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An investigation into the potential mutagenicity of South African traditional medicinal plants

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Declaration

I, ___________________________, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Dedicated to Paramadayalan, Dayalan and Ashaylin
for their overwhelming support and encouragement without which I would never have overcome the hurdles encountered in this thesis.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

AA	Aristolochic acid
2-AAF	2-acetamidofluorene
CHO	Chinese hamster ovary cells
CHP	Cumene hydroperoxide
CPA	Cyclophosphamide
CTA	Chromatid aberration
CSA	Chromosome aberration
CYP	cytochrome
DCM	Dichloromethane
DHP	6,7 dihydro-7-hydroxy-1-(hydroxymethyl)-5H-pyrrolizine
DMSO	Dimethyl sulfoxide
FACS	Flow activated cell sorting
GSH	Glutathione
LPS	Lipopolysaccharide layer
MGA	Minimum glucose agar
MMS	Methylmethanosulphate
PCB	Polychlorinated biphenyl
PHA	Phytohaemagglutinin
RBCs	Red blood cells
S.D.	Standard deviation
SULTS	Sulfotransferases
VOD	Veno-occlusive liver disease
WBCs	White blood cells

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ABSTRACT

The potential mutagenicity and clastogenicity (ability to cause chromosomal damage) of five South African traditional medicinal plants: *Acokanthera oppositifolia (Lam.)*; *Pelargonium sp. cf. inquinans (L.) L' Herit*; *Pteridium aquilinum subsp aquilinum; Rumex lanceolatus Thunb.* and *Zantedeschia aethiopica (L.) Sg*, were investigated using two *in vitro* tests in both a bacterial and a mammalian cell system. The *Salmonella* reverse mutation assay and chromosomal aberration test, two frequently used and accepted pharmacological bioassays, were selected for the investigation. The rat liver extract (S9), containing CYP P450 and other liver enzymes, was added to the *in vitro* cell system to detect pro-mutagens that require metabolic activation in order to exert mutagenicity or clastogenicity – directly acting mutagens do not require metabolic activation.

A significant mutagenic potential ($p \leq 0.01$) was evident with the *Salmonella* reverse mutation assay for three of the aqueous plant extracts: (i) *R. lanceolatus* (in strains TA97a, TA98, TA100 and TA102) with and without metabolic activation (S9), (ii) *P. aquilinum* in TA100 with S9 and in TA102 without S9 and (iii) *Pelargonium* (in TA102) without S9. Furthermore, *R. lanceolatus* and *P. aquilinum* were clastogenic in the chromosomal aberration test but this effect was reduced with S9. *Z. aethiopica* demonstrated clastogenicity, which was reduced with S9, but the extract was not mutagenic.

Since the chromosomal aberration test is dependent on cells entering the cell cycle (G_o-G_1, S and G_2-M) and chromosome visibility with light microscopy only occurs at metaphase, the clastogenicity of *A. oppositifolia* and *Pelargonium* could not be detected because these extracts inhibited mitosis (M). A DNA analysis of cultures treated with *A. oppositifolia* and *Pelargonium* by Flow Activated Cell Sorting (FACS) indicated a blockage in the G_o/G_1 phase of the cell cycle.

The *in vitro* mutagenicity and clastogenicity tests served as a preliminary investigation into the safety of five traditional plants. In addition to mutagenicity testing, it is suggested that further scientific evaluation, validation, standardisation and regulation of South African traditional medicine is essential in order to prevent the adverse acute and chronic effects of plant ingestion.

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CHAPTER 1
INTRODUCTION

1.1 Background

A major part of the population of both urban and rural communities in South Africa is reliant on traditional medicine as opposed to westernised medicine (Van Wyk *et al*., 1997 and Eldeen *et al*., 2005). This is because herbal medicines are an important part of the culture and tradition of African people and are generally more accessible and affordable. However, many investigations have shown that plants used as food or in traditional medicine have mutagenic and carcinogenic effects in *in vitro* and *in vivo* assays as well as in humans (Stickel and Seitz, 2000, Zhang *et al*., 2004, Zhou *et al*., 2004, Meinl *et al*., 2006 and Reid *et al*., 2006).

The *Salmonella* reverse mutation assay (Maron and Ames, 1983) is widely used in determining possible gene mutations caused by extracts (Reid *et al.,* 2006). A chemical that is mutagenic in *Salmonella* is presumed to be a rodent carcinogen (and potential human carcinogen) (Zeiger, 2001). The chromosome aberration test is commonly used as a biomarker for the early effects of genotoxic carcinogens.

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Plant extracts are prepared from the aerial parts of the plant or roots and the most commonly used solvents are water, dichloromethane and methanol. Such extracts contain only the soluble fractions of the plant material (usually about 20% of the total weight) and the non-soluble (fibrous) residues (about 80%) are discarded (Gurib-Fakim, 2006). A plant extract that produces a mutagenic or clastogenic response is a result of one or more phytochemicals in the extract.

1.2 Phytochemicals

Phytochemicals that are produced from primary metabolism are associated with the fundamental life processes common to all plants, such as photosynthesis, the pentose cycle, glycolysis and the citric acid cycle. The secondary plant metabolites contribute to the taxonomic and biochemical differentiation, and diversity within the plant kingdom. Furthermore, secondary plant metabolites evolved as chemical defences to repel, stun, poison or kill other species or predators; therefore it would be naive to think that every plant extract is necessarily safe for human consumption. The beneficial and adverse effects of phytochemicals are discussed in the following sections.

1.2.1 Beneficial effects

Traditional plants have been used for dietary and medicinal purposes for centuries and continue to be popular. There are many examples of the beneficial effects of traditional plants such as their chemopreventative activity when herbal constituents form reactive intermediates capable of irreversibly inhibiting various cytochrome P450s and their antibacterial and anti-inflammatory properties (Zhou *et al*., 2004, Eldeen *et al*., 2005, and Moshi and Mbwambo, 2005) and a variety of

other beneficial effects (Table 1). The pharmacological screening of medicinal plants is very important because it provides a scientific basis for the continued use of plants and could be potential sources of new, effective and safe drugs (Eldeen *et al*., 2005).

Table 1: Plant compounds and extracts with beneficial medicinal effects

Chemical compound/extract	Botanical source	Biological activity	Reference
Scopolamine (alkaloid)	*Datura stramonium*	Anti-muscarinic agent, smooth muscle relaxant and anti-nauseant	Van Wyk *et al*., 1997, 2002, and Dictionary of Natural Products, 2003
Resperine	*Rauwolfia serpentina*	Used for the treatment of high blood pressure	Craig, 1999
Taxol	*Taxus brevifolia*	A chemotherapeutic agent	Craig, 1999
Atropine (alkaloid)	*Datura stramonium*	Reduces rigidity in parkinsonism and is used as an antidote to poisoning with parasympathomimetic agents e.g. nerve gases and organophosphorous insecticides	Dictionary of Natural Products, 2003
Morphine and codeine (opium alkaloids)	*Papaver somniferum* (Poppy plant)	Narcotic analgesic (pain relief) and treatment of coughs.	
Vinblastine and vincristine (alkaloids)	*Catharanthus roseus* (Periwinkle)	Anti-cancer drugs that interfere with the turbulines of the mitotic and meiotic spindle that inhibit cell division.	
EPs®7630, Umckaloabo® (root extract)	*Pelargonium sidoides*	*In vitro* antibacterial, antiviral, and immunomodulatory properties in several studies. Efficacy has been proved in numerous clinical trials. Therapeutic effect in acute bronchitis, tonsillopharyngitis, sinusitis and symptoms of the common cold.	Bereznoy *et al*., 2003, Beil and Kilian, 2007, Agbabiaka *et al.,* 2008 and Brendler and van Wyk, 2008
Rooibos, Honeybush (leaf extracts)	*Aspalathus linearis*, *Cyclopia intermedia*	(i) *In vitro* antimutagenicity, (ii) *in vivo* antioxidant, immune-modulating and chemopreventative actions.	Van der Merwe *et al*., 2006 and McKay and Blumberg, 2006

Despite the beneficial effects of plant compounds and extracts many adverse effects continue to be reported (Stickel and Seitz, 2000, Zhang *et al*., 2004, Zhou *et al*., 2004, Meinl *et al*., 2006 and Reid *et al*., 2006).

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1.2.2 Adverse effects

Although a few plant extracts and numerous bioactive compounds that have been isolated from plants have medicinal value, the ingestion of some plants and/or their extracts has been linked to acute (short-term) or chronic (long-term) effects.

1.2.2.1 Acute effects

The symptoms of acute effects are toxicity (mainly acute hepato-toxicity), poisoning and acute veno-occlusive liver disease (VOD), which is a non-thrombotic obliteration of small hepatic veins leading to cirrhosis and, eventually, liver failure, which is diagnosed from a liver biopsy and histopathology. Acute effects usually result from the ingestion of large quantities of plant material over a short period:

(i) *Datura stramonium*, traditionally used for the treatment of gout, boils, wounds and asthma and to reduce pain, is extremely toxic because of two main alkaloids, namely atropine and scopolamine, which have been linked to deaths and poisoning for centuries (van Wyk *et al.*, 1997, Miraldi *et al.*, 2001, Steenkamp *et al.*, 2004 and Friedman, 2004). However, both atropine and scopolamine have pharmaceutical significance (refer to Table 1).

(ii) VOD is related to the ingestion of plants containing various pyrrolizidine alkaloids, such as *Symphytum officinale*, commonly referred to as comfrey (Stickel and Seitz, 2000). In South Africa many VOD cases have been reported and genera known to contain pyrrolizidine alkaloids are *Senecio* and *Crotalaria* (Steenkamp *et al.*, 2000).

(iii) The high oxalic acid content of *Rumex* sp (leaves and root) used in traditional medicine has been implicated in oxalic intoxication mainly in children. However, when oxalic acid is used medicinally as a 5% solution in 5% malonic acid it is beneficial as a haemostatic agent (The review of natural products, 2002, and van Wyk and Wink, 2004).

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1.2.2.2 Chronic effects

A chronic effect results from the ingestion of small quantities of plant material over a long period. This can cause organ damage such as in the liver and kidneys. Carcinogenesis can also result from the ingestion of plant material.

(i) A case-control study on the ‘usual consumption’ of the plant *Solanum nigrum* in the Transkei has been associated with an irritating effect on the oesophageal mucosal lining (Sammon, 1992) and oesophageal cancer (IARC Scientific Publication 2003, No.153).

(ii) The use of Comfrey leaves (*Symphytum officinale*) has been recognised as a substantial health hazard, with hepatic toxicity in humans and carcinogenic potential in rodents resulting from its pyrrolizidine alkaloids: lasiocarpine and symphytine, and their related N-oxides (Stickel and Seitz, 2000). The South African traditional medicine, *Senecio latifolius,* which is known to contain toxic pyrrolizidine alkaloids, is a potential teratogen and carcinogen, as observed from the long-term low-dose treatment of human HuH-7 cells

(Steenkamp *et al.*, 2001). Pyrrolizidine alkaloids are ubiquitous in the plant kingdom but to date only the retronecine, heliotridine and otonecine types exhibit high toxicity and tumourigenicity in experimental animals (the metabolism of these three types are the most studied) (Fu *et al.*, 2004).

(iii) The use of herbal preparations of *Aristocholia* sp led to progressive nephropathy and urothelial cancer in humans owing to the formation of DNA adducts in human target tissue (Zhang *et al.*, 2004; Zhou *et al.*, 2004 and Meinl *et al.*, 2006).

(iv) The use of *Pteridium aquilinum* has been related to mutagenicity and carcinogenicity in *in vitro, in vivo* and epidemiological studies owing to its bioactive compound, ptaquiloside, and its metabolic intermediate, pteridienone, elaborated in section 1.7.1. Metabolic activation may influence the mutagenic potential of a phytochemical, as in the case of pteridienone which was found to have more pronounced activity over ptaquiloside (Matsuoka *et al.*, 1989). Therefore, mutagenicity tests are generally performed with and without metabolic activation by rat liver microsomes (S9).

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1.3 Metabolism of chemical carcinogens

The liver is the major detoxification centre in the body where chemicals undergo biotransformation to a more "excretable" (i.e. water-soluble) form. Cytochrome P450 and liver enzymes (transferases) have critical roles in the metabolism of steroids and eicosanoids, and other biosynthetic processes, and are involved in activation of pro-carcinogens as the first step to their removal. Pro-carcinogens or pro-mutagens are not carcinogenic/mutagenic on their own but require metabolic activation to intermediates in order to exert their effect. Mutagenicity tests incorporate CYP P450 in the form of rat liver homogenate S9 for the detection of pro-mutagens in plant extracts. The metabolism of drugs or xenobiotics in the liver occurs in two steps known as Phase I and Phase II reactions.

Phase I reactions: Cytochrome P450 enzymes, 1A1, 1A2, 1B1, 2A6, 2E1 and 3A4, play a prominent role in the metabolism of carcinogens, both in bioactivation and detoxification (Guengerich and Shimada, 1991 and Guengerich, 2001). Carcinogens have reactive electrophilic groups that can bind to DNA directly and cause mutations and cancer. However, pro-carcinogens are enzymatically activated by CYP P450 into reactive electrophiles (e.g. ptaquiloside to pteridienone) that can bind to DNA and also cause mutations and cancer. It is therefore imperative that *in vitro* mutagenicity tests incorporate CYP P450 or liver microsomes for the detection of pro-mutagens that are potential pro-carcinogens (confirmed by a two-year *in-vivo* study). Most *Phase II* enzymes deactivate pro-carcinogens. Plant compounds may initiate the carcinogenic process through DNA mutations and a variety of other genotoxicities, which are discussed in the following sections.

1.4 Plant extract-induced DNA mutations and genotoxicities

Plant extracts or compounds may cause DNA damage through point mutations, chromosomal alterations and other genotoxicities (e.g. DNA adducts, DNA crosslinking). Point mutations involve one or more nucleotides (e.g. frameshift mutations and base pair substitutions) while chromosomal alterations encompass several nucleotides.

1.4.1 Point mutations

A variety of traditional plant extracts and compounds produce point mutations:

(i) Aristolochic acid, the active compound from *Aristolochia* species, is mutagenic with the *Salmonella* reverse mutation assay in strains TA98 and TA100, indicative of frameshift and base-pair substitution mutations respectively, and in the mouse lymphoma assay (Zhang *et al*., 2004).

(ii) Two extraction procedures were used in a sequential manner to extract polar (methanol-water soluble) and apolar (dichloromethane) compounds from *Cyrtanthus falcatus* and *Cyrtanthus suaveolens* (Family: Amaryllidaceae), which are commonly used in SA traditional medicine (Elgorashi and van Staden, 2004). The dichloromethane (DCM) extracts of the leaves and roots of *C. falcatus*, and the bulbs/roots and leaves of *C. suaveolens* induced mutation in the *Salmonella* reverse mutation assay in strain TA98, while none of the 90% methanol extracts of the different plant parts of the two species induced mutagenicity in TA98. Since the DCM extracts of *C. falcatus* and *C. suaveolens* induced frameshift mutations detected by TA98, the traditional medicine should be used with caution since rigorous toxicological investigations are required.

(iii) *Helichrysum simillimum, Helichrysum herbaceum* and *Helichrysum rugulosum*, extensively used in SA traditional medicine in the treatment of coughs, colds, fever, infections, headache and menstrual pain (Van Wyk *et al*., 1997), showed mutagenicity in the *Salmonella* reverse mutation assay in strains TA98 and TA100 with the 90% methanol extracts, which is indicative of frameshift and base-pair substitution mutations respectively (Reid *et al*., 2006).

Plant extractions using both polar and apolar solvents would add to scientific knowledge; however, the validation of the plant extract would be compromised because traditional healers mainly use water as a solvent in the preparation of plant extracts, mostly as decoctions and/or infusions.

1.4.2 Chromosomal alteration

(i) South African traditional medicinal plant extracts, *Afzelia quanzensis Welw.; Bersama lucens (Hochst.) Szyszyl; Ocotea bullata; Siphonochilus aethiopicus* and *Tetradenia riparia (Hochst.) Codd (Lamiaceae)*, induced DNA damage as detected in the comet assay (Taylor *et al*., 2003).

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(ii) Extracts of *Antidesma venosum E. Mey. Ex Tul.; Balanities maughamii Sprague, Catunaregan spinosa (Thunb.) Tirveng.; Chaetacme aristata, Croton sylvaticus Hochst.; Diospyros whyteana (Hiern) F. White; Euclea divinorum Hiern; and Gardenia volkensii K. Schum* caused both DNA damage and chromosomal aberrations in human white blood cells as detected by the comet assay and micronucleus tests respectively (Taylor *et al*., 2003). The micronucleus test detects chromosome breakage and/or chromosome loss.

(iii) The dichloromethane extracts of twigs/bark of *Gardenia volkensii* and *Spirostachys africana* common South African traditional medicinal plants, were found to be genotoxic in the micronucleus test (Verschaeve *et al*., 2004).

(iv) Aristolochic acid from the *Aristolochia* species caused an increase in chromosomal aberrations in Chinese hamster ovary (CHO) cells, with and without metabolic activation by S9 (Zhang *et al*., 2004).

1.4.3 Other genotoxicities

(i) Pyrrolizidine alkaloids are naturally occurring phytochemicals that are common constituents of hundreds of plant species worldwide. Some pyrrolizidine alkaloids exhibit a variety of genotoxicities, including DNA binding, DNA cross-linking and DNA-protein cross-linking (Reed *et al*., 1988, Hincks *et al*., 1991 and Coulombe *et al*., 1999).

(ii) Aristolochic acid, the active compound from *Aristolochia* species, produces DNA adducts in human target tissue. Aristolochic acids undergo reduction of the nitro group by hepatic cytochrome P450 (CYP1A/2) or peroxidases in extrahepatic tissues to reactive cyclic nitrenium ion that are capable of reacting with DNA and proteins, has been shown to activate the *H-ras* oncogene (Zhou *et al*., 2004).

Several plants used in South African traditional medicine have the potential to cause long-term damage to genetic material in patients when administered as medicinal preparations (Fennell *et al*., 2004). The generation of DNA damage, which can arise from a direct or indirect mechanism, is considered an important initial event in carcinogenesis. Mutagenicity tests generally detect those chemicals that cause direct DNA damage.

1.5 Mechanism of DNA damage

Direct mechanisms refer to DNA damage arising from point mutations, chromosome or genome alteration, while indirect mechanisms refer to the interaction of chemical compounds with non-DNA targets leading to genotoxic effects such as lipid peroxidation and protein adducts (Kirsch-Volders *et al*., 2003). Carcinogenicity caused by *Aristolochia* sp is induced by direct and indirect DNA damaging mechanisms. The metabolic intermediate of aristolochic acid produces DNA adducts (direct mechanism) and protein adducts (indirect mechanism). Examples of chemical compounds and extracts shown to damage DNA via direct mechanisms are presented in Table 2.

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Table 2: Plant extracts and chemical compounds shown to cause direct DNA damage

Extract/Compound	**Direct DNA damage**				**Reference**
	Point Mutation	**Chromosome**	**Genome**	**DNA-adducts**	
P. aquilinum, extract (bioactive compound ptaquiloside)	[e]Mutagenic in the *Salmonella* reverse mutation assay with TA100 and TA98	[a]Peritoneal and bone marrow cells of Swiss mice showed structural chromosome aberrations	[a]Peritoneal and bone marrow cells of Swiss mice showed numerical chromosome aberrations	[b]Organ specific ptaquiloside-DNA alkylations *in vivo*	[e]Matoba *et al*., 1987, [b]Alonso-Amelot and Avendano, 2002 and [a]Almeida Santos *et al*., 2006.
Riddelliine, a pyrrolizidine alkaloid	-	-	-	Riddelliine induced DNA adduct formation *in vitro* and *in vivo*	Yang *et al*.,2001a, 2001b
C. suaveolens extract	Mutagenic in the *Salmonella* reverse mutation assay with strain TA98, indicating a frameshift mutation	-	-	-	Elgorashi and van Staden, 2004
Aristocholia sp (bioactive compound aristolochic acid)	[c]Mutagenicity in the *Salmonella* reverse mutation assay with TA98 & TA100, indicating frameshift and base-pair substitution mutations	[c]Chromosomal aberrations in CHO cells	-	[d]Forms DNA adducts in human target tissue	[c]Zhang *et al*., 2004, [d]Zhou *et al*., 2004 and [d]Meinl *et al*., 2006

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1.6 Known plant carcinogens

Many plant carcinogens have been identified from phytochemical and related studies; however, only pyrrolizidine alkaloids and aristolochic acid are discussed in this section.

1.6.1 Pyrrolizidine alkaloids

Pyrrolizidine alkaloids are categorised into retronecine (e.g. riddelliine, senecionine), heliotridine (e.g. lasiocarpine, heliotrine) and otonecine (e.g. clivorine) types. These are metabolised by the cytochrome P450 CYP3A subfamily, in particular CYP3A4 isoenzyme, which is the primary

metabolising enzyme responsible for the metabolic activation by human liver microsomes. Metabolic pathways involving N-oxidation and hydrolysis of these pyrrolizidine alkaloid types produce chemical end products that are reactive toward protein or DNA to form dehydropyrrolizidine (DHP)-derived adducts (Fu *et al.*, 2004). Male rats were reported to be more susceptible than female rats to pyrrolizidine alkaloids, with a higher mortality when riddelliine was administered at 1mg/kg/day, five days per week leading to the termination of the study at 72 weeks, whereas the same study in female rats lasted for 104 weeks (Chan 2001).

The reasons for the marked gender difference in rats was ascribed to: (i) the production of toxic metabolites of riddelliine, such as pyrrolic esters, bound pyrrole and DHP-derived DNA adducts in the male rat; (ii) male-specific isoenzymes CYP3A and CYP2C11 that catalyse biotransformations in senecionine toxicity; and (iii) the lack of CYP3A1 and CYP3A2 activities in female rats leading to a significantly low metabolic activation rate of clivorine, making them less susceptible to intoxication (Williams *et al.*, 1989, Chan 1993, Chan *et al.*, 1994 and Chan 2001).

There are no reports on a gender difference in the susceptibility of humans to pyrrolizidine alkaloid intoxication; it is unlikely that such a gender difference occurs in humans because there is no evidence exhibiting significant variations of human CYP3A4 expression (Fu *et al.*, 2004). However, the abundance of CYP3A4 in the liver varies significantly between individuals and in different ethnic groups, which may result in marked variations in the metabolic profile and thus different susceptibilities to pyrrolizidine alkaloid intoxication (Fu *et al.*, 2004).

1.6.2 Aristolochic acid

The *Aristolochia* species contain the active compound aristolochic acid, an alkaloid that plays a major role in DNA adduct formation in human target tissues. Products containing aristolochic acid were withdrawn from the market in the early 1980s because it was found to be a potent carcinogen (Zhang *et al.*, 2004). The cyclic nitrenium ion metabolic intermediate produced from aristolochic acid metabolism (Fig 1.) interacts with DNA and the use of herbal preparations containing *Aristolochia* species has led to progressive nephropathy and urothelial cancer in humans (Meinl *et al.*, 2006).

Only a minority of the users of *Aristolochia*-containing products develop nephropathy and cancer, suggesting differences in individual susceptibility. Human sulfotransferases (SULTS) reinforce the mutagenic activity of aristolochic acids through activation by SULT1A1 and SULT1B1 (Meinl *et al.*, 2006). A SULT1A1 inhibitor reduces the mutagenic effect of aristolochic acid in a mammalian cell system. SULT1A1 and SULT1B1 are expressed in the human kidney but at levels substantially lower than in liver, and it is therefore conceivable that reactive sulphuric acid conjugates may be

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produced in other tissues and transferred to the target organs such as the kidney (Meinl *et al.*, 2006).

Aristolochic acid has demonstrated mutagenicity (i) in the *Salmonella* reverse mutation assay in strains TA98 and TA100 indicating frameshift and base-pair substitution mutations, (ii) in the mouse lymphoma assay and (iii) an increase in chromosomal aberrations in Chinese hamster ovary cells was observed, with and without S9 (Zhang *et al.*, 2004).

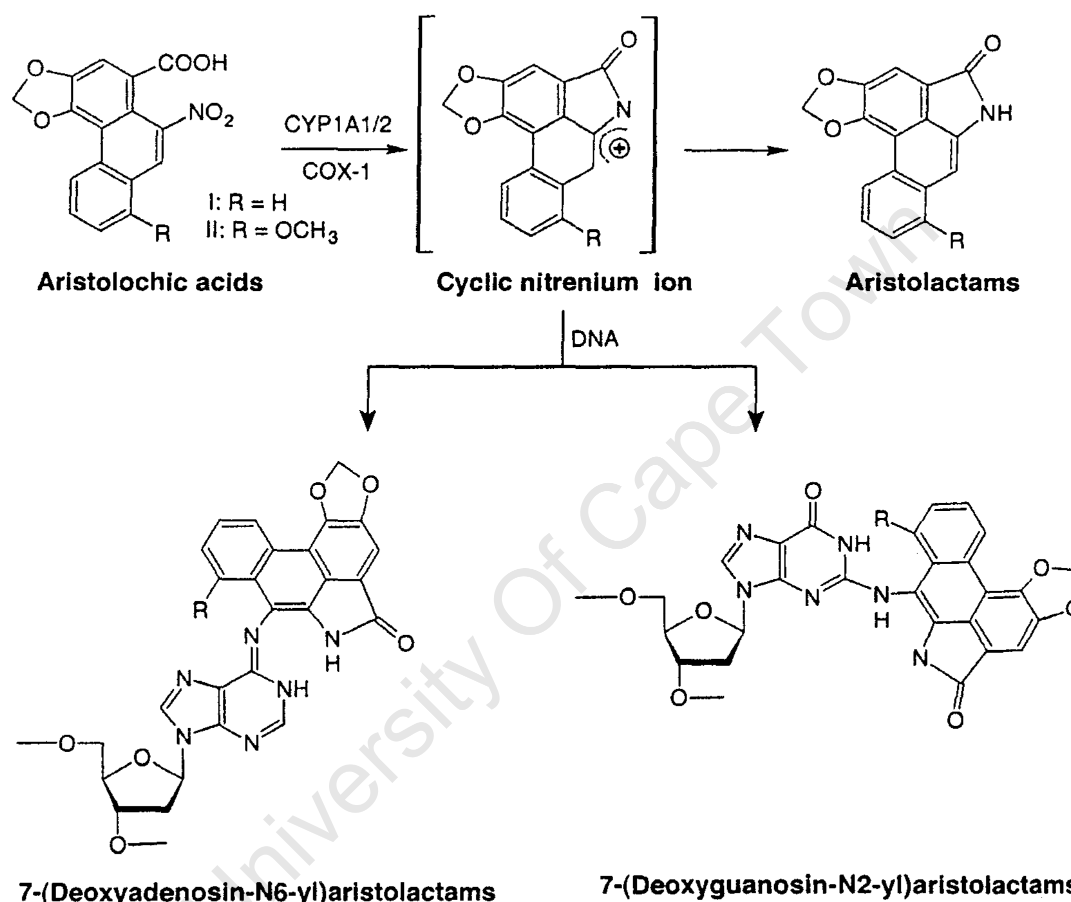

Fig1. Proposed bioactivation of aristolochic acids (AA). The reduction of the nitro group of AAI (R = H) and AAII (R = OCH_3) to reactive cyclic nitrenium ions is catalysed by oxidative enzymes (such as hepatic microsomal CYP1A1/2, NADPH:CYP reductase, DT-diaphorase, cyclooxygenase-1 and other peroxidases). The cyclic nitrenium ions are capable of reacting with DNA and/or proteins, leading to adduct formation. The formation of DNA adducts has been found to decrease or increase through the addition of inhibitors or inducers of CYP1A1/2 respectively. DNA-aristolactam adducts [e.g. (7-deoxyadenosin-N^6-yl) aristolactam I or II and (7-deoxyguanosin-N^2-yl) aristolactam I or II] have been detected in the kidney and ureter tissues of patients taking herbs containing AAs several months or years after cessation of the herbal consumption (Zhou *et al.*, 2004).

The use of simplified *in vitro* tests: (i) *Salmonella* reverse mutation assay (Ames *et al.*, 1975 and Maron and Ames, 1983); (ii) mouse lymphoma mutagenicity test (Clive *et al.*, 1987) and (iii) chromosomal aberration test (Galloway *et al.*, 1994), has demonstrated that the genotoxicity of aristolochic acid would have been easily detected if simple screening versions of the *in vitro*

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genotoxicity assays had been used during early product development (Zhang *et al.*, 2004). Furthermore, such screening tests provide a rapid and economical way of obtaining preliminary genotoxicity profiles of new substances or products as an aid to decision making for further development.

1.7 Phytochemical content of the five plants investigated

The plants currently used in traditional medicine and investigated in the current study were *Pteridium aquilinum subsp aquilinum, Zantedeschia aethiopica (L.), Acokanthera oppositifolia (Lam.)*, *Rumex lanceolatus Thunb.* and *Pelargonium sp. cf. inquinans (L.) L' Herit*.

1.7.1 *Pteridium aquilinum*

P. aquilinum, commonly known as bracken fern, belongs to the Family: Dennstaedtiaceae. It is found throughout the southern and eastern part of South Africa, and is common in moist regions and at high altitudes, where it often forms dense stands, particularly after a fire (Van Wyk *et al.,* 2002). Bracken poisoning in bovine, caprine and equine livestock occurs when the plant is interspersed in grazing fields or is a contaminant in fodder. Young fronds and rhizomes are cooked and eaten by the Zulu and Tswana (Roberts, 1990).

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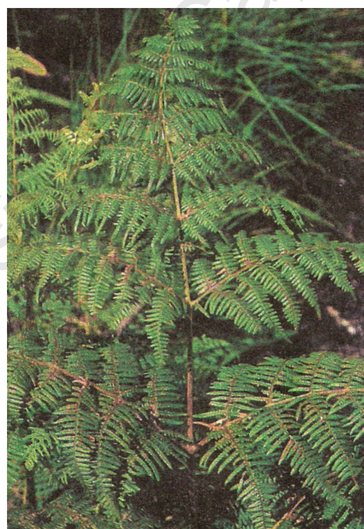

Fig 2. *P. aquilinum* is a fern with numerous firm-textured leaves, which are called fronds. Such fronds are generally 1 to 2 feet long and 1 to 2 feet wide. Young leaves are characteristically coiled in a bud (appear as hook-shaped tips) which uncurls during leaf development. Ferns are flowerless, seedless vascular plants that reproduce by means of spores, have roots that emanate from a rhizome and fronds that uncurl upward (van Wyk *et al.*, 2002).

1.7.1.1 Animal carcinogenicity, *in vitro* studies and *in vivo* studies

P. aquilinum contains: (i) thiaminase I, which gives rise to a neurological disorder in horses and sheep; (ii) a carcinogen responsible for neoplasia particularly of the urinary bladder of cattle; and (iii) a cyanide-producing glycoside, prunasin (Kofod and Eyjolfsson, 1966 and Kellerman *et al.*, 1988). After several years of investigating the components of the carcinogenic fraction of bracken,

the first of these, named ptaquiloside, was finally isolated simultaneously in Japan and The Netherlands (Niwa *et al.*, 1983 and Van der Hoeven *et al.*, 1983). Bracken is most often implicated as a cause of haemorrhagic tendencies and bone marrow suppression in cattle.

Ptaquiloside destroys bone marrow, therefore it reduces: (i) the production of blood platelets which leads to internal bleeding; and (ii) white blood cells, which makes the animal susceptible to infections (Vahrmeijer, 1981). It is possible to reproduce the following: (i) acute bracken poisoning in calves; (ii) induce papillary, anaplastic and adenocarcinomas in mammary glands and ileum of Sprague-Dawley rats; (iii) induce mutations in *Drosophila melanogaster* and (iv) chromatid exchange-type chromosomal aberrations in hamster lung fibroblast cells (Hirono *et al.,* 1984a, 1984b; Matsuoka *et al.*, 1989 and Sato *et al.*, 1991). The consumption of bracken fern as food is associated with a high incidence of cancer in humans and animals (Almeida Santos *et al.*, 2006).

The cytogenetic effects of bracken fern extract (hexane extract-HE, ethanol extract-EE, hot water extract and cold water extract-CWE) on the chromosomes of the peritoneal and bone-marrow cells of Swiss mice were investigated by Almeida Santos *et al.* (2006). The peritoneal cells were found to be susceptible to structural (clastogenic) chromosome aberrations with all four treatments and numerical (aneugenic) chromosome aberrations with the EE treatment. On the other hand, bone marrow cells showed only structural chromosome aberrations with HE and CWE treatments. Bracken fern extracts can be clastogenic or aneugenic, depending on the tissue cell assay, and it might be speculated that bracken fern induced cancer can be modulated by chromosome aberrations (Almeida Santos *et al.,* 2006).

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1.7.1.2 Human carcinogenicity

In Japan, the crosiers (young bracken leaves with coiled hook-shaped tips) are eaten with soda ash (sodium bicarbonate) to remove the bitterness caused by the high tannin content and the presence of a cyanogenic glucoside, prunasin, in considerable quantity. The culinary name for this dish is Warabi; the local industrial production is from the crosiers of the toxic *latiusculum* variety, which may contain13 to 169kg of pure ptaquiloside per 13000 metric tons of crosiers (Alonso-Amelot and Avedano, 2002). Although bracken ptaquiloside is sensitive to heat and alkali, it was possible to show that after such preparation, the crosiers from this treatment still retained part of its carcinogenic potential in rats (Hirono *et al.*, 1972). A case-control study of upper alimentary tract cancer, comprising of 98 cases and 480 age-matched controls, revealed that the daily consumption of Warabi was associated with an almost threefold greater risk of developing oesophageal cancer (Kamon and Hiryama, 1975 and Hirayama, 1979).

In some regions of Brazil *P. aquilinum* is consumed as “broto da Samambaia” where it is prepared by boiling the crosiers three to four times without alkali to remove astringency. In one locality called

Ouro Preto in Minas Gerais, a case-control study was undertaken in view of the high incidence of gastric and oesophageal cancers in people living in the area (Marliere *et al.*, 1998, 2000). Examination by endoscopy of eaters and non-eaters of bracken (86 cases) indicated a risk for the development of upper alimentary tract neoplasias in those who consumed bracken. Furthermore, a weak but significant cancer protecting activity of bracken crosiers was found when eaten with a frequency of one meal per month or less. However such an association would need to be confirmed in a larger study, with greater power. Nevertheless, the correlations obtained thus far are sufficiently strong to associate direct bracken consumption by people to the onset of cancer of the upper alimentary tract (Alonso-Amelot and Avendano, 2002).

1.7.1.3 Structure of ptaquiloside, key metabolites and related carcinogenicity

Ptaquiloside has the structure of an illudane with a glucose moiety attached at carbon C4. The younger and softer the frond (crosier) the greater the content of this illudane (Fig 3. compound 1). Ptaquiloside is unstable

(i) when separated from the plant
(ii) under mild base or acid conditions
(iii) in acidic pH, ptaquiloside is converted to the harmless pterosin B (Fig 3. compound 4a).

Under conditions (i) and (ii) above, ptaquiloside (Fig 3. compound 1) converts with the loss of the glucose moiety into pteridienone, also referred to as illudane-dienone or dienone (Fig 3. compound 2) or to ptaquiloside's aglycone ptaquilosin (Fig 3. compound 3) (Kigoshi *et al.*, 1989, 1993 and Padwa *et al.*, 1994). Ptaquilosin is also unstable under the same conditions (i and ii) and converts into the same dienone, hence suggesting that other driving forces must be contributing to this transformation (Alonso-Amelot and Avendano, 2002).

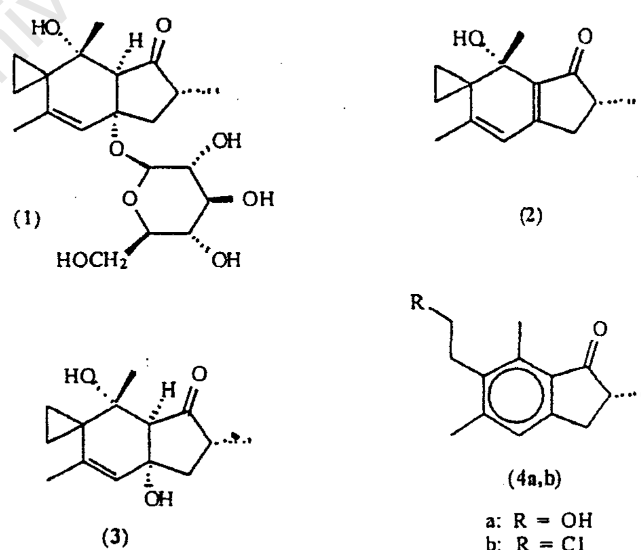

Fig 3. Structures of the carcinogenic component of bracken, key metabolites and a synthetic analogue: (1) ptaquiloside; (2) key metabolite is dienone commonly referred to as illudane-dienone or pteridienone; (3) synthetic analogue ptaquilosin (4a) is pterosin B and (4b) is pterosin F, which is possibly an artefact and not a natural bracken pterosin (Alonso-Amelot and Avendano, 2002)

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Pteridienone has a more pronounced activity over ptaquiloside, which is in line with its increased alkylating capacity (Matsuoka *et al.*, 1989).

Ptaquiloside is designed to cross both lipid and hydrophilic barriers, as its aglycone portion enables its solubility in chloroform, methylene chloride, acetonitrile and methanol, whereas the glucose fragment facilitates its dilution in water. It is therefore conceivable that ptaquiloside can penetrate deeply into tissue and cells to reach the endoplasmic reticulum and nuclear DNA. This has led to the development of experiments to detect *in vivo* DNA-ptaquiloside alkylation adducts in the intestine and urinary bladder of ruminants.

A P1-nuclease enhanced ^{32}P-postlabelling assay revealed that ptaquiloside-DNA alkylation occurred only in specific organs, such as the ileum and urinary bladder, precisely the organs more prone to bracken-induced carcinogenesis in cattle (Reddy and Randerath, 1987 and Alonso-Amelot and Avendano, 2002). The contents of the ileum and bladder are alkaline and provide ideal conditions for the transformation of ptaquiloside reaching the area into the strongly alkylating pteridienone. The ptaquiloside induction of carcinogenesis in ruminants occurs in organ-provided conditions (alkaline) for dienone formation followed by early *H-ras* mutations, and the true carcinogen is pteridienone rather than ptaquiloside.

The *H-ras* gene in the target organ has shown an adenine to pyrimidine mutation on codon 61 (N3 position) and guanines have been alkylated at N7 but at a reduced rate. Intravenous administration of pteridienone in rats induced tumours and a ^{32}P postlabelling assay indicated that the ileum of all treated rats contained DNA adducts (Shahin *et al.*, 1998). The genotoxicity and onset of cancer by *P. aquilinum* is illustrated in Fig 4. Depurination and DNA fragmentation is a destructive consequence of ptaquiloside assault that DNA synthetases find difficult to repair (Alonso-Amelot and Avendano, 2002).

Once the structure of ptaquiloside became known, a reliable and sufficiently sensitive analytical procedure for its chromatographic detection became available (Agnew and Lauren, 1991). The HPLC chromatographic method was used to quantify the carcinogen in milk from cows feeding on bracken. Since the illudane is prone to decomposition by moderate heat, base or acid, the detection of ptaquiloside in milk from cows fed bracken fronds was accomplished by forcing the decomposition of ptaquiloside in milk to pterosin B, by treating with dilute alkali followed by mild acid and then analysing for pterosin B. The pasteurisation of milk may reduce the concentration of this illudane to harmless levels so milk reaching the general public is likely to be much safer cancer-wise.

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In summary, the three possible routes of entry for carcinogens into the human body are through the consumption of milk and plant material and the inhalation of spores (powdery brown mass on the lower side of leaves). If bracken does contribute to the incidence of cancer it should be associated chiefly with two organs: (i) the respiratory organs, causing respiratory neoplasias resulting from the inhalation of spores that are windborne in large masses; and (ii) the organs of the upper alimentary tract in which swallowed spore carcinogens and bracken frond illudanes would have a higher chance of forming adducts with epithelium cells before being destroyed by acidic and/or alkaline media along the mid-digestive tract (Alonso-Amelot and Avendano, 2002). Bracken fern is important in this study because there is overwhelming evidence from *in vitro, in vivo* and epidemiological studies spanning three decades which shows a risk for the development of cancer of the upper alimentary tract from the ingestion of this plant.

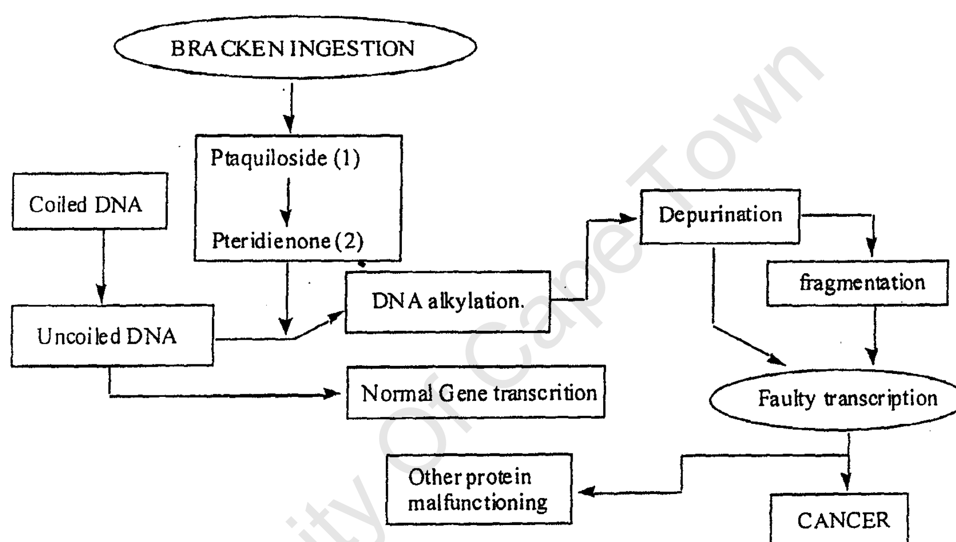

Fig 4. Flowchart of genotoxicity and onset of cancer by *P. aquilinum* illudanes (Alonso-Amelot and Avendano, 2002)

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1.7.2 *Zantedeschia aethiopica*

Z. aethiopica belongs to the Family: Araceae. It is exceptionally common and occurs over a large part of South Africa, predominantly in wetlands. The leaves are used to cover wounds and the flowers are frequently used for ornamental purposes (van Wyk *et al.*, 1997). The leaves of *Z. aethiopica* have a unique mannose-binding lectin with the ability to bind to cell membranes that are often toxic and occur mainly in seeds (van Wyk *et al.*, 1997 and Chen *et al.,* 2004). Mannose-specific lectins are widely distributed in higher plants and are believed to play a role in the recognition of high-mannose type glycans of foreign microorganisms or plant predators (Barre *et al.*, 2001). Catalase (E.C 1.11.1.6) has been purified from the leaves of *Z. Aethiopica*. This is a common enzyme found in nearly all living organisms and, in the textile industry, it is applied to fabric that has undergone bleaching with hydrogen peroxide for the elimination of hydrogen peroxide residues (Trindade *et al.,*1988 and Amorim *et al.*, 2002).

1.7.2.1 Active ingredients and pharmacological effects

The active principle varies from species to species but always includes calcium oxalate. The harmful effects of oxalate produced upon ingestion depend on (i) concentration and (ii) whether oxalate is present in a soluble or insoluble form. Low to moderate levels of soluble oxalate are less toxic, however at high levels in the plant, the soluble oxalate is very harmful when eaten since it is readily absorbed from the digestive tract, reacts with blood calcium, causes ionic imbalance and decreases the coagulatory properties of the blood leading to internal haemorrhage, nephritis and renal failure (owing to a rapid accumulation of insoluble calcium oxalate crystals in the kidney tubules) and eventual death (Blackwell, 1990 and Lewis, 1998). When plants containing the insoluble form of oxalate are consumed, the crystals may become lodged in the mucous lining of the mouth and throat causing burning, irritation and swelling with the symptoms disappearing within a few days (Blackwell, 1990). All parts of *Z. aethiopica* are toxic and can cause severe irritation of the mucous membranes, with swelling of the tongue and throat, salivation, nausea, vomiting and diarrhoea (Lewis, 1998; van Wyk *et al.*, 2002).

Fig 5. *Z. aethiopica* is commonly referred to as the arum lily and is found from the Western Cape through the Eastern Cape, KwaZulu-Natal, Mpumalanga and into the Northern Province. The flowers are large and are produced in spring, summer and autumn, with a pure white spathe and a yellow spadix, and are often used for ornamental purposes (Van Wyk *et al.*, 1997).

1.7.3 *Acokanthera oppositifolia*

A. oppositifolia is a member of the family, Apocynaceae, which is well-known for its alkaloidal content, indole alkaloids derived from tryptamine and steroidal alkaloid (C_{21} alkaloids) derived from pregnane, as well as for their potent pharmacological activity (Van Wyk *et al.*, 2002 and Gurib-Fakim, 2006).

A. oppositifolia is well known throughout Africa as a traditional source of extremely toxic arrow and spear poisons (van Wyk and Wink, 2004). Secondary poisoning may arise from the consumption of

meat from an animal that has died from cardiac glycoside poisoning. The poison obtained from roots, leaves or wood can lead to death in humans within 15 minutes (van Wyk *et al.*, 2002).

Fig 6. *A. oppositifolia* is a medium to large woody shrub with attractive hardy dark green leaves. Clusters of pinkish white, sweetly scented flowers (B) are borne in late winter and spring and are followed by large plum-coloured berry-like fruits (A) which are relished by birds (van Wyk *et al.*, 2002).

The leaves of *A. oppositifolia* are used in the form of snuff to treat headaches and in infusions for abdominal pain, convulsions (van Wyk and Gericke, 2000) and snakebite (Watt and Breyer-Brandwijk, 1962). *A. oblongifolia (Hochst.)* Codd, a different but related species, prepared by sequential extraction with dichloromethane followed by 90% methanol induced DNA damage in human white blood cells using the alkaline comet assay (Taylor *et al.*, 2003).

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1.7.3.1 Active ingredients and pharmacological effects

A. oppositifolia are characteristic for their cardiac glycosides, the plants indigenous to South Africa have the following chemical composition:
(i) seeds: the major component is water-insoluble acovenoside A (Fig 7.), two polar water-soluble glycosides, acolongifloroside K and acovenoside C, and trace amounts of ouabain (or absent).
(ii) stems and twigs: large amounts of acovenoside A and acolongifloriside K (Neuwinger, 1996).
The amount of the minor component, ouabain (Fig 7.) obtained from *Acokanthera* sp, varies in different parts of Africa: trace amounts of ouabain (or absent) from *A. oppositifolia* in South Africa and Kenya/Nairobi, whereas a higher content of ouabain is present in *A. schimperi* from Kenya/Nairobi (Neuwinger, 1996).

In a study carried out by Wangenheim and Bolcsfoldi (1988), ouabain was positive for mutagenicity in a mouse lymphoma L5178 thymidine kinase assay in the absence of metabolic activation at a dose of 0.103-8.22mmol/l; with 2-4 fold increase in the mutation frequency. From their study, potent mutagenic/carcinogenic compounds tested had greater than 4-fold increases in the mutation frequency, whilst weak carcinogens or compounds not known to be carcinogenic that was positive in the assay gave increases of between 2- and 4- fold. In addition, a less than 4-fold

increase in the mutation frequency was associated with a lower predictivity for carcinogenicity. A high *in vitro* cytotoxic activity was obtained for acovenoside A and acolongifloroside K, but no *in vivo* activity was obtained for these compounds (Kingsten and Reichstein, 1974).

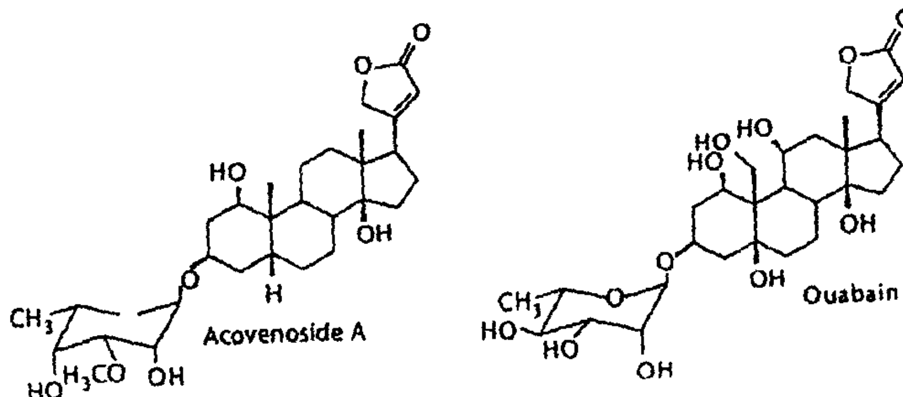

Fig 7. Molecular structures of acovenoside A and ouabain (van Wyk *et al.*, 2002)

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Ouabain has pharmaceutical significance as a cardiac glycoside for the treatment of heart ailments and is administered intravenously because it is very poorly absorbed in the digestive tract (van Wyk and Wink, 2004). Cardiac glycosides can be divided into two classes, cardenolides and bufadienolides, the former being extremely toxic with doses being carefully adapted to individual needs by health care professionals. The plant extract is lethal because the concentration and dosage of the active principles cannot be controlled.

1.7.4 ***Rumex lanceolatus***

Fig 8. *R. lanceolatus* is a weed found in seasonally wet places and occurs in many parts of southern Africa (van Wyk *et al.*, 1997)

Rumex sp (Family: Polygonaceae) occurs widely in southern Africa and the leaves are popular as a dietary supplement and are served as "sorrel" or cooked with porridge. Both *R. lanceolatus* and *R. crispus* are equally popular as medicinal plants (van Wyk *et al.*, 1997).Infusions and decoctions of this plant are traditionally used to treat constipation, liver problems and arthritis, with the root being used as a remedy for internal parasites (tapeworm and roundworm). It is also regarded as a cleansing herb and used to treat chronic skin problems. The whole plant is said to be widely employed for vascular disorders and internal bleeding (van Wyk and Wink, 2004). Externally it is applied to ulcers, boils and tumours.

1.7.4.1 Active ingredients and pharmacological effects

Many *Rumex* sp contain anthraquinones, with the exception of one species having been found to date, namely *R. induratus* (Ferreres *et al.*, 2006). *R. lanceolatus* is a mild purgative containing chrysophanic acid, emodin (Fig 9.) and a small amount of volatile oil; neither alkaloid nor glucoside are present (Watt and Breyer-Brandwijk, 1962). Roots of *Rumex* sp contain glycosides of emodin and chrysophanol (such as chrysophanein, Fig 9.) and tannins (van Wyk and Wink, 2004). The laxative effect of the roots of *Rumex* sp is due to chrysophanol and related anthracene glycosides.

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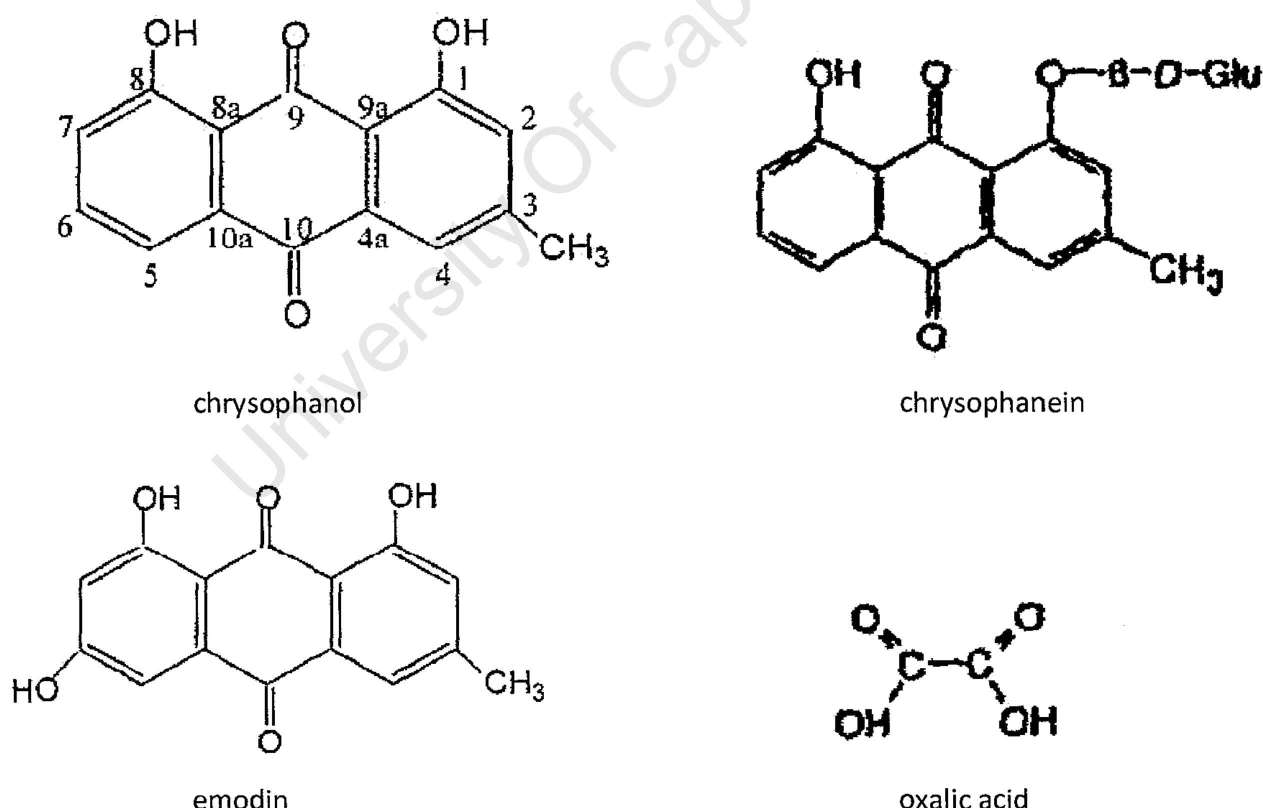

Fig 9. Molecular structures of chrysophanol, chrysophanein, emodin and oxalic acid (van Wyk *et al.*, 1997 and Lee *et al.*, 2005)

Oxalic acid (Fig 9.) is typical of *Rumex* sp and the leaves contain large amounts of it (van Wyk *et al.*, 1997 and van Wyk and Wink, 2004). Oxalic acid is toxic in high concentrations, but is used

medicinally as a haemostatic agent in the form of a 5% solution in 5% malonic acid (van Wyk and Wink, 2004).

Rumex species are related to the well-known medicinal plants known as rhubarbs, which are a group of plants that belong to the genus *Rheum* in the family Polygonaceae (van Wyk and Wink, 2004). Several monomeric anthraquinones, such as emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) and related derivatives, are constituents of rhubarb (an oriental medicine for diarrhoea) that exhibit mutagenicity in the *Salmonella*/microsomes test and cultured tumour cells (Morita *et al*., 1988 and Ueno, 1991). However, emodin from the aerial parts of *R. acetosa* strongly inhibits the proliferation of tumour cell lines, and shows antimutagenicity (in strains TA 98 and TA100) and antigenotoxic activity (Lee *et al*., 2005).The OH group at the C-6 position in the anthraquinone nucleus may play an important role for their cytotoxicities, and an introduction of OH^- or OCH_3 group at C-6 position is necessary for their antimutagenic activities (Lee *et al*., 2005).

The rhizome of *R. crispus L.* contains volatile oil, resin, tannin, rumicin, sulphur, starch, various other salts, lapathin, oxymethylanthraquinone, emodin and chrysophanic acid (Watt and Breyer-Brandwijk, 1962). The tannin content of the rhizome is so high that the plant was at one time considered a possible commercial source of tannin. The phytochemical screening of the powdered root of *Rumex steudlii* Hochst shows the presence of polyphenols, pytosterols, O-anthraquinone glycoside, tannins, hydrolysable tannins and saponins, but the test for alkaloids shows a negative result (Gebrie *et al*., 2004).

1.7.5 *Pelargonium* sp.

Pelargonium sp (Family: Geraniaceae) is used for its commercial and medicinal value. Rose-scented geranium (*Pelargonium* species) is a high-value, aromatic plant cultivated for its essential oil, which is widely used in the fragrance industry, in aromatherapy and for the extraction of commercial rhodinol (mixture of linalool, citronellol and geraniol) (Rao *et al*., 2002).

Fig 10. Leaves of *Pelargonium sp. cf. inquinans (L.) L' Herit. Pelargonium* sp. are cultivated for their medicinal and commercial value

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The essential oil is obtained using steam or water as well as steam distillation of shoot biomass (Rao *et al.*, 2002). Root extracts are used in preparations to treat acute and chronic infections of the nose, ears and chest, and have been found to be particularly effective for bronchitis in children and as a supportive treatment for tuberculosis and chronic bronchitis (van Wyk and Wink, 2004). The leaves and roots are also used for the treatment of dysentery and syphilis. Isoquercetrin and rutin, respectively the major and minor flavonoids obtained from *Pelargonium radula (Cav.) L'Herit,* have been shown to have antimicrobial activity (Pepeljnjak *et al.*, 2005).

1.7.5.1 Active ingredients and pharmacological effects

The main ingredients of *Pelargonium* sp. are coumarins (Fig 11.); mainly 7-hydroxy-5,6-dimethoxycoumarin, that co-occur with at least seven other coumarins, gallic acid derivatives (including gallic acid and gallic acid methyl ester), oligomeric proanthocyanidins, and flavonoids: flavan-3-ols (e.g. catechin) and flavon-3-ol (e.g. quercetin) (Middleton *et al.*, 2000 and van Wyk and Wink, 2004).

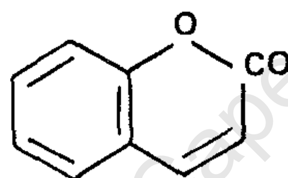

Fig 11. Chemical structure of coumarin (Culvenor and Jago, 1979)

Pelargonium oils contain a variety of monoterpenoids, such as geraniol, (+)-isomethone, citronellol and phenylethyl alcohol (responsible for the rose smell). The gallic acid derivatives and other phenolic compounds in the roots have powerful antibacterial and antiviral activity, and together with coumarins provide a rationale for the proven immunomodulatory activity.

The five South African (SA) plants discussed above have not been previously evaluated for mutagenicity. In countries where *P. aquilinum* has been consumed as a food, in-depth scientific investigations have concluded that ptaquiloside and its metabolically activated intermediate, pteridienone, play a role in the carcinogenic process. It is inevitable that mutagenicity and clastogenicity will be anticipated for the SA *P. aquilinum*, which can be evaluated with the selected *in vitro* mutagenicity tests.

1.8 *In vitro* mutagenicity assays

1.8.1 *Salmonella* reverse mutation assay

The *Salmonella* reverse mutation assay is an initial screen to determine the mutagenic potential of new chemicals and drugs, as there is a high predictive value for rodent carcinogenicity when a

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mutagenic response is obtained. In the *Salmonella* reverse mutation assay, the test substance is added to the top agar (minimal medium) containing S9, a bacterial tester strain and a minimal amount of histidine to support a few cycles of replication, but not enough to permit colonies to form. The molten top agar is poured onto solid media and incubated (48hrs/37°C). In the presence of the mutagen, the strain reverts to wild type (his^{+}) thereby allowing colonies to form on the minimal medium. The number of colonies can be related to controls and to the amount of the test substance used to give a quantitative result. A twofold rule usually applies when the number of colonies of the test chemical is twice the amount on the control plate and the test chemical is mutagenic.

Bacterial strains were originally derived from *Salmonella typhimurium* LT2. The bacteria carry a mutation (his^{-}) that makes them nutritionally deficient because they cannot synthesise one of the enzymes needed to manufacture histidine, which is supplemented in the top agar. The genotypes and related characteristics of the primary tester strains are indicated in Table 3. The *Salmonella* strains used in the test have different mutations in various genes of the histidine operon; each of these mutations is designed to be responsive to mutagens that act via different mechanisms (Mortelmans and Zeiger, 2000). TA97a and TA98 detect frameshift mutagens, whilst TA100 detects mutagens that cause base-pair substitutions (Maron and Ames, 1983). TA102 detects mutagens that cause oxidative DNA damage, and being a DNA repair proficient strain, detects cross-linking agents such as bleomycin and mitomycin C (Mortelmans and Zeiger, 2000).

Table 3: Genotypes for the primary tester strains used in the *Salmonella* reverse mutation assay

Histidine Mutation				**LPS**	**Repair**	***R-factor plasmid pKM101**
hisD6610	***hisD3052***	***hisG46***	***hisG428* **(pAQ1)**			
Strain TA97a	Strain TA98	Strain TA100		*rfa* deletion	*uvrB* deletion	+R
			Strain TA102	*rfa* deletion	*uvrB* intact	+R
Reversion event: Frameshift	Reversion event: Frameshift	Reversion event: Base-pair substitution	Reversion event: Transitions & transversions			

(*R-factor plasmid pKM101 confers ampicillin resistance, **pAQ1 confers tetracycline resistance. LPS: lipopolysaccharide layer prevalent in gram negative bacteria, *rfa* deletion makes the cell wall more permeable to the penetration of chemicals). Source: adapted from Maron and Ames (1983) and Mortelmans and Zeiger (2000)

A chemical can be scrutinised for its ability to achieve one or more kinds of genetic alteration, resulting in reversion from his^{-} to his^{+}, and is taken as a measure of an agent's mutagenic and carcinogenic potential. Since many substances are unable to pass through bacterial cell walls, the strains used carry a mutation that makes the wall more penetrable to chemicals (*rfa*). The strains TA97a, TA98 and TA100 also carry another mutation that causes a defect in the DNA repair mechanism (*uvrB*). Therefore bacterial strains (with the exception of TA102) are repair

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incompetent, and permit the easier detection of various kinds of alteration in the DNA, since a defect will not be repaired.

1.8.2 Chromosomal aberration test

The effects of plant extracts on chromosome structure (clastogenicity) were evaluated in peripheral blood lymphocyte cultures with and without S9. DNA damage is assessed from metaphase-arrested cells (Fig 12.), therefore chemicals that inhibit mitosis or are indirect mutagens via non-DNA targets such as the spindle inhibitors cannot be analysed with the chromosomal aberration test. A structural chromosomal aberration comprises two types: chromosome aberration (CSA) when both chromatids are damaged at the same loci and chromatid aberrations (CTA) when one chromatid is affected. The CSA and CTA types arise from cell-cycle progression (G_1, S, G_2 and M) and in relation to S-phase independent or dependent clastogens.

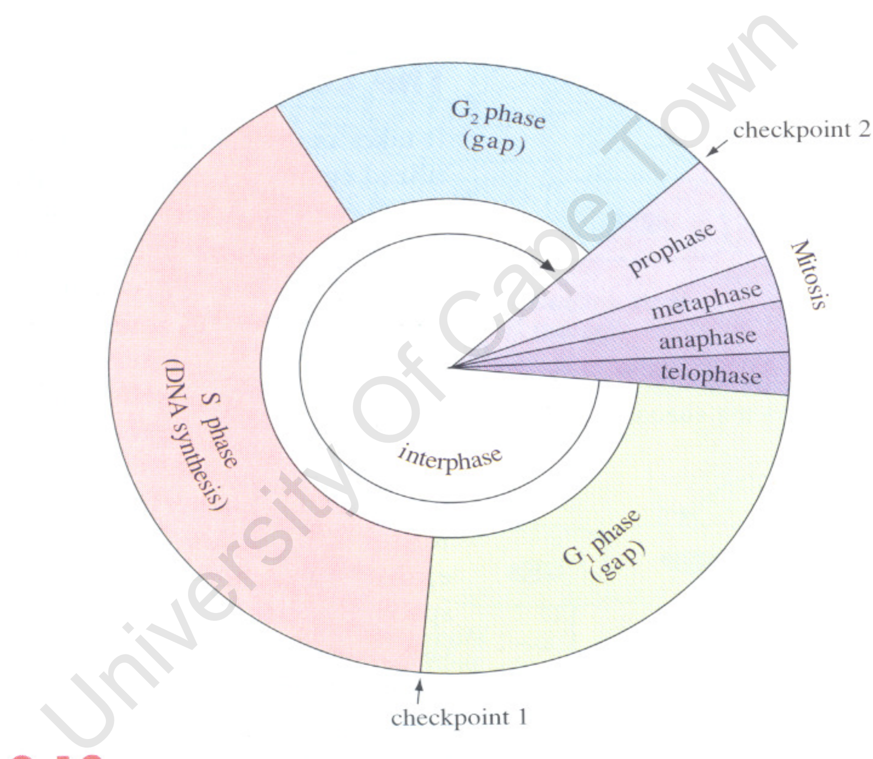

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Fig 12. The cell cycle is divided into the interphase, when cellular components are replicated, and mitosis, when the cell splits into two, distributing its contents into two progeny cells. The interphase is divided into two gap phases (G_1 and G_2) when the cell duplicates specific molecules and structures, and a synthesis phase (S) when it replicates its DNA. Checkpoints ensure that the cycle proceeds correctly. The first checkpoint, between the G_1 and S phases, monitors signals from inside and outside the cell to determine whether conditions are right for DNA duplication. The second checkpoint, just before mitosis, ensures that the chromosomes are completely replicated and that errors in the new DNA sequences are repaired (Lewis, 1997).

The mechanisms for the formation of CSAs and CTAs viewed at metaphase are cited in various publications (Savage 1975, Albertini *et al.*, 2000, Savage 2004, Hagmar *et al.*, 2004 and Mateuca *et al.*, 2006) and are summarised below:

(i) CSAs result from incompletely repaired or unrepaired double-strand breaks (DSBs) mostly generated *in vivo* in G_o–G_1 lymphocytes by the S-phase independent clastogens

(e.g. ionising radiation). After DNA synthesis and chromosome duplication at S-phase, the aberrations formed in G_o–G_1 are seen as chromosome-type breaks in the metaphase.

(ii) CTAs (e.g. chromatid-type breaks) arise predominantly *in vitro* during the G_2-phase of the cultured lymphocytes in response to base modifications and single-strand breaks (SSBs) induced by S-phase-dependent clastogens (e.g. chemicals).

The extensive use of the chromosomal aberration assay over the last 30 years has resulted in the accumulation of analytical data in many European laboratories and has enabled the examination of the potential association between previously measured structural chromosomal aberration frequency and subsequent cancer outcome (Bonassi and Au, 2002). The impact of CSAs and CTAs from smoking and other environmental carcinogens on human cancer risk has been recently assessed, because originally the study did not have sufficient power and/or follow-up time for a conclusive result with respect to cancer predictivity. In the Taiwanese and Nordic cohorts, CSAs but not CTAs predict cancer risk; however, in the Italian cohort no clear-cut difference in cancer predictivity between CSA and CTA biomarkers was observed (Liou *et al*., 1999 and Hagmar *et al*., 2004). Evidence from a recent study conducted by Norppa *et al.* (2006) indicates that both chromatid-type and chromosome-type aberrations predict cancer; even though some data suggest that chromosome-type aberrations may have a more pronounced predictive value than chromatid-type aberrations.

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<u>1.8.2.1 The distinction between gap junction and chromosome breaks</u>

Light microscopy is used to detect structural chromosomal aberrations (gaps and breaks) from metaphase-arrested lymphocytes.

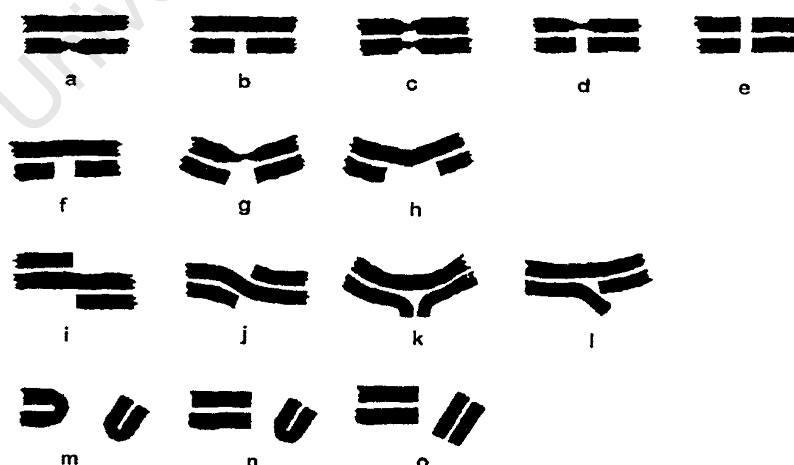

Fig 13. Structural chromosome aberrations by light microscopy of metaphase arrested cells: (a) chromatid constriction; (b) chromatid gap; (c) chromosome constriction; (d) constriction/gap; (e) chromosome gap; (f–h) examples of aligned chromatid breaks; (i–l) examples of dislocated chromatid breaks; (m) chromosome break with complete sister-union (SU); (n) chromosome break with incomplete sister-union (Nup or Nud); (o) chromosome break with no sister-union (Nupd), or a chromosome-type terminal deletion (Savage, 2004)

The distinguishing features of gaps and breaks applied to the scoring of metaphase cells was according to Savage (2004). The following features are characteristic of a *break*: (i) the non-staining region is larger than the width of a chromatid; (ii) the distance between the broken "ends" of a chromatid has no visible connection (Fig 13. f–h); and (iii) there is non-alignment, or dislocation, of the two segments (examples Fig 13. i–l). An aberration is referred to as a *gap* when the non-staining region is smaller than the width of a chromatid and little chromatin may be evident. The visual non-staining region of a gap does not imply that chromatin is absent; in such instances, chromatin is seen in the non-staining region under the electron microscope.

1.9 Overview of the study

Traditional plants or extracts consumed for their medicinal or dietary value are assumed to be safe because they occur naturally. This study looked at the potential mutagenicity of five commonly consumed traditional plants, as there is a huge body of evidence that disproves the theory that natural equates to safe. In addition, there is a high incidence of late-onset oesophageal cancer among the population of the Eastern Cape Province of South Africa where traditional plants/extracts are more frequently consumed. The rationale and justification for this research are that the five traditional plants in this study are part of the diet in this population; there is no prior research in this field with the exception of a South African epidemiological study (Sewram, 2006) which supplements this dissertation.

The research problem (hypothesis) is to identify those traditional plant extracts that damage DNA (mutagenic agents) and chromosomes (clastogenic agents) using *in-vitro* quantitative test methods. The five traditional plants investigated were *Acokanthera oppositifolia (Lam.)* Codd; *Pelargonium cf. inquinans (L.)* L' Herit; *Pteridium aquilinum subsp aquilinum; Rumex lanceolatus Thunb.* and *Zantedeschia aethiopica (L.)*. This is a screening study of the potential mutagenicity of aqueous extracts and not a phytochemical study. Simplified versions of two of the three *in vitro* genotoxicity screening assays currently recommended by the International Conferences on Harmonisation of Technical requirements for registration of Pharmaceuticals for Human Use (ICH Guidance S2B): (i) a bacterial mutation screening test (*Salmonella* reverse mutation assay) and (ii) a chromosomal aberration screening test in Chinese hamster ovary (CHO) cells (Zhang *et al.*, 2004) were selected, however in the present study peripheral blood lymphocytes were used instead of CHO cells. The tests were done with and without metabolic activation (S9 mix).

Limitations of the *Salmonella* reverse mutation assay include that it cannot detect the mutagenicity of large molecules that cannot pass through the cell wall of the tester strain and it also fails to detect those carcinogens (e.g. Fumonisin B_1) that are not mutagenic. The chromosomal aberration test was performed on peripheral blood lymphocytes, the limitations include interference from red blood cells, and the small size and low concentration of lymphocytes in the blood relative to

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monocytes and granulocytes, which resulted in very few metaphase cells for chromosomal analysis. The lymphocytes were stimulated to enter the cell cycle (G1, S, G2 and M) using phytohaemagglutinin followed by metaphase arrest using colcemid. The test was not suitable for extracts that inhibited the cell cycle, which was an additional limitation. Following Savage (2004), approximately 50 metaphase cells were scored for chromosomal aberrations (gaps and breaks).

The data were collected by measuring the two endpoints (mutagenicity and clastogenicity) at increasing concentrations of aqueous plant extracts with and without metabolic activation (refer to section 1.3). In the *Salmonella* reverse mutation assay, a reversion to wild type with an associated three- to fivefold increase in the number of revertant colonies indicated mutagenicity. Four *Salmonella* tester strains (TA97a, TA98, TA100 and TA102) were selected, each indicative of a particular kind of mutation: base-pair substitution, frameshift or oxidative DNA damage. The baseline revertant colonies for TA102 were the highest (fivefold) relative to the other three tester strains. Batch TA102 complied with the supplier's release criteria; the baseline reversion frequency had increased over the years when compared to that of the original tester strains (email correspondence with the supplier, Appendix C). The mutagenicity data were analysed by a professional statistician using gamma statistics, whilst the clastogenicity data were not statistically analysed owing to the low number of metaphase cells. In light of the evidence provided the mutagenic potential of five South African plants currently being used in traditional medicine and/or as dietary supplements were investigated.

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CHAPTER 2
MATERIALS AND METHOD

2.1 Collection of plants

The plants *Acokanthera oppositifolia (*Lam.*); Pelargonium sp. cf. inquinans (L.) L' Herit*; *Pteridium aquilinum subsp aquilinum; Rumex lanceolatus Thunb.* and *Zantedeschia aethiopica (*L.*)* Sg. were obtained from Port St Johns in the Eastern Cape, and the Silverglen medicinal plant nursery in KwaZulu-Natal during November 2002. The identities of the plants were authenticated by comparison with reference specimens at the Kei Herbarium (Walter Sisulu University) and Natal Herbarium (National Botanical Institute, KwaZulu-Natal), and voucher specimens (see Table 5) were deposited in these herbariums for future reference. The leaves of all plants were air-dried, crushed by hand or cut with an Anvil pruner before they were milled into a fine powder using a Kenwood blender. A facemask was used to avoid inhalation of the dust during the handling and milling of plant material. The powdered material was weighed into brown paper bags, labelled and stored at room temperature.

2.2 Preparation of plant extracts

Milled plant material (30g) was weighed into a conical flask (1L), and then boiled in water (500ml). The plant material was agitated using a magnetic stirrer and boiled for 1hr. The plant extracts were cooled to room temperature and filtered twice using a Rundfilter MN617 filter paper. The filtrate was transferred to a round bottom flask (1L) and refrigerated at -20°C overnight. The frozen filtrate was thereafter lyophilised and stored at 4 to 5°C in a dessicator; the mass of lyophilisate obtained is recorded in Table 4.

Table 4. Plant processing and yield of freeze-dried extract (lyophilisate)

Botanical name	Part of plant dried and milled	Extraction 75–85°C/1hr	Mass of lyophilised product	%Yield
Pteridium aquilinum subsp.aquilinum	Leaves	30g/500ml	4.33g	14.43
*Acokanthera oppositifolia (*Lam*).*	Leaves & stems	30g/500ml	5.27g	17.57
Pelargonium L. Herit	Leaves & stems	30g/500ml	4.18g	13.93
Rumex lanceolatus Thunb.	Leaves & stems	30g/500ml	4.76g	15.87
*Zantedeschia aethiopica (*L.*) Sg*	Leaves & stems	30g/500ml	5.29g	17.63

The freeze-dried material (0.8g) was dissolved in sterile distilled water (10ml), sonicated for 20min, centrifuged at 4000rpm for 10min at 0°C and the supernatant decanted into a 25ml beaker. The supernatant was transferred into a scintered glass filtration apparatus containing a 5-micron cellulose filter. The filtrate was re-filtered through a 1.2-micron filter. The solution was finally filter sterilised through a 0.2µm syringe filter into a sterile bijou bottle from which dilutions were made using sterile water by aseptic handling to avoid test sample contamination. The following dilutions were prepared: (i) 0.005; 0.01; 0.02 and 0.04g/ml for *A. oppositifolia*, *Pelargonium*, *P. aquilinum*

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Table 5: Tabulation of the plant family, vernacular name, location, uses and voucher number

Plant	Family	Vernacular name [1]	Location	Uses	Ref	Voucher number	Collector	Herbarium
Acokanthera oppositifolia (Lam.)	Apocynaceae	iNxinene (X), inhlunguyembe (Z)	Silverglen medicinal plant nursery	Treatment of headache, epilepsy, amnesia, eye disease, syphilis, scabies, leprosy, Tinea capitis, wound, eczema, warts and swelling	Tadeg *et al* (2005);	130277	V. Sewram	South African National Botanical Institute - Natal
Pelargonium sp. cf. inquinans (L.) L' Herit	Geraniaceae	iNdlebe ye-bokwe (X), amanzemnyama, ishwaga (Z)	Silverglen medicinal plant nursery	Leaves used as an antispasmodic and a colic and diarrhoea remedy. Used for wounds	Watt and Breyer-Brandwijk (1962)[2]	130278	V. Sewram	South African National Botanical Institute - Natal
Pteridium aquilinum subsp. aquilinum	Dennstaedtiaceae		Port St Johns	Food	Watt and Breyer-Brandwijk (1962)[2]; Almeida Santos *et al*. (2006)	PA01VS	V. Sewram	Kei Herbarium, Department of Botany, Walter Sisulu University
Rumex lanceolatus Thunb.	Polygonaceae	Idolo lenkonyana (X)	Port St Johns	Treatment of wounds, sterility, tapeworm, roundworm and as a purgative. The rhizome and leaf are used as decoctions	Watt and Breyer-Brandwijk (1962)[2]	RL01VS	V. Sewram	Kei Herbarium, Department of Botany, Walter Sisulu University
Zantedeschia aethiopica (L.) Sg	Araceae	Intebe (X, Z)	Port St Johns	Treatment of sores, boils and insect bites	Watt and Breyer-Brandwijk (1962)[2]	ZA01VS	V. Sewram	Kei Herbarium, Department of Botany, Walter Sisulu University

Note: 1. X refers to isiXhosa and Z to isiZulu; 2. Watt and Breyer-Brandwijk (1962) is a compilation of South African traditional plant information which remains very reputable and highly acclaimed with citations in very recent publications as an invaluable source for ethnobotanical and ethnopharmacology research

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and *R. lanceolatus*, and (ii) 0.00125; 0.0025; 0.005; 0.01 and 0.02g/ml for *Z. aethiopica* as a result of the highly viscous nature of this extract.

2.3 Preparation of S9

2.3.1 Induction of rat liver enzymes

Approval for the experimental protocol was granted by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council, Tygerberg, South Africa. Fifteen rats weighing 200 to 300g were administered with Aroclor 1254, a polychlorinated biphenyl (PCB) and a known carcinogen, based on a procedure by Czygan *et al.* (1973). Aroclor 1254 was warmed in a 20°C waterbath, and 3.0g weighed into a 25ml measuring cylinder. Warm sunflower oil was added to the measuring cylinder to a volume of 15ml and homogenised by aspiration with a pipette. This yielded a 200mg/ml Aroclor 1254 working stock solution which was dispensed into two 7ml bottles fitted with rubber bungs and sealed with metal rings. The bottles were covered in foil to avoid degradation of the light-sensitive PCBs. All pipettes and glassware, including the 25ml measuring cylinder, were discarded as carcinogenic waste. The viscous Aroclor 1254 working stock was immersed in warm water during intraperitoneal injection into rats. The Aroclor was administered at 500mg/kg body weight using a 26G needle for intraperitoneal injection. This procedure was repeated for all fifteen rats.

2.3.2 Preparation of liver homogenate S9 fraction

On the seventh day after induction the rats were sacrificed by cervical dislocation and the livers removed by midline incision, according to Maron and Ames (1983). Instruments for the dissection (forceps and scissors) were sterilised by autoclaving (121°C/20min) and iodine was used as a disinfectant during excision of livers. Preparation of the liver S9 fraction is based on the procedure of Garner *et al.* (1972). All steps were carried out on ice and using sterile solutions (cold). Glassware and centrifuge tubes were autoclaved prior to use. The centrifuge tube caps were sterilised in 70% ethanol as they were not autoclavable. Frequent disinfection of surfaces during S9 preparation was attained by application of 70% aqueous ethanol.

The excised livers were washed in pre-weighed 1L beakers containing 400ml cold sterile KCl (0.15M) to remove haemoglobin, covered with foil and kept on ice. The livers (a total mass of 206.5g) were transferred into a beaker containing 3ml of chilled 0.15M KCl per gram of wet liver, and cut into smaller pieces. The liver was homogenised for 5min with a Polytron homogeniser (PT 3000, Kinematica AG). The liver homogenate was then filtered through sterile double-layered cheesecloth into a 500ml beaker to remove larger pieces of tissue while working on ice. The filtrate collected in the beaker was transferred to an all-glass dounce homogeniser, and dounced by 10 strokes of the plunger to release the microsomes. The homogenate was then centrifuged at 9000g for 10min at 4°C using a Sorvall Type SS-34 fixed angle rotor. The supernatant (S9 fraction) was

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dispensed in 3ml and 5ml aliquots into sterile bijou bottles and immediately stored at -70°C, and the pellets were discarded. The S9 was tested for sterility, protein concentration and CYP P450 activity. The absence of colonies on a nutrient broth agar plate after inoculation and incubation at 37°C for 24hrs indicated a sterile S9 preparation.

2.3.3 Protein concentration of S9

The protein concentration was determined by the Bradford method (1976). A standard curve was constructed using known amounts of bovine serum albumin (BSA). One hundred microlitres of a 1:100 S9 dilution was used for the Bradford test. The BSA standards 8, 16, 24, 32 and 40ug and the S9 sample were prepared in duplicate, and measured at 595nm with an Uvikon 923 spectrophotometer. The protein concentration of the S9 fraction was 27.1mg/ml.

2.3.4 CYP P450 concentration of S9

The CYP P450 concentration was determined by a method adapted from Omura and Sato (1964). The S9 was diluted 20x in a volume of 8ml by combining 400µl S9 with 7600µl Tris buffer (0.1M, pH7.4). The sample was saturated with a stream of carbon monoxide for 1min and placed in two cuvettes (4ml capacity) marked as reference and sample. The baseline absorbance was read at 500-400nm at a sensitivity of 0.5 and 0.1 with a double beam Beckman spectrophotometer UV5260.

Sodium dithionite (0.01298g) was added to the sample cuvette and dissolved by inversion several times. The observed bubbling, which was indicative of CYP P450, stopped before the difference spectrum was monitored (500nm and 400nm). The CYP P450 activity was determined from the 450nm absorption peak using the following formula and the mmol extinction coefficient (91) of the compound.

CYP P450 activity = ($A_{450\text{-}490}$ - Correction of baseline)/100 X sensitivity X 1000/91 X 1/mg per ml protein

= 75.5 - (-8.6) / 100 X 0.1 X 10.989 X 1/27.1 X 20 (dilution factor)

= 0.7nmol/mg

The protein concentration (27.1mg/ml) was inserted into the formula. The value for "($A_{450\text{-}490}$ - correction of baseline)" was obtained from the difference spectra chromatograph (section 3.1.1). The CYP P450 activity calculated was 0.7nmol/mg. This was within the recommended range (0.6-0.8nmol/mg), and therefore suitable for use in the mutagenicity assay.

2.4 Verification of genotypes of *Salmonella* tester strains

The verification of genotypes was done according to the method prescribed by Maron and Ames (1983) and Mortelmans and Zeiger (2000). Five microlitres of *Salmonella* tester strains: TA97a; TA98; TA100 and TA102 (Xenometrix, CA, USA) was seeded into Oxoid nutrient broth No. 2

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(4.9ml) that was supplemented with antibiotics according to the procedure in Fig 14. Genotype verification was performed for *his*⁻, *uvrB*, *rfa* and antibiotic resistance markers. (Note: all solvents and media are listed in Appendix A.)

Strains TA97a, TA98, TA100 and TA102 contain plasmid pKM101, which (i) confers ampicillin resistance that serves as a convenient marker for detecting the presence of the plasmid, and (ii) enhances chemical and UV-induced mutagenesis. In addition to pKM101, strain TA102 contains multicopy plasmid pAQ1. Plasmid pAQ1 confers tetracycline resistance and is a convenient marker for detecting the presence of the plasmid. Furthermore, the *his*G428 mutation was inserted into pAQ1 with the aim of amplifying the number of target sites.

2.4.1 Histidine dependence

An aliquot of overnight culture (0.1ml) from each tube at step 4 (Fig 14.) was plated onto MGA plates supplemented with two drops of 0.01% w/v biotin. Because all *Salmonella* strains are histidine dependent, there should be no growth on the plates after incubation at 37°C for 16 to 24hrs. Plates that display growth indicate that the colony from which the broth culture has arisen (in the masterplate) is unsuitable for the preparation of working cultures for the *Salmonella* reverse mutation assay.

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2.4.2 Biotin and histidine dependence

MGA plates were supplemented with two drops of 0.01% w/v biotin and two drops of 0.5% w/v histidine by the spread plate technique described in Fig 14, step 2. A loopful of culture from each of the ten tubes was streaked onto the MGA plates and incubated at 37°C for 16 to 24hrs. Growth was expected to be observed with all strains since they are all histidine dependent.

2.4.3 Biotin dependence

MGA plates were supplemented with two drops of 0.5% w/v histidine. A loopful of culture from each of the ten tubes was streaked onto the MGA plates and incubated at 37°C for 16 to 24hrs. There should be no growth on the plate except for TA102, which is biotin independent since it has an intact *bio* gene, unlike the other tester strains where the *uvrB* gene deletion extends through the *bio* gene making them biotin dependent.

2.4.4 *rfa* marker

Sterilised low melting top agar (100ml) was supplemented with a sterile 0.5mM histidine/biotin solution (12ml). Aliquots of top agar (2.5ml) were dispensed into ten test tubes (with spring-loaded Cap-O-Test) and kept in a 45°C waterbath. A 0.1ml inoculum of culture was added to 2.5ml molten top agar in each of the test tubes. The top agar was vortexed and poured onto nutrient broth agar

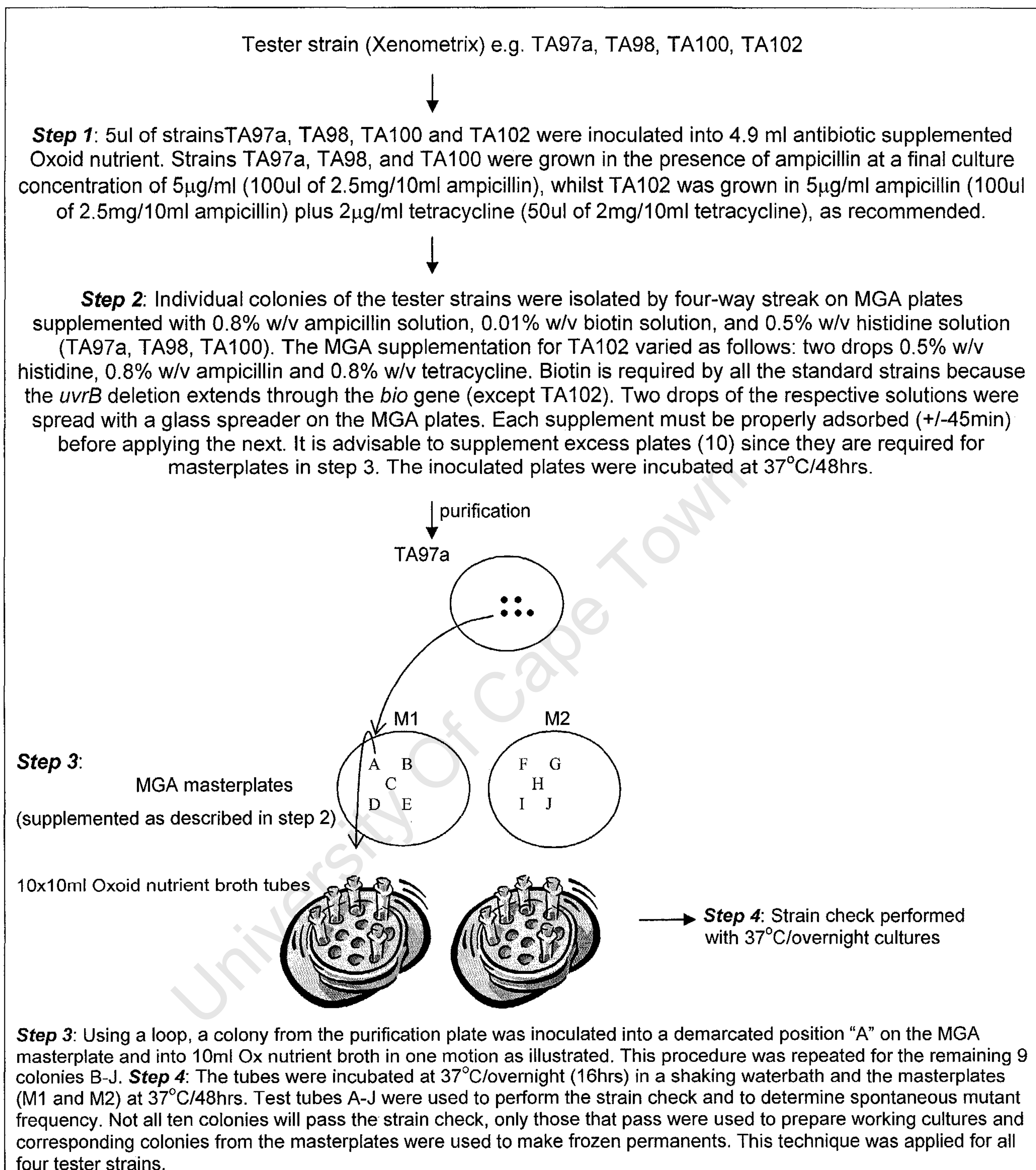

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Fig 14. Flow diagram for genotype verification of *Salmonella* tester strains
Step 1: the tester strains were inoculated into antibiotic-supplemented oxoid nutrient broth.
Step 2: individual colonies from the broth culture were isolated on MGA plates supplemented with histidine, biotin and ampicillin (for strains TA97a, TA98 and TA100), and on MGA plates supplemented with histidine, biotin, ampicillin and tetracycline (for strain TA102) by a four-way streak.
Step 3: ten colonies were transferred to a designated spot on MGA masterplates M1 and M2 marked "A-J" and into 10ml oxoid nutrient broth tubes marked "A-J".
Step 4: after incubation the broth cultures were used for the genotype test whilst the masterplates were stored at 4°C.

(NBA) plates using a pour-plate technique. Paper discs (0.6mm) were made from Whatman No. 1 using a paper punch and sterilised by autoclaving at 121°C for 20min. A single disc was placed in the centre of each plate and 5µl of 0.1% crystal violet were added to the disc. The plates were incubated at 37°C for 16 to 24hrs. A zone of inhibition indicates a positive result. The *rfa* deletion allows large molecules to penetrate the cell wall making it more efficient at detecting mutagens.

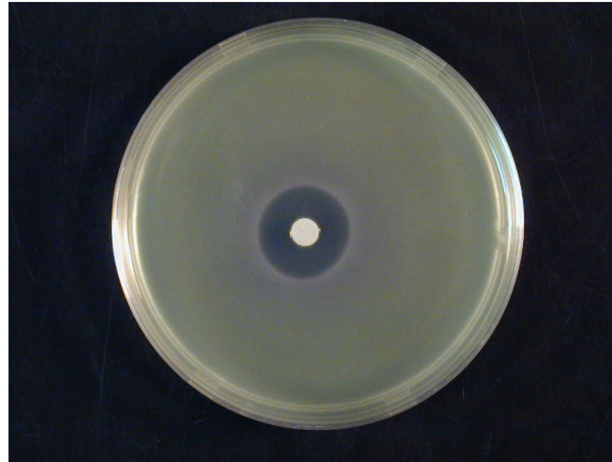

Fig 15. The zone of inhibition indicates crystal violet sensitivity. The *rfa* mutation permits large molecules such as crystal violet to enter and kill the bacteria. Wild-type strains are not inhibited because the crystal violet cannot penetrate the cell. All *Salmonella* strains should show a zone of growth inhibition surrounding the disc.

2.4.5 *uvrB* deletion

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Fig 16. Strains with *uvrB* deletion will grow only on the un-irradiated side of the plate as seen for TA97a. The deletion mutation stretches across the *bio-uvrB* region of the chromosome. It shows that the tester strains are defective in the accurate DNA repair pathway due to the *uvrB* deletion.

A loopful of inoculum from each of the ten tubes was streaked onto NBA plates. Covering half the plate with cardboard, irradiation with a 15W UV germicidal lamp (43.5cm in length) was applied for 8 seconds at a distance of 33cm. No growth on the irradiated portion of the plate should be observed for TA97a, TA98 and TA100, since they do not have an intact *uvrB* gene. However, TA102 does have an intact *uvrB* gene and growth is observed in the UV-exposed region. During

irradiation, petri plate lids were removed because the UV radiation does not penetrate the plastic. Plates were incubated at $37^{o}C$ for 16 to 24hrs.

2.4.6 Testing for the presence of plasmid pKM101

Fig 17. Ampicillin resistance is a convenient marker for testing for the presence of the plasmid pKM101 since specific regions of the pKM101 DNA are essential for enhancing UV and chemical mutagenesis. With the sample on the right, the zone of inhibition indicates loss of the plasmid and thus is unsuitable for working culture preparation.

Ampicillin resistance was used as a marker to test for the presence of plasmid pKM101, since specific regions of the pKM101 DNA are essential for the enhancement of UV and chemical mutagenesis. The pour plate technique described in section 2.4.4 was applied. An ampicillin disc (BBL Sensi-Disc Antimicrobial Susceptibility Test Discs, Ampicillin 10µg) was placed in the centre of each plate and incubated at $37^{0}C$ for 16 to 24hrs.The absence of a zone of inhibition around the disc demonstrates a positive result.

2.4.7 Spontaneous mutation frequency

A 0.1ml inoculum from each of the 10 culture tubes was seeded into top agar supplemented with a 0.5mM histidine/biotin solution and overlayed onto MGA plates. The plates were incubated at $37^{o}C$

Table 6: The spontaneous revertant laboratory values in comparison to the values stipulated in the literature and by the supplier

Strain	**Batch no.**	**Spontaneous revertant colonies**			
		Laboratory value ***Minus S9***	[b]**Values cited in the literature**		[a]**Values from supplier** ***Minus S9***
			Plus S9	***Minus S9***	
TA97a	Lot 517	*220 ± 28*	75-200	*100-200*	*196-256*
TA98	Lot 518	*39 ± 3*	20-50	*20-50*	-
TA100	Lot 519	*187 ± 14*	75-200	*75-200*	-
TA102	Lot 520	*564 ± 78*	100-300	*200-400*	*500-588*

[a] Values from the supplier's (Xenometrix CA, USA) quality testing
[b] The literature by Mortelmans and Zieger (2000) is recommended by the supplier. However, the exception is for TA97a and TA102 where the supplier values exceed the literature values (minus S9). The supplier indicates that the high values for TA97a and TA102 is because the spontaneous revertant frequencies have increased since Maron and Ames (1983).

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for 48hrs. A lawn of bacterial growth was observed at 24hrs and revertant colonies at 48hrs.The colonies were hand-counted using a Quebec darkfield colony counter. The spontaneous mutation frequencies of the tester strains cited in the literature (Mortelmans and Zieger, 2000) by the supplier and the values obtained in the laboratory are tabulated in Table 6.

2.5 Preparation working culture and frozen permanents

A colony that complied with the genotype verification and spontaneous mutant frequency from each tester strain was removed from the masterplate and seeded into oxoid nutrient broth No. 2 (5.5ml) and incubated at 37°C overnight in an orbital shaker (Culture A). Working cultures were made by seeding Culture A (1ml) into sterile oxoid nutrient broth No. 2 (50ml) and incubated at 37°C overnight in an orbital shaker. DMSO (5ml) was added to the working culture (50ml) and aliquots (1ml) were dispensed into sterile cryovials kept on ice and transferred to a -70°C freezer. For mutagenicity testing an aliquot of working culture (1ml) was thawed, seeded into oxoid nutrient broth (50ml) and incubated as previously described. Five “frozen permanents” were made by adding DMSO (0.5ml) to culture A (4.5ml), dispensed into 1ml aliquots kept on ice and stored at -70°C.

2.6 Dose-response of tester strain to mutagen

The dose-response of tester strains TA97a, TA98 and TA100 to increasing concentrations of 2-acetamidofluorine (2-AAF) was done in quadruplicate using a standard pour plate technique. The purpose of the dose response was to (i) select a single concentration from the monotonic region of the graph that produced a two- to fivefold increase in revertant colonies than the spontaneous revertants for the purpose of a positive control and (ii) examine the strain’s responsiveness to mutagens. An overnight working culture (0.1ml) was seeded into histidine/biotin supplemented with top agar (2ml), followed by 2-AAF (0.1ml) and 4% S9 mix (0.5ml). The top agar was overlayed onto minimal glucose agar (MGA) plates and incubated at 37°C for 48hrs. Revertant colonies were hand-counted and the average of quadruplicate plates was plotted (refer to the results in section 3.1.2). The dose-response in TA102 was done with cumene hydroperoxide (recommended by supplier), which is a directly acting carcinogen and as such requires no metabolic activation (S9). Dose response for TA102 was done in a similar manner with the exception that 2.5ml top agar was used instead of 2ml.

2.7 *Salmonella* reverse mutation assay

The *Salmonella* reverse mutation assay was performed in quadruplicate with TA97a, TA98, TA100 and TA102 (with and without S9) for the following reasons: (i) maximum detection of mutagenic compounds; (ii) is favourable for statistical analysis; and (iii) allows for comparisons to be drawn. The tester strains, TA97a and TA98, are designed to detect frameshift mutations, TA100 for the detection of base-pair substitutions and TA102 for the detection of oxidative DNA damage, DNA

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crosslinking and transition transversion reactions. The use of four tester strains increased the likelihood of detecting a test substance capable of mutagenesis by either one or more of the mechanisms described above. Bacteria are unable to metabolise chemicals via cytochrome P450, as in mammals and other vertebrates, and a key component for making the bacterial mutagenicity test useful was the inclusion of an exogenous mammalian metabolic activation system consisting of an extract of rat liver (S9).

An extract was mutagenic if the average number of revertants per plate (mean of 4 or 3 replicate plates) was at least doubled over the spontaneous revertant frequency and is in keeping with the two- to threefold rule. If the revertant count of a dose-response curve of test chemical exceeds twice or thrice the spontaneous background rate, the two- to threefold rule determines the test result positive. When the maximum revertant count does not exceed two to three times the spontaneous background rate, this rule concludes that the test is negative.

A standard volume of 0.1ml test sample per plate was applied across all strains. The test sample (0.08g/ml) was prepared by dissolving lyophilised extract (0.57g) in 7ml distilled water. The following concentrations were tested: (i) 0.005, 0.02 and 0.08g/ml for *A. oppositifolia*, *Pelargonium*, *P. aquilinum* and *R. lanceolatus*; and (ii) 0.00125; 0.005 and 0.02g/ml for *Z. aethiopica* as a result of problems with viscosity. The working culture (1ml) of tester strains TA97a, TA98, TA100 and TA102 was seeded into sterile oxoid nutrient broth (50ml) and incubated at 37°C overnight on an orbital shaker. Top agar was supplemented with histidine/biotin (0.5mM) and maintained at 45°C in a waterbath. The working culture (0.1ml) was added to 2ml top agar, followed by plant extract (0.1ml) and 4% S9 mix (0.5ml) for the analysis of promutagens (chemicals requiring metabolic activation). The top agar was overlayed onto minimum glucose agar (MGA) plates and incubated at 37°C for 48hrs. For the positive control the plant extract was replaced with 2-AAF (in strains TA97a, TA98 and TA100, with S9) and cumene hydroperoxide (in strain TA102 without S9). The extracts were tested in the absence of S9 in a similar manner with the exception that 2.5ml top agar was used instead of 2ml.

The condition of the background lawn is an indicator of toxicity: (i) presence of the background lawn in agar plates indicates non-toxicity of extracts (Reid *et al*, 2006) and (ii) when there is "thinning" or complete absence of the background lawn, this indicates the toxicity of the test chemical. A decrease in the number of revertant colonies to levels below the spontaneous reversion level may on occasion be seen together with thinning (Mortelmans and Zeiger, 2000). Occasionally, numerous small "pinpoint" non-revertant colonies are present on the plate that are histidine-dependent bacteria, have survived high chemical toxicity and are readily visible to the naked eye and may be mistaken for revertant colonies (Mortelmans and Zeiger, 2000).

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2.7.1 Statistical analyses

The gamma test was performed on the mean quadruplicate values and Kruskal's gamma coefficient (γ) was calculated. In the *Salmonella* reverse mutation assay the gamma statistic is very useful for measuring trends and determining significance. A positive γ statistic is indicative of an upward trend or positive dose-response relationship, while a negative γ statistic is indicative of a decreased response (due to toxicity). A p-value ≤0.01 is an indicator of significance and the trend is significant at the 1% level.

2.8 Chromosomal aberration test

Chromosomal aberrations in normal chromosome structure or number (e.g. aneuploidy, polyploidy) occur spontaneously or as a result of chemical or radiation treatment (Mateuca *et al.*, 2006). Structural chromosomal aberrations in peripheral blood lymphocytes, as assessed by the chromosome aberration assay, have been used for over 30 years in occupational and environmental settings as a biomarker of the early effects of genotoxic carcinogens (Hagmar *et al.*, 2004). The chromosomal aberration test is a cytogenetic procedure for investigating the clastogenic effects of chemicals on mammalian cells. Peripheral blood lymphocytes are most frequently used owing to their easy availability. Normal circulating lymphocytes do not divide under routine culture conditions, but are stimulated to proliferate by phytohaemagglutinin (PHA).

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2.8.1 Cultures

The culture method was adapted from Fonseca *et al.* (2000). The blood was collected by venupuncture into lithium heparin tubes (self-donated). Whole blood (0.3ml) was added to 5ml RPMI-1640 with L-glutamine (Invitrogen Corporation, Gibco) and phytohaemagglutinin (100µl, m-form, Invitrogen Corporation, Gibco). Blood was cultured at 37°C in 20ml tubes for 48hrs. The treatment with aqueous extract was done at three concentrations, that is, 0.005, 0.02 and 0.08g/ml for *A. oppositifolia*, *Pelargonium*, *P. aquilinum, R. lanceolatus* respectively and at 0.00125; 0.005 and 0.02g/ml for *Z. aethiopica* in duplicate at 37°C for 24hrs. One hour before culturing ended (i.e. 23hrs), Colcemid (10µg/ml, Karyomax, Invitrogen Corporation) was added to a final culture concentration of 19.6µl/ml for metaphase arrest and incubated at 37°C for a further 1hr. Fixation was performed by standard cytological procedures. The cells were exposed to a hypotonic shock (10ml, warmed 75mM KCl solution) for 20min at 37°C. The cells were then fixed in 5ml acetic acid:methanol (1:5v/v). The fix washes were done four times before a small quantity of fix (0.2ml) was transferred to the cells. The cell suspension was then dropped from a height of 30cm onto cold pre-cooled glass slides (pre-washed with fix).The slides were air-dried, incubated at 59°C for 1hr and stained with Giemsa:Buffer solution (1:10v/v, Giemsa, BDH and Buffer, pH6.86, +/- 0.05, at 20°C, Cameron Chemical Consultants). Slides were coded to ensure a blinded evaluation. As a positive control, the cells were treated with methylmethanosulphate (10mM, MMS dissolved in 0.5% DMSO). The scoring of fifty metaphases was adequate (Lee *et al.*, 1996; Chang *et al.*, 2005)

and carried out by the method recommended by Savage (2004). The *in vitro* tests were also carried out in the presence of the S9 fraction (4%) for 24hrs. The S9 mix was filtered through a 0.45µm filter prior to use. As a positive control, the cells were treated with cyclophosphamide (2.5mM dissolved in water).

2.9 DNA content (flow cytometry)

Owing to the absence of metaphase spreads in the chromosomal aberration assay for *Pelargonium* and *A. oppositifolia* extracts, an investigation into cell cycle arrest was initiated.

2.9.1 Cultures

The blood was collected by venupuncture into lithium heparin tubes (self-donated). Whole blood (1.2ml) was added to RPMI-1640 (5ml, BioWhittaker, Lonza) and phytohaemagglutinin (100µl, m-form, Invitrogen Corporation, Gibco). Blood was cultured at 37°C in 20ml tubes for 48hrs. The cultures were treated with 0.08g/ml *Pelargonium* and *A. oppositifolia* extracts (0.1ml) and incubated at 37°C for 24hrs. Untreated culture comprising whole blood (1.2ml) added to 5ml RPMI-1640 (BioWhittaker, Lonza) and phytohaemagglutinin (100µl, m-form, Invitrogen Corporation, Gibco) was included as a control. The experiment was performed in duplicate and the *in vitro* tests were also carried out in the presence of the S9 fraction (4%) for 24hrs.

The cells were washed twice with cold 1x PBS (10ml aliquot) and the red blood cells (RBCs) were lysed in RBC lysis buffer (15ml) at room temperature for 15min. Centrifugation was carried out at 2200rpm for 5min at 5°C. The treatment with the RBC lysis buffer was repeated until the pellet was white to translucent in colour and barely visible. The white blood cells (WBCs) were washed twice with cold 1x PBS (10ml aliquots) and the WBCs were re-suspended in 1xPBS (1ml) and transferred to FACS analysis tubes (Falcon 352052, 5ml round bottom, 12 x 75mm non-pyrogenic, Becton Dickinson). An aliquot (50µl) from each sample was diluted (30x) by the addition of 1x PBS (1450µl) and counted on a haemocytometer, the WBC count ranged from 55 to 95 X10^4 cells/ml. The remaining 9950µl WBC suspension was centrifuged at 2200rpm for 5min at 5°C. The pellet was treated with 10µg/ml RNaseA (100µl) at 37°C for 15min to remove RNA that would interfere with DNA analysis.

To determine viability, the 30x diluted WBC sample (10µl) was added to trypan blue (100µl) and PBS (90µl). This 1:20 dilution was placed on a hemocytometer slide and counted. The viability was ≥95% for *Pelargonium* and *A. oppositifolia* treated cells with and without metabolic activation.

2.9.2 Staining cells for FACS analysis

Cells were stained with propidium iodide. The amount of propidium iodide taken up by the cell is proportional to the amount of DNA present. Twenty minutes before FACS analysis, 900µl staining

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solution (Appendix B) was added to the RNaseA treated white blood cells. Analysis was performed on a FACS Calibur Becton Dickinson cell sorter.

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CHAPTER 3
RESULTS

3.1 *Salmonella* reverse mutation assay

3.1.1 The CYP P450 concentration from difference spectra

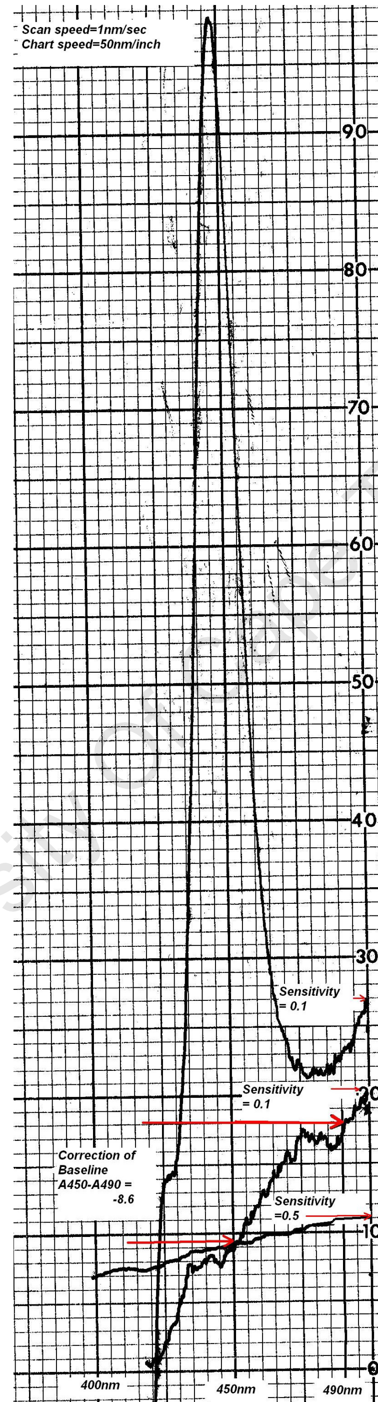

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Fig 18. CYP P450 activity of S9 was measured by difference spectra using a double beam Beckman spectrophotometer UV5260. The scan speed was 1nm/sec and UV Chart 50nm/IN. The baseline was run at a sensitivity of 0.1 and 0.5 as a trial, and the former was selected. From the above graph (i) the CYP P450 absorbance $A_{450\text{-}490}$ is 97-21.5 = 75.5 and (ii) the correction of baseline is 9.4-18 = -8.6. These values were inserted into the formula in 2.3.4 and the CYP P450 activity calculated was 0.7nmol/mg, which was within the recommended range (0.6-0.8nmol/mg) for the mutagenicity assay.

3.1.2 Dose-response and range-finding study

The dose-response of tester strains to known mutagens indicates that the test system is functional and responsive to mutagens. This experiment doubled up as a range-finding study for a positive control concentration that produced a two- to fivefold increase over the spontaneous revertant laboratory control values. The 2-acetamidofluorine (2-AAF) concentration range varied for each of the strains TA97a, TA98 and TA100 to obtain a good monotonic dose-response. The mutagens 2-AAF and cumene hydroperoxide (CHP) were prepared in DMSO (see Appendix A).

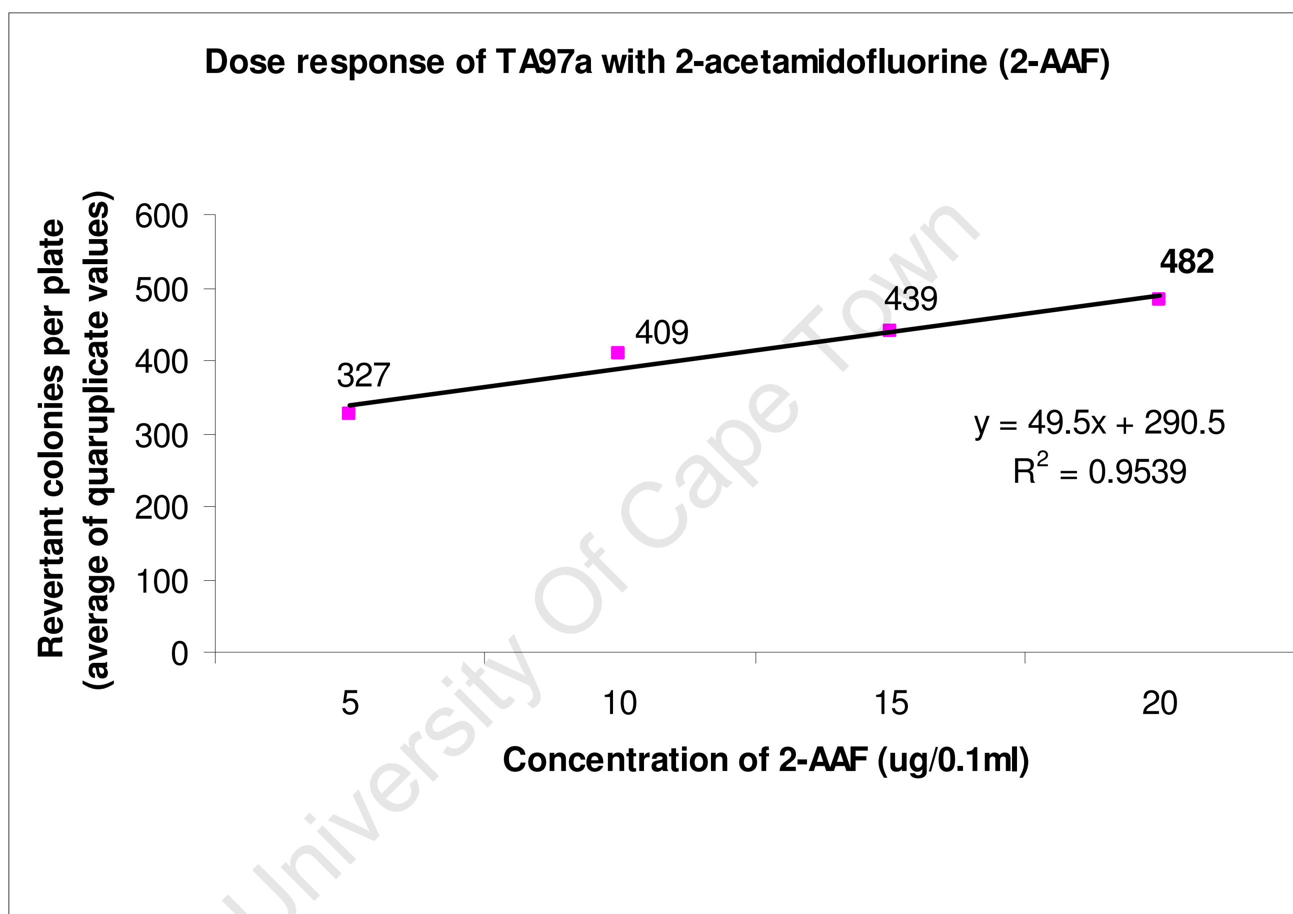

Fig 19. Dose response of strain TA97a to increasing concentrations of 2-AAF. The plot of the means of quadruplicate values against increasing concentrations of mutagen is indicated above.

The monotonic dose-response was observed for all strains over the concentration range tested. For TA97a (Fig 19) a positive control of 20µg/0.1ml 2-AAF was suitable because it produced a twofold increase in the number of revertant colonies (482) when compared to the spontaneous reversion laboratory control values (220±28) in Table 6. In order to establish whether a monotonic dose-response was present across the concentration range tested, the spontaneous reversion laboratory control values could not be plotted onto the graph but can be found in Table 6. For TA98 (Fig 20), it can be seen that a positive control of 1.5µg/0.1ml 2-AAF was suitable because it produced a fivefold increase in the number of revertant colonies (216) when compared to the spontaneous reversion laboratory control values (39±3).

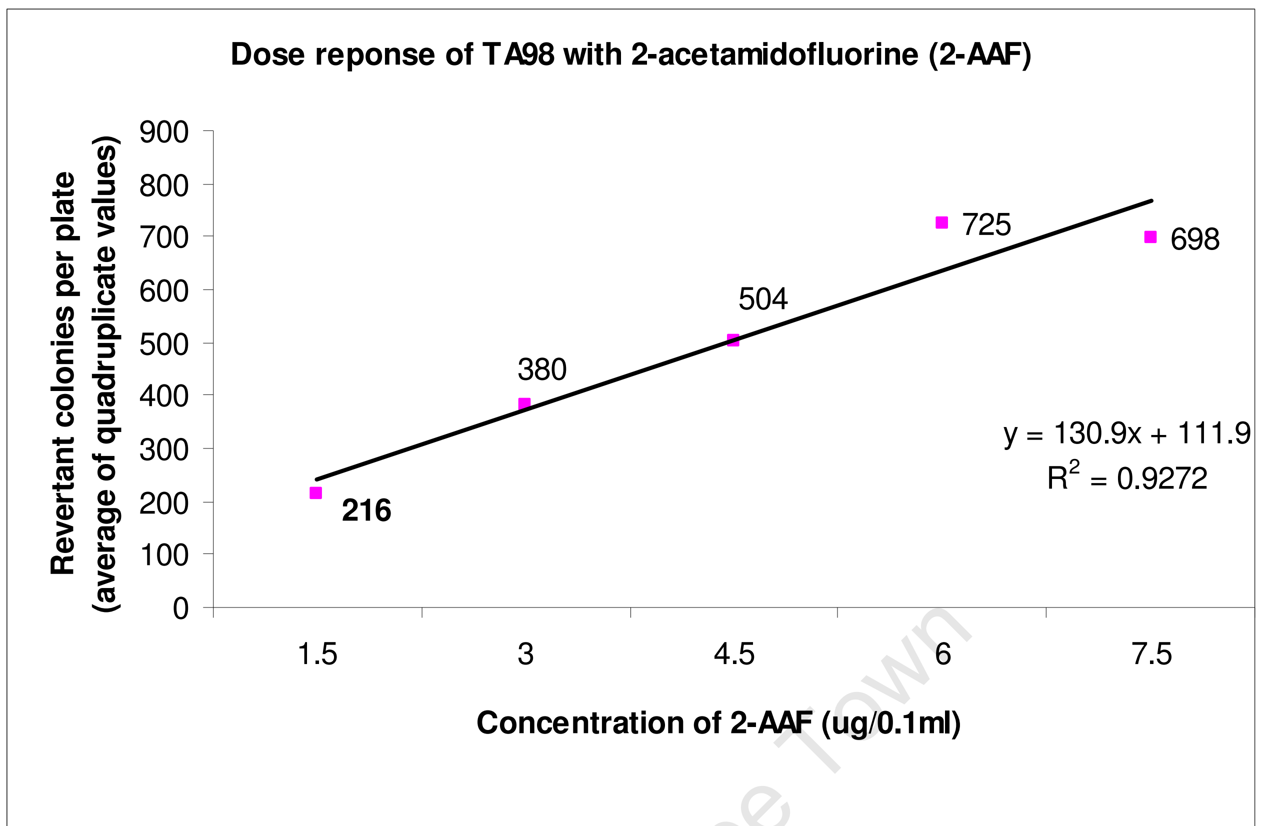

Fig 20. Dose response of strain TA98 to increasing concentrations of 2-AAF
The number of spontaneous revertants increased over the concentration range tested.

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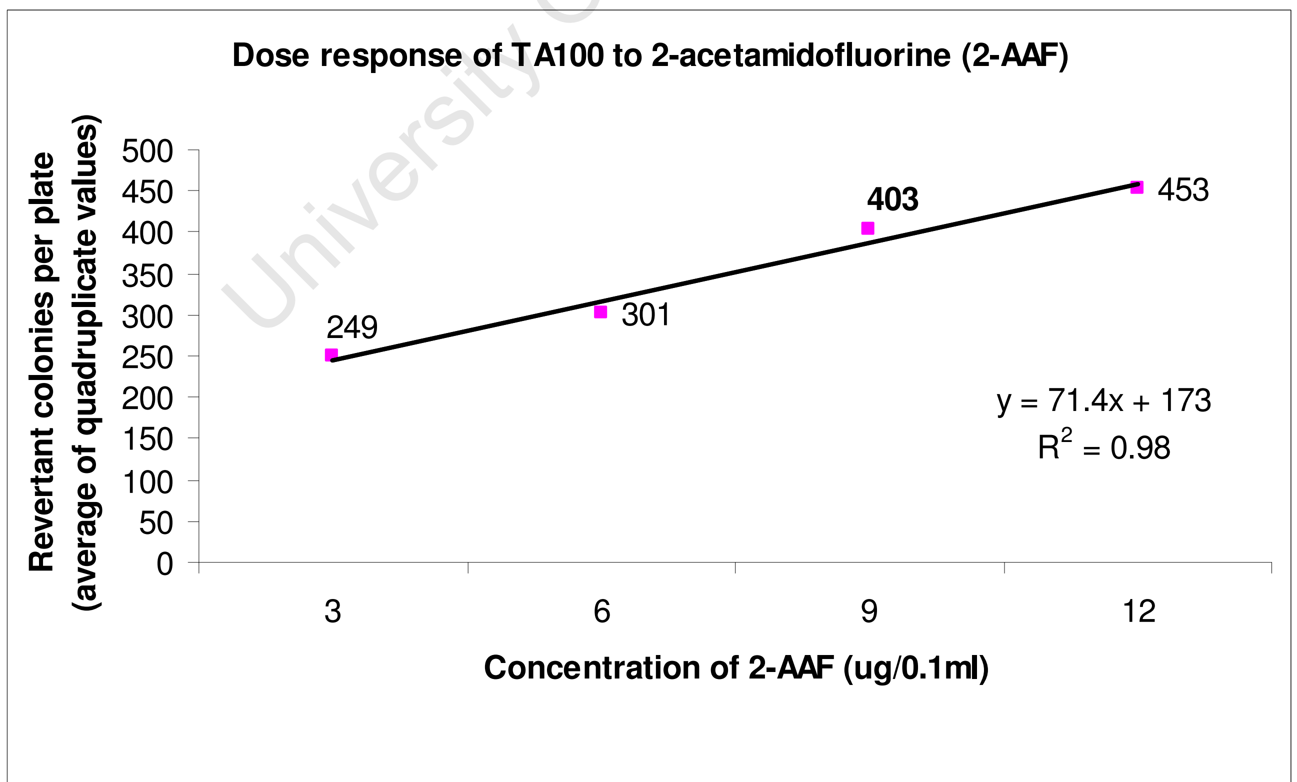

Fig 21. Dose response of strain TA100 to increasing concentrations of 2-AAF

A positive control of 9µg/0.1ml 2-AAF was selected for TA100 (Fig 21) because it produced a twofold increase in the number of revertant colonies (403) when compared to the spontaneous reversion laboratory control values (187±14).

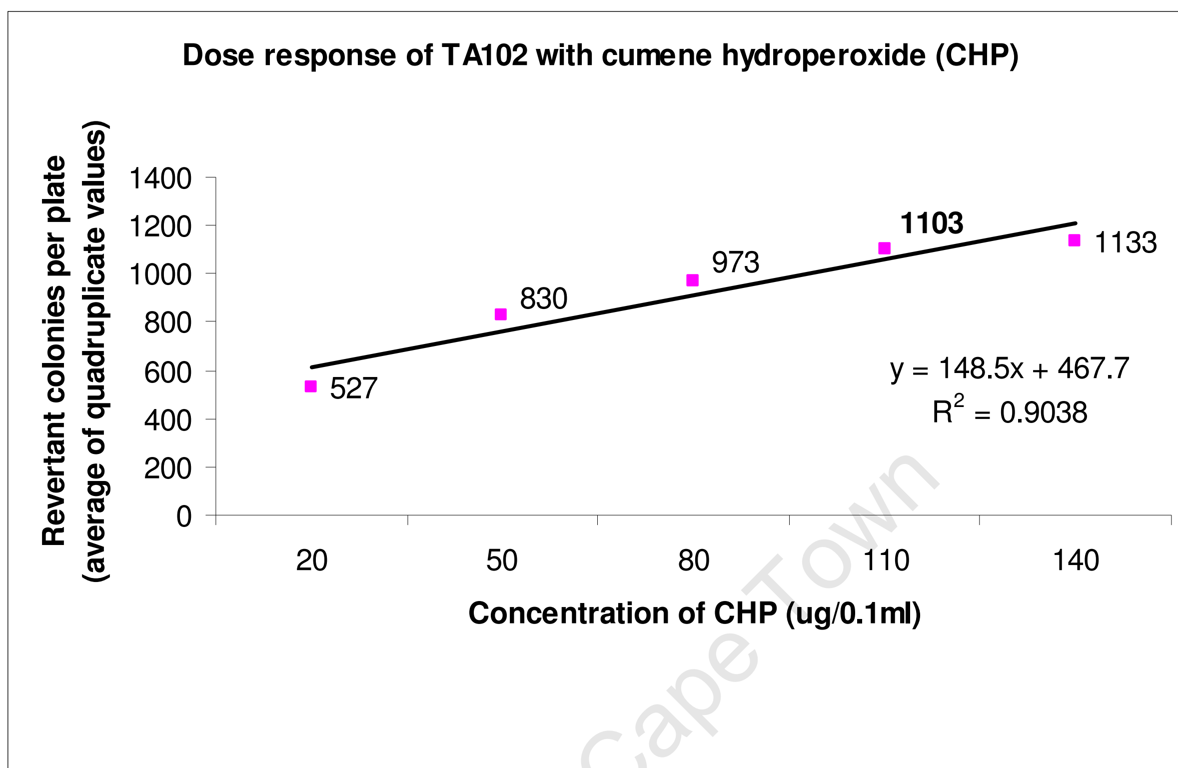

Fig 22. Dose response of tester strain TA102 to increasing concentrations of CHP, a directly acting mutagen

A positive control of 110µg/0.1ml CHP was selected for TA102 (Fig 22) because it produced a twofold increase in the number of revertant colonies (1103) when compared to the spontaneous reversion laboratory control values (564±78). The results of the range-finding study is summarised in Table 7.

Table 7: Positive control concentrations from the range-finding study

Strain	2-AAF (+S9)	Cumene hydroperoxide (-S9)
TA97a	20µg/0.1ml	-
TA98	1.5µg/0.1ml	-
TA100	9µg/0.1ml	-
TA102	-	110µg/0.1ml

3.1.3 *Salmonella* reverse mutation assay

Chemicals are judged to be non-mutagenic when they have been tested in at least four *Salmonella* tester strains: a positive response in any single strain either with or without metabolic activation is sufficient for designating a chemical as a mutagen and often causes the substance to be branded a presumptive carcinogen until definitive results have been obtained from a two-year carcinogenicity study (Zeiger, 1998; 2001). In the *Salmonella* reverse mutation assay, gamma

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statistics are very useful for measuring trends and significance. A positive gamma statistic is indicative of an upward trend and a positive dose response relationship. A negative gamma statistic is indicative of a decreased response possibly due to (i) toxicity, which may be associated with thinning or complete absence of the background lawn, or (ii) antibacterial activity in the test substance. Plant extracts were prepared as described in Chapter 2 on materials and method. It is important to note that the aqueous extracts were prepared from the aerial part of the plant (leaves), and are most often consumed in this manner as medicine and dietary supplements. The data, including standard deviation and statistical significance, are tabulated in Tables 8 and 9.

3.1.4 *Acokanthera oppositifolia (*Lam.*)* and *Zantedeschia aethiopica (*L.*)*

A. oppositifolia and *Z. aethiopica* proved to be non-mutagenic with all four tester strains with or without S9. Although *Z. Aethiopica* was non-mutagenic, it produced a significant decrease in the number of revertants in TA97a with S9 ($p \leq 0.01$). Visual inspection of the plates showed an intact background lawn with small pinpoint colonies, easily mistaken for revertant colonies. This indicated that *Z. aethiopica* was toxic but only in the presence of S9. Calcium oxalate, a chemical constituent of *Z. aethiopica*, is known to produce toxicity. The leaves of *Z. aethiopica* have a unique mannose-binding lectin that binds to cell membranes that are often toxic (van Wyk *et al*., 1997 and Chen *et al*., 2004). One can speculate that the toxicity in TA97a with S9 may be attributed to (i) metabolic intermediates of calcium oxalate and lectins or (ii) phytochemical metabolites of *Z. aethiopica* that may have potential antibacterial activity. What is rather alarming is that only one tester strain was affected in this manner when all strains carry the *rfa* deletion, making their cell wall more permeable to the penetration of chemicals. Since it is generally accepted that mutagenicity in any one of the tester strains with or without S9 is a positive response, based on a similar principle, toxicity in any one of the tester strains with or without S9 should be sufficient to brand the chemical as a potential toxicant.

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3.1.5 *Pelargonium sp. cf. inquinans (* L.*) L' Herit*

The results indicate that (i) in TA102 (Fig 23B) phytochemicals in the *Pelargonium* extract were mutagenic by one or more of the following mechanisms: transition and transversion reactions, DNA crosslinkage and oxidative DNA damage and (ii) in TA97a (Fig 23A) the cytoxicity of the *Pelargonium* extract in the presence of S9 is undoubtedly due to a phytochemical metabolite.

Furthermore, phytochemical metabolites of *Pelargonium* may have potential antibacterial activity. Coumarin, a chemical constituent of *Pelargonium* sp, may contribute to the cytotoxicity observed at increasing concentrations. With regards to a potential risk related to the coumarin content, a NOEL (no observed effect level) value of more than 750mg/kg body weight has been established in dogs and rats in a *Pelargonium sidoides*-related toxicological study (Brendler *et al*., 2008).

Table 8a: Mutagenicity of aqueous extracts from traditional medicinal plants towards *S. typhimurium* TA97a, TA98 and TA100 in the absence of S9

Extract	His^{+} revertants per plate[a] (-S9)									γ[e]
	Strain	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)							
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
A. oppositifolia	TA97a	170±13	-	-	177±12	158±4	156±11	161±13	167±18	-0.2696
Pelargonium	TA97a	109±32	-	-	150±26	166±19	177±21	166±4	129±27	0.2100
P. aquilinum	TA97a	204±25	-	-	181±14	169±10	178±5	163±20	188±15	-0.1802
R. lanceolatus	TA97a	161±27	-	-	179±15	183±32	216±8	241±13	307±51	0.8739*
Z. aethiopica	TA97a	179±18	163±35	178±8	187±29	181±7	178±8	-	-	0.0932
A. oppositifolia	TA98	24±8	-	-	23±2	25±1	23±6	26±6	27±7	0.1150
Pelargonium	TA98	21±4	-	-	21±2	23±6	22±7	23±3	24±1	0.2480
P. aquilinum	TA98	23±11	-	-	26±6	23±6	25±6	23±4	23±3	-0.1207
R. lanceolatus	TA98	20±3	-	-	23±5	30±5	33±7	50±7	78±6	0.8979*
Z. aethiopica	TA98	26±2	36±6	27±3	29±7	25±2	25±6	-	-	-0.2735
A. oppositifolia	TA100	120±11	-	-	135±11	133±6	126±24	145±14	140±21	0.2658
Pelargonium	TA100	121±17	-	-	123±14	126±17	121±23	138±8	154±21	0.3950
P. aquilinum	TA100	269±10	-	-	263±39	232±16	259±28	253±15	294±42	0.0796
R. lanceolatus	TA100	134±18	-	-	131±16	140±15	158±7	183±16	205±28	0.7815*
Z. aethiopica	TA100	148±9	133±19	140±16	137±7	135±10	152±22	-	-	0.0345

[a] data are means ±S.D. of four plates
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1ml extract per plate
[e]= Kruskal's gamma coefficient, *= the trend is significant at the 1% level ($p \leq 0.01$)

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Table 8b: Mutagenicity of aqueous extracts from traditional medicinal plants towards *S. typhimurium* TA97a, TA98 and TA100 in the presence of S9

Extract	His^{+} revertants per plate[a] (+S9)										γ[e]
	Strain	Control (-)[b]	Control (+)[d]	Concentration of plant extract per plate[c] (g/ml)							
				0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
A. oppositifolia	TA97a	161±17	480±37	-	-	132±18	131±15	140±16	158±22	133±6	-0.1224
Pelargonium	TA97a	143±16	492±23	-	-	134±18	133±19	120±13	85±19	66±8	-0.7370*
P. aquilinum	TA97a	192±14	462±17	-	-	172±6	171±10	164±16	144±5	144±6	-0.7056*
R. lanceolatus	TA97a	154±13	600±98	-	-	155±17	148±15	154±4	193±41	295±51	0.4576*
Z. aethiopica	TA97a	175±15	646±95	170±18	165±14	141±14	170±14	134±16	-	-	-0.4359*
A. oppositifolia	TA98	37±8	193±20	-	-	37±3	36±4	41±7	31±4	39±6	-0.0442
Pelargonium	TA98	35±4	173±14	-	-	24±2	21±3	20±3	23±5	26±5	-0.2750
P. aquilinum	TA98	37±3	200±40	-	-	40±8	34±4	29±5	31±3	34±7	-0.3391
R. lanceolatus	TA98	26±3	181±31	-	-	29±7	38±3	47±8	73±14	107±3	0.9409*
Z. aethiopica	TA98	37±8	228±18	36±8	38±2	43±11	35±5	35±6	-	-	-0.0553
A. oppositifolia	TA100	118±7	355±24	-	-	127±9	126±17	140±8	132±6	127±11	0.1864
Pelargonium	TA100	152±9	375±25	-	-	128±9	130±9	138±4	127±15	158±31	-0.0040
P. aquilinum	TA100	284±12	430±25	-	-	276±40	288±25	326±11	315±31	328±17	0.5459*
R. lanceolatus	TA100	155±14	508±104	-	-	149±6	165±13	179±19	202±20	221±23	0.7384*
Z. aethiopica	TA100	141±17	411±75	149±11	153±10	152±16	136±17	148±10	-	-	-0.0088

[a] data are means ±S.D. of four plates
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1ml extract per plate
[d] Control (+) = number of revertants for positive control
[e]= Kruskal's gamma coefficient, *= the trend is significant at the 1% level ($p \leq 0.01$)

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Table 9a: Mutagenicity of aqueous extracts from traditional medicinal plants towards *S. typhimurium* TA102 in the absence of S9

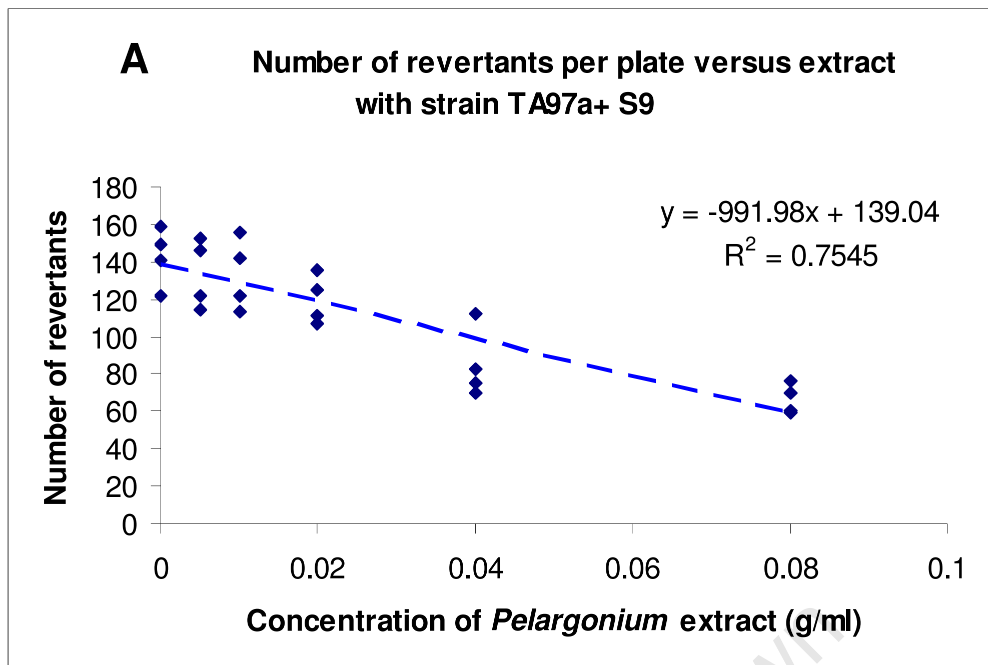
Extract	His+ revertants per plate[a] (-S9)										γ^e
	Strain	Control (-)[b]	Control (+)[d]	Concentration of plant extract per plate[c] (g/ml)							
				0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
A. oppositifolia	TA102	430±36	1613±85	-	-	470±39	415±24	435±20	468±10	423±26	-0.0796
Pelargonium	TA102	433±30	1538±131	-	-	455±24	448±36	450±64	546±33	575±30	0.6880*
P. aquilinum	TA102	405±21	1575±126	-	-	443±32	458±17	430±24	475±21	500±45	0.6195*
R. lanceolatus	TA102	402±16	1323±79	-	-	392±19	417±27	480±14	488±45	577±15	0.7908*
Z. aethiopica	TA102	445±47	1403±134	423±26	420±59	408±31	443±41	430±86	-	-	-0.0702

[a] data are means ±S.D. of four plates
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1ml extract per plate
[d] Control (+) = number of revertants for positive control
[e]= Kruskal's gamma coefficient, *= the trend is significant at the 1% level ($p \leq 0.01$)

Table 9b: Mutagenicity of aqueous extracts from traditional medicinal plants towards *S. typhimurium* TA102 in the presence of S9

Extract	His+ revertants per plate[a] (+S9)									γ^e
	Strain	Control (-)[b]	Concentration of plant extract per plate[c] (g/ml)							
			0.00125	0.0025	0.005	0.01	0.02	0.04	0.08	
A. oppositifolia	TA102	475±24	-	-	410±34	460±34	438±35	418±43	418±29	-0.3036
Pelargonium	TA102	413±56	-	-	440±22	415±39	430±26	460±20	452±63	0.3050
P. aquilinum	TA102	463±69	-	-	450±57	458±46	452±56	493±77	398±42	-0.1342
R. lanceolatus	TA102	386±33	-	-	392±19	440±29	450±16	545±38	623±33	0.8814*
Z. aethiopica	TA102	400±47	455±70	423±59	403±77	430±28	393±19	-	-	-0.0940

[a] data are means ±S.D. of four plates
[b] Control (-) = number of spontaneous revertants without plant extracts
[c] 0.1ml extract per plate
[e]= Kruskal's gamma coefficient, *= the trend is significant at the 1% level ($p \leq 0.01$)



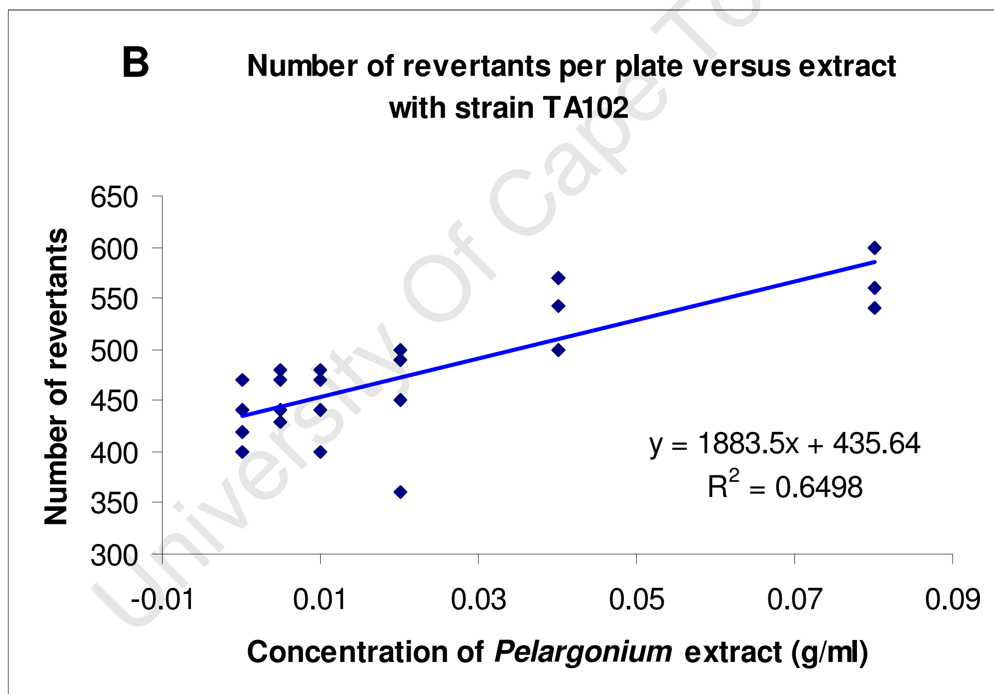


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Fig 23. *Salmonella* reverse mutation assay of *Pelargonium sp*. (A). The significant downward trend at a level of 1% ($p \leq 0.01$) with TA97a in the presence of S9 was observed. (B) The upward trend observed for PL in TA102 in the absence of S9 is significant ($p \leq 0.01$), indicating strong mutagenicity.

P. sidoides root extracts have a track record of success as a plant-based medicine and the plant is currently commercialised as EPs®7630 (Umckaloabo) (refer to Table 2 for its beneficial effects). In general, *Pelargonium* sp is used for the manufacture of rose-scented geranium oil that is used mainly in aromatherapy and in the fragrance industry. Traditionally, the leaves and root of *Pelargonium* sp are taken as tea or for the treatment of medical ailments such as dysentery and syphilis. Further toxicological studies of *Pelargonium* extracts are required.

3.1.6 *Pteridium aquilinum subsp aquilinum*

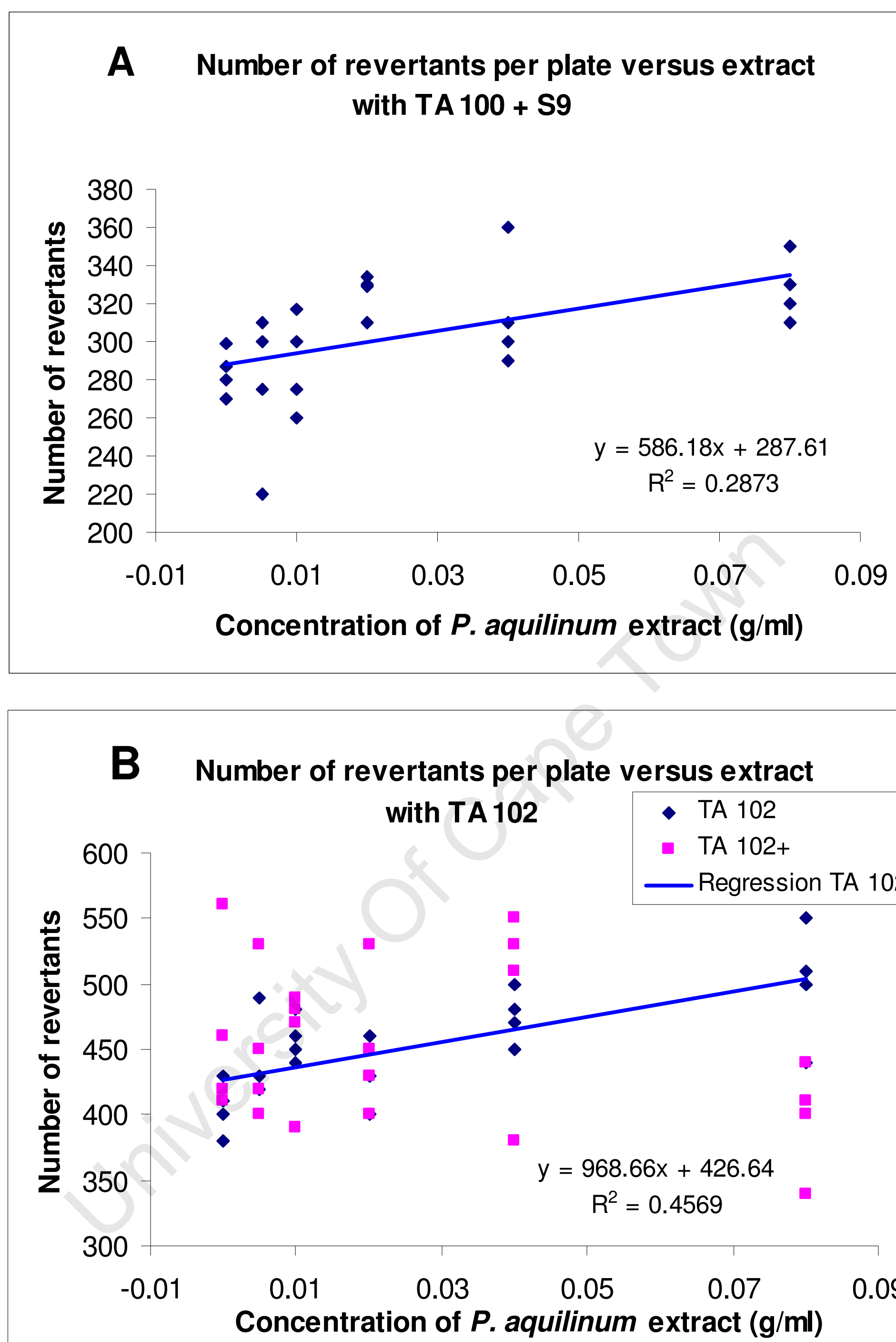

Fig 24. *Salmonella* reverse mutation assay of *P. aquilinum* indicates a mutagenic reaction with two of the four tester strains ($p \leq 0.01$) (A) in TA100 with S9 metabolic activation and (B) TA102 in the absence of S9.

P. aquilinum is mutagenic with (i) TA100 in the presence of S9 and (ii) TA102 in the absence of S9, which is evidence of promutagens and mutagens respectively. The mutagenicity, clastogenicity, teratogenicity and carcinogenicity of the major bracken carcinogen, ptaquiloside, a norsesquiterpene glucoside, has been convincingly demonstrated. Ptaquiloside is quite unstable once it is separated from the plant or under mild base or acid, and decomposes rapidly with the

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loss of the glucose moiety into pteridienone, which has been found to have more pronounced activity over ptaquiloside (Matsuoka *et al.*, 1989).

The decomposition of ptaquiloside is pH dependent, and studies have shown that ptaquiloside and its related compounds produce mutagenicity in the *Salmonella* reverse mutation assay in TA98 and TA100 under weakly basic conditions (pH8.5) (Nagao *et al.*, 1989). However, the pH in the current experiment was not controlled and mutagenicity was produced in (i) TA100 by base-pair substitution exerted by metabolic intermediates and (ii) in TA102 possibly by the crosslinkage reactions of ptaquiloside and pteridienone with DNA. These compounds have a strong alkylating capacity and react with DNA.

3.1.7 *Rumex lanceolatus* Thunb.

This extract contains compounds that are potent mutagens and promutagens, as detected with all four tester strains with and without metabolic activation (Fig 25). The mechanisms of DNA damage are by frameshift mutations, base-pair substitution, transition and transversion mutations, DNA crosslinkage and oxidative DNA damage.

Rumex species are related to the well-known medicinal plants known as rhubarbs (*Rheum* species) (van Wyk and Wink, 2004). Several monomeric anthraquinones, such as emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) and related derivatives that are constituents of rhubarb (an oriental medicine for diarrhoea) exhibit mutagenicity in the *Salmonella*/microsomes test and cultured tumour cells (Morita *et al.*, 1988 and Ueno, 1991). Emodin is a constituent of *R. lanceolatus* (Watt and Breyer-Brandwijk, 1962), which may be responsible for the potent mutagenic response observed.

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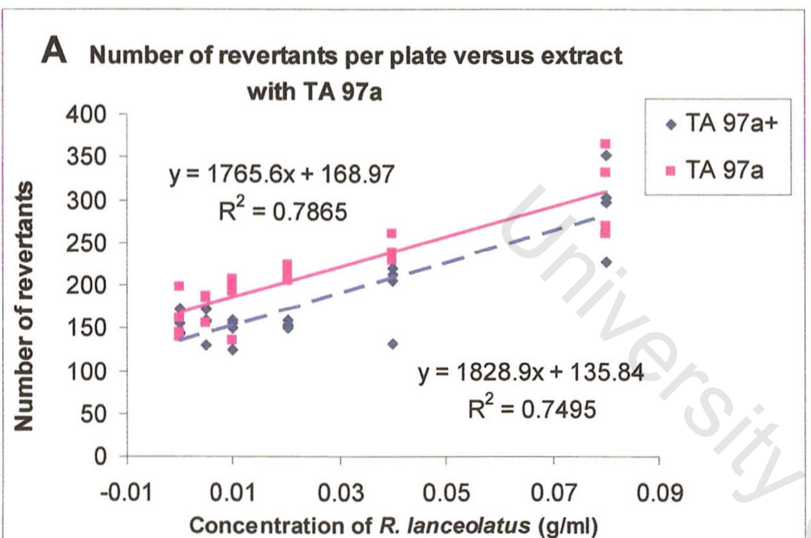
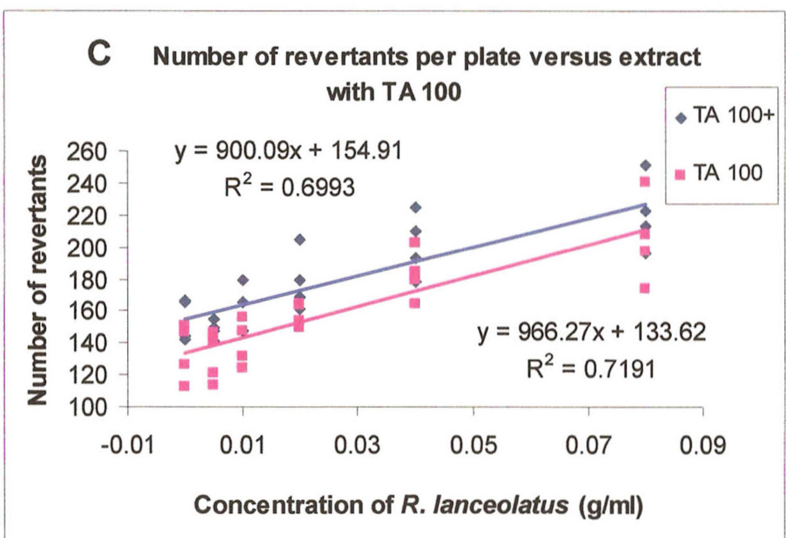
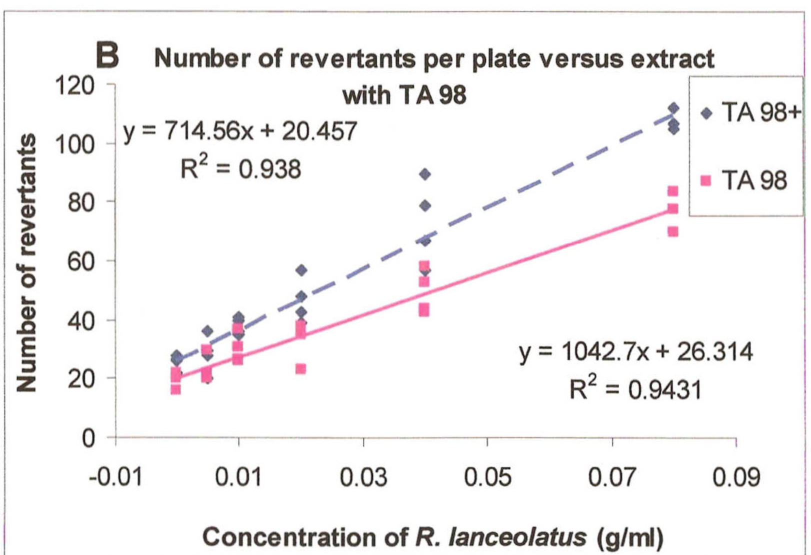
3.2 Chromosomal aberration test

The *in vitro* chromosomal aberration test was used to measure the clastogenicity of aqueous plant extracts in peripheral blood lymphocytes. The chromosomal aberration test was done in duplicate with S9 activation and non-activation at three concentrations of plant extract: (i) concentrations of 0.005, 0.02 and 0.08g/ml *P. aquilinum* and *R. lanceolatus* and (ii) concentrations of 0.00125; 0.005 and 0.02g/ml for *Z. aethiopica* because of problems with viscosity. The positive control used in the non-activation analysis was methanemethanosulphate (MMS), soluble in 0.5% DMSO for which a solvent control was included. The positive control used with S9 activation was cyclophosphamide (CPA), which is water soluble. Chromosomal aberrations were scored from metaphase arrested cells that had been fixed, dropped from a height of 30cm onto microscope slides and Giemsa stained. However, this method is not suitable for estimating numerical chromosomal aberrations, as artefactual chromosome loss may occur (Mateuca *et al.*, 2006) and its use for this purpose was not considered in this investigation. The KARY workstation (microscope, personal computer and







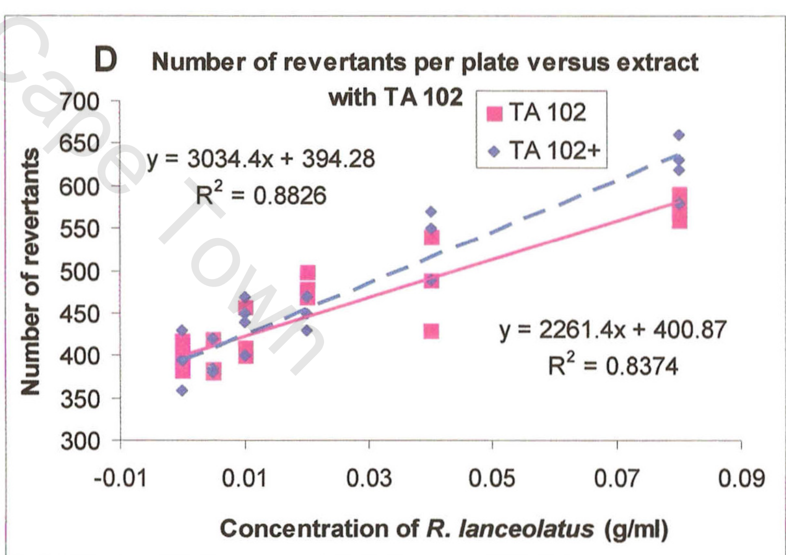


Fig 25. **A-D**: *Salmonella* reverse mutation assay for *R. lanceolatus* indicates that the extract contains compounds that are mutagenic in all four tester strains in the presence and absence of S9 ($p \leq 0.01$) and has the highest dose-response.

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printer) facilitated image capture via a microscope mounted video camera, digitisation of the captured image, storage on the computer's hard disk or on compact disc (CD) as a photomicrograph to be imported or printed.

Extensive research has been done by means of light microscopy from which categories of structural chromosome aberrations for metaphase cells have been established (Fig 13). Metaphase cells were scored as recommended by Savage (2004) and categorised into chromosome-type (CSA) and chromatid-type (CTA) aberrations. The CSA and CTA types arise from cell cycle progression (G_1, S, G_2 and M) and in relation to S-phase independent or dependent clastogens. CSAs result from incompletely repaired or unrepaired double-strand breaks mostly generated *in vivo* in G_o–G_1 lymphocytes by S-phase independent clastogens (e.g. ionising radiation). After DNA synthesis and chromosome duplication, the aberrations formed in G_o–G_1 were doubled and chromosome-type breaks were seen in metaphase. CTAs (e.g. chromatid type breaks) arose predominantly *in vitro* during the G_2-phase or late S-phase of the cultured lymphocytes in response to base modifications and single-strand breaks (SSBs) induced by S-phase-dependent clastogens (e.g. chemicals) (Savage 1975, 2004; Albertini *et al*., 2000, Hagmar *et al*., 2004 and Mateuca *et al*., 2006).

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3.2.1 *Pteridium aquilinum subsp aquilinum*

This test was done in duplicate and the average values from scoring approximately 50 metaphases was tabulated (Table 10). Photomicrographs depict chromatid-type aberrations (CTAs) observed at metaphase in Fig 26a, b and c. A chromatid aberration results from a single strand DNA break (SSB) occurring in the G_2 phase of the cell cycle by S-phase dependent clastogens in the 0.005 and 0.02g/ml *P. aquilinum* extracts.

The clastogenicity is attributed to ptaquiloside and pteridienone. Liver enzymes and CYP P450 play a major role in the detoxification of drugs and xenobiotics in the body. This was observed by a reduction of aberrations to 2% in *P. aquilinum* treated cultures with S9 under the current *in vitro* experimental conditions. The clastogenicity decreased in the presence of S9 as a result of detoxification; however, metabolically activated clastogens were still evident. A graphical representation of the data is given in Fig 27.

Table 10: Chromosomal aberrations from the treatment of cultures with *P. aquilinum*

Treatment	Dose (g/ml)	% cells with chromatid aberrations (CTAs)		% cells with chromosome aberrations (CSAs)	
		Gaps	Breaks	Gaps	Breaks
Nonactivation					
P. aquilinum	0.005	10	2	6	0
	0.02	5	0	5	3
	0.08	3	3	0	5
Negative control (untreated blood)	-	0	0	0	0
0.5% DMSO		2	4	2	2
10mM MMS		14	4	6	6
S9 activation (24 hr exposure)					
P. aquilinum	0.005	0	2	2	0
	0.02	2	2	0	0
	0.08	0	0	0	0
Negative control (untreated blood)		0	0	0	0
2.5mM CPA		15	10	3	3

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Fig 26a. A chromatid gap after treatment of peripheral blood lymphocytes with 0.005g/ml *P. aquilinum* without S9, implying direct clastogenicity [Slide Z1, vernier 65 X 92.5]

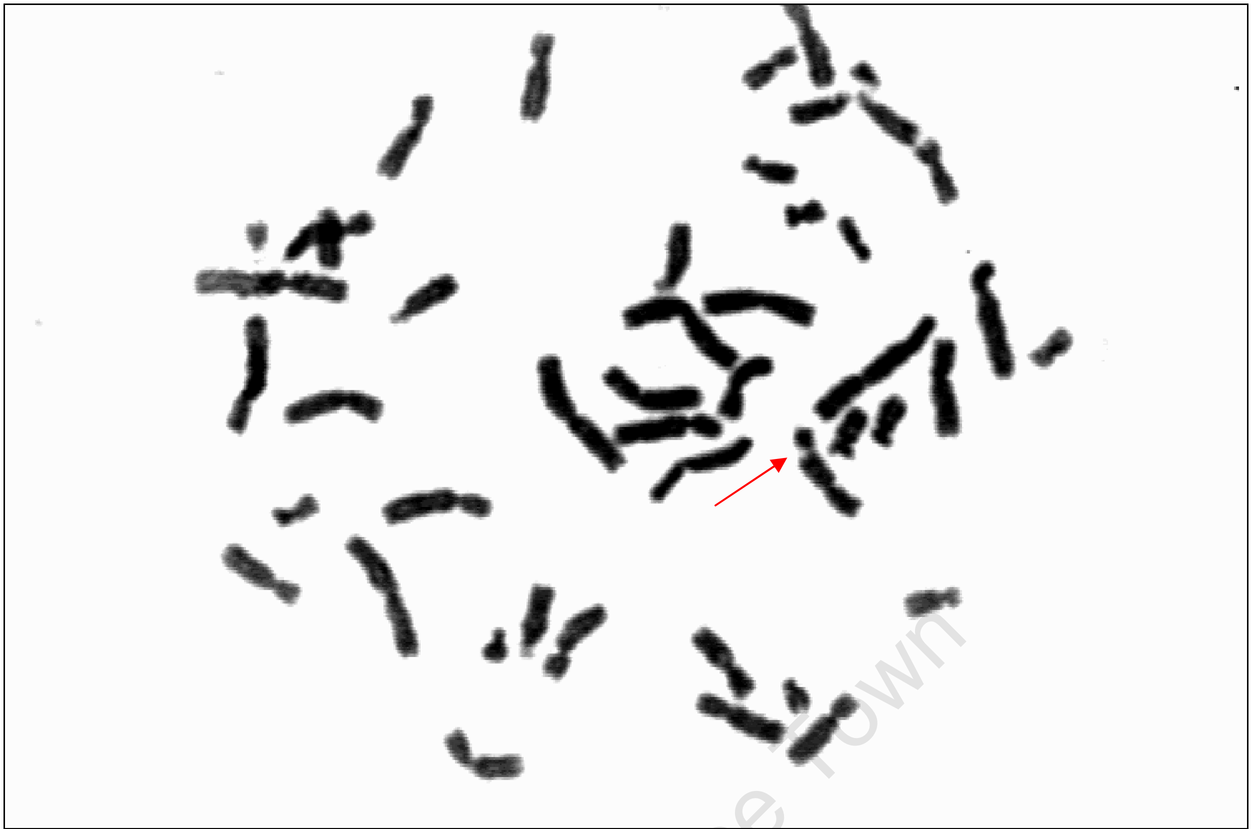

Fig 26b. Chromatid break caused after treatment of lymphocytes with 0.005g/ml *P. aquilinum* with S9. A reduction in chromosomal aberrations was observed after metabolic activation with S9 owing to detoxification reactions [Slide ZD12, vernier 61 X 93.5, aberration ZD12.5]

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Fig 26c Chromatid break caused by treatment of lymphocytes with 0.02g/ml *P. aquilinum* with S9 [slide ZD14, vernier 57 X 81, aberration ZD14.6]

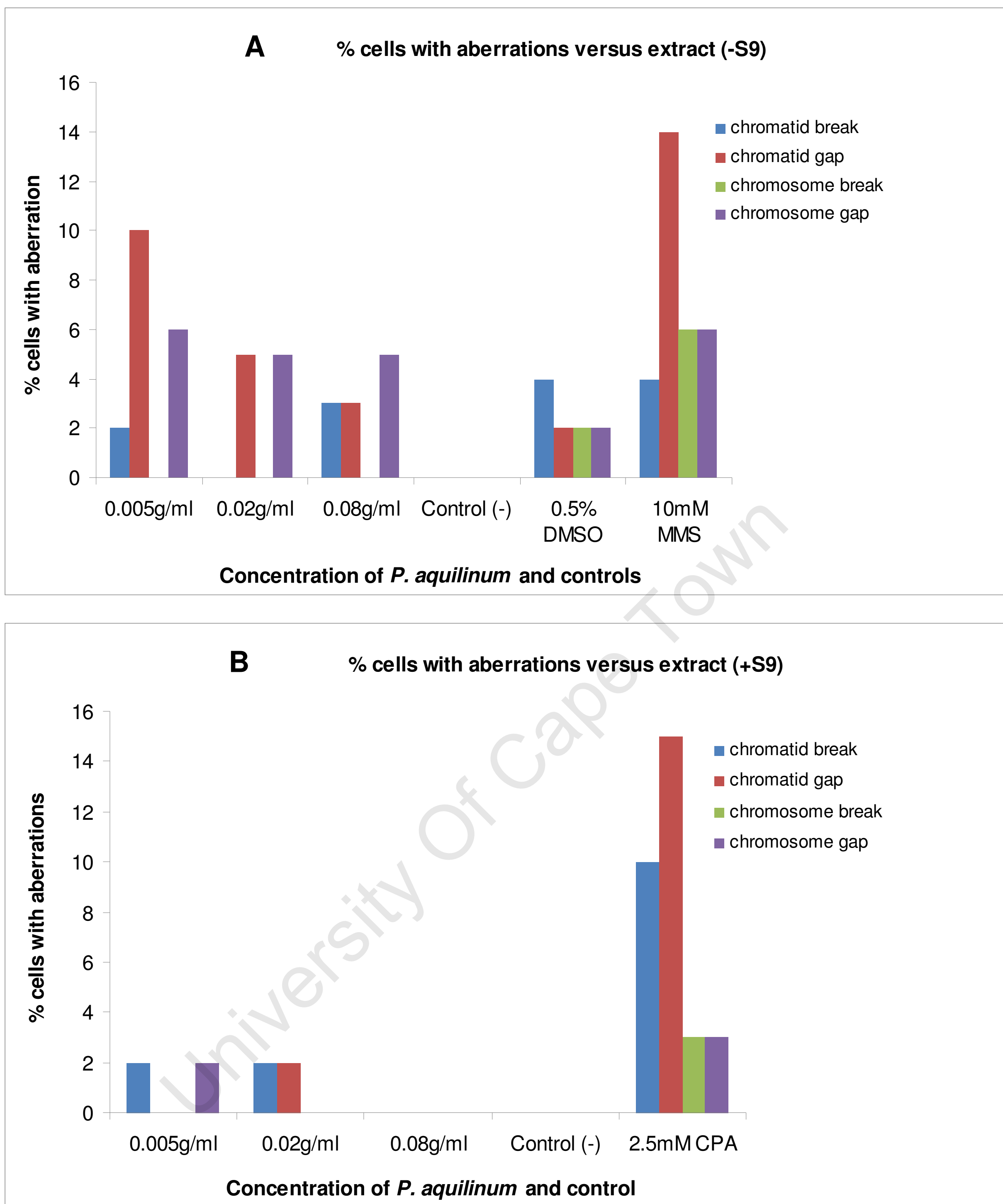

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Fig 27. The treatment of lymphocytes with *P aquilinum* at three increasing concentrations, 0.005; 0.02 and 0.08g/ml, in (A) the absence of S9 and (B) presence of S9. The reduction in genotoxicity is evident with CYP P450 and liver enzymes. The 10mM methylmethanosuphate (MMS) is soluble in 0.5% DMSO and cyclophosphamide (CPA) is water soluble.

3.2.2 *Rumex lanceolatus* Thunb.

This test was done in duplicate and the average values from scoring approximately 50 metaphases was tabulated (Table 11). *R. lanceolatus* generated CTAs (Fig 28) and CSAs at 0.005, 0.02 and 0.08g/ml without S9. However, only CSAs were generated at 0.005 and 0.02g/ml in the presence of S9 at a reduced level and no aberrations were detected at 0.08g/ml (Fig 29). The percentage of

cells with aberrations decreased in the presence of S9 as a result of phase II enzymes that deactivate the reactive species. However, the chromosome breaks evident at 0.005 and 0.02g/ml in the presence of S9 are attributed to S-phase independent clastogenic metabolites, possibly 2-hydroxy-emodin.

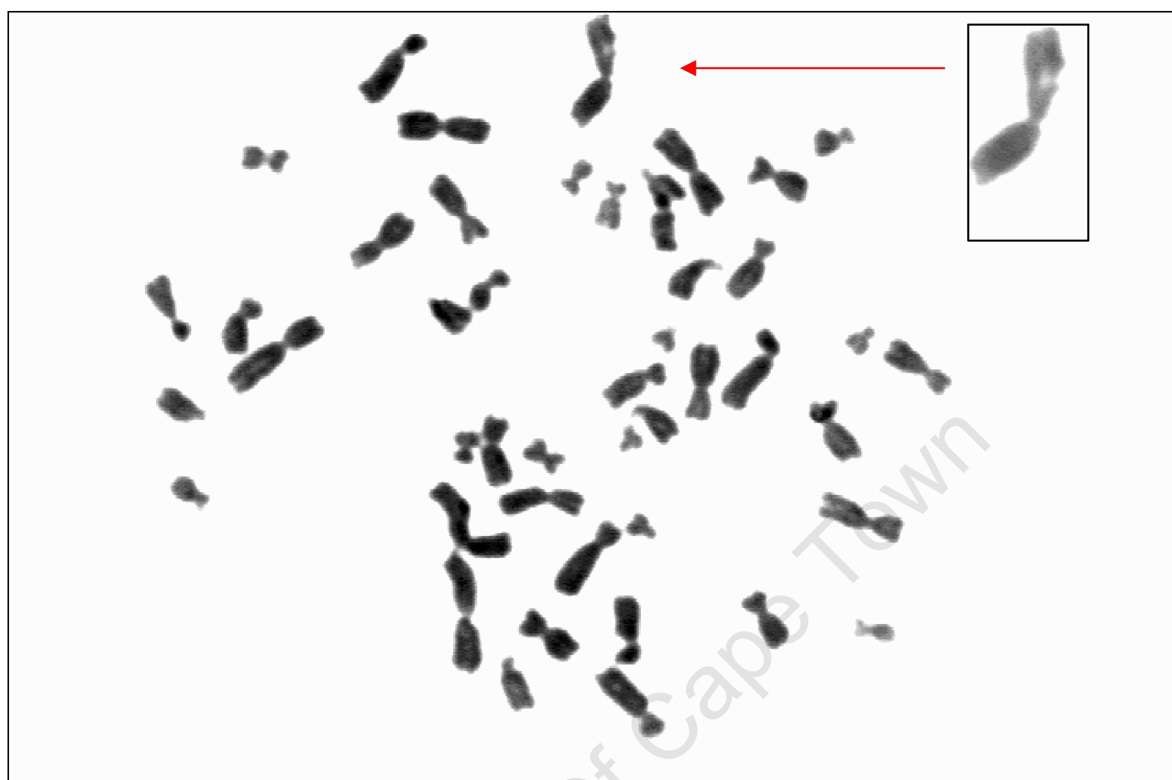

Fig 28. A chromatid gap by treatment of lymphocytes with 0.02g/ml *R. lanceolatus* without S9, indicating direct genotoxicity. The CTA was generated at the G_2 phase of the cell cycle by S-phase dependent phytochemical clastogens in the *R. lanceolatus* extract [Slide Z5, vernier 61 X 103, aberration Z5.1].

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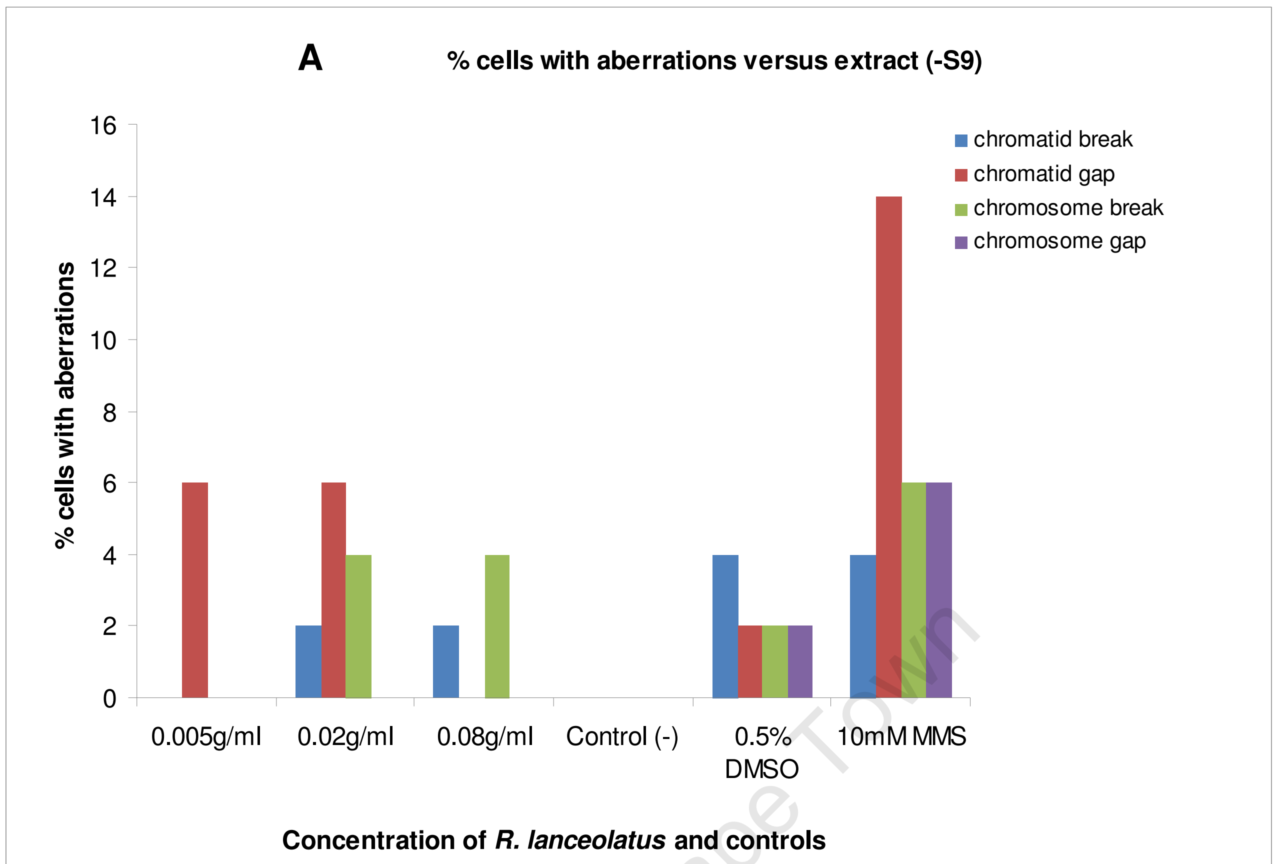
Table 11: Chromosomal aberrations from the treatment of cultures with *R. lanceolatus*

Treatment	Dose (g/ml)	% cells with chromatid aberrations (CTAs)		% cells with chromosome aberrations (CSAs)	
		Gaps	Breaks	Gaps	Breaks
Nonactivation					
R. lanceolatus	0.005	6	0	0	0
	0.02	6	2	0	4
	0.08	0	2	0	4
Negative control (untreated blood)	-	0	0	0	0
0.5% DMSO		2	4	2	2
10mM MMS		14	4	6	6
S9 activation (24hr exposure)					
R. lanceolatus	0.005	0	0	0	2
	0.02	0	0	0	2
	0.08	0	0	0	0
Negative control (untreated blood)		0	0	0	0
2.5mM CPA		15	10	3	3



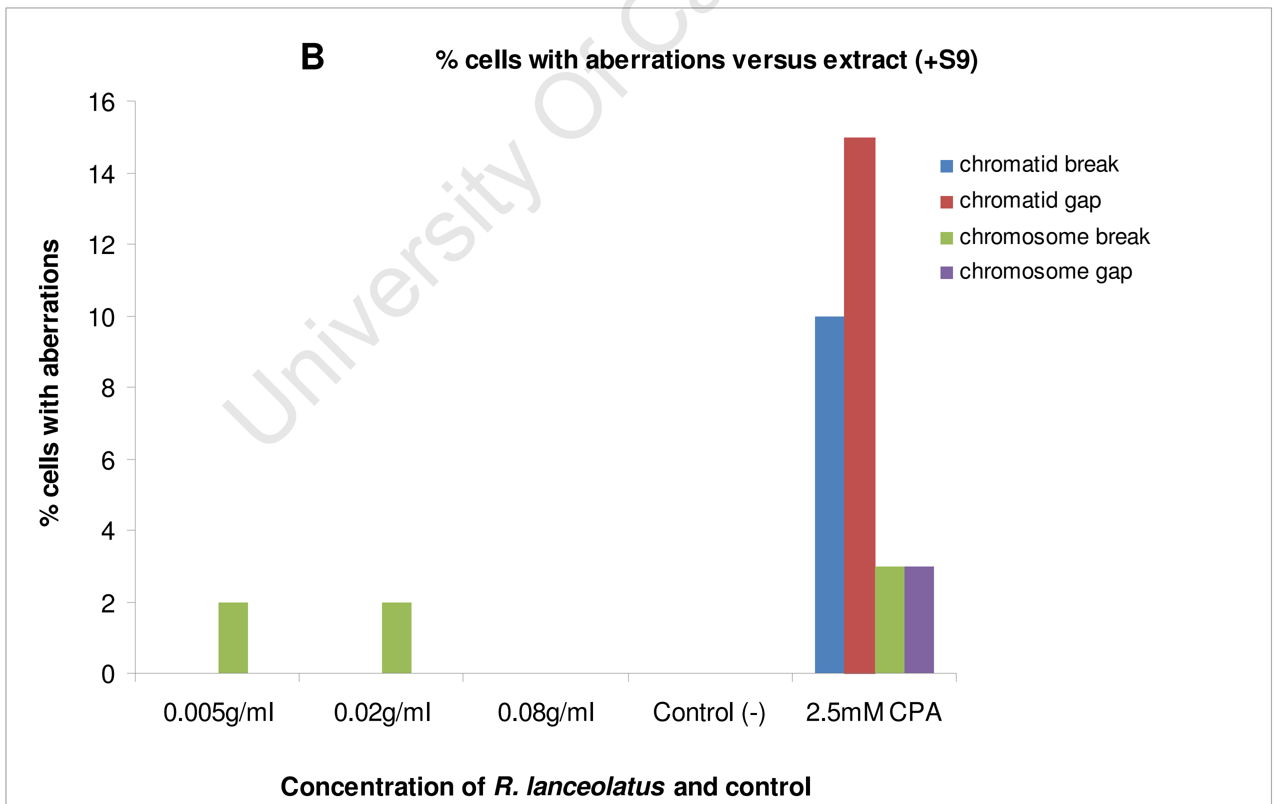


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Fig 29. The treatment of lymphocytes with *R lanceolatus* at three increasing concentrations 0.005, 0.02 and 0.08g/ml in (A) the absence of S9 and (B) the presence of S9 where the chromosomal breaks are reduced from 4 to 2%. Chromosome breaks may translocate or rejoin with a loss of genetic integrity.

3.2.3 *Zantedeschia aethiopica* (L.)

Fig 30a. Chromosome gap caused by treatment of lymphocytes with 0.005g/ml *Z. aethiopica* in the absence of S9 [slide X2, vernier73 X 93, aberration X2.1]

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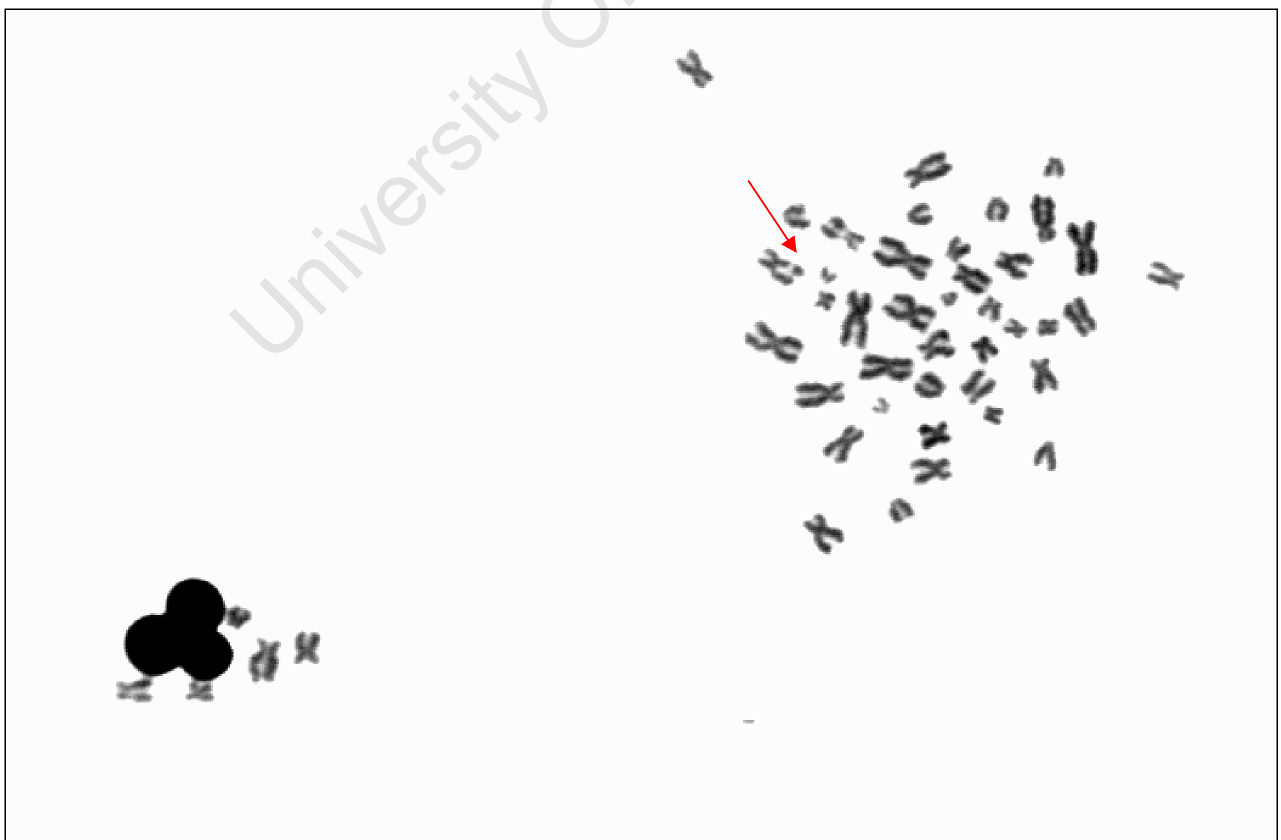

Fig 30b. Chromatid break caused by treatment with 0.005g/ml *Z aethiopica* in the absence of S9 [slide X23, vernier 63 X 86.5]

Table 12: Chromosomal aberrations from the treatment of cultures with *Z. aethiopica*

Treatment	Dose (g/ml)	% cells with chromatid aberrations (CTAs)		% cells with chromosome aberrations (CSAs)	
		Gaps	Breaks	Gaps	Breaks
Nonactivation					
Z. aethiopica	0.00125	0	0	0	0
	0.005	2	2	1	0
	0.02	2	4	2	0
Negative control (untreated blood)	-	0	0	0	0
0.5% DMSO		2	4	2	2
10mM MMS		14	4	6	6
S9 activation (24hr exposure)					
Z. aethiopica	0.00125	0	0	0	0
	0.005	0	0	0	0
	0.02	0	4	0	0
Negative control (untreated blood)		0	0	0	0
2.5mM CPA		15	10	3	3

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The chromosome-type aberrations (CSA) seen in Fig 30a above were generated in the G_o-G_1 phase of the cell cycle from an S-phase independent phytochemical clastogen. The chromatid-type aberration (CTA) seen in Fig 30b above was generated in S or G_2 of the cell cycle from an S-phase dependent phytochemical clastogen. CTAs and CSAs were generated over the concentration range tested, but were reduced in the presence of S9 (Fig 31a,b).

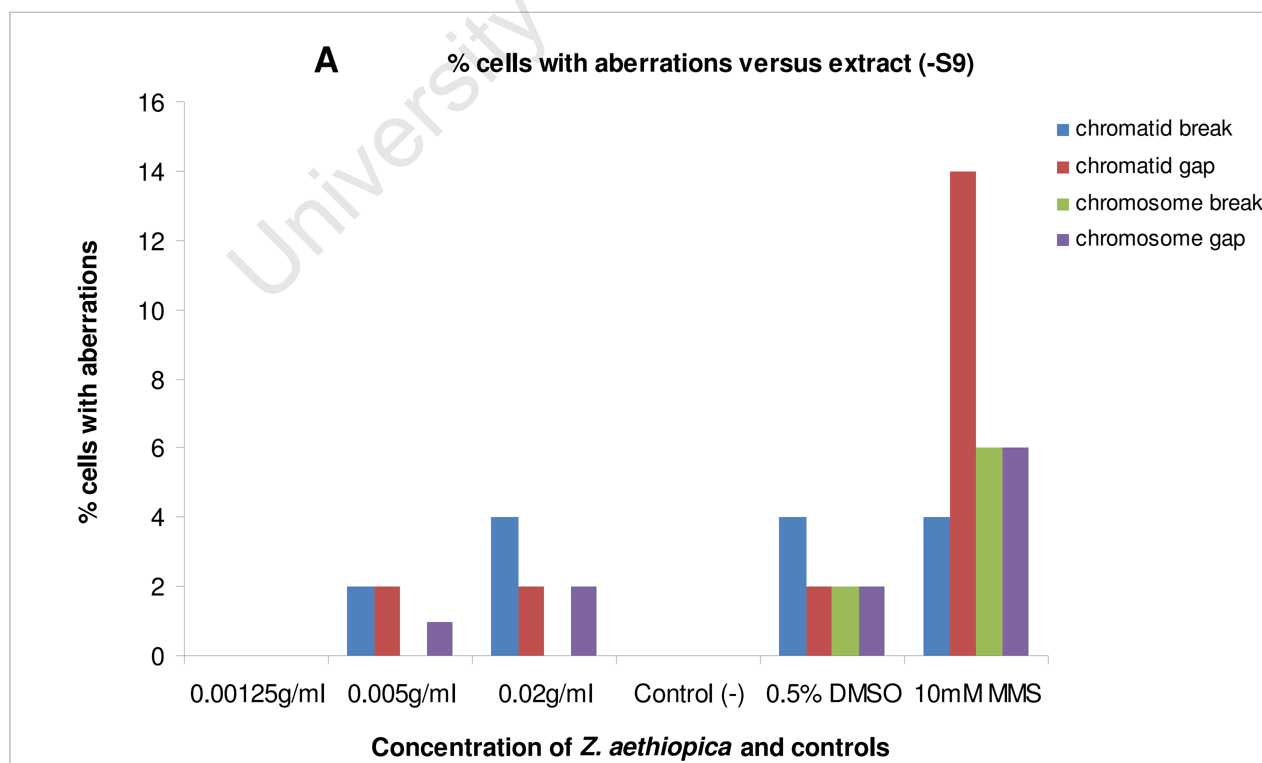

Fig 31a. A dose related increase in chromatid breaks and chromosome gaps was observed with 0.005g/ml and 0.02g/ml *Z. aethiopica* in the absence of S9

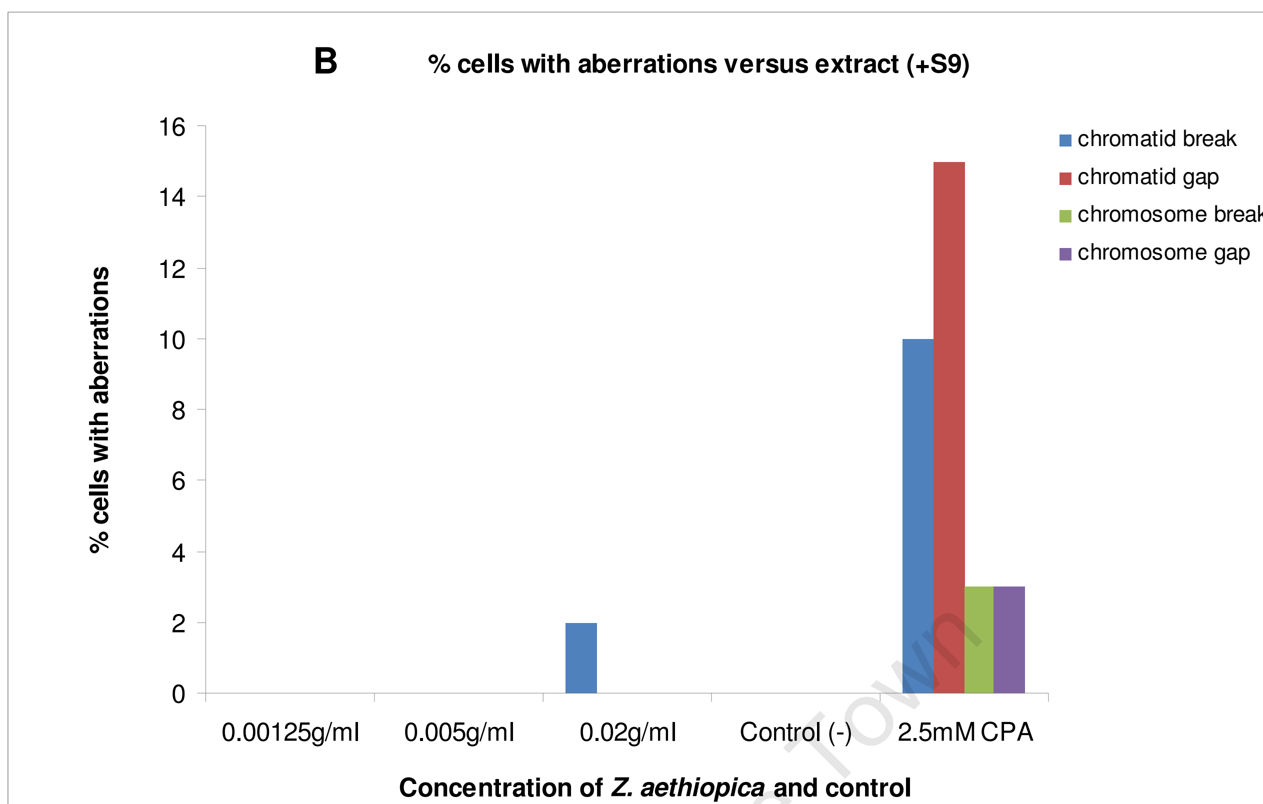

Fig 31b. A reduction in genotoxicity in the presence of S9 was observed for *Z. aethiopica*

3.3 Flow cytometry (DNA content)

A. oppositifolia and *Pelargonium* inhibited mitosis in the chromosomal aberration test and produced no scorable metaphases. Cell cycle arrest was investigated by flow cytometry. Experiments for DNA content were carried out in duplicate at the highest concentration, 0.08g/ml. Since lymphocytes are quiescent (i.e. in G_o phase they are not actively dividing cells), the cultures were stimulated with phytohaemagglutinin (PHA) to enter the cell cycle (G_1, S, G_2-M). After 72hrs at 37°C, the red blood cells were lysed and the DNA content of the white blood cells was determined by flow cytometry. In both treated and untreated cells, ≥95% viable white blood cells remained at the end of the 24hr treatment period. Three distinct populations of white blood cells based on their difference in size lymphocytes<monocytes<granulocytes were visualised. The instrument was configured to measure the DNA content of lymphocytes and the data tabulated in Table 13.

Table 13. DNA content of lymphocytes (average of duplicate values is indicated)

Microculture	PHA	%G_o-G_1	%S	%G_2-M
Blood + PHA (control)	100µl	81±1	19±1	0.1±0
A. oppositifolia 0.08g/ml, -S9	100µl	86±0	14±0	0
A. oppositifolia 0.08g/ml, +S9	100µl	99±1	2±1	0
Pelargonium 0.08g/ml, -S9	100µl	98±1	2±1	0
Pelargonium 0.08g/ml, +S9	100µl	89±1	11±1	0

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The control (blood + PHA) produced 0.1% G_2-M phase cells which was lower than expected (i) due to the fact that lymphocytes are at a much lower concentration in the blood when compared to monocytes and granulocytes; (ii) since lymphocytes are extremely small they were lost during centrifugation at 1500rpm for 5min (recommended for cell lines), which was modified to 2200rpm for 5min at 5°C, but some loss of cells was still experienced; and (iii) because the propidium iodide stain was taken up by all the white blood cells; it did not differentiate the monocytes, granulocytes and lymphocytes and the separation was reliant on size.

The studies of DNA content confirmed an increase in the percentage of the G_o-G_1 phase of the cell cycle in *A. oppositifolia* and *Pelargonium* treated cells with a concomitant decrease in the percentage of S-phase cells when compared to the control (Fig 32). Furthermore, no G_2-M phase

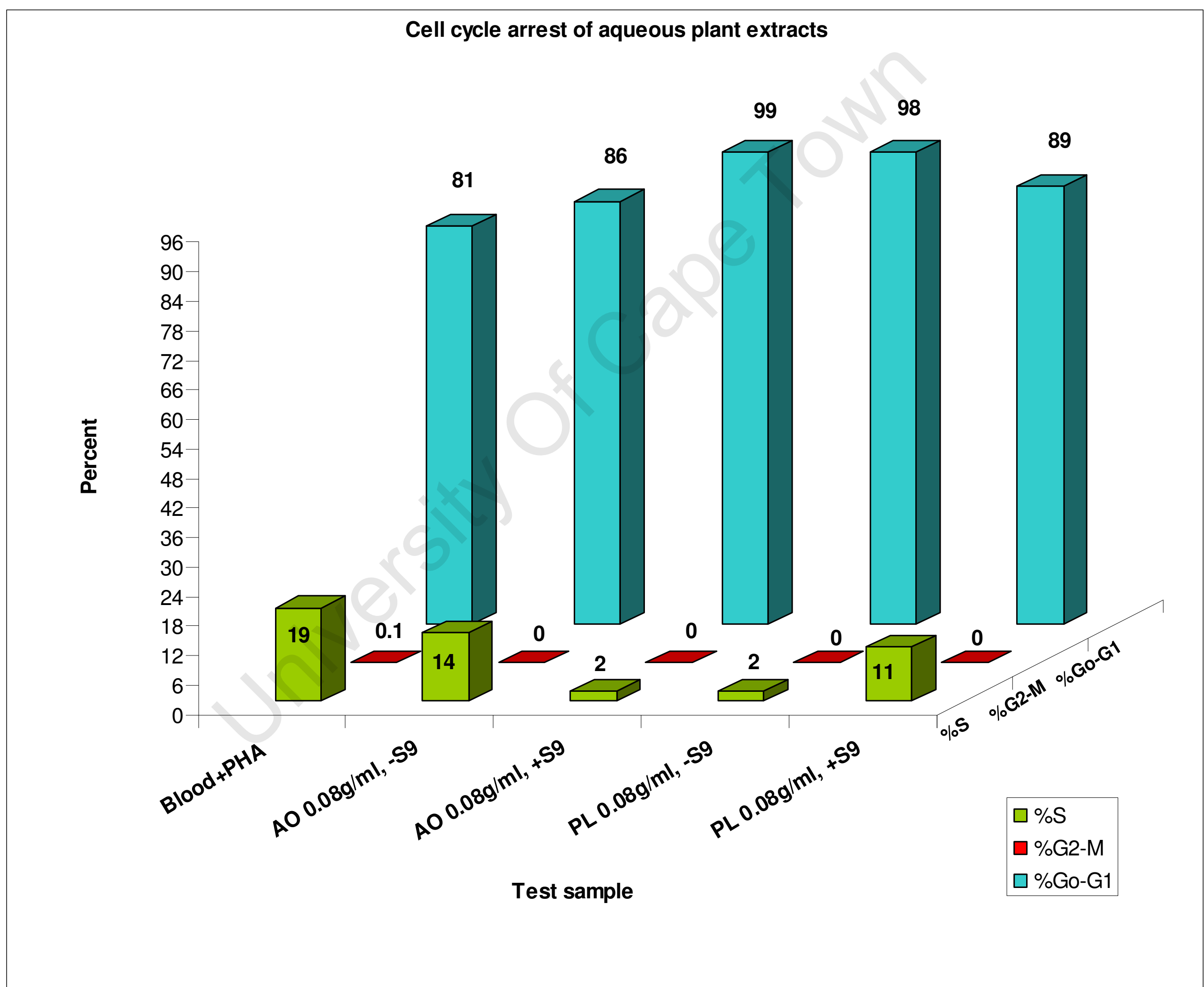

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Fig 32. DNA content of lymphocytes from *A. oppositifolia* and *Pelargonium* sp treatment with and without S9. The G_2-M phase of the cell cycle was absent in all treatment groups with the exception of the control (blood + PHA) which had 0.1% G_2-M phase.

was evident for any of the treated cells. This indicates a G_o/G_1 blockage in the cell cycle and is the reason for the absence of G_2/M phase. *A. oppositifolia* with S9 produced a greater G_o/G_1 blockage (99±1) in the cell cycle when compared to without S9 (86±0). This is indicative that phytochemical

metabolites of *A. oppositifolia* are more potent cell cycle arresting substances. *Pelargonium* without S9 produced a greater G_o/G_1 blockage (98±1) when compared to with S9 (89±1), indicating that phytochemicals in the extract are potential cell cycle arresting substances. These plants should be further investigated for their cancer chemopreventative properties. The studies of DNA content in *A. oppositifolia* and *Pelargonium* confirmed the absence of the G_2-M phase of the cell cycle, indicating a blockage in the G_o/G_1 phase of the cell cycle.

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CHAPTER 4
DISCUSSION

The *Salmonella* reverse mutation assay was used as a primary screen for measuring the ability of plant extracts or metabolites to induce DNA mutations. The extract was judged to be non-mutagenic when tested in at least four *Salmonella* tester strains. A positive response in any single strain either with or without metabolic activation is considered sufficient for designating the extract as a mutagen and a presumptive carcinogen until definitive carcinogenicity studies are performed. The *Salmonella* reverse mutation assay identifies chemicals that react with cellular DNA to produce mutations, whereas the carcinogenicity test identifies only those chemicals that can induce tumour cells that survive and replicate to form visible tumours.

Structural chromosomal aberrations in the form of gaps and breaks were detected in metaphase cells and comprise both chromosome aberrations (CSAs) and chromatid aberrations (CTAs). These aberrations arise from cell cycle progression and in relation to S-phase-independent or dependent clastogens as follows: CSAs result from incompletely repaired or unrepaired double-strand breaks mostly generated *in vivo* in G_o–G_1 lymphocytes by S-phase-independent clastogens (e.g. ionising radiation) (Mateuca *et al*., 2006). After DNA synthesis and chromosome duplication, the aberrations formed in G_o–G_1 are replicated and chromosome-type breaks (CSAs) are seen in metaphase, chromatid-type breaks (CTAs) arise predominantly *in vitro* during the G_2 or late S-phase of the cultured lymphocytes in response to base modifications and single-strand breaks (SSBs) induced by S-phase-dependent clastogens (e.g. chemicals) (Albertini *et al*., 2000 and Hagmar *et al*., 2004).

Evidence from a recent study conducted by Norppa *et al*. (2006) indicates that both chromatid-type and chromosome-type aberrations predict cancer, even though some data suggest that chromosome-type aberrations may have a more pronounced predictive value than chromatid-type aberrations. The relationship between the presence of a high frequency of chromosome aberrations and a predisposition to cancer has been well established in chromosome instability syndromes (e.g. ataxia telangiectasia, Fanconi's anaemia and Bloom's syndrome) (Barrios *et al.,* 1991). Furthermore, structural and numerical chromosome aberrations are evident in bladder carcinomas (gaps or breaks = 25.37%, major structural chromosome abnormalities = 67.16% and numerical chromosome aberrations = 7.46%) (Barrios *et al.,* 1990).

Although peripheral blood lymphocytes are frequently used in the chromosomal aberration assay, the use of whole blood cultures has many disadvantages. The initial concentration of the different cell types in whole blood is unknown at the time of seeding. Red blood cells cause interference during flow cytometry, and the several lysis steps required to remove them result in a decrease in

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the concentration of lymphocytes. In a "whole blood culture environment", there is potential interference between blood components and the extract. In addition, the donor's genetic predisposition to chromosomal aberration frequency will impact on the results. However, although this was not controlled in the current experiment, it is recommended that the test be performed with blood from different donors. Supporting evidence comes from studies showing a decrease in chromosomal aberration frequency in individuals with homozygous genotype for the XPD 751Gln variant allele (Vodicka *et al,* 2004 and Mateuca, 2006). Therefore, an individual's susceptibility to chromosomal aberrations may impact on the test, as peripheral blood lymphocytes from such individuals will be "resilient" to genotoxic agents. Since a high frequency of structural chromosomal aberrations in lymphocytes is predictive of increased cancer risk, it is advisable to use blood from more than one source for the chromosomal aberration test to circumvent the problem encountered with genetic resilience. Cell lines, such as Chinese hamster ovary cells, which were used for the detection of clastogenicity in *Aristolochia* sp. (Zhang *et al*., 2004) would have averted the many problems encountered. The lack of automated systems for colony counting in the *Salmonella* reverse mutation assay and scoring metaphase cells in the chromosomal aberration test presented difficulty. The results of the mutagenicity and clastogenicity studies under the current experimental conditions are discussed in the following sections.

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4.1 *R. lanceolatus*

The aqueous extract prepared from the aerial parts of *R. lanceolatus* contains potent mutagens and pro-mutagens. Mutagenicity was detected with all four tester strains TA97a, TA98, TA100 and TA102 in the presence and absence of S9 ($p \leq 0.01$). Metabolic activation produces strong electrophiles that have a high affinity for DNA, resulting in DNA damage. The phytochemicals and metabolites of *R. lanceolatus* cause DNA damage through a variety of mechanisms: (i) frameshift mutations detected by TA97a and TA98; (ii) base-pair substitutions detected by TA100; and (iii) oxidative DNA damage, transition and transversion reactions (Fig 33) and cross-linking reactions detected by TA102. The compounds present in the *R. lanceolatus* extract are presumptive rodent carcinogens (and potential human carcinogens).

Emodin, a monomeric anthraquinone constituent of *R. lanceolatus* (Watt and Breyer-Brandwijk, 1962), may be responsible for the mutagenicity observed in TA97a, TA98, TA100 and TA102 in this study. Supporting evidence comes from extensive studies conducted in the 1980s, which show that many monomeric anthraquinones, such as emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) and related derivatives that are constituents of *Rheum* (rhubarb), exhibit mutagenicity in the *Salmonella* reversion/microsome tests and cultured tumour cells (Morita *et al*., 1988 and Ueno, 1991). The genus *Rumex* (sorrel, dock) and *Rheum* belong to the same family, Polygonaceae. Emodin is a promutagen and is converted to its active form 2-hydroxy-emodin in the presence of

microsomes (Masuda and Ueno, 1983 and Masuda *et al.*, 1985). CYP P450 catalyses this biotransformation (Ueno 1985 and Masuda *et al.*, 1985).

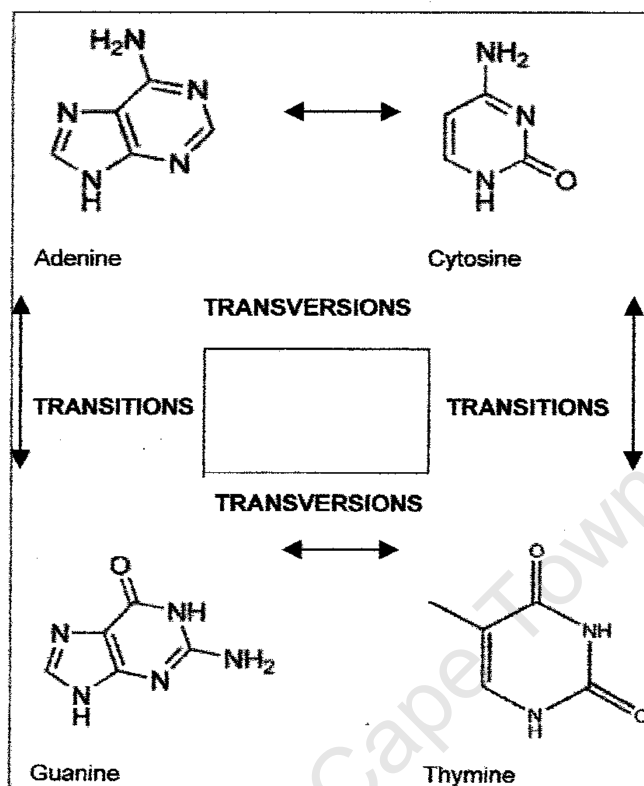

Fig 33. Base substitution reactions by transitions and transversion. Transitions are interchanges of purines (A↔G) or pyrimdines (C↔T); they therefore involve bases of similar shape. Transversions are interchanges between purine and pyrimidine bases, which involve the exchange of one-ring and two-ring structures.

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2-Hydroxy-emodin has a strong electron-spin resonance in a mixture with DNA, which indicates the participation of free radicals derived from anthraquinone in DNA damage (Kodama *et al.*, 1987) and may account for the oxidative DNA damage observed with TA102 in the current investigation. Since hydroxy-radical-forming agents selectively hydroxylate deoxyguanosine residues of DNA at C-8 in *in vivo* and *in vitro* systems, it was presumed that the anthraquinones cause genotoxicity by their potential for hydroxy radical formation (Hayatsu, 1990). These anthraquinones show characteristic colours in solutions and on TLC (thin layer chromatography) plates and this physical property is applied for identification and quantitation in TLC (Ueno, 1982). Furthermore, dimeric anthraquinones, such as luteoskyrin and rugulosin, are hepatotoxic and hepatocarcinogenic in mice (Uraguchi *et al.*, 1972 and Ueno *et al.*, 1980).

In the chromosomal aberration test, *R. lanceolatus* extract produced CTAs and CSAs over the concentration range tested in the presence and absence of S9; however there was a reduction of chromosomal aberrations in the presence of S9, which is ascribed to detoxification. Since CTAs

occur at G_o/G_1 and CSAs at G_2 or the late S-phase of the cell cycle, *R. lanceolatus* extract has both S-phase-dependent and independent clastogens. The study by Schwartz (1989) indicates that alkylating agents are S-phase-dependent clastogenic agents, that is, chromosomal aberrations are not observed unless the treated cells have first undergone a round of replicative DNA synthesis. It can be speculated that CTAs were generated by S-phase-independent chemical compounds originating in the extract or from metabolism. A decreased percentage of cells with aberrations in the presence of S9 indicated the detoxification of clastogenic phytochemicals by CYP P450. In the presence of S9, the 2% CSAs produced at 0.005g/ml and 0.02g/ml is due probably to S-phase-dependent clastogenic metabolites, possibly 2-hydroxy-emodin. Supporting evidence comes from the fact that 2-hydroxy-emodin caused strand breaks in circular phage DNA (Kodama *et al*., 1987) and may be the cause of the clastogenicity observed in the presence of S9 at 0.005 and 0.02g/ml. However, in light of the results from the study by Lee *et al*. (2005), which demonstrated that emodin from the aerial parts of *Rumex acetosa* inhibited the proliferation of tumour cells, was antimutagenic and antigenotoxic, the *R. lanceolatus* extract tested requires further investigation.

The beneficial effects of *Rumex induratus* as a good dietary source of nutrients owing to its agreeable taste, and a high phenolic content cannot be ruled out; however, it has to be approached with caution owing to its high oxalic acid content. No high doses can be consumed because oxalic acid has a predicted LD_{50} value (the mg/kg dose required to kill 50% of a test population) of 375mg/kg body weight (Ferreres *et al*., 2006).

Other effects of *Rumex* sp. are anti-fertility action (*R. steudelii)*, antibacterial and anti-inflammatory activities (*Rumex nervosus* and *Rumex abyssinicus*), antidiarrheal effects (*Rumex maritimus*) and antiviral activities (*Rumex bequaerti)* (Getie *et al*., 2002; Rouf *et al*., 2002; Cos *et al*., 2002 and Gebrie *et al*., 2004). The lessons from the "*Aristolochia* incident" in the early 1980s indicate that mutagenicity tests, such as the *Salmonella* reverse mutation assay and chromosomal aberration, are good indicators of potential carcinogenicity. Extracts of *R. lanceolatus* have produced both mutagenic and clastogenic effects under the current experimental conditions and must be further investigated.

4.2 *Pelargonium sp*.

Perlargonium extract proved to be mutagenic with TA102 in the absence of S9 ($p \leq 0.01$), implicating directly acting mutagens in DNA damage by any of the following: transition/transversion reactions, oxidative DNA damage or cross-linking reactions. *Perlargonium* is known to contain coumarins that interfere with excision repair processes in UV-damaged DNA and with host cell

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reactivation of UV-irradiated phage T1 in *E coli* (Grigg, 1972). TA102 has an intact excision repair system unlike the other three *Salmonella* tester strains, TA7a, TA98 and TA100, which may have been targeted by coumarin in the *Perlargonium* extract. Toxicity was observed at a level of 1% ($p \leq 0.01$) with TA97a in the presence of S9, and was attributed to a metabolically activated phytochemical. Alternatively, this effect may have arisen from antibacterial activity since isoquercetrin and rutin, respective major and minor flavonoids obtained from *Pelargonium radula (Cav.) L'Herit,* were shown to have antimicrobial activity (Pepeljnjak *et al.*, 2005). Under the current experimental conditions *Pelargonium* is a presumptive carcinogen (mutagenic with TA102), and showed *in vitro* cytotoxicity and/or antibacterial activity.

Rose-scented geranium oil from *Pelargonium* sp. is of high value in the fragrance industry, while *P sidoides* root extract commercialised as EPs®7630 (Umckaloabo) has several benefits (refer to Table 2). In a *Pelargonium sidoides* related toxicological study on potential risk related to the coumarin content, a NOEL (no observed effect level) value of more than 750mg/kg body weight was established in dogs and rats (Brendler *et al.*, 2008). NOEL values are generated from the dose-response curves of various agents by plotting "percent of population affected versus dose (mg/kg)". When examining such graphs, there is often a minimum concentration (or threshold) of dose below which there is no observable effect in the test population (e.g. dogs, rats) and this is referred to as the NOEL. The NOEL dose information obtained from animal experimentation is often used to establish safe levels for humans. Similar toxicological studies are warranted for use of *Pelargonium* as a traditional medicine in South Africa due to the toxicity observed with TA97a.

In the chromosomal aberration test, *Pelargonium* caused cell-cycle inhibition of peripheral blood lymphocytes. The studies of DNA content in *Pelargonium* confirmed the absence of G_2-M phase of the cell cycle, indicating a blockage in the G_o/G_1 phase of the cell cycle, therefore the clastogenicity of this extract should be investigated with other test methods. Since the aqueous extract of the aerial parts of this plant caused cell-cycle inhibition further studies for its potential as a cancer chemotherapeutic agent should be undertaken.

4.3 *P. aquilinum*

P. aquilinum produced highly significant mutagenicity with TA102 in the absence of S9 ($p \leq 0.01$), implicating directly acting mutagens in DNA damage by any of the following mechanisms: transition/transversion reactions (Fig 33), oxidative DNA damage and cross-linking reactions. Furthermore *P. aquilinum* proved to be mutagenic with TA100 in the presence of S9 ($p \leq 0.01$), implicating pro-mutagens in DNA damage by means of a mechanism of base-pair substitution. The toxicity observed in TA97a with S9 was significant ($p \leq 0.01$), and since this toxicity was not evident in TA97a in the absence of S9, it was attributed to a chemical that was metabolically activated.

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The experimental results for *P. aquilinum* (bracken fern) indicate both mutagenic and promutagenic activity. Ptaquiloside is responsible for adenine to pyrimidine transversion in the *H-ras* gene on codon 61 (N3 position) (Alonso-Amelot and Avendano, 2002), and therefore it is conceivable that ptaquiloside carries out mutagenicity by purine to pyrimidine transversions in TA102 or by cross-linkage reactions, since both ptaquiloside and pteridienone in bracken fern are alkylating agents. Pteridienone was found to have more pronounced alkylating activity over ptaquiloside (Matsuoka *et al*., 1989). Other chemical constituents of bracken fern, hypolosides and illudins M and S, produced mutagenicity in the *Salmonella* reversion test with TA98 and TA100 (Nagao *et al*., 1989).

It was found that *P. aquilinum* generated CTAs and CSAs in the presence and absence of S9. Schwartz (1989) reported that alkylating agents are S-phase-dependent clastogenic agents, that is, chromosomal aberrations are not observed unless the treated cells have first undergone a replicative DNA synthesis. Therefore, CSAs must be attributed to ptaquiloside and pteridienone, which are alkylating agents. In the presence of S9 the aberrations were reduced by CYP P450 detoxification; however, metabolically activated S-phase-independent clastogens are still evident. Supporting evidence comes from the structural chromosomal aberrations produced in the peritoneal cells of female Swiss mice treated with a variety of bracken fern extracts, including hot and cold water extracts (Almeida Santos *et al*., 2006). Hypoloside B and C are clastogenic at a dose of 4.5µg/ml, and illudin M and S are potent clastogens and induced aberrations at much lower doses than ptaquiloside (Matsuoka *et al*., 1989). The clastogenicity, mutagenicity, carcinogenicity and teratogenicity of *P. aquilinum* has been convincingly demonstrated in prior studies (Kofod and Eyjolfsson, 1966; Kamon and Hiryama, 1975; Hirayama, 1979; Hirono *et al.,* 1984a,1984b; Reddy and Randerath, 1987; Kellerman *et al*., 1988; Matsuoka *et al*., 1989; Sato *et al*., 1991; Shahin *et al*., 1998; Marliere *et al*., 1998, 2000; Alonso-Amelot and Avendano, 2002 and Almeida Santos *et al*., 2006). There is a potential risk for the development of cancer of the upper alimentary tract if this plant is ingested.

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4.4 A. oppositifolia

A. *oppositifolia* proved to be non-mutagenic with all four *Salmonella* tester strains (TA97a, TA98, TA100 and TA102) in the presence and absence of S9. In a study conducted by Wangenheim and Bolcsfoldi (1988), ouabain proved to be mutagenic in a mouse lymphoma assay, however the quantity of ouabain varies in *Acokanthera* sp.; it is present in trace amounts or completely absent in the South African *A. oppositifolia* (Neuwinger, 1996), explaining the non-mutagenicity observed in the current study.

The clastogenicity of *A*. *oppositifolia* was not established owing to the cell-cycle inhibition of peripheral blood lymphocytes in the chromosomal aberration test. The studies of DNA content in *A. oppositifolia* confirmed the absence of G_2-M phase of the cell cycle, indicating a blockage in the

G_o/G_1 phase of the cell cycle, therefore the clastogenicity of this extract should be investigated with other test methods.

Since the South African *A. oppositifolia* contains trace amounts of ouabain (or completely absent); the water-soluble acolongifloriside K showed no *in vivo* activity in the study conducted by Wangenheim and Bolcsfoldi (1988); the aqueous extract of the aerial parts of this plant is non-mutagenic and inhibits mitosis as observed in the current study, this plant extract is a good candidate for further studies into its cancer chemotherapeutic value.

4.5 *Z. aethiopica*

Z. aethiopica proved to be non-mutagenic with all four strains in the presence and absence of S9. *Z. aethiopica* was toxic with TA97a in the presence of S9 and the trend was significant at the 1% level ($p \leq 0.01$). Since this toxicity was not evident in the absence of S9, it was attributed to a chemical that was metabolically activated. *Z. aethiopica* was clastogenic and produced CTAs and CSAs which were reduced in the presence of S9; however, metabolically activated S-phase-dependent clastogens, giving rise to a level of 2% chromatid breaks, were still evident with S9.

Z. aethiopica has characteristic lectins that have the ability to bind to cell membranes, which probably accounts for the precipitation or agglutination of red blood cells observed in the cultures. Further toxicological studies are required since toxicity was observed with TA97a.

4.6 Conclusion and future prospects

It was found that *R. lanceolatus* and *P. aquilinum* are mutagenic in the *Salmonella* reverse mutation assay in the presence and absence of metabolic activation ($p \leq 0.01$), with *R. lanceolatus* showing the highest dose-response. *Pelargonium,* meanwhile, was found to be mutagenic in the absence of metabolic activation ($p \leq 0.01$). Definitive carcinogenicity studies should be conducted on these three extracts, since a positive response in any single strain, either with or without metabolic activation, is considered sufficient for designating the extract a mutagen and a presumptive carcinogen. *In vitro* cytoxicity was observed for *Z. aethiopica* and *Pelargonium* with TA97a in the presence of metabolic activation (S9), therefore toxicology studies would seem to be indicated. *R. lanceolatus*, *P. aquilinum* and *Z. aethiopica* were all found to be clastogenic in the chromosomal aberration test with peripheral blood lymphocytes in the presence and absence of S9; however, a reduction in the number of aberrations was observed in the presence of S9 as a result of detoxification reactions. Both chromatid-type and chromosome-type aberrations are predictive of cancer. Although the number of metaphase cells counted was inadequate for statistical analysis, *Z. aethiopica* should also be included in the carcinogenicity study.

Pelargonium and *A. oppositifolia* could not be examined for clastogenicity in the chromosomal aberration test since they inhibited the cell-cycle; therefore the clastogenicity of this extract should

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be investigated with other test methods. The studies of DNA content of peripheral blood lymphocytes treated with the *A. oppositifolia* and *Pelargonium* extracts confirmed the absence of the G_2-M phase and a blockage in the G_o/G_1 phase of the cell cycle. The South African *A. oppositifolia* is known to contain traces of ouabain (which can be completely absent); in addition, the plant is non-mutagenic and inhibits mitosis as observed in the current study. Other researchers have found no *in vivo* activity in the water-soluble acolongifloriside K, therefore this plant extract is a good candidate for further studies on its potential as a cancer chemotherapeutic agent.

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APPENDIX A (Materials: *Salmonella* reverse mutation assay)

1. 0.5mM Histidine/Biotin solution,

D-Biotin	0.12215g
L-Histidine	0.07758g
Distilled water	to final volume of 1000ml

2. Top Agar

Bacto difco agar	1.5g
Sodium chloride (NaCl)	1.25g
Distilled water	250ml

3. Oxoid nutrient broth

Oxoid nutrient broth No. 2	1.25g
Distilled water	50ml

4. 0.1M NADP

NADP-sodium salt, Sigma ($C_{21}H_{27}N_7O_{17}P_3Na$)	0.7466g or
NADP-disodium salt, Roche ($C_{21}H_{26}N_7O_{17}P_3N_2$)	0.7880g
Cold sterile distilled water	10ml

The solution was filter sterilised in a laminar flow cabinet with a 0.22µm syringe filter. Aliquots of 2ml and 0.4ml were dispensed into cryovials and stored at -20°C.

5. 25X Vogel Bonner Medium E (VB salts), used in minimal agar

Magnesium sulphate 7 hydrate, $MgSO_4.7H_2O$ (BDH)	5g
Citric acid monohydrate, $C_6H_8O_7.H_2O$ (BDH)	50g
Di-potassium hydrogen phosphate, anhydrous, HK_20_4P	250g
Ammonium sodium hydrogen orthophosphate	
$NH_4NaHPO_4.4H_2O$	87.5g
Warmed distilled water (45°C)	to final volume of 1000ml

Dispense two 500ml aliquots into two 1L Schott bottles, and autoclave at 121°C for 20min.

6. 40% Glucose solution

D-(+)-glucose, BDH	400g
Distilled water	to final volume of 1000ml

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Dissolve; divide into two 500ml aliquots using 1L Schott bottles and autoclave at 121°C for 20min.

7. Minimal glucose agar plates (MGA)

Bacto Difco agar	15g
25x VB salts	40ml
40% glucose	50ml
Distilled water	to 1L

Autoclave at 121°C for 20min, cool slightly, add 40ml 25X VB salts and 50ml 40% glucose. Ensure that the solution is thoroughly stirred. Pour 30ml into each 90mm petri plate. Allow to set and store at 4°C.

8. 1M Glucose-6-phosphate (used in the preparation of S9 mix)

D-glucose-6-phosphate (disodium salt hydrate, anhydrous m.wt 304)	2.82g
sterile distilled water	10ml

The solution was filter sterilised with a 0.22µm syringe filter. Aliquots of 1ml and 2ml were dispensed into cryovials and transferred to storage at -4°C.

9. 0.2M Sodium phosphate buffer pH7.4

9.1) 0.2M sodium dihydrogen phosphate, $NaH_2PO_4.H_20$ (m.wt=137g/mol, BDH)	13.8g
9.2) 0.2M disodium hydrogen orthophosphate, anhydrous Na_2HPO_4 (m.wt=142)	14.2g
Distilled water	to final volume of 1000ml

The two solutions were prepared separately and then combined as follows: 60ml of 9.1 and 440ml of 9.2. The pH of the solution was adjusted to 7.4 by adding 9.1, transferred to a 1L Schott bottle and autoclaved at 121°C for 20min. Store the cooled solution in a refrigerator.

10. 2-Acetamidofluorene (2-AAF)

2-AAF	0.00506g
DMSO	2.53ml

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Reagent	2-AAF working standards for mutagenicity assay		
	TA97a (20µg/0.1ml)	**TA98 (1.5µg/0.1ml)**	**TA100 (9µg/0.1ml)**
2-AAF stock (0.002g/ml)	400µl	30µl	180µl
DMSO	3.6ml	3.97ml	3.82ml
Total volume	4ml	4ml	4ml

11. Cumene hydroperoxide

Cumene hydroperoxide (80% solution in cumene, Sigma) 100µl

DMSO to final volume of 50ml

Reagent	CHP working standards for dose response verification				
	20 µg/0.1ml	**50 µg/0.1ml**	**80 µg/0.1ml**	**110 µg/0.1ml**	**140 µg/0.1ml**
CHP stock 1.6mg/ml	0.13ml	0.31ml	0.5ml	0.69ml	0.88ml
DMSO	0.87ml	0.69ml	0.5ml	0.31ml	0.12ml
Total volume	1ml	1ml	1ml	1ml	1ml

12. Salt solution (1.65M KCl + 0.4M $MgCl_2$), used in S9 mix for mutagenicity assay

Potassium chloride (KCl) 61.5g

Magnesium chloride ($MgCl_2.6H_2O$) 40.7g

Distilled water to final volume of 500ml

Autoclave at 121°C for 20min and store at 4°C.

13. Preparation of S9 mix (standard S9 mix, 4%)

Salt solution $MgCl_2$.KCl 1ml

1M Glucose-6-phosphate 0.25ml

0.2M Phosphate buffer pH7.4 25ml

Sterile distilled water 19.75ml

0.1M NADP 2ml

Rat liver S9 2ml

Add the ingredients in the order indicated so that the rat liver S9 will be added to a buffered solution. Thaw a 2ml aliquot of S9 and keep on ice. Preparation is done in a laminar flow hood and all ingredients should be chilled, whilst the S9 mix is prepared on crushed ice. Any left over S9 or S9mix should be discarded. Never refreeze S9.

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14. Nutrient broth agar plates (NBA)

Oxoid nutrient broth No. 2	25g
Bacto difco agar	15g
Distilled water	1000ml

Autoclave at 121°C for 20min, dispense into 90mm petri plates, cool and store at 4°C.

15. Crystal violet solution (0.1%, w/v)

Crystal violet	100mg
Distilled water	100ml

Dissolve the crystal violet in the 100ml of water. Mix well and store at 4°C in a brown glass bottle to protect against light.

16. Ampicillin solution (0.025% w/v)

Ampicillin	2.5mg
Distilled water	10ml

Dissolve the ampicillin in warm (65°C) water. Filter sterilise using a 0.2µm filter and store at 4°C.

18. Ampicillin solution 0.8%, w/v

Ampicillin	0.00295g
Warm sterile distilled water	36.875ml

Filter sterilise using a 0.2µm filter and store at 4°C. Minimal glucose agar (MGA) plates are supplemented with two drops of 0.8% w/v ampicillin solution by spread plating.

17. Tetracycline solution (0.022% w/v)

Tetracycline (Sigma)	0.0022g
Hydrochloric acid (0.02N)	10ml

Oxoid nutrient broth No. 2 (4850ul) was supplemented with 50ul 0.022% w/v tetracycline and 100ul 0.025% w/v ampicillin solution

18. Tetracycline solution (0.8%, w/v)

Tetracycline	0.00140g
Hydrochloric acid (0.02N)	17.5ml

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Dissolve the tetracycline in the 0.02N HCl, filter sterilise with a 0.2µm syringe filter into a sterile bottle, wrap with foil and store at 4°C (tetracycline is light sensitive).

19. <u>Biotin solution (0.01%, w/v)</u>

D-biotin	10mg
Distilled water	100ml

Filter sterilise using a 0.45µm filter and store at 4°C.

20. <u>Histidine solution (0.5% w/v)</u>

L-Histidine	500mg
Distilled water	100ml

Dissolve the histidine in the water, autoclave for 15min at 121°C and store at 4°C.

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APPENDIX B (Materials: Flow activated cell sorting)

1. Rnase Working solution (10µg/ml)

A dilution (1:100) of Rnase was made by adding 40µl Rnase stock (10mg/ml) to 3.96ml 1x PBS. Ensure that there is sufficient Rnase working solution for the number of samples to be analysed. Aliquots of 0.1ml Rnase working solution is added to the pellet.

2. Phosphate Buffered Saline (10x stock)

Sodium chloride (NaCl)	80g
Potassium chloride (KCl)	2g
$Na_2HPO_4.7H_2O$	11.5g
KH_2PO_4	2g

Add water to 1000ml

3. Staining solution

An aliquot of 1ml staining solution per sample is required. Forty millilitre of staining solution is made as follows:

Stock	Stock concentration	Volume for 40ml
Triton X-100	100%	40µl
$MgCl_2$	1M	80µl
NaCl	5M	0.8ml
PIPES	0.1M	4ml
PI (propidium iodide)	1mg/ml	0.4ml
Total		5.32ml
H_2O		34.68ml

4. 0.4% Trypan Blue stain (for viability)

0.4% trypan blue was prepared in 1x PBS

5. Cell lysis buffer

NH_4Cl	8.34g
EDTA	0.037g
$NaHCO_3$	1.00g

Dissolve in 1L distilled water, filter sterilise and store at 4^oC.

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APPENDIX C (*Salmonella* tester strain query)

Subject: FW: Ames strain query (Customer id XMEDI)
Date: Mon, 25 Nov 2002 20:38:22 -0800
From: pgee@discoverypartners.com
To: vikash.sewram@mrc.ac.za
CC: bkuenstler@discoverypartners.com, tcrouch@discoverypartners.com

Dear Dr. Sewram,

The spontaneous in TA102 depends to a large extent to the copy number of the pBR322 plasmid carrying the mutation. I would encourage you to grow the strain under decreasing concentrations of Tet until you get the spontaneous that you want. If you do that however you will also decrease the sensitivity of the strain to mutagens. We routinely divide the plate into sectors and count only a sampling on the plate and average out. We in fact cultivated the strain in such a way to maximize the sensitivity to weak mutagens.

Sometimes it helps to grow the overnight for shorter periods of time, approximately 11-12 hours and the spontaneous for this strain drops also without seemingly sacrificing its sensitivity to weak and very fast reacting chemical species.

Unfortunately we do not have another lot available at this time to send you and will contact you immediately if we have one in the future. We do not have a definite time for when that might be, and whether the next lot that we make will not have similar characteristics.

Sincerely,

Pauline
-----Original Message-----
From: Vikash Sewram [mailto:vikash.sewram@mrc.ac.za]
Sent: Thursday, November 21, 2002 7:53 AM
To: bkuenstler@discoverypartners.com
Subject: Re: Ames strain query (Customer id XMEDI)

Dear Bonnie Kuenstler
Thank you for your prompt reply. I am concerned as to why these spontaneous revertant counts have more than doubled (TA 102). This obviously makes manual counting quite tedious when mutagens are detected and more prone to error. Are there any batches with counts close to the historical values i.e. values quoted by Maron & Ames. We would very much prefer to work with a batch that has values close to those quoted by Maron & Ames as these will compare quite well to tests that we have conducted in the past. I look forward to your response.
Regards
Vikash Sewram

bkuenstler@discoverypartners.com wrote:
> *Dear Vakash Sewram,*
>
> *Our Quality testing shows:*
>
> *TA97a Lot #517 had a range of 196-256 with an average of 226*
> *TA102 Lot #520 had a range of 500-588 with an average of 544*
>
> *So your tests are within the acceptable range. We have found since the*
> *Maron and Ames in 1983 that the numbers have increased and we consider*
these
> *to be good.*
>
> *If you have any further questions please feel free to contact me.*
>
> *Best regards*
>
> *Bonnie Kuenstler*

University of Cape Town

Subject: RE: Ames strain query (Customer id XMEDI)
Date: Wed, 20 Nov 2002 09:23:34 -0800
From: bkuenstler@discoverypartners.com
To: vikash.sewram@mrc.ac.za

Dear Vakash Sewram,

Our Quality testing shows:

TA97a Lot #517 had a range of 196-256 with an average of 226
TA102 Lot #520 had a range of 500-588 with an average of 544

So your tests are within the acceptable range. We have found since the Maron and Ames in 1983 that the numbers have increased and we consider these to be good.

If you have any further questions please feel free to contact me.

Best regards

Bonnie Kuenstler
Material Manager

-----Original Message-----
From: Vikash Sewram [mailto:vikash.sewram@mrc.ac.za]
Sent: Wednesday, November 20, 2002 6:06 AM
To: bkuenstler@discoverypartners.com
Subject: Ames strain query (Customer id XMEDI)
Importance: High

Dear Bonnie Kuenstler
We received our Ames Strains promptly, however I have a few queries which I wish to raise. The
strains were tested for spontaneous reversion and all but TA 97a and TA102 were off-spec. According
to Maron and Ames (1983), the spontaneous revertant counts without S9 should be as follows:
TA97a: 90-180
TA102: 240-320

Mortelmans and Zeiger (2000)
TA97a: 75-200
TA102: 100-300

Our average results are as follows: (-S9)
TA97a: 231 (range 224 - 238)
TA102: 533 (range 521 - 565)

These values are higher than what we have previously obtained on strains that had been supplied to
us by Prof Ames. Please can you check the quality control and let us know as soon as possible on
this batch (lot 517 for TA97a and lot 520 for TA102, Manuf. 09/01), as we do not wish to continue
with these strains until we have recieved some feedback.

Regards
Vikash Sewram
--
****
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REFERENCES

Agbabiaka T.B., Guo R. and Ernst E. (2008) *Pelargonium sidoides* for acute bronchitis: a systematic review and meta-analysis. Phytomedicine 15, p378-385

Agnew M.P. and Lauren D.R. (1991) Determination of ptaquiloside in bracken fern (*Pteridium esculentum*). Journal of Chromatography A. 538, p462-468

Albertini R. J., Anderson D., Douglas G.R., Hagmar L., Hemminki K., Merlo F., Natarajan A.T., Norppa H., Shuker D.E., Tice R., Waters M.D. and Aitio A. (2000) IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans, International Programme on Chemical Safety, Mutat. Res. 463, p111-172.

Almeida Santos Mde F., Dorea J.G. and Luna H. (2006) Bracken-fern extracts can be clastogenic or aneugenic depending on the tissue cell assay. Food and Chemical Toxicology 44, p1845-1848

Alonso-Amelot M.E. and Avendano M. (2002) Human carcinogenesis and brackern fern: a review of the evidence. Current Medicinal Chemistry 9, p675-686

Ames B.N., Mc Cann J. and Yamasaki J. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/Mammalian-microsome mutagenicity test. Mutation Research 31, p347-364

Amorim A.M., Gasques D.G., Andreaus J. and Scharf M. (2002) The application of catalase for the elimination of hydrogen peroxide residues after bleaching of cotton fabrics. Annals of the Brazilian Academy of Sciences (Anais da Academia Brasileira de Ciencias) 74, p433-436

Barre A., Bourne Y., Van Damme E.J.M., Peumans W.J. and Rouge P. (2001) Mannose-binding plant lectins: different structural scaffolds for a common sugar-recognition process. Biochimie 83, p645-651

Barrios L., Miro R., Caballin M.R., Fuster C., Guedea F., Subias A., and Egozcue J. (1990) Chromosome instability in bladder carcinoma patients. Cancer Genet Cytogenet. 49, p107-11

Barrios L., Miro R., Caballin M.R., Miro R., Fuster C., Guedea F., Subias A. and Egozcue J. (1991) Chromosomal instability in breast cancer patients. Human Genet. 88, p39-41

Beil W. and Kilian P. (2007) EPs® 7630, an extract of *Pelargonium sidoides* roots inhibits adherence of Helicobacter pylori to gastric epithelial cells. Phytomedicine 14, p5-8

Bereznoy V.V., Riley D.S., Wassmer G. and Heger M. (2003) Efficacy of the extract of *Pelargonium sidoides* in children with acute non-group A beta-hemolytic streptococcus tonsillopharyngitis: a randomized, double-blind, placebo-controlled trial. Alternative Therapies in Health and Medicine 9, p68-79

Blackwell W.H. (1990) Poisonous and Medicinal Plants. Edited by Szyferblatt G. Prentice-Hall, Inc. Chapters 3-4, p46-47; p65-66

Bonassi S and Au W.W. (2002) Biomarkers in molecular epidemiology studies for health risk prediction, Mutat. Res. 511, p73-86.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, p248-254

Brendler T. and van Wyk B.-E. (2008) A historical, scientific and commercial perspective on the medicinal use of *Pelargonium sidoides* (Geraniaceae). Journal of Ethnopharmacology 119, p420-433

Chan P.C. (1993) NTP technical report on toxicity studies of riddelliine (CAS No. 23246-96-0) administered by Gavage to F344/N rats and B6C3F_1 mice. NIH Publication 94-3350; U.S. Government Printing Office: Washington D.C.

Chan P.C., Mahler J., Bucher J.R., Travlos G.S. and Reid J.B. (1994) Toxicology and carcinogenicity of riddelliine following 13 weeks of treatment to rats and mice. Toxicon 32, p891-908

Chan P.C. (2001) NTP technical report on toxicology and carcinogenesis studies of riddelliine, in F344/N rats and B6C3F_1 mice. NTP TR, 508

University of Cape Town

Chang J.Y., Komaki R., Sasaki R., Liao Z., Stevens C.W., Lu C., Fossella F.V., Allen P.K., Cox J.D., Spitz M.R. and Wu X. (2005) High mutagen sensitivity in peripheral blood lymphocytes predicts poor overall and disease-specific survival in patients with Stage III non-small cell lung cancer treated with radiotherapy and chemotherapy. Clin Cancer Res. 11, p2894-2898

Chen Z., Sun X. and Tang K. (2004) Genomic cloning and characterization of a novel lectin gene from *Zantedeschia aethiopica*. Bioscience Reports 24, p225-234

Clive D., Caspary W., Kirby P.E., Krehl R., Moore M., Mayou J. and Oberly T.J. (1987) Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity. Mutation Research 189, p143-156

Cos P., Hermans N., Bruyne T., De Apers S., Sindambiwe J.S., Witvrouw M., Clerq E., De Berghe D.V., Pieters L. and Vlietnick A.J. (2002) Antiviral activity of Rwandan medicinal plants against human immunodeficiency virus type-1 HIV-1. Phytomedicine 9, p62-68.

Coulombe R.A. Jr, Drew G.L. and Stermitz F.R. (1999) Pyrrolizidine alkaloids crosslink DNA with actin. Toxicol. Appl. Pharmacol. 154, p198-202

Craig W.J. (1999) Health promoting properties of common herbs. Am J Clin Nutr. 70, p491-499

Culvenor C.C.J. and Jago M.V. (1979) Chapter 6: Carcinogenic Plant Products and DNA. In Chemical Carcinogens and DNA, Volume I, Edited by Grover P.L., CRC Press, Inc. P176

Czygan P., Griem H., Garro A.J., Hutterer F., Schaffner F., Popper H., Rosenthal O. and Cooper D.Y. (1973) Microsomal metabolism of dimethylnitrosamine and the cytochrome P450 dependency of its activation to a mutagen. Cancer Research 33, p2983-2986

Dictionary of Natural Products, CRC Press, CD-ROM, Version 11.2 (2003)

Eldeen I.M.S., Elgorashi E.E. and van Staden J. (2005) Antibacterial, anti-inflammatory, anti-cholinesterase and mutagenic effects of extracts obtained from some trees used in South African traditional medicine. Journal of Ethnopharmacology 102, p457-464

Elgorashi E.E. and van Staden J. (2004) Pharmacological screening of six Amaryllidaceae species. Journal of Ethnopharmacology 90, p27-32

Fennell C.W., Lindsey K.L., McGaw L.J., Sparg S.G., Stafford G.I., Elgorashi E.E., Grace O.M. and van Staden J. (2004) Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. Journal of Ethnopharmacology 94, p205-217

Ferreres F., Ribeiro V., Izquierdo A.G., Rodrigues M.N., Seabra R.M., Andrade P.B. and Valento P. (2006) *Rumex induratus* Leaves: Interesting dietary source of potential bioactive compounds. Journal of Agricultural and Food Chemistry 54, p5782-5789.

Fonseca C.A.S, Otto S.S., Paumgartten F.J.R. and Leitao A.C. (2000) Nontoxic, mutagenic, and clastogenic activities of Mate-Chimarroa (*Ilex paraguariensis*). Journal of Environmental Pathology, Toxicology and Oncology 19, p333-346

Friedman M. (2004) Analysis of biological active compounds in potatoes (*Solanum tuberosum*), tomatoes (*Lycopersicon esculentum*), and jimson weed (*Datura stramonium*) seeds. Journal of Chromatography A 1054, p143-155

Fu P.P., Xia Q., Lin G. and Chou M.W. (2004) Pyrrolizidine Alkaloids: genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. Drug Metabolism Reviews 36, p1-55

Galloway S.M., Aardema M.J., Ishidate M. Jr, Ivett J.L., Kirkland T., Morita T., Mosesso P. and Sofuni T. (1994) Report from working group on in vitro tests for chromosomal aberrations. Mutation Research 312, p241-261

Garner R.C., Miller E.C. and Miller J.A. (1972) Liver microsomal metabolism of aflatoxin B1 to a reactive derivative toxic to *Salmonella typhimurium* TA1530. Cancer Res. 32, p2058-2066

University of Cape Town

Gebrie E., Makonnen E., Debella A. and Zerihun L. (2004) Phytochemical screening and pharmacological evaluations for the antifertility effect of the methanolic root extract of *Rumex steudelii*. Journal of Ethnopharmacology 96, p139-143

Getie M., Gebre-Mariam T., Rietz R., Hohne C., Huschka C., Schmidtke M., Abate A. and Neubert R.H.H. (2002) Evaluation of the anti-microbial and anti-inflammatory activities of the medicinal plants *Dodonaea viscose, Rumex nervosus and Rumex abyssinicus.* Fitoterapia 74, p139-143

Grigg G.W. (1972) Effects of coumarins, pyronin Y, 6,9-dimethyl-2-methylthiopurine and caffeine on excision repair and recombination repair in *Escherichia coli*. J. Gen. Microbiol. 70, p221

Guengerich F.P. and Shimada T. (1991) Oxidation of Toxic and carcinogenic chemicals by human Cytochrome P450 enzymes. Chemical Research in Toxicology 4, p391-407

Guengerich F.P (2001) Forging the links between metabolism and carcinogenesis. Mutation Research 488, p195-209

Gurib-Fakim A. (2006) Medicinal plants: Traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine 27, p1-93

Hagmar L., Stromberg U., Bonassi S., Hansteen I.-L, Knudsen L.E., Lindholm C. and Norppa H. (2004) Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts. Cancer Res. 64, p2258-2263

Hayatsu H. and Sorsa V. (1990) Mutagens in Food: Detection and Prevention, CRC Press, p120-125

Hincks J.R., Kim H.Y., Segall H.J., Molynuex R.J. and Stermitz F.R. (1991) DNA cross linking in mammalian cells by pyrrolizidine alkaloids: structure-activity relationships, Toxicol. Appl. Pharmacol. 111, p90-98

Hirayama T. (1979) Diet and Cancer. Nutrition and Cancer 1, p67

Hirono I., Shibuya C., Shimizu M. and Fushimi K. (1972) Carcinogenic Activity of Processed Bracken Used as Human Food. J. National Cancer Inst 48, p1245-1250

Hirono, I., Kono Y., Takahashi K., Yamada K., Niwa H., Ojika M., Kigoshi H., Niyama K. and Uosaki Y. (1984a) Reproduction of acute bracken poisoning in calf with ptaquiloside, a bracken constituent. Vet Rec. 115, p375

Hirono I., Aiso S., Yamaji T., Mori H., Yamada K., Niwa H., Ojika M., Wakamatsu K., Kigoshi H., Niiyama K., Uosaki Y. (1984b) Carcinogenicity in rats of ptaquiloside isolated from bracken. Gann: Japanese Journal of Cancer Research 75, p833

IARC Scientific Publications No 153 (2003) Cancer in Africa: Epidemiology and Prevention. Edited by Parkin D.M, Ferlay J., Hamdi-Cherif M., Sitas F., Thomas J., Warbinga H. and Whelan S.L., p345-352

Kamon S. and Hirayama T. (1975) Proc. Jpn. Cancer Assoc. 34, p211

Kellerman T.S., Coetzer J.A.W. and Naude T.W. (1988) Plant Poisonings and Mycotoxicoses of Livestock in Southern Africa. Oxford University Press, Cape Town. p62-63; 86 and 204-206

Kigoshi H., Imamura Y., Niwa H. and Yamada K. (1989) Total synthesis of ptaquilosin: the aglycon of ptaquiloside, a potent bracken carcinogen. J. Am. Chem. Soc. 111, p2302-2303

Kigoshi H., Imamura Y, Mizuta K., Niwa H. and Yamada K. (1993) Total synthesis of natural (-)-ptaquilosin, the aglycon of a potent bracken carcinogen ptaquiloside, and the (+)-enantiomer and their DNA cleaving activities. J. Am. Chem. Soc. 115, p3056-3065

Kingston David G.I. and Reichstein T. (1974) Cytotoxic cardenolides from *Acokanthera longiflora* stapf. and related species. Journal of Pharmaceutical Sciences 63, p462-464

Kirsch-Volders M., Vanhauwaert A., Eichenlaub-Ritter U. and Decordier I. (2003) Indirect mechanisms of genotoxicity. Toxicology Letters 140-141, p63-74.

University of Cape Town

Kodama M., Kamioka Y., Nakayama T., Nagata C., Marooka N. and Ueno Y. (1987) Generation of free radicals and hydrogen peroxide from 2-hydroxyemodin, a direct acting mutagen, and DNA strand breaks by active oxygen. Toxicol. Lett. 37, p149.

Kofod H. and Eyjolfsson R. (1966) The isolation of cyanogenic glycoside prunasin from *Pteridium aquilinum* (L.) Kuhn. Tetrahedron Lett. 12, p1289-1291

Lee J.J., Trizna Z., Hsu T.C., Spitz M.R. and Hong W.K. (1996) A statistical analysis of the reliability and classification error in application of the mutagen sensitivity assay. Cancer Epidemiol Biomarkers Prev. 5, p191-197.

Lee N.J., Choi J.H., Koo B.S., Ryu S.Y., Han Y.H., Lee S.I. and Lee D.U. (2005) Antimutagenicity and cytotoxicity of the constituents from the aerial parts of *Rumex acetosa*. Biol Pharm Bull. 28, p2158-2161

Lewis R. (1997) Human Genetics: concepts and applications, 2nd Ed. Wm. C. Brown Publishers, Editor: E Sievers, p32

Lewis R.A. (1998) Lewis' Dictionary of Toxicology. CRC Press, p108

Liou S.H., Lung J.C., Chen Y.H., Yang T., Hsieh L.L., C.J. Chen, Wu T.N. (1999) Increased chromosome-type chromosome aberration frequencies as biomarkers of cancer risk in a blackfoot endemic area. Cancer Res. 59, p1481-1484

Marliere C.A., Santos R.C., Galvao M.A.M, Soares J.F., Evangelista C.L.M. and Gomes R.F.Q. (1998) Rev. Bras. Cancerol. 44, p225

Marliere C.A., Wathern P., Freitas S.N., Castro M.C. and Galvao M.A.M. (2000) In Bracken fern toxicity, biology and control. Edited by Taylor J.A. and Smith R.T. International Bracken Group Special Publication No 4. Manchester, UK, p144

Maron D.M. and Ames B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. Mutation Research 113, p173-215

Masuda T. and Ueno Y. (1983) Microsomal transformation of emodin into a direct mutagen. Mutation Res 125, p135

Masuda T., Haraikawa K., Marooka N., Nakano S. and Ueno Y. (1985) 2-Hydroxyemodin, an active metabolite of emodin in the hepatic microsomes of rats. Mutation Res.149, p327.

Mateuca R., Lombaert N., Aka P.V., Decordier I. and Kirsch-Volders M. (2006) Chromosomal changes: induction, detection methods and applicability in human biomonitoring. Biochimie 88, p1515-1531

Matoba M., Saito E., Saito K., Koyama K., Natori S., Matsushima T. and Takimoto M. (1987) Assay of ptaquiloside, the carcinogenic principle of bracken, *Pteridium aquilinum*, by mutagenicity testing in *Salmonella typhimurium*. Mutagenesis 2, p419-423

Matsuoka A., Hirosawa A., Natori S., Iwasaki S., Sofuni T., Ishidate M. Jr (1989) Mutagenicity of ptaquiloside, the carcinogen in bracken, and its related illudane-type sesquiterpene II. Chromosomal aberration tests with cultured mammalian cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 215, p179-185

McKay D.L. and Blumberg J.B. (2006) A review of the bioactivity of South African herbal teas: rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*). Phytotherapy Research 21, p1-16

Meinl W., Pabel U.; Osterioh-Quiroz M.; Hengstler J.G. and Glatt H. (2006) Human sulphotransferases are involved in the activation of aristolochic acids and are expressed in renal target tissue. Int. J. Cancer 118, p1090-1097

Middleton E. Jr, Kandaswami C. and Theoharides T.C. (2000) The effects of Plant Flavonoids on Mammalian Cells: Implications for Inflammation, Heart Disease, and Cancer. Pharmacological Reviews 52, p673-751

University Of Cape Town

Miraldi E., Masti A., Ferri S. and Comparini I.B. (2001) Distribution of hyoscyamine and scopolamine in *Datura stramonium*. Fitoterapia 72, p644-648

Morita H., Umeda M., Masuda T. and Ueno Y. (1988) Cytotoxic and mutagenic effects of emodin on cultured mouse carcinoma EM3A cells. Mutation Research 204, p329

Mortelmans K. and Zieger E. (2000) The Ames *Salmonella*/microsome mutagenicity assay. Mutation Research 455, p29-60

Moshi M.J. and Mbwambo Z.H. (2005) Some pharmacological properties of extracts of *Terminalia sericea* roots. Journal of Ethnopharmacology 72, p247-263.

Nagao T., Saito K., Hirayama E., Uchikoshi K., Koyama K., Natori S., Morisaki N., Iwasaki S. and Matsushima T. (1989) Mutagenicity of ptaquiloside, the carcinogen in bracken, and its related illudane-type sesquiterpenes. I. Mutagenicity in *Salmonella typhimurium*. Mutat. Res. 215, p173-178

Neuwinger H.D. (1996) African Ethnobotany. Poisons and drugs. Chapman and Hall, London. p78-80

Niwa H., Ojika M., Wakamatsu, K., Yamada K., Hirone I. and Matsushita K. (1983) Ptaquiloside, a novel norsesquiterpene glucoside from bracken. Tetrahedron Letters 24, p4117-4120

Norppa H., Bonassi S., Hansteen I.-L, Hagmar L., Stromberg U., Rossner P., Boffetta P., Lindholm C., Gundy S., Lazutka J., Cebulska-Wasilewska A., Fabianova E., Sram R.J., Knudsen L.E., Barale R. and Fucic A. (2006) Chromosomal aberrations and SCEs as biomarkers of cancer risk. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 600, p37-45

Omura T. and Sato R. (1964) The carbon monoxide-binding pigment of liver microsomes. The Journal of Biological Chemistry 239, p2370-2378

Padwa A., Sandanayaka V.P. and Curtis E.A. (1994) Synthetic studies toward illudins and ptaquilosin. A highly convergent approach via the dipolar cycloaddition of Carbonyl Ylides. J. Am. Chem. Soc. 116, p2667-2668

Pepeljnjak S., Kalodera Z. and Zovko M. (2005) Antimicrobial activity of flavonoids from *Pelargonium radula (Cav.) L' Herit*. Acta Pharm. 55, p431-435

Rao Rajeswara B.R., Kaul P.N., Syamasundar K.V. and Ramesh S. (2002) Water soluble fractions of rose-scented geranium (*Pelargonium* species) essential oil. Bioresour Technol. 84, p243-246

Reddy M.V. and Randerath K. (1987) ^{32}P-postlabelling assay for carcinogen-DNA adducts: nuclease P_1-mediated enhancement of its sensitivity and applications. Environmental Health Perspectives 76, p41-47

Reed R.L., Ahern K.G., Pearson G.D. and Buhler D.R. (1988) Crosslinking of DNA by dehydroretronecine, a metabolite of pyrrolizidine alkaloids. Carcinogenesis 9, p1355-1356

Reid K.A., Maes J., Maes A., van Staden J., De Kimpe N., Mulholland D.A. and Verschaeve L. (2006) Evaluation of the mutagenic and antimutagenic effects of South African plants. Journal of Ethnopharmacology 106, p44-50

Roberts M. (1990) Indigenous Healing Plants. Southern Book Publishers, Halfway House

Rouf A.S.S., Islam M.S. and Rahman M.T. (2002) Evaluation of anti-diarrheal activity of *Rumex maritimus* root. J. Ethnopharmacol. 84, p307-310

Sammon A.M. (1992) A case-control study of diet and social factors in cancer of the esophagus in Transkei. Cancer 69, p860-865

Sato T., Indaba H., Kawai K., Furukawa H., Hirono I., Miyazawa T. (1991) Low-level chemiluminescence from *Drosophila melanogaster* fed with chemical mutagens polycyclic aromatic hydrocarbon quinines and a carcinogenic bracken fern. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 251, p91-97

University of Cape Town

Savage J.R.K. (1975) Classification and relationships of induced chromosomal structural changes. Journal of Medical Genetics 12, p103-122

Savage J.R.K. (2004) On the nature of visible chromosomal gaps and breaks. Cytogenet Genome Res. 104, p46-55

Schwartz J.L. (1989) Monofunctional alkylating agent-induced S-phase dependent DNA damage. Mutat Res. 216, p111-8

Sewram V. (2006) Risk factors for oesophageal cancer in the Eastern Cape Province of South Africa. PhD (Public Health) in the School of Public Health and Family Medicine, Faculty of Health Science, University of Cape Town

Shahin M, Smith B.L., Worral S. and Moore M.R. (1998) Bracken fern carcinogenesis: multiple intravenous doses of activated ptaquiloside induce DNA adducts, monocytosis, increased TNF∝ levels, and mammary gland carcinoma in rats. Biochemical and Biophysical Research Communications 244, p192-197

Steenkamp V., Stewart M.J. and Zuckerman M. (2000) Clinical and analytical aspects of pyrrolizidine poisoning caused by South African traditional medicines. Ther Drug Monit. 22, p302-306

Steenkamp V., Stewart M.J., van der Merwe S., Zuckerman M. and Crowther N.J. (2001) The effect of *Senecio latifolius* a plant used as a South African traditional medicine, on a human hepatoma cell line. Journal of Ethnopharmacology 78, p51-58

Steenkamp P.A., Harding N.M., van Heerden F.R. and van Wyk B.-E (2004) Fatal *Datura* poisoning: Identification of atropine and scopolamine by high performance liquid chromatography/photodiode array/mass spectrometry. Forensic Science International 145, p31-39.

Stickel F. and Seitz H.K. (2000) The efficacy and safety of comfrey. Public Health Nutrition 3, p501-508

Tadeg H., Mohammed E., Asres K. and Gebre-Mariam T. (2005) Antimicrobial activities of some selected traditional Ethiopian medicinal plants used in the treatment of skin disorders. Journal of Ethnopharmacology 100, p168-175

Taylor J.L.S, Elgorashi E.E., Maes A., Van Gorp U., De Kimpe N., van Staden J. and Verschaeve L. (2003) Investigating the safety of plants used in South African traditional medicine: testing for genotoxicity in the micronucleus and alkaline comet assays. Environmental and Molecular Mutagenesis 42, p144-154

The review of natural products (2002) Facts and comparison s (3rd Edition): St Louis, MO, p772

Trindade H., Karmali A. and Pais M.S. (1988) One-step purification and properties of catalase from leaves of *Zantedeschia aethiopica*. Biochimie 70, p 1759-1764

Ueno Y., Sato N., Ito T., Ueno I., Enomoto M. and Tsunodo H. (1980) Chronic toxicity and hepatocarcinogenicity of (+) rugulosin, anthraquinoid mycotoxin from *Penicillium* sp: Preliminary surveys in mice. J. Toxicol. Sci. 5, p295.

Ueno Y. (1982) Thin-layer chromatographic determination of luteoskyrin in rice grains, in Environmental Carcinogens Selected Methods of Analysis, Vol 5, Egan H. Ed. International Agency for Research on Cancer, Lyon, France, 450.

Ueno Y. (1985) Biotransformation of mycotoxins in the reconstituted cytochrome P450 system. Proc. Jpn. Assoc. Mycotoxicol. 22, p28.

Ueno Y. (1991) Chapter 6.2: Mycotoxins, In *Mutagens in Food: Detection and Prevention*, Edited by H. Hayatsu, CRC Press, p120-121

Uraguchi K., Saito M., Noguchi Y., Takahashi K., Enomoto M. and Tatsuno T. (1972). Chronic toxicity and carcinogenicity in mice of purified mycotoxins luteoskyrin and cyclochlorotine. Food Cosmet. Toxicol. 10, p193.

Vahrmeijer J. (1981) Poisonous Plants of South Africa that cause stock losses. Tafelberg Publishers Limited, Cape Town. p28-29

University of Cape Town

Van der Hoeven, Lagerweij W.J., Posthumus M.A., van Veldhuizen A. and Holterman H.A.J (1983) Aquilide A, a new mutagenic compound isolated from bracken fern (*Pteridium aquilinum* (L.) Kuhn). Carcinogenesis 4, p1587-1590

Van der Merwe J.D., Joubert E., Richard E.S., Manley M., Snijman P.W., Marnewick J.L. and Gelderblom W.C. (2006) A comparative study on the antimutagenic properties of aqueous extracts of *Aspalathus linearis* (rooibos), different *Cyclopia* spp. (honeybush) and *Camellia sinensis* teas. Mutation Research 611, p42-53

Van Wyk B-E., van Oudstoorn B. and Gericke N. (1997) Medicinal plants of South Africa. 1st Ed, edited by Smit J., Briza Publications, p8, 10, 12, 14, 16, 20, 22, 26, 102-103, 218, 280, 288-289

Van Wyk B-E. and Gericke N. (2000) People's Plants. 1st Ed, Edited by Smit J., Briza Publications, p74, 132, 202, 238

Van Wyk B.-E.; van Heerden F. and van Oudtshoorn B. (2002) Poisonous Plants of South Africa. 1st Ed, edited by Ferreira R., Briza Publications, p6-7, 18-28, 34, 172, 261-271

Van Wyk B.-E and Wink M. (2004) Medicinal Plants of the World. Edited by Ferreira R., Briza Publications. p231, 278, 310, 387, 395-397, 420-425

Verschaeve L., Kerstens V., Taylor J.L.S., Elgorashi E.E., Maes A., Van Puyvelde L., De Kimpe N. and Van Staden J. (2004) Investigation of the antimutagenic effects of selected South African medicinal plant extracts. Toxicology In Vitro 18, p29-35

Vodicka P., Kumar R., Stetina R., Sanyal S., Soucel P., Haufroid V., Dusinska M., Kuricova M., Zamecnikova M., Musak L., Buchancova J., Norppa H., Hirvonen A., Vodickova L., Naccarati A., Matousu Z. and Hemminki K. (2004) Genetic polymorphisms in DNA repair genes and possible links with DNA repair rates, chromosomal aberrations and single-strand breaks in DNA. Carcinogenesis 25, p757-763

Wangenheim J. and Bolcsfoldi G. (1988) Mouse lymphoma L5178Y thymidine kinase locus assay of 50 compounds. Mutagenesis 3, p193-205

Watt J.M. and Breyer-Brandwijk M.G. (1962) The Medicinal and Poisonous Plants of Southern and Eastern Africa. 2nd Ed. E&S Livingstone LTD, London, U.K., p115-116; 453-455; 867; 1089-1093

Williams D.E., Reed R.L., Kedzierski B., Dannan G.A., Guengerich F.P. and Buhler D.R. (1989) Bioactivation and detoxification of the pyrrolizidine alkaloid senecionine by cytochrome P450 enzymes in rat liver. Drug Metab. Dispos. 17, p387-392

Yang Y.C., Yan J., Doerge D.R., Chan P.C., Fu P.P. and Chou M.W. (2001a) Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation *in vivo*. Chem. Res. Toxicol. 14, p101-109

Yang Y.C., Yan J., Churchell M., Berger R., Chan P.C., Doerge D.R., Fu P.P. and Chou M.W. (2001b) Development of a 32P-postlabelling/HPLC method for detection of dehydroretronecine-derived DNA adducts *in vivo* and *in vitro*. Chem. Res. Toxicol. 14, p91-100

Zhang H., Cifone M.A., Murli H., Erexson G.L., Mecchi M.S. and Lawlor T.E. (2004) Application of simplified *in vitro* screening tests to detect genotoxicity of aristolochic acid. Food and Chemical Toxicology 42, p2021-2028

Zhou S., Koh H-L., Gao Y., Gong Z-Y. and Lee E.J.D. (2004) Minireview. Herbal bioactivation: the good, the bad and the ugly. Life Sciences 74, p935-968

Zeiger E. (1998) Identification of rodent carcinogens and non-carcinogens using genetic toxicity tests: premises, promises, and performance. Regul. Toxicol. Pharmacol. 28, p85-95

Zeiger E. (2001) Mutagens that are not carcinogens: faulty theory or faulty tests. Mutation Research 492, p29-38

University of Cape Town