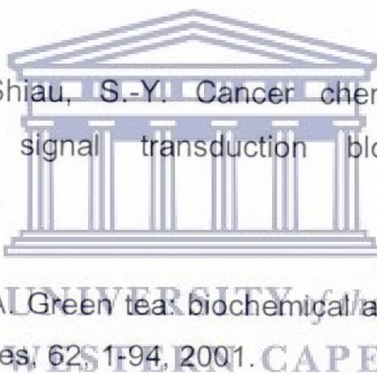

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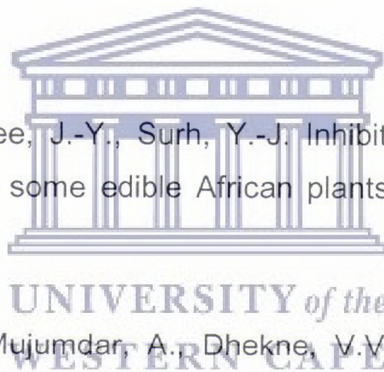
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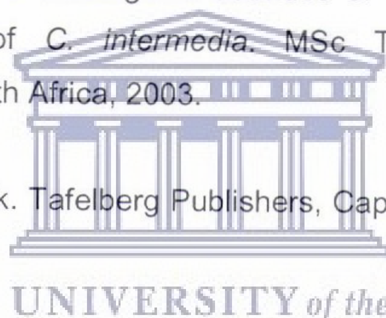
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CHAPTER 3

Modulating effect of rooibos and honeybush teas, and a *Sutherlandia* infusion in the development of oesophageal cancer in male Fischer F344 rats

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Abstract

The modulating properties of various teas and a herbal infusion were assessed in a rat (Fischer 344) oesophageal cancer model utilising the site-specific carcinogen, N-methylbenzylnitrosamine (MBN). Male rats were randomly divided into ten experimental groups and subcutaneously injected with MBN, after which the treatment groups were given preparations of green and black teas (*Camellia sinensis*); unfermented and fermented rooibos teas (*Aspalathus linearis*); unfermented and fermented honeybush teas (*Cyclopia intermedia*); and an infusion of *Sutherlandia frutescens* subspecies *microphylla* as their sole source of drinking fluid for twenty-five weeks. A methanol extract of unfermented rooibos tea (RgM) extract was also prepared. The control groups received normal tap water. Detailed tea intake profiles and body weight gain parameters were collected and the total polyphenol (TPP), flavanol and flavonol content of the different teas and infusions determined, while the major monomeric polyphenols of the herbal teas were quantified by HPLC. After 25 weeks the rats were terminated, and the number and size of oesophageal papillomas determined. Ninety to 100% of the MBN control and the MBN treated rats receiving the different teas and the *Sutherlandia* infusion developed papillomas. None of the rats receiving the solvent control developed papillomas. Depending on the different size categories, *Camellia sinensis* teas, as well as the herbal teas significantly ($P<0.05$) reduced the mean total number of papillomas. Rats receiving unfermented rooibos, unfermented honeybush, and fermented honeybush failed to developed larger papillomas (>10 and >20 mm^3), while green tea and the *Sutherlandia* infusion failed to develop papillomas greater than 20 mm^3. When considering the mean papilloma size, only unfermented rooibos and honeybush teas exhibited a marginal reduction. A total polyphenol (TPP) intake (>7 mg/100 g body weight) and a specific ratio of polyphenolic subgroups appear to be important in the chemopreventive properties of the *Camellia sinensis* and herbal teas. Modulation of the growth kinetics of these early neoplastic lesions by the herbal infusions, presumably the polyphenolic constituents, is likely to reduce their proliferative capacity.

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Keywords: Oesophageal cancer, chemoprevention, rooibos, honeybush, N-methylbenzylnitrosamine, papillomas.

3.1 Introduction

Oesophageal cancer ranks the ninth most common malignancy in the world and recent evidence has shown that its incidence is increasing (McCabe and Dlamini, 2004). The incidence of oesophageal cancer occurs in different geographical areas worldwide (Sewram *et al.*, 2003). Although epidemiological data suggests that the major causative factor for the incidence of cancer, in general, is the environment (Safdar *et al.*, 2003), the occurrence and development of oesophageal squamous cell carcinoma (SCC) is a result of interactions between environmental and genetic factors (Stoner *et al.*, 2001).

Certain mycotoxins, which are secondary metabolites produced by fungi, have been associated with disease conditions among rural populations around the world and, more recently, fumonisin B_1 (FB_1) has been implicated in the etiology of oesophageal cancer in the former Transkei, South Africa (Marasas *et al.*, 1981 and 1988; Rheeder *et al.*, 1992; Chelule *et al.*, 2001; Myburgh *et al.*, 2002). Exposure to mycotoxins present in food and feed crops has been recognized as a serious threat to human and animal health (Miller and Marasas, 2002). As maize is the staple diet of the South African rural population, mycotoxin contamination is associated with an increased risk of developing cancer (Marasas *et al.*, 1981 and 1988; Rheeder *et al.*, 1992; Chelule *et al.*, 2001).

Another risk factor for oesophageal cancer is the formation of nitrosamines via a chemical reaction between nitrosating agents and nitrosatable amines. Various constituents in food can affect nitrosamine formation, for example, nitrosodimethylamine is formed as a result of the direct-fire drying process (Scanlan, 1983). Many foods, such as cured meats, some cheeses, primarily cooked bacon and sometimes fish, have been shown to contain volatile nitrosamines (Sen *et al.*, 1972; Gough *et al.*, 1976; Gough *et al.*, 1977; Fiddler *et*

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al., 1978). N-nitroso compounds are formed in the human body even when levels of precursors that are considered to be normal are ingested (Bartsch and Montesano, 1984). Nitrosamines and other nitroso-compounds are known to induce oesophageal and other cancers in experimental animals, and the occurrence of these compounds and their precursors in water and food samples has been investigated (Yang, 1980). Traditionally, most people relied on water from dry wells or man-made ponds, often contaminated with microorganisms, as well as human and animal waste. In the mid-1970's nitrate and nitrite were found in most of the drinking water samples, collected from random wells in a high-incidence area in China. When food (rice, corn, millet, sweet potato) samples from high- and low-incidence areas were analyzed for nitrosamines, a substantial amount (29 out of 124 samples) from a high-incidence area tested positive in the nitrosamine assay, whereas only one of eighty-six samples tested positive in a low-incidence area. The diet in most parts of China, especially in high-incidence areas is simple, consisting mainly of corn, wheat, millet, rice and some seasonal vegetables (Yang, 1980). Most families cook on coal stoves and, due to poor ventilation, the inhabitants inhale the smoke. The polycyclic aromatic hydrocarbon constituents of smoke are carcinogenic and, in the presence of nitrosamines, benzo(a)pyrene has been shown to have a synergistic effect in pulmonary carcinogenesis in rats (Rubin, 2001). Although nitrosamines are primary suspects for oesophageal cancer, mycotoxins and other carcinogens also play a role either acting as co-carcinogens or cancer promoters (Yang, 1980).

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A number of inhibitors of *N*-nitrosation in the diet that could be used in decreasing the levels of exposure of humans to nitroso-compounds have been characterized. Epidemiological studies indicate that the frequent intake of certain food components plays an important role in reducing the risk for cancer development in humans (Chen, 1992; Gao *et al.*, 1994). Dietary constituents can selectively modulate the drug-metabolizing enzymes thereby altering the metabolic fate of the carcinogen in the cell (Chen, 1992; Gao *et al.*, 1994). Thus, diets containing substances that can modulate or prevent cancer may play a role in the general

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health of humans, especially if they are cost-effective and easily available (Marnewick *et al.*, 2000). The protective role of tea (*Camellia sinensis*) against mutagenesis and carcinogenesis has been well established in literature (Suganuma *et al.*, 1999; Steele *et al.*, 2000; Sun *et al.*, 2002), while the inhibition of oesophageal tumours by certain polyphenolic compounds from green and black teas have been well documented (Han and Xu, 1990; Hirose *et al.*, 1993). Tea is a widely consumed beverage throughout the world and the popularity of herbal/health teas increased significantly during the past two decades (Chen, 1992; Gao *et al.*, 1994). Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp*), two South African teas, indigenous to the Cape "fynbos" plants, are consumed both locally and abroad and might have several health-promoting properties (Marnewick *et al*, 2003). Although herbal teas are not regarded as true teas since they are not derived from the original tea plant (Trevisanato and Young-In, 2000), they are ingested in the same manner as green and black teas.

Considerable evidence is now available showing that teas can prevent tumour induction by a variety of chemical carcinogens in experimental animals, which is mainly attributed to the polyphenolic constituents of tea. These polyphenols possess antioxidant activity and interfere with carcinogen activation, demonstrate anti-mutagenic and anti-genotoxic properties, exhibit anti-tumour activity and alter certain events in signal transduction pathways. Certain tea polyphenols are known to exhibit cytotoxic effects, which could lead to apoptosis in various human tumor cell lines, or it could inhibit growth that may lead to cell cycle arrest (Roy *et al.*, 2001). Rooibos and honeybush teas contain no caffeine, and have a low tannin content (Von Gadow *et al.*, 1997; Kamara *et al.*, 2003). Because the phenolic constituents of the two South African herbal teas differ from those of green and black teas, the mechanisms involved in protection against DNA damage could also differ (Marnewick *et al.*, 2003). These herbal teas may represent a promising tool for chemoprevention against cancer in humans as they are consumed on a regular basis locally, and are becoming increasingly important as a nutraceutical product globally.

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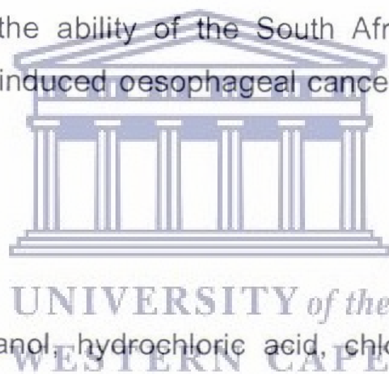
Another herbal plant, *Sutherlandia frutescens* (Cancer Bush), attracted attention for its possible chemopreventive properties due to its anti-proliferative effects towards several human cancer cell lines (Tai et al., 2004; Chinkwo, 2005). The characteristics of the active ingredients of this plant is mostly unknown, although L-canavanine, a non-protein amino acid and an arginine antagonist, have been demonstrated (Swaffar *et al*., 1994), both known to exhibit anti-proliferative activity in cancer cells (Rosenthal, 1997).

Evaluation of the cancer modulating properties of the two SA herbal teas and *Sutherlandia,* and their polyphenol and/or other constituents could have important implications for the possible use as chemopreventive agents against various cancers. In the present study the ability of the South African herbal teas and *Sutherlandia,* to modulate MBN-induced oesophageal cancer development in rats, have been evaluated.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3.2 Materials and methods

3.2.1 Chemicals and reagents

Analytical grade methanol, ethanol, hydrochloric acid, chloroform ($CHCl_3$), and sodium carbonate (Na_2CO_3) were purchased from Merck Chemicals (South Africa). Gallic acid (GA), quercetin, *p*-dimethylaminocinnamaldehyde (DAC), (+)-catechin, dimethylsulfoxide (DMSO), and Folin-Ciocalteau (F-C) reagents were obtained from Sigma-Aldrich (South Africa). Prof P Preussman, German Cancer Research Centre, Heidelberg, Germany provided the N-methylbenzylnitrosamine (MBN). The authentic polyphenol standards used for HPLC quantification was obtained from Extrasynthése (Genay, France).

3.2.2 Plant material

Green tea (*Camellia sinensis*) was purchased from Vital Health Foods (Kuils River, Cape Town), and the black tea (*Camellia sinensis)* was obtained from a retail outlet in Cape Town, South Africa. Fermented and unfermented rooibos teas (*Aspalathus linearis*) were a gift from Rooibos Ltd (Clanwilliam, South Africa) and the fermented and unfermented honeybush teas (*Cyclopia intermedia*) were obtained from

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Coetzee & Coetzee Distributors (Blackheath, Cape Town). Parceval Pharmaceuticals (Wellington, South Africa) was the supplier of the wild type *Sutherlandia frutescens* subspecies *microphylla*.

3.2.2.1 Preparation of tea samples/infusion

Stock solutions of aqueous extracts of the rooibos, honeybush, *Sutherlandia*, and black teas were prepared by adding freshly boiled tap water to the dried plant material (10 g/100 ml). The teas were allowed to steep for 30 minutes at room temperature, filtered through cheesecloth, Whatman No. 4 and finally through and Whatman No. 1 filter paper. After cooling the teas were dispensed into plastic bottles and stored at -20°C until used. Prior to use the tea was thawed at 40°C in a water bath for 20-25 minutes. The *Camellia sinensis* and rooibos teas were diluted with tap water after defrosting to the equivalent of 2% plant material per volume for black and rooibos teas, 4% for honeybush teas and 1% for *Sutherlandia frutescens*. The diluted extracts were prepared daily, except in the case of green tea, which was prepared at 2% every alternate day due to difficulty in filtering the 10% stock solution.

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3.2.2.2 Preparation of methanol extract of unfermented rooibos tea (RgM extract)

500 g of unfermented Rooibos tea leaves (Rooibos Ltd) was weighed out in a 5 L Erlenmeyer flask and extracted with chloroform (3 L) by stirring for 20 hours. The mixture was filtered and 1.5 L of chloroform was added to the plant material, stirred for 7 hours, and filtered again. Whatman No. 41 filter paper was used for all filtrations. The procedure was repeated overnight and the chloroform extracts discarded. The residual material was extracted with methanol (3 L), by stirring for 20 hours and filtered. The process was repeated using 1.5 L of methanol for 7 hours and filtering, followed by another extraction (1.5 L) overnight. All the filtrates were pooled and evaporated to dryness at 40°C using a Buchï rotavapor (Labortechnik, Switzerland). The dried methanol extract was weighed and stored at 4°C until used. For the rat experiments the methanol extract of unfermented

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rooibos (RgM) tea was diluted with tap water, after defrosting, to the equivalent of 0.1% plant material per volume.

3.2.3 Chemical analysis of herbal tea samples

3.2.3.1 Soluble solid determination

The soluble solid content of each tea sample was determined at three different time points during this investigation. Small glass vials were labelled and placed in a drying oven (120°C) overnight at and stored in a dessicator. Each vial was weighed and 1ml tea, in triplicate, per sample was added. Samples were placed in a drying oven (at 120°C) overnight, and cooled in a dessicator. The samples were weighed and the soluble solids determined per 1ml of the tea.

3.2.3.2 Determination of total polyphenol content

The total polyphenol content of the various extracts was determined by using the Folin-Ciocalteau method with gallic acid as the standard (Singleton & Rossi, 1965). The reagents for the assay were prepared by making a 10% solution of Folin-Ciocalteau reagent, a 7.5% solution of sodium carbonate (Na_2CO_3), and a gallic acid stock solution of 0.1%. The Folin reagent and gallic acid solutions were prepared fresh daily. The gallic acid standards were prepared (Table 1(a), Addendum) to obtain a dilution range using the 0.1% stock solution. Extracts were prepared, in triplicate, by diluting with water to give an absorbance reading between 0.3 and 0.7 in the assay. One ml each of the following was dispensed into separate tubes: tea sample, gallic acid and dH_2O (negative control) (Table 1(b), Addendum). Folin reagent (5 ml) and 7.5% Na_2CO_3 (4ml) was added, the solution mixed thoroughly on a vortex mixer and samples incubated in a water bath at 37°C for 2 hours. Once cooled, the solution was vortexed again, transferred to disposable cuvettes and the absorbance read, spectrophotometrically, at 765 nm. The results were expressed as mg gallic acid equivalents per litre of diluted extracts (as fed to the rats).

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3.2.3.3 Determination of flavonol/flavone content

The flavonol/flavone content was determined by a method of Mazza *et al.* (1999) modified by De Beer (2002), using a stock solution (0.004%) of quercetin prepared in 95% ethanol (EtOH). Extracts were diluted in water to give an absorbance reading between 0.06 and 0.5. The standards and samples were prepared as summarised in Table 2a (Addendum). The absorbances of the sample control (EtOH only), standards and extracts were determined at 360 nm. Results were expressed as mg quercetin equivalents per litre of diluted extracts.

3.2.3.4 Total flavanol/proanthocyanidin content

The standard for flavanol/proanthocyanidin determination was prepared as summarised in Table 3a (Addendum) using (+)-catechin as the standard. Various concentrations of the extracts were prepared, in triplicate. A solvent control was prepared for each extract since the dilutions, as well as the incubation times of each one differed (Table 3b, Addendum). For the flavanol/proanthocyanidin determination, solvent controls, catechin standards and extracts were prepared in triplicate (Table 3c, Addendum). The absorbance of the standards was read at 640 nm after 2 minutes, after adding *p*-dimetylaminocinnamaldyhyde (DAC) (McMurrough and McDowell, 1978). The absorption was recorded separately for each extract as an allowance was made for each one to react until a steady state was reached. Total flavanol/proanthocyanidin content of the various extract was determined and the results expressed as mg catechin equivalents per litre of diluted extract.

3.2.4 Flavonoid analyses of the herbal teas by HPLC

3.2.4.1 Rooibos tea

High performance liquid chromatography (HPLC) was performed using the method of Joubert (1996) with slight modifications of the mobile phase gradients. The instrument used was an Agilent 1200 HPLC with diode-array detector (Agilent, Wilmington. Delaware, USA) using a LiChrorspher 100 RP-18 column. The mobile phase gradient (A = 2% acetic acid; B = acetronitrile) were as follows: 20% B from

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0 to 5 min.; change to 28% B after 25 min.; 28% B to 35 min.; change to 35% B after 50 min.; change to 40% B after 60 min.; change to 50% B after 70 min.; change to 60% b after 80 min.; change to 80% B after 90 min.; change to 60% B after 100 min.; change to 20% B after 115 min.; equilibration for 15 min. The flow rate was 0.4 mL/min, but changed to 1.1 mL/min between 100 and 115 min and remained at 1.1 mL/min during equilibration. Peak identity was determined by means of retention time and UV spectra that were recorded for all samples in the range 200-400 nm and compared to that of authentic polyphenol standards. Quantitation of aspalathin and nothofagin was conducted at 288 nm, while the other compounds were quantified at 350 nm.

3.2.4.2 *Honeybush tea*

HPLC analysis was performed using a Waters LC Module I equipped with a Waters 2996 photodiode array detector using Millenium32 version 4.0 software (Waters, Milford, MA) (Joubert *et al.*, 2003). Separation was achieved on a Synergi Max-RP 150 x 4.6 mm column (4 μ particle size; 80 Á pore size) from Phenomenex (Temperrance, CA, USA). The separation gradient was as follows: 0-6 min. (12% B), 7 min (18% B), 14 min (25%B), 19 min (40% B), 24 min (50% B), 29 min (12% B) (solvent A = 2% acetic acid in aqueous solution (v/v) and solvent B = acetonitrile). Peak identity was determined by means of retention time and UV spectra that were recorded for all samples in the range 200-400 nm and compared to that of authentic polyphenol standards. Quantitation of mangiferin and isomangiferin was conducted at 320 nm, while the other compounds were quantified at 288 nm.

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3.2.5 Animals and treatments

The study was approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council. A protocol was submitted to the ethical review committee with detailed information on the determination of sample numbers, level of severity of the experiment, and motivation for the use of animals. One hundred and twenty male Fischer 344 rats, weighing between 150-200 g, were obtained from the Primate Unit of the Medical Research Council, and housed

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separately under a 12 hour photoperiod, with 50% humidity and a constant room temperature of 23-25°C. Rats had free access to rat feed pellets (Epol, SA), and fresh water and extracts were provided every alternate day. All the rats were weighed at least once a week. Detailed fluid intake profiles and bodyweight gain parameters were monitored. Random aliquots of the "left-over" extracts were collected after two, four and six months, and stored at -20°C for imminent chemical analyses.

3.2.5.1 Modulation of oesophageal papillomas by MBN

Rats were randomly divided into ten experimental groups and injected subcutaneously with methylbenzylnitrosamine (MBN), dissolved in 20% DMSO/H_2O), at a dose of 0.5 mg/kg body weight, three times a week for five weeks. The control group was injected with the solvent vehicle (20% DMSO/H_2O). One week following the MBN treatment, the treatment groups were given various extracts (green, black, unfermented and fermented rooibos, a methanol extract of unfermented rooibos, and unfermented and fermented honeybush), and an infusion infusion (*Sutherlandia frutescens* subspecies *microphylla*) as their sole source of drinking fluid for twenty-five weeks (Fig 1).

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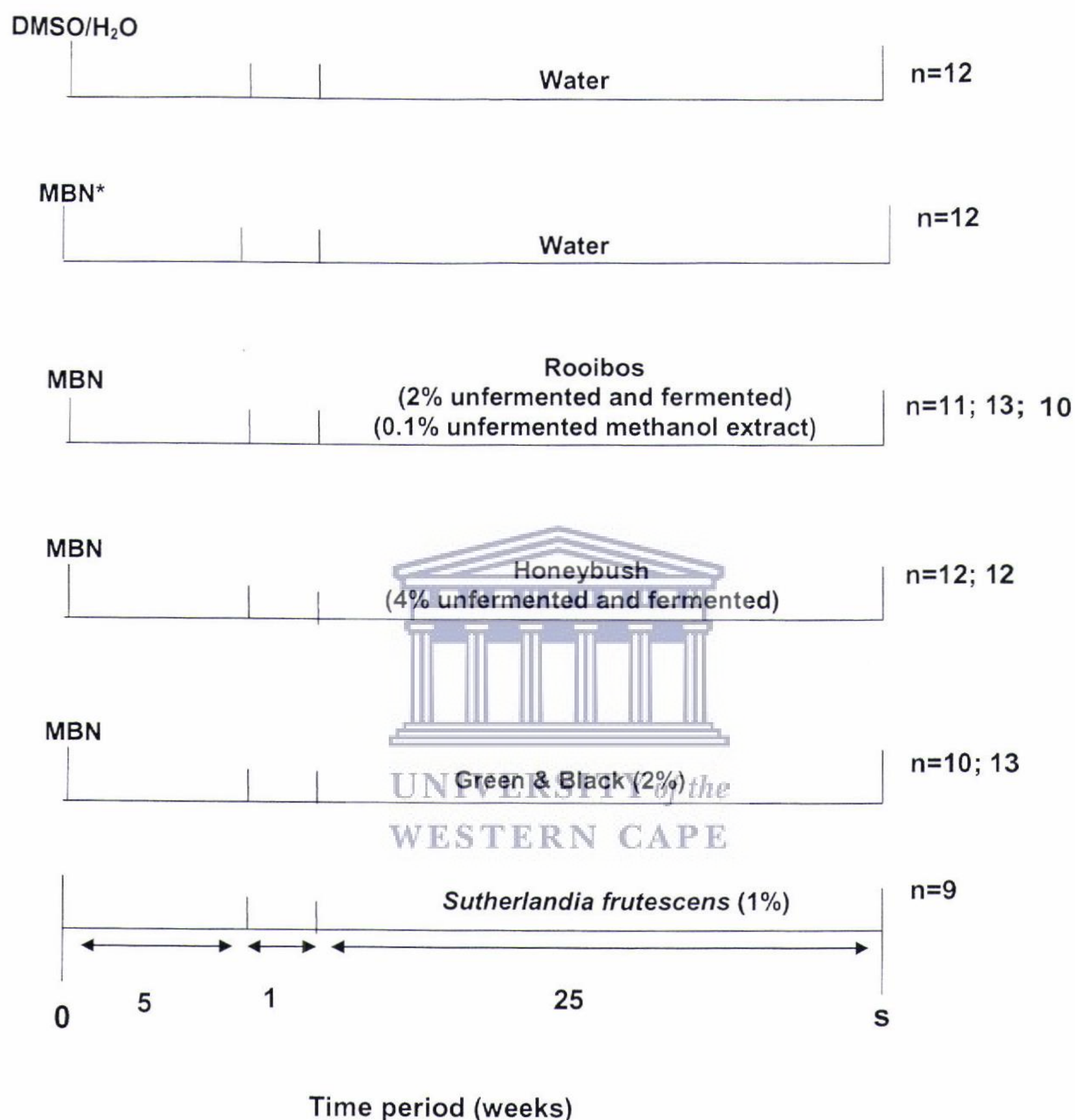

Fig .1 Experimental design for the oesophageal cancer model. *Positive control; MBN= subcutaneous injection (0.5 mg/kg bw per rat) 3 x weekly for five weeks. n = rats per group; s = sacrifice; 20% DMSO/H_2O = solvent control.

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Upon termination the rats were sacrificed under pentobarbital anaesthesia, and the oesophagi excised, weighed, and opened longitudinally with the mucosa uppermost. Quantitation of oesophageal papillomas was performed using a

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dissecting microscope (1.5 x magnificaiton) (Nikon SMZ-10 Stereo Microscope, Japan) under an optical fibre light. The number of papillomas was recorded and their size determined by measuring the length, width and height. The volume was calculated using the formula $[(l \times w \times h)\pi]/6$ (Janik *et al.*, 1975). Papillomas were grouped into various subclass sizes, namely, >0.01 mm^3; 0.01-0.1 mm^3; 0.1-10 mm^3; 10-20 mm^3; >20 mm^3.

3.3 Statistical analysis

For the tea intake profiles, the data were checked for normality using both the Kolmogorov-Smirnov test and doing a visual assessment using box plots. No transformations were necessary.

3.3.1 Repeated analysis to assess tea intake parameters

Mixed effects regression models were fitted on the repeated parameters (three measurements per rat) to estimate and compare the group differences and time effects. The two fixed effects in the models were group and time, and the random effects model within each rat was based on the time measurement. The group by time interaction was included in all models to test for a difference in slopes, which would indicate that the different groups reacted differently over time. The Satterthwaite degrees of freedom specification was used for the fixed effects testing. Least Squares Means (LSMeans) were used to estimate group differences at times 2, 4 and 6. Group comparisons and 95% confidence intervals for the effects and differences were obtained using the Tukey-Kramer adjustment, as the data were unbalanced. The default a=0.05 was used throughout.

3.3.2 Analysis without repeats (time effects)

Analysis of Variance (ANOVA) tests (GLM in SAS) were used on all these parameters to test for any differences-in-group means. With Levene's test homogeneity of variances were tested, and if it was rejected, Welch's ANOVA was used instead of the usual F-test ANOVA. The Tukey-Kramer test was used as the multiple comparison procedure for all pairwise comparisons. The default a=0.05 was used throughout.

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3.3.3 Modulation of MBN-induced oesophageal papillomas

All statistical analyses were performed using the NCSS statistical computer package (Hintze, 2007).

3.4 Results

3.4.1 Characterisation of the different tea and infusion parameters

3.4.1.1 Soluble solid content

Unfermented honeybush tea had the highest soluble solid content, followed by green tea, while fermented honeybush and black teas contained similar levels. Unfermented rooibos tea had a higher soluble solid content than its fermented counterpart but it was significantly ($P<0.05$) lower than fermented honeybush and black teas. The soluble solid content of the *Sutherlandia* infusion did not differ significantly from that of fermented rooibos tea, but it was significantly ($P<0.05$) lower that all the other teas. The soluble content of the fermented herbal teas was significantly ($P<0.05$) lower than their unfermented counterparts. The RgM extract had the lowest soluble content (Table1).

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3.4.1.2 Total polyphenol content

The methanol extract of unfermented rooibos tea had the highest level of total polyphenols (TPP) per 100 mg soluble solids, while the *Sutherlandia* infusion contained the lowest level. The total TPP content of the green and black teas did not differ from each other, but was significantly ($P<0.05$) lower than unfermented honeybush and the rooibos teas, which did not differ significantly from each other. The TPP content was significantly ($P<0.05$) lower in fermented honeybush tea when compared to the unfermented tea while no difference was noticed between the rooibos teas.

3.4.1.3 Flavonol/flavone content

Fermented rooibos tea had the highest ($P<0.05$) flavonol/flavone content per 100 mg soluble solids followed by the RgM extract, unfermented rooibos and honeybush teas. Fermented honeybush tea, black tea and the *Sutherlandia*

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infusion had similar flavonol/flavone levels while the lowest content was noticed in green tea.

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Table 1 Amount of soluble solids, total polyphenols, total flavonols, and total flavanols of the different teas fed to the rats over six months.

	Level in drinking water	Soluble solids (mg/ml)	TPP (mg gallic acid equiv/100mg soluble solids)	Flavo/flavone (mg quercitin equiv/100mg soluble solids)	Flava/proanthocyanidin (mg catechin equiv/100mg soluble solids)
Gr	2%	6.63 ±1.06a	23.27 ±3.39d	1.94 ±0.11f	9.84 ±2.43a
Bl	2%	5.84 ±0.33ab	26.65 ±1.95cd	3.13 ±0.39e	5.93 ±1.23b
Rg	2%	3.66 ±0.13d	33.26 ±3.03b	5.89 ±0.20c	1.49 ±0.24d
Rf	2%	2.70 ±0.12c	31.75 ±1.97b	11.13 ±0.84a	0.69 ±0.10de
RgM	0.1%	0.94 ±0.06e	45.62 ±3.17a	7.12 ± 0.36b	2.97 ±0.21c
Hg	4%	9.47 ±0.59f	29.40 ±3.45bc	5.22 ±0.12d	3.28 ±0.50c
Hf	4%	5.12 ±0.95b	15.67 ±2.68e	3.22 ±0.15e	1.34 ±0.16de
Sut	1%	2.19 ±0.15c	3.771 ±0.19f	2.68 ±0.32e	0.01 ±0.0024e

Values are the means of 9 replicates ± STD. Means in a column followed by the same letter do not differ significantly. If the letters differ, then P<0.05. TPP: total polyphenols; Flavo : flavonol/flavone and Flava : flavanol/proanthocyanidin. Values are the means of three months' samples. Gr = green tea; Bl = black tea; Rg = unfermented rooibos; Rf = fermented rooibos; RgM = unfermented rooibos methanol extract; Hg = unfermented honeybush; Hf = fermented honeybush; Sut = *Sutherlandia frute*

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3.4.1.4 Flavanol/proanthocyanidin content

Green tea had the highest (P<0.05) flavanol/proanthocyanidin content followed by black tea. Unfermented honeybush, as well as the RgM extracts had similar levels, followed by unfermented rooibos and fermented honeybush teas. Fermented

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rooibos tea had the lowest flavanol/proanthocyanidin content of the herbal teas, while the *Sutherlandia* infusions contained very low levels.

3.4.2 Fluid intake profiles and body weight gain

There was a significant (P<0.05) decrease in the average tea fluid intake profiles over the six months period when expressed per 100g body weight. The monthly inter tea comparison indicated that green tea was consumed at a higher rate during the first month while *Sutherlandia* showed a decreased intake for the first two months. This resulted in green tea having the highest average intake over the 6-month period. No visible clinical signs of illness and/or weight loss were detected in any of the rats in the various groups as a result of the intake of different teas over the six-month period. There was no significant difference in body weight gain of the rats in the different groups. (Addendum, Table 4).

3.4.3 Quantification of the different tea intake parameters

The mean monthly tea intake profiles of the different groups are summarised in Table 2. The different tea intake parameters over the 6-month period were compared bi- monthly for statistical analysis.

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3.4.4 Kinetic variation of the tea and herbal infusion intake parameters

3.4.4.1 Fluid intake

There was a significant (P<0.05) group and time interaction when considering the intake of green, fermented and unfermented honeybush teas as it significantly (P<0.05) decreased over time (Table 2).

When observing the groups as the main effect while combining time, there was no significant difference in the tea intake between the different treatments. However, when the effect over time was considered by combining all the groups, there was a significant (P<0.05) decrease in the tea intake between 2 and 4 months and 4 and 6 months.

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Table 2 Inter-group/Intra-group comparison of tea, soluble solids, total polyphenols, flavonols and flavanols intakes.

	Month 2					Month 4					Month 6				
ID	TI (ml/100g bw)	Sol	TPP (mg/100g bw)	Flavo (mg/100g bw)	Flava (mg/100g bw)	TI (ml/100g bw)	Sol	TPP (mg/100g bw)	Flavo (mg/100g bw)	Flava (mg/100g bw)	TI (ml/100g bw)	Sol	TPP (mg/100g bw)	Flavo (mg/100g bw)	Flava (mg/100g bw)
Gr (2%)	10.82 ±0.88a^{a}	77.18 ±6.26a^{a}	16.47 ±1.34a^{a}	1.57 ±0.13a^{a}	7.97 ±0.65a^{a}	9.55 ±0.5a^{b}	50.06 ±2.68a^{b}	10.45 ±0.56a^{b}	0.90 ±0.05a^{b}	4.55 ±0.24a^{b}	8.34 ±0.40a^{c}	63.02 ±3.02a^{c}	17.39 ±0.83a^{c}	1.26 ±0.06a^{c}	6.37 ±0.37a^{c}
Bl (2%)	9.84 ±0.91bb^{a}	57.78 ±5.35b^{a}	15.73 ±1.46a^{a}	2.06 ±0.19b^{a}	3.93 ±0.36b^{a}	8.71 ±1.02c^{b}	52.07 ±6.11b^{ab}	12.97 ±1.52a^{b}	1.54 ±0.18b^{b}	2.88 ±0.34b^{b}	8.11 ±0.81a^{b}	45.93 ±4.61b^{b}	13.55 ±1.36b^{b}	1.40 ±0.14b^{b}	2.66 ±0.27b^{b}
Rg (2%)	9.35 ±0.93b^{a}	35.50 ±3.51c^{a}	10.88 ±1.08c	2.07 ±0.20b^{a}	0.59 ±0.06c^{a}	8.97 ±0.99b^{ab}	32.73 ±3.60c^{a}	10.40 ±1.14c^{a}	1.95 ±0.21c^{b}	0.51 ±0.06c^{b}	8.21 ±1.07d^{b}	28.92 ±3.79c^{b}	10.60 ±1.39c^{a}	1.67 ±0.22c^{b}	0.36 ±0.05c^{c}
Rf (2%)	9.95 ±0.78bb^{a}	26.24 ±2.05d^{a}	7.65 ±0.60d^{a}	2.91 ±0.23d^{a}	0.19 ±0.01d^{a}	8.99 ±0.65d^{b}	24.46 ±1.77d^{ab}	7.01 ±0.51b^{a}	2.61 ±0.19d^{ab}	0.17 ±0.01d^{b}	8.16 ±0.67d^{b}	22.39 ±1.83d^{b}	7.67 ±0.63d^{a}	2.33 ±0.19d^{b}	0.13 ±0.01d^{c}
RgM (0.1%)	9.73 ±0.52b^{a}	9.05 ±0.49e^{a}	4.22 ±0.23b^{a}	0.69 ±0.04c^{a}	0.28 ±0.01e^{a}	8.83 ±0.98d^{ab}	8.12 ±0.90e^{a}	3.52 ±0.39d^{a}	0.59 ±0.07e^{ab}	0.25 ±0.03d^{a}	8.07 ±1.37d^{b}	5.92 ±1.01e^{b}	3.61 ±0.61e^{a}	0.50 ±0.08e^{b}	0.21 ±0.04e^{b}
Hg (4%)	10.54 ±1.14a^{a}	100.73 ±10.86f^{a}	29.25 ±3.15e^{a}	5.29 ±0.57c^{a}	3.33 ±0.36b^{a}	9.35 ±0.99a^{b}	93.80 ±9.88f^{a}	23.81 ±2.51e^{b}	4.61 ±0.49f^{b}	2.90 ±0.31b^{b}	7.87 ±0.92b^{c}	69.33 ±8.12f^{b}	22.08 ±2.59f^{c}	3.57 ±0.42f^{c}	2.24 ±0.26b^{c}
Hf (4%)	10.12 ±1.14ab^{a}	46.96 ±5.29g^{a}	6.01 ±0.68b^{a}	1.39 ±0.16a^{a}	0.57 ±0.06c^{a}	8.78 ±0.89b^{b}	40.25 ±4.10g^{a}	5.92 ±0.60b^{b}	1.29 ±0.13g^{a}	0.53 ±0.05c^{a}	7.53 ±0.92b^{b}	50.96 ±6.23b^{b}	9.46 ±1.16g^{b}	1.68 ±0.21d^{b}	0.71 ±0.09f^{b}
Sut (1%)	9.34 ±1.04bb^{a}	19.68 ±2.20h^{a}	0.71 ±0.08f^{a}	0.51 ±0.06c^{a}	0.0005 ±0.0001f^{a}	8.35 ±0.91bc^{b}	19.63 ±2.13h^{a}	0.60 ±0.07f^{b}	0.45 ±0.05e^{b}	0.0037 ±0.0004e^{b}	7.36^{b} ±0.86bc	15.51 ±1.80g^{b}	0.63 ±0.07h^{b}	0.43 ±0.05e^{b}	0.0017 ±0.0002g^{c}

Values are the means of 9-13 determinations ± STD (values in parenthesis). Means in a column followed by the same letter do not differ significantly. If the letters differ, then $P<0.05$. TI: tea intake; Sol: soluble solids; TPP: total polyphenols; Flavo: flavonol/flavone and Flava: flavanol/proanthocyanidin. Time is indicated as month 2, 4 and 6. Tea intake is the mean for the month when the tea was sampled. Gr= green; Bl = black; Rg = unfermented rooibos; Rf = fermented rooibos; RgM = unfermented rooibos methanol extract; Hg = unfermented honeybush; Hf = fermented honeybush; Sut = *Sutherlandia.* Values in superscript indicates significant differences in individual groups over time.

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When considering inter group comparisons as a function of each time interval (2, 4 and 6 months) the intake of green tea was significantly ($P<0.05$) higher than that of fermented and unfermented rooibos teas, RgM rooibos extract, black tea and *Sutherlandia* after 2 months. The intake of unfermented honeybush tea was significantly ($P<0.05$) higher than black tea, unfermented rooibos tea, RgM extract and *Sutherlandia*. At the end of the fourth month, the green tea intake was significantly higher than fermented honeybush and unfermented rooibos teas. The intake of *Sutherlandia* was significantly ($P<0.05$) lower than green, unfermented honeybush and fermented rooibos teas. The intake of green tea was significantly ($P<0.05$) higher than fermented honeybush tea at the sixth month, while the consumption of *Sutherlandia* was significantly ($P<0.05$) lower than green, fermented rooibos and unfermented rooibos teas. No significant differences were noted in the other tea groups.

3.4.4.2 Soluble solid intake

There was a significant group and time interaction when considering the intake of black, green and unfermented honeybush tea soluble solids as it significantly ($P<0.05$) decreased over time (Table 2).

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There was a significant group effect in the soluble solid intake when the time points were combined. There was no difference in the soluble solid intake between black tea and fermented honeybush tea or between fermented rooibos tea and *Sutherlandia*, while all the other teas displayed significant ($P<0.05$) differences. When the groups were combined to monitor the time effect, a significant decrease in the soluble solid intake was noted.

When considering inter group comparisons as a function of each time interval (2, 4 and 6 months), there was a significant ($P<0.05$) reduction of the soluble solid intake of the teas in the order: unfermented honeybush, green, black, fermented honeybush, unfermented rooibos, fermented rooibos, *Sutherlandia*, and RgM extract after two months. All the groups displayed a significant ($P<0.05$) reduced intake in the soluble solid intake after 4 months. Unfermented honeybush tea had

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the highest intake, followed by black, green, fermented honeybush, unfermented rooibos, fermented rooibos teas, *Sutherlandia*, and the RgM extract. At the end of the sixth month no difference in the soluble solid intake was noted between black tea and fermented honeybush tea. The soluble solid intake of all the other groups differed significantly (P<0.05) from each other, and followed a similar pattern as described for the two and four–month intervals. Unfermented honeybush had the highest soluble solid intake, followed by green, fermented honeybush, black, unfermented rooibos, fermented rooibos teas, *Sutherlandia*, and the RgM extract.

3.4.4.3 Total polyphenol (TPP) intake

There was a significant group and time interaction for unfermented honeybush tea as the intake significantly (P<0.05) decreased over the different treatment intervals (Table 2).

When considering the groups as the main effect, combining time, there was no difference in the TPP intake between black and green teas, as well as between fermented honeybush and fermented rooibos tea. The TPP intake of the rats from the other groups differed significantly (P<0.05). When considering time as the main effect, the TPP intake significantly (P<0.05) decreased at month 4 when compared to months 2 and 6.

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Inter tea comparisons after 2 months showed that the highest (P<0.05) TPP intake was noticed for the rats treated with unfermented honeybush tea, followed by green, black, unfermented rooibos, fermented rooibos, fermented honeybush teas, RgM extract, and *Sutherlandia*. No significant differences were noted between black and green tea, as well as fermented honeybush tea and RgM extract. There was no difference in the TPP intake between the rats treated with black and green teas or between fermented rooibos and fermented honeybush teas after 4 months. The TPP intake of the treatment groups decreased (P<0.05) in the order: unfermented honeybush, black, green, unfermented rooibos, fermented rooibos, fermented honeybush, and *Sutherlandia*. At the end of month 6, the TPP intake of all the groups differed significantly (P<0.05) from each other with rats receiving the

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unfermented honeybush having the highest intake, followed by green, black, unfermented rooibos, fermented honeybush, fermented rooibos teas, RgM extract and *Sutherlandia*.

3.4.4.4 Flavonol/Flavone intake

Black, unfermented honeybush and fermented rooibos tea intake significantly decreased over time, displaying a significant group and time interaction, (Table 2).

When considering the groups as the main effect, combining time, there was no significant difference in the total flavonol/flavone intake of the rats consuming black and fermented honeybush teas, black and unfermented rooibos teas, as well as green and fermented honeybush teas. The flavonol/flavone intake of the other treatments differed significantly ($P<0.05$). When considering time as the main effect the total flavonol/flavone intake of the rats consuming green and fermented honeybush teas was significantly ($P<0.05$) decreased at month 4 when compared to months 2 and 6. The flavonol/flavone intake of the rest of the treatment groups was lowest at month 6.

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Inter tea comparisons after 2 months showed that the highest ($P<0.05$) flavonol/flavone intake was noticed fot the rats treated with unfermented honeybush followed by fermented rooibos, unfermented rooibos, black, green, fermented honeybushs, RgM extract and *Sutherlandia*. No differences were noted between the groups treated with, green and fermented honeybush teas and black and unfermented rooibos teas as well as RgM extract and *Sutherlandia*. The flavonol/flavone intake of the groups decreased ($P<0.05$) in the order: unfermented honeybush, fermented rooibos, unfermented rooibos, black, fermented honeybush, green teas, RgM extract and *Sutherlandia* with no difference between the latter two treatments after 4 months. After sixth months, the group consuming unfermented honeybush tea had the highest flavonol/flavone intake, followed by fermented rooibos, fermented honeybush, unfermented rooibos, black, green tea, RgM extract and *Sutherlandia*. The flavonol/flavone intake of the rats consuming

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fermented honeybush and unfermented rooibos teas, as well as RgM extract and *Sutherlandia* did not differ from each other.

3.4.4.5 Flavanol/Proanthocyanidin intake

There was a significant (P<0.05) group and time interaction for black, green, unfermented honeybush teas and *Sutherlandia* over the different treatment intervals (Table 2).

When considering the groups as the main effect, combining time, there was no difference in the flavanol/proanthocyanidin intake of the rats consuming fermented honeybush and unfermented rooibos teas; fermented rooibos and RgM extract; and fermented rooibos tea and *Sutherlandia*. The flavanol/proanthocyanidin intake of the rats from the other groups differed significantly (P<0.05). When considering time as the main effect the flavanol/proanthocyanidin intake of the rats consuming green and fermented honeybush teas was significantly (P<0.05) decreased at month 4 when compared to month 6, while for *Sutherlandia*, the flavanol/proanthocyanidin intake was increased at month 4 when compared to months 2 and 6. The flavanol/proanthocyanidin intake of rats receiving *Sutherlandia* was considerably less (>100x) than for the other teas.

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Inter tea comparisons after 2 months showed that the highest (P<0.05) flavanol/proanthocyanidin intake was noticed for the rats that consumed green tea, followed by black, unfermented honeybush, unfermented rooibos, fermented honeybush teas, RgM extract, fermented rooibos tea, and *Sutherlandia*. No differences were noted in the flavanol/proanthocyanidin intake between the rats treated with fermented honeybush and unfermented rooibos teas. There was no difference in the flavanol/proanthocyanidin intake between the rats treated with fermented honeybush and unfermented rooibos teas, as well as fermented rooibos tea and RgM extract after 4 months. The flavanol/proanthocyanidin intake of the other groups decreased (P<0.05) in the order: green, unfermented honeybush, black, fermented honeybush, unfermented rooibos, RgM extract, fermented

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rooibos, and *Sutherlandia*. At the end of month 6, the flavanol/proanthocyanidin intake of all the other treatment groups differed (P<0.05), with rats receiving the green tea having the highest flavonol/proanthocyanidin intake, followed by black, unfermented honeybush, fermented honeybush, unfermented rooibos, RgM extract, fermented rooibos and *Sutherlandia*. The flavanol/proanthocyanidin intake of the rats consuming black and unfermented honeybush teas as well as fermented rooibos tea and RgM extract did not differ from each other.

3.4.4.6 Average tea and herbal infusion intake parameters

The tea intakes were not statistically different between the various treatments, except for the *Sutherlandia* intake that was significantly (P<0.05) lower than green tea. Major differences exist when considering the soluble solid intake with the rats consuming *Camellia sinensis* teas having a significantly (P<0.05) higher intake when compared to the rooibos teas. The highest intake, however, was noticed for the rats consuming unfermented honeybush tea, while the intakes of RgM and *Sutherlandia* were the lowest. This aspect also impacted on the TPP and flavonol/flavone intake of the rats when compared to the other teas. The TPP intake of the *Camellia sinensis* and unfermented rooibos tea was approximately 50% of that obtained with unfermented honeybush tea that provided the highest intake. The flavonol/flavone intake of the rats receiving the *Camellia sinensis* and fermented honeybush teas was similar, while the intake of the rooibos teas was significantly (P<0.05) higher. The flavanol/proanthocyanidin intake of the rats receiving the *Camellia sinensis* teas was significantly (P<0.05) higher than the other teas with unfermented honeybush group having the highest intake of the herbal teas. The flavonol/flavone to flavanol/proanthocyanidin (Flavo/Flava) ratio increased with fermentation with the most dramatic effect obtained with rooibos tea (Table 3). The Flavo/Flava ratios of the honeybush, unfermented rooibos teas and the RgM extract were similar while the *Camellia sinensis* teas had the lowest. Rats receiving the *Sutherlandia* infusion had the lowest intake of all the different phenolic groups of compounds (Table 3).

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Table 3 Inter-group comparison of the average tea, soluble solids, total polyphenols, flavonols and flavanols intakes over the 6 month period.

Treatments	TI (ml/100g bw)	Sol (mg/100g bw)	TPP	Flavo	Flava	Flavo/Flava ratio
Gr (2%)	9.57 ±0.40a	63.42 ±2.70a	14.77 ±0.60a	1.24 ±0.05a	6.30 ±0.27a	0.2
Bl (2%)	8.89 ±0.86ab	51.92 ±5.01b	14.08 ±1.35a	1.66 ±0.16b	3.16 ±0.30b	0.5
Rg (2%)	8.84 ±0.89ab	32.38 ±3.25c	10.63 ±1.08b	1.89 ±0.19c	0.49 ±0.05c	3.9
Rf (2%)	9.04 ±0.41ab	24.36 ±1.10d	7.45 ±0.34c	2.62 ±0.12d	0.16 ±0.01d	16.4
RgM (0.1%)	8.88 ±0.84ab	7.70 ±0.70e	3.78 ±0.38d	0.59 ±0.05e	0.25 ±0.02d	2.4
Hg (4%)	9.25 ±0.93ab	87.95 ±8.86f	25.05 ±2.52e	4.49 ±0.45f	2.82 ±0.28f	1.6
Hf (4%)	8.81 ±0.88ab	46.06 ±4.62g	7.13 ±0.72c	1.46 ±0.15ab	0.60 ±0.06c	2.4
Sut (1%)	8.35 ±0.86b	18.27 ±1.88h	0.65 ±0.07f	0.46 ±0.05e	0.0021 ±0.0002e	230

Values are the means of 9-13 determinations ± STD (values in parenthesis). Means in a column followed by the same letter do not differ significantly. If the letters differ then P<0.05. TI: tea intake; Sol: soluble solids; TPP: total polyphenols; Flavo: flavonol/flavones and Flava: flavanol/proanthocyanidin. Gr= green; Bl = black; Rg = unfermented rooibos; Rf = fermented rooibos; RgM = unfermented rooibos methanol extract; Hg = unfermented honeybush; Hf = fermented honeybush; Sut = *Sutherlandia*. Flavo/Flava ratio = flavonol/flavone to flavanol/proanthocyanidin ratio.

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3.4.5 Quantification of known herbal tea flavonoids

3.4.5.1 Rooibos tea

HPLC analyses indicated that aspalathin was the major flavonoid present in unfermented rooibos followed by nothofagin, orientin and iso-orientin. Only trace amounts of luteolin and chrysoeriol were present. Orientin was the major flavonoid of fermented rooibos tea, followed by rutin/isoquercetin and iso-orientin. Aspalathin, isovitexin, and vitexin were present in low amounts, while trace amounts of hyperoside, nothofagin, chrysoeriol and luteolin were detected (Table 4).

3.4.5.2 Flavonoid intake

The rats consuming the unfermented rooibos tea had a higher intake of aspalathin, followed by isoquercetin/rutin, nothofagin, orientin, iso-orientin, hyperoside, isovitexin, vitexin, luteolin and chrysieriol. Orientin intake was highest in the rats consuming the fermented rooibos tea, followed by isoquercetin/rutin, iso-orientin, aspalathin, isovitexin, vitexin, hyperoside, nothofagin, chrysoeriol and luteolin.

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3.4.5.3 Honeybush tea

Mangiferin was the major monomeric polyphenol present in the unfermented honeybush tea, followed by isomangiferin, hesperidin and hesperetin. Narirutin was present in trace amounts in the unfermented honeybush tea. Isomangiferin was the major flavonoid in the fermented honeybush tea, followed by mangiferin and hesperetin. The presence of hesperidin and narirutin could not be shown in the fermented honeybush tea (Table 5).

3.4.5.4 Flavonoid and polyphenol intake

The daily intake of mangiferin was the highest in the rats fed the unfermented honeybush tea. This was followed by isomangiferin, hesperidin, hesperetin, and trace amounts of narirutin. Isomangiferin was

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the major polyphenol consumed by the rats receiving fermented honeybush tea, followed by mangiferin and hesperetin.

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Table 4 Mean intake profiles of major flavonoids of rooibos as a function of mean amount of soluble solids and different polyphenol intake parameters

Rooibos Tea	Processed		Unprocessed	
Soluble Solids (mg/ml)	2.74 ± 0.01		3.59 ± 0.09	
Total Polyphenols (mg/100mg soluble solids)	31.72 ± 2.91		34.52 ± 3.16	
Total Flavonol/flavone (mg/100mg soluble solids)	5.92 ± 0.20		10.63 ± 0.55	
Total Flavanol/proanthocyanidin (mg/100mg soluble solids)	1.41 ± 0.25		0.63 ± 0.09	
Phenolic Compounds	**% of soluble solids**	**Mean daily Intake (mg/100g BW)**	**% of soluble solids**	**Mean daily Intake (mg/100g BW)**
Aspalathin	0.48	0.12 ± 0.02	14.728	4.83 ± 0.58
Nothofagin	0.05	0.01 ± 0.002	1.121	0.36 ± 0.04
Orientin	0.88	0.23 ± 0.03	1.010	0.33 ± 0.04
Iso-orientin	0.70	0.18 ± 0.02	0.985	0.32 ± 0.04
Vitexin	0.14	0.04 ± 0.005	0.191	0.06 ± 0.01
Iso-vitexin	0.16	0.04 ± 0.01	0.214	0.07 ± 0.01
Hyperoside	0.08	0.02 ± 0.003	0.220	0.07 ± 0.01
Rutin/Isoquercitrin	0.80	0.21 ± 0.03	2.008	0.66 ± 0.08
Luteolin	0.01	0.002 ± 0.0003	0.004	0.0013 ± 0.0002
Chrysoeriol	0.01	0.003 ± 0.0004	0.004	0.0012 ± 0.0002

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Table 5 Mean intake profiles of major polyphenols of honeybush as a function of mean amount of soluble solids and different polyphenol intake parameters

Honeybush Tea	Processed		Unprocessed	
Soluble Solids (mg/ml)	4.55 ± 0.33		9.61 ± 0.01	
Total Polyphenols (mg/100mg soluble solids)	16.62 ± 3.58		19.73 ± 11.04	
Total Flavonol/flavone (mg/100mg soluble solids)	3.24 ± 0.24		3.46 ± 1.37	
Total Flavanol/proanthocyanidin (mg/100mg soluble solids)	1.35 ± 0.11		1.89 ± 1.37	
Phenolic Compounds	**% of soluble solids**	**Daily Intake (mg/100g BW)**	**% of soluble solids**	**Daily Intake (mg/100g BW)**
Mangiferin	0.42 ± 0.05	0.18 ± 0.03	4.59 ± 0.20	4.26 ± 0.72
Iso-Mangiferin	0.51 ± 0.02	0.22 ± 0.09	1.92 ± 0.10	1.78 ± 0.30
Hesperidin	nd	-	0.38 ± 0.10	0.35 ± 0.06
Hesperitin	0.11 ± 0.03	0.05 ± 0.01	0.10 ± 0.02	0.09 ± 0.02
Narirutin	nd	-	Trace	

nd = not detected

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3.4.6 Induction of papillomas by MBN

The negative control group, treated with 20% DMSO/H_2O did not develop any lesions (Fig. 2 a). Ninety percent of the rats in the MBN control group developed papillomas that were visible macroscopically when considering all the different sizes of lesions (Fig. 2 b-f). Of the different size categories 90% of the rats had papillomas ranging from 0.01 to 0.1 mm^3 size, 50% papilloma size between 0.1 to 10 mm^3, and 10 % each of the sizes 10 to 20 mm^3 and >20 mm^3.

3.4.7 Modulation of papilloma development by the different teas and herbal infusion

None of the teas at the concentrations used in this study showed any toxic effects in the rats. Between 90 to 100 % of the rats in the tea treated rats developed papillomas. When considering the 0.1 to 10 mm^3 size classes of papillomas, 50 to 80% of the rats treated with the different teas and herbal infusion developed papillomas, which were similar to that of the control group. Of the bigger size papillomas (≥10 mm^3) none of the rats treated with fermented and unfermented honeybush, as well as unfermented rooibos tea developed papillomas. In the size class >20 mm^3, none of the rats receiving green tea, fermented and unfermented honeybush, and unfermented rooibos tea developed papillomas. Black tea, fermented rooibos and the RgM extract developed larger papillomas although the average size was smaller than that observed in the positive control (Table 6).

3.4.7.1 Average total size and different size categories

The means of the total papilloma size categories indicated that the positive control group displayed the biggest papillomas. Although all the treatments reduced the average total size as compared to the control, only the unfermented rooibos and honeybush displayed a marginal ($P<0.1$) reduction (Table 6; Fig. 3 a). Of the other sizes, no effect was noticed for the 0.01 to 0.1mm^3 size category (Fig. 3 c), while a marked reduction was

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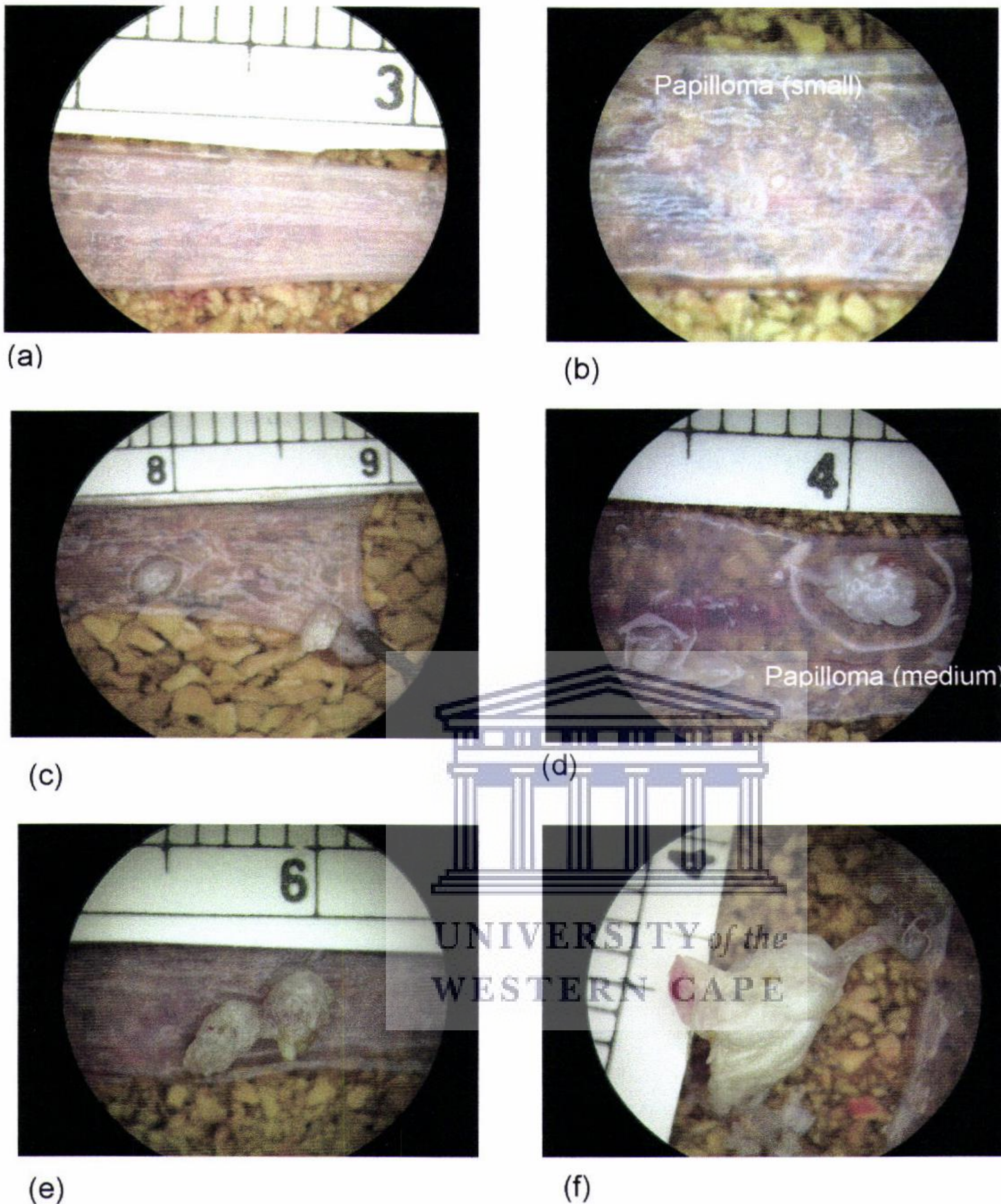

(a) (b) (c) (d) (e) (f)

Fig. 2 Appearance of oesophageal papillomas (X1.5) at termination of the study. Normal oesophagus (a); Papillomas ranging from small to large (b – f). Some of the larger papillomas have become vascularised (e) and (f).

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Table 6 Effect of *Camellia sinensis* and different herbal teas on the total mean and different size categories oesophageal papillomas

Group	Papillomas									
	0.01 to 0.1mm³		0.1 to 10mm³		10 to 20 mm³		>20		Tot (>0.01)	
	Number	Size	Number	Size	Number	Size	Number	Size	Number	Size
Neg Ctrl Tap water	-	-	-	-	-	-	-	-	-	-
Pos Ctrl* Tap water	6.08±3.70 (11/12)a	0.03±0.02	1.33 ± 2.31 (6/12)a	1.92 ± 2.50	0.08 ± 0.29 (1/12)	11.15	0.17 ± 0.58 (1/12)	75.64 ± 48.41	7.67 ± 4.64 (11/12)a	2.10 ± 12.18a
Gr (2%)	2.50±2.84 (7/10)b	0.03±0.02	1.00 ± 0.67 (8/10)a	2.30 ± 3.26	0.10 ± 0.32 (1/10)	16.87	-	-	3.80 ± 3.36 (9/10)b	1.10 ± 3.28a
Bl (2%)	3.92±1.89 (13/13)b	0.03±0.02	0.69 ± 0.75 (7/13)a	1.34 ± 2.54	0.15 ± 0.38 (2/13)	10.96 ± 0.30	0.08 ± 0.28 (1/13)	26.27	4.85 ± 2.44 (13/13)b	1.00 ± 3.92a
Rg (2%)	3.82±2.32 (10/11)b	0.03±0.02	1.91 ± 2.63 (6/11)a	0.81 ± 1.08	-	-	-	-	5.64 ± 3.98 (10/11)a	0.29 ± 0.72(b)
Rf (2%)	3.77±2.74 (13/13)b	0.03±0.02	1.08 ± 1.26 (8/12)a	1.91 ± 2.44	0.23 ± 0.44 (3/12)	12.88 ± 0.58	0.08 ± 0.28 (1/12)	22.05	5.15 ± 3.80 (13/13)a	1.14 ± 3.61a
RgM (0.1%)	4.50±2.51 (9/10)a	0.03±0.02	1.20 ± 1.14 (7/10)a	2.11 ± 2.74	-	-	0.20 ± 0.42 (2/10)	42.52 ± 17.82	5.90 ± 2.51 (10/10)a	1.90 ± 8.15a
Hg (4%)	3.33±2.35 (10/12)b	0.03±0.02	0.83 ± 0.94 (6/12)a	0.60 ± 0.53	-	-	-	-	4.17 ±2.69 (12/12)b	0.14 ± 0.32(b)
Hf (4%)	4.25±2.26 (12/12)a	0.03±0.02	1.33 ± 1.44 (8/12)a	2.18 ± 2.72	-	-	-	-	5.58 ± 2.91 (12/12)a	0.54 ± 1.59a

*MBN treatment (cancer initiation) during weeks 1 – 5, after which tea was given as the sole drinking fluid for 25 weeks, 1 week after initiation. Values in parenthesis are number of rats with a lesion. Means ± STD in the column followed by the same letter does not differ significantly. If letters differ, then $P<0.05$. Green tea (Gr); Black tea (Bl); Rooibos unfermented (Rg); Rooibos fermented (Rf); Honeybush unfermented (Hg); Honeybush fermented (Hf); Rooibos unfermented methanol extract (RgM).

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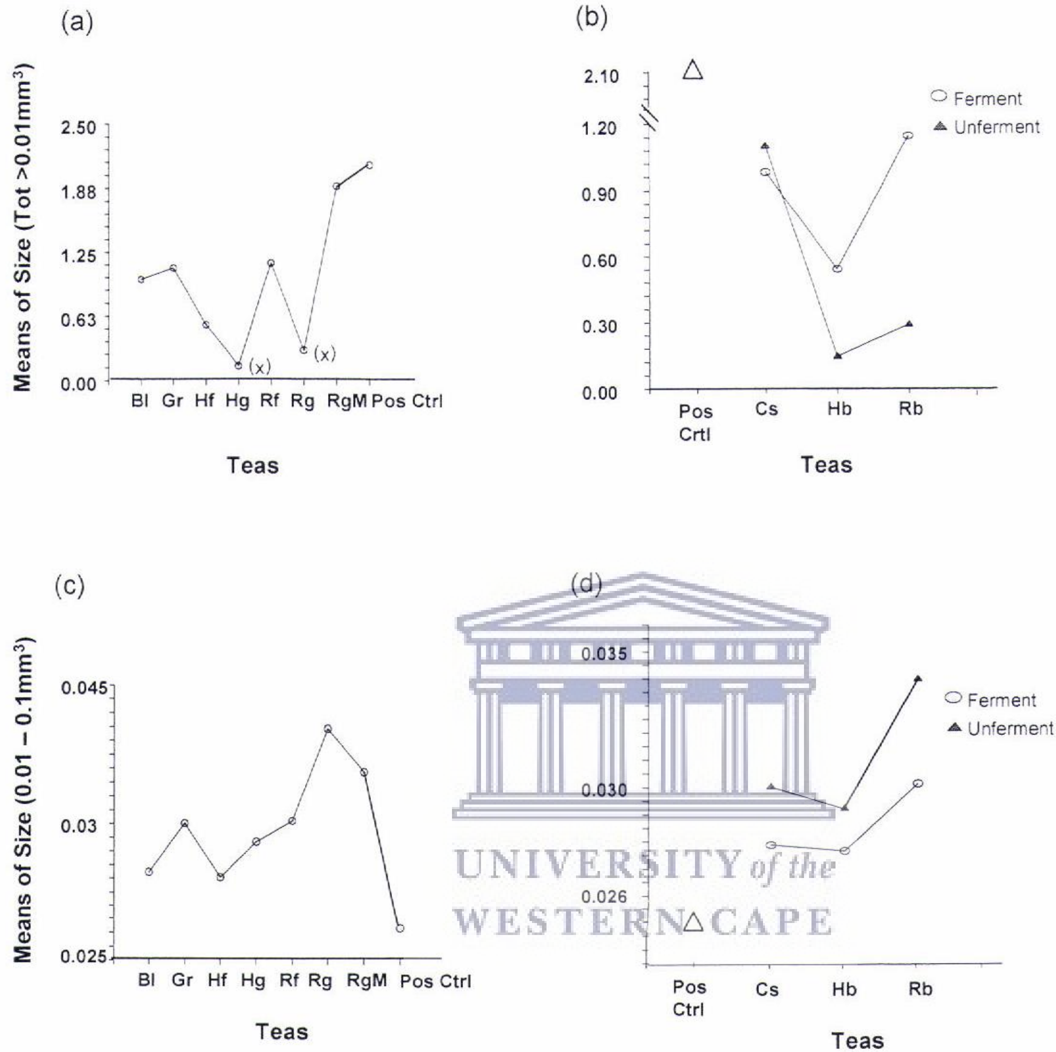

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Fig. 3 Interactive plots illustrating the combined (a, c, e, g) and separate (b, d, f) effects of fermented and unfermented teas on the papilloma sizes in the categories, > 0.01 and 0.01-0.1cm^3, 0.1-10 and 10-20cm3. (x) denotes marginal differences. [Bl = black tea; Gr = green tea; Hf = fermented honeybush tea; Hg = unfermented honeybush tea; Rf = fermented rooibos tea; Rg = unfermented honeybush tea; RgM = unfermented rooibos methanol extract; Pos ctrl = positive control]. [Cs = *Camellia sinensis;* Hb = honeybush; Rb = rooibos].

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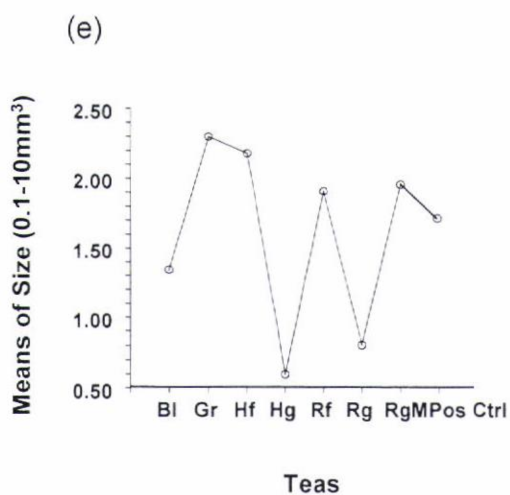
(e)



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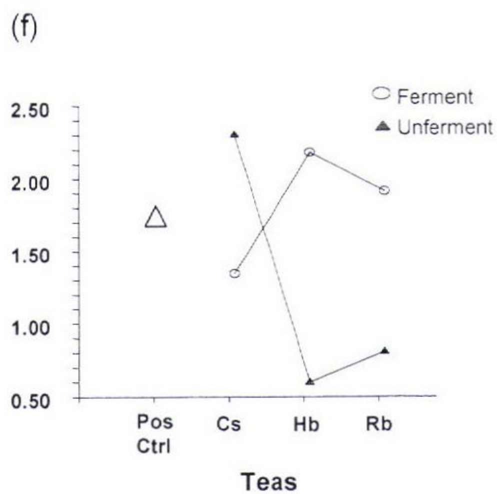

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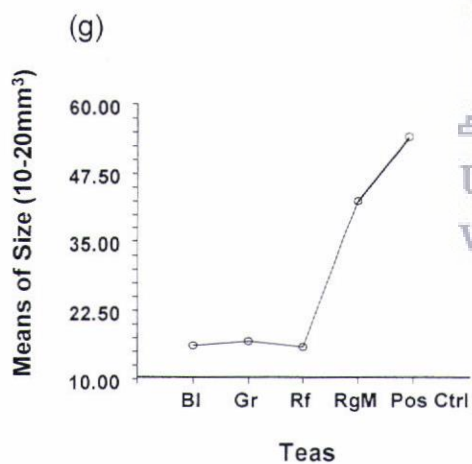

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noticed for the 0.1 to 10 mm^3 category (Fig. 3 e) by unfermented rooibos and honeybush. The latter teas, as well as fermented honeybush, inhibited the development of larger size papillomas (>10 mm^3). Green tea inhibited the formation of papillomas >20 mm^3 (Table 6).

Interactive plots showed that fermentation counteracted the reduction of the total mean size of the papillomas (Fig. 3 b). Although the means size of the sub category (0.01 to 0.1 mm^3) was similar (Fig. 3 d) between fermented and unfermented teas, fermentation markedly increased the size in the 0.1 to 10 mm^3 subclass (Fig. 3 f). Treatment with unfermented rooibos and honeybush, as well as fermented honeybush tea prevented the induction of larger (> 10 mm^3) papillomas (Fig. 3 g). Fermented rooibos did not prevent the induction of papillomas in this size category.

3.4.7.2 Mean total number (> 0.01 mm^3)

The highest mean number of papillomas was observed in the positive control group (Table 6). Green, black and unfermented honeybush teas significantly ($P<0.05$) reduced the number of papillomas. Both rooibos teas, fermented honeybush tea and the RgM extract also markedly reduced the number of papillomas, although not significantly. The number of papillomas in the subcategory 0.01 to 0.1 mm^3 was significantly ($P<0.05$) reduced by green, black, unfermented and fermented rooibos and unfermented honeybush tea. A marked reduction was noticed with fermented honeybush tea and the RgM extract. A similar effect (not significantly) was noticed for black tea and unfermented honeybush tea in reducing the number of papillomas of the subcategory 0.1 to 10 mm^3. The RgM extract, both honeybush teas and unfermented rooibos did not show papillomas in the subcategory 10 to 20 mm^3. In the subclass >20 mm^3 there were no papillomas in the rats consuming green, unfermented rooibos, unfermented honeybush and fermented honeybush teas. In the group receiving the RgM extract two rats developed papillomas >20 mm^3.

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When considering the interactive plots combining the papilloma incidence of green and black, fermented and unfermented rooibos and fermented and unfermented honeybush, all the teas significantly ($P<0.05$) lowered the total number (Fig. 4 a) and the number of the 0.01 to 0.1 mm^3 size category (Fig. 5 a). The combined effects were considered to increase the statistical power of the results. No significant difference was noticed for the other size categories (Fig. 6 a, 8a, 9a), with the exception of the 10 to 20 mm^3 (Fig. 7 a) and >20 mm^3 (Fig. 8 a) size categories, where no papillomas were obtained for the unfermented rooibos and honeybush teas, and fermented honeybush tea.

The separate effects of the different teas showed that, except for rooibos, fermentation minimised the effect that the unfermented teas had (Fig. 4 b) when considering the total mean number of papillomas (>0.01 mm^3). Green tea and unfermented honeybush tea showed the lowest papilloma score when compared to their fermented counter parts. The mean number of the size subclass 0.01 to 0.1 mm^3 showed that green, black, unfermented honeybush teas and fermented and unfermented rooibos teas significantly reduced the mean number of papillomas (Fig. 5 b). Again green tea and unfermented honeybush tea showed the lowest number of papillomas. No significant changes were notice in the number of papillomas for the 0.1 to 10 mm^3 size category (Fig. 6 b) although unfermented honeybush tea showed a lower number than fermented honeybush tea. When considering the subcategories (10 to 20 and >20 mm^3), no papillomas were observed in any of the rats treated with unfermented rooibos and honeybush teas and fermented honeybush tea (Fig. 7 b, 8 b). When compared to their unfermented counterparts, fermented rooibos and black teas exhibited a higher papilloma count in these size categories.

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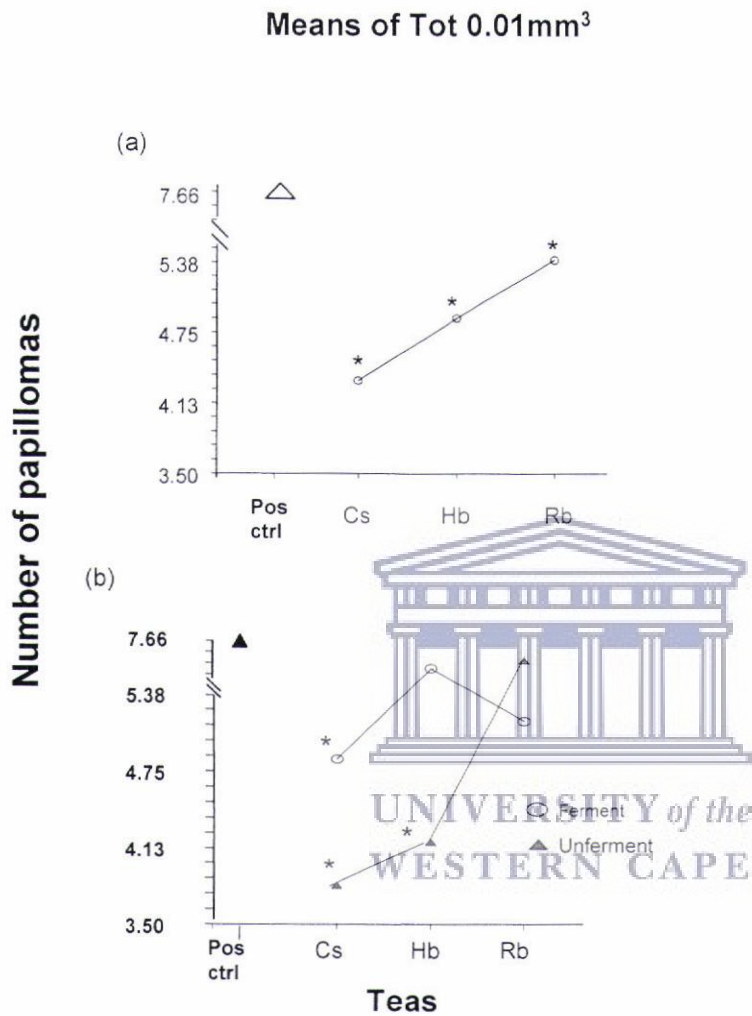

Fig. 4 Interactive plots illustrating the combined (a) and separate (b) effects of fermented and unfermented teas on the induction of the total number of oesophageal papillomas (>0.01mm3). *denotes significant ($P<0.05$) difference compared to the control. [Pos ctrl = positive control; Cs = *Camellia sinensis;* Hb = honeybush; Rb = rooibos].

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Means of Tot 0.01 – 0.1mm³

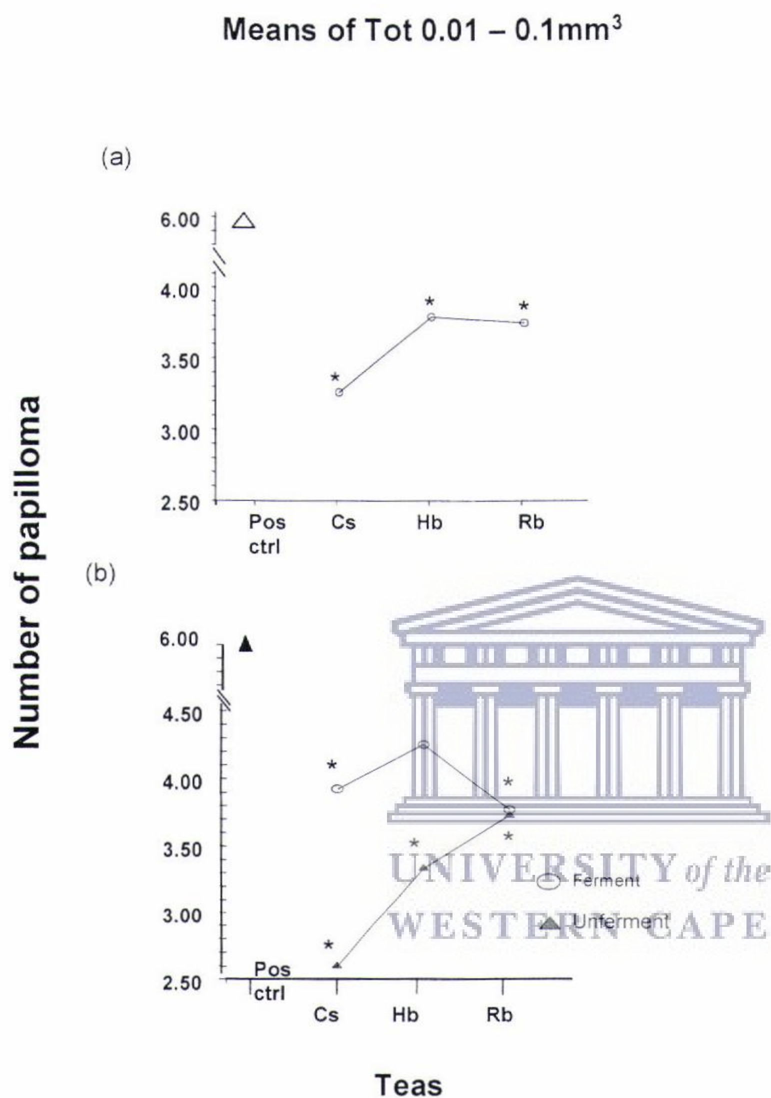

Fig. 5Interactive plots illustrating the combined (a) and separate (b) effects of fermented and unfermented teas on the induction of the total number of oesophageal papillomas (0.01-0.1mm³). * denotes significant ($P<0.05$) difference compared to the control. [Pos ctrl = positive control; Cs = *Camellia sinensis;* Hb = honeybush; Rb = rooibos].

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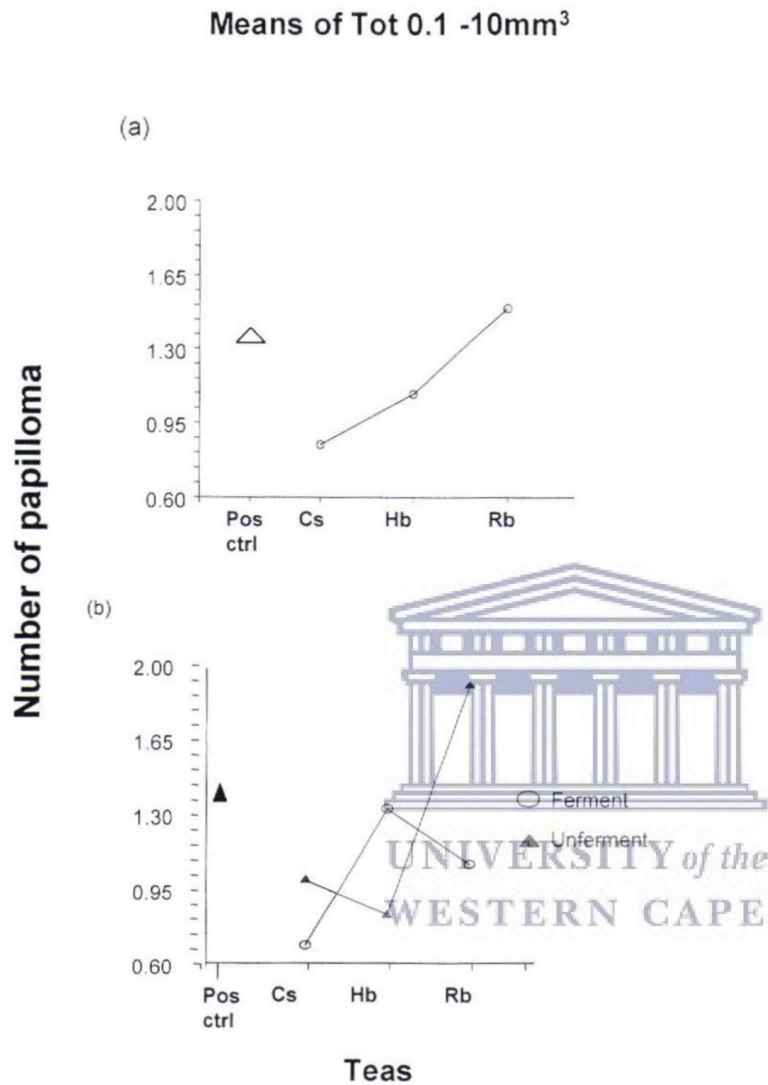

Fig. 6. Interactive plots illustrating the combined (a) and separate (b) effects of fermented and unfermented teas on the induction of the total number of oesophageal papillomas (0. 1-10mm^3). [Pos ctrl = positive control; Cs = *Camellia sinensis;* Hb = honeybush; Rb = rooibos].

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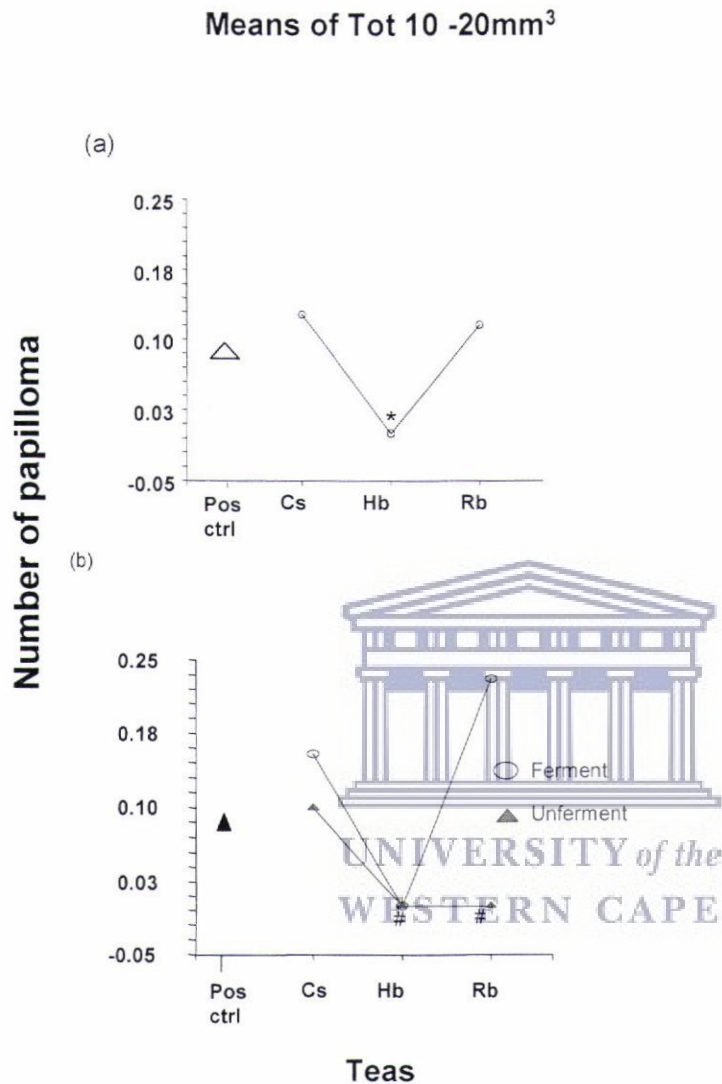

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Fig. 7 Interactive plots illustrating the effects of combined (a) and separate (b) effects of fermented and unfermented teas on the induction of the total number of oesophageal papillomas (10-20mm^3). * denotes significant ($P<0.05$) difference compared to the control. # denotes absence of large papillomas. [Pos ctrl = positive control; Cs = *Camellia sinensis;* Hb = honeybush; Rb = rooibos].

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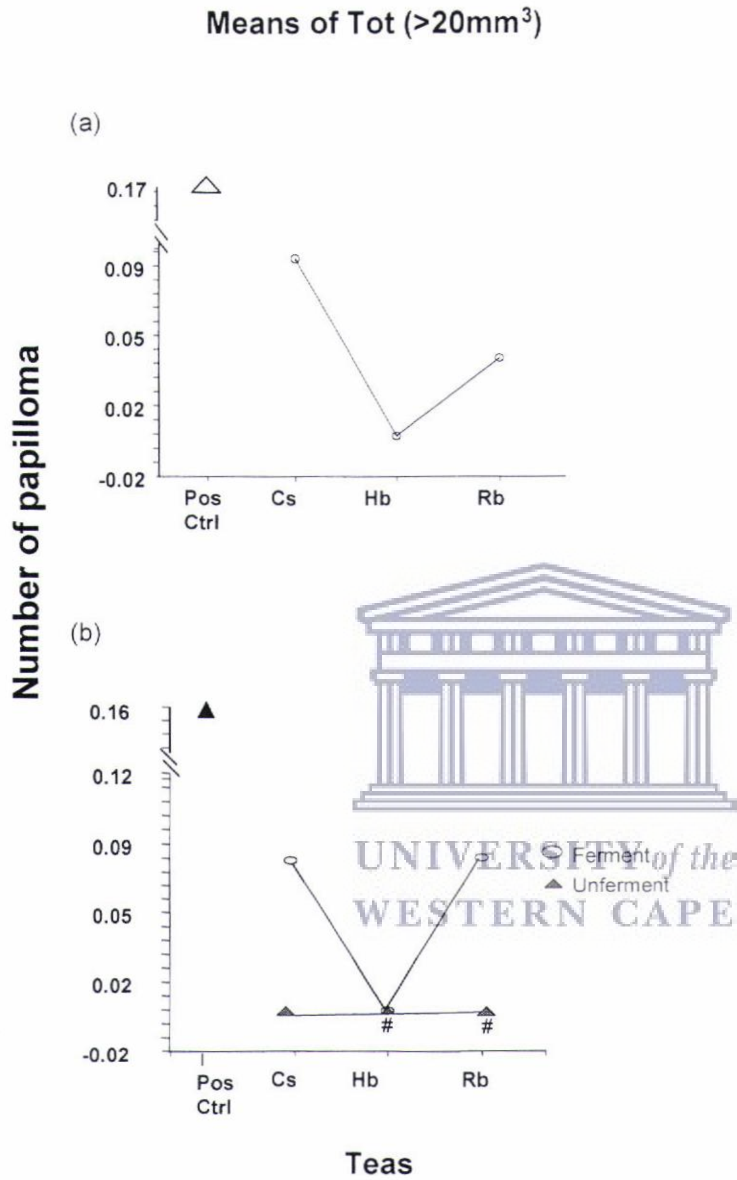

Fig.8 Interactive plots illustrating the effects of combined (a) and separate (b) effects of fermented and unfermented teas on the induction of the total number of oesophageal papillomas (>20mm^3). # denotes absence of large papillomas. [Pos ctrl = positive control; Cs = *Camellia sinensis;* Hb = honeybush; Rb = rooibos].

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3.4.8 Modulation by *Sutherlandia* infusions

Sutherlandia infusion, at the concentration (1%) used did not produce any known toxic or side effects in the rats throughout the study. Ninety percent of the rats developed papillomas, while only 33% of the rats developed papillomas in the size category 0.1 to 10 mm^3 when compared to the 50% of the control rats. The total means, as well as the number of papillomas in the 0.01 to 0.1 mm^3 size category, were marginally decreased (Table 7). No significant changes were noticed regarding the size of the papillomas, although there was a marked reduction in the 0.1 to 10 mm^3 size category, while no papillomas >20 mm^3 were recorded (Table 7).

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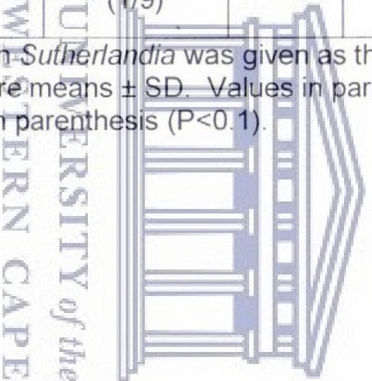
Table 7 Effect of *Sutherlandia frutecens* on the total mean and different size categories oesophageal papillomas

Group	Papillomas									
	0.01 to 0.1mm^3		0.1 to 10mm^3		10 to 20 mm^3		>20		Tot (>0.01)	
	Number	Size	Number	Size	Number	Size	Number	Size	Number	Size
Ctrl Tap water	-	-	-	-	-	-	-	-	-	-
Pos Ctrl* Tap water	6.08±3.70 (11/12)a	0.03±0.02	1.33 ± 2.31 (6/12)	1.92 ± 2.50	0.08 ± 0.29 (1/12)	11.15	0.17 ± 0.58 (1/12)	75.64 ± 48.41	7.67 ± 4.64 (11/12)a	2.10 ± 12.18
Sut 1%	3.89±3.69 (8/9)(b)	0.03±0.02	0.78 ± 1.30 (3/9)	0.67 ± 0.69	0.11 ± 0.33 (1/9)	13.78	-	-	4.78 ± 4.68 (8/9)(b)	0.44 ± 2.09

*MBN treatment (cancer initiation) during weeks 1 – 5, after which *Sutherlandia* was given as the sole drinking fluid for 25 weeks, 1 week after initiation. *Sutherlandia frutescens* subspecies *microphylla* (Sut). Values are means ± SD. Values in parenthesis are number of rats with a lesion. Means in a column followed by the same letter does not differ significantly. Letters in parenthesis ($P<0.1$).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3.5 Discussion

The anticancer properties of tea (*Camellia sinensis*) has been studied extensively in various carcinogenesis models in animals including skin, lung, oesophagus, stomach, liver, small intestine, pancreas, colon and mammary gland (Lambert et al., 2003a). Many of these chemopreventive properties are attributed to the biochemical and pharmacological properties of the tea polyphenolic constituents, the catechins, which constitute between 30 to 40% of their dry weight (Gupta et al., 2001; Lambert and Yang, 2003b).

In the present study, the ability of South African herbal teas and an infusion of *Sutherlandia* in modulating MBN-induced oesophageal carcinogenesis in F344 rats was compared to green and black teas. A recent study showed that rooibos and honeybush tea exhibit anticancer properties in a mouse skin model (Marnewick *et al.*, 2005), which merit further studies to elucidate their chemopreventive properties. Apart from studies in cell culture systems, very little is known about the anticancer properties of *Sutherlandia* (Tai *et al.*, 2004; Chinkwo, 2005; Stander *et al.*, 2007). The chemopreventive properties of some principle compounds, characterised from the herbal plant, including L-canavanine, a non-protein alpha-amino acid, pinitol, and gamma amino butyric acid (van Wyk *et al.*, 1997) are not particularly well-known at present.

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Different batches of the teas were prepared during the course of the experiments, which could account for variations in the intake of soluble solids, total polyphenol, flavonol/flavone and flavanol/proanthocyanidin intake parameters. These variations could have arisen during the preparation of the stock solutions, despite that fact the same source of raw material was used for each tea. In general, a decrease in the intake profiles of the different parameters, expressed per 100 g body weight, was noticed during the 6 month period, mainly due to the increase in rat body weight. Unfermented honeybush tea had the highest soluble solid content followed by green and black tea, unfermented rooibos, fermented rooibos and honeybush, *Sutherlandia* and RgM extract. The reason for using a higher level for honeybush (4%) was that its aqueous extracts and polyphenols exhibited weaker antioxidant

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potency than rooibos and the *Camellia sinensis* teas (Van der Merwe, 2006; Joubert *et al*., 2008). The RgM extract was used at a concentration of 0.1% to avoid any possible toxic effects.

A recent *in vitro* study showed that an ethyl acetate fraction of unfermented rooibos, as well as an aspalathin enriched extract exhibited prooxidant effects (Joubert *et al*., 2005). It is also known that the total polyphenol content could affect prooxidant effects (Galati *et al*., 2002). However, a 4 to 5 times higher level of the RgM extract should have been considered as the TPP intake profiles was approximately 3 times less than that of the rats receiving unfermented rooibos tea. The selection of a specific concentration of *Sutherlandia* in the drinking water was based on the soluble solid content, which was similar to fermented rooibos tea, while the flavonol/flavone concentration was similar to unfermented honeybush and black teas. However, the bitter taste of the extract (1%) seems to hamper the fluid intake of the rats, as it was significantly lower than green tea. A higher concentration (>1%) could have adversely affected the tea intake profiles. Future studies in experimental animals could include detailed TPP analyses before feeding commences in order to provide a more balanced polyphenol intake profile.

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The oesophageal specific carcinogen, MBN, induces a variety of epithelial lesions, including preneoplastic (squamous cell hyperplasia and dysplasia) or neoplastic (squamous cell papilloma and carcinoma) (Xiang, 1995; Zgodzinski, 2003; Szumilo, 2007), and is therefore routinely used in rat oesophageal tumorigenesis. Regardless of the route of administration, multiple oesophageal lesions develop promptly in response to chemical carcinogens over several months (Siglin *et al*., 1996). In the present study, all the tea preparations exhibited an inhibitory effect on the number of oesophageal papillomas.

When considering possible mechanisms involved whereby the herbal teas inhibited papilloma development, different tea intake parameters should be considered. The tea intake did not differ significantly between the different treatments. Rats receiving the unfermented honeybush tea had the highest intake of soluble solids,

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TPP and flavonol/flavone, while the flavanol/proanthocyanedin intake exceeded that of the other herbal teas. Although the intake of fermented rooibos soluble solids, TPP and flavonol/flavone were similar to unfermented rooibos, the flavanol/proanthocyanidin intake was almost 3 times lower. The *Camellia sinensis* teas had similar intakes regarding soluble solids, TPP and flavonol/flavone, while the flavanol/proanthocyanidin intake was 2 times higher than in black tea. The flavonol/flavone intakes of black and green tea were in the same order as fermented and unfermented rooibos and fermented honeybush due to the high soluble solid intake. It has to be kept in mind that the flavonol/flavone content (mg/100 mg soluble solids) of the aqueous extracts of the rooibos teas is considerably higher than the *Camellia sinensis* teas, but the soluble solid intake was 50% higher in the latter teas. When considering the different intake parameters of the *Camellia sinensis* and herbal teas, it would appear that the TPP intake plays a critical role in the inhibition of papilloma development. It can be assumed that a TPP intake of >7 mg/100g body weight, and a specific ratio of different polyphenolic constituents appear to be important, as the RgM extract, which had a TPP intake of 3.78 mg/100 g body weight did not affect papilloma development, although the flavonol/flavone to flavanol/proanthocyanidin (Flavo/Flava) ratio compared to unfermented rooibos and honeybush teas.

Another aspect to consider is the fermentation process, which may be important when considering the level of TPP intake, as it is related to the soluble solid content. Taking rooibos tea as an example, the chemopreventive properties of unfermented rooibos are related to a specific level of TPP intake (10.6 mg/100 mg body weight), which is significantly reduced during fermentation (7.5 mg/100 g body weight). Due to the low concentration of RgM extract used, the TPP intake was very low (3.8 mg/100 g body weight), which may account for the lack of any protective effects. Both fermented rooibos and the RgM extract did not affect tumour multiplicity when compared to unfermented rooibos, although fermented rooibos significantly reduced the number of the smaller papillomas. When considering the polyphenolic subclasses, the flavanol/pro-anthocyanidins were significantly reduced, while the flavonol/flavone content of the soluble solids was

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increased, resulting in an increased Flavo/Flava ratio (16.4) during fermentation, again implying the role of specific polyphenolic ratios. Even though the TPP intake of fermented honeybush tea was reduced, the high soluble solid intake due to the preparation of the 4% tea extract countered the reduction, thus having a similar TPP intake as the rats fed fermented rooibos tea. The ratio, however, was similar to that of unfermented rooibos tea. The TPP intake, however, was significantly lower than unfermented honeybush and therefore exerted a weaker chemoprotective effect with respect to papilloma development.

Fermentation does not dramatically affect the TPP intake of green and black teas. They have however, a higher soluble solid content compared to the rooibos teas. Far higher levels of flavanol/proanthocyanindins were consumed while the TTP and flavonol/flavone intake levels compared favourably to that of the rooibos teas. Even though the rats in the green tea group did not have papillomas bigger than 20 mm^3, the rats consuming black tea had smaller sized papillomas (0.1 to 10 mm^3) than in the green tea group. Since fermentation changes the chemical structure of the tea flavonoids by reducing the catechins (Babich *et al.*, 2006; Wang and Li, 2006), black tea would be expected to have a lesser inhibitory action of the papillomas than green tea, if solely dependent on the catechins for an effect. Although green tea exhibited a higher protective effect with respect to papilloma number, the high TPP content of both green and black tea resulted in a similar chemopreventive effect in the present study, suggesting that compositional changes, i.e. loss of catechins and formation of theaflavins, thearubigins and theafulvins, as a result of fermentation had little effect. It was reported that the inhibitory action on papilloma induction in rats by green tea is attributed to the catechin content, which consist of EC, ECG, EGCG (Nishikawa *et al.*, 2006; Thangapazham *et al.*, 2006). The black tea catechins, theaflavins and thearubigins or a combination of two or more, is exhibiting similar chemopreventive properties to green tea (Morse *et al.*, 1997).

Unfermented rooibos and honeybush teas contained a higher level of phenolic compounds than their fermented counterparts, resulting in a higher mean daily

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intake (mg/100 g body weight). The major flavonoid of unfermented rooibos, when considering the daily intake was aspalathin, followed by equal amounts of nothofagin, orientin, iso-orientin and rutin/isoquercetin, which was almost 10 times lower. Orientin, iso-orientin and rutin/isoquecitrin was the major flavonoids consumed by the rats receiving the fermented rooibos. Based on these intake parameters, aspalathin, constituting about 14% of the soluble solids of unfermented rooibos, is likely to impact on the chemopreventive properties. This becomes evident as fermented rooibos contains considerably less aspalathin, resulting in a lower intake, and a reduction in the chemopreventive properties of rooibos tea. At present very little is known about the chemopreventive properties of aspalathin, although it is known to be the major antioxidant of rooibos (Von Gadow *et al.*, 1997; Joubert *et al.*, 2004), while it only exhibited moderate antimutagenic properties (Snijman *et al.*, 2007). However, apart from aspalathin, other unidentified compounds or changes in Flavo/Flava ratios could be responsible for the chemopreventive properties of rooibos against oesophageal cancer.

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Rats consuming the unfermented honeybush tea, like unfermented rooibos, had the smallest mean sized papillomas, while it significantly reduced the number of papillomas. However, despite the high flavonol/flavone and flavanol/proanthocyanidin intake, the major polyphenols was the xanthone, mangiferin followed by isomangiferin, which constitute almost 6% of the soluble solids. This inhibitory effect on papilloma development may be brought about either by one compound, or a combination of constituents, some of which could be unknown. This becomes evident as fermented honeybush contained considerably less mangiferin and Isomangiferin than unfermented honeybush, which coincided with a reduction in the protective effect against the number and size of papillomas. Mangiferin constitute the major antioxidant of honeybush (Hubbe *et al.*, 2000a, b), while it also exhibited anti-inflammatory properties (Beltràn *et al.*, 2004). Mangiferin also did not contribute to the flavanol/proanthocyanidin content, since it rails to react with DAC (Marnewick *et al.*, 2005), whereas other flavanol-like compounds such as the proanthocyanidins and/or polymeric tea tannis would.

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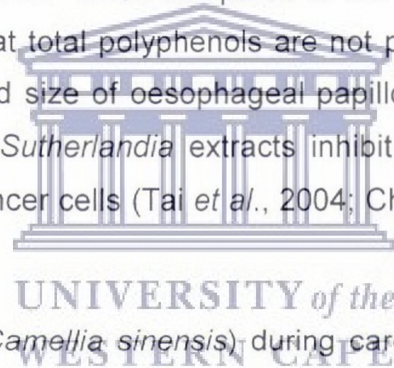
The *Sutherlandia* infusion intake profiles are of interest when compared to the *Camellia* and herbal teas. Analysis of teas showed low soluble solid, total polyphenols and flavonol/flavone content, while only trace amounts of flavanols/proanthocynidins were detected. Although the mean fluid intake was significantly lower than green tea and similar when compared to the other teas, the TPP only represent approximately 4% of the soluble solid intake. However, when looking at papilloma multiplicity, there was a marginal ($P<0.1$) decrease in the total mean papilloma number when compared to the positive control, due to a marginal decrease in the smaller papillomas (0.01 to 0.1 mm^3). The rats failed to developed papillomas larger than 20 mm^3. There was a marked decrease in total mean size and the size category 0.1 to 10 mm^3 when compared to the positive control. From the intake profiles, it is clear that total polyphenols are not playing a major role in the reduction of the number and size of oesophageal papillomas in these rats. *In vitro* studies have shown that *Sutherlandia* extracts inhibit cell proliferation and induce apoptosis in different cancer cells (Tai *et al*., 2004; Chinkwo, 2005; Stander *et al*., 2007).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The modulating effect of tea (*Camellia sinensis*) during carcinogenesis in animal models is unique, as no other agent tested for possible chemopreventive effects have shown such efficacy at the concentrations usually consumed by humans (Dreosti *et al*., 1997). Studies reported on the beneficial effect of green tea as a cancer prevention tool in animal cancer models and epidemiological studies in humans (Klaunig, 1992; Katiyar and Mukhtar, 1996). The present study demonstrated that, both unfermented herbal teas significantly inhibit papilloma development in rats. An infusion of *Sutherlandia* also provided evidence, for the first time, of chemopreventive properties against papilloma development *in vivo*. Since it is improbable that the polyphenolic compounds are principlally responsible for the inhibitory action of *Sutherlandia*, the bioactive constituents that may play a significant role still needs to be identified. Modulation of the growth kinetics of the early neoplastic lesions by rooibos and honeybush teas, presumably by the polyphenolic constituents, is likely to reduce their proliferative activity and interfere with the cancer promotion/progression stages of carcinogenesis. It seems that

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multiple mechanisms exist by which the tea constituents elicit their inhibitory effects, but the relevant mechanisms still have to be determined to assess whether it can be used in developing chemopreventive strategies in humans. The key differences regarding the *Camellia sinensis* and herbal teas would appear to be the intake of a specific level (>7 mg/100 g body weight) and ratio of the polyphenol components. This is not the case regarding *Sutherlandia,* which has a total polyphenol content of less than 1 mg/100g body weight, suggesting that the protective effect may be ascribed to non-polyphenol compounds.

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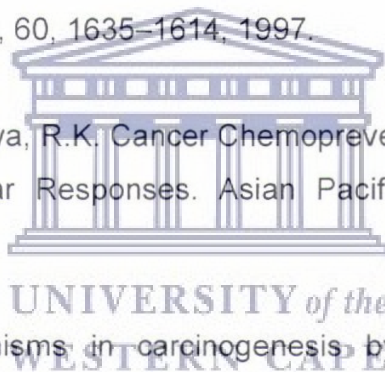
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CHAPTER 4

Comparative cytotoxic and anti-proliferative properties of South African herbal teas and an infusion in a human oesophageal cancer cell line

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Abstract

The cytotoxic effects of freeze-dried aqueous extracts (FDE) of green and black (*Camellia sinensis*), unfermented and fermented rooibos (*Aspalathus linearis*) *and honeybush* (*Cyclopia intermedia*) teas, as well as an aqueous infusion of *Sutherlandia frutescens* were investigated in a human oesophageal cancer (WHCO5) cell line. The effect on the cell proliferation was monitored using green tea and the unfermented herbal teas. The cytotoxic parameters of the *Sutherlandia* infusion could not be determined due to plant matrix and media interactions. Colour interference by the different teas also prohibited the use of the lactate dehydrogenase (LDH) and the tetrazolium bromide (MTT) cytotoxicity assays. Cell viability assays utilising flow cytometry and the CellTiter Glo® Luminescent Cell Viability assay indicated that green and black teas exhibited higher cytotoxic effects than rooibos and honeybush teas when considering the IC_{50} values. Differences in the cytotoxicity (IC_{50} values) could be explained by the total polyphenol (TPP) content of the different teas and herbal tea extracts and, more specifically, the flavonoid and/or xanthone/flavanones subgroups. Aspalathin the major flavonoid in unfermented rooibos tea and some of the constituents in fermented rooibos tea could be important factors affecting cell viability, while the xanthones, mangiferin and isomangiferin appear to be the key determinant in honeybush tea. Unfermented rooibos tea was the most active in inhibiting cell proliferation, followed by green and honeybush teas. Differences seem to exist between the type of polyphenol involved in cytotoxicity and inhibition of cell proliferation. The selective cytotoxic and inhibition of cell proliferation effects in cancer cells could be important in developing flavonoid-enriched extracts as possible tools for chemoprevention.

Keywords

Herbal teas, oesophageal cancer cells, cytotoxicity, cell proliferation, total polyphenols.

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4.1 Introduction

Oesophageal cancer occurs worldwide with its most common form being squamous cell carcinoma (SCC) (Stoner *et al.*, 2007). In 2002 oesophageal cancer was the eighth most common cancer, with nearly 400 000 new cases diagnosed annually (Parkin, 2005). Although many carcinogens are found in the environment, no single substance has been found to be responsible for oesophageal cancer. Any carcinogen may thus play a role, either alone or in synergy with other factors in the development of the disease. In addition to smoking tobacco, alcohol is also a risk factor for oesophageal cancer. Alcohol may increase susceptibility of other carcinogens, contribute to deficiencies in the diet, or it may act as a direct irritant to the oesophageal mucosa (Holmes and Vaughan, 2006). A wide variety of dietary substances is carcinogenic and reported to stimulate the development of tumours in experimental animals (Surh, 2003). Salt-cured, salt-pickled and mouldy foods as a result of contamination with N-nitrosamine carcinogens due to the presence of fungal toxins have been implicated (Li *et al.*, 1986; Rheeder *et al.*, 1992; Sammon, 2006). Studies in China suggested that nitroso compounds and their precursors contribute to the development of oesophageal carcinoma in the high-risk areas (Lu *et al.*, 1991).

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Regular consumption of fruit and vegetables may decrease the risk of specific cancers (Surh, 2003). Thus, individual or collective components in some plant-based diets possess anti-carcinogenic and anti-mutagenic properties. Carcinogenesis is a multi-step process during which distinct molecular and cellular alterations occur in cell-signalling pathways that regulate cell proliferation and differentiation (Hartwell and Kastan, 1994; Lin *et al.*, 1999). Plant-based substances may protect against these carcinogen-induced cellular changes thereby intervening with the initiation and/or promotion stages of carcinogenesis, or retard the progression of precancerous cells into cancer. Effective chemopreventive agents would either inhibit further growth of the lesions or delay the progression of the lesions into carcinoma (Stoner *et al.*, 2007).

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Although progress has been made regarding our understanding of the process of carcinogenesis, the mechanisms of action exerted by various components in the diet are not fully elucidated (Surh, 2003). The risk of cancer may be reduced if the carcinogens identified can be eliminated or exposure to them be minimized, so that primary prevention or intervention may be implemented (Surh, 2003). Despite advances in the diagnosis and treatment of oesophageal cancer, the prognosis remains poor. Chemoprevention is therefore an important approach to reduce the incidence and mortality of this disease and studies could provide important leads as to new preventive strategies.

The chemopreventive properties of green tea have been studied extensively and studies in cancer cell lines showed that the tea catechins and/or polyphenolic extracts inhibit their growth (Valcic *et al*., 1996; Yang *et al*., 1998; Steel *et al*., 2000; Chen et al., 2004; Ma *et al*., 2007). The tea catechins target many cellular pathways within the cell, leading to alterations in proliferative and/or apoptotic function (Paschka *et al*., 1998; Bode & Dong, 2004; Ramos *et al*., 2005), and their role as antimutagenic agents is well known (Kuroda & Hara, 1999; Gupta *et al*., 2002). Studies on the chemopreventive properties of South African herbal teas as well as *Sutherlandia*, and/or their constituents, have recently been conducted and reviewed (Tai *et al*., 2004; Marnewick, *et al*., 2004; 2005; McKay and Blumberg, 2006). Extracts of *Sutherlandia frutescens* were reported to exhibit anti-proliferative effects and induces apoptosis in cancer cells (Swaffer *et al*., 1994; Chinkwo, 2005; Stander *et al*., 2007). However, no studies on the anti-proliferative effects of the herbal teas in cancer cell lines have been conducted as yet.

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The present study was conducted to evaluate the cytotoxic and growth inhibitory properties of *Camellia sinensis*, *Aspalathus linearis*, *Cyclopia intermedia*, and *Sutherlandia frutescens* in a human oesophageal cancer cell line utilising different *in vitro* assays.

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4.2 Materials and methods

4.2.1 Reagents and disposable plastics

Cell culture materials were purchased from Whitehead Scientific (South Africa) and consisted of Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin versene and Hank's balanced salt solution (HBSS). Tissue culture flasks and clear 96-well microtiter plates (MPs) were purchased from The Scientific Group (South Africa), while disposable plastic pipettes were obtained from LASEC (South Africa). The black and white 96-well MPs were purchased from Whitehead Scientific. The MTT powder (M5655-1g) and solvents, except for 70% ethanol (Rob Dyer Surgical), were obtained from Sigma-Aldrich and Merck Chemicals (South Africa), respectively. The CytoTox 96® Non-Radioactive Cytoxicity Assay and CellTiter Glo® Luminescent Cell Viabiltiy Assay (Promega) was obtained from Whitehead Scientific (South Africa), while the Cell Proliferation ELISA, BrdU (Chemiluminescent) assay kit was purchased from Roche (Germany).

4.2.2 Human cancer cell line

Human derived oesophageal squamous cell carcinoma, WHCO5, was kindly donated by Prof Iqbal Parker, Institute of Infectious Disease and Molecular Medicine (IIDMM), Faculty of Health Sciences, University of Cape Town. The cells were maintained in DMEM, with 10% fetal bovine serum (FBS) and L-glutamine (1%), in a humidified atmosphere of 5% CO_2 at 37°C.

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4.2.3 Tea preparations and polyphenol analyses:

Aqueous tea extracts of the herbal teas and infusion, as well as the methanol extract of unfermented rooibos tea (RgM extract) were prepared as described in Chapter 3. Determination of the different tea polyphenolic groups, as well as the HPLC analyses, was conducted as described in Chapter 3 (sections 3.2.3).

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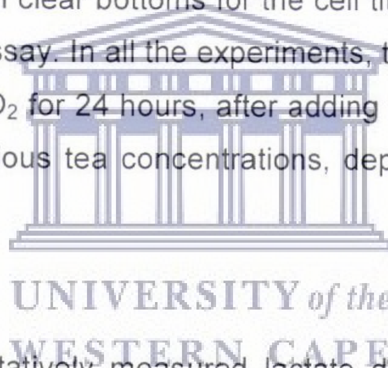
4.2.4 Cell culture studies

The aqueous extracts (10%) were freeze-dried and kept desiccated in airtight containers. Stock solutions (10% soluble solids) of the freeze-dried extracts (FDE) were prepared in 10% DMSO/H_2O, except for green tea and *Sutherlandia*, which was dissolved in 20% DMSO/H_2O due to difficulty in solubility and filtering the solution. The stock solutions were filtered through 0.2µm syringe filters before diluting with culture medium. Different concentrations of the extract were investigated, depending on the assay used, in order to obtain an IC_{50} value.

For each *in vitro* assay the cells were seeded (5 000 cells/well) in DMEM (200µl) and allowed to attach for approximately 15 hrs to reach 60 to 70% confluency, before adding the tea dilutions. Clear microtiter plates were used for LDH and MTT, opaque-walled white, with clear bottoms for the cell titer, and opaque black for the BrdU cell proliferation assay. In all the experiments, the cells were allowed to incubate at 37°C with 5% CO_2 for 24 hours, after adding fresh medium (100µl) containing 0.5% FBS, and various tea concentrations, depending on the assay implemented.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4.2.4.1 <u>LDH Assay</u>

This colorimetric assay quantitatively measured lactate dehydrogenase (LDH) release upon cell lysis. LDH leakage into cell culture medium was measured 24 hrs after the addition of different dilutions of the various tea extracts. The cytotoxicity assay performed was adapted from the CytoTox 96® Non-Radioactive Cytoxicity Assay Kit protocol (Whitehead Scientific), where the amount of colour formed is directly proportional to the number of lysed cells. The assay is based on the conversion of lactate to pyruvate in the presence of LDH, with the reduction of NAD to NADH. In short, lysis solution (10-15µl) was added to untreated cells in DMEM for maximum LDH release, as well as to the volume correction control, and incubated for 45 min at 37°C. For the LDH and MTT assays the extracts were diluted in the DMEM as follows: 0.5, 2.5, 5 and 10 mg FDE/ml for green, black, fermented rooibos and fermented honeybush teas; 0.1,

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0.5, 2 and 4 mg FDE/ml for unfermented rooibos and unfermented honeybush teas, as well as the unfermented rooibos methanol extract, and 5, 10, 20 and 50 mg FDE/ml for the *Sutherlandia* infusion.

The substrate mix was reconstituted in the assay buffer (12ml) at room temperature. For the LDH measurement, 50µl aliquots of the supernatant of the lysed cells were transferred to fresh flat-bottomed MPs, using a multichannel pipettor. The reconstituted substrate mix (50µl) was added and allowed to incubate, in the dark, for 30 min at room temperature. After 30 min the stop solution (50µl) was added and the absorbance recorded at 490nm, using an Opsys MR™ 96-well plate reader (Dynex Technologies). The percentage of cytotoxicity was calculated using the formula: % Cytotoxicity = $[(OD_{sample} - OD_{background})/(OD_{maximum\ control} - OD_{volume\ correction\ control}) \times 100\%]$.

4.2.4.2 MTT assay

The MTT assay is a quantitative, colorimetric-based assay that measures the reduction of a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] component (MTT) into an insoluble formazan product by the mitochondria of viable cells. The solubilised colour crystals are directly proportional to the number of viable cells. MTT (Sigma-Aldrich) was prepared fresh for every experiment, and added (10µl) to each well. After an incubation of 2 hrs at 37°C acidified isopropanol (100µl) was added, and the plate left for 25 min to solubilise. The absorbance was measured at 570-630nm using an Opsys MR™ 96-well plate reader (Dynex Technologies). The percentage of cell viability was calculated using the formula: % Cytotoxicity = $[(OD_{sample} - OD_{sample\ background})/(OD_{control} - OD_{background\ control}) \times 100\%]$.

4.2.4.3 Flow cytometry

Cells were seeded (1×10^5) in $25cm^2$ tissue culture-treated flasks and allowed to attach. The media was replaced with different tea/media dilutions (0.5, 1 and 2 mg FDE/ml of only green tea, unfermented rooibos and unfermented honeybush

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teas) after 24 hrs and the flask incubated at 37°C with 5% CO_2 for 24 hrs. Cells were trypsinized and stained according to the Guava Technologies protocol (Cat. No. 4000-0040) to obtain between 1×10^4 to 5×10^5 cells/ml. The Guava Viacount assay was selected from the desktop Guava® PCA™ flow cytometer made available by Whitehead Scientific to determine cell count and viability. The Cytosoft™ software, version 2.1.4 for data analyses was used. This assay allows for a rapid and reliable alternative to the trypan blue exclusion assay for cell count and viability. The Viacount reagent differentially stains for viable and non-viable cells determined by their permeability to the DNA-binding dyes in the reagent. The assay allows for the quantitative analysis of live, apoptotic and dead nucleated cells, by determining the exact numbers of each.

4.2.5 Determination of cell viability

This assay determined the number of viable cells in culture, based on the quantitation of ATP. With the addition of a single reagent cell lysis occurred and the luminescent signal produced would be proportional to the amount of ATP present. The signal develops due to the enzymatic (luciferase) conversion of luciferin into the luminescent product, oxyluciferin, in the presence of magnesium and ATP. The assay used in this experiment was adapted from the CellTiter Glo® Luminescent Cell Viabiltiy Assay protocol (Whitehead Scientific). The experimental cells were treated with 0.1, 0.5 and 1 mg FDE/ml of all the teas and/or infusions in the cell titer assay. Assay buffer was thawed and added (10ml) to the CellTiter Glo® substrate. The microtiter plates were removed from the incubator, allowed to equilibrate to room temperature, and the substrate (100µl) added. The contents were mixed for 2 min on an orbital plate shaker to allow for cell lysis, and the plates incubated for 10 min at room temperature after which the luminescence signal was recorded on a Veritas luminometer (Turner Biosystems). The percentage of cell viability was calculated using the formula: Luminescens = $[(RLUs_{sample} - RLUs_{sample\ background})/(RLUs_{control} - RLUs_{control\ background}) \times 100\%]$.

4.2.6 Cell proliferation assay

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This assay (Cell Proliferation, ELISA, BrdU Chemiluminescence - Roche) is designed to quantify cell proliferation by measuring the amount of bromodeoxyuridine (BrdU) incorporated into cellular DNA of proliferating cells. The luminescence signal generated in the reaction is quantified by using a micro plate luminometer. The cells were allowed to incubate with only the green tea (0.0625, 0.125, 0.25, 0.5 mg FDE/ml), unfermented rooibos (0.068, 0.138, 0.275, 0.55 mg FDE/ml) and unfermented honeybush (0.105, 0.21, 0.42, 0.84 mg FDE/ml) teas for 24 hrs and labelled with BrdU labelling solution (10μl) for 2 hrs at 37°C. The labelling solution was removed and 200μl/well FixDenat solution was added and left at room temperature for 30 min. The FixDenat was removed thoroughly and 100μl/well Anti-BrdU-POD working solution added for 90 min at room temperature. The plate was rinsed three times with washing solution (200μl) prior to adding the substrate solution (100μl/well). The plate was incubated for 3 min on an orbital plate shaker before quantifying luminescence (within 10 min after adding the substrate) with a Veritas luminometer (Turner Biosystems). Luminescens = $[(RLUs_{sample} - RLUs_{sample\ background})/(RLUs_{control} - RLUs_{control\ background}) \times 100\%]$; where sample is the medium/tea + cells + BrdU labelling solution + Anti-BrdU POD; sample background is the medium/tea + cells + Anti-BrdU POD; control is medium + cells + BrdU labelling solution + Anti-BrdU POD; and control background is medium only.

4.2.7 IC_{50} determination

The IC_{50} is a measure of effectiveness of the teas and/or infusions. It is indicative of the amount of substance necessary to inhibit a given biological process (cell growth, cell death, etc), or a component thereof, by half. The IC_{50} of the teas were determine constructing a dose-response curve and examining the effect of various concentrations on cytotoxicity, cell viability and cell proliferation *in vitro*. The IC_{50} values were obtained employing the equation y=mx+c, where m represents the gradient and c represents the y-intercept.

4.3 Statistical analysis

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The data, having only one factor (tea group) were analyzed using the F-test in a one-way ANOVA. The post hoc Tukey's Studentized Range Test was used to test for multiple pairwise comparisons between the means of the different levels of the group factor. As the data were unbalanced, the Tukey–Cramér adjustment was made automatically. Where variances were not equal the Welch test was substituted for the F test. Statistical significance was considered at $P < 0.05$.

4.4 Results

4.4.1 Cytotoxicty assays

4.4.1.1 LDH assay

WCHO5 cells were exposed to various doses of different teas and/or infusions to assess the cytotoxic effects. The cytotoxicity of the *Camellia sinensis* teas could not be determined as an increased dose resulted in colour interference with the absorbance readings (Table 1; Addendum, Table 1; Fig. 1 a, b, e, f). A similar trend was noted with the rooibos teas, with the exception of the unfermented rooibos methanol extract, which seemed to show increasing cytotoxicity with increasing doses of tea, although some interference existed at the highest concentration (4 mg/ml) (Table 1). Both honeybush teas, and the *Sutherlandia* infusion also exerted a dose response effect, regarding colour interference, at higher concentrations. However, the colour interference (%) was less when compared to the other teas (Addendum, Table 1; Fig. 1c, d, g, h). Data obtained from this study suggests that cytotoxicity could not be determined by using the Cytotox 96 non-radioactive assay kit due to colour interference of the teas at certain concentrations.

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4.4.1.2 MTT assay

The viability of WHCO5 cells was also monitored against different concentrations of teas using the MTT assay. As with the LDH cytotoxicity assay, the cell viability could not be accurately determined due to the percent of colour interference (Table 2; Addendum, Table 2, Fig. 2a -h). The extent of colour interference of all

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the tea preparations and herbal infusion was similar to that obtained in the LDH assay.

4.4.1.3 Flow cytometry

The cytotoxicity of the different unfermented tea preparations on WHCO5 cells were monitored by flow cytometry. The mean percentage of viable, apoptotic and dead cells were determined (Fig. 1) after exposure to various concentrations of green (a), unfermented rooibos (b) and unfermented honeybush (c) freeze-dried (FDE) extracts for 24 hrs. Each dot plot represents the different conditions (live, apoptotic and dead) in the cell population per FDE concentration [0.5(i), 1(ii), and 2 (iii) mg FDE/ml] of each tea. The top left-hand quadrant represents all viable cells; top middle the amount of apoptotic cells and the top right-hand the dead cells. When observing any one of the three teas (a, b, or c) at the different concentrations, a decrease in % cell viability and an increase in % apoptotic and dead cells is noticed. At 0.5mg FDE/ml the cluster of cells is more in the top left-hand sector; while at 1mg/ml cells migrated into the apoptotic and dead cell quadrants. At 2mg FDE/ml of green and unfermented rooibos teas, a large percentage of the cells are dead, and is represented in the top right-hand quadrant. At the same concentrations, cells exposed to unfermented honeybush tea showed only a small percentage of cells undergoing apoptosis (6.24%), and cell death (18.30%) while the amount of viable cells is still high (75.47%) (Fig.1c (iii); Table 3).

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All the teas portrayed decreasing cell viability with increasing concentrations of the FDE, with concomitant increases in the mean percentage of apoptotic and dead cells (Table 3). At 0.5 mg FDE/ml, viability of cells exposed to green tea was 82.5%, followed by unfermented rooibos (84.8%) and honeybush (91.8%).

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Table 1: LDH release as a percentage of the control, in WHCO5 cells exposed to various teas.

Gr		Bl		Rg		RgM		Rf		Hg		Hf		Sut	
mg FDE/ ml	**% cytox**	**mg FDE/ ml**	**% cytox**	**mg FDE/ ml**	**% cytox**	**mg FDE/ ml**	**% cytox**	**mg FDE/ ml**	**% cytox**	**mg FDE/ ml**	**% cytox**	**mg FDE/ ml**	**% cytox**	**mg FDE/ ml**	**% cytox**
0.5	5.3 (2.9)	**0.5**	6.6 (2.2)	**0.1**	7.0 (1.8)	**0.1**	12.2 (1.2)	**0.5**	15.8 (2.3)	**0.1**	7.7 (1.4)	**0.5**	10.8 (2.1)	**5**	10.0 (2.4)
2.5	-0.8 (1.2)	**2.5**	4.5 (1.4)	**0.5**	5.1 (3.3)	**0.5**	17.9 (1.9)	**2.5**	15.8 (4.1)	**0.5**	15.6 (1.7)	**2.5**	16.9 (3.5)	**10**	28.8 (9.8)
5	-7.2 (0.9)	**5**	-5.6 (1.1)	**2**	-9.0 (1.1)	**2**	32.4 (0.9)	**5**	4.8 (1.9)	**2**	19.3 (3.0)	**5**	16.2 (2.7)	**20**	39.4 (9.9)
10	-16.5 (0.6)	**10**	-5.8 (2.1)	**4**	-20.1 (2.6)	**4**	37.6 (1.3)	**10**	-16.0 (3.2)	**4**	23.7	**10**	12.4 (2.1)	**50**	34.0 (8.8)

Values are means of six replicates ± standard deviation. Gr = green tea; Bl = black tea; Rg = unfermented rooibos; Rf = fermented rooibos; Hg = unfermented honeybush; Hf = fermented honeybush; RgM = unfermented rooibos methanol extract; Sut = *Sutherlandia frutescens*. FDE (10%) was dissolved in DMSO/H_2O and further diluted in cell culture medium for each assay.

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Table 2: Cell viability utilising the MTT colorimetric assay, as a percentage of the control in WHCO5 cells exposed to various teas.

Gr		Bl		Rg		RgM		Rf		Hg		Hf		Sut	
mg/ FDE ml	% viable	mg/ FDE ml	% viable	mg/ FDE ml	% viable	mg/ FDE ml	% viable	mg/ FDE ml	% viable	mg/ FDE ml	% viable	mg/ FDE ml	% viable	mg/ FDE ml	% viable
0.5	23.7 (6.6)	**0.5**	19.4 (8.0)	**0.1**	158.6 (30.5)	**0.1**	157.0 (39.8)	**0.5**	111.3 (29.5)	**0.1**	202.8 (26.7)	**0.5**	135.3 (19.4)	**5**	411.0
2.5	8.3 (3.3)	**2.5**	13.6 (2.7)	**0.5**	166.1 (9. 7)	**0.5**	680.2 (44.5)	**2.5**	49.7 (23.9)	**0.5**	166.8 (14.1)	**2.5**	34.7 (37.9)	**10**	249.2
5	5.9 (3.5)	**5**	22.6 (8.9)	**2**	53.0 (15.1)	**2**	144.2 (32.4)	**5**	43.7 (17.7)	**2**	37.6 (15.0)	**5**	35.0 (16.5)	**20**	80.9 (452.9)
10	-7.0 (7.9)	**10**	16.0 (11.0)	**4**	44.5 (41.2)	**4**	nd	**10**	68.7 (41.39)	**4**	29.4 (11. 4)	**10**	92.9 (39.2)	**50**	nd

Values are means of six replicates ± standard deviation. Gr = green tea; Bl = black tea; Rg = unfermented rooibos; Rf = fermented rooibos; Hg = unfermented honeybush; Hf = fermented honeybush; RgM = unfermented rooibos methanol extract; Sut = *Sutherlandia frutescens*. Nd = not detected. FDE (10%) was dissolved in DMSO/H_2O and further diluted in cell culture medium for each assay.

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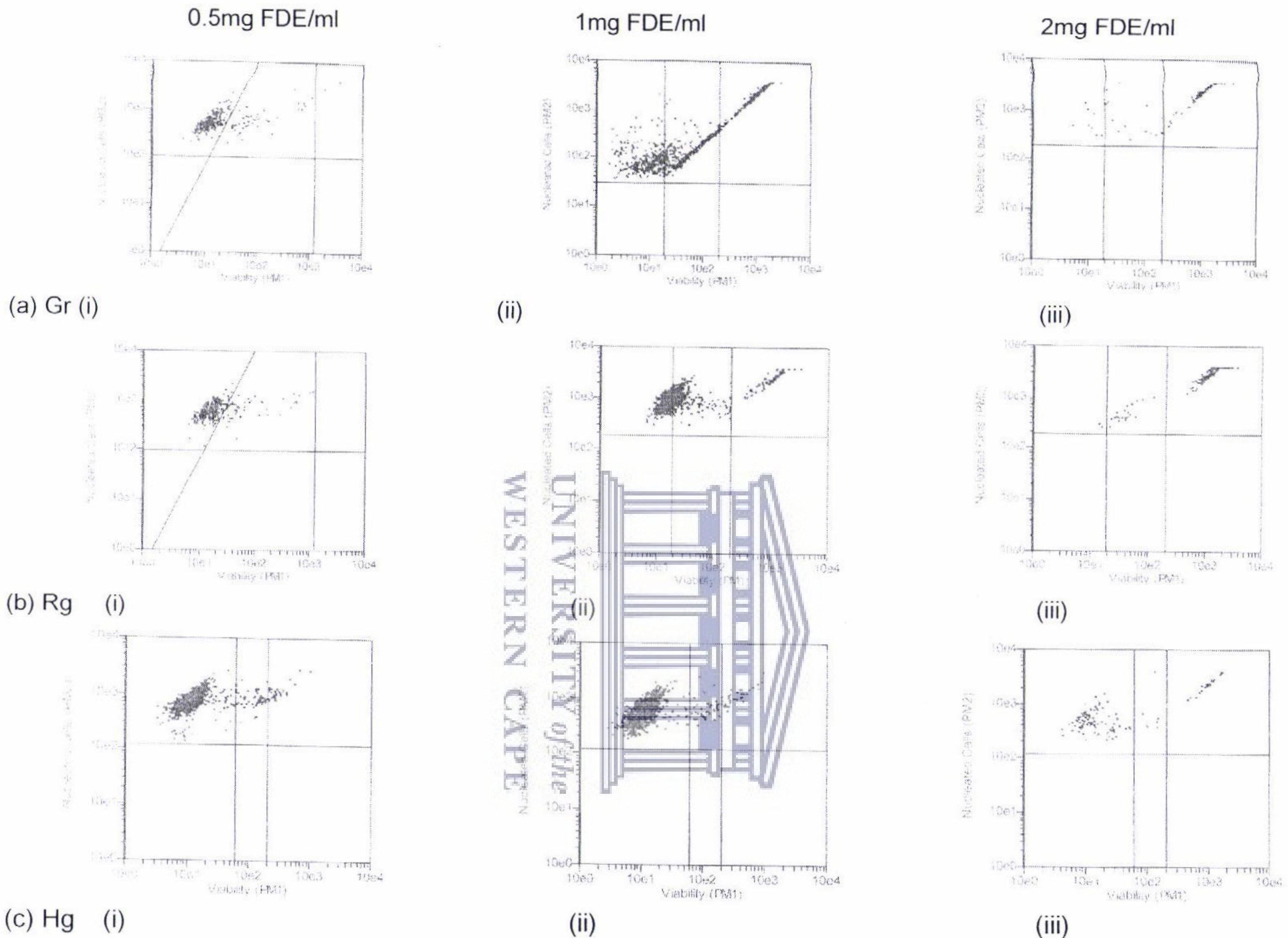

Fig. 1 Flow cytometric diagrams illustrating viable, apoptotic and dead WHCO5 cells exposed to **green (a)** and **unfermented rooibos (b)** and **unfermented honeybush (c)** teas at 0.5, 1 and 2 mg/ml. FDE = freeze dried extract.

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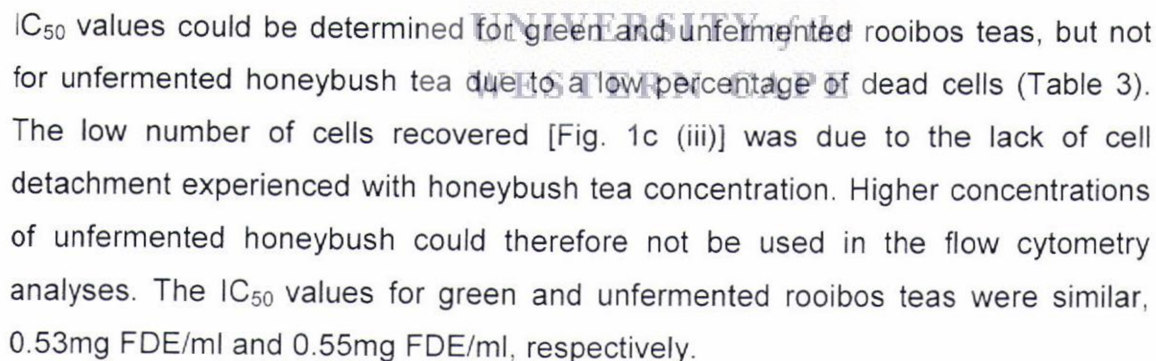
At 1mg/ml, green tea (34.7%) showed the lowest cell viability followed by unfermented rooibos tea (50.3%) and honeybush tea (93.7%). Unfermented honeybush tea exhibited the highest viability (75.5%) at 2mg FDE/ml, followed by green tea (7.5%) and unfermented rooibos tea (1.0%). When considering the apoptosis at 0.5mg FDE/ml, green (16.7%) and unfermented rooibos (15.1%) teas exhibited similar effects, which was higher than unfermented honeybush tea (5.8). At 1mg FDE/ml, green (31.6%) and unfermented rooibos (33.9) teas exhibited a similar pattern, while no effect was noticed with unfermented honeybush tea (3.4%). The cells exposed to 2 mg FDE/ml unfermented honeybush tea, again, showed no increase in apoptosis although the amount of dead cells (18.3%) increased. The mean percentage of dead cells also increased in a dose dependent manner for the green and unfermented rooibos teas, with unfermented rooibos tea (86.3%) exhibiting a higher toxic effect than green (74.2%) tea at the highest concentration (Table 3). It is important to note that the amount of apoptotic cells decreased when the concentration of green and unfermented teas were increased between 1 and 2 mg FDE/ml, while the amount of dead cells increased.



IC_{50} values could be determined for green and unfermented rooibos teas, but not for unfermented honeybush tea due to a low percentage of dead cells (Table 3). The low number of cells recovered [Fig. 1c (iii)] was due to the lack of cell detachment experienced with honeybush tea concentration. Higher concentrations of unfermented honeybush could therefore not be used in the flow cytometry analyses. The IC_{50} values for green and unfermented rooibos teas were similar, 0.53mg FDE/ml and 0.55mg FDE/ml, respectively.

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Table 3: Flow cytometric results illustrating cell viability, apoptosis and cell death of WHCO5 cells exposed to various concentrations of green and unfermented herbal teas.

Flow Cytometry (mean percentage of totals)									
Teas	Gr			Rg			Hg		
Concentration (mg FDE /ml)	**0.5**	**1**	**2**	**0.5**	**1**	**2**	**0.5**	**1**	**2**
% Viable	82.5 (3.7)	34.7 (7.6)	7.5 (1.8)	84.9 (2.7)	50.3 (1.7)	1.02 (0.9)	91.8 (7.5)	93.7 (1.9)	75.5 (2.7)
% Apoptotic	16.7 (3.4)	31.6 (2.1)	18.6 (5.2)	15.2 (2.8)	33.9 (4.8)	12.7 (2.0)	5.8 (6.9)	3.4 (2.2)	6.3 (2.1)
% Dead	0.9 (0.6)	33.7 (6.6)	74.2 (5.4)	0.03 (0.1)	15.8 (5.7)	86.3 (2.6)	2.4 (1.0)	3.0 (0.6)	18.3 (3.4
% Cytotoxicity (IC50) (mg FDE/ml)	0.53			0.55			Nd		

Values are means of 4 replicates ± standard deviation. Gr = green tea; Rg = unfermented rooibos; Hg = unfermented honeybush; FDE = freeze dried extract. nd = not detected (see Results section).

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4.4.2 Cell viability

The ATP content of the cells was determined after a 24 hr treatment with different concentrations of the various teas. All the teas, as well as the *Sutherlandia* infusion exhibited a significant ($P<0.05$) dose response effect with decreased ATP production as a function of increased tea concentration (Table 4). At 0.1mg FDE/ml, there were no significant differences in the inhibitory effects on ATP production by any of the FDE of the tea and herbal teas tested. The % ATP inhibition was significantly lower when compared to *Sutherlandia*. At 0.5mg FDE/ml, *Sutherlandia* (81.9%) exhibited the smallest ($P<0.05$) effect on ATP production followed by fermented (72.2%) and unfermented honeybush teas (62.1%). Green (51.7%), fermented rooibos (50.1%), unfermented (47.3%) rooibos and black (41.4%) teas exhibited similar effects on ATP production.

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Unfermented honeybush tea did not differ significantly from green, fermented and unfermented rooibos and black teas. A similar pattern was noticed at 1mg/ml, with the *Sutherlandia* infusion exhibiting (74.2%), the lowest ($P<0.05$) effect on ATP production, followed by fermented (56.7%) and unfermented honeybush (42.9%). The RgM extract (22.3%), unfermented (21.5%) and fermented (28.7%) rooibos were significantly ($P<0.05$) lower, while black (7.7%) and green tea (2.6%) exhibited the highest inhibitory effects on ATP production.

Based on the IC_{50}-values of the different teas (mg FDE/ml), the cells were more sensitive to black (0.49) and green (0.5) teas, followed by unfermented rooibos tea (0.55) and the RgM extract (0.57), that exhibited similar inhibitory effects (Table 4). The cells were more resistant to unfermented (0.84) and fermented (1.24) honeybush teas, while the IC_{50}-value of the *Sutherlandia* infusion could not be determined due to matrix interferences with the assay procedures. During the 24 hr incubation period the media in the microtiter plate tended to form a gel in the presence of the extract, which was likely to interfere with the enzymatic conversion of luciferin.

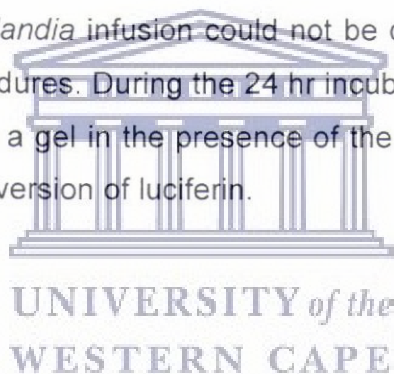
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Table 4 Dose response cytotoxic effects of *Camellia sinensis*, herbal teas and the *Sutherlandia* infusion obtained in cell TiterGglo® assay, depicting percentage ATP inhibiton and IC_{50} concentration in mg FDE/ml.

Treatments	mg FDE/ml medium 0.1	0.5	1	IC_{50} (mg FDE/ml)
Green tea	87.1 (13.2)a	51.7 (5.4)ae	2.6 (1.5)a	0.50
Black tea	85.6 (8.9)a	43.4 (17.2)a	7.7 (3.0)a	0.49
Rooibos unfermented tea	81.1 (12.4)a	47.3 (8.0)ae	21.5 (7.2) b	0.55
Rooibos fermented tea	83.0 (10.2)a	50.0 (7.0)ad	28.7 (4.6)b	0.60
Rooibos unfermented MeOH extract	80.0 (6.9)a	45.3 (5.7)a	22.2 (5.0)b	0.57
Honeybush unfermented tea	85.5 (9.8)a	62.1 (12.9)ad	42.9 (9.0)c	0.84
Honeybush fermented tea	90.4 (6.7)a	72.2 (5.3)bd	56.7 (8.6)c	1.24
Sutherlandia frutescens*	109.2 (14.6)b	81.9 (5.8)c	71.1 (50.9)#	Nd*

Values are means of six replicates ± standard deviation. FDE = freeze-dried extract; *nd = not detected. #Data not repeatable at higher concentrations due to gel formation during 24hr incubation period (See Result section). If letters differ, then $P<0.05$.

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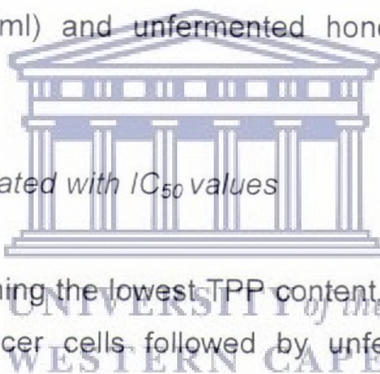
4.4.3 Cell proliferation

The concentrations of the FDE used were based on the IC_{50} values obtained from the flow cytometry and ATP luminescence assays. Green tea, as well as unfermented rooibos and honeybush teas inhibited cell proliferation in a dose-dependent manner (Table 5). The percentage proliferation for WHCO5 cells treated with green tea was 0.39%, 9.06%, 54.6%, and 67.7% at 0.5, 0.25, 0.125, and 0.0625mg FDE/ml respectively (Table 5). The cells exposed to unfermented rooibos tea displayed a 15.2% proliferation at 0.55 mg FDE/ml, while the inhibitory effect was significantly decreased at lower concentrations. Unfermented honeybush tea showed a similar anti-proliferative activity at 0.84mg/ml (12.0%). When considering the IC_{50} values, unfermented rooibos tea (0.08mg/ml) exhibited the highest inhibitory effect on cell proliferation, followed by green (0.11mg/ml) and unfermented honeybush (0.15mg/ml) teas.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4.5 Polyphenol groups associated with IC_{50} values

4.5.1 Cell viability assay

Green and black teas, containing the lowest TPP content, exhibited the lowest IC_{50} values against WCHO5 cancer cells followed by unfermented and fermented rooibos teas (Table 6). Fermented honeybush tea exhibited the highest IC_{50} value while its TPP content was comparable to fermented rooibos tea. Unfermented honeybush tea exhibited a higher IC_{50} value with a TPP content slightly lower than the RgM extract. Fermentation increased the IC_{50} of honeybush tea, but only slightly with the rooibos tea, while no effect was noticed with green and black teas. The RgM extract contained far higher TPP content but the IC_{50} value was similar to unfermented rooibos tea. When considering the flavonol/flavone and flavanol/proanthocyanindin content, honeybush tea showed that reduced levels were associated with an increased IC_{50} value. Fermented rooibos tea had an increased and decreased flavonol/flavone and flavanol/proanthocyanindin content, respectively, which coincided with a lower IC_{50}. The opposite effect was noticed with green and black teas, whilst the IC_{50} values were similar.

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Table 5 Dose response showing inhibitory effects and IC_{50} values of green, unfermented rooibos and honeybush on cell proliferation as monitored by the incorporation of BrdU.

Parameters	mg FDE/ml											
	Gr				Rg				Hg			
	0.5	0.25	0.125	0.0625	0.55	0.275	0.138	0.068	0.84	0.42	0.21	0.105
% Prolif	0.39	9.06	54.06	67.70	15.23	28.30	40.69	56.01	12.01	17.76	21.16	48.13
IC50 (mg FDE/ml)	0.11				0.08				0.15			

Values of % proliferation are the means of duplicate determinations of 6 replicates each. Gr = green tea; Rg = unfermented rooibos; Hg = unfermented honeybush. FDE = freeze dried extract

Table 6 Comparable soluble solids, total polyphenols, flavonol/flavone, and total flavanol/proanthocyanidin contents of the different teas associated with the IC_{50} of the ATP activity in the WHCO5 cells.

	mg FDE/ml (IC_{50})	TPP (µg)*	Flavo (µg)*	Flava (µg)*
Gr	0.5	116.35	9.70	49.20
Bl	0.49	130.59	15.34	29.60
Rg	0.55	182.93	32.40	8.20
Rf	0.6	190.50	66.78	4.14
RgM	0.57	260.03	40.58	16.93
Hg	0.84	246.96	43.85	27.55
Hf	1.24	194.68	39.93	16.62

IC_{50} = mg soluble solids; TPP = total polyphenols; Flavo = flavonols/flavones; Flava = flavanols/proanthocyanidins; Gr = green tea; Bl = black tea; Rg = unfermented rooibos; Rf = fermented rooibos; Hg = unfermented honeybush; Hf = fermented honeybush; RgM = unfermented rooibos methanol extract. FDE = freeze dried extract. * Data obtained from Chapter 3 (Table 1).

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When considering the major polyphenolic constituents, fermented rooibos tea had substantially less aspalathin, nothofagin, hyperoside and rutin/isoquercitin content than unfermented rooibos tea (Table 7). In contrast the levels of orientin and iso-orientin were similar. The levels of mangiferin and isomangiferin were lower in fermented honeybush tea, compared to unfermented honeybush tea (Table 8). The level of hesperetin was markedly higher in the fermented honeybush tea.

4.5.2 Anti-proliferative effects utilizing the BrdU incorporation assay

In contrast to the cell cytotoxicty assay, unfermented rooibos tea exhibited the highest activity followed by green tea and unfermented honeybush tea (Table 9). The rooibos TPP content was almost 50% of honeybush tea, which also had higher levels of flavonols/flavones and flavanol/proanthocyanidins. The major rooibos tea flavonoid was aspalathin, with nothofagin, orientin iso-orientin and rutin/isoquercitin present in equal but lower quantities. Mangiferin and isomangiferin represented the major unfermented honeybush tea polyphenols.

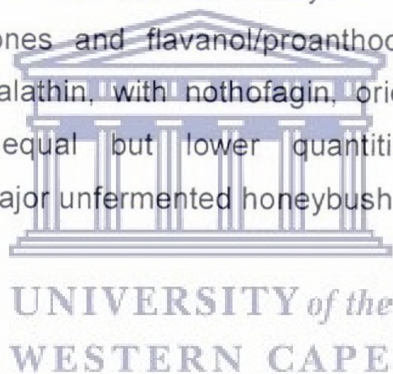
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Table 7 Major flavonoids expressed as a function of the IC_{50} values of unfermented and fermented rooibos teas associated with cell viability as determined by the TiterGglo® assay

Rooibos Tea	Fermented	Unfermented
IC_{50} (mg FDE/ml)	0.6	0.55
Total Polyphenols (µg)	190.5	182.93
Total Flavonol/flavone (µg)	66.78	32.4
Total Flavanol/proanthocyanidin (µg)	4.14	8.2
Phenolic Compounds	**µg/ml**	
Aspalathin	2.85	81.004
Nothofagin	0.312	6.1655
Orientin	5.268	5.555
Iso-orientin	4.212	5.4175
Vitexin	0.858	1.0505
Isovitexin	0.942	1.177
Hyperoside	0.486	1.21
Rutin/Isoquercitrin	4.806	11.044
Luteolin	0.054	0.022
Chrysoeriol	0.06	0.022

FDE = freeze-dried extract [Values of phenolic compounds expressed as µg/ml].

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Table 8 Major polyphenols expressed as a function of the IC_{50} values of unfermented and fermented honeybush teas associated with cell viability as determined by the TiterGglo® assay.

Honeybush Tea	Fermented	Unfermented
IC_{50} (mg FDE/ml)	1.24	0.84
Total Polyphenols (µg)	0.195	0.247
Total Flavonol/flavone (µg)	0.04	0.044
Total Flavanol/proanthocyanidin (µg)	0.017	0.026
Phenolic Compounds	**µg/ml**	
Mangiferin	5.208	38.556
Isomangiferin	6.324	16.128
Hesperidin	nd	3.192
Hesperitin	1.364	0.84
Narirutin	nd	Trace

nd = not detected. FDE = freeze-dried extract. [Values of phenolic compounds expressed as µg/ml]. Only trace amounts of narirutin were found in the unfermented honeybush tea.

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Table 9 Major rooibos flavonoids and honeybush polyphenols expressed as a function of the IC_{50} values associated with the inhibition of cell proliferation.

Rooibos Tea (unfermented)		**Honeybus Tea (unfermented)**	
IC_{50} (mg FDE/ml)	0.08	IC_{50} (mg FDE /ml)	0.15
Total Polyphenols (µg)	26.61	Total Polyphenols (µg)	44.10
Total Flavonol/flavone (µg)	4.71	Total Flavonol/flavone (µg)	7.83
Total Flavanol/proanthocyanidin (µg)	1.19	Total Flavanol/proanthocyanidin (µg)	4.92
Phenolic Compounds	**µg/ml**	**Phenolic Compounds**	**µg/ml**
Aspalathin	11.7824	Mangiferin	6.89
Nothofagin	0.8968		
Orientin	0.808	Isomangiferin	2.88
Iso-orientin	0.788		
Vitexin	0.1528	Hesperidin	0.57
Isovitexin	0.1712		
Hyperoside	0.176	Hesperitin	0.15
Rutin/Isoquercitrin	1.6064		
Luteolin	0.0032	Narirutin	Trace
Chrysoeriol	0.0032		

nd = not detected. FDE = freeze dried extract. Only trace amounts of narirutin were detected. [Values expressed as µg/ml].

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4.6 Discussion

A host of naturally occurring plant products have the ability to act as chemopreventive agents (Bode and Dong, 2004). Many of these preventive properties have been ascribed to the phenolic compounds that are found in a wide variety of human foods and beverages. Tea polyphenols modulate carcinogen metabolizing enzymes, inhibit cell proliferation, and play a role in cell cycle arrest and the induction of apoptosis (Wang *et al.*, 1989; Xu *et al.*, 1992; Ahmed *et al*, 1997; Lin and Lin, 1997). Green and black teas prevent tumour induction by a variety of chemical carcinogens in animal models, which was mainly attributed to the polyphenolic constituents (Roy *et al.*, 2001). However, the chemopreventive activity of tea is suggested to be a combined effect of several constituents, acting synergistically, rather than one specific compound (Suganama *et al.*, 1999).

Although studies have reported on the protective role of green tea against human cancers, there is limited evidence on the anticancer effect of black tea (Katiyar and Mukhtar, 1996; Kohlmeier *et al.*, 1997; Khan and Mukhtar, 2007), and only anecdotal evidence on the South African herbal teas and *Sutherlandia*. Most of the biological properties of rooibos and honeybush teas are ascribed to their unique polyphenolic constituents. Although their phenolic constituents differ from the *Camellia sinensis* teas, they exhibited similar properties in various *in vitro* and *in vivo* biological models monitoring antioxidant, antimutagenic and anticancer properties (Marnewick *et al.*, 2003, 2005; van der Merwe *et al.*, 2006, Snijman *et al.*, 2007). In the present study, the cytotoxic and anti-proliferative properties of aqueous extract of the herbal teas and a *Sutherlandia* infusion was compared, for the first time, with green and black teas utilising an oesophageal cancer cell line.

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The LDH and MTT colorimetric assays could not be used to determine the IC_{50} values due to colour and/or other plant constituent interferences. It has been reported that some herbal extracts interact with the MTT in the absence of cells, resulting in false–positive or false-negative results (Shoemaker *et al.*, 2004). Since the LDH and MTT assays are based on enzymatic reactions, they may also be

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influenced by specific enzyme inhibitors (Weyerman *et al*., 2005). Polymeric polyphenolic compounds, known as tannins, may also form perceptible complexes with some protein (Hageman and Carlson, 1998).

Cytotoxicity performed with the flow cytometry provided more detailed results regarding the toxicity of the unfermented extracts without any colour interference as observed with the LDH and MTT assays. It provided the status of each cell and thus determined the percentage of live, apoptotic and dead cells. However, flow cytometry was not appropriate to determine the IC_{50} of the unfermented honeybush tea, mainly due to the lack of detachment of the WHCO5 cells following trypsination. This could presumably be due to the inhibition of the activity of the protease enzyme by tea polyphenols (He and Yao, 2006). Thus, when assays in cytotoxicity screening are dependent on enzymatic reactions, it was recommended to perform a battery of tests to acquire the most appropriate results (Weyermann *et al*., 2005; Fotakes and Timbrell, 2006).

No toxicity was established when the cells were exposed to the *Sutherlandia* infusion due to various factors influencing the outcome of the assays. Apart from the forming of sediments during the preparation of the FDE, addition of the different dilutions resulted in the media taking on a gel-like consistency, with the formation of thread-like precipitates. The reagents and/or substrate added to perform each assay did not mix thoroughly with the cells, presenting false results. Under the current conditions, no cytotoxic and/or anti-proliferative data could be determined.

When considering the chemiluminescent assay utilising the mitochondrial function, no colour interference was observed, as the signal was not affected. This can be deduced from the fact that green and unfermented rooibos tea had similar IC_{50} values when monitored by flow cytometry analyses. The total polyphenols and flavonol/flavones of black tea were higher than the green tea extract, while the flavanol/proanthocyanidin was not. The same pattern was observed in rooibos tea, while the total polyphenols, flavonol/flavones and flavanol/proanthocyanidin

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content of fermented honeybush tea was lower. Black and green teas' constituents, presumably the catechins, seem to be more reactive than the rooibos tea polyphenols when considering cell death. The IC_{50} values obtained for green (0.50mg FDE/ml), black (0.49mg FDE/ml), unfermented (0.55mg FDE/ml), and fermented (0.6mg FDE/ml) rooibos teas, imply that the changes in phenolic composition, taking place during fermentation did not alter the cytotoxic effects of the *Camellia sinensis* teas and only slightly in the case of rooibos tea. It is known that the tea catechins are converted into the thearubigans and theaflavins during fermentation (Gupta *et al.*, 2002; Luczaj and Skrzydlewska, 2005). The level of aspalathin and nothofagin were 20 to 30 times lower in fermented rooibos, while the levels of all flavones were not markedly different. When considering the flavonol/flavone content, the level was two times that of unfermented rooibos tea, implying that this flavonoid subclass exhibited far lower cytotoxic effects. A similar outcome was reported recently when considering the antioxidant properties of aqueous extracts of fermented and unfermented rooibos teas utilising different antioxidant assays (Joubert *et al.*, 2008). However, when rooibos teas were tested in the *Salmonella* mutagenicity assay using aflatoxin B_1 and 2-acetylaminofluorene, fermented rooibos tea exhibited stronger antimutagenicity than its unfermented counterpart (van der Merwe *et al.*, 2006). Aspalathin, the major dihydrochalcone, and/or unknown products may play a major role in the cytotoxic effects of unfermented rooibos tea against WHCO5 oesophageal cancer cells. When considering the honeybush teas, fermented honeybush tea had a higher IC_{50} value (0.84 mg FDE/ml) than unfermented honeybush tea (1.24 mg FDE/ml), indicating a reduced cytotoxic effect as a result of the reduction of the known polyphenolic constituents or newly formed compounds of lower potency. The major polyphenolic compound, mangiferin and isomangiferin could therefore be important determinants as they decreased in accordance with the TPP content during fermentation. This is also noted when considering other biological parameters such as antioxidant and antimutagenic properties, indicating that honeybush tea TPP are less active than rooibos and black teas, while fermentation decreased these parameters (van der Merwe et al., 2006; Joubert et al., 2008).

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It would be important to note that synergistic interaction between the different tea constituents and/or specific flavonol/flavanol ratios will affect different cellular processes. This would imply that the most abundant constituent is not necessarily the most active or effective when considering a biological effect. It would also imply that the results obtained from various experiments would be affected by the assay system implemented, as well as the type of tea used. However, further studies using unfermented and fermented plant material from the same tea batches and purified polyphenolic compounds need to be conducted before the specific role of individual constituents could be evaluated.

Possible mechanisms for the cytotoxic and anti-proliferative effects could also be related to the polyphenolic content of the FDE extract. It has been suggested that the growth-inhibitory effect is ascribed to the polyphenolic constituents in the teas (Yang et al., 1998; Uesato et al., 2001). Even though tea polyphenols have antioxidant activities, they are also known to exhibit pro-oxidative properties under certain conditions that could increase oxidative stress resulting in cytotoxicty (Luczaj and Skrzydlewska, 2005). It is known that cancer cells exhibited a low oxidative status, and that increased oxidative status resulted in cell death (Das, 2002). Recent *in vitro* experiments indicated that EGCG produces reactive oxygen species while a green tea extract, as well as EGCG, causes oxidative stress-related responses in yeast (Maeta *et al.*, 2007). EGCG and other tea polyphenols produce H_2O_2 when added to cell culture media in the absence of cells (Yang *et al.*, 1998, 2000; Dashwood *et al.*, 2002). EGCG is also known to cause apoptosis in cancer cells via the induction of reactive oxygen species (Azam *et al.*, 2004). When considering the South African herbal teas, aspalathin contributes a substantial percentage of the total antioxidant capacity of the aqueous extracts of unfermented rooibos tea (Shultz et al., 2003), while its content is significantly decreased during fermentation (von Gadow *et al*, 1997; Standley *et al.*, 2001). A recent study showed that a flavonoid-enriched fraction of unfermented rooibos tea, containing high levels of aspalathin, displayed *in vitro* pro-oxidant activity (Joubert *et al.*, 2005). In this study green tea displayed similar cytotoxic effect to the

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unfermented rooibos tea with reference to the flow cytometry results. This is expected since the major catechin in green tea, ECGC, is a potent antioxidant that displays prooxidant properties. In this regard, it would be expected that aspalathin also display prooxidant activity, since it has antioxidant potency comparable to ECGC (Snijman, 2007).

The disruption of mitochondrial function and cytotoxicity seems to be closely related events. In contrast, the inhibition of cell proliferation appears to be an early event prior to any cytotoxic effects. This is also in accordance with the flow cytometry data, as increased apoptosis is noticed at lower concentrations when considering the IC_{50} levels for cell death. Different tea constituents could be involved in triggering the cytotoxic effects and the inhibition of cell proliferation. Unfermented rooibos tea was more reactive as an inhibitor of cell proliferation than green tea, while the opposite was found with respect to cytotoxicity. The major polyphenolic groups of rooibos tea consist of the dihydrochalcones as compared to the flavanol/proanthocyanidins (catechins) of green tea. This could suggest that catechin (EGCG) is a more reactive cytocidal component, the dyhydrochalcone aspalathin, is a more effective inhibitor of cell proliferation via a different mechanism. EGCG is known to be overtly cytotoxic toward cancer cells (Valcic et al., 1996; Chen et al., 2004; Galati et al., 2006), whilst very little is known about the cytotoxic effects of aspalathin, the major rooibos flavonoid.

The study provides the first experimental *in vitro* evidence that the extracts of rooibos and honeybush teas exhibit cytotoxic, anti-proliferative, and probable apoptotic effects in a oesophageal cancer cell line. The selective effects of the herbal teas can be ascribed to the TPP content, while the role of specific polyphenolic constituents and fermentation still need to be further elucidated.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

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A variety of plant and/or other phenolic compounds exhibit chemoprotective properties by disrupting the multi-step process associated with cancer development. Although research has been carried out on the possible mechanisms involved, it is still not known how these compounds exert their action (Roy *et al*, 2001). Tea extracts and their polyphenolic constituents exhibited inhibitory and/or modulating properties during the initiation, promotion and progression stages of chemical carcinogenesis, presumably via different metabolic pathways. A polyphenol could react with electrophilic carcinogenic species to form a tea polyphenol-carcinogen adduct that could result in the prevention of cellular damage and hence, carcinogenesis (Kim and Masuda, 1997).

Animal studies offer unique opportunities to investigate the biological properties that tea and tea polyphenols may have in a physiological system. *In vivo* studies showed preventive properties of green and black tea (*Camellia sinsesis*) extracts and/or green tea catechins against oral and gastrointestinal (Cardeni *et al.*, 2000; Li *et al.*, 2002), lung (Sazuka *et al.*, 1995; Cao *et al.*, 1996), liver (Cao *et al.*, 1996; Sai *et al.*, 1998), mammary (Tanaka *et al.*, 1997; Kavanagh *et al.*, 2001), and prostate (Gupta *et al.*, 2001) cancer. The polyphenols from green or black tea have been reported to decrease experimental carcinogenesis in various rodent organs such as skin, lung, liver and oesophagus (Yang and Wang, 1993; Wang *et al.*, 1994; Han and Xu, 1990; Weisburger, 1999). The present study provides evidence that the South African herbal teas, rooibos and honeybush, exhibits cancer preventive properties against oesophageal cancer in rats. When compared to the *Camellia sinensis* teas a similar effect was noticed regarding the reduction in the number of oesophageal papillomas. The unfermented herbal teas were, however, more effective in reducing the papilloma size as it inhibited the development of larger papillomas. Fermentation seems to reduce the protective effects of the herbal teas when considering the number and size of papillomas. Green tea and the *Sutherlandia* infusion also prevented the development of larger papillomas and, although no significant changes in the mean total size were recorded, although it was markedly decreased. *Sutherlandia*, however, marginally reduced the total

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average number of papillomas. This is of interest, since the infusion contained very low levels of TPP and flavonol/flavones and only traces of flavanol/proanthocyanidins. In view of this, it would seem that the tumour-inhibitory action of *Sutherlandia* is due to one or more of its active compounds, either acting independently, or in synergy. Future studies regarding the role of L-canavanine, known to exhibit anti-proliferative properties to cancer cells (Rosenthal, 1997), should be conducted.

A TPP threshold for the prevention of papilloma development is suggested when considering the rat intake profiles of the different treatments. This became evident when considering the effects of rooibos tea versus the RgM extract of unfermented rooibos tea, suggesting that a TPP intake of <7mg/100g body weight lacks any protective effects. The TPP intake was also significantly reduced when using the fermented teas, which coincided with a reduction of the protective effects of rooibos and honeybush teas. Treatment with unfermented honeybush tea provided the highest TPP intake (25.05 mg/100g body weight) and protective effects, which was significantly ($P<0.05$) reduced during fermentation, and coincided with a reduction in the TPP intake profiles (7.13 mg/100g body weight). The effect of green and black teas are of interest as a TPP intake of >7 mg/100g body weight, significantly reduced the mean total number of papillomas but not the mean size, although it was markedly reduced. This is in accordance with previous studies where green and black teas inhibited papilloma development in NMBA-treated rats (Yang and Wang 1993; Wang *et al.,* 1995; Li *et al.*, 2002). These studies also provided evidence that the major green tea catechin, EGCG, and the black tea theaflavins exhibited protective effects. It can therefore be assumed that, in the present study, the green and black tea catechins and theaflavins provided the protective effects, respectively. At present the effect of aspalathin and mangiferin, the major rooibos and honeybush tea polyphenolic compounds, on the development of oesophageal papillomas is not known. However, a study showed that hesperidin, one of the major honeybush tea polyphenols inhibit the development of oesophageal cancer in NMBA-induced rats (Tanaka, 1997). The

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intake of both aspalathin and mangiferin are significantly reduced during fermentation (Joubert and de Villiers, 1997), which coincides with a reduction in the protective effects, hence their role as the reactive constituents in the herbal teas should be evaluated.

The present study also evaluated the cytotoxic and anti-proliferative activity of the different teas on human oesophageal cancer cells. *In vitro* cytotoxic assays that use different parameters associated with cell death and proliferation have been developed which can be used to predict human toxicity (Weyermann *et al.*, 2005). These assays can therefore be used to screen chemicals implied in human toxicology (Fotakis and Timbrell, 2006). However, studies have shown that various cytotoxic assays give different results, depending on the test agent used and the method employed. The results obtained when using the LDH and MTT assays in the present study were satisfactory when using diluted concentrations of the teas but no true cytotoxic or viability effects could be determined. However, when using lower dilutions the intense tea colours influenced the absorbance reading, providing false positive results. In order to avoid overestimation or underestimation of cytotoxic endpoints the toxicity of the different teas were evaluated in more than one assay thereby increasing the reliability of the results as was suggested by Fotakes and Timbrell (2006). Flow cytometry and a cell viability assay monitoring ATP production were shown to be applicable to determine the cell cytotoxic parameters of the different teas against WHCO5 oesophageal cancer cells. Some plant matrix/protease interactions when using unfermented honeybush tea, however, seems to interfere during flow cytometry screening as the detachment of cells was prohibited, presumably due to the high TPP content. It has been reported that polyphenols exhibited protease inhibitory potential (He, *et al.*, 2006). *Sutherlandia* could also not be evaluated for cytotoxicity with the LDH and MTT assays, or the cell viability assay monitoring ATP production due to plant matrix/media interactions.

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Although green and unfermented rooibos teas exhibited similar IC_{50} values using flow cytometry, the cell viability assay showed that green and black teas were more effective in reducing ATP production. Fermented and unfermented rooibos teas exhibited a higher cytotoxic effect than the honeybush tea counterparts. As discussed above, fermentation also seems to reduce the cytotoxic effects of the herbal teas, especially when considering the honeybush teas. The weaker response of honeybush tea, when compared to the rooibos tea, is in accordance with the reduced activities of honeybush tea polyphenols in different *in vitro* assays (van der Merwe *et al.*, 2006; Snijman *et al.*, 2007; Joubert *et al.*, 1997, 2008). This was one of the main reasons for using a higher concentration of honeybush tea for the *in vivo* oesophageal cancer model. Based on the present study it would appear that the TPP of *Camellia sinensis* teas are more cytotoxic than rooibos tea to the WHCO5 cells. However, when considering inhibition of cell proliferation unfermented rooibos tea exhibited a lower IC_{50} value than green tea. It was suggested that the type of compound(s) involved might differ when considering the cytotoxic or anti-proliferative effects. In this regard unfermented honeybush tea also exhibited a far more active response towards cell proliferation as to cytotoxicty. The *in vitro* cytotoxic and anti-proliferative properties of green tea, monitored in many different cell culture systems (Valcic *et al.*, 1996; Chen *et al.*, 2004; Galati *et al.*, 2006), were ascribed to the presence of EGCG. As no information regarding the cytotoxicity of the major rooibos and honeybush tea constituents in cell cultures is available, the specific role of the major antioxidants, aspalathin and mangiferin should be investigated.

The exact mechanism involved in the cytotoxic effects of the *Camellia sinensis* and herbal teas are not known at present. Polyphenolic compounds are known to act both as antioxidants, protecting cells against oxidative damage, and as pro-oxidants,resulting in cytotoxic effects (Nemeikaitė-Čėnienė *et al.*, 2005). Studies conducted with the green tea polyphenol, EGCG, showed that when used at low concentrations, it functions as a scavenger of reactive nitrogen species (ROS). At higher concentrations it functions as a producer of ROS, especially in the presence

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of organic salts such as iron, which leads to oxidative stress and cell cytotoxicity and/or DNA damage (Chai *et al.*, 2003; Babich *et al*, 2005). Apart from the observation that EGCG is oxidised in DMEM with the formation of H_2O_2 (Chai *et al.*, 2003), studies conducted by Nakagawa *et al.* (2004) also observed that hydrogen peroxide was produced in a sodium phosphate buffer amended with EGCG. This would imply that the mechanism, although unknown, is not totally dependent on iron for the generation of H_2O_2 (Babich *et al.*, 2005). In the present study green and unfermented rooibos teas exerted a higher toxic effect to the WHCO5 cells than the unfermented honeybush tea. This could be due to the catechins, specifically EGCG in green tea, and the production of H_2O_2 in the DMEM. Unfermented rooibos tea displayed cytotoxic activity similar to green tea, possibly as a result of aspalathin, a strong antioxidant, which demonstrates pro-oxidant activity in enriched extracts *in vitro* (Joubert *et al.*, 2003). Although there are very limited studies regarding honeybush tea in physiological systems, mangiferin was shown to be one of the major compounds in honeybush tea (De Nysschen *et al.*, 1996; Joubert *et al.*, 2003). In a study by Amazzal *et al.* (2007), mangiferin protected murine neuroblastoma cells against 1-methyl-4-phenyl-pyridine ion (MPP^+) by quenching reactive oxygen intermediates. Mangiferin could therefore, like other polyphenols, exhibit a biphasic response, by acting either as an antioxdiant or a pro-oxidant, ultimately resulting in cell toxicity.

Tea polyphenolic compounds are known to exhibit anticancer potential in various animal model systems (Yang *et al.*, 1997). It was suggested that the growth inhibitory activities are directly related to the tea polyphenol-induction of H_2O_2 and subsequent mediation of apoptosis (Yang *et al.*, 1998). In the present study, green tea exhibited anti-proliferative effects in a dose-dependent manner, which was associated with an increase in apoptosis and cell death. This may be attributed to the cytotoxic effects of EGCG, which may lead to cell cycle arrest and ultimately inhibition of proliferation. When considering the herbal teas, no *in vitro* studies have been conducted on rooibos and honeybush teas *per se* however, quercetin, a minor rooibos tea flavonoid, was reported to have a biphasic response, depending

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on the concentrations, and modulates proliferation and differentiation of the human colorectal cell line (van der Woude *et al.*, 2003; Dihal *et al.*, 2006). Mangiferin has been shown to reduce cell proliferation in colonic mucosa of F344 rats induced with azoxymethane (AOM) (Yoshimi *et al.*, 2001). In this study the unfermented rooibos tea displayed greater inhibitory action that the green and unfermented honeybush teas. When considering the above characteristics of the various teas, one can assume that they exert their action via different signalling pathways, either as a single compound, or a combination of two or more in synergy. Again, as discussed previously, the relative amount of compounds in the tea is important, as this could determine whether proliferation would be stimulated or inhibited, thereby suppressing or inducing apoptosis. The inhibitory effect of rooibos and honeybush teas against cell proliferation in WHCO5 cells could explain their chemoprotective effect in the *in vivo* oesophageal cancer model.

The South African herbal teas displayed chemopreventive properties comparable to green tea in both the *in vivo* and *in vitro* experiments in the present study. The current data on the chemopreventive properties of rooibos and honeybush tea further substantiate claims regarding their possible health benefits.

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Future studies

Future studies would include the chemical and biological characterization of the potential anticancer constituents of rooibos and honeybush extracts through activity-guided fractionation by:

(i) Investigating the anti-oxidative, anti-inflammatory, pro-apoptotic and anti-proliferative properties of tea extracts, selected fractions and/or purified compounds utilizing oesophageal cancer cell lines. These investigations will be performed using various cell survival parameters including: IC_{50} determination using the Cell titer-proliferation and flow cytometry assays, caspase-3-apoptosis enzyme analyses and DNA laddering-DNA fragmentation assays.

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(ii) Evaluating the anti-oxidant properties of selected extracts, fractions and purified herbal tea flavonoids in oesophageal tissue fractions and cell cultures. Specific antioxidant parameters will be monitored including gluthathione status, antioxidant and anti-inflammatory enzymes and lipid peroxidation.

(iii) Evaluating the validity of the in vitro biological parameters in an *in vivo* oesophageal cancer model in rats to develop biomarkers to critically assess the chemopreventive properties of extracts, fractions or purified flavonoids. These aspects will play an critical role in developing nutraceutical herbal products

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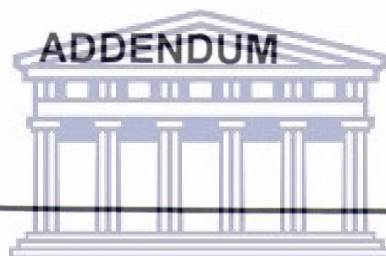
ADDENDUM



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Chapter 3

Table 1a Determination of total polyphenol assay using Folin-Ciocalteau reagent for various fermented and unfermented teas. The gallic acid dilution range was made according to the table below.

Conc. of dilution (mg/l)	Vol. of Stock Solution (µl)	Volume dH_2O (ml)	Final volume (ml)
10	50	4.95	5
20	100	4.90	5
40	200	4.80	5
60	300	4.70	5
80	400	4.60	5
100	500	4.50	5

dH^2O = distilled water

Table 1b The table below was used as a guide to prepare the control, diluted standards and tea samples.

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Tube	Sample (ml)	Amount dil. Gallic acid (ml)	dH_2O (ml)	F-C reagent (ml)	7.5% Na_2CO_3 (ml)	Total volume (ml)
blank			1.0	5.0	4.0	10
10		1.0		5.0	4.0	10
20		1.0		5.0	4.0	10
40		1.0		5.0	4.0	10
60		1.0		5.0	4.0	10
80		1.0		5.0	4.0	10
100		1.0		5.0	4.0	10
sample	1.0			5.0	4.0	10

F-C = Folin-Ciocalteau reagent; Na_2CO_3 = sodium carbonate
The order in which the reagents are added is important and should be done from left to right as listed in the table above

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Table 2a Spectrophotometric determination of flavonols were determined using quercitin as the standard, and various concentrations of the different teas.

Test-tube	Quercetin Stock (ml)	95% EtOH (ml)	Quercetin mg/l EtOH	Final Volume
Blank	0	4.0		4.0
S1	0.8	3.2	8	4.0
S2	0.4	3.6	4	4.0
S3	0.2	3.8	2	4.0
S4	0.1	3.9	1	4.0

EtoH = ethanol

Table 2b The table below indicates the suggested dilutions for the different concentrations of the various tea samples used in flavonol determination.

Tea	Dilution	Tea (ml)	Solvent (ml)	Total volume (ml)
Blank		0	3.0	3.0
Green	5X	0.6	2.4	3.0
	10X	0.3	2.7	3.0
	15X	0.2	2.8	3.0
Black	5X	0.6	2.4	3.0
	10X	0.3	2.7	3.0
	25X	0.12	2.88	3.0
Rooibos unfermented	5X	0.6	2.4	3.0
	10X	0.3	2.7	3.0
	25X	0.12	2.88	3.0
Rooibos fermen.	10X	0.3	2.7	3.0
	15X	0.2	2.8	3.0
	25X	0.12	2.88	3.0

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Honeybush unfermented	10X	0.3	2.7	3.0
	25X	0.12	2.88	3.0
	35X	0.0857	2.9143	3.0
Honeybush ferm.	5X	0.6	2.4	3.0
	10X	0.3	2.7	3.0
	25X	0.12	2.88	3.0
Sutherlandia	5X	0.6	2.4	3.0
	10X	0.3	2.7	3.0
	15X	0.2	2.8	3.0
Rooibos unferm MeOH	5X	0.6	2.4	3.0
	10X	0.3	2.7	3.0
	15X	0.2	2.8	3.0

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Table 3a Spectrophotometric determination of flavanols were determined using catechin as the standard, and prepared using the table below.

Test-Tube	Catechin Stock (µl)	MeOH (ml)	Catechin µg/ml MeOH	Final Dilution	Final Volume
S1	25	4.975	5	200x	5.0
S2	50	4.950	10	100x	5.0
S3	100	4.900	20	50x	5.0
S4	150	4.850	30	33.33x	5.0
S5	200	4.800	40	25x	5.0

* The standards concentrations can be adjusted to accommodate teas with very low levels of flavanols (eg. *Sutherlandia*)

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Table 3b Suggested incubation time necessary for the different concentrations of the various tea samples used in flavanol determination.

Tea	Dilution	Tea (ml)	Solvent (ml)	Total vol (ml)	Suggested incubation time (min)
Blank		0	5.0	5.0	
Green	150X	0.0333	4.967	5.0	5
	180X	0.0278	4.972	5.0	5
	200X	0.0250	4.975	5.0	5
Black	80X	0.0625	4.9375	5.0	6
	100X	0.050	4.950	5.0	6
	120X	0.0417	4.9583	5.0	6
Rooibos unfermented	100X	0.050	4.950	5.0	15
	110X	0.0455	4.9545	5.0	15
	120X	0.0417	4.9583	5.0	15
Rooibos fermenented	15X	0.333	4.667	5.0	20
	30X	0.167	4.833	5.0	20
	45X	0.111	4.889	5.0	20
Honeybush unfermented	60X	0.0833	4.917	5.0	28
	100X	0.050	4.950	5.0	28
	120X	0.0417	4.9583	5.0	28
Honeybush fermented	15X	0.333	4.667	5.0	40
	30X	0.167	4.833	5.0	40
	45X	0.111	4.889	5.0	40
Sutherlandia	1X	5.000	0	5.0	33
	1.5X	3.333	1.667	5.0	33
	2X	2.500	2.500	5.0	33
Rooibos unferm MeOH	5X	1.000	4.000	5.0	12

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	10X	0.500	4.500	5.0	12
	15X	0.333	4.667	5.0	12

- The dilutions may vary with varying concentrations of tea

Table 3c The control, catechin-diluted standards and tea samples were prepared using the table below as a guide.

Test Tube	Catechin Std (ml)	Samples (ml)	Solvent* (ml)	DAC (ml)	HCL-MeOH (ml)	Total Vol (ml)
Blank			1.0	5.0		6.0
Standard	1.0			5.0		6.0
Sample		1.0		5.0		6.0
Sample blank		1.0			5.0	6.0

DAC = *p*-dimethylaminocinnamaldehyde; HCl-MeOH = hydrochloric acid/methanol

- Solvent that the samples are dissolved in.

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Table 4 Monthly fluid intake (ml/100g body weight) and body weight gain over 6 months

ID	Mean monthly fluid intake (TI) over the 6 month period (ml/100g BW)						Mean Fluid intake (ml/100g BW)	Body Weight Gain (g)
	TI (1)	TI(2)	TI(3)	TI(4)	TI(5)	TI(6)		
NEG	9.93 $\pm 0.67b^{a}$	8.87 $\pm 0.65b^{ab}$	9.57 $\pm 1.35a^{a}$	8.48 $\pm 0.84a^{bc}$	8.23 $\pm 0.80a^{bc}$	7.62 $\pm 0.8a^{c}$ 3	8.79 ±0.50a	91.25 (13.83)
POS	10.48 $\pm 1.54b^{a}$	9.72 $\pm 1.16a^{ab}$	9.78^{b} $\pm 0.85a^{a}$	9.30 $\pm 0.98a^{ab}$	9.16 $\pm 1.04a^{ab}$	8.42 $\pm 1.23a^{b}$	9.48 ±1.03a	89.25 (24.75)
Gr (2%)	12.98 $\pm 1.02a^{a}$	10.82 $\pm 0.88a^{b}$	10.39 $\pm 0.72a^{bc}$	9.55 $\pm 0.51a^{cd}$	8.84 $\pm 0.31a^{de}$	8.34 $\pm 0.40a^{e}$	10.15 ±0.37b	100.10 (16.13)
Bl (2%)	10.64 $\pm 1.50b^{a}$	9.84 $\pm 0.91a^{ab}$	9.84 $\pm 1.06a^{ab}$	8.71 $\pm 1.02a^{bc}$	8.71 $\pm 0.83a^{bc}$	8.11 $\pm 0.81a^{c}$	9.31 ±0.88a	92.46 (24.07)
Rg (2%)	10.19 $\pm 0.92b^{a}$	9.35 $\pm 0.93b^{abc}$	9.49 $\pm 0.75a^{ab}$	8.97 $\pm 0.99a^{bc}$	8.63 $\pm 0.86a^{bc}$	8.21 $\pm 1.08a^{c}$	9.14 ±0.80a	87.00 (22.84)
Rf (2%)	10.77 $\pm 1.302b^{a}$	9.95 $\pm 0.78a^{ab}$	9.67 $\pm 0.73a^{b}$	8.99 $\pm 0.65a^{bc}$	8.61 $\pm 0.72a^{c}$	8.16 $\pm 0.67a^{c}$	9.36 ±0.48a	99.92 (26.91)
RGM (0.1%)	10.02 $\pm 0.99b^{a}$	9.73 $\pm 0.52a^{a}$	9.29 $\pm 0.77a^{ab}$	8.83 $\pm 0.98a^{ab}$	8.40 $\pm 0.958a^{b}$	8.07 $\pm 1.37a^{b}$	9.06 ±0.67a	94.20 (23.80)
Hg (4%)	11.35 $\pm 1.46a^{a}$	10.54 $\pm 1.14a^{ab}$	10.21 $\pm 1.37a^{ab}$	9.35 $\pm 0.99a^{bc}$	8.61 $\pm 1.03a^{cd}$	7.87 $\pm 0.92a^{d}$	9.66 ±1.05a	98.92 (19.01)
Hf (4%)	10.99 $\pm 1.25b^{a}$	10.12 $\pm 1.14a^{a}$	9.91 $\pm 0.87a^{ab}$	8.78 $\pm 0.89a^{bc}$	8.39 $\pm 0.957a^{cd}$	7.53 $\pm 0.92a^{d}$	9.29 ±0.90a	95.75 (15.64)
Sut (1%)	9.78 $\pm 1.16b^{a}$	9.34 $\pm 1.04b^{ac}$	10.74 $\pm 5.02a^{ac}$	8.35 $\pm 0.91a^{bc}$	8.01 $\pm 0.79a^{bc}$	7.36 $\pm 0.86a^{bd}$	8.939 ±1.22a	89.78 (26.48)

Values are the means of 9-13 determinations ± STD. Means in a column followed by the same letter do not differ significantly. If the letters differ, then $P<0.05$. TI: fluid intake. Fluid intake is the mean of each month for the six months. Neg = negative control; Pos = positive control; Gr = green; Bl = black; Rg = unfermented rooibos; Rf = fermented rooibos; RGM = unfermented rooibos methanol extract; Hg = unfermented honeybush; Hf = fermented honeybush; Sut = *Sutherlandia frutescens*.

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Table 1 Absorbance (490nm) values of LDH release in WHCO5 cells exposed to various concentrations of the different teas in the Cytotox 96 LDH assay.

	Gr (mg/ml)				Bl (mg/ml)			
	0.5	**2.5**	**5**	**10**	**0.5**	**2.5**	**5**	**10**
Tea	0.119	0.339	0.583	1.035	0.120	0.435	0.796	1.201
Tea & cells	0.277	0.315	0.377	0.571	0.312	0.502	0.638	1.034
% color	42.7	107.6	154.6	181.3	38.5	86.7	124.8	116.2
	Rg (mg/ml)				**Rf (mg/ml)**			
	0.1	**0.5**	**2**	**4**	**0.5**	**2.5**	**5**	**10**
Tea	0.219	0.698	1.630	2.410	0.153	0.725	1.233	2.268
Tea & cells	0.417	0.981	1.643	2.105	0.483	1.128	1.344	2.134
% color	52.5	71.2	99.2	114.5	31.7	64.3	91.7	106.3
	Hg (mg/ml)				**Hf (mg/ml)**			
	0.1	**0.5**	**2**	**4**	**0.5**	**2.5**	**5**	**10**
Tea	0.197	0.656	2.006	2.980	0.105	0.323	0.594	1.011
Tea & cells	0.431	1.170	2.599	3.451	0.410	0.795	1.013	1.304
% color	45.7	56.1	77.2	86.4	25.6	40.6	58.6	77.5
	RgM (mg/ml)				**Sut (mg/ml)**			
	0.1	**0.5**	**2**	**4**	**5**	**10**	**20**	**50**
Tea	0.127	0.218	0.489	0.886	0.125	0.145	0.221	0.313
Tea & cells	0.443	0.442	0.900	1.152	0.415	1.092	1.110	1.411
% color	28.7	49.3	54.3	76.9	30.1	13.3	20.1	22.2

Gr = green tea; Bl = black tea; Rg = unfermented rooibos; Rf = fermented rooibos; Hg = unfermented honeybush; Hf = fermented honeybush; RgM = unfermented rooibos methanol extract; Sut = *Sutherlandia frutescens*. Tea = tea dilutions in media; Tea & cells = cells exposed to various tea dilutions.

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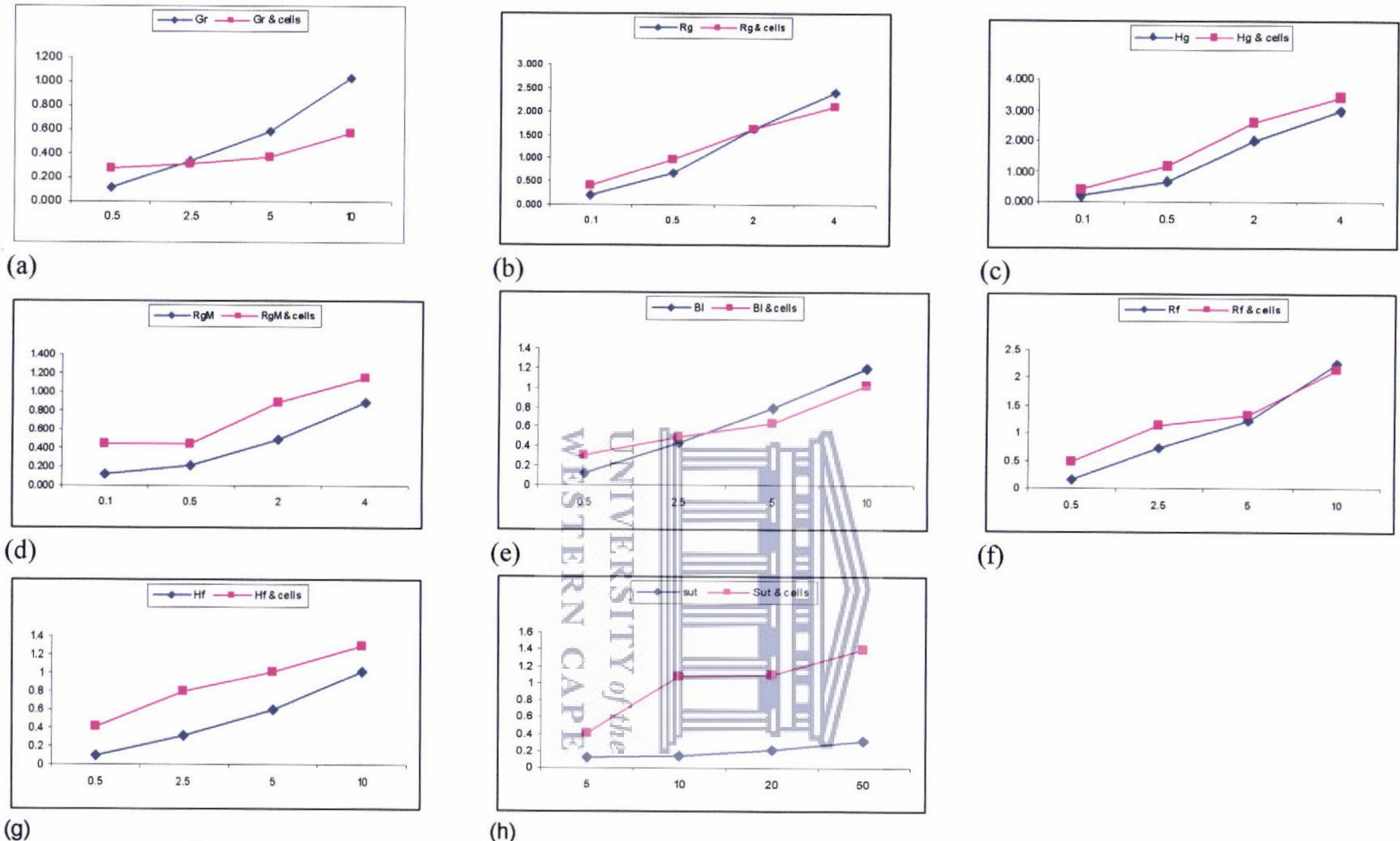

Gr = green tea; Bl = black tea; Rg = unfermented rooibos; Rf = fermented rooibos; Hg = unfermented honeybush; Hf = fermented honeybush; RgM = unfermented rooibos methanol extract; Sut = *Sutherlandia frutescens*.

Fig. 1 Graphic representation of color interference when using the LDH colorimetric assay to determine cytotoxicity of various concentrations of teas in WHCO5 cells.

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Table 2: Absorbance (570-630nm) values of viability in WHCO5 cells exposed to various concentrations of the different teas in MTT assay.

	Gr (mg/ml)				Bl (mg/ml)			
	0.5	2.5	5	10	0.5	2.5	5	10
Tea	0.030	0.092	0.164	0.327	0.041	0.120	0.201	0.381
Tea & cells	0.067	0.105	0.173	0.316	0.070	0.141	0.235	0.405
% color	44.8	87.6	94.8	103.5	58.6	85.1	85.5	94.1
	Rg (mg/ml)				Rf (mg/ml)			
	0.1	0.5	2	4	0.5	2.5	5	10
Tea	0.140	0.460	0.854	1.203	0.270	0.776	1.170	1.515
Tea & cells	0.381	0.668	0.935	1.271	0.439	0.852	1.237	1.619
% color	36.7	68.9	91.3	94.6	61.5	91.1	94.6	93.6
	Hg (mg/ml)				Hf (mg/ml)			
	0.1	0.5	2	4	0.5	2.5	5	10
Tea	0.072	0.220	0.458	0.717	0.157	0.479	0.725	0.947
Tea & cells	0.380	0.473	0.515	0.761	0.362	0.532	0.778	1.088
% color	18.9	46.5	88.9	94.2	43.4	90.0	93.1	87.0
	RgM (mg/ml)				Sut (mg/ml)			
	0.1	0.5	2	4	5	10	20	50
Tea	0.239	1.407	2.336	3.509	2.886	3.131	3.079	3.509
Tea & cells	0.476	1.090	2.118	3.509	3.083	3.509	3.202	3.509
% color	50.2	5.3	110.3	100	93.6	89.2	96.2	100

Gr = green tea; Bl = black tea; Rg = unfermented rooibos; Rf = fermented rooibos; Hg = unfermented honeybush; Hf = fermented honeybush; RgM = unfermented rooibos methanol extract; Sut = *Sutherlandia frutescens*. Tea = tea dilutions in media; Tea & cells = cells exposed to various tea dilutions.

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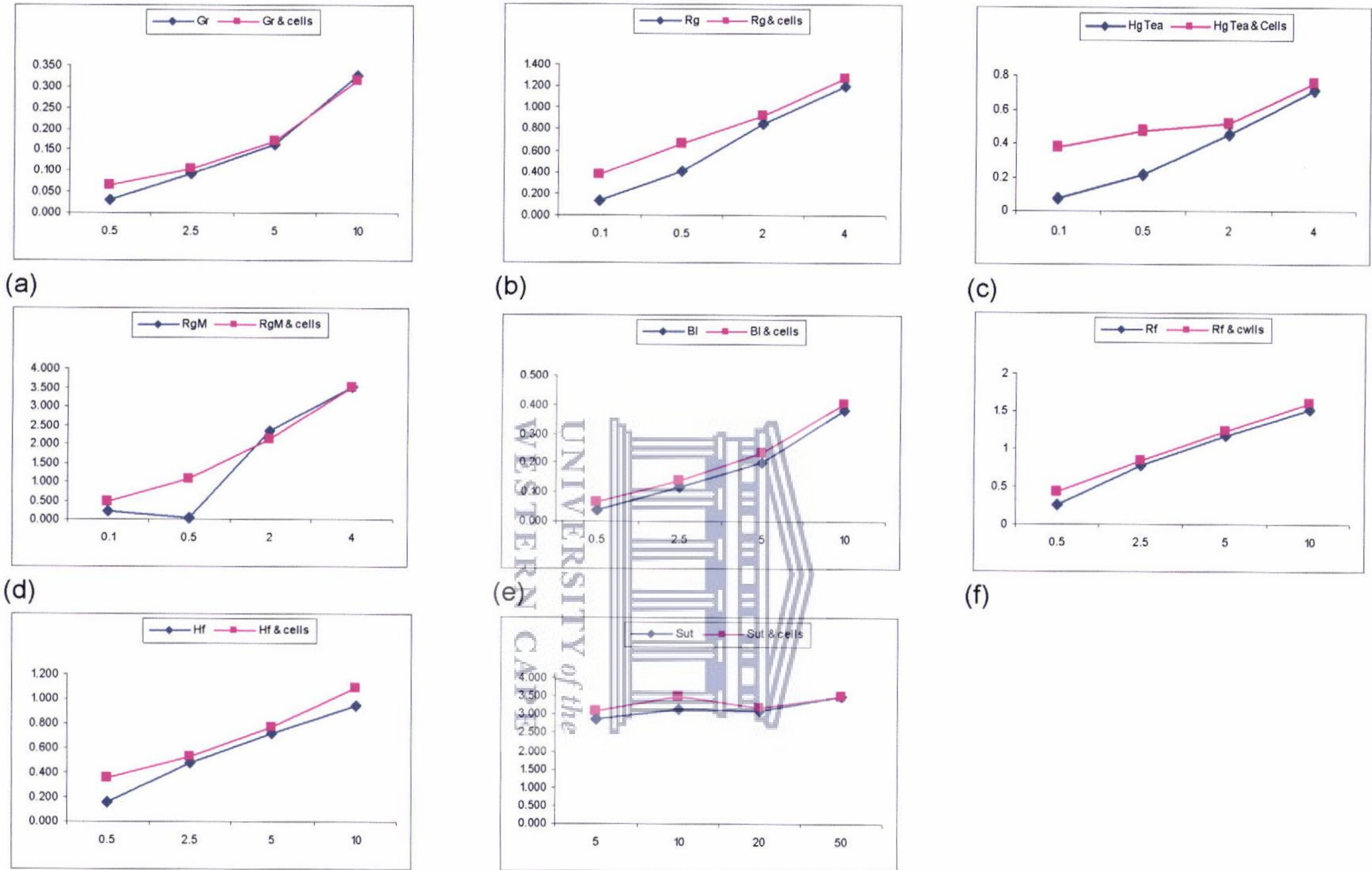

Gr = green tea; Bl = black tea; Rg = unfermented rooibos; Rf = fermented rooibos; Hg = unfermented honeybush; Hf = fermented honeybush; RgM = unfermented rooibos methanol extract; Sut = *Sutherlandia frutescens*.

Fig. 2 Graphic representation of color interference when using the MTT colorimetric assay to determine viability of WHCO5 cells exposed to different concentrations of teas.

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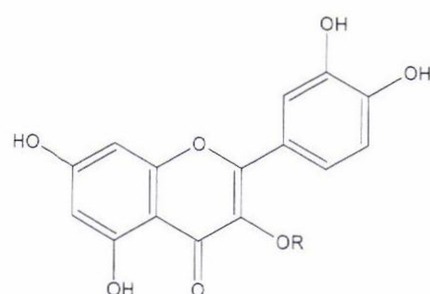
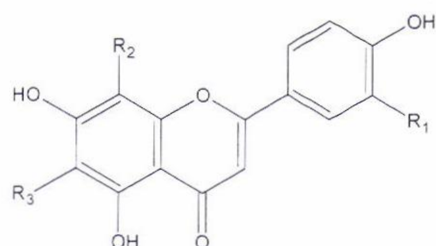


Flavonol
quercetin R=H
isoquercitrin R= β-D-glucopyranosyl
rutin R= rutinosyl



C-C linked flavone glycosides
iso-orientin R_1=OH, R_3= glycopyranosyl (glucose)
isovitexin R_3= glycopyranosyl (glucose)
orientin R_1= OH, R_2=glycopyranosyl (glucose)
vitexin R_2=glycopyranosyl (glucose)

Fig. 3 Structures of the flavonols quercetin, isoquercitrin and rutin, and four C-C linked flavone glycosides, iso-orientin, isovitexin, orientin and vitexin (Rabe *et al.*, 1994).

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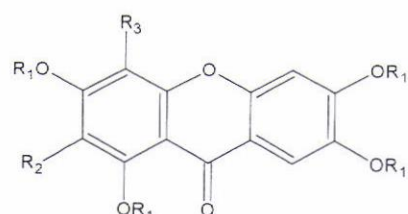
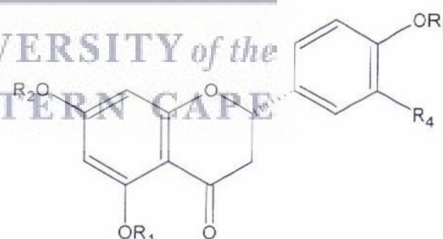


Xanthone
mangiferin R_1=R_3=H,
R_2=2-β-*D*-glucopyranosyl
isomangiferin R_1=R_2=H,
R_3= 2-β-*D*-glucopyranosyl



Flavanones
hesperetin R_1=R_2=H, R3=Me, R_4=OH
hesperidin R_1=H, R_2 = rutinosyl, R_3=Me, R_4=OH
narirutin R_1= R_3= R_4, R_2 = rutinosyl

Fig. 4 Structures of the major compounds in *C. intermedia;* mangiferin and hesperidin (Joubert *et al.*, 2003) and other phenolic compounds that have been isolated from fermented *C. intermedia* (Ferreira *et al.*, 1998).

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