The *in vitro* inhibition of genotoxicity by plant extracts and the isolation and characterization of antimutagenic compounds from *Combretum microphyllum* (Combretaceae)

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The *in vitro* inhibition of genotoxicity by plant extracts and the isolation and characterization of antimutagenic compounds from *Combretum microphyllum* (Combretaceae)

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(BSc Hons Biochemistry, MSc Vet.Sci.)

Thesis submitted in fulfilment of the requirements for the degree of

Philosophiae Doctor

(PhD)

Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria

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UNIVERSITY OF PRETORIA
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Co-Promoters: Dr E.E. Elgorashi & Dr L.J. McGaw

December 2014

Dedicated to my loving parents and my sisters.

I will forever be grateful for your love, encouragement and support.

DECLARATION

I declare that this thesis hereby submitted to the University of Pretoria for the degree Philosophiae Doctor contains results of my own investigations, and that all inputs of others contained herein have been duly acknowledged. This work has not previously been submitted by me for a degree at this or any other university.

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Mr T. J. Makhafola

ACKNOWLEDGEMENTS

First and foremost, I thank **GOD** Almighty, for all His mercies and continued blessings: **Psalm 107 V 1-5**.

I thank **Prof JN Eloff,** my supervisor, for his invaluable support and dedication. I am grateful for his guidance and involvement from my MSc and now my PhD studies.

To **Dr LJ McGaw** and **Dr EE Elgorashi**, thank you for seeing potential in me and for converting my mistakes into lessons. I have learnt a lot from both of you. I am happy that our professional relationship evolved into true friendships. It would not have been possible to get this degree without your assistance. **Dr McGaw**, Your compliments on my progress and performance always inspired me. **Dr M. Awouafack**, thank you for your help with structure elucidation, much appreciated. **Prof L Verschaeve** and his team **(Dr A Maes** and **Mr R Anthonissen)** at the Scientific Institute of Public Health in Brussels, Belgium, thank you for teaching me the techniques with patience and dedication.

Prof CJ Botha and all my former colleagues in the Pharmacology and Toxicology Section, thank you all for the motivation. **Dr L Meyer**, thank you for helping with statistical analysis and lending me your kids' laptop. Thank them on my behalf. I apologise for the gameless days and nights. **Dr L Le Roux-Pullen** and **Ms A Venter**, thank you for the friendship. I needed the chats and advices. Many thanks to Ms **T De Winnaar** for all her help. You are the best. Thank you.

Financial support from the National Research Foundation (NRF) and the University of Pretoria is acknowledged.

This study would not have been possible without the encouragement of the Phytomedicine postgraduate students, thank you all for the friendship. To my sisters**, Kgaogelo (Sesi)** and **Thapelo (Rakgadi)**, your love keeps me going. I love you very much.

ABSTRACT

The possibility of moderating the response of cells to a particular mutagen by phytomedicines opens new horizons in cancer prevention. On this basis, the search for antimutagens presents many possibilities for the discovery of new anticarcinogenic compounds. Determination of the antimutagenic potential of plant extracts is an important step in the discovery of new effective cancer chemopreventive agents. The main aim of this study was to isolate and characterize antimutagenic compounds active against 4-nitroquinoline 1-oxide (4NQO), mitomycin-C (MMC) and ethyl methanesulfonate (EMS) *in vitro*.

Antioxidant compounds play a preventive role against mutation related diseases and thus may have potential antimutagenic activity. It was for this reason that methanol leaf extracts of 120 plant species from the existing plant material collection of tree leaves in the Phytomedicine Programme of the University of Pretoria were assayed for qualitative antioxidant activity. Almost 98% of the extracts (117) had well defined antioxidant compounds. From these 117 species, 31 were selected for investigation of qualitative antioxidant activity, total phenolic content, mutagenic and antimutagenic activity.

Methanol extracts of the selected 31 species effectively reduced the DPPH free radical with EC_{50} values ranging from 1.20 ± 0.22 to 19.07 ± 1.50 µg/ml and total phenolic content measured in gallic acid equivalents (GAE) ranged from 5.17 ± 0.97 to 18.65 ± 3.86 mgGAE/mg extract. In some instances, the plant extracts had better antioxidant activity than the positive control, L-ascorbic acid (vitamin C) with EC_{50} value of 2.28 ± 0.02 µg/ml. Only one plant (*Halleria liucida*) extract was mutagenic in the Ames test using *Salmonella typhimurium* TA98 and TA100. Upon investigating antimutagenicity, the percentage inhibition of 4-NQO in the Ames test ranged from 8.8 ± 2.4 to 76.7 ± 4.7% in *S. typhimurium* TA98 and from 0.8 ± 6.9 to 99.00 ± 2.9% in TA100. There was a direct correlation between the presence of antioxidant activity and antimutagenic activity of the plant extracts confirming the initial hypothesis of the study. Some of the plant extracts had a comutagenic effect as they potentiated the mutagenic effects of 4-NQO.

From the 31 plant species investigated, 4 species (2 antimutagenic and 2 comutagenic) were selected for in-depth genotoxicity (mutagenicity and antimutagenicity) studies using the Ames test, cytokinesis block micronucleus/cytome assay and alkaline single-cell gel electrophoresis/comet assay. These species were: *Combretum microphyllum* Klotzsch (Combretaceae), *Leucospermum erubescens* Rourke (Proteaceae), *Kirkia wilmsii* Engl (Simaroubaceae) and *Thespisia acutiloba* (Baker f) Exell & Mendonça (Malvaceae).

No plant extract was mutagenic in the Ames test and micronucleus/cytome assay. However, some extracts were slightly mutagenic in the comet assay and this may be attributed to cytotoxicity rather than genotoxic effects. The extracts of *C. microphyllum* and *L. erubescens* inhibited the mutagenic effects of 4-NQO (*S. typhimurium* TA98 and TA100) and MMC (*S. typhimurium* TA102) with values from 10% to more than 30% in the Ames test. However, extracts of *K. wilmsii* and *T. acutiloba* enhanced the mutagenic effects of 4-NQO and MMC in all tester strains. Extracts of *C. microphyllum* and *L. erubescens* prevented micronuclei induction by up to 65.9%, chromosomal rearrangements by 51.9% and gene amplification by 86.1% in the micronucleus/cytome assay. In the comet assay, there was a clear dose dependent decrease in comet tail length.

Based on the preliminary screening results, in depth genotoxicity investigation results and availability of plant material, *C. microphyllum* was selected for the isolation of antimutagenic compounds. Bioassay-guided fractionation of the crude methanol leaf extract, using the Ames test (*S. typhimurium* TA98, TA100 and TA102) as an indicator of antimutagenicity was used for the isolation of antimutagenic compounds. To simplify the isolation of the antimutagenic compounds, the methanol leaf extract of *C. microphyllum* was first separated into four fractions based on polarity using a solvent-solvent fractionation procedure. The solvents used were: hexane, ethyl acetate, water and butanol. The fractions were subjected to antimutagenicity testing in the Ames test. The ethyl acetate fraction was the most active in all three tester strains i.e. *S. typhimurium* TA98, 100 and 102 with percentage antimutagenicity of up to 32.7 ± 2.1, 30.6 ± 3.8% and 21.4 ± 3.1% respectively at the highest concentration (5 mg/ml) assayed. The activity was dose dependent. Bioactivity-guided fractionation of the ethyl acetate fraction by open column chromatography led to the isolation of three compounds. The structures of the compounds were determined using NMR and were identified as n-tetracosanol **(C1),** eicosanoic acid **(C2)** and olean-12-ene-28-oic acid (arjunolic acid) **(C3)**.

The antimutagenic activity in the Ames test using *S. typhimurium* TA98, 100 and 102, and the cytotoxicity on C3A human hepatocarcinoma cell line of the isolated compounds were determined. In the Ames test, the compounds were assayed at concentrations 10 times lower than the concentrations used for the crude extract and fraction. Arjunolic acid was more active in all three tester strains with percentage antimutagenicity of up to 41.9 ± 9.6%, 35.8 ± 1.5% and 43.8 ± 0.18% in *S. typhimurium* TA98, 100 and 102 respectively, followed by eicosanoic acid and lastly n-tetracosanol. Overall, the compounds had much higher antimutagenic activity than the crude extract and the fractions. The quantities of the isolated compounds were not sufficient to allow testing in the micronucleus/cytome assay and comet assay.

The compounds were not cytotoxic at the highest concentration tested i.e. 200 µg/ml. n-Tetracosanol and eicosanoic acid had LC_{50} values > 200 µg/ml (with percentage cell viability of 59.7 ± 7.2and 50.1 ± 6.2% at the

highest concentration respectively) and arjunolic acid had LC_{50} value of 106.4 ± 5.1 µg/ml. Arjunolic acid was the only compound with pronounced antioxidant activity with an EC_{50} value of 6.3 ±0.3 µg/ml. This was moderate antioxidant activity compared to that of vitamin C. n-Tetracosanol and eicosanoic acid did not have antioxidant activity. The antimutagenic activity of arjunolic acid at least in part may be attributed to its antioxidant activity resulting in the detoxification of reactive oxygen species produced during mutagenesis, but other mechanisms were probably involved with the other compounds. These results also show that it would not have worked by just isolating antioxidant compounds and testing these for genotoxicity.

Combretum microphyllum has potential antimutagenic activity and protective effects against cancer since the crude extract of this plant species effectively inhibits the genotoxic end-points induced by 4-nitroquinoline 1-oxide (4NQO), mitomycin-C (MMC) and ethyl methanesulfonate (EMS) *in vitro*. This conclusion is supported by the fact that chromosomal biomarkers of genomic instability are relevant to cancer and that genotoxicity involving gene mutations, chromosomal aberrations and rearrangements and DNA strand breakages play a major role in cancer initiation. Pure compounds isolated from the ethyl acetate fraction of this extract had antimutagenic effects in the Ames test using *S. typhimurium* TA98, 100 and 102. The compounds had better activity compared to the crude extract and fractions at concentrations 10 times lower. The compounds were not cytotoxic against C3A human hepatocarcinoma cells at the highest concentration tested of 200 µg/ml.

Overall, these types of studies on plant extracts may provide leads to the discovery of chemopreventive agents that can be used to develop pharmacologically active agents for prevention of chronic degenerative diseases. The compounds isolated in this study have been previously isolated from other plant species and are known to possess numerous biological activities, including amongst others: antioxidant activity, antimicrobial activity, antitumor effects and anticholinesterase activity. Even when new chemical structures are not found in medicinal plant research studies and drug discovery, known compounds with new biological activities can provide important drug leads

Some of the extracts, fractions or compounds may be used as elements in nutraceuticals, functional foods and other applications as antimutagens to limit the possibility of mutations. Antimutagens and anticarcinogens play a major role in the primary prevention of mutations and cancer development by lowering the frequency or rate of mutations. This is the first report of the isolation of n-tetracosanol, eicosanoic acid and arjunolic acid from *C. microphyllum*. We also report for the first time the potential antigenotoxic effects of the crude extract, fractions and the compounds isolated from *C. microphyllum*.

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4-NQO	4-Nitroquinoline 1-oxide
ANOVA	Analysis of variance
BEA	Benzene, ethyl acetate, ammonia (90:10:1; v/v/v)
But	Butanol
CC	Column chromatography
CEF	Chloroform, ethyl acetate, formic acid (5:4:1; v/v/v)
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydraxyl radical
EC_{50}	Effective concentration on inhibition of 50% DPPH
EtOAC	Ethyl acetate
EMS	Ethyl methane sulphonate
EMW	Ethyl acetate, methanol, water (40:5.4:4; v/v/v)
GAE	Gallic acid equivalents
H_2O	Water
Hex	Hexane
KNBG	Kirstenbosch National Botanical Garden
LC_{50}	Lethal concentration on 50% of cells
LMP	Low melting point
MMC	Mitomycin-C
NDCI	Nuclear Division Cytotoxicity Index
NMP	Normal melting point
NMR	Nuclear Magnetic Resonance
NRU	Neutral Red Uptake
OD	Optical Density

OECD	Organization for Economic Co-operation and Development
PBS	Phosphate buffered saline
PRE	Pretoria National Botanical Garden
PRU	HGWJ Schweickerdt Herbarium
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TLC	Thin Layer Chromatography
UP	University of Pretoria
USA	United States of America
WHO	World Health Organization

Papers prepared from this thesis

Makhafola T.J., Elgorashi E.E., McGaw L.J., Verschaeve L. and Eloff J.N. Antimutagenic potential of 31 plant extracts with good antioxidant activity in the Ames test. Prepared for submission to *BMC Complementary and Alternative Medicine*.

Makhafola T.J., Elgorashi E.E., McGaw L.J., Verschaeve L. and Eloff J.N. Antigenotoxicity of *Combretum microphyllum* and *Leucospermum erubescens. Inhibition of the genotoxic effects of 4-nitroquinoline-1-oxide, mitomycin C and ethyl methano-sulphonate in vitro*. Prepared for submission to *Food and Chemical Toxicology*.

Makhafola T.J., Elgorashi E.E., McGaw L.J., Awouafack, M.D., Verschaeve L. and Eloff J.N. Antimutagenicity, cytotoxicity and antioxidant activities of n-tetracosanol, eicosanoic acid and arjunolic acid; compounds isolated from *Combretum microphyllum*, (Combretaceae). Prepared for submission to *Planta Medica*.

Conference presentations

Makhafola T.J., Elgorashi E.E., Verschaeve L. and Eloff J.N. Investigation of the genotoxic and antigenotoxic effects of selected South African medicinal plants. *37th Annual Conference of the South African Association of Botanists*. 17-19 Jan 2011, Rhodes University, Grahamstown, South Africa.

Makhafola T.J., Elgorashi E.E., McGaw L.J., Verschaeve L. and Eloff J.N. *Combretum microphyllum* and *Leucospermum erubescens* inhibit genotoxic effects of 4-NQO, MMC and EMS *in vitro*. 46th Annual Congress of the SASBCP in association with Department of Family Medicine and TOXSA. 30 September-02 October 2012 University of Pretoria, South Africa.

Makhafola T.J., Elgorashi E.E., McGaw L.J., Verschaeve L. and Eloff J.N. Antimutagenic potential of *Combretum microphyllum* methanol leaf extract. 39th *Annual Conference of the South African Association of Botanists*, 20-24 January 2013. Drakensberg, South Africa.

CHAPTER 1

Introduction

1.1. Background

Plants are chemically diverse and provide a practically unlimited and valuable source of pharmacologically active compounds. These compounds can be useful for the development of newer and effective chemotherapeutic agents. Many useful chemotherapeutics have been sourced from medicinal plants (Philipson, 2001).

The aim of this study was to investigate the antimutagenic potential of several plant extracts and to isolate and characterize antimutagenic/antigenotoxic compounds from the plant extracts. The search for antimutagens is important since mutations play a major role in the pathogenesis and development of several chronic degenerative diseases including cancer (De Flora and Ferguson, 2005). It follows, therefore, that the incidence of mutation related diseases can be reduced by decreasing the rate of mutations. Investigation of the antimutagenic potential of traditional medicinal plants and compounds isolated from plant extracts provides one of the tools that can be used in the identification of compounds with potential anticancer properties (Arora *et al.,* 2005, Reid *et al.,* 2006).

Induction of mutagenesis occurs mainly through damage of DNA by free radicals and other reactive oxygen species (ROS) (Namiki, 1990). Numerous mutagens act through generation of ROS. Antioxidants which are inhibitors of oxidation are therefore an important part of a strategy to minimize mutation related diseases. Living organisms have numerous antioxidant defences, but these defences are not perfect thus the consumption of dietary antioxidants may be important in the prevention of oxidation induced DNA damage (Ames *et al.,* 1993). It is for this reason that this study focuses on the antioxidant activity and phenolic content of the selected plant extracts as a crucial step in the selection of plants with antimutagenic activity related to decreasing oxidative free radicals.

Antimutagens may be useful in the prevention of mutations; a phenomenon termed chemoprevention. They provide multiple points of intervention for the pharmacological prevention of diseases in which mutations plays a pathogenic role by lowering the frequency or rate of mutations or blocking initiation of carcinogenesis. (De Flora *et al.,* 2001). Chemoprevention strategies target numerous steps including anti-initiation strategies (e.g. DNA repair, detoxification, free radical scavenging and carcinogen metabolism) anti-promotion/anti-progression strategies (e.g., free radical scavenging, proliferation suppression, differentiation induction, immunity

enhancement, inflammation reduction, increase in apoptosis, altered gene expression and decrease in angiogenesis) (Greenwald, 2002).

Although plant extracts have been used for centuries for the treatment of ailments, scientific research has also shown that some of the chemical substances present in plants may be potentially toxic or carcinogenic. Therefore, mutagenicity testing is important in this study. Mutagens derived from plants can also be useful as an anticancer tool, as most anticancer drugs are mutagenic (e.g. the spindle-disturbing compounds taxol and vinblastine) (Verschaeve and Van Staden, 2008). However, presence of mutagenic compounds in plant extracts raises concern about the carcinogenic hazards resulting from the long-term use of the plants as medicines or food. Plants with clear mutagenic properties should be considered as potentially unsafe whereas plants with obvious antimutagenic potential can be considered interesting for therapeutic use.

This study aims to add valuable information to the ongoing search for chemopreventive agents. It is generally acceptable that the use of antimutagens and anticarcinogens in everyday life is the most effective procedure for preventing human cancers and other genetic disorders (Ferguson *et al.,* 2004).
There are at least 250 000 species of higher plants worldwide. It is therefore logical to presume that many more useful drugs will be found in the plant kingdom (Farnsworth and Soejarto, 1985).

1.2. Literature review

1.2.1. Mutations and their role in the pathogenesis of diseases

Mutations are caused by permanent transmissible changes in the DNA structure and have been implicated in the etiopathology of cancer, neurodegenerative diseases and other degenerative diseases (Ames et al., 1973). These changes may involve individual genes, blocks of genes or whole chromosomes, and are heritable. DNA damage alters the genetic message carried by genes involved. Agents or substances that cause alteration of DNA are termed mutagens and can range from chemicals to radiation and sunlight (Klaassen, 2008). All mutagens elicit a genotoxic response, hence they are also known as genotoxins. DNA damage can be in the form of single and double strand breaks, point mutations and structural and numerical chromosomal aberrations (Tao, 2010).

Chronic degenerative diseases such as cancer, cardiovascular diseases, atherosclerosis etc. share common risk factors and common pathogenic determinants such as DNA damage, oxidative stress and chronic inflammation (DeFlora *et al.,* 1996). For instance, frame-shift mutations are apparent in severe genetic diseases such as Tay-Sachs disease and cystic fibrosis (Zimmerman *et al*., 1997). Mutations are also involved in the inception of degenerative diseases including hepatic disorders, neurodegenerative disorders, cardiovascular disorders, diabetes, arthritis, chronic inflammation and the process of aging. These diseases are the leading causes of diseases in developed countries (De Flora *et al.,* 1996, Dhillon and Fenech, 2014).

DNA damage is present both in the circulating cells of patients with atherosclerosis and atherosclerotic plaques (Mercer *et al*., 2009). DNA strand breaks, oxidized pyridines and altered purines (related to environmental exposure to genotoxic chemicals) are higher in patients with coronary artery disease (Binkova *et al.,* 2002). There is considerable evidence that gene and chromosomal mutations are important factors in carcinogenesis (Fenech, 2002, Solomon *et al.,* 1991). Even though only certain mutations lead to cancer, most mutagens that were tested and identified by Ames *et al.,* (1973) are classified as potential carcinogens. The field of genetic toxicology remains an important tool in the development of new pharmaceuticals (Sarasin, 2003, Klaassen, 2008). As a consequence of mutations, several *in vitro* and *in vivo* tests have been developed to assess the potential DNA damaging effects of chemicals.

1.2.2. Antimutagens and their role in chemoprevention

Antimutagens are chemical agents that reduce or counteract the mutagenicity of physical and chemical mutagens, either by inactivating the mutagen or by preventing the reaction between a mutagen and DNA (Mitscher et al., 1986, Bhattacharya, 2011). Since mutagens are involved in the initiation and promotion of several human diseases, research focusing on the identification of novel bioactive phytocompounds that reduce mutagenicity and counteract mutagenesis has gained credence in recent years (Aqil *et al.*, 2008, Słoczyńska *et al*., 2014).

It is generally accepted that antimutagenic compounds have chemopreventive properties. Chemoprevention was first defined as the inhibition or reversal of carcinogenesis by the use of non-cytotoxic nutrients or pharmacological compounds that protect against the development and progression of mutant clones of malignant cells (Sporn, 1976). Chemoprevention 'with respect to mutations' is the pharmacological approach that uses either natural or synthetic chemical agents to inhibit, reverse, suppress or prevent and arrest mutagenesis (Greenwald, 2002, Woolf *et al.*, 2008).

Antimutagens play a major role in the primary prevention of mutations and cancer development by lowering the frequency and/or rate of mutations, or blocking initiation of carcinogenesis, a chemopreventive role (De Flora *et al.,* 2001). The use of antimutagens and anticarcinogens in everyday life may be effective in the prevention of human cancers and chronic diseases that share common pathogenetic mechanisms such as DNA damage, oxidative stress and chronic inflammation (De Flora *et al*., 1991, De Flora and Ferguson, 2005). It is thus evident that cancer and other mutation-related diseases can be prevented not only by avoiding exposures to recognised risk factors but also by favouring intake of protective factors. Most chemopreventive agents have antioxidant activity and detoxifying properties (Shankel *et al*., 2000). In addition to their antimutagenic activity and anticarcinogenic properties, they exert additional health benefits including antiproliferative and anti-inflammatory properties (Mukhtar *et al.*, 2012).

Antimutagenic agents have different mechanisms of action including but not limited to antioxidant potency, inhibition and deactivation of mutagens, and blocking interaction of mutagens with DNA, while others possess multiple mechanisms of action (Słoczyńska *et al*., 2014). The major mechanisms of antimutagens broadly include chemical or enzymatic inactivation, prevention of formation of active species and antioxidant and free radical scavenging (Ishaq *et al.,* 2003). Based on their mechanism of action, antimutagens are divided into two major groups, namely bioantimutagens and desmutagens (Kada *et al*., 1982, Ferguson, 1994).

- Bioantimutagens are antimutagens that act as modulators of DNA replication and repair processes in cells. This group of antimutagens act by preventing fixation of premutagenic lesions into mutations, resulting in a decline in mutation frequency. Bioantimutagens are considered to be “true” antimutagens.
- Desmutagens are antimutagens that inactivate mutagens or prevent their interaction with DNA. They may be antimutagens as they indirectly fully or partially inactivate the mutagen. Among desmutagenic agents, antioxidants are of special interest because they are implied in inhibition of all stages of carcinogenesis. The mechanism of inhibition of mutagenesis and initiation of carcinogenesis by antioxidants include scavenging of reactive oxygen species and inhibition of mutagen/carcinogen binding to DNA.

1.2.3. Antioxidants and their role in chemoprevention

Many mutagens act through generation of reactive oxygen species (ROS) which induce oxidative stress in living cells. Many mutations related to oxidative stress, or DNA damage and repair, have been identified in human disease syndromes (Beckman and Ames, 1998). Oxidative stress is involved in more than 100 common diseases including cancer, all inflammatory diseases (arthritis, vasculitis lupus etc.), autoimmune diseases, diabetes, emphysema, catactogenesis and macular degeneration, gastric ulcers, hemochromatosis, hypertension, heart diseases, and neurologic diseases (multiple sclerosis, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, muscular dystrophy etc.) (Sies, 1998, Schafer and Buettner, 2001).

Oxygen free radicals or more generally reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of cellular metabolism and are common in biological systems. ROS and RNS harms living systems by inducing oxidative damage to cell structures and biomolecules such as lipids, nucleic acids and proteins. Normally there is a balance between the amount of free radicals generated in the body and the defence systems that scavenge or quench these free radicals, preventing them from causing deleterious effects in the body. When there is a shift or imbalance in the pro-oxidation and antioxidation homeostatic phenomena resulting from excessively high levels of these oxidative species in the body, either due to environmental conditions or being produced within the body, these free radicals, increase the burden in the body leading to oxidative stress which results in tissue injury and subsequent diseases (Finkel and Holbrook, 2000, Castro and Freeman, 2001).

In recent years, there has been an increased interest in the application of antioxidants in the health sector (Adams and Adams, 2002). Widespread attention is currently being given to the identification of novel potent antioxidant compounds. Antioxidants are chemical substances that are capable of slowing or preventing the oxidation of other molecules. They have a wide application in the health sector due to the pathological role of free radicals in a variety of diseases and in the food sector because free radicals result in deterioration of food products (Benzie and Strain, 1999). Antioxidants are widely used as ingredients in dietary supplements in the hope of preventing diseases such as cancer and coronary heart disease. Prevention of cancer and cardiovascular disease has been linked to the intake of vegetables, fruits and teas rich in natural antioxidants (Johnson, 2001).

Kaur *et al,* (2006) reviewed the antimutagenic and anticarcinogenic potential of polyphenols and concluded that polyphenolic compounds have a major place in the chemoprotection against cancer. They conclude that it is worth investigating what place these compounds have in the prevention of cancer.

Plant phenolic compounds such as those occurring in wine could protect against degenerative diseases involving oxidative damage due to their antioxidant action. The role of phenolic compounds from food and beverages in the prevention of free radical-mediated diseases has become more important. The emphasis placed by the European Commission on enhancing the nutrient content of food crops through traditional plant breeding as well as food-processing technologies confirms the importance of phenolic compounds in terms of health benefits to the international community (Lindsay, 2000; Chisholm and Steinberg, 2000).

The application of new, sensitive techniques of analytical chemistry has confirmed the importance of endogenous oxidative DNA damage in the etiology of many human cancers. Permanent modification of genetic material resulting from "oxidative damage" incidents represents the first step in mutagenesis, carcinogenesis, and ageing. DNA damage can result in either arrest or induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with carcinogenesis. Since oxidative DNA damage can play a significant role in mutagenesis, the decrease of oxidative stress seems to be the best strategy for the prevention of development of mutation related diseases (Valko *et al.,* 2007).

1.2.4. Medicinal plants as sources of new pharmaceuticals

Natural product research continues to provide a variety of lead structures which are used as templates for the development of new drugs by the pharmaceutical industry (Lall, 2001). Numerous useful drugs have been developed from lead compounds discovered from medicinal plants (Paulsen, 2010). To date, plants still remain an essential route for the discovery of new pharmaceuticals. There is growing research interest in the use of medicinal plants as dietary supplements and for the development of new medicinal products (Newman *et al.,* 2003).

Plants offer excellent opportunities for the discovery of new therapeutic products (Farnsworth and Soejarto, 1985, Cox and Balick, 1994). They are considered to be a rich source of medicines as they produce a host of pharmacologically active compounds recognised by pharmacologists to have reactions towards sickness (Van Wyk et al., 1997). The enormous chemical diversity of plant secondary metabolites presents a valuable resource for possible development of new pharmaceuticals.

Approximately 80% of populations in developing countries use medicinal plants to help meet their health care needs (WHO, 2008). In South Africa alone, a large percentage of the population rely fully on medicinal plants for their health care needs and food security (Fennell *et al*., 2004). The use of plants for medicinal purposes is a

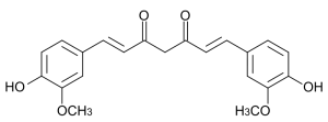
worldwide practice and is recognised by the World Health Organization as an essential component of health care (Van Wyk, *et al*., 1997; WHO, 2008).

Drug discovery from medicinal plants led to the isolation of numerous useful drugs (Newman et al., 2000, Butler, 2004). Farnsworth and Soejarto (1985) listed a detailed summary of drugs derived from plants that are currently used. A few of the drugs listed in their study are simple synthetic modifications of naturally obtained substances. Below are examples of important plant compounds developed for the benefit of human health (Paulsen, 2010).

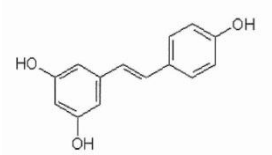
- **Opium alkaloids**- Isolated from *Papaver somniferum*, from which morphine, codeine, noscapine and papaverine were derived.
- **Salicin** from *Salix* species resulted in the production of Aspirin, Albyl and Disprilused as pain killers, fever reducing agents and as an anti-coagulant.
- **Atrakurium** is a registered medicine used as a muscle relaxant developed from **tubocurarine and strychnine**. Tubocurarine and strychnine were isolated from *Chondrodendron tomentosum* and *Strychnos nux-vomica* respectively.
- **Atropine, hyscyamine and scopolamine**- All these compounds are present in *Atropa belladonna, Hyoscyamus niger* and *Datura stramonium* and are used in various medical conditions including treatment of asthma and ophthalmological disorders.
- **Cardiac glycosides**- Digitoxin isolated from *Digitalis purpurea* is one of the cardiac glycosides that are still used to treat certain heart conditions.
- *Artemisia annua* and *Cinchona* species are the sources of the well-known remedies for malaria, **artemisinin and quinine.** Quinine was isolated from *Cinchona* species and has been in use for a long time in the treatment of malaria, and artemisinin from *Artemisia annua* is used both in the prophylaxis and treatment of malaria.
- **Podophyllotoxin**- a compound isolated from the roots of *Podophyllum peltatum* served as a lead compound for the development of cancer chemotherapeutic agents teniposide and eposide.
- Vinblastin and vincristin are successful antineoplastic agents developed from **vinblastine and vincristine** isolated from *Catharanthus roseus*.

Higher plants are known to synthesize structurally varied biologically active secondary metabolites that have shown various therapeutic potential as well as antimutagenic and anticarcinogenic properties (Mitscher *et al.,* 1992, Mitscher *et al.,* 1996). Much attention and research has been focused on screening of higher plants for the presence of antimutagenic compounds (Arora *et al.,* 2003). Antimutagens can play a major role as chemopreventive agents (Bhattacharya, 2011).

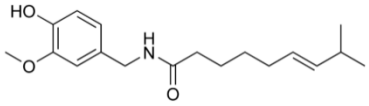
Below are structures of representative chemopreventive phytochemicals (Surh, 2003).



Curcumin



Resveratrol



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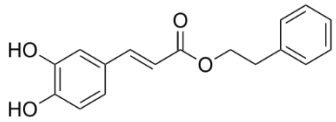

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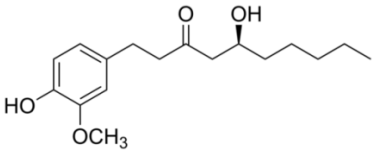
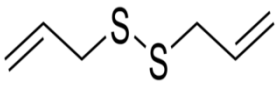
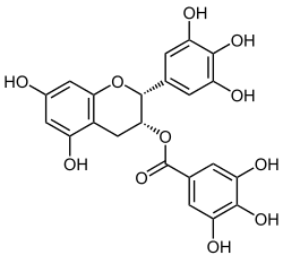


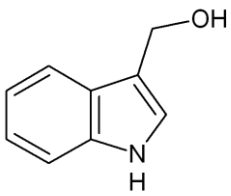
Gingerol



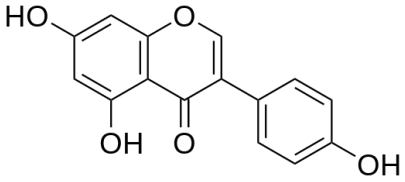
Diallyl sulphide



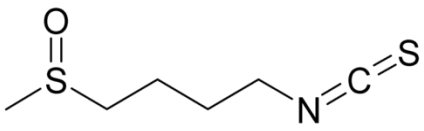
epigallocatechin-3-gallate



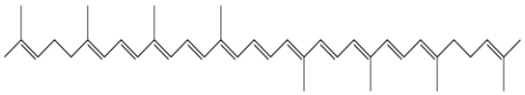
Indole-3-carbinol



Genistein



Sulphoraphane



Lycopene

Figure 1.1. Representative chemopreventive phytochemicals from dietary sources

1.2.5. Mutagens used in the study

In order to detect the various mechanisms of mutations, different mutagens and different *in vitro* assays were used to investigate the antimutagenic effects of the selected plants. Three mutagens (4-nitroquinoline 1-oxide, mitomycin-C and ethyl methane sulphonate) were used. 4-Nitroquinoline 1-oxide (4-NQO) and mitomycin C (MMC) were used in both the Ames test and micronucleus/cytome assay whereas ethyl methane sulphonate (EMS) was used in the comet assay.

4-Nitroquinoline 1-oxide is a potent mutagen that induces intracellular oxidative stress by generating reactive oxygen species (ROS) and exerts potential intracellular oxidative stress. The metabolic products of 4-NQO bind to DNA, contributing to tumour promotion (Nunoshiba and Demple, 1993). These forms of DNA damage are similar to damage imposed by several carcinogens. In studies of the development of biomarkers and chemopreventive agents using 4-NQO, microgram quantities of 4-NQO induced carcinogenesis in most animals and the histological and molecular changes observed were similar to those observed in human carcinogenesis (Kanojia and Vaidya, 2006).

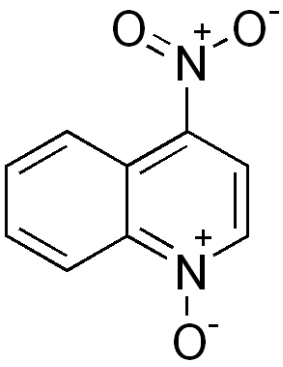


Figure 1.2. Chemical structure of 4-NQO (www.chemsources.com)

Mitomycin-C is an aziridine-containing natural compound isolated from *Streptomyces* spp. Mitomycin C is a potent DNA cross linker and is used as an antitumour agent. Mitomycin C generates free radicals when metabolised (Ortega-Gutierrez *et al*., 2009).

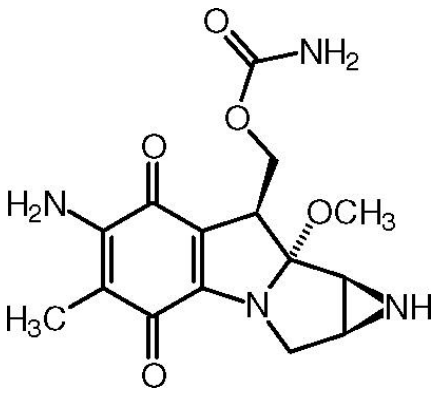


Figure 1.3. Chemical structure of mitomycin C (www.chemsources.com)

Ethyl methane sulphonate (EMS) is a mutagenic and teratogenic compound. It produces random mutations in the genetic material by nucleotide substitution and is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals. It is a well-known genotoxic agent that has been extensively used as an alkylating model compound in genotoxicity experimental work. It is a direct DNA damaging agent and is used experimentally as a mutagen, teratogen and carcinogen (HSDB, 2000).

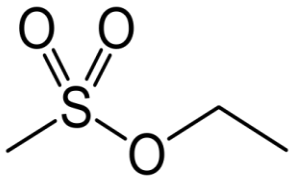


Figure 1.4. Chemical structure of ethyl methane sulphonate *(*www.chemsources.com)

Exposure to EMS induces gene mutations and chromosomal aberrations in many test systems. It appears to have no organ specific DNA damaging effects (Hartmann *et al.,* 2004, Smith *et al.,* 2008). Several studies provide evidence that EMS induces similar levels of DNA damage in different tissues and organs.

1.2.6. Overview of methods used for cytotoxicity, genotoxicity and antigenotoxicity testing

1.2.6.1. Neutral red uptake

The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. It is one of the most widely used cytotoxicity tests with many biomedical and environmental applications. It is used in basic and applied research and it is also included in the first non-genotoxicity *in vitro* assay accepted for the regulatory evaluation of chemicals. The neutral red uptake assay (NRU) is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosome. Lysosomal integrity, with the concomitant binding of the neutral red dye, is a highly sensitive indicator of cell viability. The dye is then extracted from the viable cells and the absorbance of the solubilized dye is quantified using a spectrophotometer. The quantity of the dye extracted from the lysosomes is directly proportional to the number/percentage of viable cells (Repetto *et al.,* 2008). For the purpose of this study, this assay was used as a toxicity screening assay to select concentration ranges to use in further studies after selection of plant species

1.2.6.2. Tetrazolium-based MTT cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a commonly used colorimetric assay for the detection of cytotoxicity and for assessing cell viability following exposure to toxic substances. MTT is a water soluble yellow tetrazolium salt which is converted to an insoluble purple formazan in viable cells with active mitochondrial dehydrogenases enzymes. The formazan product is impermeable to the cell membrane and therefore accumulates in healthy cells. In essence, the intensity of the MTT formazan produced by living metabolically active cells is directly proportional to the number of live cells present (Mosmann, 1983).

1.2.6.3. Ames *Salmonella*/microsome mutagenicityassay

The *Salmonella* mutagenicity test is designed to detect chemically induced mutagenesis. With minor modifications, this assay can be used to detect substances with antimutagenic activity. The Ames test is a widely accepted short term bacterial reverse mutation assay designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. The test employs several *Salmonella* strains with pre-existing mutations in various genes in the histidine operon that renders the bacteria histidine dependant. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. In genotoxicity/mutagenicity testing, *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine. Only those bacteria that revert to histidine independence are able to form

colonies. When a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner (Maron and Ames, 1983, Mortelmans and Zeiger, 2000).

The number of spontaneously induced revertant colonies per plate is relatively constant. For antigenotoxicity/antimutagenicity testing, a variation of the Ames test is used where the mutagen in combination with the presumed antimutagen are added to the plate. When a mutagen is incubated with a presumed antimutagen the number of revertant colonies in the plates with mutagen alone vs number of revertant colonies in the plate with a combination of the mutagen and test sample (presumed antimutagen) provides a measure of antimutagenicity. In essence, in the presence of an antimutagenic substance, the number of colonies will decrease when compared to the number of revertant colonies in the plate with mutagen alone. In this study, *Salmonella typhimurium* tester strains TA98, TA100 and TA102 were used for both mutagenicity and antimutagenicity studies. Strain TA98 gives an indication of frame-shift mutations, TA100 indicates base-pair substitutions and TA102 indicates transitions/transversions (Mortelmans and Zeiger, 2000).

1.2.6.4. Cytokinesis-block micronucleus cytome assay

The cytokinesis-block micronucleus cytome (CBMN) assay is a comprehensive cytogenetic test for genetic toxicology testing. This system is used to measure DNA damage, cytokinesis and cytotoxicity. Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ration (Fenech, 2000). DNA damage events are scored specifically in once-divided binucleated cell and include

- Micronuclei (MNi) – a biomarker of chromosome breakage and/or whole chromosome loss.
- Nucleoplasmic bridges (NPBs) – a biomarker of DNA misrepair and /or telomere end-fusions.
- Nuclear buds (NBs) – a biomarker of elimination of amplified DNA and DNA repair complexes (Fenech, 2007).

The micronucleus (MNi) originate from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase during mitosis. This happens when chromosomes do not attach properly with the mitotic spindle and lag behind at anaphase during nuclear division. These displaced chromosomes or chromosome fragments are eventually enclosed by a nuclear membrane and, except for their smaller size, are morphologically similar to nuclei after conventional nuclear staining (Fenech et al., 2011). In the CBMN assay, once-divided cells are recognised by their binucleated appearance after blocking cytokinesis with cytochalasin-B. Cytochalasin-B is an inhibitor of microfilament ring assembly required for the completion cytokinesis (Fenech, 2000). When measuring micronuclei alone, this test can detect the clastogenic (chromosome breaking) and aneugenic (spindle

disturbances, genome mutations) events when combined with fluorescence in situ hybridization (Verschaeve and Van Staden, 2008).

Nucleoplasmic bridges (NPBs) originate during anaphase when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell during mitosis. In the absence of breakage of the anaphase bridge, the nuclear membrane eventually surrounds the daughter nuclei and the anaphase bridge and in this manner, a NPB is formed. NBPs are usually broken in during cytokinesis but they can be accumulated in cytokinesis-blocked cells using the cytokinesis inhibitor cytochalasin-B (Fenech *et al*., 2011).

Nuclear buds (NBs) form as a result of excessive gene amplification. Amplified DNA may be eliminated from chromosomes through recombination between homologous regions within amplified sequences forming mini-circles of acentric and atelomeric DNA. The NBs are characterised by having the same morphology as micronuclei with the exception that they are connected to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process (Fenech *et al*., 2011).

The cytokinesis-block micronucleus cytome assay also allows the evaluation of the mitogenic response of cells and cytostatic effects of test samples. This is achieved by calculation of the nuclear division index (NDI) (Eastmond and Tucker, 1989). The NDI provides a measure of the proliferative state of the viable cells.

$$\mathbf{NDI = (M1 + 2(M2) + 3(M3) + 4(M4))/N}$$

Where NDI = nuclear division index, M1–M4 = number of viable cells with 1–4 nuclei and N = total number of cells scored (viable and non-viable). The NDI method however excludes necrotic and apoptotic cells in the calculation. A more accurate assessment of nuclear division status is obtained when necrotic and apoptotic cells are included in the calculations, in which case the calculation measures nuclear division cytotoxicity index (NDCI).

$$\mathbf{NDCI = (Ap + Nec + M1 + 2M2 + 3M3 + 4M4) / N}$$

Where NDCI = nuclear division cytotoxicity index, Ap = number of apoptotic cells, Nec = number of necrotic cells, M1–M4 = number of viable cells with 1–4 nuclei and N = total number of cells scored (viable and non-viable). The lowest possible value is 1.0 which occurs if all viable cells have failed to divide during the cytokinesis-block period and are therefore all mononucleated. If all viable cells complete one nuclear division and are all binucleated, the value is 2.0. (Fenech, 2000, Fenech, 2007). This is of great importance in assessing the cellular toxic effects of the test samples as opposed to their genetic toxicity.

1.2.6.5. Alkaline single-cell gel electrophoresis/Comet assay

The alkaline single-cell gel electrophoresis/comet assay is a rapid and sensitive procedure for quantitating DNA single and double strand breaks and alkali labile sites in single cells (Singh *et al*., 1988). This assay is widely used to detect chemical genotoxicity *in vitro* and *in vivo*. The assay combines DNA gel electrophoresis with fluorescence microscopy to visualise migration of DNA from individual embedded cells (Olive and Banath, 2006). In essence, individual cells are embedded in a thin agarose gel on a microscope slide, lysed and the DNA is allowed to unwind in an alkaline buffer. Following the unwinding, the DNA is subjected to electrophoresis, allowing the broken DNA fragments or damaged DNA to migrate away from the nucleus. After staining with a DNA-specific fluorescent dye such as ethidium bromide, the gel is visualised for amount of fluorescence in head and tail and length of tail using fluorescence microscopy and comet image analysis software (Tice *et al*., 2000). During electrophoresis, relaxed and broken DNA fragments move further from the nucleus than intact DNA, generating a comet-like band with a distinct head and tail. The head is composed of intact DNA, while the tail consists of damaged (single strand or double strand breaks). The extent of DNA damage can be measured by the fluorescence intensity of the comet tail. Different parameters are used to measure the extent of DNA damage. In this study, DNA damage was quantified by measuring the tail length, percentage DNA in the tail and tail moment (Tice *et al*., 2000).

1.3. Aims and objectives

1.3.1. Aim(s)

To investigate the antimutagenic potential of several plant extracts with high antioxidant activity based on hypothesis that antioxidant activity may be related to antimutagenic/antigenotoxic activity and to isolate and characterize pure compound(s) from plant species with a high antimutagenic/antigenotoxic activity.

1.3.2. Specific Objectives

- Screening of 120 tree leaf extracts for qualitative antioxidant activity to determine antioxidant compounds separated by thin layer chromatography as a preliminary step to identify antimutagenic plant species.
- Determining the quantitative antioxidant activity and total phenolic content of plant species with well-defined antioxidant compounds.
- Investigation of the mutagenic and antimutagenic activity and cytotoxicity of plant extracts with high antioxidant activity in the Ames test. Selection of active plant species for further studies.
- In depth study of the selected species for antimutagenic/antigenotoxic activity in the Ames test, micronucleus/cytome assay and comet assay.
- Isolation and chemical characterization of antimutagenic compounds from highly active plant species.
- Comparing the antimutagenic activity, cytotoxicity and antioxidant activity of isolated compounds.

1.4. Hypothesis and Justification

There is a correlation between antioxidant activity and some antimutagenic activities. Using *in vitro* methods it is possible to isolate and characterize compounds with antimutagenic activity from plant extracts with good antioxidant activity. Since oxidative DNA damage plays a role in the pathogenesis of several chronic degenerative diseases, the decrease of oxidative stress is a good possible strategy for the prevention of these diseases. Antioxidant compounds may prevent mutation-related diseases and thus have potential antimutagenic effects. To test the hypothesis plants were initially selected based on antioxidant activity and the antimutagenicity/antigenotoxicity was determined. Compounds were isolated from plant extracts with high antimutagenic activities by bioassay guided fractionation and relevant biological activities were determined.

1.5. Structure of the thesis

Chapter 1 provides the background of the study and literature review.

Chapter 2 reports on the preliminary screening of 120 South African plant species for qualitative antioxidant activity and the selection of 31 extracts with good antioxidant activity for further assays. The selected 31 plant extracts were further analysed for quantitative antioxidant activity and total phenolic content. This study was carried out to establish whether there is a correlation or relationship between antioxidant activity, presence of phenolic compounds and antimutagenic activity of the plant extracts.

In **Chapter 3**, the safety of the 31 selected plant species based on genotoxicity and cytotoxicity were determined. In addition, antimutagenic activity of the extracts in the Ames test and micronucleus/cytome assay were determined. The results obtained in this study were used to select the plant species with potential antimutagenic and/or antigenotoxic effects for further investigation. Four plant species (*Combretum microphyllum, Leucospermum erubescens, Thespisia acutiloba and Kirkia wilmsii)* were selected for further studies.

Chapter 4 describes an in-depth study on the genotoxic and antigenotoxic activity of the four plant species using the Ames/microsome test, cytokinesis block micronucleus/cytome assay and the single cell gel electrophoresis/comet assay.

In **Chapter 5**, the isolation and chemical characterization of antimutagenic compounds from *C. microphyllum* is described. This species was selected from the four plant species studied in Chapter 4 based on its activity and availability.

Chapter 6 deals with the antimutagenic, cytotoxic and antioxidant activity of the three compounds isolated in Chapter 5.

Finally, **Chapter 7** summarises the motives of the entire project, and provides the general discussion and conclusions. Furthermore, recommendations for future research based on the findings of this study are provided.

CHAPTER 2

The antioxidant activity and total phenolic content of 120 South African plant species as a preliminary step in identifying antimutagenic plant species

2.1. Introduction

Antioxidant activity of higher plants is commonly associated with the presence of phenolic compounds. A review article found that a large part of the isolated active antioxidant compounds from plants were different low and high molecular weight plant polyphenolics (Moure *et al.,* 2001). Phenolic compounds are characterized by the presence of at least one aromatic ring (C6) bearing one or more hydroxyl groups. Phenols are divided into several different groups distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton. They have various functions in plants and the enhancement in their metabolism can be observed under different environmental factors and stress (Macheix *et al.,* 1990).

The antioxidant activity of phenolic compounds is well-known. Phenolics help maintain membrane integrity by preventing the access of deleterious molecules to the hydrophobic region of the bilayer. Other *in vitro* studies have shown that flavonoids (a group of phenolics) can directly scavenge molecular species of active super oxides, hydrogen peroxide, hydroxyl radical and singlet oxygen or peroxyl radical. Many herbal infusions frequently used in domestic traditional medicine have antioxidant and pharmacological properties connected with the presence of phenolic compounds, especially flavonoids (Bors *et al.,* 1990).

Natural antioxidants, rather than synthetically produced ones, are considered to be more beneficial for the maintenance of good health (Morton *et al.,* 2000). Studies have shown that synthetic antioxidants and their metabolic products might have toxic side effects. Moreover, the long term use of synthetic antioxidants may aid in modifying the acute toxicity of several carcinogenic and mutagenic chemicals and lead to chronic side effects (Benzie, 2003). Scientific information on the antioxidant properties of plants, especially those that are less widely used for culinary applications and in medicine, is still rather scarce. Of the estimated 22 000 plant species occurring in South Africa, only a small percentage has been investigated phytochemically. The assessment of such properties remains a useful tool or method of finding new sources of natural antioxidants. These are some of the reasons the antioxidant activities of several plant species were targeted in the present

study to investigate their potential applicability in the development of antimutagenic agents of natural origin.

2.2. Materials and methods

2.2.1. Plant material collection and Extraction

The plants used for screening were obtained in a powdered form from the Phytomedicine Programme data base, University of Pretoria (Pauw and Eloff, 2014). The leaves were collected from the the Pretoria National Botanical Gardens in Pretoria, the Lowveld National Botanical Gardens in Nelspruit, the Kirstenbosch National Botanical Gardens in Cape Town and Manie van der Schijff Botanical Gardens (University of Pretoria). Voucher specimens of the plants are kept in the HCW Schweikert Herbarium of the University of Pretoria.

The leaves were dried in the dark at room temperature, pulverized into fine powder and stored in glass bottles until use. The dried plant samples form part of a collection from the Tree Screening Project conducted within the Phytomedicine Programme. To extract phytochemicals from the leaves, a direct extraction method was employed. The dried plant materials were extracted with methanol following a ratio of 1:10 of plant material to extractant. Methanol was selected because it extracts more antioxidant compounds than acetone (Masoko and Eloff, 2007). Separate aliquots of 2 g of the powdered leaves were weighed into 50 ml polyester centrifuge tubes followed by the addition of 20 ml methanol. The tubes were shaken vigorously on a Labotec shaking machine for 30 minutes. The tubes were then centrifuged at 300 x g for 15 minutes and the extracts were decanted into preweighed glass vials by filtering through Whatman No.1 filter paper and concentrated to dryness under a stream of cold air. After drying, the vials were weighed to determine the quantity extracted.

2.2.2. Qualitative antioxidant assay (Thin Layer Chromatography)

This method was used to determine the number of antioxidant compounds in extracts (Masoko and Eloff, 2007). The dried plant extracts were resuspended in methanol to a stock solution of 10 mg/ml to be used in subsequent bioassays. From the stock solution, 10 µl samples containing 100 µg of the extract were loaded onto Thin Layer Chromatography (TLC) plates (Merck, Kieselgel 60 F254) in a 1 cm band and developed using the solvent system EMW (ethyl acetate/methanol/water) (Kotze and Eloff, 2002), one of the polar mobile phases developed and used in the Phytomedicine laboratory of the University of Pretoria. After development, the plates were visualized under UV light and thereafter sprayed with 0.2% DPPH in methanol reagent spray to detect antioxidant compounds (Glavind and Holmer, 1967).

2.2.3. Quantitative antioxidant assay

For quantitative antioxidant activity of the selected plant species, the quantitative 2,2 diphenyl-1-picryhydrazyl (DPPH) free radical scavenging spectrophotometric method described by Mensor *et al.,* (2001) and modified by Aderogba *et al.* (2006) was used. Reactions were carried out in 96-well microtitre plates and each of the crude extracts was tested at varying concentrations. Final concentrations of 250.0, 125.0, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 µg/ml were prepared from 1000 µg/ml initial stock solutions of each extract. Twenty µl of 0.25 mM DPPH in methanol was added to 50 µl of each concentration of sample tested and allowed to react at room temperature in the dark for 30 minutes. Blank solutions were prepared with sample solution (50 µl) and 20 µl of methanol only while the negative control was DPPH solution (20 µl plus 50 µl methanol). Methanol served as a blank for the microplate reader and the decrease in absorbance was measured at 515 nm. Percentage antioxidant activity (AA%) values were calculated from the absorbance values using the formula:

$$\mathbf{AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}}$$

(Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control). L-ascorbic acid (vitamin C) was used as a positive control (antioxidant agent). The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the separate linear regression of plots of the mean percentage of the antioxidant activity against concentration of the test extracts (µg /ml) obtained from the three replicate assays. The results are expressed as EC_{50} values obtained from the regression plots.

2.2.4. Total phenolic content

The Folin-Ciocalteu colorimetric method described by Singleton and Rossi (1965) was used to determine the total phenolic content of the 31 methanol plant extracts. The Folin-Ciocalteu method uses gallic acid as a standard phenolic compound. Firstly, 0.1 ml of the samples at 1 mg/ml was mixed with 0.9 ml of distilled water and 0.1 ml Folin–Ciocalteu reagent. After 5 min, 1 ml of 7% sodium carbonate solution was added and the volume was made up to 2.5 ml with distilled water. The absorbance of the resulting blue-coloured solution was measured at 765 nm after 2 hours with intermittent shaking. Quantitative measurements were performed, based on a standard calibration curve of seven points from 0.0078 to 1 mg/ml of gallic acid in methanol. The total content of phenolic compounds in the plant extracts in gallic acid equivalents (GAE) were calculated using the following formula :**C= c. V/m**

Where C is the total content of phenolic compounds, mg/g plant extract, in GAE; c is the concentration of gallic acid established from the calibration curve, mg/ml; V is the volume of extract, ml; and m is the mass of pure plant methanol extract from 1 g of plant material (Miliauskas *et al.,* 2004).

2.3. Results and discussion

Thin Layer Chromatography using DPPH reagent spray was used to screen for the presence of potential antioxidant compounds in the crude extracts. The method involves the chromatographic separation of the crude extracts using TLC, after which the developed chromatogram is sprayed with a coloured radical solution (DPPH). The presence of antioxidant compounds is indicated by bleaching of the purple radical colour. The TLC-DPPH antioxidant screening method indicated the presence of antioxidant compounds in almost all the extracts. These antioxidant compounds are observed as yellow bands on the violet/purplish background (Figure. 2.1).

Of the 120 plant extracts assayed, 117 had antioxidant compounds. After plates were sprayed with DPPH, fast reacting spots with a high intensity yellow colour appeared on the plates (55 extracts), but after two hours of incubation at room temperature, several faint spots started appearing in the remaining 62 samples. This indicates that the nature of antioxidant compounds present in the extracts varies since plant extracts contain mixtures of compounds and with different functional groups, polarity and chemical behaviour. The difference in the intensity of the yellow spots and reaction speed of the compounds also suggests that the different antioxidant compounds may possess different properties, some of which have a fast radical scavenging capacity reducing DPPH radicals very rapidly while others take a longer time to react (Tepe *et al.,* 2004)

This method represents a fast and simple technique to determine the presence of free radical scavenging of compounds in crude extracts. Another added advantage in using the DPPH to assay for antioxidant activity is that DPPH is not specific to any particular class or group of antioxidants and thus provides the overall qualitative antioxidant capacity of each sample (Choi *et al.,* 2002).

Because only one solvent system (EMW) was used to develop the TLC plates, there may be more antioxidant compounds present in the extracts. The extractant used, methanol is polar, thus its components should separate better in a polar eluant system. However, not all compounds moved from the origin. These extracts probably contained some highly polar polyphenolic/tannin compounds. From the 120 plant species assayed for qualitative antioxidant activity, the 31 most active plant species (generally containing several well-separated antioxidant compounds) were selected for further assays (Table. 2.1). In some cases the TLC plates may have been overloaded leading to poor separation and a smearing of compounds

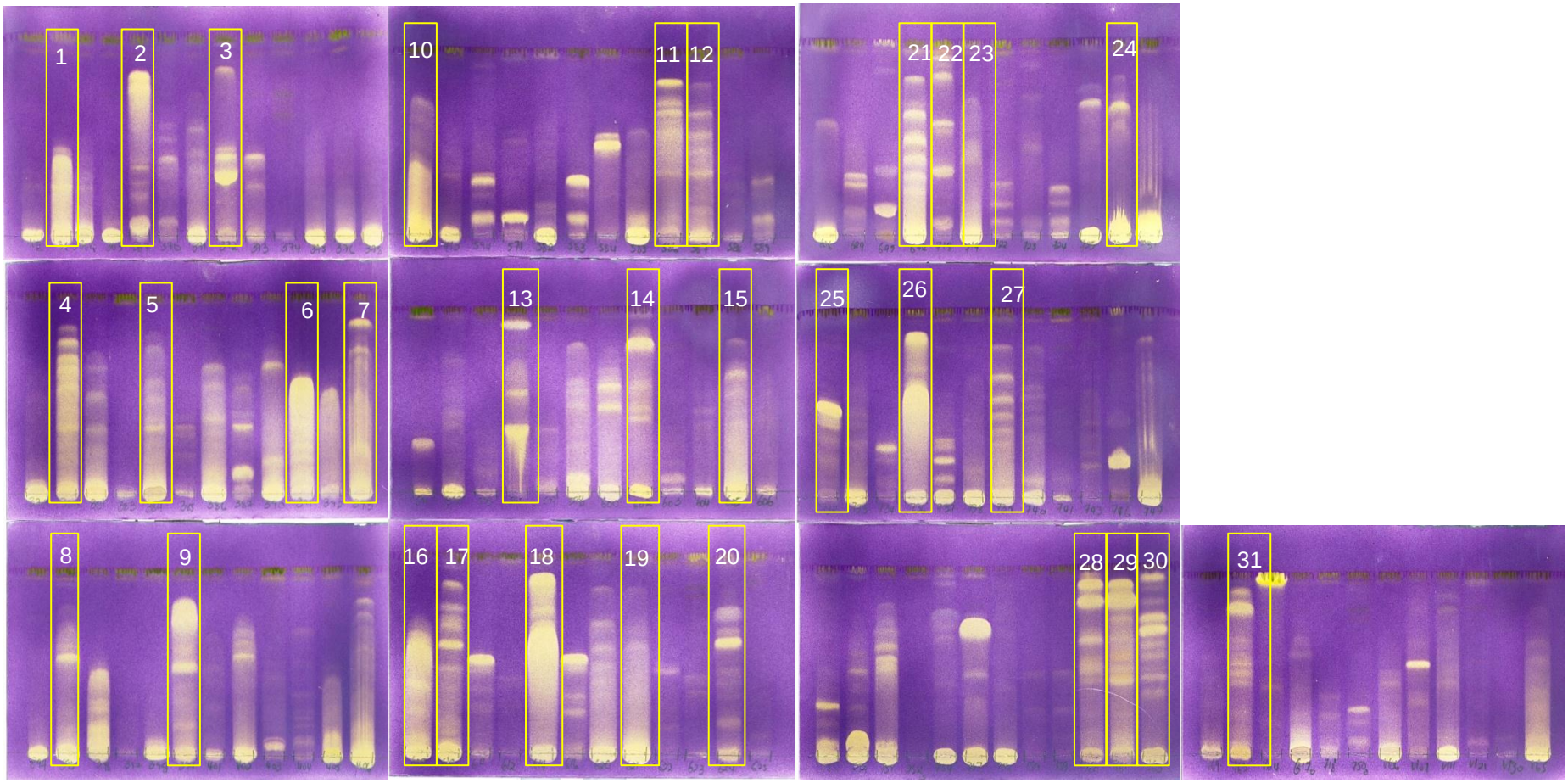


Figure 2.1. TLC chromatograms of 120 methanol plant extracts developed in EMW and sprayed with DPPH for the qualitative antioxidant activity. The blocked/highlighted extract profiles are the 31 plants that were selected for further work.

Table 2.1. List of 31selected plant species with well-defined antioxidant bands in the qualitative assay.

A= sample number corresponds to the numbers of the extract profiles in figure 2.1. **PRU** = HGWJ Schweickerdt Herbarium, **PRE** = Pretoria National Botanical Garden, **KNBG** = Kirstenbosch National Botanical Garden, **UP** = Manie van der Schiff Botanical Garden

A Sample number/ Plant name	Family	Voucher specimen number
1. *Acalypha glabrata* Thunb.	Euphorbiaceae	PRU 1144674
2. *Dalbergia nitidula* Baker	Fabaceae	PRU 114678
3. *Halleria lucida* L.	Scrophulariaceae	PRU 119037
4. *Puttelikra restripinosa* (L.) Szyszyl.	Celastraceae	PRU 114689
5. *Thespesia acutiloba* (Baker f.) Exell & Mendonça	Malvaceae	PRU 114692
6. *Alchomea hirtella* Benth. forma hirtella	Euphorbiaceae	PRU 114699
7. *Androstachys johnsonii* Prain	Picrodendraceae	PRU 114701
8. *Agromuellera macraphylla* Pax.	Acalyphoidaceae	PRU 114703
9. *Brachystegia spiciformis* Benth.	Fabaceae	PRU 114705
10. *Kirkia wilmsii* Engl.	Kirkiaceae	PRE 580129
11. *Elaeodendron transvaalense* (Burtt Davy) R.H. Archer	Celastraceae	PRU 119038
12. *Cassinopsis illicifolia* (Hochst.) Kuntze	Icacinaceae	PRU 119039
13. *Dais cotinifolia* L.	Thymelacaceae	PRE 578648
14. *Faurea saligna* Harv.	Proteaceae	PRU 119040
15. *Harpephyllum caffrum* Bernh. ex Krauss	Anacardiaceae	PRU 119041
16. *Combretum microphyllum* Klotzsch	Combretaceae	LNBG 259/1995
17. *Leucospernum erubescens* Rourke	Proteaceae	PRU 119042
18. *Loxostylis alata* A. Spreng. ex. Rchb.	Anacardiaceae	PRE 584183
19. *Podocarpus henkelii* Stapf ex. Dallim. & A.B. Jacks.	Podocarpaceae	PRE 818945
20. *Protea rubropilosa* Beard	Proteaceae	PRU 1109043
21. *Ochna gamostigmata* Du Toit	Ochnaceae	KNBG 1425/14
22. *Buxus natalensis*(Oliv.) Hutch.	Buxaceae	PRU 1109044
23. *Morella serrata* (Lam.) Killick	Acanthaceae	PRU 1109045
24. *Gomphostigmata virgatum* (L.f.) Baill.	Buddlejaceae	UP 4192
25. *Ochna serrulata* (Hochst.) Walp.	Ochnaceae	UP 302
26. *Mimetes cucculatus* (L.) R.Br.	Proteaceae	PRU 1109046
27. *Protea mundii* Klotzsh	Proteaceae	PRU 1109047
28. *Protea cyanroides* (L.) L.	Proteaceae	PRU 1109048
29. *Protea neriifolia* R.Br.	Proteaceae	PRU 119049
30. *Protea nitida* Mill.	Proteaceae	PRU 119050
31. *Soralea pinnota* L.	Fabaceae	PRU 119051

To further evaluate the antioxidant activities of the 31 selected plant species, their quantitative radical scavenging capacity against the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined using a colorimetric spectrophotometry method. This is a rapid, reproducible and inexpensive method to qualitatively measure antioxidant capacity. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods.

All the extracts had a concentration dependent radical scavenging activity. A decrease in absorbance was observed as the radical was reduced by the antioxidant compounds present in the extracts. Results are presented as EC_{50} values for clear comparison (Table. 2.2). All the extracts effectively reduced the DPPH free radical with EC_{50} values ranging from 1.2±0.2 µg/ml to 19.1±1.5 µg/ml. The scavenging properties of the extracts serve as a clear indication of their potential antioxidant properties. Out of the 31 plant species assayed, 17 had activity higher than that of L-ascorbic acid (vitamin C, reference standard). The higher antioxidant activity demonstrated by these plant extracts could not only be due to the concentration of numerous antioxidant compounds present in the plant extracts. Because the same mass 100 µg was separated in each case, these extracts must have contained compounds with higher antioxidant activity than ascorbic acid such as bibenzyls in *Combretum woodii* (Eloff *et al*., 2005; Masoko and Eloff, 2007)

Phenolic and polyphenolic compounds constitute the main class of natural antioxidants present in plants, food, and beverages. The antioxidant activity of plant extracts in several studies was found to be mostly due to the phenolic compounds present in the extracts (Bors *et al.,* 1990, Moure *et al.,* 2001). As plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant species. The total phenolic contents of the 31 methanol extracts ranged from 5.2±0.9 to 18.6±3.9 mgGAE/mg of extract.

Table 2.2. Percentage extract yield, total phenolic content and DPPH free radical scavenging activity (EC_{50} (µg/ml) of methanol extracts of the 31 selected plant species. Extracts with higher activity than ascorbic acid are printed in bold.

Sample	% Extract yield	Total phenolic content mg GAE/ mg of extract	Antioxidant activity EC_{50} (µg/ml)
1	14.45	8.56±1.75	2.48±1.11
2	14.74	7.66±0.88	**1.94±0.44**
3	17.93	7.43±0.46	**1.97±0.24**
4	20.10	7.03±1.21	3.88±0.64
5	20.41	9.21±0.233	**1.81±0.40**
6	9.24	14.58±4.09	**1.52±0.30**
7	16.62	11.40±1.67	**1.87±0.08**
8	9.11	8.75±0.81	**1.20±0.22**
9	11.58	10.61±3.07	**1.76±0.28**
10	6.22	10.39±0.74	**1.93±0.86**
11	11.43	9.43±0.95	2.81±1.10
12	10.94	6.53±0.59	8.36±1.37
13	9.47	8.71±1.34	**1.61±0.27**
14	15.97	8.32±3.03	3.88±0.64
15	10.04	13.61±7.47	**1.52±0.59**
16	15.70	17.66±3.00	**1.30±0.10**
17	24.43	8.73±2.80	**1.54±0.52**
18	19.69	18.54±1.43	**1.58±0.54**
19	17.76	8.51±3.30	4.02±0.43
20	21.42	8.40±1.12	8.18±0.72
21	17.1	16.35±1.97	**1.62±0.21**
22	18.77	6.73±1.86	8.69±0.03
23	10.69	8.04±2.64	3.38±0.08
24	8.19	7.93±1.26	8.23±0.84
25	12.67	18.65±3.86	4.20±3.39
26	16.78	16.08±1.93	**1.62±0.01**
27	23.29	15.60±2.06	**1.45±0.64**
28	28.97	10.32±4.24	**1.48±0.30**
29	25.23	7.64±0.25	3.25±2.15
30	16.13	12.35±0.40	12.14±1.11
31	17.72	5.17±0.97	19.07±1.50
Ascorbic acid		-	**2.28±0.02**

The total phenolic content of the plant extracts correlated well with the respective antioxidative activity of the plant extracts (Figure 2.2). Good correlation was found between the mg GAE/mg and the logarithm of EC_{50} values (R^2 > 0.9447). Polyphenols have been reported to be responsible for the antioxidant activity in plant extracts (Piluzza and Bullitta, 2011). Phenolic constituents react with active oxygen radicals such as hydroxyl radical, superoxide anion radical and lipid peroxyl radical (Husain *et al.,* 1987, Afanaslev *et al.,* 1989). These compounds have a broad spectrum of chemical and biological activities including radical scavenging properties.

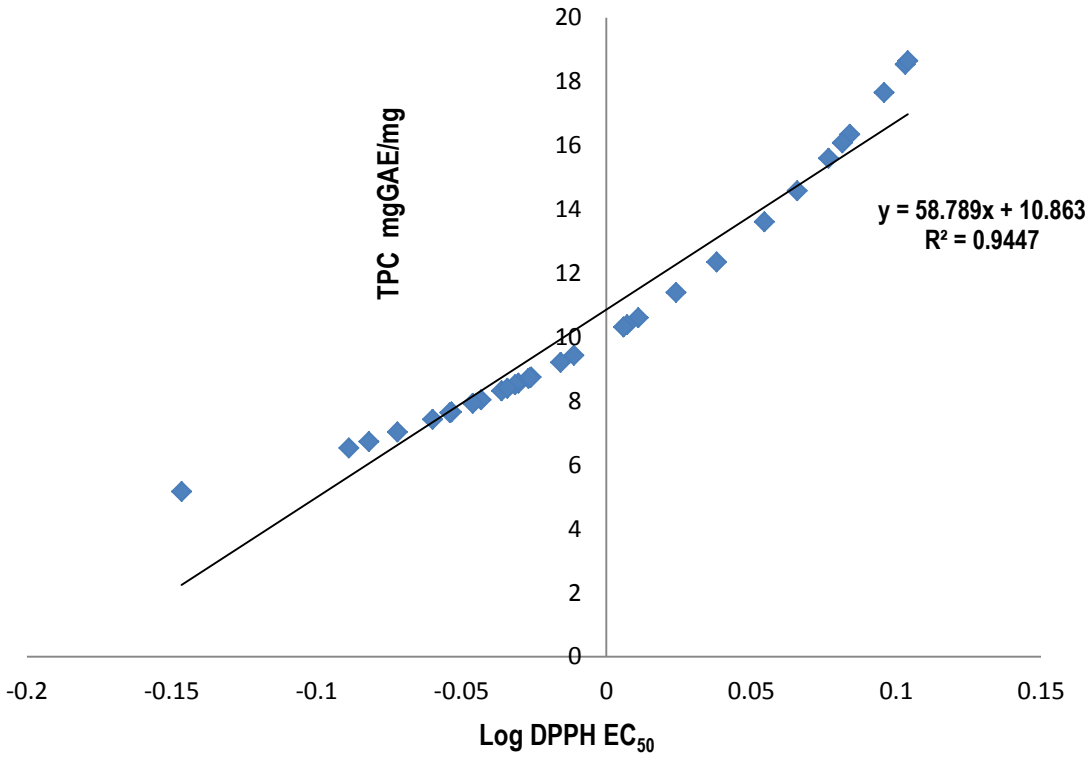


Figure 2.2. Correlation between antioxidant activity and total phenolic content of methanol extracts of the 31 selected plant species.

2.4. Conclusion

Since oxidative DNA damage can play a significant role in mutagenesis, cancer, aging and other human pathologies, a decrease in oxidative stress is a logical strategy to protect cell structures from oxidative damage and people from cancer and other ROS-dependent morbid conditions. Considering the radical scavenging capacity of the assayed plant extracts, and their total phenolic contents as indices of antioxidant activity of the extracts, these findings reveal the potential of the extracts as sources of natural antioxidants. It indicates that these plant species could be promising agents in scavenging free radicals and could contribute to the prevention and treatment of diseases related to free radical reactions. This could also contribute to understanding the molecular basis of therapeutic properties of many medicinal plants (Aluyi *et al.,* 2003).

In this study, methanol leaf extracts of 31 plant species had different radical scavenging activities and the majority had higher activities than ascorbic acid. Their antioxidant activity could be attributed to the presence of phenolic compounds present in the extracts. In the next chapter, the genotoxic effects of these extracts will be investigated, focusing specifically on their potential to prevent mutations i.e. antimutagenic effects.

CHAPTER 3

The mutagenic, antimutagenic and cytotoxic activities of 31 plant species with high antioxidant activity

3.1. Introduction

Validating the efficacy and long-term safety of herbal preparations is a vital step towards making them acceptable from a first-world perspective and hence in obtaining government approval and financial support for traditional medical systems (Taylor *et al.,* 2003). Plant extracts and isolated compounds may interact directly or indirectly with DNA, causing changes that affect cell function and in the long term cause malignant transformation. It is thus important to detect the action of these extracts on genetic material using different biomarkers of effect (Carballo *et al.,* 1992).

In this study, the bacterial Ames test (Maron and Ames, 1983) and micronucleus test (Fenech and Morley, 1985) were used to determine the genotoxic and antigenotoxic activity of 31 plant extracts. These plant species were selected based on their high antioxidant activity and high concentration of phenolic compounds because there may be a correlation between antioxidant activity and antigenotoxicity/genotoxcicity. These plant species may have applications as probes for development of antimutagenic agents of natural origin (Kaur *et al.* 2006).

We started by investigating the potential genotoxic effects of the extracts before antigenotoxic effects because most of the traditional medicinal plants have never been subjected to exhaustive toxicological tests as are required for modern pharmaceutical compounds. Based on their traditional use for long periods of time they are often assumed to be safe, however, research has shown that many plant species which are used as food or in traditional medicine have *in vitro* mutagenic (Dèciga-Campos *et al.,* 2007; Mohd-Fuat *et al.,* 2007) or toxic and carcinogenic (De Sá Ferreira and Ferrão Vargas, 1999) properties. This raises concerns about the potential mutagenic or genotoxic hazard resulting from the use of such plant species. In some cases, their use has been correlated with a high rate of tumour formation (Ames, 1986, Schimmer *et al.,* 1994). It is therefore necessary to evaluate the cytotoxicity and genotoxicity of plant products to determine whether they are able to act as bioactive agents without causing adverse effects.

Plants with clear mutagenic properties should be considered as potentially unsafe and certainly require further testing before their continued use can be recommended (Verschaeve and Van Staden, 2008). Tests that measure DNA damage can be used as potential predictors of cancer. Moreover, assays that indicate DNA protective effects of test samples can be used to investigate cancer chemoprevention. We also measured cytotoxicicty of the extracts using the neutral red uptake assay (Repetto *et al.,* 2008). In addition to its value in determining safety it will also assist in finding the dosage for samples that have antigenotoxic activity.

3.2. Materials and methods

3.2.1. Ames test

The Ames test (Maron and Ames, 1983) was performed with *S. typhimurium* strain TA98 (for detection of frame-shift mutations), TA100 (for detection of base-pair substitutions) and TA102 (for detection of transitions and transversions). The well-known plate incorporation procedure described by Maron and Ames (1983) was used. One hundred µl of bacterial stock were incubated in 20 ml of Oxoid Nutrient broth for 16 h at 37 °C on a rotary shaker. Of this overnight culture, 0.1 ml were added to 2.0 ml of top agar (containing traces of biotin and histidine) together with 0.1 ml test solution (test sample at concentrations of 5000, 500 and 50 µg/ml, solvent control or positive control) and 0.5 ml phosphate buffer The top agar mixture was poured over the surface of a minimal agar plate. The plate was incubated for 48 h at 37°C. After incubation the number of revertant colonies (mutants) were counted. All cultures were made in triplicate (except the solvent control where five replicas were made). Absence of toxicity was confirmed by observing the background bacterial growth, which should be normally present.

For antimutagenicity testing, a variation of the Ames test was used. Here, 50µl test solution at each concentration was added to 0.5 ml phosphate buffer, followed by addition of 50 µl of a mutagen (4-NQO) before addition of 0.1 ml of the overnight bacterial culture. After incubation, revertant colonies were counted and antimutagenicity was expressed as percentage inhibition of mutagenicity calculated using the formula below:

$$\% \text{ inhibition} = \left[\frac{M-T}{M}\right] X\ 100$$

Where T is the number of revertants per plate in the presence of mutagen and the test solution and M is the number of revertants per plate in the positive control. All cultures were prepared in triplicate (except the solvent control where five replicates were used). Absence of toxicity was confirmed when a background layer of bacterial growth was observed, which should be normally present. The positive control used in this study was 4-nitroquinoline 1-oxide (4-NQO) at a concentration of 2 µg/ml (for TA98) and 1 µg/ml (for TA100).

3.2.2. Neutral red uptake (NRU) assay

The NRU assay (Borenfreund and Puerner, 1985, Repetto *et al.,* 2008) was carried out to measure the cytotoxicity of the 31 plant extracts. Cells were maintained in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% foetal calf serum. Cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.2 × 10^6 cells/ml i.e. 40 000 cells/200 µl into each well of a 96-well microtitre plate and incubated for 24 h at 37°C in a 5% CO_2 incubator. After incubation, the subconfluent cells in the microtitre plate were incubated with the test samples for a further 24 h for the cytotoxicity assay. The test sample was assayed at 2.5, 0.5 and 0.1 mg/ml to establish a nontoxic concentration to confirm genotoxicity at a later stage. After the 24 hour incubation, the test sample was removed and cells were washed with 200 µl of a phosphate buffered saline (PBS) solution. To each well, 200 µl of medium containing 0.05 mg/ml neutral red dye were added and the plates were incubated for 3 hours. The neutral red was aspirated and the cells were washed with 200 µl of PBS. The dye was extracted from the cells using a 200 µl acetic acid-ethanol (50/1) mixture (destaining solution) for approximately 1 hour. Using a microtitre plate shaker, the plates were agitated for at least 90 minutes until a homogenously stained medium was obtained. Optical density (OD) was measured with a spectrophotometer. The OD_{620} measured as a reference value was subtracted from the OD_{540} which is the optical density at the wavelength at which maximal absorption of neutral red occurs. Absorption of non-treated cells was given a 100% value to which data from exposed cells were compared. Sodium dodecyl sulphate (SDS) was used as a positive control. Results are expressed as percentage cell viability

3.2.3. Cytokinesis-block micronucleus/cytome assay

The micronucleus test was performed as described by Fenech and Morley (1985) on C3A cells which are a clonal derivative of HepG2 cells (human hepatocellular carcinoma). The cells were grown in DMEM growth medium supplemented with 10% foetal bovine serum and 1% L-glutamine. The cells were cultured at 37°C in a 24 well plate with 5% carbon dioxide for 24 h. After 24 h, the cells (200000 cells/ml) were treated with different concentrations of the plant extracts at concentrations of 2.5, 0.5 and 0.1 mg/ml and incubated further for another 24 h. At this point, 15 µl of cytochalasin B (0.6 µg/ml) was added to the 24 well plates and the plates were incubated for a further 24 h. 4-Nitroquinoline 1-oxide (4-NQO) was used as a positive control at a concentration of 1 µg/ml. After incubation, the cells were trypsinized, transferred into centrifuge tubes and centrifuged at 1000 rpm for 10 minutes. The supernatant was then removed and the pellet was resuspended in cold fixation solution (1:3 acetic acid/methanol) with the addition of 37% formaldehyde. The centrifugation was repeated and the pellet resuspended in fixation solution twice more. Finally, the pellets were resuspended in 4 ml of fixation solution and stored at -20°C for three days. After three days, cells were resuspended in fresh fixation solution and mounted on microscope slides. The slides were stained with May-Grünwald stain for 2

minutes followed by Giemsa stain for 5 minutes. The slides were viewed under a microscope and the micronuclei, nucleoplasmic bridges and nuclear buds were counted per 2000 binucleated cells counted. Samples were considered positive for mutagenicity testing if the number of micronuclei, nucleoplasmic bridges and nuclear buds were significantly higher than the negative control and positive in the antimutagenicity testing when the number of mucronuclei, nucleoplasmic bridges and nuclear buds were significantly lower than the positive mutagen (4-NQO).

For antigenotoxicity testing, a variation of the cytokinesis-block micronucleus cytome method described above was used. Here, the cells were incubated in the presence of a combination of 4-NQO and the test sample (Plant extract) at each concentration. Antigenotoxicity was therefore measured as a decrease in the number of micronuclei, nucleoplasmic bridges and nuclear buds per 2000 binucleated cells in the positive control and the test sample.

3.3. Results and discussion

In this chapter, the mutagenic, antimutagenic and cytotoxicity activity of 31 plant extracts were determined in the Ames/microsome test, cytokinesis block micronucleus/cytome assay and neutral red uptake assay respectively. Numerous studies have shown that a large proportion of carcinogens identified as mutagens by the Ames test (Zeiger, 2001). Screening is required to identify and limit the use of all mutagenic plants. To assay for antimutagenic activity and/or potential of the plant extracts to prevent DNA damage by 4-NQO (positive mutagen/carcinogen), plant extracts were incubated together with 4-NQO in both the Ames test and cytome assay.

The results of the mutagenic effects of 31 plant leaf extracts in the Ames test (*S. typhimurium* TA98 and TA100) are summarised in Table 3.1. The two strains used allow the detection of frame shift mutations (TA98) and base-pair substitutions (TA100). The two strains used to assay for mutagenicity and antimutagenicity effects were selected because they are the most commonly used strains within the pharmaceutical industry (Purves *et al.,* 1995). Furthermore, there was a consensus agreement that these strains can be used routinely as they are sensitive and detect a large proportion of known bacterial mutagens (Gatehouse *et al.,* 1994), and they have relatively low colony forming rates making the colony counting procedure easier when numerous samples are assayed.

Only one plant extract; *Halleria lucida* (#3) was mutagenic in TA98 (Table 3.1). Negative mutagenicity results mean that the extracts were not able to enhance the number of His- to His+ revertants by a factor of two or more above the spontaneous (solvent/negative control) background level. This background level as well as positive control values were in all cases within the normal limits found in the literature (Mortelmans and Zeiger, 2000). This confirms that the test system was sensitive enough even though the assay was carried out without metabolic activation. The absence of mutagenic response by plant extracts against *Salmonella typhimurium* bacterial strains in the Ames test is a positive step forward in determining the safe use of plants in traditional medicine (Reid *et al.,* 2006). An extensive data base has shown that many chemicals that are positive in this test also have mutagenic activity in other tests. Moreover, the proportion of carcinogens identified as mutagens by the Ames test ranges from about 50% to 90% (Maron and Ames, 1983, OECD, 1997, Mortelmans and Zeiger, 2000).To confirm absence of toxicity, the background layer of bacterial growth was observed in all experiments. Although the bacterial lawn was present,the low numbers of revertant colonies in *S. typhimurium* TA100 may indicate toxicity since the tester strain TA100 is more sensitive to toxic substances than strain TA98 (Prival and Zeiger, 1998) (i.e. sample with an average number of revertant colonies ≤100). In some cases, the number of colonies in test sample (sample 21) is lower than the negative control by more than 50%.

Table 3.1. Mean number of revertants per plate (± SD) in *S. typhimurium* TA 98 and TA 100 exposed to different concentrations of the plant extracts.

Sample #	TA 98			TA 100		
Conc.(mg/ml)	5	0.5	0.05	5	0.5	0.05
1	33.33±5.03	36.33±4.04	43.33±5.51	134.00±16.09	139.00±18.52	135.33±7.63
2	25.33±4.50	42.33±7.30	36.00±6.12	151.00±16.64	139.67±7.50	136.67±9.86
3	53.67±3.51	63.33±4.93	58.33±6.35	124.33±6.43	137.67±4.93	155.00±3.60
4	23.00±6.24	20.00±4.04	24.00±5.20	137.67±11.59	143.00±5.57	143.67±2.52
5	28.67±7.10	16.90±5.69	18.67±3.78	135.33±5.13	117.67±6.80	127.67±15.95
6	23.67±2.52	23.33±3.05	19.00±1.00	109.33±10.12	127.33±4.93	145.00±3.51
7	32.33±2.52	29.67±7.02	19.67±3.51	111.33±10.07	137.00±12.12	138.67±11.72
8	34.67±4.51	24.67±7.23	21.33±2.52	113.00±3.61	134.00±4.04	120.33±4.04
9	29.33±5.03	28.67±5.51	25.67±3.21	112.67±2.04	126.00±7.58	122.67±11.37
10	27.00±2.00	26.67±1.53	26.67±2.08	101.67±5.13	119.67±4.51	104.67±5.69
11	28.00±3.60	29.00±6.24	25.67±4.51	101.33±4.16	90.00±3.61	93.83±2.52
12	22.33±6.81	23.67±4.04	25.67±3.05	101.33±1.53	115.00±12.06	127.33±3.21
13	19.33±6.35	24.67±6.43	19.00±3.61	109.33±9.71	104.00±5.00	116.00±4.58
14	15.00±5.29	29.00±9.54	29.33±5.69	100.67±4.16	101.67±7.37	96.67±9.71
15	22.67±1.15	28.33±7.50	23.67±1.53	92.67±6.43	96.33±3.21	99.33±3.06
16	19.67±4.16	22.00±2.64	20.67±4.16	91.67±3.21	85.00±3.00	90.33±2.31
17	24.33±4.51	25.00±4.00	22.67±5.13	104.00±5.29	91.00±4.60	98.00±2.00
18	24.00±2.00	21.67±2.08	26.67±6.03	95.67±2.52	82.67±3.79	96.67±1.53
19	26.00±5.57	23.00±6.00	25.33±8.14	89.33±10.69	81.33±1.52	102.33±2.08
20	28.67±6.11	24.33±1.15	21.33±3.05	70.00±7.55	84.33±1.53	74.00±7.80
21	23.33±6.03	21.33±2.08	25.00±2.64	49.33 3.79	84.00±10.15	77.00±8.08
22	16.00±1.00	19.67±3.05	18.67±5.51	87.67±8.62	83.33±4.73	86.33±3.3.22
23	18.00±4.36	19.67±4.04	20.33±1.53	88.00±1.73	88.33±14.50	89.00±3.06
24	21.33±3.21	25.00±6.24	19.33±3.05	82.33±7.37	83.33±2.89	86.00±2.65
25	40.00±3.60	35.00±8.54	35.33±2.89	102.00±7.94	127.33±10.69	119.00±11.27
26	38.67±11.06	33.33±9.50	34.33±6.66	114.00±14.00	104.00±6.00	92.50±7.00
27	36.67±8.14	42.67±2.08	36.66±2.08	122.67±11.59	107.33±6.03	124.00±4.58
28	37.00±2.64	36.67±6.02	34.67±4.51	119.00±6.00	94.00±4.16	104.00±8.50
29	28.33±9.71	29.00±6.00	33.00±9.16	97.50±5.13	86.50±5.69	100.00±8.72
30	33.33±6.11	34.67±4.04	29.67±6.81	98.67±5.03	91.66±9.45	98.67±5.51
31	25.33±4.04	35.00±3.60	33.00±11.00	82.67±2.52	104.67±5.51	104.00±9.64
Solvent blank		24.7±6.59			119.90±9.85	
Positive control		239.44±17.31			1082.34±63.91	

Not all classes of mutagens can be detected by the Ames test. Factors such as mutagenic end-points and differences in metabolic activation may enhance the sensitivity of the bacterial reverse mutation test that may lead to either overestimation or underestimation of mutagenic activity. In some cases, extracts with high antibacterial activity against *Salmonella* may not be appropriate for testing in this assay. In the case of strain TA98, antibacterial activity that could mask mutagenic activity, was probably not relevant based on the low concentration of the extracts that had antimutagenic activity (Gatehouse *et al.,* 1994). In the case of strain TA100, toxicity may have contributed to the observed antimutagenicity activity. It is possible that some of the plant extracts may have had antibacterial activity resulting in lower numbers of colonies, especially at the higher concentrations tested. Results obtained in bacterial mutagenicity tests may not always be predictive of genotoxicity in eukaryotic systems (Walmsley and Billinton, 2011). It was therefore important to also assay the mutagenic effects of the selected plant extracts in mammalian cells (hepatocytes) using the cytokinesis block micronucleus/cytome assay. Human hepatocytes (C3A) cells were used as a mammalian model to study the genotoxic effects of the selected plant extracts. C3A cells are derived from HepG2 cells. They retain many functions of normal human hepatocytes. They have the essential structural, biochemical and growth features of normal human liver cells and have conserved both phase I and phase II metabolic activities (Kelly, 1994). This model thus provides a system where indirectly acting mutagenic plant extracts which may have been missed in the Ames test can be identified.

Results of the cytokinesis block micronucleus/cytome assay are summarised in Table 3.2. In all cases, the background frequency of micronuclei, nucleoplasmic bridges and nuclear buds is similar in all the negative controls. These are the three parameters that were used to measure genotoxicity in the cytome assay. A high proportion of the plant extracts gave negative results. Only samples 12, 13 and 22 had much higher micronuclei compared to the negative control, whereas samples 1, 3, 17, 24, 27 and 29 had a higher incidence of nucleoplasmic bridges. Lastly, samples 4, 5, 12, 13 and 22 had a high incidence of nuclear buds. Only 14 out of 31 (45%) plant extracts produced one or more chromosomal abnormalities. It is thus not possible to generally conclude that these plants are completely genotoxic based on this assay. Most of the results are in agreement with those obtained in the Ames bacterial reverse mutation test where all extracts were not mutagenic.

Micronuclei are expressed in dividing cells that either contain chromosomal breaks lacking centromeres and/or whole chromosomes. By scoring the number of micronuclei induced in a population of dividing cells, the potential of a test sample to break chromosomes or to disrupt cell division and cause aneuploidy is assessed (Taylor *et al.,* 2003). Nucleoplasmic bridges provide a measure of chromosome rearrangement whilst nuclear buds indicate induction of gene amplification by DNA damaging agents. These are all chromosomal biomarkers of genomic instability relevant to cancer (Fenech, 2000, Fenech 2002).

Table 3.2. Number of micronuclei, nucleoplasmic bridges and nuclear buds per 2000 binucleated cells in C3A cells exposed to different concentrations of the plant extracts.

Sample #	*Conc. (mg/ml)*	*Micronuclei*	*Nucleoplasmic bridges*	*Nuclear buds*
1	2.5	-	-	-
	0.5	12	24	12
	0.1	8	14	20
2	2.5	-	-	-
	0.5	4	14	7
	0.1	10	18	21
3	2.5	-	-	-
	0.5	7	8	8
	0.1	4	9	9
Blank	**0**	**6**	**6**	**8**
4	2.5	-	-	-
	0.5	6	9	14
	0.1	4	7	4
5	2.5	6	10	11
	0.5	8	12	15
	0.1	9	10	17
6	2.5	-	-	-
	0.5	6	9	11
	0.1	5	7	6
Blank	**0**	**7**	**9**	**7**
7	2.5	-	-	-
	0.5	6	7	12
	0.1	4	4	8
8	2.5	7	9	6
	0.5	6	9	9
	0.1	4	11	13
9	2.5	10	10	11
	0.5	4	10	8
	0.1	5	11	9
Blank	**0**	**5**	**10**	**7**
10	2.5	-	-	-
	0.5	4	11	5
	0.1	5	9	6
11	2.5	-	-	-
	0.5	-	-	-
	0.1	5	10	9
12	2.5	-	-	-
	0.5	14	13	26
	0.1	10	20	18
13	2.5	-	-	-
	0.5	19	18	27
	0.1	7	13	11
14	2.5	-	-	-
	0.5	6	9	9
	0.1	5	10	9
15	2.5	-	-	-
	0.5	7	11	10
	0.1	6	12	9
Blank	**0**	**5**	**12**	**8**
16	2.5	-	-	-
	0.5	7	15	7
	0.1	5	11	9
17	2.5	-	-	-
	0.5	8	28	16
	0.1	3	18	12
18	2.5	-	-	-

	0.5	7	10	9
	0.1	5	9	8
Blank	**0**	**6**	**10**	**11**
19	2.5	-	-	-
	0.5	8	10	12
	0.1	7	10	8
20	2.5	7	15	15
	0.5	7	17	14
	0.1	6	22	9
21	2.5	-	-	-
	0.5	-	-	-
	0.1	8	12	10
Blank	**0**	**5**	**15**	**12**
22	2.5	-	-	-
	0.5	9	10	20
	0.1	14	24	23
23	2.5	-	-	-
	0.5	5	15	8
	0.1	7	14	9
24	2.5	5	31	12
	0.5	8	43	11
	0.1	5	38	8
Blank	**0**	**4**	**15**	**9**
25	2.5	-	-	-
	0.5	6	16	10
	0.1	5	14	9
26	2.5	-	-	-
	0.5	4	17	7
	0.1	6	18	11
27	2.5	-	-	-
	0.5	5	45	16
	0.1	6	40	16
Blank	**0**	**7**	**17**	**11**
28	2.5	3	13	8
	0.5	4	10	10
	0.1	3	11	9
29	2.5	-	-	-
	0.5	7	20	12
	0.1	6	19	12
30	2.5	4	15	7
	0.5	3	17	6
	0.1	4	17	11
31	2.5	-	-	-
	0.5	-	-	-
	0.1	6	31	11
Blank	**0**	**5**	**11**	**10**

- = no binucleated cells due to toxicity

Screening for antimutagenic activities in plant extracts is important in the discovery of new effective cancer preventing agents. Antimutagenic compounds can possibly also be anticarcinogens. But then, many chemicals can contribute to the carcinogenesis process without inducing mutations (Verschaeve and van Staden, 2008). One of the most important characteristics of antimutagens is their universality regarding inhibition of mutations induced by different stressing agents (Alekperov, 1982). Antimutagenicity determination of plant extracts is important in the discovery of new effective anti-carcinogenic treatments. Plant extracts indicating antimutagenicity are not necessarily anticarcinogens, but this is an indication of a possible anticarcinogen (Reid *et al.,* 2006). There are no reports on the antimutagenic activities of plants investigated in this study.

Almost 50% of the plant extracts (sample # 3, 6, 9, 16, 17, 18, 20, 21, 22, 23, 24, 25, 27, 30 and 31) reduced the mutagenic effects of 4-NQO in the Ames test using *S. typhimurium* TA98 (Figure 3.1). In *S. typhimurium* TA100, samples 1, 2, 3, 4, 5, 6, 7, 12, 13, 14, 15, 16, 17, 18, 22, 23, 24, 28, 29, 30 and 31 inhibited the mutagenic effects of 4-NQO (Figure 3.3 and 3.4). The remaining plants increased the mutagenic effects of the mutagen 4-NQO, a phenomenon known as comutagenicity. Extracts 1, 2, 4, 5, 7, 8, 10, 11, 12, 13, 14, 15, 19, 26, 28 and 29 enhanced the mutagenic effects of carcinogen 4-NQO in *S. typhimurium* TA98 (Figure 3.2) and samples 8, 9, 10, 11, 19, 20, 21, 25, 26 and 27 enhanced the mutagenic effects of carcinogen 4-NQO in *S. typhimurium* TA100 (Figure 3.3 and 3.4). A comutagenic effect is observed in instances where, when tested alone, plant extracts don't have any mutagenic effects, but in the presence of a positive mutagen, these extracts enhance or increase the mutagenicity of the positive mutagen.

Not all antimutagenic plant extracts had activity in both *S. typhimurium* TA98 and *S. typhimurium* TA100. Only samples 3, 6, 16, 17, 18, 22, 23, 24, 30 and 31 had antimutagenic effects in both strains. The differences in the observed activities in the other plant extracts may be because compounds contained within the extracts can only prevent, inhibit and/or reverse frame shift or base-pair substitutions. Extracts active in both strains may be general non-specific antimutagens. These extracts reduced the number of mutant colonies in *S. typhimurium* TA98 and *S. typhimurium* TA100, protecting against the mutagenicity induced by 4-NQO either by frame-shift mutations and base pair substitutions. Still in this case, the question of possible antibacterial effects of the plants is important. It is a known phenomenon that higher plants often produce antimicrobial agents and these can kill the tester strain. However, for screening purposes, concentrations employed are generally established by trial and error in each individual laboratory (Mitscher *et al.,* 1992). Plant extracts that had antimutagenic effects in TA100 demonstrated a clear dose response which was not observed against TA98. However, the dose response in TA100 may be a result of toxicity as this tester strain seems to be sensitive to toxic substances than TA98

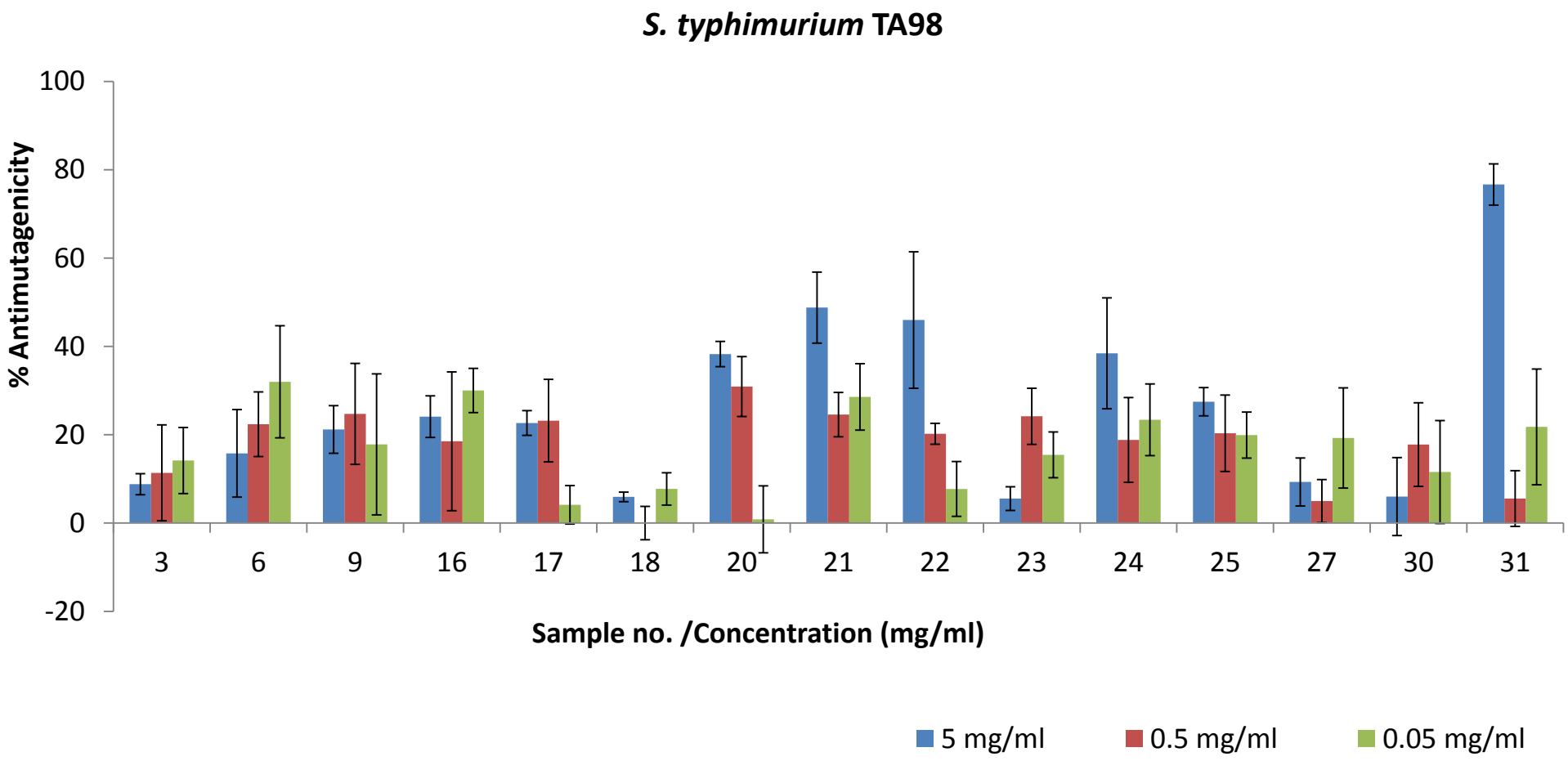


Figure 3.1. Antimutagenic activity of 15 methanol plant extracts in the Ames test using *S. typhimurium* TA98 (percentage inhibition of mutagenic effects of 4-NQO)

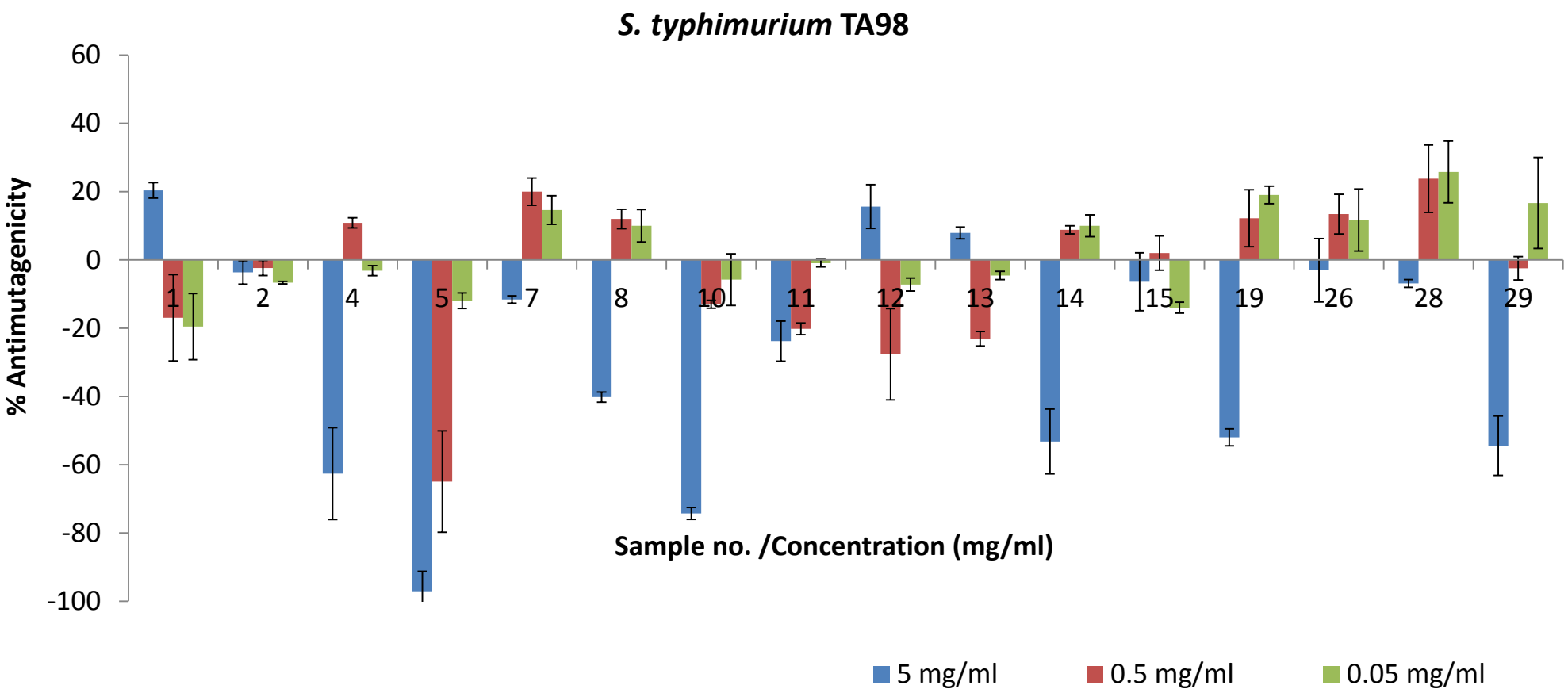


Figure 3.2. Antimutagenic activity of 16 methanol plant extracts in the Ames test using *S. typhimurium* TA98 (percentage inhibition of mutagenic effects of 4-NQO)

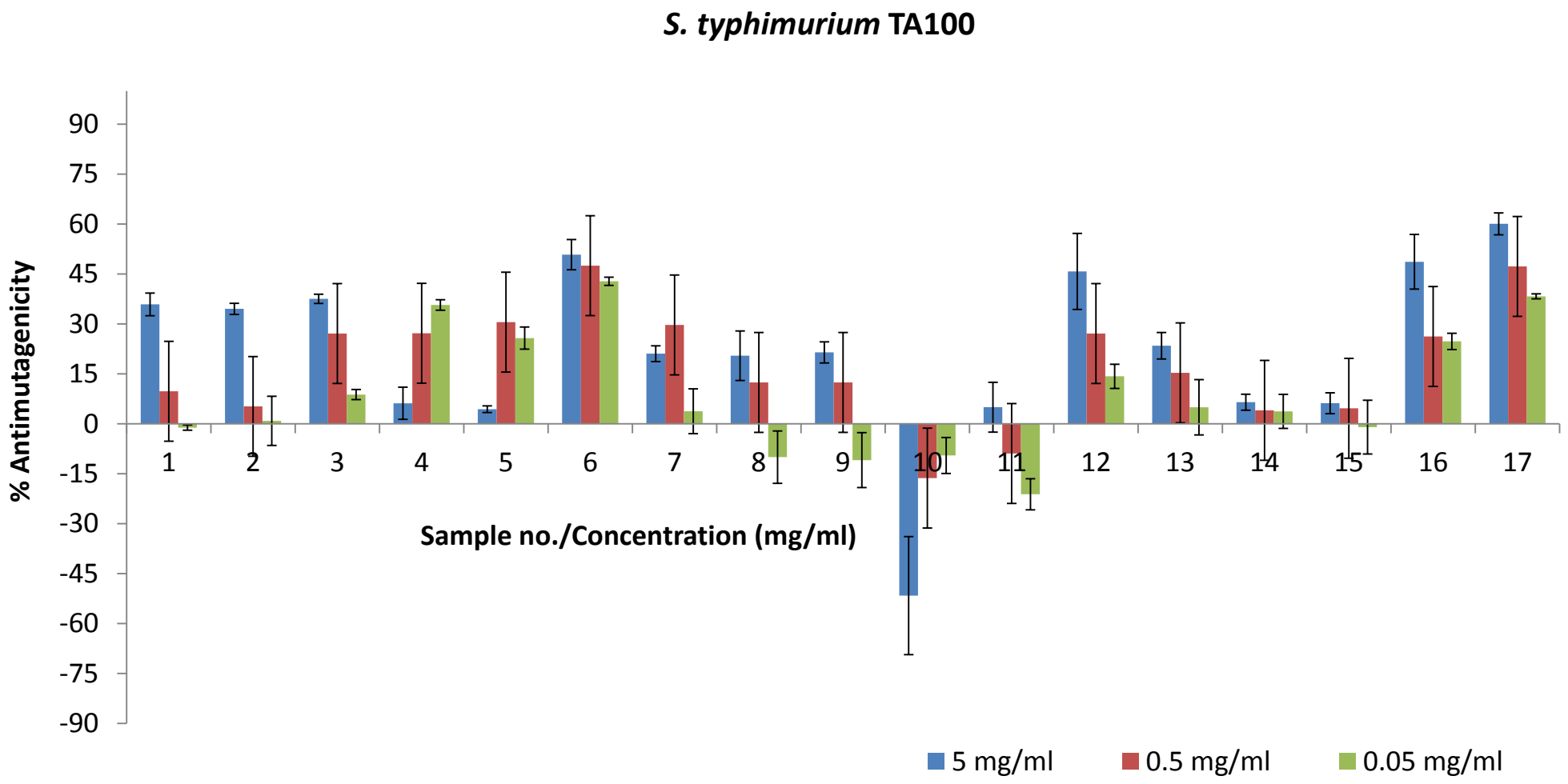


Figure 3.3. Antimutagenic activity of 17 methanol plant extracts in the Ames test using *S. typhimurium* TA100 (percentage inhibition of mutagenic effects of 4-NQO)

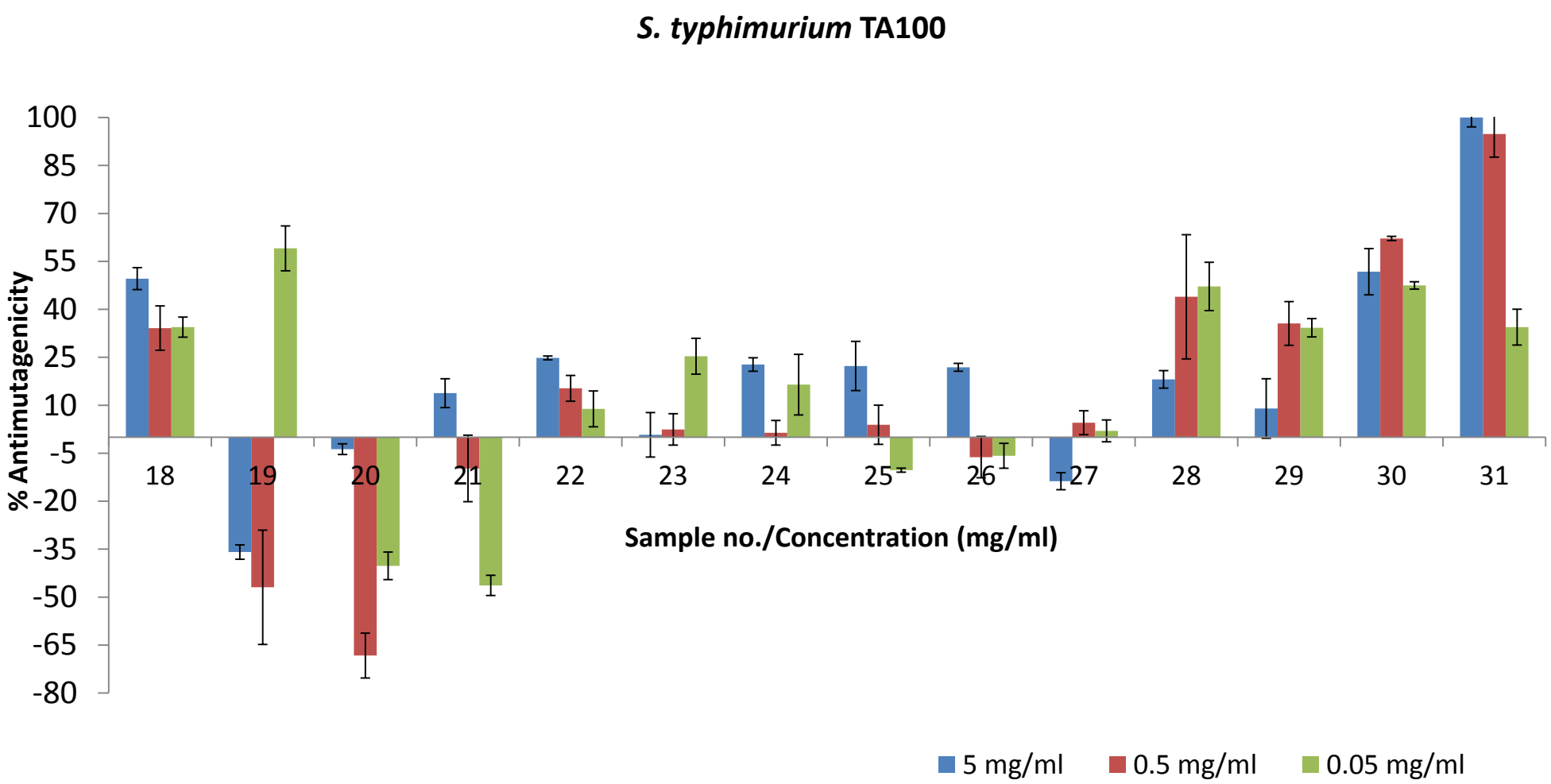


Figure 3.4. Antimutagenic activity of 14 methanol plant extracts in the Ames test using *S. typhimurium* TA100 (percentage inhibition of mutagenic effects of 4-NQO)

The percentage inhibition of mutagenic effects of 4-NQO (antimutagenicity) of the plant extracts ranged from 8.79 ± 2.39–76.67 ± 4.67% in *S. typhimurium* TA98 and 0.77 ± 6.95-99.00 ± 2.95% in *S. typhimurium* TA100. In *S. typhimurium* TA98, eight (8) extracts had more than 25% antimutagenic effects. Only 3 extracts had 45% and more antimutagenic effects even at the highest concentration tested. In *S. typhimurium* TA100, 9 plant extracts had more than 25% antimutagenic effects and only 7 extracts had 45% and more antimutagenic effects.

It appears the tested plant extracts have a more desired effect in *S. typhimurium* TA100 than in *S. typhimurium* TA98 which may to some extent explain the mode and/or mechanism of the antimutagenic effects. In the Ames test, the antimutagenic effect is considered moderate when the inhibitory effect is between 25 and 40% and strong when more than 45%. Inhibitory effects of less than 25% are considered weak (Negi *et al.,* 2003, Verschaeve and Van Staden, 2008). Based on Negi and colleagues' conclusion, most of the plant extracts assayed in this study may be considered to have weak or no antimutagenic effects. The real value of a screen cannot be judged until several cycles have been run and the results evaluated (Mitsher *et al.,* 1992).

Results for the antimutagenic effects of the 31 plant extracts in the micronucleus/cytome assay are presented in Table 3.3. All plant extracts assayed in the micronucleus/cytome assay had cytotoxic effects at the highest concentration assayed. This was evident as there were less than 1000 binucleated cells in each case. Out of the 31 plant extracts, *Harpephyllum caffrum* (15) reduced induction of micronuclei, *Androstachys johnsonii* (7) reduced frequencies of nucleoplasmic bridges and *Faurea saligna* (14) reduced frequencies of nuclear buds. Only samples *Puttelikra restripinosa* (4), *Cassinopsis illicifolia*, (12), *Combretum microphyllum* (16), *Leucospernum erubescens* (17) and *Protea cyanroides* (28) had an antigenotoxic effect by reducing occurrence of all the genotoxic endpoints measured in this assay. This is a clear indication that these plant species may protect against chromosomal damage resulting from chromosomal breakages and aneuploidy, chromosomal rearrangements and alteration on gene expression. All the others that indicated an antigenotoxic response either prevented one or two of the measured aberrations. Because of the cytotoxic effects, we therefore carried out a viability assay to find the most suitable dose to use in further experiments.

Table 3.3. Number of micronuclei, nucleoplasmic bridges and nuclear buds per 2000 binucleated cells in C3A cells exposed to different concentrations of the plant extracts and mutagen 4-NQO.

Sample	*Conc. (mg/ml)*	*Micronuclei*	*Nucleoplasmic bridges*	*Nuclear buds*
1	2.5	-	-	-
	0.5	20	16	18
	0.1	18	18	15
2	2.5	-	-	-
	0.5	8	18	14
	0.1	6	21	15
3	2.5	-	-	-
	0.5	5	10	8
	0.1	4	11	14
4NQO	**1 µg/ml**	**14**	**20**	**25**
4	2.5	-	-	-
	0.5	6	9	17
	0.1	6	20	10
5	2.5	10	15	25
	0.5	13	10	20
	0.1	12	14	23
6	2.5	-	-	-
	0.5	16	18	20
	0.1	8	19	11
4NQO	**1 µg/ml**	**13**	**19**	**23**
7	2.5	-	-	-
	0.5	6	10	10
	0.1	7	6	8
8	2.5	4	6	4
	0.5	6	12	7
	0.1	12	23	24
9	2.5	5	5	5
	0.5	4	12	7
	0.1	8	24	22
4NQO	**1 µg/ml**	**11**	**20**	**19**
10	2.5	-	-	-
	0.5	9	22	15
	0.1	8	11	9
11	2.5	-	-	-
	0.5	-	-	-
	0.1	14	23	24
12	2.5	-	-	-
	0.5	8	14	16
	0.1	5	12	13
4QNO	**1 µg/ml**	**12**	**21**	**23**
13	2.5	-	-	-
	0.5	15	17	18
	0.1	16	14	15
14	2.5	-	-	-
	0.5	9	22	19
	0.1	11	19	21
15	2.5	-	-	-
	0.5	10	17	28
	0.1	11	19	28
4NQO	**1 µg/ml**	**12**	**18**	**21**

Sample	Conc. (mg/ml)	Micronuclei	Nucleoplasmic bridges	Nuclear buds
16	2.5	-	-	-
	0.5	4	6	5
	0.1	8	16	7
17	2.5	-	-	-
	0.5	10	5	12
	0.1	8	10	13
18	2.5	-	-	-
	0.5	11	16	22
	0.1	12	15	21
4NQO	**1 µg/ml**	**13**	**20**	**22**
19	2.5	-	-	-
	0.5	10	33	15
	0.1	8	22	8
20	2.5	15	17	11
	0.5	10	18	9
	0.1	4	22	5
21	2.5	-	-	-
	0.5	-	-	-
	0.1	12	17	21
4NQO	**1 µg/ml**	**14**	**27**	**23**
22	2.5	-	-	-
	0.5	10	12	14
	0.1	14	17	22
23	2.5	-	-	-
	0.5	8	19	22
	0.1	5	18	17
24	2.5	7	45	14
	0.5	5	31	5
	0.1	9	30	5
4NQO	**1 µg/ml**	**12**	**17**	**20**
25	2.5	-	-	-
	0.5	6	22	20
	0.1	12	17	20
26	2.5	-	-	-
	0.5	6	32	16
	0.1	6	43	11
27	2.5	-	-	-
	0.5	5	41	6
	0.1	6	33	14
4NQO	**1 µg/ml**	**15**	**19**	**22**
28	2.5	3	11	4
	0.5	4	26	15
	0.1	9	32	16
29	2.5	-	-	-
	0.5	5	24	10
	0.1	8	30	10
30	2.5			
	0.5	5	28	12
	0.1	6	26	15
31	2.5	-	-	-
	0.5	-	-	-
	0.1	7	49	9
4NQO	**1 µg/ml**	**11**	**30**	**24**

- = no binucleated cells due to toxicity

One problem with genotoxicity and antigenotoxicity testing is that many higher plants produce toxic agents which may affect the proliferation of bacterial and mammalian cells used in the assay. Dead cells do not mutate and thus do not perpetuate DNA damage (Thilly, 1985). As a result, to eliminate possible interference of cytotoxicity of the plant extracts with their genotoxic and antigenotoxic effects, the extracts were evaluated for cytotoxicity using the neutral red uptake assay (NRU). The neutral red uptake test measures cell viability based on the property of living cells to be able to take up neutral red dye in their lysosomes (Reppetto *et al.,* 2008). This is one of the most commonly used cytotoxicity tests with many biomedical and environmental applications. Dying cells have altered membrane properties and therefore they cannot anymore take up neutral red. The assay quantitates cell viability and can be used to measure cell replication, cytostatic effects or cell death.

The results of the neutral red uptake test are shown in Figure 3.5 and 3.6 where percentage cell viability of the three tested concentrations (2.5, 0.5, and 0.1 mg/ml) was calculated. The LC_{50} values of the 31 plant species are presented in Table 3.4. The LC_{50} values ranged from 0.19 to >2.5 mg/ml. Most plant extracts had LC_{50} values less than 0.5 mg/ml. The cell viability assay was performed as a control measure of toxicity (cytotoxicity) and to serve for dose-finding for samples that have a positive indication of antigenotoxic effects. At the highest concentration tested, only 1 sample (sample number 5) led to a cell viability more than 50% (Figure 3.4). All the other samples inhibited cell proliferation significantly. At the lowest concentration, cell viability was 80% for 27 out of 31 plant extracts. This assay was carried out to allow us to choose the right concentration to further assay selected plant extracts for mutagenic, antimutagenic and comutagenic effects in the subsequent studies in chapters to follow.

From the results obtained in the study of cytotoxic effects, it is clear that further assaying for both mutagenicity and antimutagenicity should be carried out at a highest concentration of 0.5 mg/ml giving an average cell viability of 40%. This is because some genotoxic carcinogens are not detectable in *in vitro* genotoxicity assays unless the concentrations tested induce some degree of cytotoxicity. It is also apparent that excessive toxicity often does not allow a proper evaluation of the relevant genetic endpoints, implying that a wide range of concentrations should be tested (OECD, 1997). In this case, concentrations of up to 0.2 mg/ml or less with average percentage viability of 89% should be included in the assay allowing coverage of both cytotoxic and non-cytotoxic concentration ranges for both bacterial and mammalian cell line genotoxicity assays i.e. Ames, micronucleus/cytome assay and comet assay (carried out to confirm results from the other two assays). Any growth inhibiting ingredients in a complex mixture such as a plant extract might mask the mutagenicity of the extracts and give false antimutagenic effects.

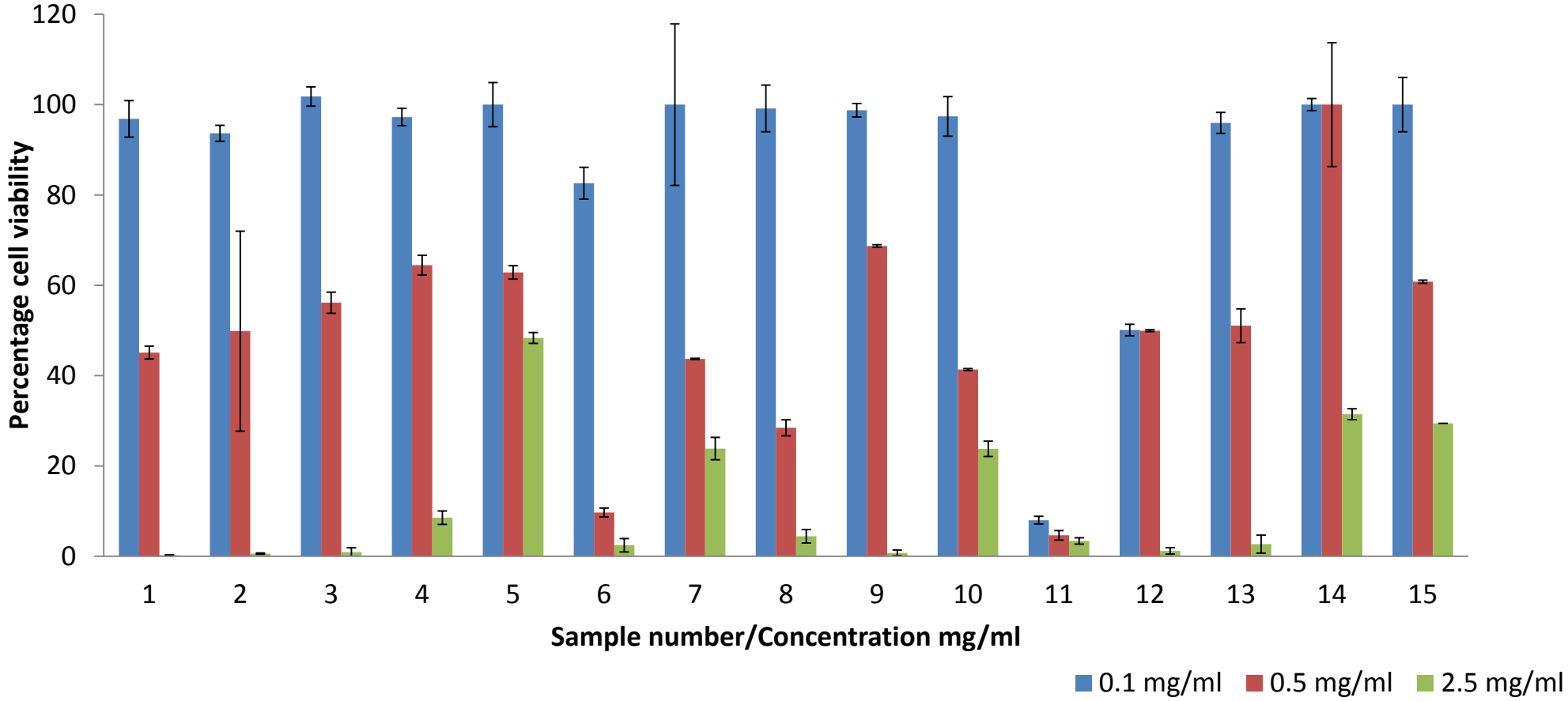


Figure 3.5. Percentage cell viability of C3A cells exposed to different concentrations of methanol leaf extracts of 15 plant species.

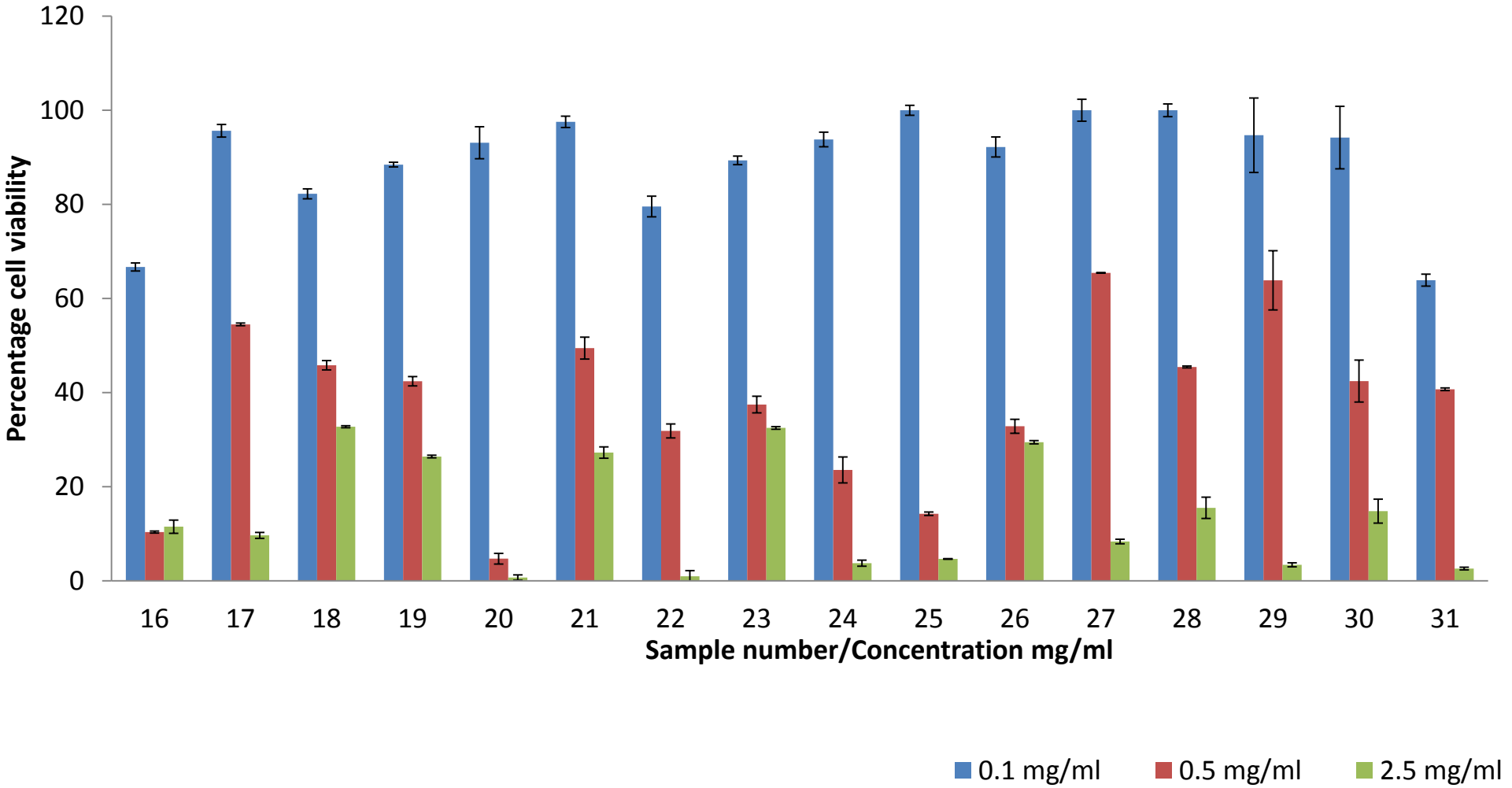


Figure 3.6. Percentage cell viability of C3A cells exposed to different concentrations of methanol leaf extracts of 16 plant species

Table 3.4. LC_{50} values (mg/ml) of 31 plant extracts in the neutral red uptake (NRU) assay.

Sample	LC_{50} (mg/ml)
Acalypha glabrata Thunb.	0.19 ± 0.03
Dalbergia nitidula Baker	1.81 ± 0.009
Halleria lucida L.	0.52 ± 0.02
Puttelikra restripinosa (L.) Szyszyl.	0.60 ± 0.19
Thespesia acutiloba (Baker f.) Exell & Mendonça	0.36 ± 0.03
Alchomea hirtella Benth. forma hirtella	0.23 ± 0.007
Androstachys johnsonii Prain	0.63 ± 0.02
Agromuellera macraphylla Pax.	0.38 ± 0.02
Brachystegia spiciformis Benth.	0.60 ± 0.10
Kirkia wilmsii Engl.	0.56 ± 0.02
Elaeodendron transvaalense (Burtt Davy) R.H. Archer	>2.5 ± 0.00
Cassinopsis illicifolia (Hochst.) Kuntze	0.45 ± 0.01
Dais cotinifolia L.	0.40 ± 0.04
Faurea saligna Harv.	0.83 ± 0.01
Harpephyllum caffrum Bernh. ex Krauss	0.67 ± 0.05
Combretum microphyllum Klotzsch	0.26 ± 0.07
Leucospernum erubescens Rourke	0.41 ± 0.09
Loxostylis alata A. Spreng. ex. Rchb.	0.42 ± 0.07
Podocarpus henkelii Stapf ex. Dallim. & A.B. Jacks.	0.26 ± 0.11
Protea rubropilosa Beard	0.38 ± 0.08
Ochna gamostigmata Du Toit	0.31 ± 0.03
Buxus natalensis(Oliv.) Hutch.	0.29 ± 0.02
Morella serrata (Lam.) Killick	0.40 ± 0.10
Gomphostigmata virgatum (L.f.) Baill.	0.31 ± 0.02
Ochna serrulata (Hochst.) Walp.	0.24 ± 0.06
Mimetes cucculatus (L.) R.Br.	0.33 ± 0.08
Protea mundii Klotzsh	0.24 ± 0.08
Protea cyanroides (L.) L.	0.33 ± 0.07
Protea neriifolia R.Br.	0.32 ± 0.06
Protea nitida Mill.	0.26 ± 0.07
Soralea pinnota L.	0.60 ± 0.02
SDS (positive control)	0.19 ± 0.02 mM

3.4. Conclusions

The results obtained in this chapter identified plant extracts which were not mutagenic, cytotoxic and that had an indication of antimutagenic activity in either the Ames test or micronucleus/cytome assay. Based on the findings from this chapter, plant extracts that had antimutagenic effects will be further investigated. Only one plant extract (*Halleria lucida* i.e. sample 3) was mutagenic in strain TA98. Almost no methanol plant extract assayed in the Ames test induced a mutagenic response when using *S. typhimurium* TA98 and TA100. It appears the negative mutagenicity results in strain TA100 may be attributed to the toxic i.e. antibacterial effects of the assayed plant extracts. The tester strain TA100 is more sensitive to toxic substances than strain TA98 (Prival and Zeiger, 1998). The absence of a mutagenic response in the Ames test is a positive step forward in the safe use of those plants used in traditional medicine. Only 7 plant extracts (22%) showed mutagenic response in micronucleus/cytome assay.

Some plant extracts had potential antimutagenic effects in the Ames test using *S. typhimurium* TA98 and TA100 as well as in the micronucleus/cytome assay. The level of antimutagenicity observed in the Ames test was below the recommended percentage to conclude whether plants have good antimutagenic effects or not. However, other research indicates that the level of antimutagenicity which must be present in order to score the extract as active differs depending on a variety of considerations. In the light of the argument presented above, the general conclusion from this study is that 48% of the plant extracts are antimutagenic when using *S. typhimurium* TA98 and 65% in *S. typhimurium* TA100 in the Ames test irrespective of their varied percentage antimutagenic effects.

There was little or no correlation between the results observed in the Ames test and micronucleus/cytome assay both in the case of mutagenicity and antimutagenicity testing. This may be a result of metabolic activation occurring in hepatocytes (C3A) or different mechanisms of activity. This cell line retains many of the properties of the normal human hepatocytes (Kelly, 1994). The Ames test was performed without metabolic activation. It is clear that the two assays measure or reflect different genetic end-points and events and hence different mechanisms of action (Verschaeve *et al.,* 2004). The lack of correlation between the assays suggests different mechanisms of either mutagenic and/or antimutagenic effects of the plants, that may be more complex than simple interception and activation or inactivation of the mutagen of interest. No statistical evaluation was carried out as this was a preliminary screening phase to investigate any possible indication of antimutagenicity and/or mutagenic response so as to aid in selecting promising plant extracts for further investigations. Based on the results from this study, four plant species; *Thespesia acutiloba Kirkia wilmsii Combretum microphyllum* and

Leucospermum erubescens (samples 5, 10, 16 and 17) were selected for further investigation. Extracts of these species had similar responses towards both strains used for the Ames test and also in the micronucleus/cytome assay for antimutagenicity assays. *Thespesia acutiloba Kirkia wilmsii* were comutagenic and *Combretum microphyllum* and *Leucospermum erubescens* were antimutageni in both assays. The two antimutagenic plants were selected for possible isolation of antimutagenic compounds that can be useful for chemopreventive purposes. On the other hand, in an attempt to broaden the scope of the project, the two co-mutagenic extracts were selected on the basis of their interaction with DNA and hence is in line with the objectives.

The following chapters detail the potential antigenotoxic effects of *Combretum microphyllum*, *Leucospermum erubescens*, *Thespesia acutiloba* and *Kirkia wilmsii*. It was clear that there was interference between the cytotoxic activity of the plant extracts and their potential antigenotoxic effects. This was the case especially in the cytokinesis block micronucleus/cytome assay where fewer binucleated cells were observed at the highest concentration assayed. The concentration of 0.5 mg/ml was chosen as the highest concentration to use in the subsequent experiments using mammalian cells, covering slightly cytotoxic as well as non-cytotoxic concentrations.

CHAPTER 4

Genotoxic and antigenotoxic activity of *Combretum microphyllum, Leucospermum erubescens, Thespesia acutiloba* and *Kirkia wilmsii* against 4-NQO, MMC and EMS

4.1. Introduction

The search for inhibitors of mutagenesis is an important tool towards the discovery of cancer preventing agents. Just as mutagens are considered carcinogens, antimutagens can be considered potential anticarcinogens (Verschaeve, 2006). Investigation of the antimutagenic potential of traditional medicinal plants and compounds isolated from plant extracts provides one of the tools that can be used in the identification of compounds with potential cancer chemopreventive properties (Arora *et al.,* 2005, Reid *et al.,* 2006).

The antimutagenic effects of certain naturally occurring compounds extracted from plants have been established in bacterial and mammalian cells. However due to the variety of DNA lesions and the complexity of repair pathways, it is difficult to identify the processes involved in antimutagenesis. Antimutagens may be effective against single mutagens or a class of mutagens, may act by multiple sometimes strictly interconnected or partially overlapping mechanisms, and may be even mutagenic at certain concentrations or in certain test systems (Kuroda, 1990, Simic´, 1997).

Because cancer can take from several years to decades to develop it is not always practical to perform prospective epidemiological studies over such long times. Therefore, there is justifiable interest in determining whether biomarkers of DNA damage may predict cancer risk. One would expect, in theory, that biomarkers that measure events on the direct causal path to cancer would be the ones most likely to be predictive. However because cancer involves multiple mutations it is not clear whether a generic biomarker that may predict genomic instability leading to hypermutation would be as important as a common event in cancer cells, such as inactivation of p53 or apoptosis. An important limitation of DNA biomarkers in human studies is the relevance of the accessible tissues in which DNA damage is measured to the cancer studied (Fenech, 2002). It is of great importance to investigate the antimutagenic properties of plants or phytocompounds in order to identify

compounds with potential anticancer activity. These compounds may be used as therapeutic agents or as supplements in functional food, or in other applications.

In this chapter, we confirm the activity of four selected plant species. Both the mutagenicity and antimutagenicity of these species were determined at a wide range of concentrations in the Ames test using *S. typhimurium* TA98, TA100 and TA102. To further confirm the results obtained in the initial screening study, the genotoxicity and antigenotoxicity of the extracts were determined in the cytokinesis block micronucleus assay and the single cell gel electrophoresis/comet assay. Genotoxicity and/or mutagenicity has different endpoints. Besides point mutations, mutagens also induce changes in chromosomal number, or chromosome structure (breaks, deletions and rearrangements) (Fenech, 2002).

4.2. Materials and methods

4.2.1. Ames test

Methods described in Chapter 3, section 3.2 were used for antimutagenicity testing. The activities of the plant extracts were determined at concentrations of 5000, 500, 50, 5, 0.5 and 0.05 µg/ml. In the Ames test, two mutagens were used: 4-NQO was used for *S. typhimurium* strains TA98 and TA100, and mitomycin C (MMC) for *S. typhimurium* strain TA102. These strains detect different types of carcinogens or mutagens including those causing frameshift and base-pair substitution mutations, as well as DNA damage associated with oxidative damage, in the presence and absence of metabolic activation.

4.2.2. Cytokinesis-block micronucleus/cytome assay

In the cytokinesis-block micronucleus cytome assay, the method described in Chapter 3, 3.2.2 was used. 4-NQO at 1 µg/ml was used as a mutagen. Based on cytotoxicity results in the neutral red uptake assay (NRU), 500 µg/ml was the highest sample concentration tested. The plant extracts were tested at concentrations of 500, 250, 125 and 62.5 µg/ml. In addition, the Nuclear Division Cytotoxicity Index (NDCI) was calculated according to the method of Eastmond and Tucker (1989). Five hundred viable cells were scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and the NDCI was calculated using the formula:

$$\mathbf{NDCI = (Ap + Nec + M_1 + 2M_2 + 3M_3 + 4M_4) / \mathit{N}}$$

Where NDCID = nuclear division cytotoxicity index, Ap = number of apoptotic cells, Nec = number of necrotic cells, M_1–M_4 = number of viable cells with 1–4 nuclei and *N* = total number of cells scored (viable and non-viable). This is important in differentiating the cellular toxic effects of the test samples from the genetic toxicity.

4.2.3. Alkaline single-cell gel electrophoresis/Comet assay

The protocol of Singh *et al.* (1988) was followed to evaluate the DNA damaging and protective effects of the four plant extracts.

Microscope slides were pre-coated by spreading 300 µl 1% normal melting point (NMP) agarose in water evenly over the slides and allowing the agarose to harden. C3A cells at a density of 200000 cells/ml were treated with different concentrations of the test sample in 24 well plates and incubated for 24 hours at 37°C in a 5% carbon dioxide incubator. Based on cytotoxicity results in the neutral red uptake assay (NRU), 500 µg/ml was the highest sample concentration tested. The plant extracts were tested at concentrations of 500, 250, 125 and 62.5 µg/ml. Ethyl methane-sulfonate (EMS) at 1 mM was used as a positive control/mutagen. For mutagenicity testing, the cells were exposed to plant extracts alone and for antimutagenicity testing, the cells were exposed to a combination of the plant extracts and 1 mM EMS.

After incubation, cells were trypsinised and 10 µl of a 10 000 cell suspension was added to 300 µl of 0.8% low melting point (LMP) agarose at 37°C. The mixture was spread on the precoated slides and allowed to harden under a coverslip on ice. Once the agarose had been prepared, the coverslips were removed and the microscope slides placed in lysis buffer overnight. Denaturation was conducted using the electrophoresis buffer at 17°C for 40 minutes. Electrophoresis was conducted using the same solution at 25V, current adjusted to 300 mA for 20 minutes. After electrophoresis, neutralization of the microscope slides was carried out in Tris buffer (pH 7.5) and dried. The slides were then placed in ice cold ethanol for 10 minutes and allowed to dry at room temperature. The gels were stained with 100 µl of 20 µg/ml ethidium bromide, left for 10 minutes and rinsed in distilled water. The slides were analysed using a fluorescence microscope supplied with a camera. The tail length, % DNA in tail and tail moment were determined using the PC image-analysis programme TriTek CometScore™. This programme allows measurement of tail length, percentage DNA in tail and tail moment as parameters to measure DNA damage in the comet assay. For mutagenicity testing, differences in parameters used to measure DNA damage (i.e. tail length, percentage DNA in tail and tail moment) were compared between sample concentration and solvent blank (negative control). For antimutagenicity testing, to measure antimutagenicity, the same parameters used for mutagenicity testing were used. In this case, the measurements in the test samples were compared to the positive control.

4.2.4. Statistical analysis

The statistical significance of differences between means was determined using Dunnet's test. Variance and differences among the means were determined by a one-way ANOVA with Newman-Keuls Multiple Comparison Test using Prism 6.0, GraphPad Instat (GraphPad Software Inc., San Diego, CA, USA).

4.3. Results and discussion

In antimutagenicity investigations, it is extremely important to test genotoxic effects of the sample first because even amongst antimutagenic compounds, many substances reported to be antimutagens or anticarcinogens have, themselves, been shown to be mutagenic or carcinogenic (Zeiger, 2003). All the samples assayed for antimutagenicity were first tested for mutagenicity in all the assays in this study. Table 4.1 summarizes the mutagenicity results of the four plant extracts in the Ames test using *S. typhimurium* TA98, TA100 and TA102.

None of the assayed plant species leaf extracts induced increased incidences in the number of revertant colonies compared to the negative control (solvent blank). The mutation frequency for all the three strains when exposed to differing concentrations of the four plant extracts did not change at any concentration tested, and none of the extracts resulted in double the number of colonies compared to the negative control. A positive mutagenic response in the Ames test is attributed to the doubling in the number of revertant colonies at any concentration of the test sample compared to the negative control (Verschaeve and van Staden, 2008).

The non-mutagenic response demonstrated by *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* against *Salmonella typhimurium* bacterial strains in the Ames test is a positive step forward in further investigating the plants for their antimutagenic potential.

Table 4.1. Mean number of revertant colonies per plate (±SD) in *Salmonella typhimurium* TA98, TA100 and TA102 exposed to methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* to measure mutagenicity of the plant extracts

Concentration µg/ml	**5000**	**500**	**50**	**5**	**0.5**	**0.05**
***S. typhimurium* TA98**						
Combretum microphyllum	29.00 ± 1.00	29.67 ± 9.02	32.00 ± 1.73	29.00 ± 2.00	29.00 ± 1.00	30.67 ± 3.05
Leucospermum erubescens	31.00 ± 2.65	30.66 ± 3.05	33.67 ± 2.52	32.67 ± 6.03	31.67 ± 2.52	28.00 ± 1.00
Kirkia wilmsii	24.67 ± 3.06	24.67 ± 6.43	25.67 ± 2.52	23.66 ± 1.53	25.67 ± 4.51	27.66 ± 1.53
Thespesia acutiloba	23.00 ± 2.00	24.33 ± 4.51	25.00 ± 4.00	26.67 ± 2.52	27.67 ± 1.53	26.00 ± 5.20
Negative/solvent blank	**28.90 ± 5.28**			**Positive control 2 µg/ml 4-NQO**		**237.42 ± 17.46**
***S. typhimurium* TA100**						
Combretum microphyllum	131.00 ± 7.22	140.33 ± 5.13	141.67 ± 20.74	123.33 ± 6.03	127.00 ± 7.00	118.33 ± 13.20
Leucospermum erubescens	153.33 ± 5.06	133.00 ± 18.68	156.33 ± 9.07	123.33 ± 14.05	134.67 ± 4.04	128.67 ± 4.16
Kirkia wilmsii	135.33 ± 5.13	117.67 ± 6.81	130.67 ± 10.50	125.00 ± 12.17	128.00 ± 8.54	127.33 ± 11.93
Thespesia acutiloba	114.67 ± 11.01	127.33 ± 3.21	122.33 ±2.31	115.33 ± 5.03	107.33 ± 6.03	124.00 ± 4.58
Negative/solvent blank	**138.80 ± 12.74**			**Positive control 1 µg/ml 4-NQO**		**628.44 ± 6.89**
***S. typhimurium* TA102**						
Combretum microphyllum	300.00 ± 11.53	263.00 ± 6.08	255.00 ± 11.53	261.67 ± 1.53	255.67 ± 6.66	261.00 ± 29.70
Leucospermum erubescens	306.33 ± 12.74	280.67 ± 11.37	271.67 ± 14.57	274.00 ± 7.81	283.00 ± 7.00	270.67 ± 18.15
Kirkia wilmsii	304.33 ± 5.86	294.00 ± 26.89	326.00 ± 16.70	272.67 ± 17.07	268.33 ± 24.01	256.67 ± 4.93
Thespesia acutiloba	305.33 ± 4.44	280.67 ± 10.69	288.67 ± 28.68	308.67 ± 14.18	274.67 ± 1.53	266.00 ± 5.29
Negative/solvent blank	**270.50 ± 5.28**			**Positive control 1 µg/ml MMC**		**1135.75 ± 9.85**

There are instances where toxic effects of the plant extracts towards the tester strains are implicated in giving false negative results in mutagenicity screening. Any substance that causes cells to die or prevent them from replicating regardless of its mechanism of action will reduce the number of revertant colonies and may wrongly lead to the conclusion that the substance is not mutagenic. This may be expected and is true for higher plants since they often produce antimicrobial secondary metabolites that can kill the tester strain (Mitscher *et al.,* 1992).

To some extent, this possible phenomenon was ruled out since six concentrations with 10-fold dilution factors were assayed and there was no increase in the number of colonies as the concentrations decreased which is what is expected in cases where toxicity may have played a role to mask mutagenic effects of the plant extracts. Additionally, to confirm absence of toxicity, the background layer of bacterial growth was present in all experiments. The absence of mutagenic effects can be seen in frame-shifts (TA98), base-pair substitutions (TA100) and transitions/transversions (TA102), indicating that the plant extracts are not mutagenic. Because of this lack of mutagenicity, these plant extracts were then investigated for their potential antimutagenic effects.

The results for the antimutagenic effects of the plant extracts in the Ames test using *S. typhimurium* TA98, TA100 and TA102 are present in Table 4.2 as number of colonies in the positive control plates and plates containing a combination of the mutagens (either 4-NQO or MMC) with the plant extracts.

The results are presented using this approach to allow for statistical comparison of the effects of the plant extracts on the mutagenicity of the two known direct acting mutagens 4-NQO and MMC. One-way ANOVA followed by Dunnet's Multiple Comparison test was used (Graph Pad Software) to compare the number of revertant colonies in the positive control (mutagen alone) and the number of revertants in the plates containing a combination of the mutagen and the different concentrations of the plant extracts.

Antimutagenicity determination of plant extracts is important in the discovery of new effective chemopreventive agents. Due to increased occurrence of development of mutation related chronic degenerative diseases worldwide, determination of chemoprevention or chemoprophylactic compounds is important in the effort to reduce the risk of cancers and other mutation related diseases (Kundu *et al.,* 2004, Reid *et al.,* 2006).

Extracts of *C. microphyllum* and *L. erubescens* inhibited the mutagenic effects of 4-NQO (TA98 and TA100) and MMC (TA102) in all the tester strains at all concentrations tested, a phenomenon referred to as antimutagenicity even though there was no clear dose response, whereas *K. wilmsii* and *T. acutiloba* enhanced the mutagenic effects of the tested mutagens in all three strains, a phenomenon referred to as comutagenicity. *Combretum*

microphyllum extracts significantly decreased the number of revertant colonies of *S. typhimurium* TA98 at the two highest concentrations and the last concentration tested compared to the positive control ($p<0.05$) and only at the highest concentration ($p<0.01$) when TA102 was used. *Leucospermum erubescens* extracts significantly decreased the number of revertant colonies at five of the six tested concentrations ($p<0.01$) when TA102 was used. There was however no significant decrease in number of colonies when *S. typhimurium* TA100 was used to test both plant extracts for antimutagenicity.

Almost similar trends were observed in the case of *K. wilmsii* where a significant increase in revertant colonies was observed only at the three highest concentrations and four high concentrations for *T. acutiloba* extracts ($p<0.01$) in TA98. The two extracts also significantly increased the frequency of revertant colonies of *S. typhimurium* TA100 only at the two highest concentrations and at the four highest concentrations tested ($p<0.01$) when TA102 was used.

Table 4.2. Mean number of revertant colonies per plate in *Salmonella typhimurium* TA98, TA100 and TA102 exposed to a combination of methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* and mutagens 4-NQO (TA98 and TA100) and MMC (TA102). The results are expressed a mean number of revertants ± standard deviation. Statistical significance is given for comparison of the extract + mutagen compared to the mutagen alone.

Concentration µg/ml	**5000**	**500**	**50**	**5**	**0.5**	**0.05**
***S. typhimurium* TA98**						
Combretum microphyllum	224.00* ± 14.00	220.00* ± 16.09	231.00 ± 16.37	255.00 ± 12.49	255.00* ± 12.49	221.33* ± 14.50
Leucospermum erubescens	157.33** ± 35.02	182.00 ± 22.07	159.00* ± 2.83	193.67 ± 20.21	172.00 ± 1.00	181.67 ± 24.19
Kirkia wilmsii	311.00** ± 22.34	303.00* ± 38.59	367.00** ± 39.69	232.67 ± 6.43	236.67 ± 4.16	231.33 ± 4.16
Thespesia acutiloba	385.33** ± 12.10	352.67** ± 19.86	339.00** ± 18.03	310.00** ± 10.00	249.00 ± 7.94	245.33 ± 6.43
2 µg/ml 4-NQO	**237.42 ± 17.46**					
***S. typhimurium* TA100**						
Combretum microphyllum	707.67 ± 67	636.33 ± 9.87	653.5 ± 4.95	631.33 ± 12.05	608.67* ± 35.57	629.67 ± 15.70
Leucospermum erubescens	591.33 ± 8.08	581.00 ± 9.02	617.33 ± 18.90	582.5 ± 2.12	607.33 ± 7.43	600.67 ± 6.90
Kirkia wilmsii	690.67** ± 6.03	678.67** ± 6.66	622.00 ± 10.44	612.00 ± 7.21	622.33 ± 21.08	614.33 ± 15.50
Thespesia acutiloba	731.67** ± 1.88	688.33* ± 12.42	659.67 ± 20.55	628.67 ± 6.81	632.33 ± 6.66	628.33 ± 6.51
1 µg/ml 4-NQO	**628.44 ± 6.89**					
***S. typhimurium* TA102**						
Combretum microphyllum	803.33** ± 3.78	945.00 ± 3.78	962.33 ± 20.60	1006.00 ± 5.29	993.33 ± 7.57	1010.33 ± 24.01
Leucospermum erubescens	626.00** ± 9.00	735.67** ± 4.77	788.00** ± 31.43	791.33** ± 18.18	766.33** ± 18.18	873.33 ± 28.87
Kirkia wilmsii	1802.00** ±3.46	1693.00** ± 9.64	1508.00**± 11.14	1460.00 ± 9.461	1329.33 ± 24.0277	1311.00 ± 10.15
Thespesia acutiloba	1880.00** ± 26.46	1820.00** ± 6.09	1708.67** ± 19.50	1722.33** ± 24.58	1338.00 ± 6.08	1340.67 ± 13.12
1 µg/ml MMC	**1135.75 ± 9.85**					

* Significant at 0.05 probability level of significance, ** Significant at 0.01 probability level of significance and *** Significant at 0.001 probability level of significance

Figures 4.1, 4.2 and 4.3 present the average percentage antimutagenicity and/or percentage inhibition of the mutagens, which is an easier way to see the potential antimutagenic effects of the plant extracts. From the figures, it is clear that *C. microphyllum* and *L. erubescens* inhibited the mutagenic effects of 4-NQO and MMC in a dose dependent manner in all the tester strains, whereas *K. wilmsii* and *T. acutiloba* enhanced the mutagenicity of the chosen mutagens, also in a dose dependent manner.

The percentage antimutagenicity of *C. microphyllum* and *L. erubescens* was not significantly different between concentrations in all tester strains but there was however a clear increasing trend of antimutagenicity as the concentrations of the plant extracts increased. It appears the two plants have different mutagen inhibitory mechanisms as, at similar concentrations, the plant extracts inhibited mutagenicity differently in different tester strains. *C. microphyllum* seems to better in preventing base-pair substitutions and deletion mutations (17% vs. 9.97% at highest concentration) compared to *L. erubescens* in TA100. However, *L. erubescens* may have a better antimutagenic activity as it had percentage antimutagenicity of more than 35% in TA98 and TA102.

Combretum microphyllum and *L. erubescens* generally have mutagen inhibiting activity in the Ames test. *C. microphyllum* is however considered to have moderate antimutagenic activity in TA102 and to have weak antimutagenic activity in both TA98 and TA100. *L. erubescens* had moderate antimutagenic eactivity in TA98 and TA102 and weak antimutagenicity in TA100. The antimutagenic activity is considered to be moderate when the inhibitory effect is 25-40%, and strong when more than 40%. Inhibitory effects of less than 25% were considered as weak and were not recognized as a positive result (Ikken *et al.,* 1999, Negi *et al.,* 2003). *L. erubescens* moderately protected against 4-NQO induced frame-shift mutations and MMC transitions/transversions induced mutations in TA98 and 102 respectively.

Both *C. microphyllum* and *L. erubescens* plant extracts had high antimutagenic effect in TA102 at the highest concentration tested. They can potentially provide lead compounds for development of chemopreventive agents that are able to inhibit mutations caused by oxidative mutagens, active forms of oxygen, alkylating DNA cross linking agents which cannot be detected in the other tester strains (Mortelmanns and Zeiger, 2000). These types of mutations are highly related to various physiological and pathological events such as inflammation, aging and carcinogenicity., Oxygen radicals may be the most important class of mutagens contributing to cancer and aging, yet a number of oxidants that have been tested are not detected as mutagens by *Salmonella* tester strains other than *S. typhimurium* TA102 (Ames, 1983).

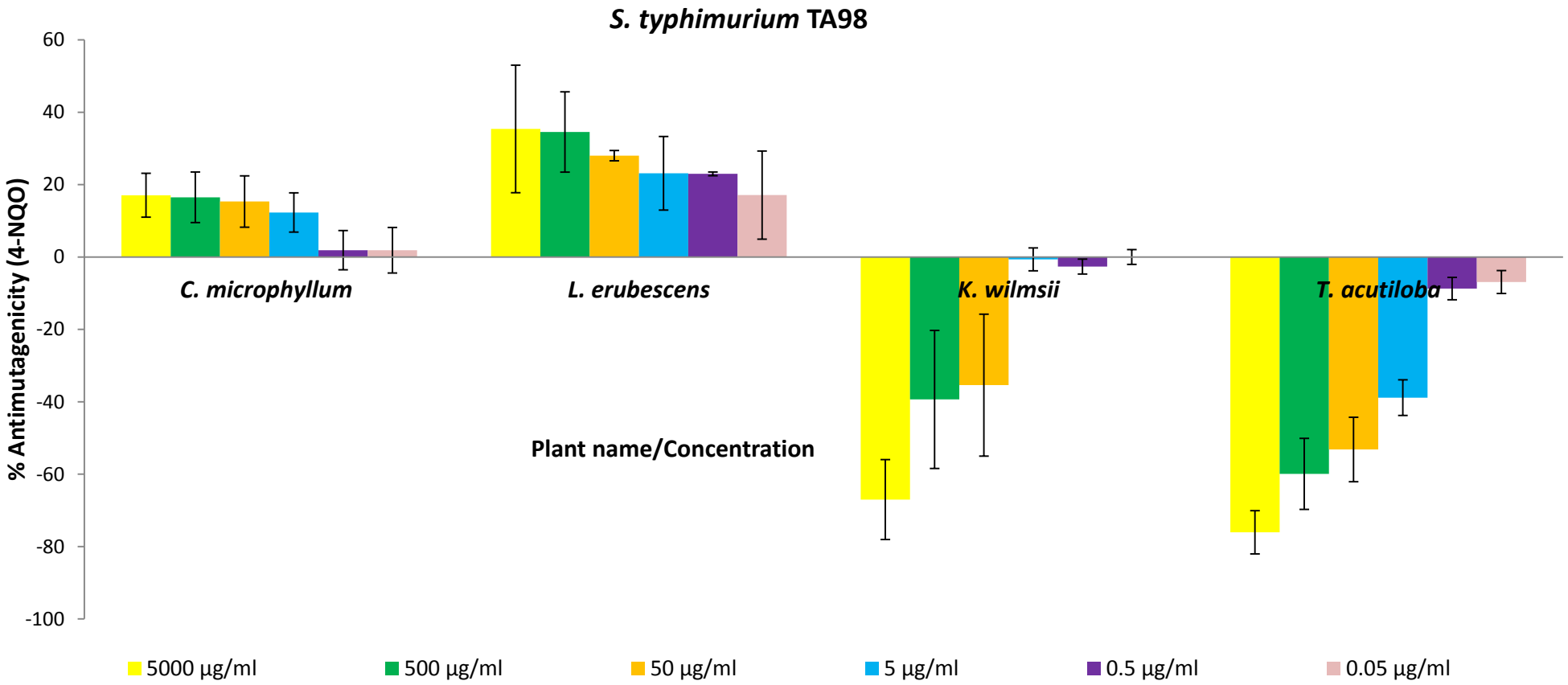


Figure 4.1. Antimutagenic activity of methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* in the Ames test using *S. typhimurium* TA98 (percentage inhibition and enhancement of the mutagenic effects of 4-NQO)

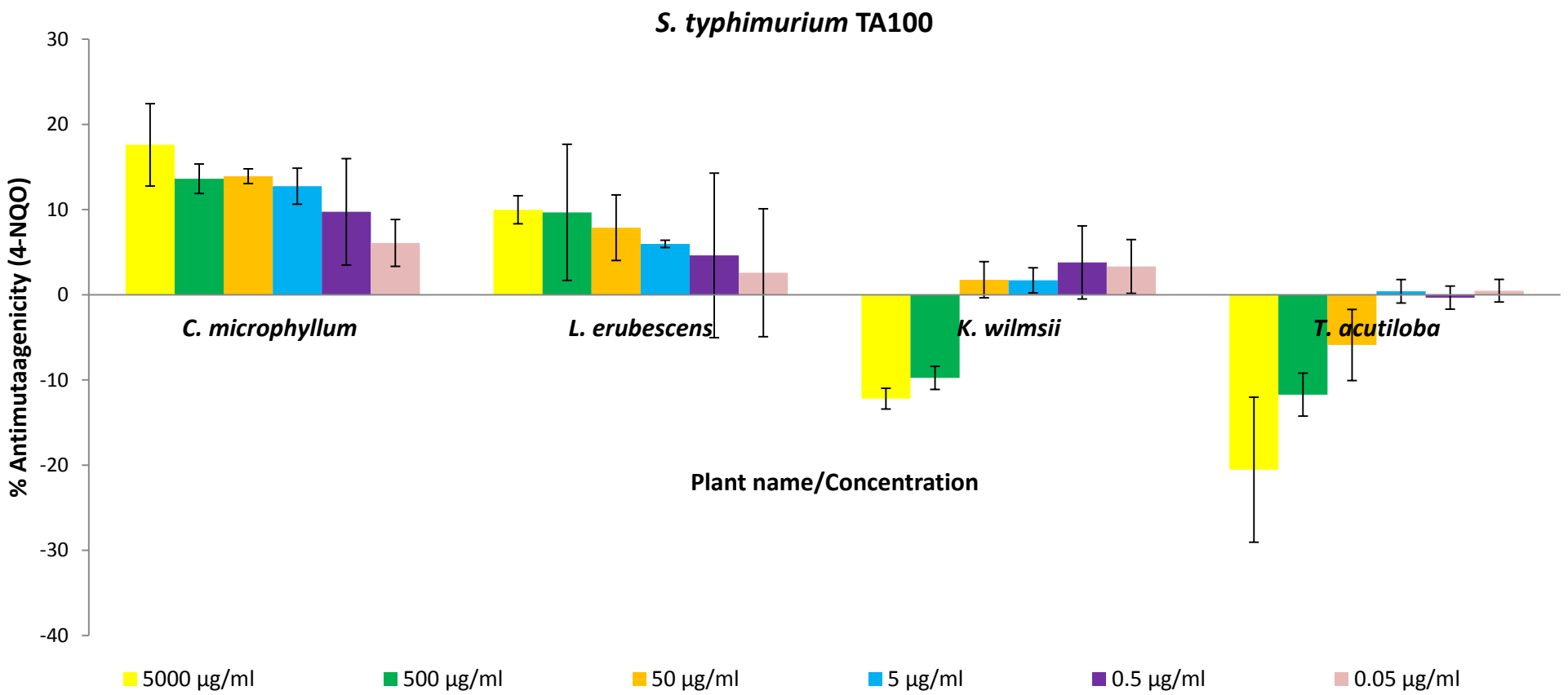


Figure 4.2. Antimutagenic activity of methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* in the Ames test using *S. typhimurium* TA100 (percentage inhibition and enhancement of the mutagenic effects of 4-NQO)

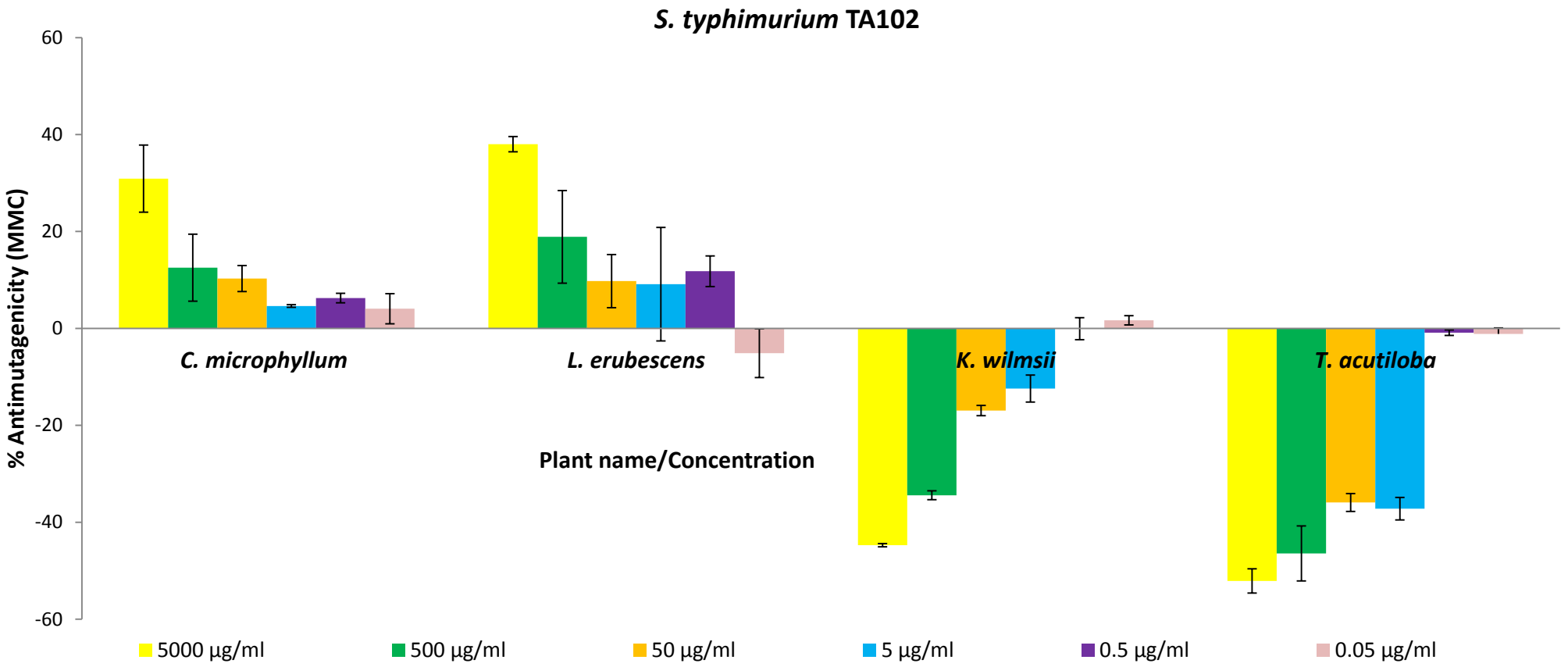


Figure 4.3. Antimutagenic activity of methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* in the Ames test using *S. typhimurium* TA102 (percentage inhibition and enhancement of the mutagenic effects of 4-NQO)

Extracts of *K. wilmsii* and *T. acutiloba* increased mutagenicity of 4-NQO in TA98 by 67% and 76% at the highest concentrations respectively. This is a phenomenon referred to as co-mutagenicity, where a non-mutagenic substance potentiates the mutagenic effects of known mutagens. In TA100, there was less than 25% potentiation of mutagenic effects of 4-NQO. The extracts increased mutagenicity of MMC by 44% and 52% in TA102at the highest concentration used. The evident high co-mutagenic effects induced by *K. wilmsii* and *T. acutiloba* in TA98 and TA102 is a clear indication that these extracts could possibly contribute to the increased development of mutation related diseases if the active compounds were present in the extracts prepared by traditional healers. Humans and animals are forever exposed to mutagenic substances through the air, water, industrial chemicals and drugs (Durnev and Seredenin, 2003).

The presence of co-mutagens in plant extracts could be a limiting factor to their medicinal use. Co-mutagens have no intrinsic mutagenic activity and are not usually detected during genotoxicological screening; as a result, the problem of co-mutagenesis receives little attention. The uncontrolled presence of co-mutagens in the environment can potentiate the negative effects of industrial, medicinal and other mutagens, which come into contact with humans (Durnev and Seredenin, 2003).

Most data about co-mutagens are obtained as a spin-off result during the search for and the study of antimutagens. Daily consumption may pose a health risk to consumers. Co-mutagenic activity of medicinal preparations is an important problem because of the wide indications and long-term usage of many medicinal plants and high probability of their interference with environmental mutagens. Moreover, in some instances, these interferences are virtually unavoidable: for example, in complex cancer therapy and autoimmune diseases or during prescription of routine drugs to persons using medicinal plants. Most antimutagens exert their antimutagenic action via improvement of the repair system. In the event where there are substances in plant extracts that inhibit the process of DNA repair, they may exhibit co-mutagenic activity (Durnev and Seredenin, 2003).

To assay extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* for mutagenic and antimutagenic effects in mammalian based system, the cytokinesis block micronucleus/cytome assay was used. The results for both mutagenicity and antimutagenicity testing in the micronucleus/cytome assay are summarized in Table 4.3. In this assay, the frequency of micronuclei, nucleoplasmic bridges and nuclear buds were analysed in 2000 binucleated cells treated with plant extracts alone compared to the negative control (mutagenicity testing) and in antimutagenicity testing, the frequency of micronuclei, nucleoplasmic bridges and nuclear buds were determined in 2000 binucleated cells treated with a combination of the plant extracts and the mutagen 4-NQO compared to the cells treated with the mutagen alone.

Micronuclei originate from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides. Nucleoplasmic bridges originate from asymmetrical chromosome rearrangements and/or telomere end fusion. They form when the centromere of dicentric chromosomes or chromatids are pulled to opposite directions at anaphase. Both micronuclei and nucleoplasmic bridges occur in cells exposed to DNA-breaking agents. The cytome assay also allows for the detection of nuclear buds, which represents mechanisms by which cells remove amplified DNA and are therefore considered a biomarker of possible gene amplification. These are all chromosomal biomarkers of genomic instability relevant to cancer (Fenech, 2002).

Extracts of *C. microphyllum* and *K. wilmsii* had no effect on the frequency of micronuclei compared to the negative control ($p>0.05$), whereas extracts of *T. acutiloba* and *L. erubescens* had a significant effect on the frequency of micronuclei compared to the negative control ($p<0.05$) at some concentrations. A significant difference in frequency of micronuclei induction was observed at 500, 250 and 62.5 µg/ml of *L. erubescens* treated cells ($p<0.05$). These results are however confusing because at 250 µg/ml, there is a decrease in number of micronuclei but at 62.5 µg/ml, the number of micronuclei increased. The lowest concentration of *C. microphyllum* had significantly less nucleoplasmic bridges compared to the negative control and there was also no difference in the frequency of nuclear buds. There was an increase in the frequency of nucleoplasmic bridges at the highest concentration (500 µg/ml) of *C. microphyllum and L. erubescens*. This increase was however statistically significant only in the case of extracts of *L. erubescens*. *Leucospernum erubescens* and *T. acutiloba* induced a significant concentration dependent increase in the frequency of nuclear buds. This is an indication of genotoxicity.

The antigenotoxic effects of all four plant extracts are also presented in Table 4.3. Extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* modified the genotoxic effects of 4-NQO in the cytome assay. Compared to the positive control, the highest concentration of *C. microphyllum* and *L. erubescens* significantly decreased the number of micronuclei induced by 4-NQO ($p<0.001$). *Leucospermum erubescens* also reduced the frequency of nuclear buds significantly ($p<0.01$). The extracts of *L. erubescens* and *T. acutiloba* significantly reduced 4-NQO induced nucleoplasmic bridges ($p<0.05$). Extracts of *C. microphyllum* (500 and 62.5 µg/ml, $p<0.001$) (250 and 125 µg/ml, $p<0.01$) and *T. acutiloba* ($p<0.01$) significantly decreased the frequency of micronuclei in a dose dependent manner. The decrease in the number of nucleoplasmic bridges in cells treated with a combination of 4-NQO and *C. microphyllum* was not significant, nonetheless this plant extract significantly reduced frequency of nuclear buds ($p<0.001$). *Kirkia wilmsii* did not modify the effects of 4-NQO on nucleoplasmic bridge induction but significantly decreased the frequency of nuclear buds ($p<0.01$). *Thespesia acutiloba* had no effect on 4-NQO induced nuclear buds.

Table 4.3. Number of micronuclei, nucleoplasmic bridges and nuclear buds per 2000 binucleated cells in human hepatocarcinoma C3A cells exposed to plant extracts alone (mutagenicity test) and a combination of 4-NQO and plant extracts (antimutagenicity test). The results are expressed a mean number of measurements ± standard deviation.Statistical significance is given for comparison of the extract compared to the solvent control (mutagenicity) and extract + mutagen compared to the mutagen alone (antimutagenicity)

MUTAGENICITY				**ANTIMUTAGENICITY**		
Combretum microphyllum				**+ 4-NQO**		
Concentration µg/ml	Micronuclei	Nucleoplasmic bridges	Nuclear buds	Micronuclei	Nucleoplasmic bridges	Nuclear buds
500	6.33 ± 1.54	14.00 ± 1.73	9.33 ± 2.08	5.00*** ± 1.00	12.33* ± 0.58	3.33*** ± 0.58
250	5.33 ± 0.58	11.00 ± 3.60	9.33 ± 1.15	9.33** ± 1.15	15.33* ± 1.53	5.67*** ± 1.15
125	4.67 ± 0.58	10.33 ± 0.58	10.33 ± 1.54	8.66** ± 2.31	17.00 ± 1.00	7.33*** ± 1.54
62.5	7.33* ± 1.15	7.33* ± 1.15	9.66 ± 0.577	7.33*** ± 1.54	20.33 ± 1.53	13.33** ± 2.89
Leucospermum erubescens				**+ 4-NQO**		
500	7.00* ± 1.00	18.33* ± 2.08	12.67* ± 1.15	7.33*** ± 1.54	6.0** ± 1.73	11.33** ± 1.15
250	3.33* ± 0.58	12.67 ± 4.61	13.33*± 2.31	10.66* ± 1.15	6.66** ± 1.15	13.00** ± 1.00
125	5.33 ± 0.58	9.67 ± 0.58	11.33 ± 1.15	11.33 ± 2.31	12.33* ± 2.08	17.67* ± 3.05
62.5	7.67* ± 0.58	9.67 ± 0.58	10.67 ± 1.15	11.33 ± 4.16	15.33* ± 0.58	17.00* ± 1.73
Kirkia wilmsii				**+ 4-NQO**		
500	6.33 ± 1.53	11.33 ± 0.58	5.33** ± 0.58	10.33* ± 3.21	21.33 ± 0.58	10.33** ± 4.16
250	4.66 ± 0.58	8.67* ± 0.58	5.33** ± 0.58	8.33** ± 3.51	22.67 ± 0.58	8.33 ***± 1.53
125	5.33 ± 0.58	8.33* ± 1.53	5.33** ± 1.15	10.11* ± 1.00	21.67 ± 1.53	13.67** ± 3.51
62.5	5.00 ± 1.00	9.33* ± 1.15	5.67** ± 0.58	7.67** ± 1.53	22.33 ± 1.16	12.33** ± 3.21
Thespesia acutiloba				**+ 4-NQO**		
500	8.00* ± 1.00	11.33 ± 1.15	14.67* ± 2.52	8.00** ± 2.00	18.68 ± 1.55	22.67 ± 2.52
250	7.33* ± 1.15	12.00 ± 2.00	12.67* ± 2.08	10.60* ± 1.15	15.00* ± 3.00	20.67 ± 1.16
125	7.67* ± 1.53	10.00 ± 2.00	10.67 ± 1.53	13.33 ± 1.54	10.67* ± 1.16	23.33 ± 1.53
62.5	5.33 ± 0.58	8.33* ± 1.15	7.33 ± 1.53	13.67 ± 0.58	11.33* ± 1.16	20.66 ± 1.16
Negative 0.00 µg/ml	**5.67 ± 1.15**	**12.00 ± 1.00**	**8.67 ± 1.14**			
Positive 1 µg/ml 4-NQO				**14.67 ± 1.52**	**25.67 ± 10.69**	**24.00 ± 5.29**

* Significant at 0.05 probability level of significance, ** Significant at 0.01 probability level of significance and *** Significant at 0.001 probability level of significance

The formation of micronuclei, nucleoplasmic bridges and nuclear buds is a direct consequence of chromosome breakages and loss, chromosomal rearrangement and excessive gene amplification respectively. All these chromosomal irregularities can lead to cancer either by forming hybrid genes or by causing disregulation of genes. Hybrid genes accelerate cell division e.g. Philadelphia chromosome associated with leukaemia (Lobo, 2008). Moreover, an increase in the occurrence of micronuclei, nucleoplasmic bridges and nuclear buds has been correlated to the break-fusion-bridge cycle mechanism of hyper-mutation during carcinogenesis (Fenech *et al.*, 2011).

To better express the antimutagenic potential of these plant extracts, the percentage reduction in the number of measured aberrations was calculated using the formula:

(1-(a/b)*100)

where a = number of measured aberrations in the test sample mixed with the mutagen and b = number of measured aberrations in the positive control (mutagen alone). For all extracts, the percentage reduction in micronuclei, nucleoplasmic bridges and nuclear buds, showing antigenotoxic activity, was calculated independently and is presented in Figures 4.4, 4.5 and 4.6. Generally, the two extracts that have antimutagenic effects in the Ames test (*C. microphyllum* and *L. erubescens*) greatly reduced the percentage of micronuclei by up to 66%, nucleoplasmic bridges by 76% and nuclear buds by 88%. *C. microphyllum* and *T. acutiloba* significantly reduced the percentage of micronuclei, nucleoplasmic bridges and nuclear buds respectively.

Extracts of *K. wilmsii* and *T. acutiloba* were not comutagenic in the micronucleus/cytome assay as was observed in the Ames test. Instead, they had a low level of antimutagenicity. When testing plant extracts and phytochemicals in biological systems, such results are sometimes expected, especially in cases where both bacterial-based and mammalian cell line based assays are used. In our case, there was no metabolic activation in the Ames test whilst a higher level of metabolic activity is maintained in C3A hepatocytes (personal communication, Elgorashi, Verschaeve). This cell line retains many of the properties of the normal human hepatocyte. They have all essential structural, biochemical and growth features of normal human liver and have conserved phase I and II metabolic capacities (Kelly, 1994, Edziri *et al.,* 2011).

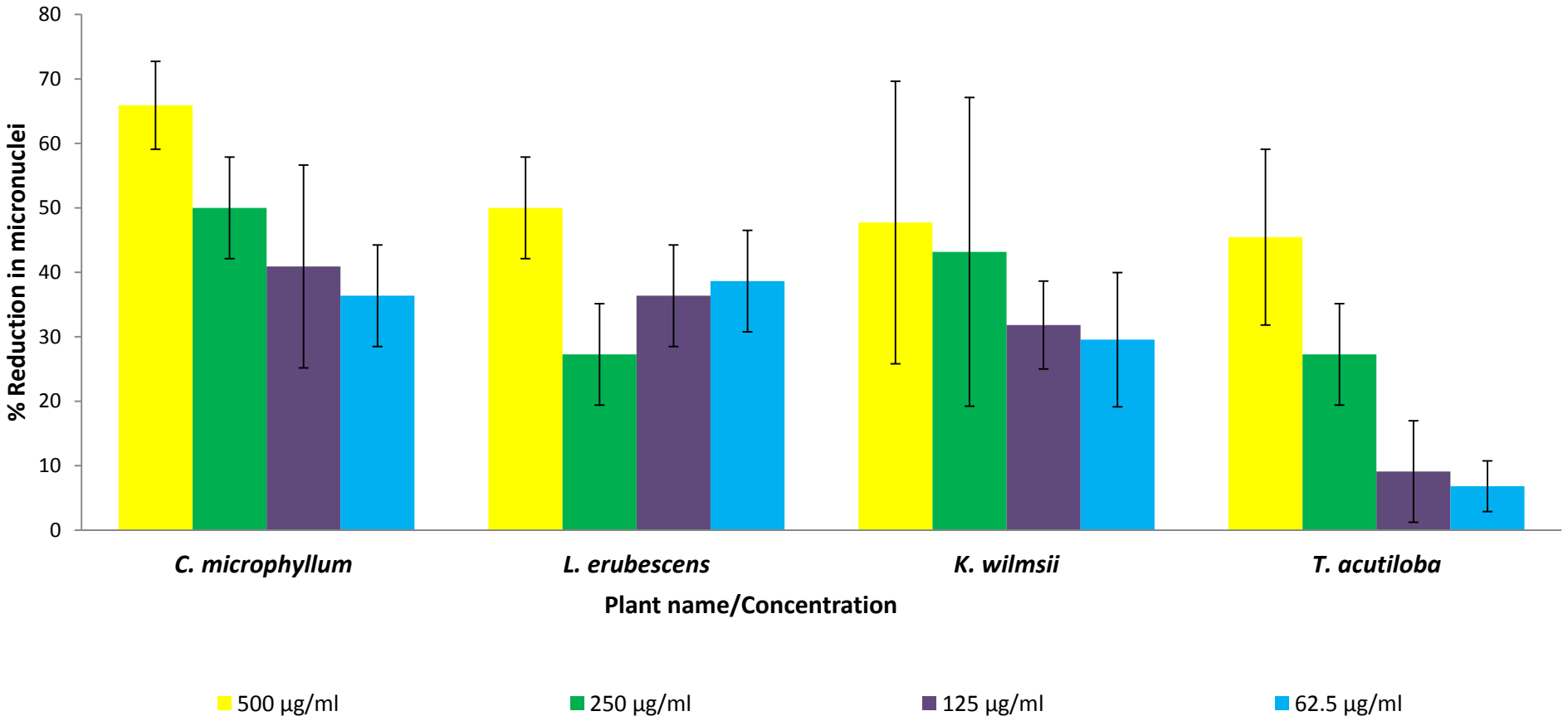


Figure 4.4. Percentage reduction in the number of micronuclei induction by methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* in C3A cells treated with 1 µg/ml 4-NQO.

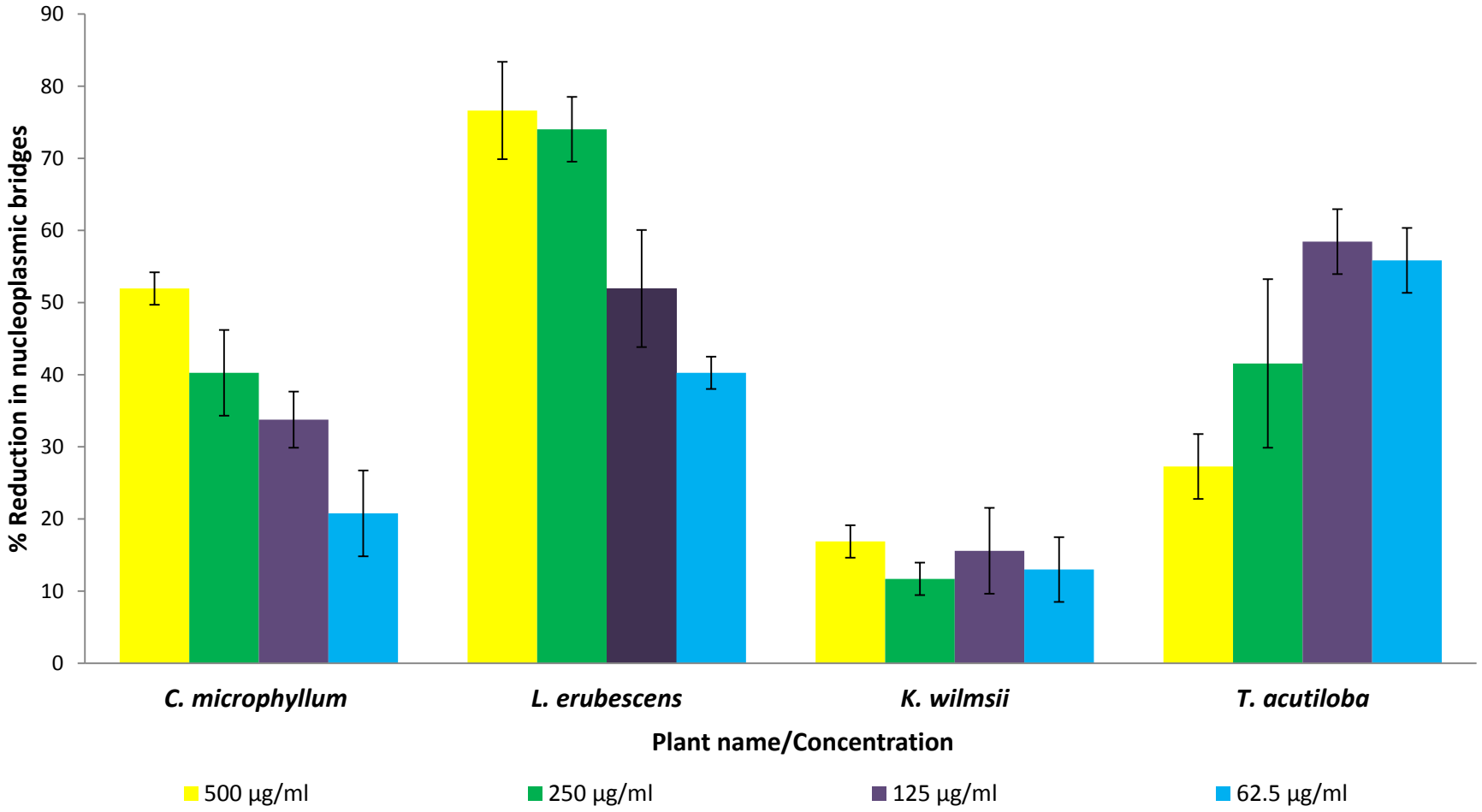


Figure 4.5. Percentage reduction in the number of nucleoplasmic bridge induction by methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* in C3A cells treated with 1 µg/ml 4-NQO.

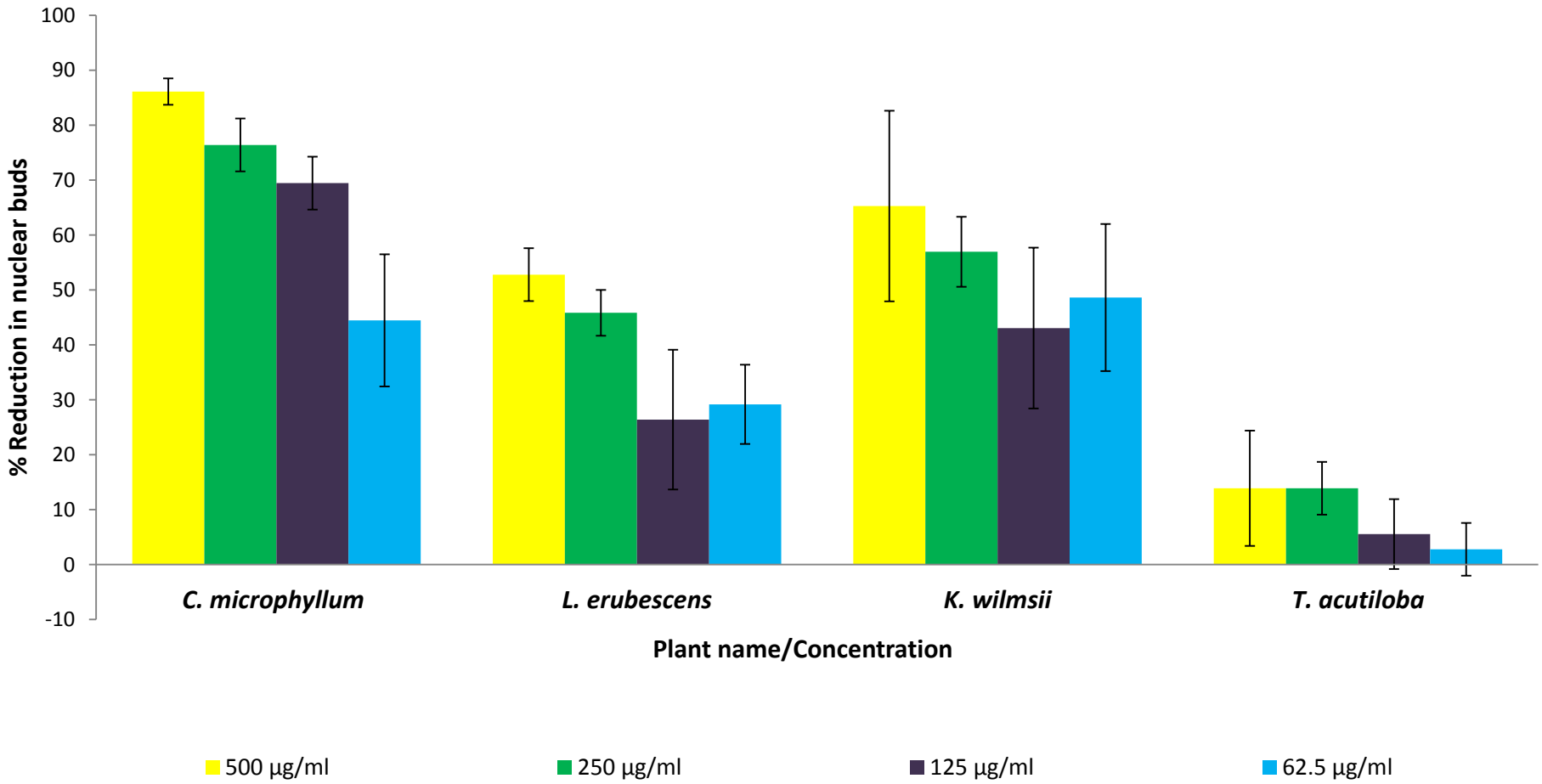


Figure 4.6. Percentage reduction in the number of nuclear buds by methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba* in C3A cells treated with 1 µg/ml 4-NQO.

It was clear that extracts of *C. microphyllum* and *L. erubescens* have antigenotoxic effects in the test systems used. Extracts of these two species may therefore have a preventive effect against mutation related diseases. It is well known that cancer results from an accumulation of multiple genetic changes that can be mediated through chromosomal changes and are cytogenetically detectable (Solomon *et al.,* 2002). Cancer is a disease of altered gene expression involving gene mutations, chromosomal rearrangements and altered chromosome numbers. The coincidence of genotoxic events with the induction of cancer is proof enough that agents that reduce genotoxic effects of known genotoxicants may be useful in cancer chemoprevention (Fenech, 2002).

The four extracts had no cytotoxic effects as evident in the nuclear division cytotoxicity index (NDCI) values (Table 4.4). The determination of the NDCI values is crucial in this study as it allows the measure of mitogenic response and cell proliferation kinetics. The NDCI is an accurate biological index in the detection of cellular toxicity or cell cycle delay (Eastwood and Tucker, 1989, Fenech, 2007).

Table 4.4. Nuclear division cytotoxicity index values (in C3A cells) of methanol leaf extracts of *C. microphyllum, L. erubescens, K. wilmsii* and *T. acutiloba*. The results are expressed a mean number of measurements ± standard deviation.

Sample/ Concentration µg/ml	**500**	**250**	**125**	**62.5**
Mutagenicity assay				
Combretum microphyllum	1.520 ± 0.092	1.627 ± 0.130	1.672 ± 0.028	1.726 ± 0.0056
Leucospermum erubescens	1.523 ± 0.0184	1.637 ± 0.0778	1.635 ± 0.038	1.693 ± 0.0266
Kirkia wilmsii	1.517 ± 0.0099	1.659 ± 0.0297	1.627 ± 0.0325	1.712 ± 01.046
Thespesia acutiloba	1.547 ± 0.0424	1.650 ± 0.0480	1.638 ± 0.2842	1.638 ± 0.0961
Negative control	**1.828 ± 0.0311**			
Antimutagenicity assay				
Combretum microphyllum	1.449 ± 0.024	1.473 ± 0.205	1.612 ± 0.011	1.681 ± 0.055
Leucospermum erubescens	1.372 ± 0.1385	1.395 ± 0.2107	1.543 ± 0.0099	1.679 ± 0.1541
Kirkia wilmsii	1.507 ± 0.0523	1.536 ± 0.0821	1.570 ± 0.0283	1.674 ± 0.0507
Thespesia acutiloba	1.339 ± 0.3152	1.401 ± 0.1909	1.526 ± 0.0905	1.542 ± 0.107
Positive control	**1.68 ± 0.0254**			

It appears there might be a slight increase in the toxicity of the extracts when tested in combination with 4-NQO. In the mutagenicity study, NDCI ≤1.517≥1.726 from highest to lowest concentration assayed, whilst the control value was 1.82 ± 0.03. In the antimutagenicity NDCI≤1.339≥1.681 from highest to lowest concentration assayed, whilst the control value was1.68 ± 0.02. This is an indication that the mutagen 4-NQO is slightly toxic.

The lowest possible NDCI value is 1.00 which occurs if all viable cells have failed to divide during the cytokinesis-block period and are therefore all mononucleated. The highest possible value is 2.00, which occurs in an event where all viable cells completed one nuclear division during the cytokinesis-block phase and are therefore all binucleated (Fenech, 2007).

The potential DNA protective effects of the extracts were also evaluated in the comet assay. The comet assay is a well-validated tool that has been used to measure DNA strand breaks in single cells, allowing evaluation of genotoxicity. The comet tail parameters measured in each cell are assumed to be related to the amount of damage in nuclear DNA (Olive and Banath, 2006). Table 4.5 presents the mean extent of DNA migration measured as tail length, percentage DNA in tail and tail moment. All these parameters were measured in both mutagenicity and antimutagenicity assays. In the mutagenicity assay (Table 4.5), the extracts of all four plant species significantly increased the mean extent of DNA damage ($p<0.001$). Additionally, in some cases, the significance in DNA damage was dose dependent ($p<0.05$).

These results are a clear indication of a genotoxic effect. However, it is clear that the DNA strand damage in the comet assay is not structurally incorporated into gene mutations as no gene mutations were detected in the Ames test. Additionally there was no significant increase in the number of structural aberrations measured in the micronucleus/cytome assay when comparing the results from all three assays. The interpretation of comet assay results is sometimes complicated because comet formation may be due to primary DNA lesions (which occur spontaneously in cells and are then repaired). It should always be established whether there is a relationship between DNA damage caused by a test sample and the biological impact of that damage. i.e. if the DNA damage is converted into biologically relevant gene mutations (Merk and Speit, 1999). This can be achieved by comparing the comet assay results with the results obtained in the Ames test and micronucleus/cytome assay in order to interpret the biological relevance of the DNA damage.

Cell death leads to DNA fragmentation. Increased DNA damage accompanies cytotoxicity resulting from necrosis and apoptosis (Tice *et al.,* 2000, Olive and Banáth, 2006). The positive genotoxic effects in this assay may be a result of cytotoxic effects which may not have been detected in the micronucleus/cytome assay as the latter assay allows for DNA damage to be incorporated into chromosomal structures during mitotic activity. This

is absent in the comet assay. The test samples were assayed at both cytotoxic and non-cytotoxic concentrations. This is because some genotoxic carcinogens are not detectable in *in vitro* assays unless the concentrations tested induce some degree of cytotoxicity (OECD, 1997). The recommended tested concentrations should be closely spaced to cover concentrations with little or no cytotoxicity to clearly cytotoxic concentrations up to 55 ±5% cytotoxicity (OECD, 2010). Contrary to ≥70 % cell viability recommended by Henderson *et al,* (1998), at 500 µg/ml (highest concentration), the average cell viability was ≥ 60% and at the lowest concentration it was above 80%.

Exposure time is an important factor affecting the outcomes of *in vitro* genotoxicity tests (Moore *et al.,* 2006). The influence of exposure time in a study like this that measures DNA strand fragmentation cannot be ruled out. In this case, genotoxicity of these plant extracts (increased comet tail length, % DNA in tail and tail moment especially at the highest concentration tested) may be a consequence of a combination of cytotoxic effects and exposure time. Long exposure times in the comet assay may lead to false positives and negatives depending on the nature of compound tested. Even though it was clearly established that during longer incubation times DNA damage is often repaired and this gives false negative tests (Moore *et al.,* 2006), we however speculate that the opposite might happen in the case of plant extracts where longer incubation time may lead to more DNA damage.

Table 4.5. Tail length, percentage DNA in tail and tail moment in human hepatocarcinoma C3A cells exposed to plant extracts alone (mutagenicity test) and a combination of EMS and plant extracts (antimutagenicity test). The results are expressed a mean number of measurements ± standard deviation. Statistical significance is given for comparison of the extract compared to the solvent control (mutagenicity) and extract + mutagen compared to the mutagen alone (antimutagenicity)

MUTAGENICITY				**ANTIMUTAGENICITY**		
Combretum microphyllum				**+ EMS**		
Concentration µg/ml	Tail length	% DNA in tail	Tail moment	Tail length	% DNA in tail	Tail moment
500	21.40***3 ± 2.50	20.73*** ± 1.28	6.05*** ± 1.56	42.57*** ± 6.93	27.26*** ± 2.22	13.84*** ± 2.95
250	21.54*** ± 6.52	22.89*** ± 0.41	6.5***4 ± 1.98	29.90*** ± 5.79	30.36*** ± 8.67	13.64*** ± 4.67
125	11.95*** ± 3.40	14.00*** ± 0.37	3.26* ± 0.99	33.86*** ± 0.57	33.20** ± 1.38	13.07*** ± 0.76
62.5	18.27*** ± 4.23	17.77*** ± 1.96	5.26** ± 1.33	43.87*** ± 7.47	31.91** ± 5.41	15.57*** ± 1.74
Leucospermum erubescens				**+ EMS**		
500	18.29*** ± 4.48	13.70**** ± 2.96	2.96* ± 0.86	47.58*** ± 7.25	39.47 ± 1.34	20.52* ± 2.97
250	19.42*** ± 5.69	16.65*** ± 1.54	4.51** ± 1.06	50.53*** ± 2.64	25.08*** ± 2.30	14.30*** ± 1.44
125	10.59*** ± 4.85	11.86*** ± 2.24	2.37* ± 1.28	42.82*** ± 3.18	23.18*** ± 0.90	11.40*** ± 0.52
62.5	20.79*** ± 8.13	5.08 ± 0.82	1.72 ± 0.68	41.46*** ± 6.57	33.47** ± 1.44	17.86*** ± 2.46
Kirkia wilmsii				**+ EMS**		
500	16.16*** ± 2.24	14.84*** ± 2.56	3.64* ± 0.51	57.61 ± 4.75	19.37*** ± 3.19	12.09 ***± 1.92
250	20.80*** ± 8.64	16.32*** ± 5.08	7.29*** ± 7.59	47.79*** ± 3.12	34.97 ± 8.42	21.07* ± 5.20
125	15.09*** ± 5.32	18.83*** ± 2.59	3.64* ± 0.49	44.31*** ± 1.62	37.38 ± 5.38	19.02** ± 2.34
62.5	13.95*** ± 3.38	16.06*** ± 2.24	2.73* ± 0.14	41.74*** ± 6.05	30.57*** ± 2.38	13.87*** ± 1.55
Thespesia acutiloba				**+ EMS**		
500	38.29*** ± 4.49	19.31*** ± 5.98	7.86*** ± 0.81	47.43*** ± 1.34	44.39 ± 2.62	26.01 ± 1.73
250	29.89*** ± 8.30	22.12*** ± 4.37	7.20*** ± 1.35	42.42*** ± 4.37	35.64 ± 5.42	16.81*** ± 5.94
125	21.35*** ± 8.82	26.85*** ± 3.94	8.06*** ± 1.75	37.39*** ± 2.20	38.61 ± 3.94	17.42*** ± 2.25
62.5	11.84*** ± 5.23	24.83*** ± 0.50	3.71* ± 1.46	42.58*** ± 1.23	37.68 ± 2.94	50.06*** ± 5.70
Negative 0.00 µg/ml	**3.35 ± 3.31**	**1.99 ± 1.42**	**1.28 ± 0.81**			
Positive 1 mM EMS				**63.57 ± 2.94**	**40.14 ± 6.40**	**26.73 ± 2.45**

* Significant at 0.05 probability level of significance, ** Significant at 0.01 probability level of significance and *** Significant at 0.001 probability level of significance.

Extracts of all four plants had antimutagenic activities in the antimutagenicity assay. When comparing the extent of DNA damage in the positive control (EMS) to the test sample (plant extract+EMS), the degree of antimutagenicity is clear. The antimutagenicity is well pronounced in a dose dependent pattern from the second highest concentration tested. There was a significant decrease in the DNA damaging effects of EMS. Extracts of *C. microphyllum* and *L. erubescens* significantly decreased tail length, percentage DNA in tail and tail moment at all tested concentrations. Even though *K. wilmsii* and *T. acutiloba* decreased tail length, percentage DNA in tail and tail moment at some concentrations, the highest concentration tested for *T. acutiloba* (500 µg/ml) had a high %DNA in tail whilst its lowest concentration (62.5 µg/ml) significantly increased the tail moment. For all measured parameters, the values in the antimutagenicity assay were much higher than in the mutagenicity assay. This may be a result of combinational DNA damaging effects of the extracts (as established in the mutagenicity assay) and EMS.

4.5. Conclusions

Methanol leaf extracts of *C. microphyllum, L. erubescens,* had antimutagenic and/or antigenotoxic activities whilst methanol extracts of *K. wilmsii* and *T. acutiloba* had comutagenic activities. This makes these plants possible candidates for further studies on the antimutagenic potential of higher plants in general. The two antimutagenic plant extracts have potential either as antimutagenic/antigenotoxic herbal preparations or as the starting point for the identification of phytocompounds that could serve as probes for the development of pharmacological candidates for cancer prevention and chemopreventive nutraceuticals. Based on the results presented in this chapter and ease of access and availability of plant material, *C. microphyllum* was selected to isolate and chemically characterize antimutagenic compounds in the next chapter.

CHAPTER 5

Isolation of antimutagenic compounds from *Combretum microphyllum*

5.1. Introduction

The complex chemical composition of plant extracts generally complicates the isolation of bioactive compounds. Plant extracts occur as a combination of various types of compounds or phytochemicals with different polarities and their separation still remains a big challenge for the purpose of identification and characterization (Kalemba and Kunicka, 2003). However, the use of many different types of separation media and solvent combinations can help to pull compounds apart more efficiently (McRae *et al.,* 2007). Isolation of biologically active compounds from plants is an important tool in drug discovery, leading to the isolation of numerous pharmacologically active compounds. Fractionation leading to isolation of bioactive compounds is the basis of lead compound discovery from naturally occurring sources and has led to the introduction of many important drugs (Houghton *et al.,* 2007). There are numerous drugs of known structure that are still extracted from higher plants and used globally in allopathic medicine (Farnsworth and Soejarto, 1985).

Drug discovery from medicinal plants led to the isolation of compounds such as cocaine, codeine, digitoxin and morphine (Newman *et al.,* 2000). It is only once a compound has been characterized that it can be assessed in terms of its potential as a lead compound, and if it is worthy of further investigation (McRae *et al.,* 2007). Chemical characterization of bioactive components in plant extracts is a crucial objective in medicinal plant research and development (Hamburger and Hostettman, 1991). Furthermore, the techniques and processes used in the isolation of active plant compounds are important in medicinal plant research (Newman *et al.,* 2000). Bioassay-guided fractionation is used for separating plant compounds and isolating only those that exhibit the desired activity. When bioactivity has been localised to a specific fraction, further separation by a combination of chromatographic methods based on size, charge and hydrophobicity of compounds is carried out (Massiot *et al.,* 1992).

The main objectives of this chapter were to:

- Fractionate the methanol leaf extract of *C. microphyllum* into different fractions based on polarity, determine the antimutagenic activity of each fraction using the Ames test and select the fraction with the highest activity.
- Use bioactivity-guided fractionation to isolate the active compounds with a combination of column chromatography (CC) and thin layer chromatography (TLC).
- Chemically characterize and identify the isolated compounds using Nuclear Magnetic Resonance (NMR).

In this study, a combination of solvent-solvent fractionation and Silica gel chromatography was used to isolate compounds of interest from the crude plant extracts. (Stoddard *et al.,* 2007).

5.2. Materials and Methods

5.2.1. Plant collection and storage

Leaves of *C. microphyllum* were collected from the Pretoria National Botanical Gardens, dried at room temperature, ground to fine powder and stored in glass bottles. The dried ground leaf material (580 g) was extracted three times with 5 litres of methanol. The extract was concentrated with a rotary evaporator to dryness and decanted into a pre-weighed glass bottle container.

5.2.2. Extraction, solvent-solvent fractionation and antimutagenicity testing

The dried extract (120.98 g) was fractionated using solvent-solvent fractionation into four fractions, namely, hexane (Hex), ethyl acetate (EtOAc), *n*-butanol (But) and water. The total mass yield of the resulting fractions were: Hex (18.00 g, 15%), EtOAc (27.81 g, 23.18%), H_2O (33.21 g, 27.68%) and But (40.01 g, 33.07%) The various fractions obtained from solvent-solvent fractionation were assayed for antimutagenic effects in the Ames test as described in Chapter 3, section 3.2.1 and Chapter 4, section 4.2.1. The most active fraction was selected for the isolation of antimutagenic compounds using column chromatography.

The fractions obtained from solvent-solvent fractionation of the methanol extract were analysed using TLC to visualize their phytochemical constituents. TLC plates were developed using three mobile systems of differing polarities (BEA: non-polar, CEF: intermediate polarity and EMW: polar) and sprayed with vanillin-sulphuric acid and 0.2% DPPH (Figure 5.1).

The antimutagenicity of all fractions were determined, the ethyl acetate fraction was generally the most active in the Ames test. This fractions was subjected to open column chromatography (CC) using silica gel as a stationary phase in a 25 cm X 73 cm column where 140 g silica was mixed with hexane to form a slurry. The slurry was packed in the column. The ethyl acetate fraction (27.81 g) was dissolved in a small volume of ethyl acetate, mixed with 7 g of silica gel, allowed to dry under a stream of air and loaded onto the packed column. The column was initially eluted with 100% hexane, subsequently increasing the polarity by 5% of the eluting solvent with ethyl acetate from 0-100% ethyl acetate.

5.3. Results and discussion

5.3.1. Solvent-solvent fractionation

The hexane and ethyl acetate fractions had similar compounds visible in all mobile phases. The ethyl acetate, butanol and water fractions contained the same potent antioxidant compounds seen in the plate developed in CEF and sprayed with DPPH (Figure 5.1).

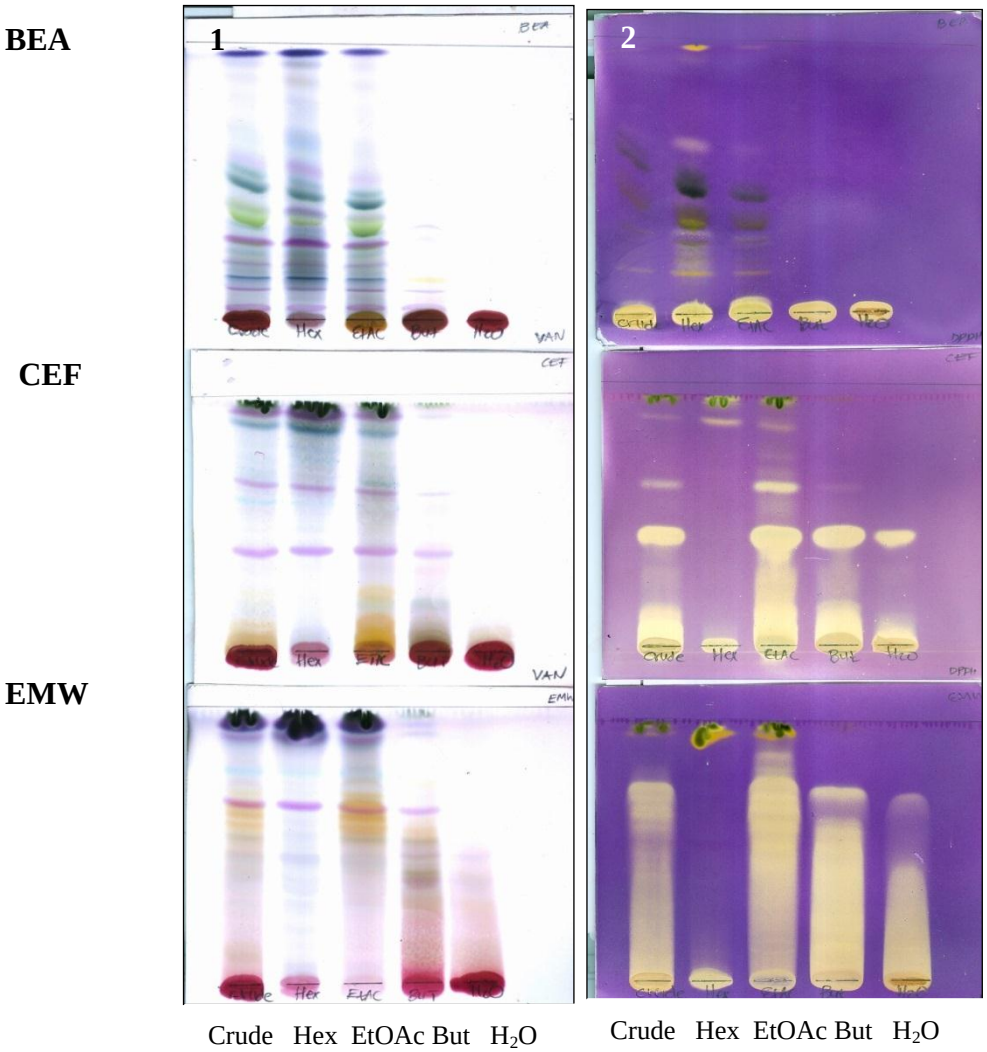


Figure 5.1. TLC chromatograms of solvent-solvent fractions of methanol leaf extract of *C. microphyllum* developed in BEA, CEF and EMW (top to bottom) and sprayed with vanillin-sulphuric acid (1) and DPPH (2).

5.3.2. Mutagenicity and antimutagenicity of solvent-solvent fractions in the Ames test

Table 5.1 shows the summarized mutagenicity results of the solvent-solvent fractions in the Ames test using *S. typhimurium* TA98, TA100 and TA102. None of the assayed fractions induced and increased incidence in the number of revertant colonies compared to the negative control (solvent blank). The mutation frequency for all the three strains when exposed to differing concentrations of the fractions did not change, as at any concentration tested, none of the extracts resulted in double the number of colonies compared to the negative control.

A positive mutagenic response in the Ames test is attributed to a doubling in the number of revertant colonies at any concentration of the test sample compared to the negative control (Verschaeve and van Staden, 2008). The non-mutagenic response demonstrated by fractions obtained from *C. microphyllum* methanol leaf extract in the Ames test is a positive step forward in further investigating the fractions for their antimutagenic potential.

The fractions were also analysed for antimutagenicity in the Ames test using *S. typhimurium* TA8, TA100 and TA102 without metabolic activation (Figure 5.2). All the fractions were active in TA100. Varying degrees of antimutagenicity was observed for all the fractions in TA100. The ethyl acetate fraction had good antimutagenicity in all tester strains. The antimutagenic effects may be due to the presence of antioxidant compounds present in this fraction. It was for this reason that it was selected for the isolation of antimutagenic compounds.

Table 5.1. Mean number of revertant colonies per plate (± standard deviation) in *Salmonella typhimurium* TA98, TA100 and TA102 exposed to solvent-solvent fractions of the methanol leaf extracts of *C. microphyllum* to measure mutagenicity of the plant extract

Concentration µg/ml	**5000**	**500**	**50**
***S. typhimurium* TA98**			
Hexane	32.00 ± 2.64	35.33 ± 5.51	33.00 ± 11.79
Ethyl acetate	25.67 ± 4.04	34.00 ± 2.00	31.00 ± 2.65
Butanol	31.00 ± 2.64	35.33± 0.57	27.67 ± 8.50
Water	27.33 ± 7.57	24.678 ±3.05	22.67 ± 2.08
Negative/solvent blank	35.00 ± 4.18		4NQO (2 µg/ml) 225.40 ± 12.70
***S. typhimurium* TA100**			
Hexane	106.00 ± 8.88	103.67 ± 9.61	107.67 ± 6.81
Ethyl acetate	117.00 ± 11.14	104.00 ± 14.00	115.00 ± 3.60
Butanol	109.00 ± 11.14	100.00 ± 12.12	95.33 ± 8.39
Water	118.67 ± 4.51	113.00 ± 12.50	97.33 ± 11.01
Negative/solvent blank	101.20 ± 3.90		4NQO (1 µg/ml) 622.67 ± 18.10
***S. typhimurium* TA102**			
Hexane	311.00 ± 22.24	303.00 ± 38.59	232.67 ± 6.42
Ethyl acetate	297.67 ± 32.72	320.00 ± 18.25	306.33 ± 5.86
Butanol	293.24 ± 24.64	276.33 ± 12.7	286.7 ± 33.31
Water	287.33 ± 17.62	272.33 ± 10.12	279.67 ± 14.22
Negative/solvent blank	296.20 ± 11.00		MMC (1 µg/ml) 1125.33 ± 6.10

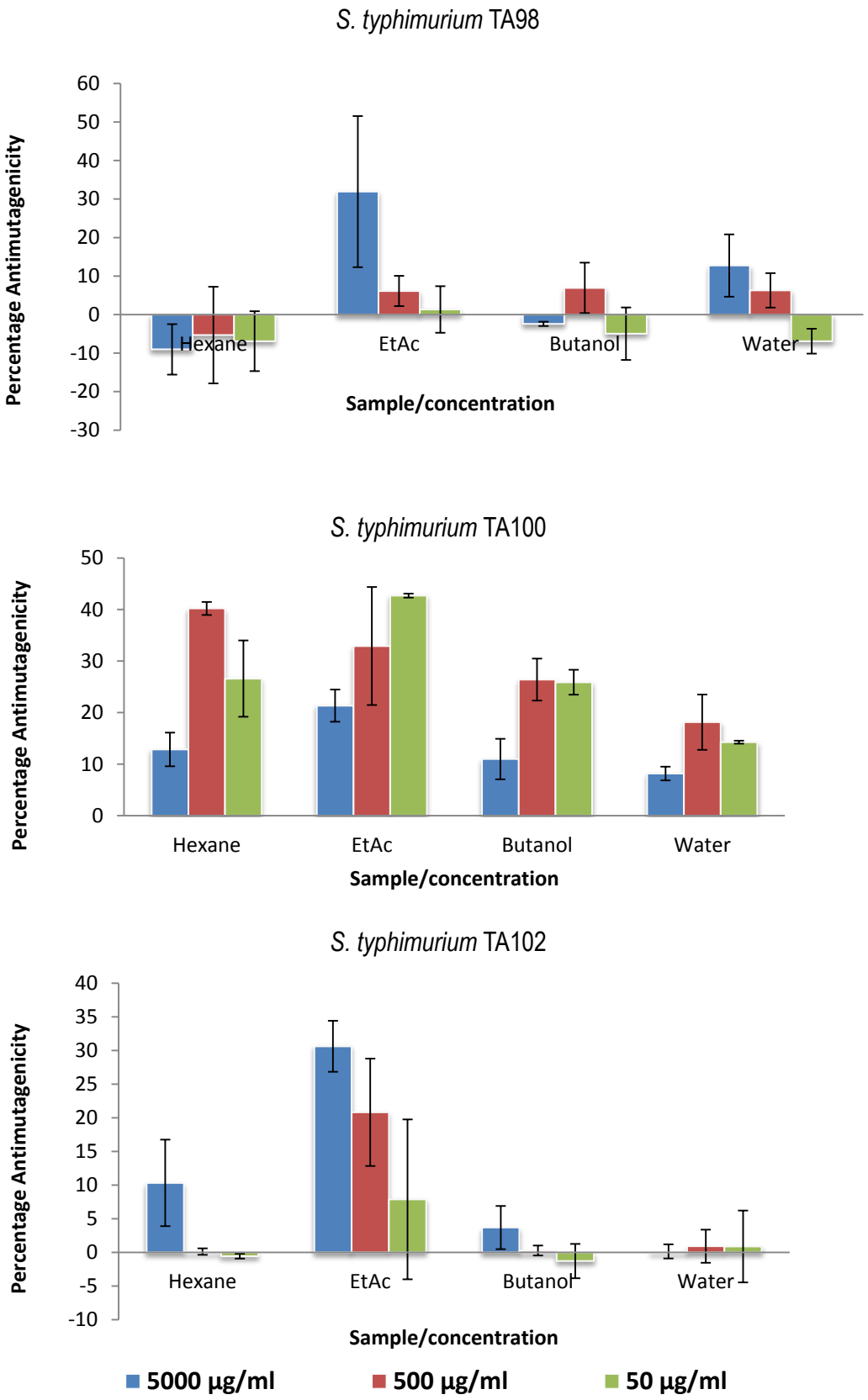


Figure 5.2. Antimutagenic activity of solvent-solvent fractions of the methanol leaf extracts of *C. microphyllum*, in the Ames test using *S. typhimurium* TA98, TA100 and TA102

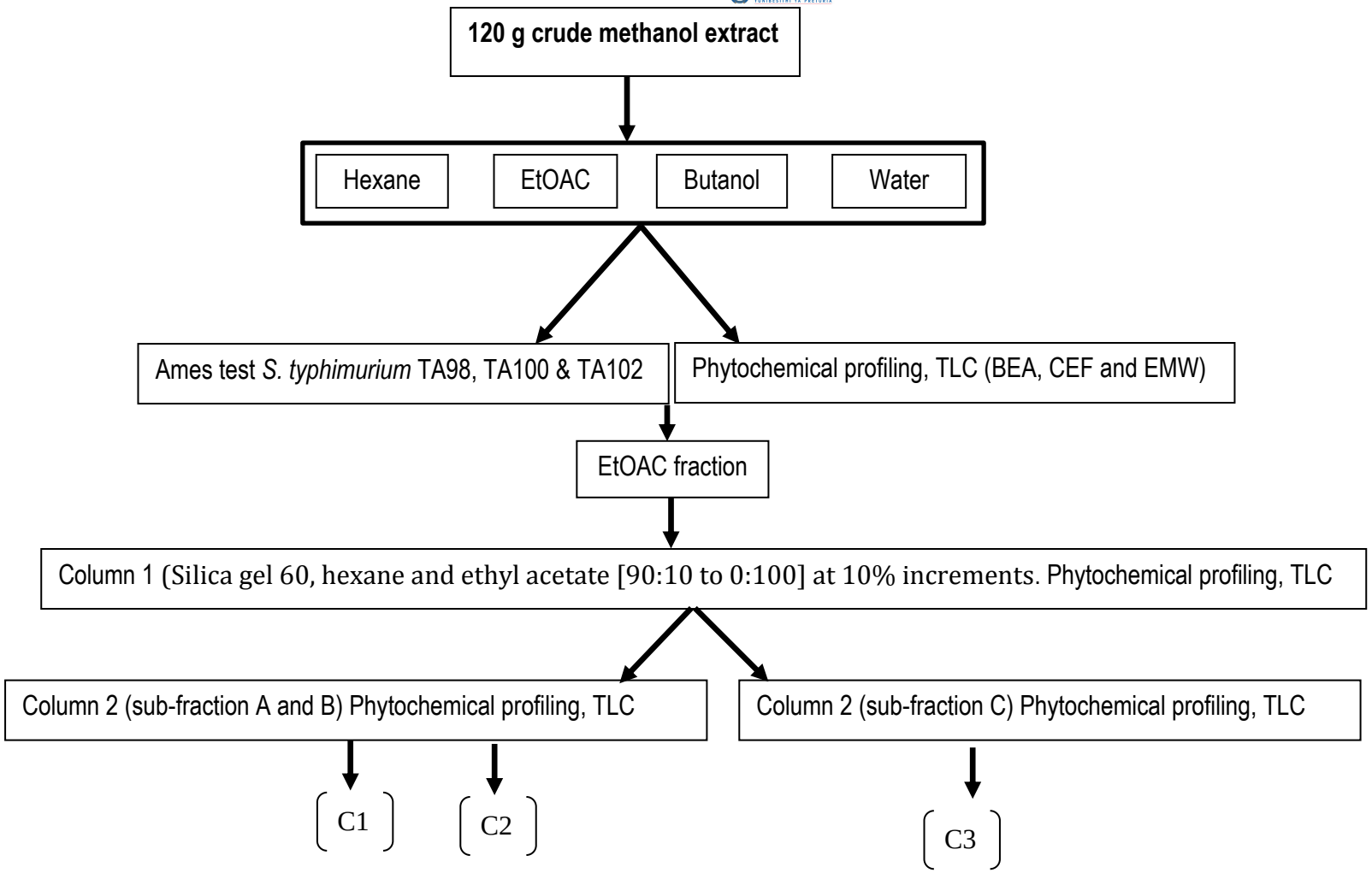


Figure 5.3. Schematic representation of the stepwise procedure followed in the isolation of possible antimutagenic compounds from *C. microphyllum*

5.3.2.1. Isolation of compounds 1, 2 and 3 from the ethyl acetate fraction

A total of 120 fractions were collected form the first column and analysed using TLC using hexane: ethyl acetate (8:2) as the mobile solvent and vanillin sulphuric acid reagent spray to visualise compounds. Fractions containing similar components/compounds were pooled together into sub-fractions and purified further. The 120 fractions were grouped into three (A, B and C) sub-fractions based on similar phytochemical profiles. Sub-fraction A contained fractions 1-20, B contained fractions 21-40 and C contained fractions 41-120. Figure 5.3 is a schematic presentation of the procedure followed in the isolation of the three compounds.

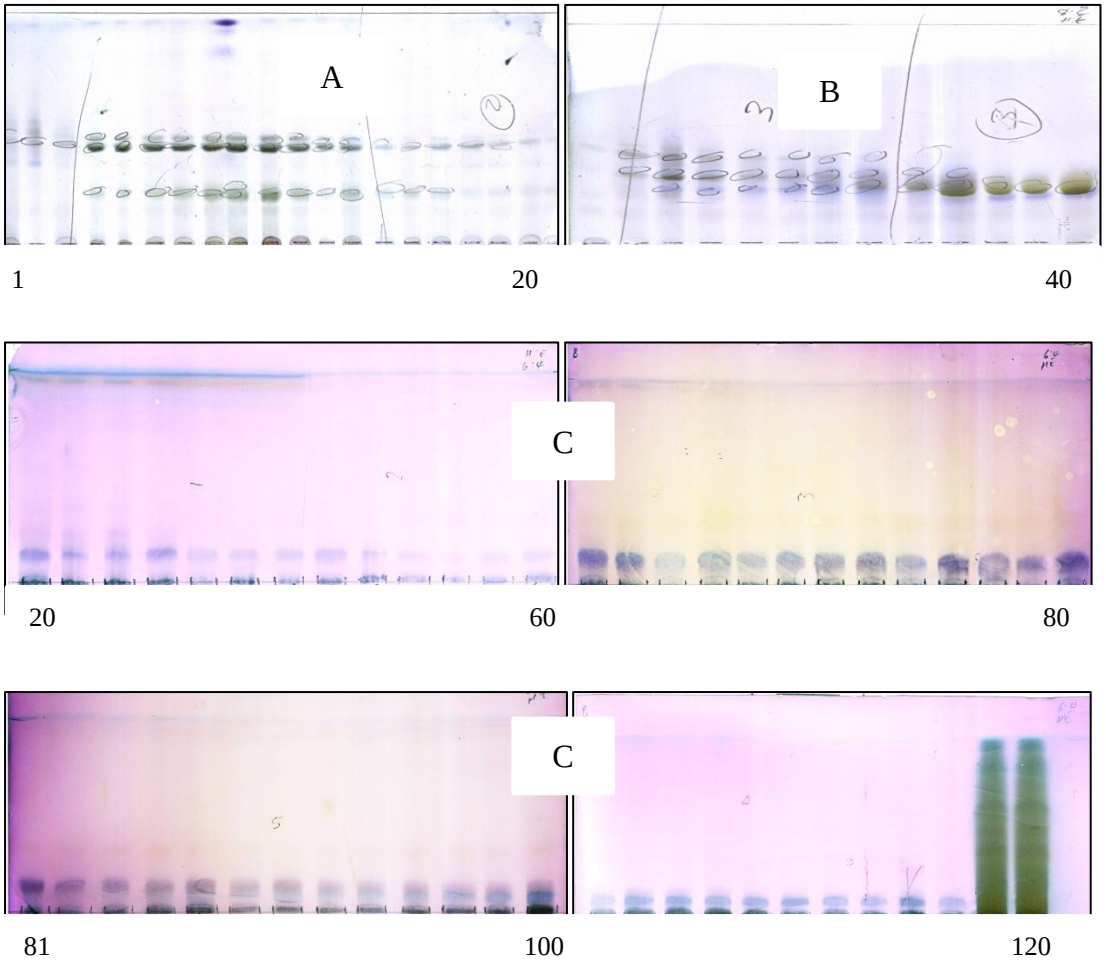


Figure 5.4. TLC chromatograms (Column 1) of fractions after column chromatography of the ethyl acetate fraction developed in hexane: ethyl acetate (8:2) and sprayed with vanillin-sulphuric acid reagent spray (fractions 1-120).

5.3.2.2. Isolation of compounds 1 and 2 (C1 and C2)

Fractions A and B were subjected to open CC separation using silica gel as stationary phase and hexane: ethyl acetate (hexane and ethyl acetate [90:10 to 0:100] at 10% increments) as the mobile phase. From fraction A, a total of 40 fractions were collected and fractions 25-35 contained pure C1. From fraction B, a total of 32 fractions were collected, and none were pure. They were combined again and upon washing the dried residue with chloroform, compound 2 precipitated out as a white powder.

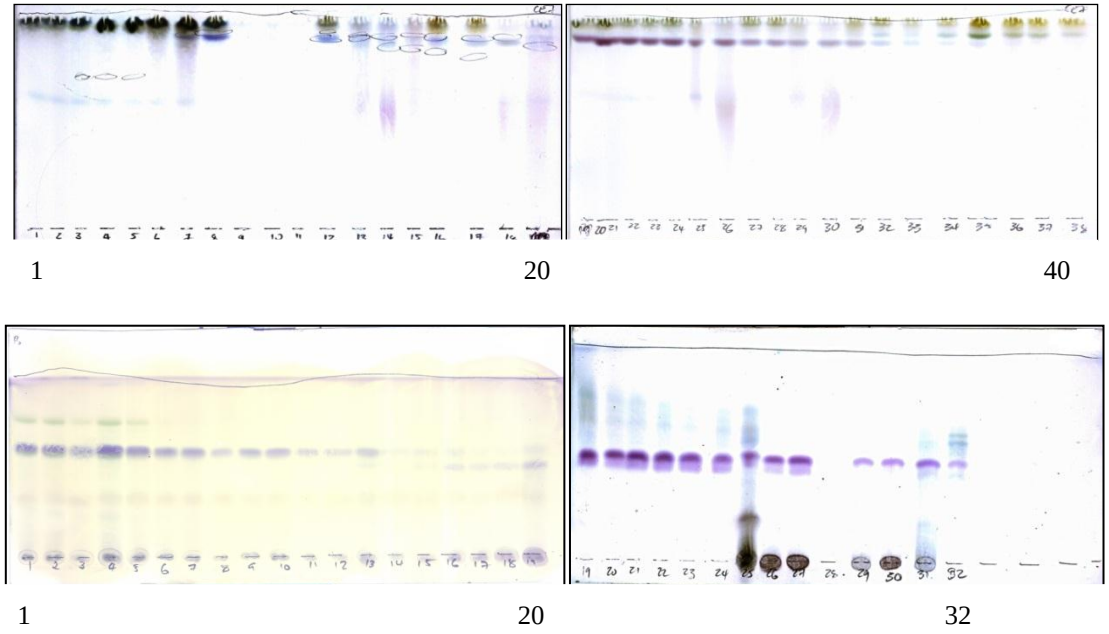


Figure 5.5. TLC chromatograms of sub-fractions A and B developed in CEF and sprayed with vanillin-sulphuric acid reagent spray.

5.3.2.3. Isolation of compound 3 (C3) from sub-fraction C.

Fraction C was subjected to open CC using silica gel and hexane combined with ethyl acetate as eluent solvent with 5% increments in polarity. A total of 60 fractions were collected and pure compound 3 was obtained from fraction 41-60 as a white powder.

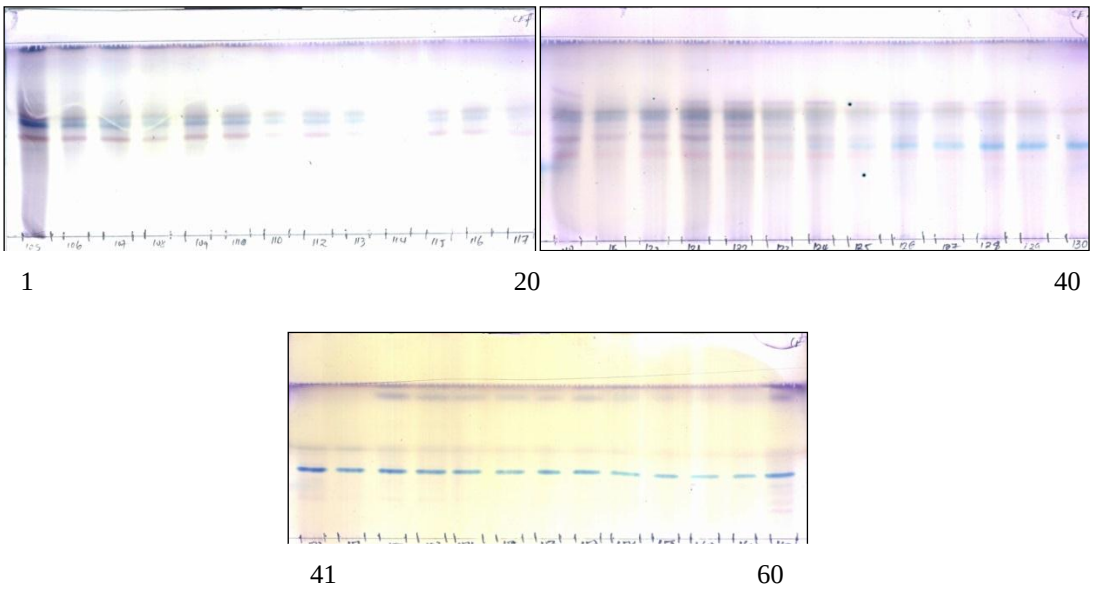


Figure 5.6. TLC chromatograms of sub-fraction C developed in CEF and sprayed with vanillin-sulphuric acid reagent spray.

TLC profiles showing the purification and isolation of the three compounds are presented in Figure 5.7.

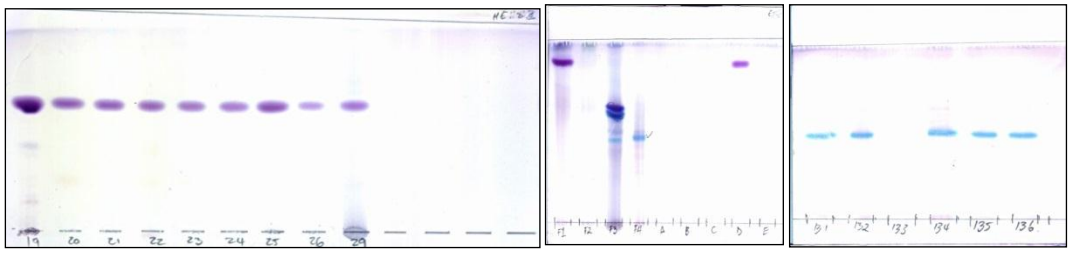


Figure 5.7. TLC chromatogram of combined fractions from sub-fraction A, B and C, developed in CEF and sprayed with vanillin-sulphuric acid spray reagent (showing purity of isolated compounds).

5.4. Structure elucidation of compounds isolated from *C. microphyllum*

Both ^{1}H-and^{13}C-NMR spectra were recorded with a Varian spectrometer at 400 MHz. Chemical shifts (δ) were quoted in parts per million (ppm) from the internal standard tetramethylsilane (TMS). Compound 1 and 2 were dissolved in deuterated chloroform and compound 3 was dissolved in deuterated dimethyl sulfoxide (DMSO).

5.4.1. Compound 1

Compound (C1) was obtained as a white powder (12 mg). The NMR spectra (^{1}H, ^{13}C, ASAP-HMQC, COSY and HMBC) are presented in figure 5.9-5.13. The ^{1}H-NMR spectrum (Table 5.2, Appendix 1) had four sets of proton signals at *δ* 3.64 (br.*t*, 4.0, 8.0 Hz, 2H), 1.56 (*m*, 2H), 1.25 (br.*s*, 42H) and 0.88 ppm (*t*, 8.0 Hz, 3H) corresponding to protons at position C-1, C-2, C-3 – C-23 and C-24, respectively. The ^{13}C-NMR spectrum (Table 5.2, Appendix 2) had characteristic signals for a fatty acid derivative at *δ* 63.1 (CH_2), 32.8 (CH_2), 31.9 ($21CH_2$) and 14.1 (CH_3) ppm corresponding to an oxygenated methylene (C-1), a methylene (C-2), a methylenic side chain (C-3 - C-23) and a methyl (C-24) groups, respectively. The analysis of the spectroscopic data (^{1}H-, ^{13}C-NMR, HMQC, COSY and HMBC) compared with those reported in the literature enabled its unambiguous identification as n-tetracosanol (**1**) (Murray and Schoenfeld, 1995).

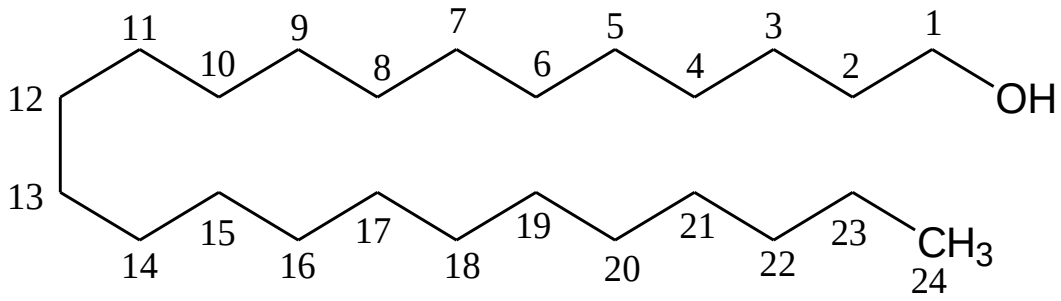


Figure 5.8. Chemical structure of n-tetracosanol

Table 5.2. ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR data of n-tetracosanol (1) in $CDCl_3$, (*δ* in ppm, *J* in Hz)

n-Tetracosanol (1)		
Position	**^{13}C**	**^{1}H**
1	63.1, (CH_2)	3.64, br.*t* (4.0, 8.0), 2H
2	32.8, (CH_2)	1.56, *m*, 2H
3-23	31.9 – 22.7, (CH_2)	1.25, br.*s*, 42H
24	14.1, (CH_3)	0.88, *t*, (8.0) 3H

5.4.2. Compound 2

Compound 2 (C2) was obtained as a white powder (11.3 mg). The NMR spectra (^{1}H, ^{13}C, ASAP-HMQC, COSY and HMBC) are presented in Appendix 6-10). The ^{13}C-NMR spectrum (Table 5.3, Appendix 7) had a characteristic signal at δ 178.1 ppm assignable to a carboxylic group of a fatty acid. The presence of an acid group was substantiated by the broad singlet observed on the ^{1}H-NMR spectrum (Table 1) at δ 10.00 ppm corresponding to the proton of the hydroxyl group. Others signals were observed on the ^{13}C-NMR spectrum at δ 33.7 (CH_2), 31.9 (CH_2), 29.7 - 22.7 (16 CH_2) and 14.1ppm (CH_3) corresponding to carbons at positions C-2, C-3, C-4 – C-19 and C-20, respectively. Similar signals as those from compound **1** described above were observed on the ^{1}H-NMR spectrum (Table 5.3, Figure 5.13) at δ 2.35 (br.*t*, 4.0, 8.0 Hz, 2H), 1.63 (*m*, 2H), 1.25 (br.*s*, 32H) and 0.88 ppm (*t*, 8.0 Hz, 3H), and corresponding to protons at positions C-2, C-3, C-4 – C-19 and C-20, respectively. All these data were in agreement with those of eicosanoic acid (**2**), also called arachidic acid, previously isolated from *Milletia laurenti* (Vieux *et al.,* 1970; Ongoka *et al.,* 2006).

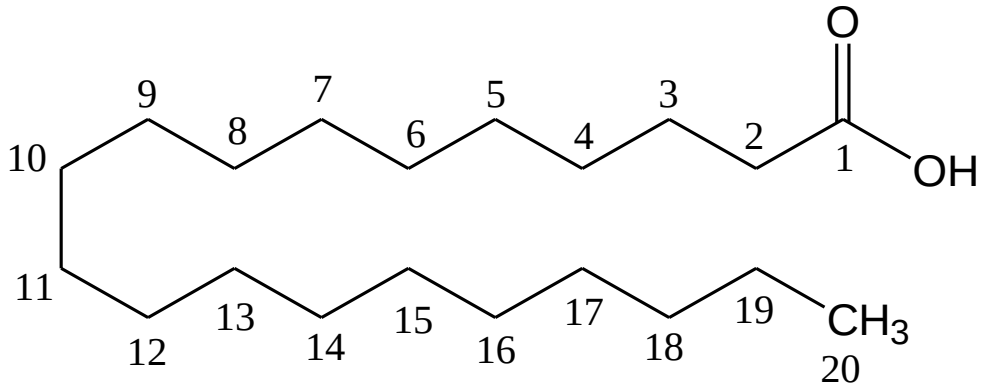


Figure 5.9. Chemical structure of eicosanoic acid

Table 5.3. ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR data of eicosanoic acid (2) in $CDCl_3$, (δ in ppm, *J* in Hz)

Eicosanoic acid (2)		
Position	**^{13}C**	**^{1}H**
1	178.1, (C=O)	
2	33.7, (CH_2)	2.35, br.*t* (4.0, 8.0), 2H
3	31.9, (CH_2)	1.63, *m*, 2H
4-19	29.7 – 22.7, (CH_2)	1.25, br.*s*, 32H
20	14.1, (CH_3)	0.88, *t*, (8.0), 3H
-		10.00, br.*s*, OH

5.4.3. Compound 3

Compound 3 (C3) was obtained as a white powder (15 mg) and responded positively to the Liebermann - Büchard test characteristic of triterpenoids. The NMR spectra (^{1}H, ^{13}C, ASAP-HMQC, COSY and HMBC) are presented in Appendix 11-15. The ^{13}C-NMR spectrum (Table 5.4 Appendix 12) had a total number of 30 carbons of which six were methyl carbon signals at δ 13.7 (C-23), 16.8 (C-25), 16.9 (C-26), 25.7 (C-27), 32.1 (C-29), 23.0 (C-30) and six downfield carbon signals at 178.6 (C-28), 144.0 (C-13), 121.5 (C-12), 75.5 (C-3), 67.4 (C-2) and 63.9 (C-24) characteristic for olean-12-ene triterpenoid (Mahato and Kundu, 1994) bearing one carboxylic acid and three hydroxyl groups. This assumption was substantiated by the presence of some characteristic proton signals on the ^{1}H-NMR spectrum (Table 2, Fig 5.21) at δ 5.17 (br.*s*, COOH-28), 3.47 (*m*, H-2), 3.17 (*m*, H-3), 2.74 (br.*d*, 8.0 Hz, H-9), 0.54 (*s*, Me-23), 0.87 (*s*, Me-25), 0.71 (Me-26), 1.10 (*s*, Me-27), 0.87 (*s*, Me-29) and 0.91 (*s*, Me-30). All the data above along with the HSQC, HMBC and COSY were in agreement with those reported for arjunolic acid (**3**) (Bag *et al.,* 2008), a constituent of the core wood of *Terminalia arjuna* (Ramesh *et al.*, 2012).

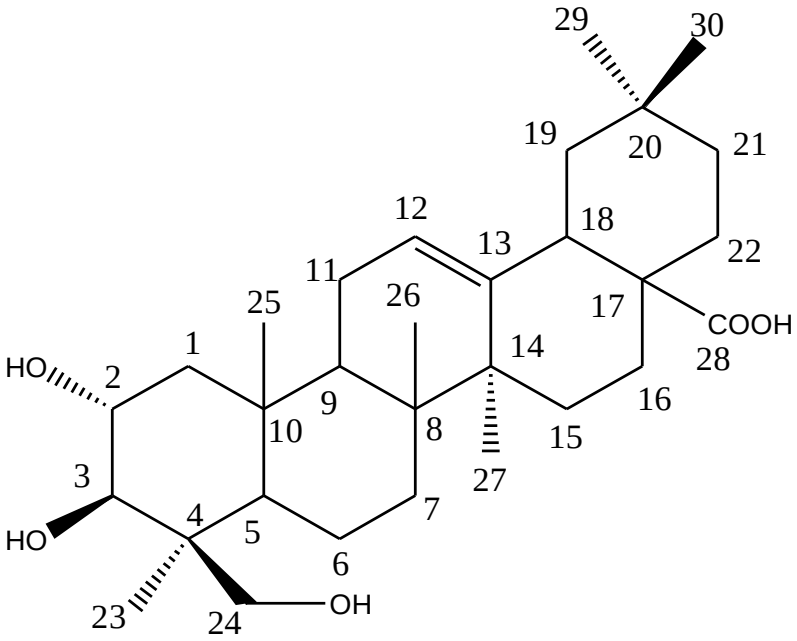


Figure 5.10. Chemical structure of arjunolic acid

Table 5.4. ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR data of arjunolic acid (3) in DMSO-d_6, (δ in ppm, J in Hz)

Position	^{13}C	^{1}H
1	45.7 (CH_2)	
2	67.4 (CH)	3.47 *m*
3	75.5 (CH)	3.17 *m*
4	42.5 (C)	
5	45.4 (CH)	
6	17.5 (CH_2)	
7	32.9 (CH_2)	
8	37.4 (C)	
9	40.8 (CH)	2.74 *bd* (8.0)
10	41.4 (C)	
11	23.4 (CH_2)	
12	121.5 (CH)	5.17 *brs*
13	144.0 (C)	
14	47.1 (C)	
15	30.4 (CH_2)	
16	27.2 (CH_2)	
17	46.7 (C)	
18	41.4 (CH)	
19	46.0 (CH)	
20	30.4 (CH)	
21	33.3 (CH_2)	
22	31.9 (CH_2)	
23	13.7 (CH_3)	0.54 *s*
24	63.9 (CH_2)	3.29 *d* (8.0), 3.03 *d* (8.0)
25	16.8 (CH_3)	0.87 *s*
26	16.9 (CH_3)	0.71 *s*
27	25.7 (CH_3)	1.10 *s*
28	178.6 (C)	
28-COOH		12.00 *brs*
29	32.1 (CH_3)	0.87 *s*
30	23.0 (CH_3)	0.91 *s*

5.5. Conclusion

Three compounds were isolated from the ethyl acetate fraction of the methanol leaf extract of *C. microphyllum* by bioassay-guided fractionation up to the solvent-solvent fractionation step using a combination of column chromatography and thin layer chromatography. The three compounds were chemically characterized using NMR and identified as n-tetracosanol (C1), eicosanoic acid (C2) and arjunolic acid (C3). Although these compounds have been isolated from other plant species before, this is the first report on these compounds occurring in *C. microphyllum.*

In the next chapter the antimutagenicity and cytotoxicity of the isolated compounds will be determined.

CHAPTER 6

Antimutagenicity, cytotoxicity and antioxidant activity of n-tetracosanol, eicosanoic acid and arjunolic acid; compounds isolated from *Combretum microphyllum*

6.1. Introduction

Antimutagens and anticarcinogens play a major role in the primary prevention of mutations and cancer development (De Flora *et al.,* 2001). The use of antimutagens and anticarcinogens in everyday life is the most effective way of preventing human cancers and genetic diseases (Kuroda *et al.,* 1990). There is increasing evidence that cancer and other mutation-related diseases can be prevented by favouring intake of protective factors and by modulating the defence mechanisms of the host organism, a strategy referred to as chemoprevention (Ferguson *et al.,* 2005). Chemoprevention of mutation related diseases is an area of increasing research.

Plants have different chemical compounds, many of which may be bioactive against various diseases, but can also be toxic. Generally, an ideal antimutagen and/or chemopreventive agent should be non-toxic, have little to no untoward side effects, have high efficacy and have a known mechanism of action (Morse and Stoner, 1993). Toxicity testing can reveal some of the risks that may be associated with the use of plants and their products (Ifeoma and Oluwakanyinsola, 2013). Investigating the potential toxicity of medicinal plants and the pure compounds isolated from them is an important consideration in medicinal plant research (McGaw *et al.,* 2007). This is because secondary metabolites obtained from plants are not benign molecules. Plants have evolved such chemicals as a defence mechanism amongst other reasons, and they may thus be poisonous (Gurib-Fakim, 2006). It was for these reasons that we determined the antimutagenic activity and cytotoxicity of compounds isolated from *C. microphyllum*.

Many mutations related to oxidative stress and DNA damage by ROS or RNS have been identified in numerous human syndromes (Sies, 1998, Schafer and Buettner, 2001). Since oxidative DNA damage plays a role in the pathogenesis of several chronic degenerative diseases, a decrease of oxidative stress is the best possible strategy for the prevention of these diseases. Antioxidants are capable of slowing or preventing the oxidation of

other molecules. They have a wide application in the health sector due to the pathological role of free radicals in a variety of diseases (Benzie and Strain, 1999). Antioxidant compounds can play a preventative role against mutation related diseases and thus have potential antimutagenic applications.

Even though the NRU assay was used for the initial toxicity testing (Chapter 3), in this chapter, the cytotoxicity of the three compounds isolated from *C. microphyllum* was determined using the MTT assay. The NRU assay for the initial screening was conducted at the Scientific Institute of Public Health, Brussels in Belgium. The NRU assay is not routinely performed in the laboratory of the Phytomedicine Programme; as a result, reagents for this assay are not readily available. On the other hand, the MTT assay is performed routinely in the Phytomedicine Programme, which is the reason why it was an assay of choice in this particular study. Although cytotoxicity is measured based on different physiological endpoints in this two assays, these assays are always in agreement on the basis of midpoint cytotoxicity values (LC_{50}) (Borenfreund et al., 1988).

6.2. Materials and methods

6.2.1. Ames test

Methods described in Chapter 3, section 3.2, were used for both mutagenicity and antimutagenicity testing. For antimutagenicity assays, two mutagens were used: 4-NQO was used for *S. typhimurium* strains TA98 and TA100, and mitomycin C (MMC) was used for *S. typhimurium* strain TA102. These strains screen for different types of carcinogens/mutagens including those causing frame-shift and base-pair substitution mutations, as well as DNA damage associated with oxidative damage, in the presence and absence of metabolic activation.

6.2.2. Cytotoxicity assay

The MTT cytotoxicity assay described by Mosmann (1983) was used to test the cytotoxic effects of the three compounds isolated from *C. microphyllum* against human hepatocellular carcinoma (C3A) cells. Doxorubicin chloride was used as a positive control. The cells of a subconfluent culture were harvested using trypsin-EDTA (Sigma) and centrifuged at 200 x g for 5 minutes and resuspended in growth medium to 5 x 10^4 cells/ml. A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a 96 well culture plate. The same amount of the growth medium was added to wells of column 1 and 12 to maintain humidity and minimize the edge effect. The plates were incubated at 37°C in a 5% CO_2 incubator overnight until the cells were in the exponential phase of growth. After incubation, the MEM was aspirated from the cells and replaced with 200 µl of different concentrations of the test samples. Each dilution of the test sample was tested in quadruplicate.

The plates were again incubated for 2 days at 37°C in a 5% incubator. A negative control (untreated cells) and positive control (cells treated with different concentrations of doxorubicin chloride (Sigma) were included. After incubation, 30 µl of 5 mg/ml MTT (Sigma) in phosphate buffered saline PBS was added to each well and the plates were incubated for a further 4 hours at 37°C. After incubation with MTT, the medium in each well was removed and the formazan crystals formed were dissolved by adding 50 µl of DMSO to each well of the plates. The plates were gently shaken until the crystals were dissolved. The amount of MTT reduction was measured immediately by detecting the absorbance using a microplate reader at a wavelength of 570 nm (VersaMax, Molecular Devices). The wells in column 1 and 12, containing medium and MTT but no cells was used to blank the microplate reader. The percentage of cell viability was calculated using the formula below:

$$\% \text{ cell viability} = \frac{\text{Mean Absorbance of sample}}{\text{Mean Absorbance of control}} \times 100$$

The LC_{50} values were calculated as the concentration of the test sample that resulted in 50% reduction of absorbance compared to untreated cells. The intensity of the MTT formazan produced by living metabolically active cells is directly proportional to the number of live cells present (Mosmann, 1983).

6.2.3. Quantitative antioxidant assay

Quantitative antioxidant activity of the three compounds isolated from *C. microphylum* was determined using the spectrophotometric method described by Mensor *et al,* (2001) and modified by Aderogba *et al,* (2006). This method is fully described in Chapter 2, section 2.2.3.

6.2.4. Preparation of compounds for bioassays

For the Ames test, all three compouds were dissolved in DMSO to a concentration of 10 mg/ml (stock solution). The compounds were tested at 500, 50 and 5 µg/ml dissolved in10% DMSO in water. To determine the cytotoxicity, 20 mg/ml , of the test samples were prepared in DMSO. The compounds were tested at 200, 150, 100, 50, 20 and 10 µg/ml dissolved in1% DMSO in growth media. from the stock solution. To determine antioxidant activity, all three compounds were prepared to a concentration of 1 mg/ml in methanol. The compounds were then serially diluted from 100 to 0.05 µg/ml with methanol.

6.3. Results and discussion

The mutagenic and antimutagenic activities of the three compounds isolated from *C. microphyllum* n-tetracosanol (C1), eicosanoic acid (C2) and arjunolic acid (C3)] were determined in the Ames test using S. *typhimurium* TA98, TA100 and TA102. Additionally, the compounds were assayed for cytotoxic effects against the human hepatocarcinoma C3A cell line and for antioxidant activity. Table 6.1 shows the summarized mutagenicity results of the three compounds in the Ames test using *S. typhimurium* TA98, TA100 and TA102.

Figure 6.1 represents antimutagenic activity of the three compounds in the Ames test using *S. typhimurium* TA98, TA100 and TA102. The results for cytotoxic effects of the compounds are presented in Figure 6.2 (percentage cell viability) and Table 6.2 (LC_{50} values). The antioxidant activity results measured as DPPH free radical scavenging activity are presented in Figure 6.3 (percentage DPPH inhibition) and Table 6.3 (EC_{50} values).

All three compounds were not mutagenic in the Ames test using *S. typhimurium* TA98, TA100 and TA102 at the concentrations tested. None of the compounds induced an increased incidence in the number of revertant colonies compared to the negative control (solvent blank). The mutation frequency/index for all the three strains when exposed to differing concentrations of the four plant extracts was less than 2, meaning none of the extracts caused double the number of colonies compared to the negative control. A positive mutagenic response in the Ames test is attributed to a doubling in the number of revertant colonies at any concentration of the test sample compared to the negative control (Verschaeve and van Staden, 2008).

Table 6.1. Mean number of revertant colonies per plate (± standard deviation) in *Salmonella typhimurium* TA98, TA100 and TA102 exposed to C1-C3 isolated from. *C. microphyllum* to measure mutagenicity of the plant extract

Concentration µg/ml	500	50	5
***S. typhimurium* TA98**			
n-Tetracosanol	30.00 ± 7.81	35.67 ± 9.71	23.67 ± 4.51
Eicosanoic acid	27.77 ± 1.53	29.33 ± 4.04	28.67 ± 5.51
Arjunolic acid	33.00 ± 2.65	28.33 ± 3.78	26.67 ± 1.15
Negative/solvent blank	28.60 ± 5.32	4NQO (2 µg/ml)	239.33 ± 33.20
***S. typhimurium* TA100**			
n-Tetracosanol	125.00 ± 8.18	121.33 ± 2.52	127.00 ± 7.21
Eicosanoic acid	108.67 ± 5.03	102.67 ± 4.73	112.33 ± 2.89
Arjunolic acid	109.00 ± 8.72	104.33 ± 2.52	107.33 ± 1.15
Negative/solvent blank	107.00 ± 4.85	4NQO (1 µg/ml)	864.00 ± 9.77
***S. typhimurium* TA102**			
n-Tetracosanol	294.33 ± 20.74	271.00 ± 4.58	286.67 ± 8.50
Eicosanoic acid	292.33 ± 5.51	278.33 ± 7.57	288.00 ± 10.82
Arjunolic acid	287.00 ± 15.39	280.67 ± 10.69	288.67 ± 28.68
Negative/solvent blank	282.40 ± 15.53	MMC (1 µg/ml)	1241.67 ± 7.77

All the compounds were antimutagenic in the Ames test (Figure 6.1). The compounds clearly have multiple mechanisms of mutation inhibition as they inhibit mutagenicity of 4-NQO in *S. typhimurium* TA98, TA100 and mutagenicity of MMC in *S. typhimurium* TA102. Moreover, these compounds may have varying mechanisms of antimutagenesis since they prevent frame-shift mutations detectable in TA98, base-pair substitutions detectable in TA100 and small in-frame deletions detectable in TA102. This is one of the many advantages of using the Ames test in antimutagenesis studies as it provides information not only of antimutagenesis but also on possible mode of action (De Flora *et al.,* 1992).

Arjunolic acid (C3) was the most active in all three tested strains with percentage antimutagenicity of up to 41.92 ± 9.59%, 35.84 ± 1.45% and 43.78 ± 0.18% in *S. typhimurium* TA98, TA100 and TA102 respectively, followed by eicosanoic acid (C2) and lastly n-tetracosanol (C1) (Figure 6.1). Against *S. thiphimurium* TA100 and TA 102 there was an excellent dose related effect and higher dose may have led to even a larger effect. The compounds had substantially higher activity compared to the crude extract and fractions assayed (Chapter 4). The compounds were more active at concentrations 10 times lower than those assayed for the crude extract and the fractions. This is in agreement with the reports that the concept of antimutagenicity on plants is complicated by the fact that the activity demonstrated by a crude extract may be generated by a small amount of a very potent constituent, a large amount of a very weakly active agent or by the cumulative effect of many components (Mitscher *et al.,* 1992).

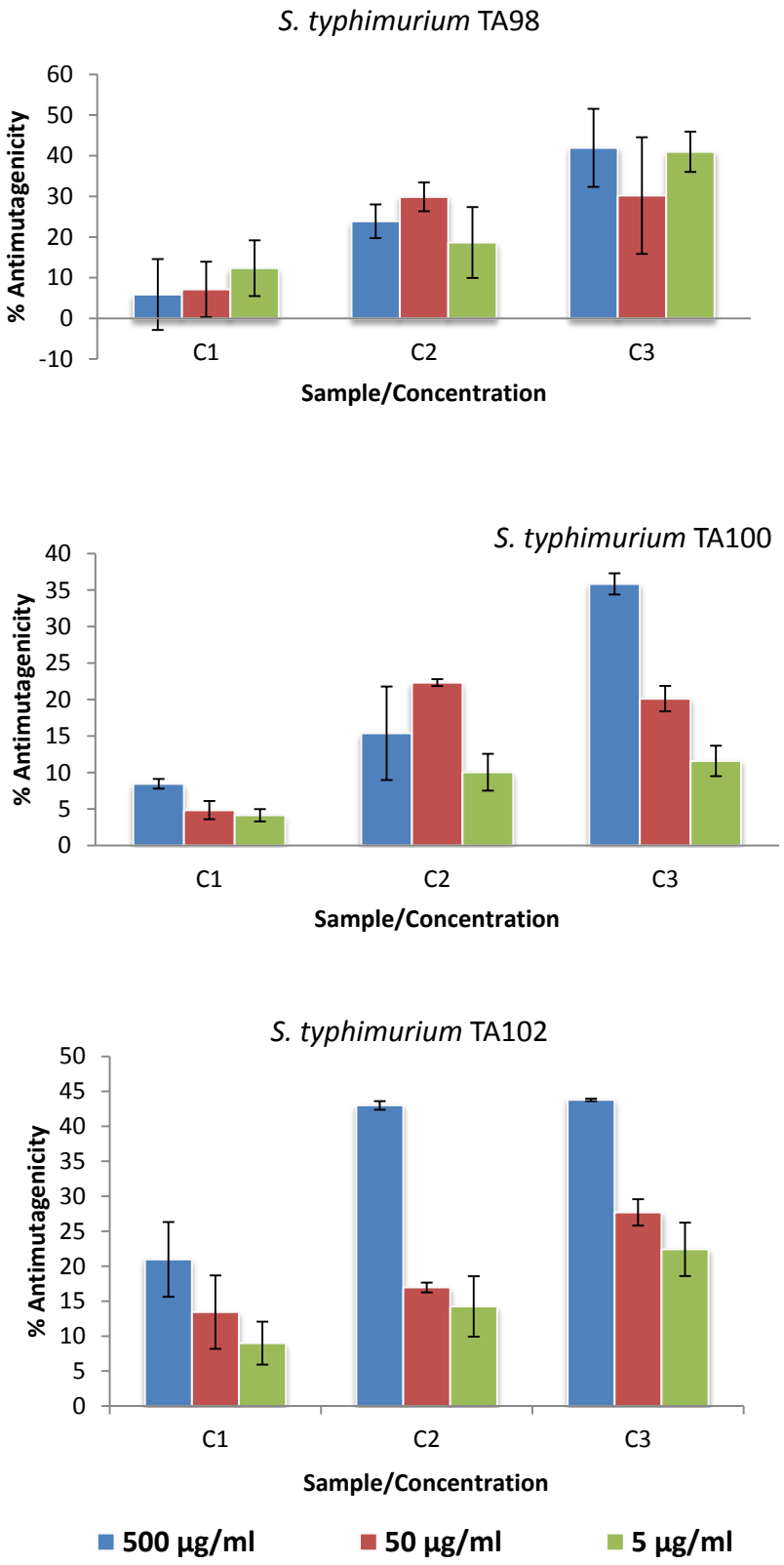


Figure 6.1. Antimutagenic activity of compounds isolated from *C. microphyllum* in the Ames test using *S. typhimurium* TA98, TA100 and TA102 (percentage inhibition and enhancement of the mutagenic effects of 4-NQO and MMC (C1 = n-Tetracosanol, C2= Eicosanoic acid C3= Arjunolic acid.

Because it is so labour intensive to determine the antimutagenicity of all the fractions in the final separation, it is pleasing that the three compounds we isolated all had some antimutagenosity activity. Arjunolic acid is a triterpenoid saponin and a major constituent present in *Terminalia arjuna* (King *et al.,* 1954, Ghosh *et al.,* 2008). Arjunolic acid was isolated from the ethyl acetate fraction and methanol extracts of *T. arjuna* core wood (Ramesh *et al.,* 2012). There are no reports on the antimutagenic activity of arjunolic acid. However, Hemalatha and colleagues (2010) reported that arjunolic acid has antimutagenic activity in a review article on the multifunctional therapeutic applications of arjunolic acid. There is, however, no data to support this report, making this study the first to investigate antimutagenicity of arjunolic acid in the Ames test.

Ever since the registration of a patent on hormonal, wound healing and bactericidal properties of arjunolic acid by Ratsimamanga and Boiteau (1963), various biological activities of this compound have been studied (Ghosh and Sil, 2013). Arjunolic acid possesses multi-functional medicinal applications including antioxidant, antiplatelet, anticoagulant, antinecrotic, anti-tumour, antinephrotoxic, antihepatotoxic, anti-inflammatory, anti-nociceptive, anticholinesterase, antidiabetic, anti-asthmatic, antimicrobial and anti-insecticidal activity (Hemalatha *et al.,* 2010, Ghosh and Sil, 2013).

n-Tetracosanol, an aliphatic alcohol with 24 carbons, was the least active compound in all the tester strains. Nonetheless, the antimutagenic activity of this compound to some extent may be correlated to the activity of other aliphatic alcohols reported in literature. C18 to C26 aliphatic alcohols have antiproliferative activity on hyper-proliferative skin lesions. These compounds had selective antiproliferative activity against hypertrophic fibroblasts (Katz *et al.,* 1991). This is a clear indication that these compounds can be used to treat cancer and prevent malignancies, an indication of anticancer and anticarcinogenic activity.

Compounds with 12 to 22 carbon atoms are used in the treatment of virus-induced inflammation. n-Docosanol (C22) has potential antiviral activity against a variety of lipid enveloped viruses in cell culture assays (Katz *et al.,* 1991). Tricontanol (C30) is used for the treatment of inflammatory disorders, herpes simplex, eczema, shingles, atopic dermatitis and psoriasis (Katz *et al.,* 1991). Aliphatic alcohols are known to have various biological activities and it is a central premise of medicinal chemistry that structurally similar molecules have similar biological activities (Martin *et al.,* 2002). There is a direct correlation between related chemical compounds and compositions and their therapeutic activities (Pope *et al.,* 1996).

There are no literature available on the medicinal or therapeutic uses of eicosanoic acid.

The antimutagenicity of the compounds could only be determined in the Ames test due to the small quantities of compounds isolated. We opted to use the Ames test because it is the most widely applied genotoxicity assay for the evaluation of potential antimutagenic activities of new compounds. Additionally, it is cheap and flexible compared to other genotoxicity assays.

The Ames test can easily provide preliminary information not only on antimutagenesis but also on the possible mode of action involved in antimutagenesis (De Flora *et al.,* 1992). It has generated interest in the development of novel therapeutics for many diseases including cancer (Ames, 1983, El-Sayed and Hussin, 2013).

The primary aim of toxicological assessment of medicinal plants and their products, either as crude extracts or pure compounds, is to identify the possibility of adverse effects and to determine the limits of exposure at which such effects occur. The isolated compounds had no pronounced cytotoxic effects, and thus are considered to be non-cytotoxic with LC_{50} values >200 µg/ml for n-tetracosanol and eicosanoic acid. Arjunolic acid had an LC_{50} value of 106.39 ± 5.11 µg/ml. The percentage cell viability for each compound at the highest concentration tested was: 59.74 ±7.23% and 50.09 ± 6.21 for n-tetracosanol and eicosanoic acid respectively.

Similar to our findings, Ramesh and colleagues (2012) found arjunolic acid to be cytotoxic to Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma (DLA) cell lines. In their investigations, arjunolic acid inhibited cell growth by up to 70% at 100 µg whilst in our present study; arjunolic acid inhibited 66% of hepatocellular carcinoma C3A cell growth at 200 µg/ml. Based on these findings, it appears that the cytotoxic effects of arjunolic acid may be cell line specific.

It is evident that aliphatic alcohols are not cytotoxic as it was the case for n-tetracosanol in this study. Most aliphatic alcohols are not cytotoxic at concentrations of up to 300 mM (Pope *et al.,* 1996). These compounds are expected to have no adverse hepatotoxic effects. Most reports of toxic effects due to the use of herbal medicines and dietary supplements are associated with hepatotoxicity, although reports of other toxic effects including kidney, nervous system, blood, cardiovascular and dermatologic effects, mutagenicity and carcinogenicity have also been published (Temple and Himmel,2002).

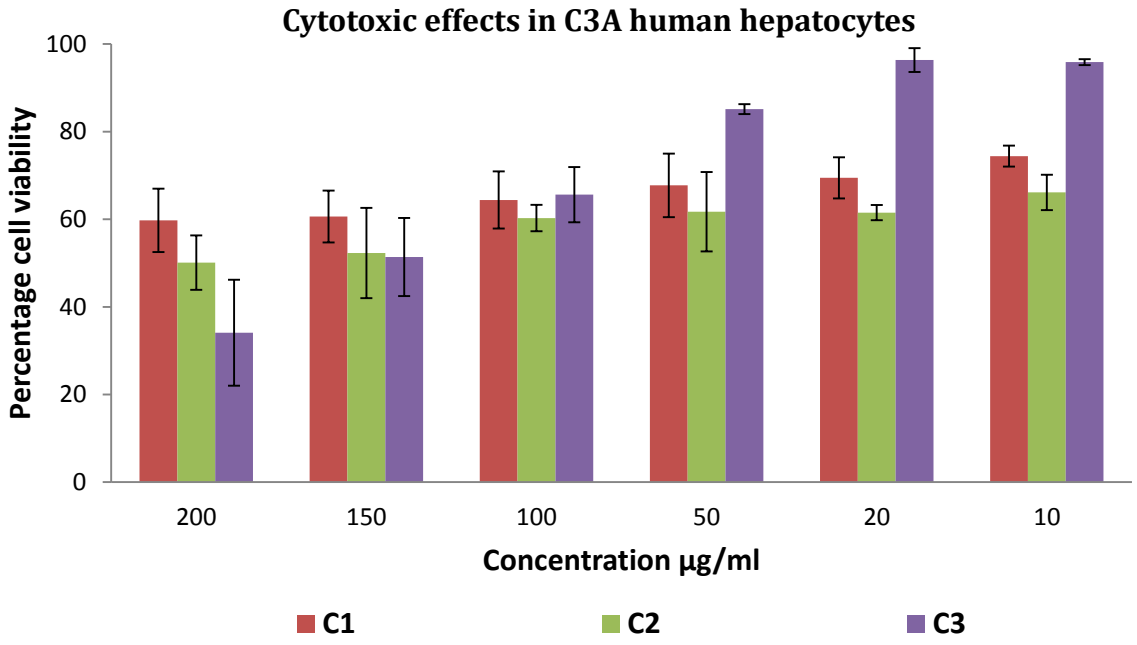


Figure 6.2. Percentage cell viability of C3A cells exposed to different concentrations of compounds isolated from *C. microphyllum.* (C1 = n-Tetracosanol, C2= Eicosanoic acid C3= Arjunolic acid)

Table 6.2. LC_{50} (µg/ml) (± standard deviation) of the three compounds isolated from *C. microphyllum*

Compound	n-Tetracosanol	Eicosanoic acid	Arjunolic acid	Doxorubicin
LC_{50}	>200	>200	106.39± 5.11	0.64 ± 0.032µM

Arjunolic acid was the only compound with DPPH scavenging activity, an indication of antioxidant activity. It effectively reduced the DPPH free radical with EC_{50} value of 6.25 ±0.29 µg/ml and vitamin C, a positive control, had an EC_{50} value of 0.514 ± 0.079 µg/ml. The antioxidant activity of arjunolic acid observed in this study is in agreement with observations reported by Manna and colleagues (2007) where high levels of antioxidant activity were recorded at concentrations ranging from 100 to 600 µg/ml in a cell-free system. The authors recorded up to 80% DPPH free radical scavenging activity of arjunolic acid at the lowest concentration of 100 µg/ml. The scavenging properties of this compound serve as a clear indication of its antioxidant potential. Based on this observation, the antimutagenic activity of arjunolic acid may, at least in part, be attributed to its antioxidant activity resulting in the detoxification of reactive oxygen species produced during mutagenesis.

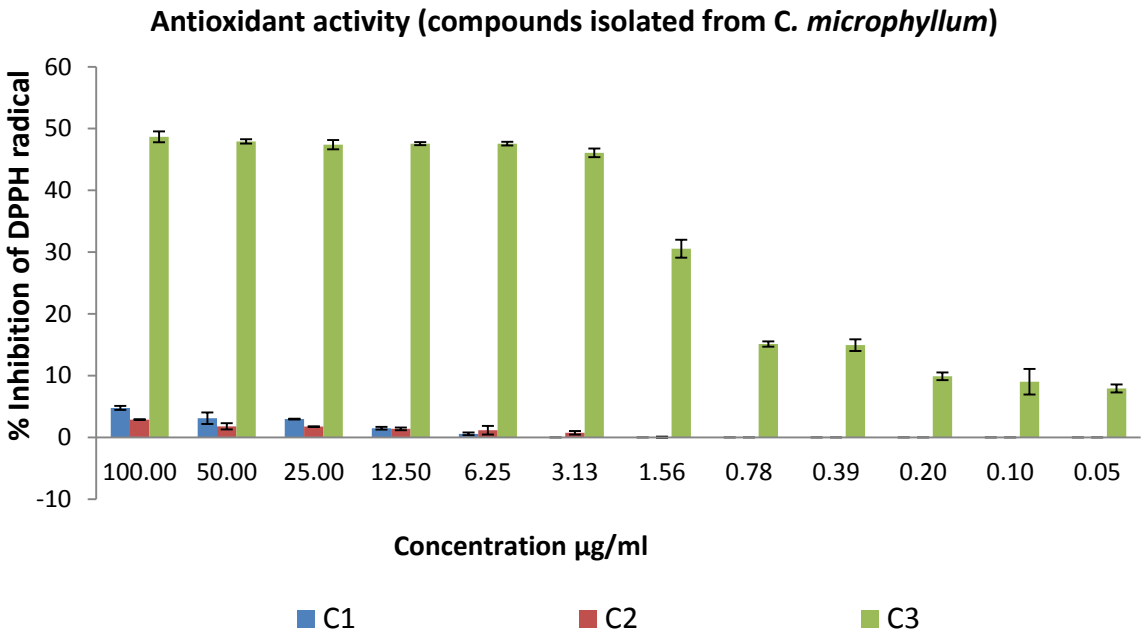
Arjunolic acid contains poly hydroxyl groups and thus can easily be oxidised during its interaction with ROS. The DPPH radical scavenging activity of arjunolic acid can further be explained by the presence of its carboxylic hydrogen atom that can easily be abstracted by any free radical like DPPH (Manna *et al.,* 2007). In comparison to vitamin C, the compound was active at concentrations 10 times higher whereas the crude extract had better activity than vitamin C (Chapter 2). The higher antioxidant activities demonstrated by the plant extracts in Chapter 2 could be due to the concentration of numerous antioxidant compounds present in the crude extract acting in synergy.

The observation that compounds 1 and 2 had reasonable antimutagentic activity without having substantial antioxidant activity is an indication that not only antioxidant activity is associated with antimutagenic activity. Although the assay for isolating antioxidant compounds by bioassay guided fractionation is much easier than by using antimutagenic activity the approach of isolating only antioxidant compounds and then determining the antimutagenic activity would have missed these compounds. It is also clear from the activities of the different solvent-solvent fractions that there could be more compounds with antimutagenic activity in *C. microphyllum* leaf extracts. The antimutagenic activity of these compounds from *Combretum microphyllum* is interesting because bibenzyls from root bark of the closely related *Combretum caffrum* contains combrestatins that have outstanding potential on treating cancer by a completely different mechanism.

Table 6.3. DPPH free radical scavenging activity (EC_{50} (µg/ml) (± standard deviation) of three compounds isolated from *C. microphyllum*

Compound	n-Tetracosanol	Eicosanoic acid	Arjunolic acid	Vitamin C
EC_{50}	>100	>100	6.25 ±0.29	0.514 ± 0.079

A



B

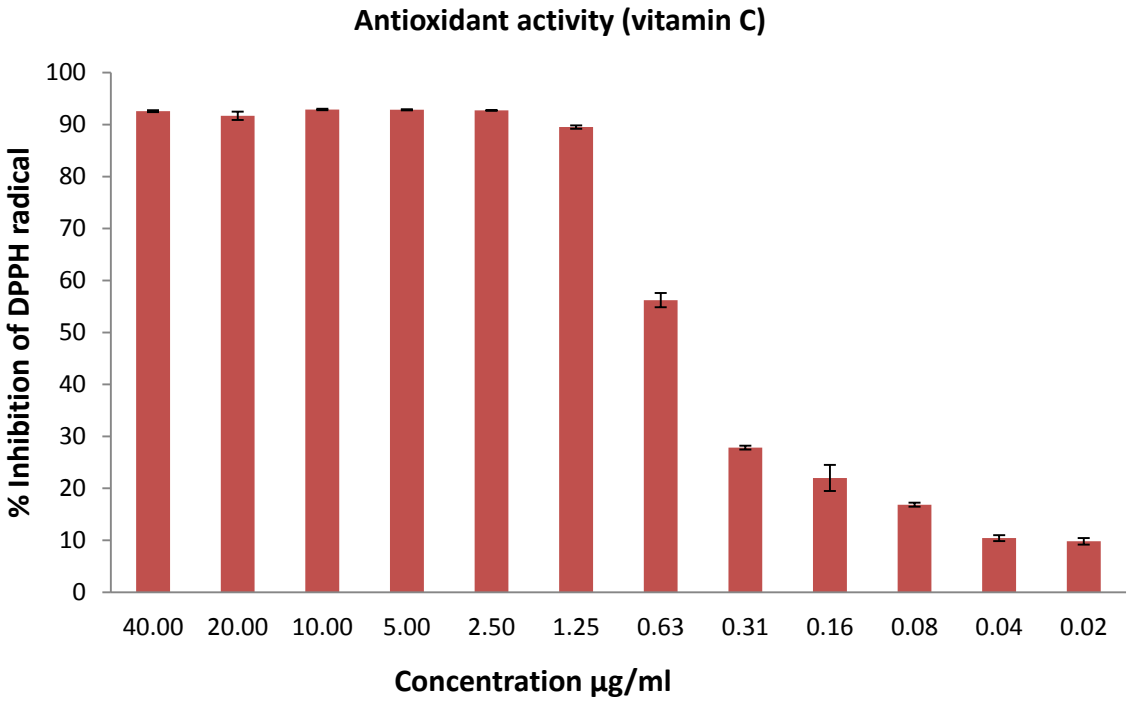


Figure 6.3. Percentage DPPH free radical scavenging activity of compounds isolated from *C. microphyllum* (A) and vitamin C (B). C1 = n-Tetracosanol, C2= Eicosanoic acid C3= Arjunolic acid

6.4. Conclusions

The threecompounds n-tetracosanol, eicosanoic acid and arjunolic acid isolated from *C. microphyllum* protect against 4-NQO and MMC induced mutations as determined by the Ames test. Only arjunolic acid was slightly cytotoxic against C3A hepatocarcinoma at the highest concentration (200 µg/ml) tested. Not any of the compounds were mutagenic. To some extent, compounds from *C. microphyllum* are safe and could be useful to humans for the purpose of chemoprevention although these assumptions would have to be confirmed using *in vivo* studies.

Since most human mutation related diseases would be delayed by a period extending beyond the average lifespan if the mutation rate could be reduced by half (Sugimura, 2000), results obtained in this study are a clear indication that compounds isolated from *C. microphyllum* have potential antimutagenic effects and may be useful in the prevention of cancer and other mutation related diseases if they are bioavailable. All stages of tumourigenesis i.e. initiation, promotion, conversion, progression and metastasis are associated with mutations and increased genetic instability (Sugimura, 2000).

Arjunolic acid was the only compound with antioxidant activity and had better antimutagenic activity compared to eicosanoic acid and n-tetracosanol. The antimutagenic activity of arjunolic acid, at least in part, may be attributed to its antioxidant activity resulting in the detoxification of reactive oxygen species produced during mutagenesis.

CHAPTER 7

General conclusions

The aim of this study was to investigate the antimutagenic potential of several plant extracts selected based on antioxidant activity and to isolate and characterize pure compound(s) with antimutagenic/antigenotoxic activity from the most active plant species. Several objectives to attain this aim were identified. The results obtained in addressing the different objectives are discussed below.

1. **Screening of 120 plant leaf extracts for qualitative antioxidant; determination of the quantitative antioxidant activity and total phenolic content of 31 selected plant species as a preliminary step to identify antimutagenic plant species**

The experiments in this section were conducted as a preliminary step in identifying antimutagenic plant species based on the assumption that antioxidant compounds may play a preventive role against mutations caused by reactive oxidant species and thus may have potential antimutagenic activity (Valko et al., 2006). Thin layer chromatography of extracts with DPPH as spray reagent was used to determine the number of antioxidant compounds in methanol leaf extracts of 120 plant species. Approximately 98% of the plant extracts had antioxidant compounds that separated well in the TLC. The 31 most active species containing well defined antioxidant compounds were selected for further assays. The quantitative antioxidant activity and total phenolic activity of these plant extracts were determined. All the extracts had a concentration dependent radical scavenging activity with 17 extracts having antioxidant activity better than that of L-ascorbic acid the positive control. All the extracts contained phenolic compoundsand there was a direct correlation between antioxidant activity and total phenolic content ($R^2 > 0.9447$). The next step was to determine the mutagenic, antimutagenic activities and cytotoxicity of the 31 selected plant species to aid in the selection of the mostpromising antimutagenic plant species for further studies.

2. **Investigation of the mutagenic and antimutagenic activity and cytotoxicity of plant extracts with good antioxidant activity in the Ames test to Select plant species for further studies**

In this section, the bacterial Ames test and cytokinesis block micronucleus/cytome assays were used to determine the genotoxic and antigenotoxic activity of 31 plant extracts. Additionally, the neutral red uptake assay was used to determine the cytotoxicity of the 31 plant species. I started by investigating the potential genotoxic activity of the extracts in both test systems to identify and limit and eliminate mutagenic/genotoxic

plant species from the selection. Of the selected plant species, only one plant extract; *Halleria lucida* (#3) was mutagenic in the Ames test. The absence of mutagenic response by plant extracts against *Salmonella typhimurium* bacterial strains in the Ames test is a positive step forward in determining the safe use of plants in traditional medicine (Reid *et al.,* 2006). It should however be kept in mind that not all classes of mutagens can be detected by the Ames test. I therefoe also tested the genotoxic effects of the plant species in mammalian cells (human hepatocytes C3A cells) using the cytokinesis block micronucleus/cytome assay. Extracts of only 7 plant species were genotoxic in the cytokinesis block micronucleus/cytome assay. This may be attributed to the metabolic activation of some plant compounds by the cells, which is otherwise absent in the Ames bacterial test system I used. The C3A hepatocytes have the essential structural, biochemical and growth features of normal human liver cells and have conserved both phase I and phase II metabolic activities (Kelly, 1994).

The absence of mutagenic response by plant extracts in *Salmonella typhimurium* bacterial strains and low genotoxic response in the cytokinesis block micronucleus/cytome assay was a positive step forward to study the antimutagenic/antigenotoxic activities of the selected of plant species. A total of 15 plant extracts (50%) were antimutagenic in the Ames test using *S. typhimurium* TA98 and 21 plant extracts (68%) were antimutagenic using *S. typhimurium* TA100. Plant extracts that had antimutagenic effects in TA100 had a clear dose response effect which was not observed against TA98.This may mean that the optimal concentration was lower than the highest concentration tested.

One problem with genotoxicity and antigenotoxicity determination is that many higher plants produce toxic agents which may affect the proliferation of bacterial and mammalian cells used in the assay**.** At the highest concentration tested, all plant extracts were toxic to the cells in the micronucleus/cytome assay. Nonetheless, out of the 31 plant extracts, *Harpephyllum caffrum*, *Androstachys johnsonii, Faurea saligna, Puttelikra restripinosa*, *Cassinopsis illicifolia*, *Combretum microphyllum*, *Leucospernum erubescens* and *Protea cyanroides* had antigenotoxic activity by reducing occurrence of all the genotoxic endpoints measured in the cytokinesis block micronucleus/cytome assay.

(NRU) To eliminate possible interference of cytotoxicity of the plant extracts with their genotoxic and antigenotoxic effects the cytotoxicity of all the extracts was determined using the neutral red uptake assay. This study was conducted to find the most suitable dose to use in further experiments. By determining the percentage cell viability and LC_{50} values. The LC_{50} values ranged from 0.19 to >2.5 mg/ml. Most plant extracts had LC_{50} values less than 0.5 mg/ml. Because some genotoxic carcinogens are not detectable in *in vitro* genotoxicity assays unless the concentrations tested induce some degree of cytotoxicity (OECD, 1997), it was evident that concentrations above 0.2 mg/ml should be included in the assay allowing coverage of both cytotoxic and non-cytotoxic concentration ranges. It was for this reason that 0.5 mg/ml was selected as the

highest concentration for all other subsequent experiments requiring mammalian cells. From these results, *Combretum microphyllum* and *Leucospermum erubescens* were selected as the most promising antimutagenic plant species and *Thespesia acutiloba* and *Kirkia wilmsii* were selected because they increased the mutagenic effects of the mutagen 4-NQO. The next step was then to conduct in-depth antigenotoxicity studies of the 4 selected species.

3. Genotoxic and antigenotoxic activity of *C. microphyllum*, *L. erubescens*, *T. acutiloba* and *K. wilmsii* against 4-NQO, MMC and EMS

Both mutagenicity and antimutagenicity of the four species were determined at a wide range of concentrations in the Ames test using *S. typhimurium* TA98, TA100 and TA102. To further confirm the results obtained in the initial screening study, the genotoxicity and antigenotoxicity of the extracts were determined in the cytokinesis block micronucleus assay and the single cell gel electrophoresis/comet assay. Extracts of *C. microphyllum* and *L. erubescens* inhibited the mutagenic effects of 4-NQO (TA98 and TA100) and MMC (TA102) in all the tester strains at all concentrations tested whereas *K. wilmsii* and *T. acutiloba* enhanced the mutagenic effects of the tested mutagens in all three tester strains.

Combretum microphyllum and *L. erubescens* greatly reduced the percentage of micronuclei by up to 66%, nucleoplasmic bridges by 76% and nuclear buds by 88%. *C. microphyllum* and *T. acutiloba* significantly reduced the percentage of micronuclei, nucleoplasmic bridges and nuclear buds respectively. Extracts of *K. wilmsii* and *T. acutiloba* were not comutagenic in the micronucleus/cytome assay as was observed in the Ames test. Instead, they had a low level of antimutagenicity. When testing plant extracts and phytochemicals in biological systems, such results are sometimes expected, especially in cases where both bacterial-based and mammalian cell line based assays are used. In our case, there was no metabolic activation in the Ames test whilst a higher level of metabolic activity is maintained in C3A hepatocytes.

The potential DNA protective effects of the extracts were also evaluated in the comet assay. There was a significant decrease in the DNA damaging effects of EMS by extracts of *C. microphyllum* and *L. erubescens.* These extracts significantly decreased tail length, percentage DNA in tail and tail moment at all tested concentrations. Additionally, extracts of *K. wilmsii* and *T. acutiloba* decreased tail length, percentage DNA in tail and tail moment at some concentrations. For all measured parameters, the values in the antimutagenicity assay were much higher than in the mutagenicity assay. This may be a result of combinational DNA damaging effects of the extracts (as established in the mutagenicity assay) and ethyl methane sulphonate EMS.

The results obtained in this study are a clear indication that extracts of *C. microphyllum* and *L. erubescens* have antigenotoxic effects in the test systems used. Extracts of these two species may therefore have a preventive effect against mutation related diseases if pharmacokinetic parameters are positive.

Based on the findings from this chapter, ease of access and availability of plant material, *C. microphyllum* was chosen for isolation and chemical characterization of antimutagenic compounds

4. Isolation of antimutagenic compounds from *Combretum microphyllum*

Bioassay-guided fractionation using a combination of solvent-solvent fractionation and chromatographic techniques was used to isolate compounds of interest from the leaves of *C. microphyllum*. ^{13}C and ^{1}H NMR spectrophotometric data led to identification of the isolated compounds as n-tetracosanol, eicosanoic acid and arjunolic acid. n-Tetracosanol is an aliphatic alcohol with 24 carbons (Murray and Schoenfield, 1995), eicosanoic acid is a saturated fatty acid with 20 carbons (Vieux *et al.,* 1970) and arjunolic acid is a triterpenoid saponin and a major constituent present in *Terminalia arjuna* (King *et al.,* 1954). These compounds have been isolated from other plant species before, but this is the first report on these compounds occurring in *C. microphyllum*. The next step was to confirm the antimutagenicity of the isolated compounds, to determine their cytotoxicity and to evaluate their antioxidant activity.

5. Antimutagenic activity, cytotoxicity and antioxidant activity of isolated compounds

The Ames test was used as an indicator for antimutagenicity in the bioassay-guided fractionation. All the compounds were antimutagenic in the Ames test. The compounds clearly have multiple mechanisms of mutation inhibition as they inhibit mutagenicity of 4-NQO in *S. typhimurium* TA98, TA100 and mutagenicity of MMC in *S. typhimurium* TA102. Moreover, these compounds may have varying mechanisms of antimutagenesis since they prevent frame-shift mutations detectable in TA98, base-pair substitutions detectable in TA100 and small in-frame deletions detectable in TA102.

Arjunolic acid was the most active in all three tested strains with percentage antimutagenicity of up to 41.92 ± 9.59%, 35.84 ± 1.45% and 43.78 ± 0.18% in *S. typhimurium* TA98, TA100 and TA102 respectively, followed by eicosanoic acid and lastly n-tetracosanol. The three compounds did not have a pronounced cytotoxic effects with LC_{50} values >200 µg/ml for n-tetracosanol and eicosanoic acid. Arjunolic acid had an LC_{50} value of 106.39 µg/ml. Arjunolic acid was the only compound with DPPH scavenging activity, an indication of antioxidant activity. It effectively reduced the DPPH free radical with EC_{50} value of 6.25 µg/ml.

Because it is so labour intensive to determine the antimutagenicity of all the fractions in the final separation, it is pleasing that the three compounds we isolated all had some antimutagenic activity in the Ames test. One of the

many advantages of using the Ames test in antimutagenesis studies as it provides information not only of antimutagenesis but also on possible mode of action (De Flora *et al.,* 1992).

The hypothesis at the beginning of the study was that plants with high antioxidant activity may be good candidates for isolating antimutagenic compounds. Based on this hypothesis it may have been feasible to isolate the antioxidant compounds, a much easier process and then determine their antimutagenic activities. The results show that this approach would have missed the isolation of two of the three antimutagenic compounds from *C. microphyllum*. It may nevertheless be an interesting approach to determine the antimutagenic activity of compounds with high antioxidant activity.

The results obtained from this study indicate that isolated compounds from plants have potential as leads for the discovery of new chemopreventive and chemotherapeutic agents. Since all stages of tumourigenesis are associated with mutations and increased genetic instability, these types of studies may yield agents that can be of therapeutic use in cancer prevention.

Possible future studies:

- To investigate and explore possible mechanisms involved in antimutagenesis and anticarcinogenesis of the most active plant extracts against 4-NQO and MMC induced mutagenesis and carcinogenesis.
- To determine whether the active plant extracts and/or compounds have desmutagenic or bioantimutagenic effects.
- To investigate the potential *in vivo* chemoprevention of carcinogenesis induced by 4-NQO and MMC.
- To investigate the potential use of comutagenic plant extracts or compounds in enhancing clinical effectiveness of cancer chemotherapeutic drugs. This is possible because most cancer drugs are mutagens and chemotherapy resistant cancers are a major problem.

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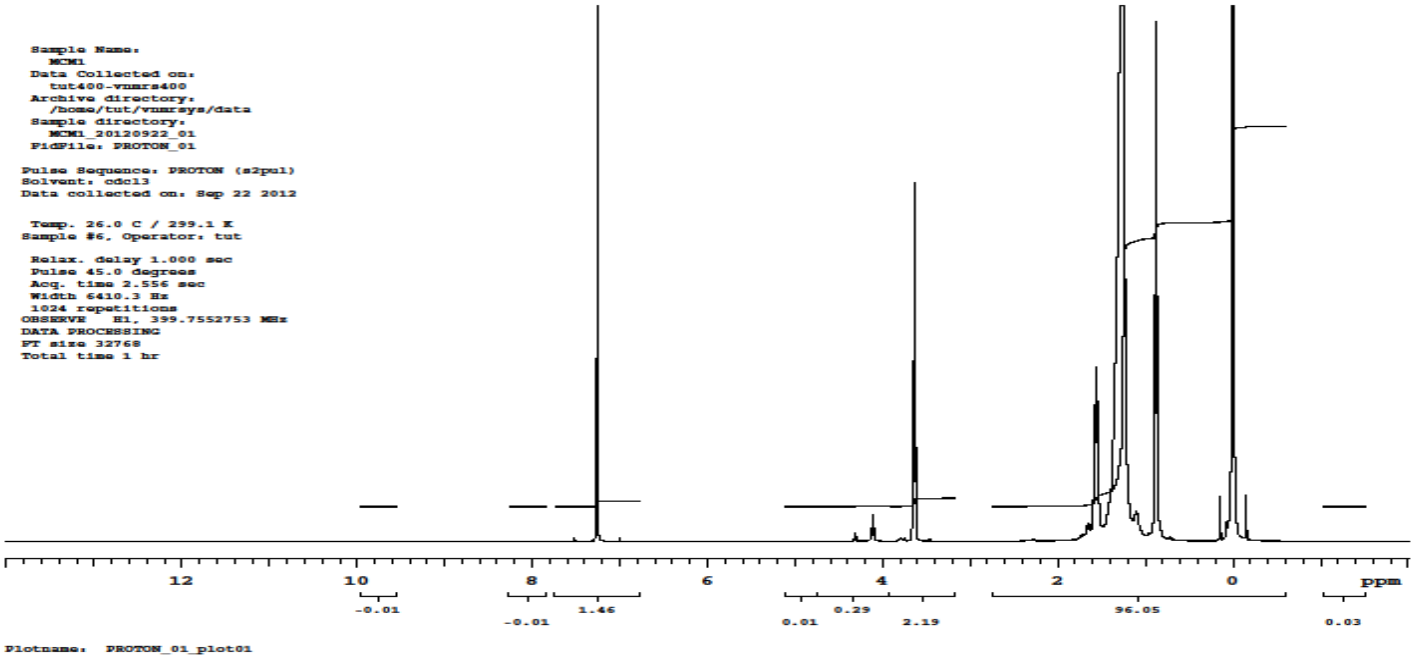
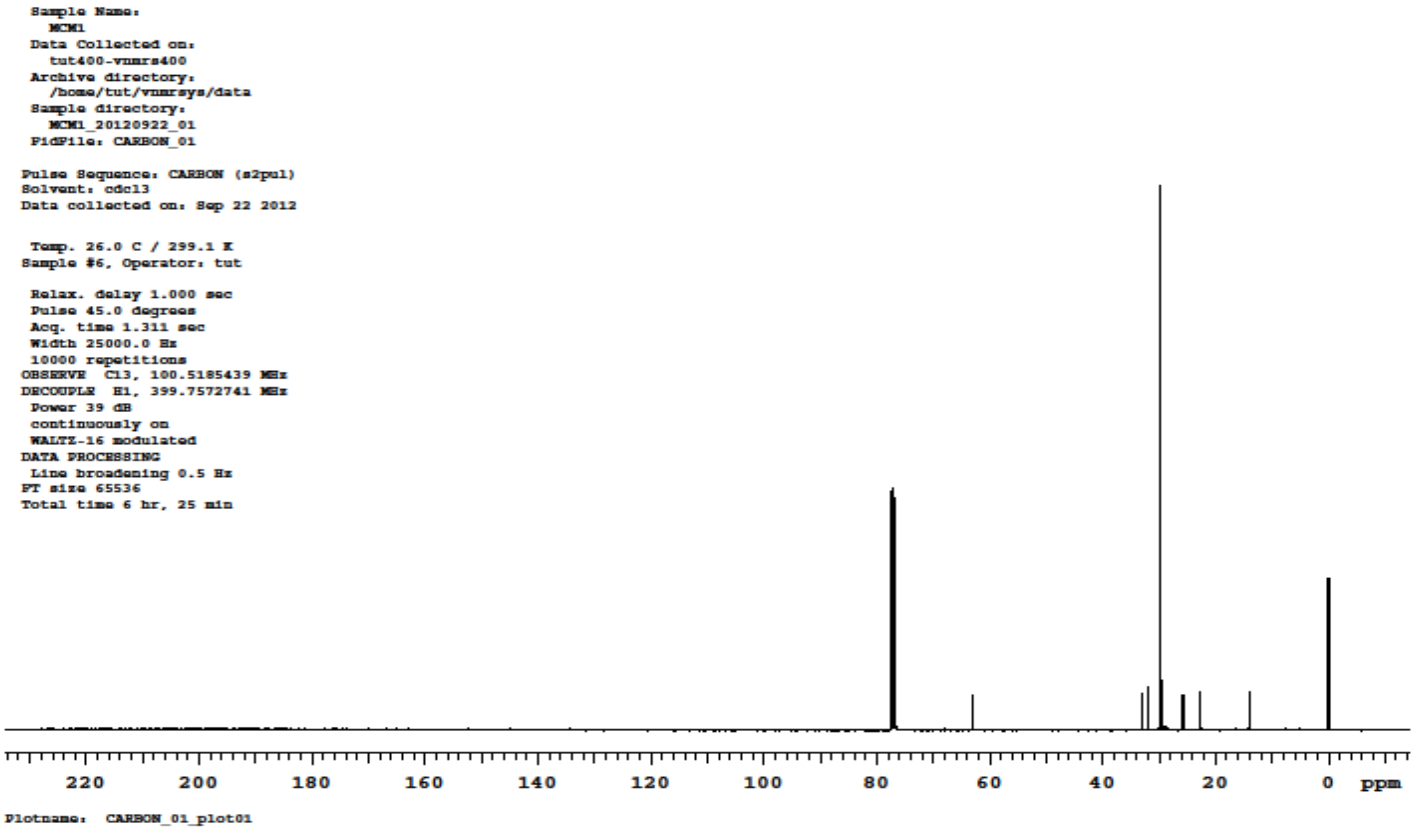
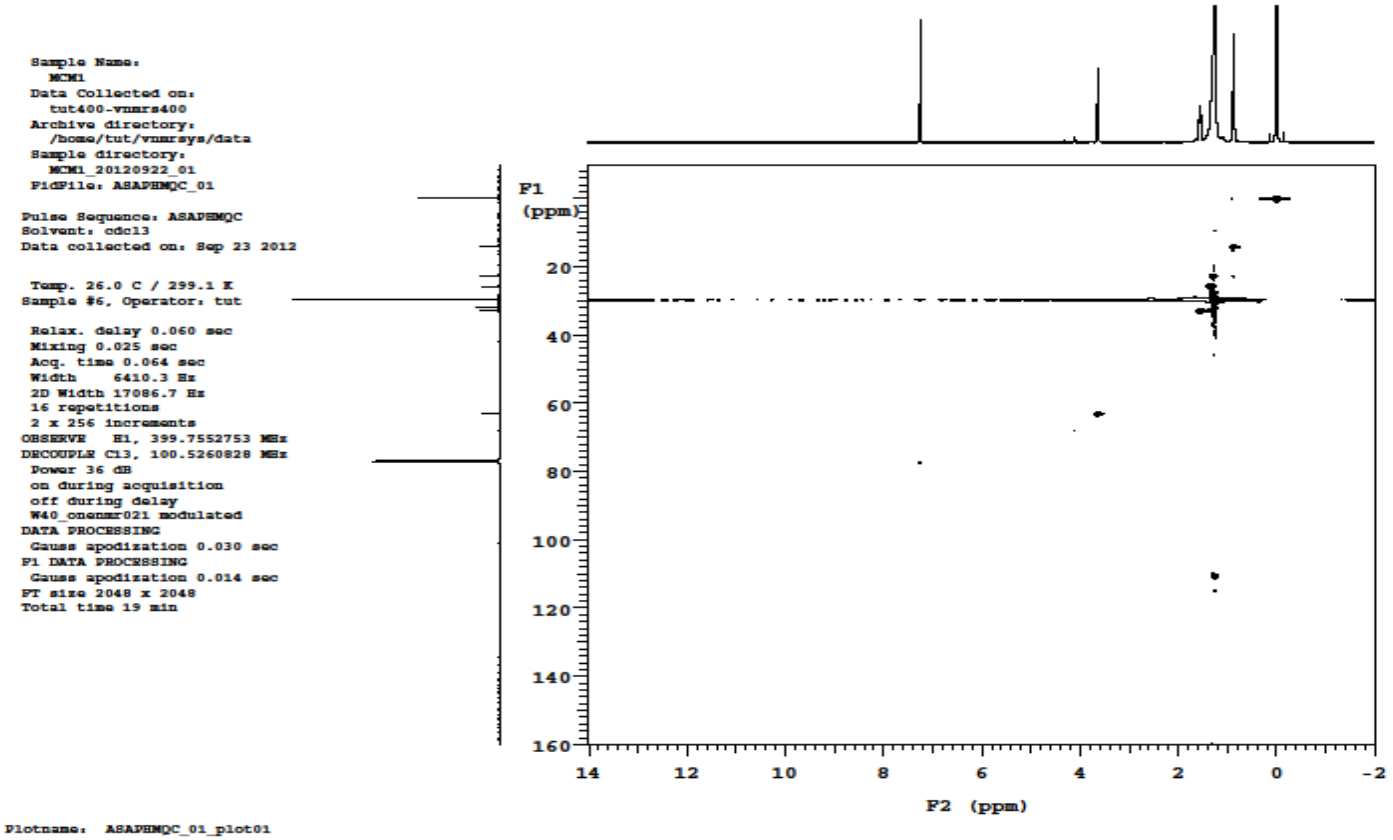
Appendix



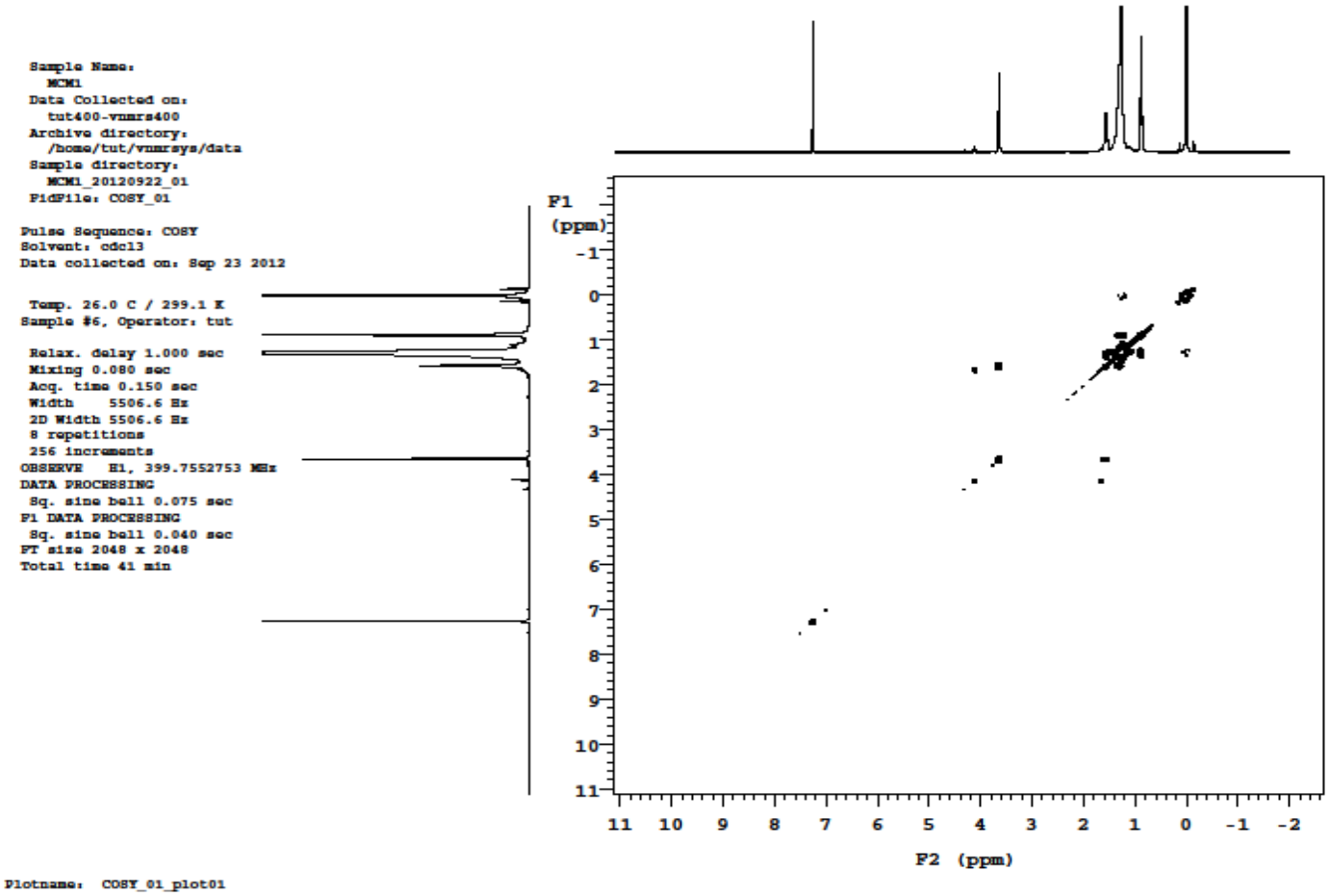
1. ^{1}H-NMR spectrum of C1



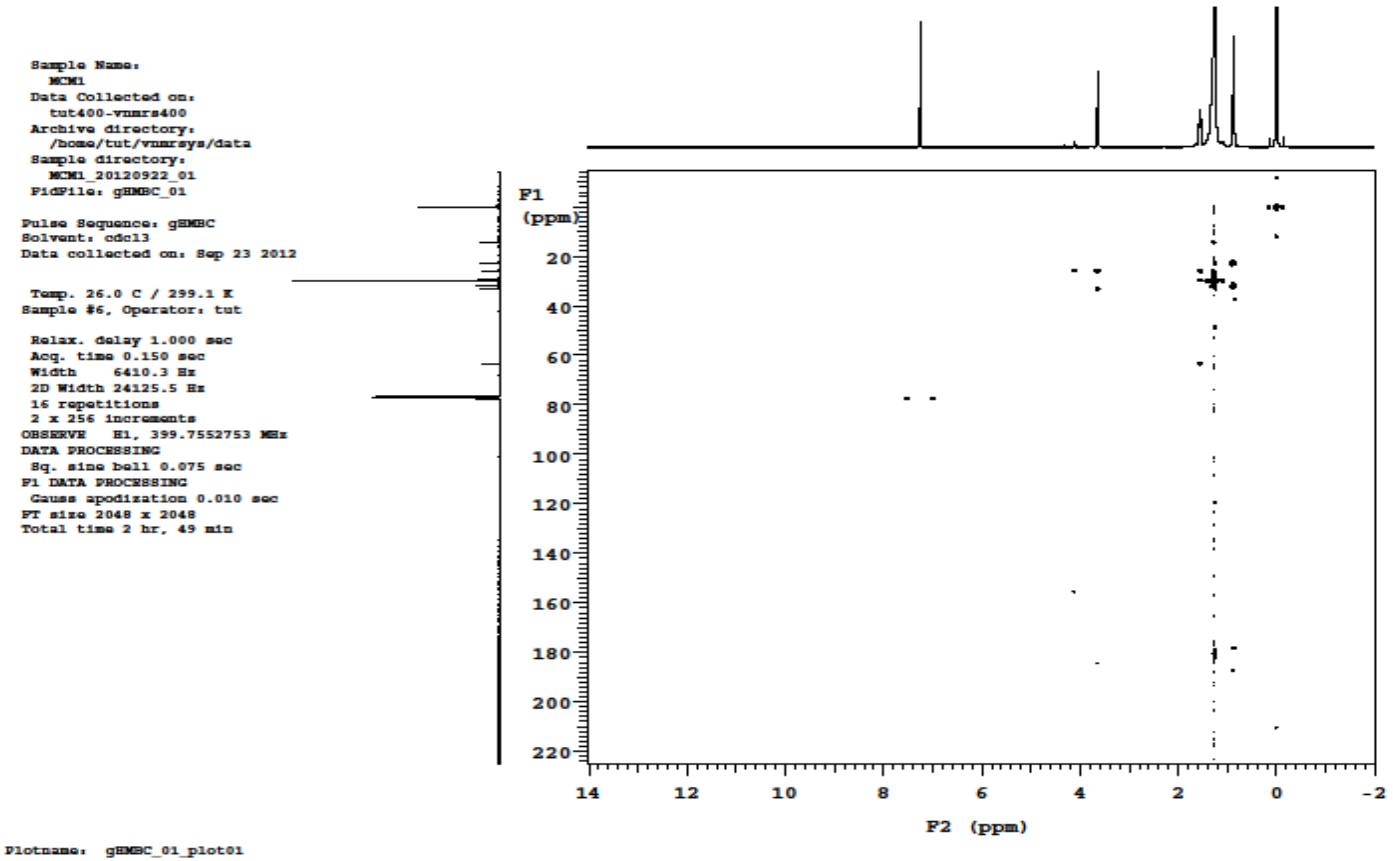
2. ^{13}C-NMR spectrum of C1



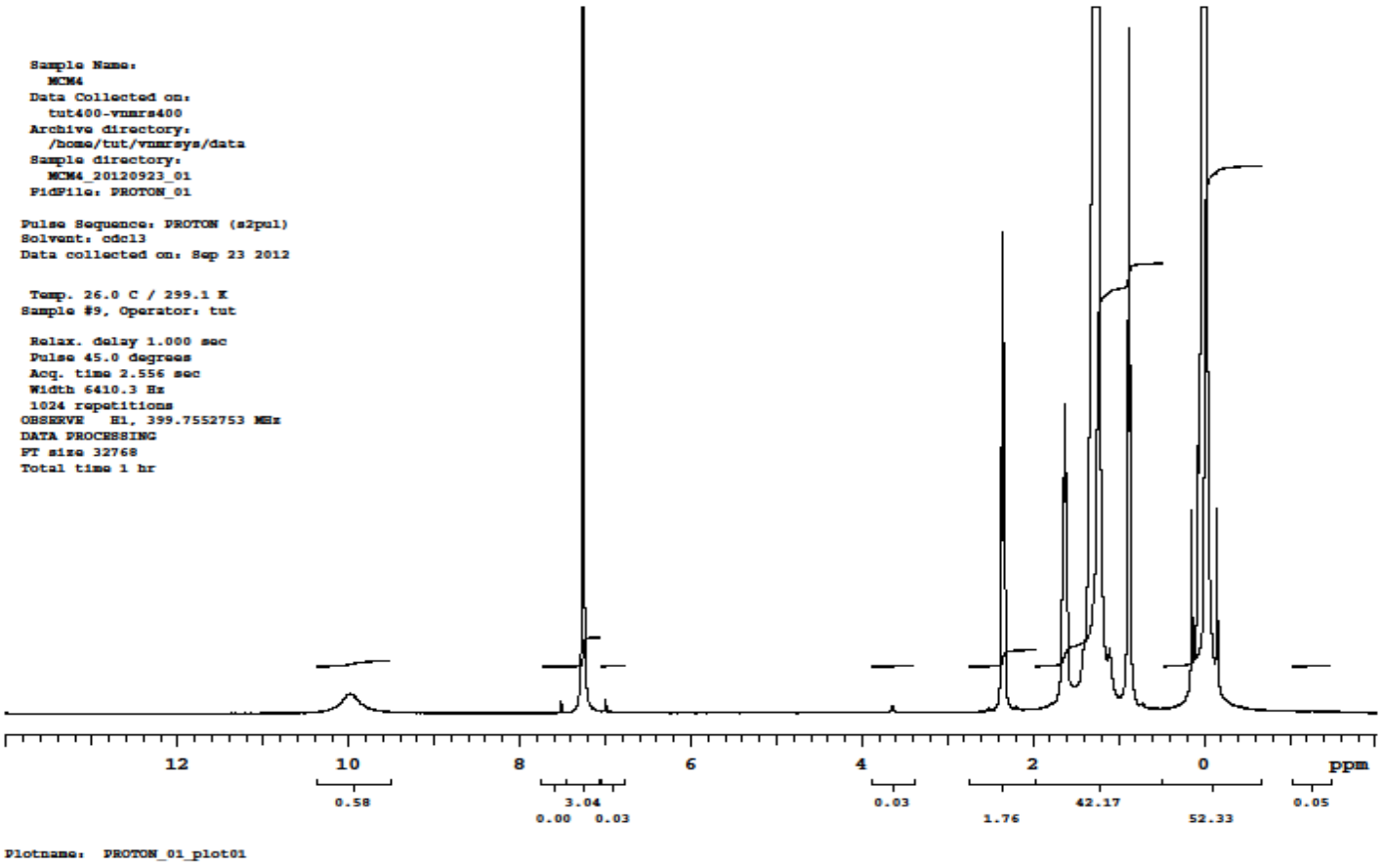
3. ASAP-HMQC-NMR spectrum of C1



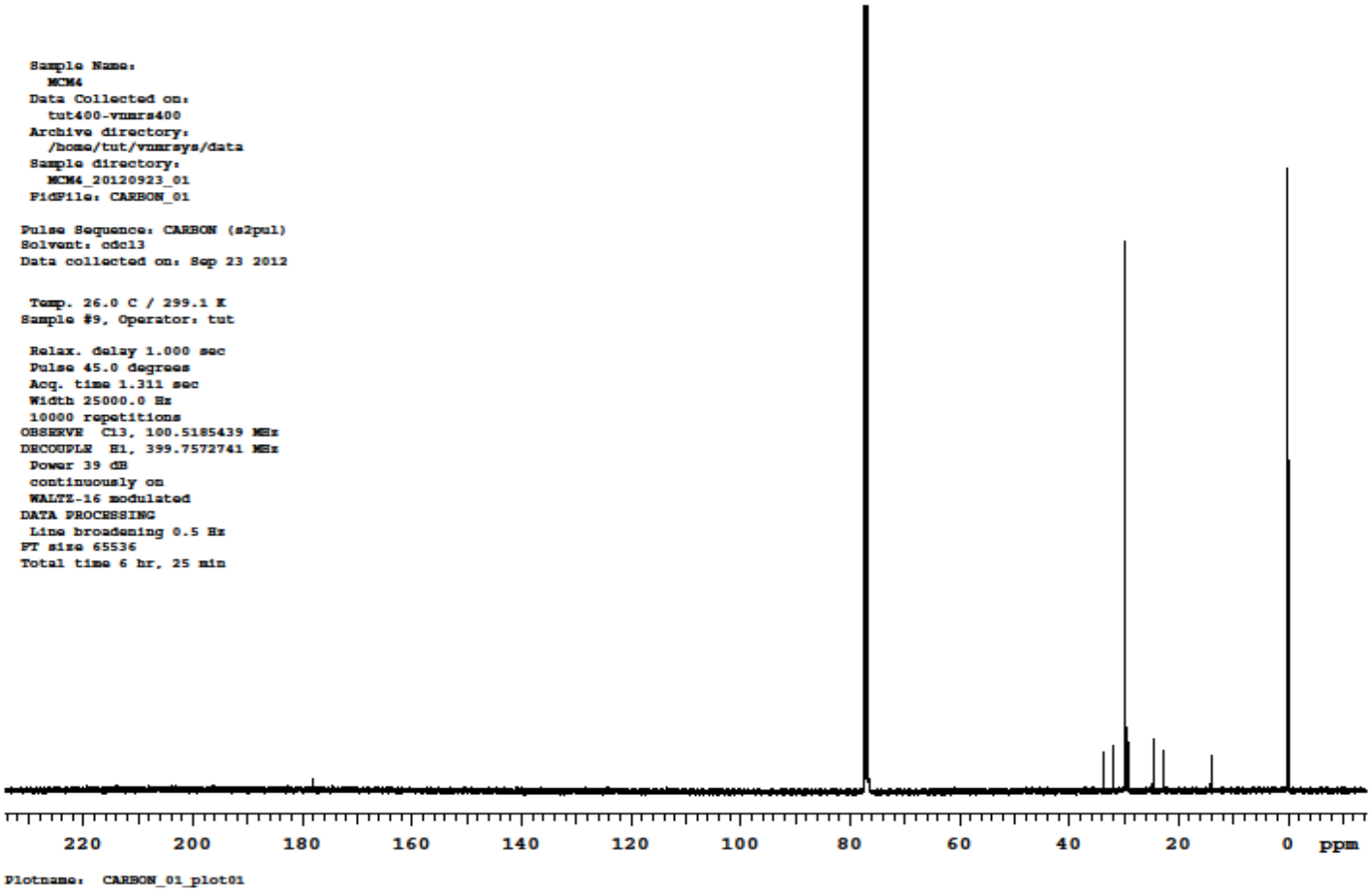
4. COSY-NMR spectrum of C1



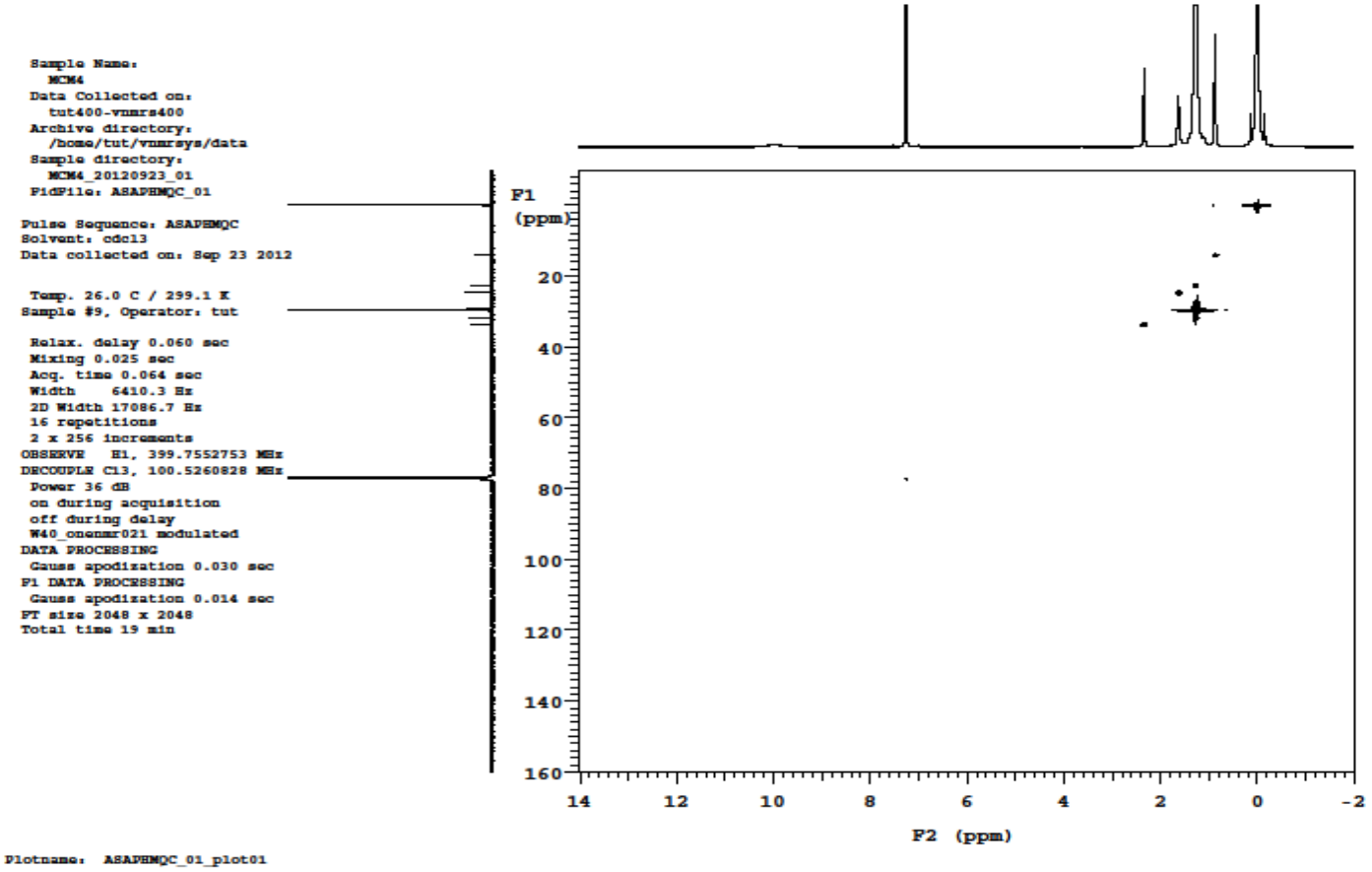
5. HMBC-NMR spectrum of C1



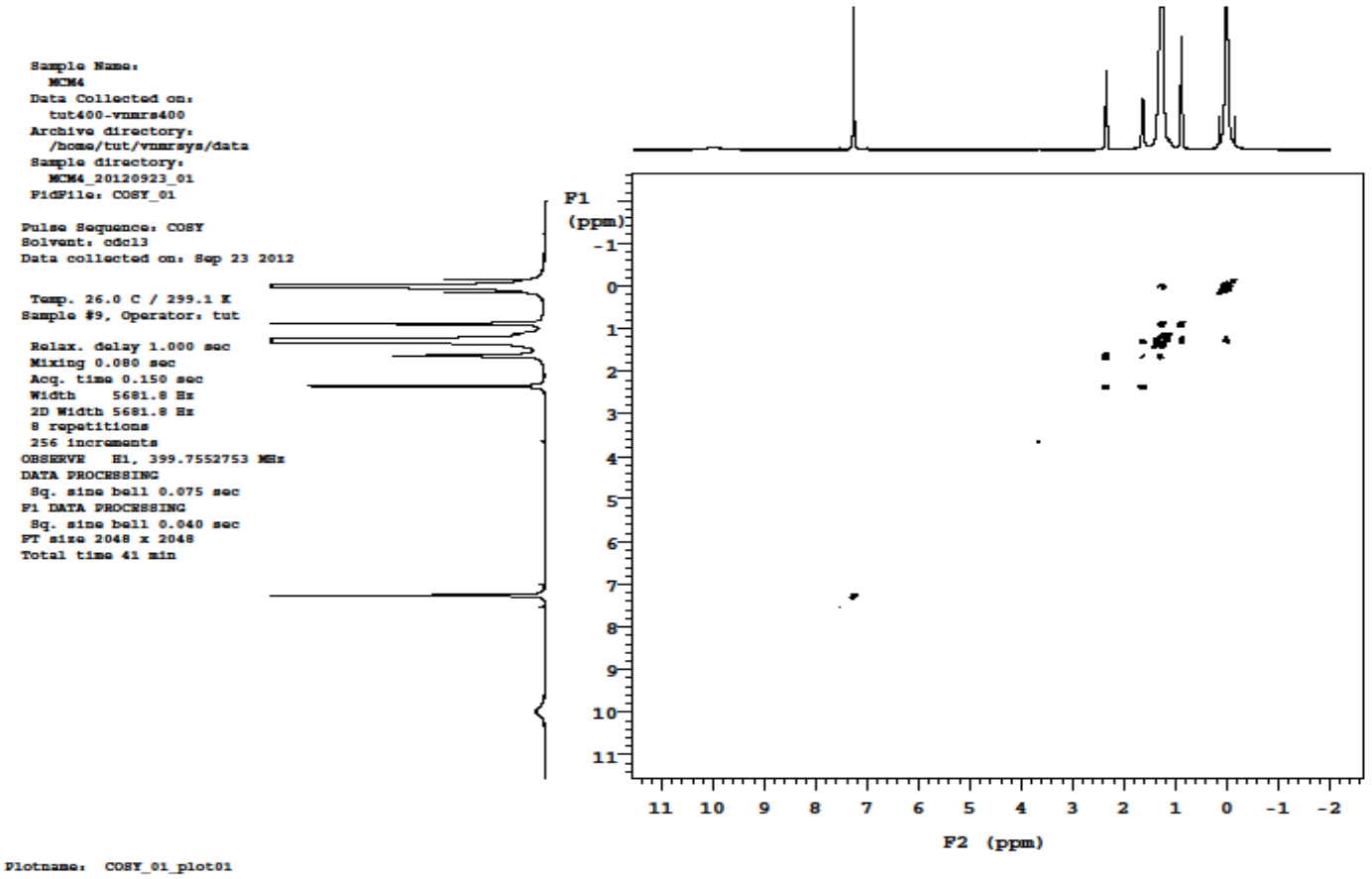
6. ^{1}H-NMR spectrum of C2



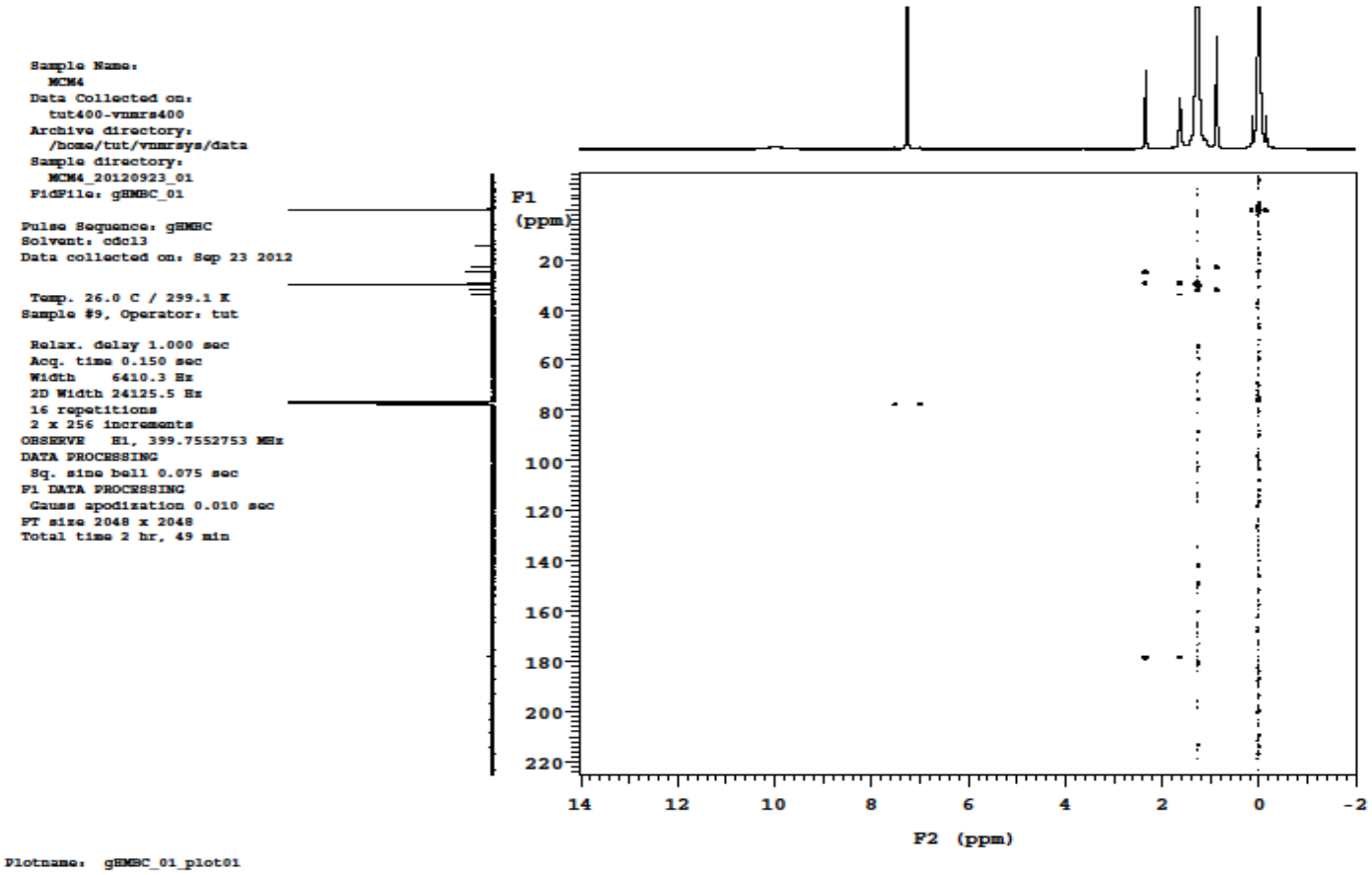
7. ^{13}C-NMR spectrum of C2



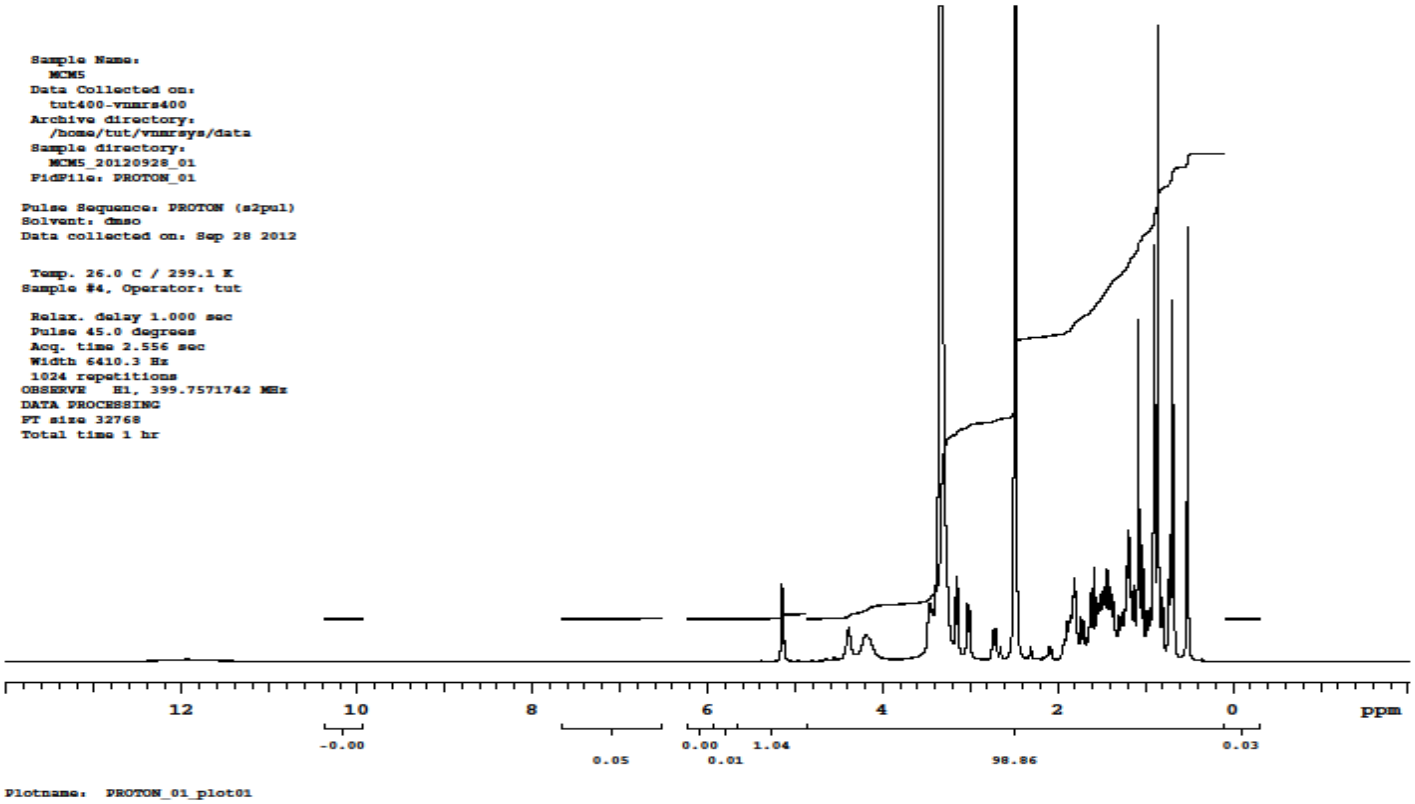
8. ASAP-HMQC-NMR spectrum of C2



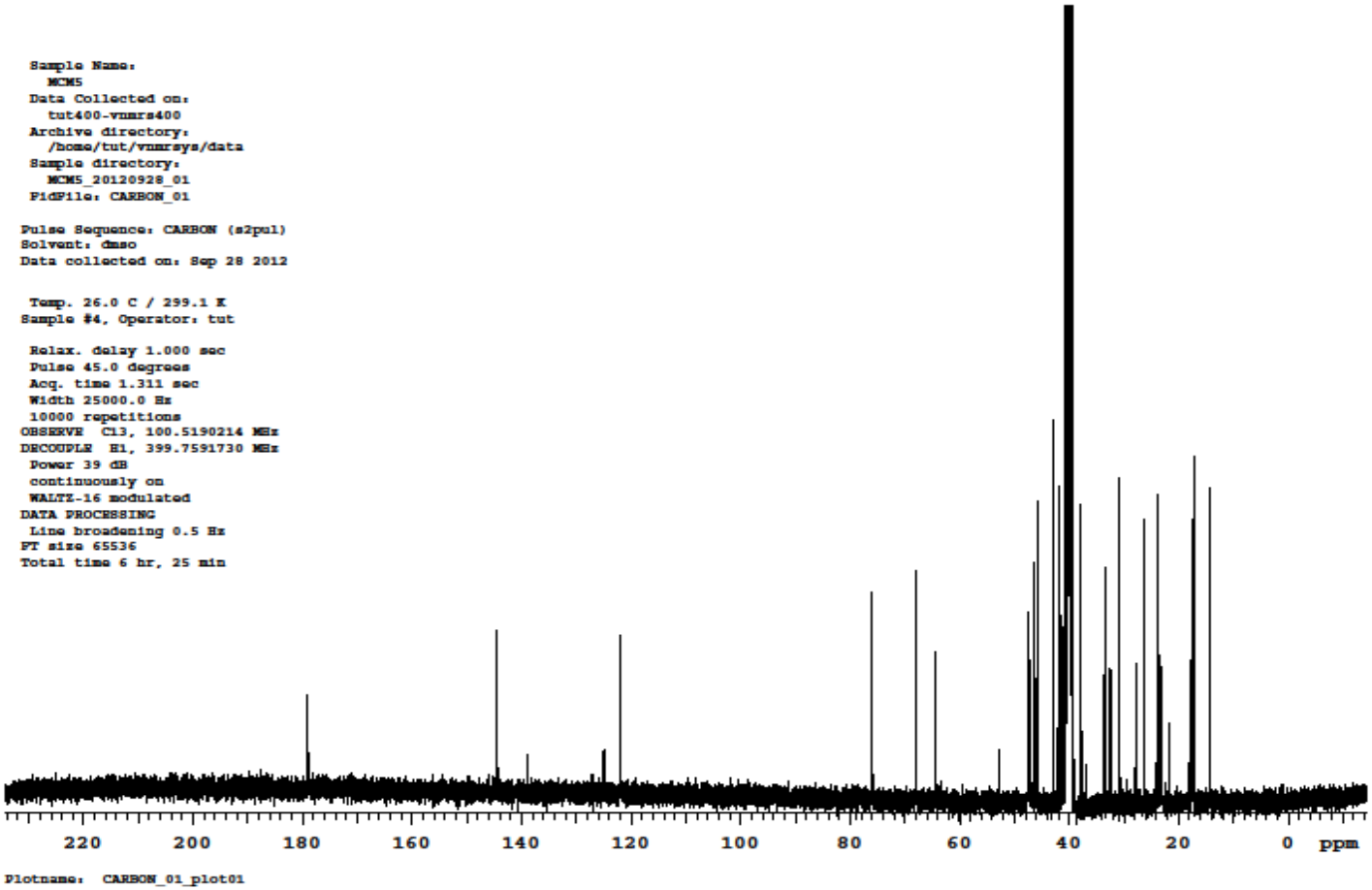
9. COSY-NMR spectrum of C2



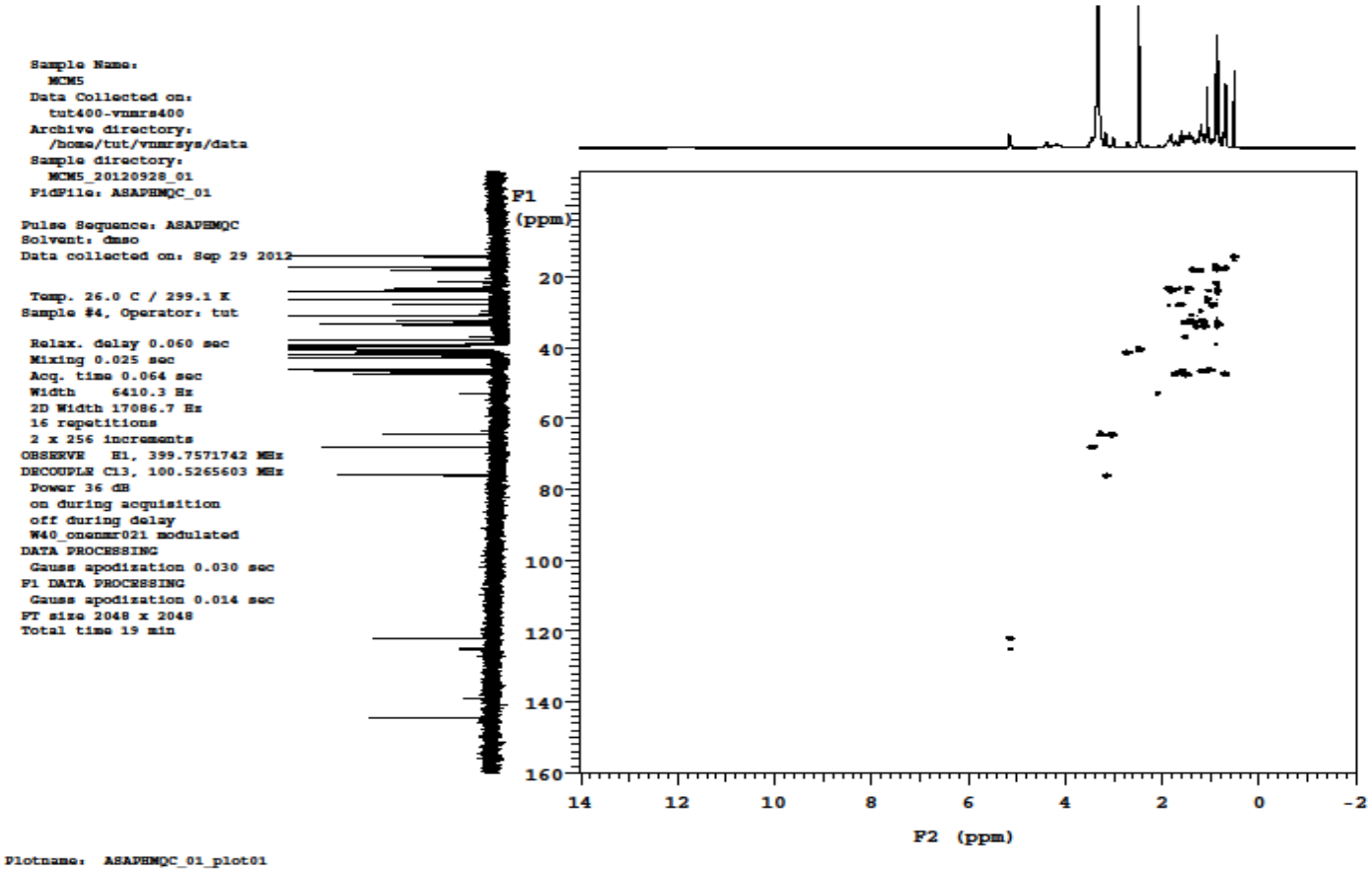
10. HMBC-NMR spectrum of C2



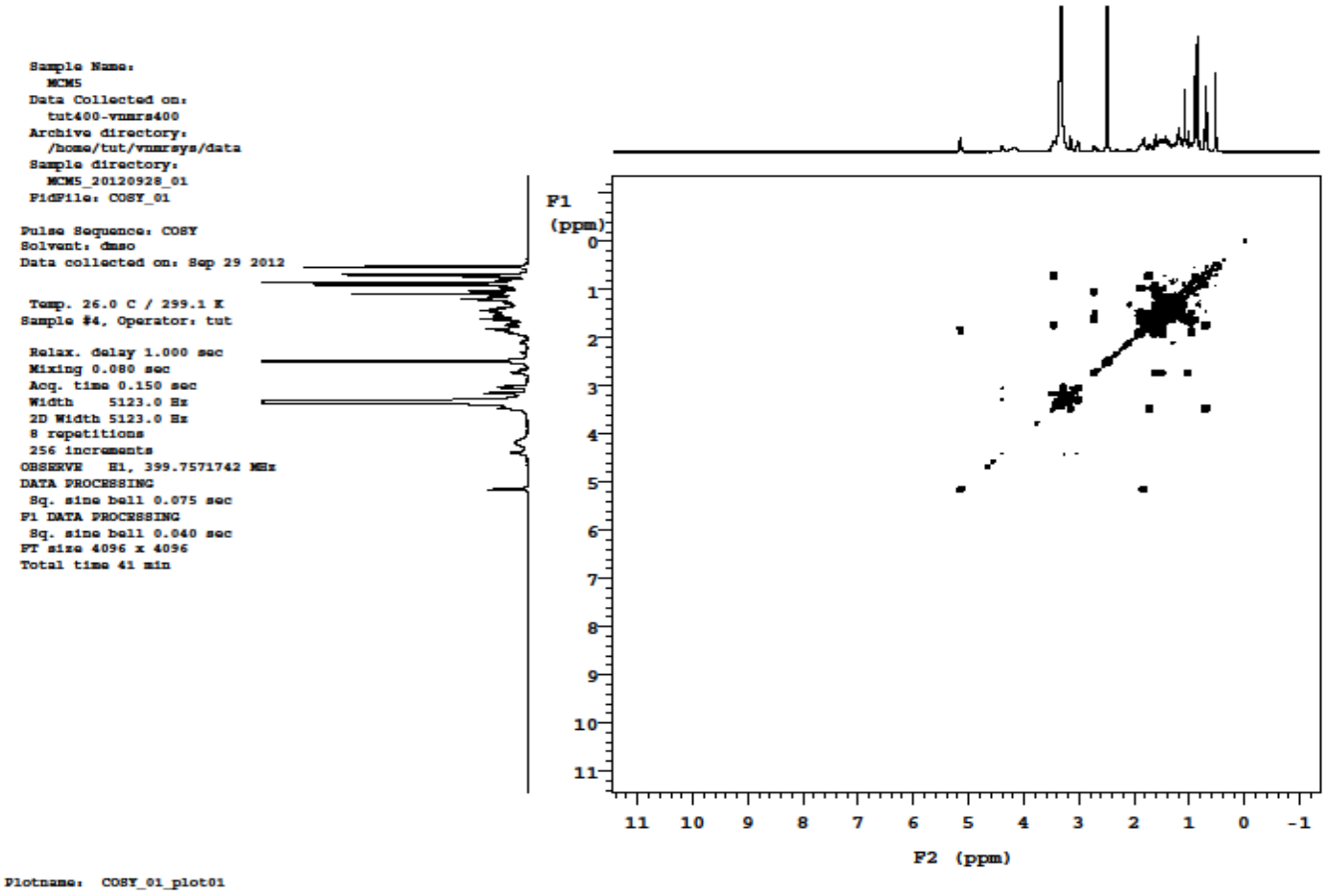
11. ^{1}H-NMR spectrum of C3



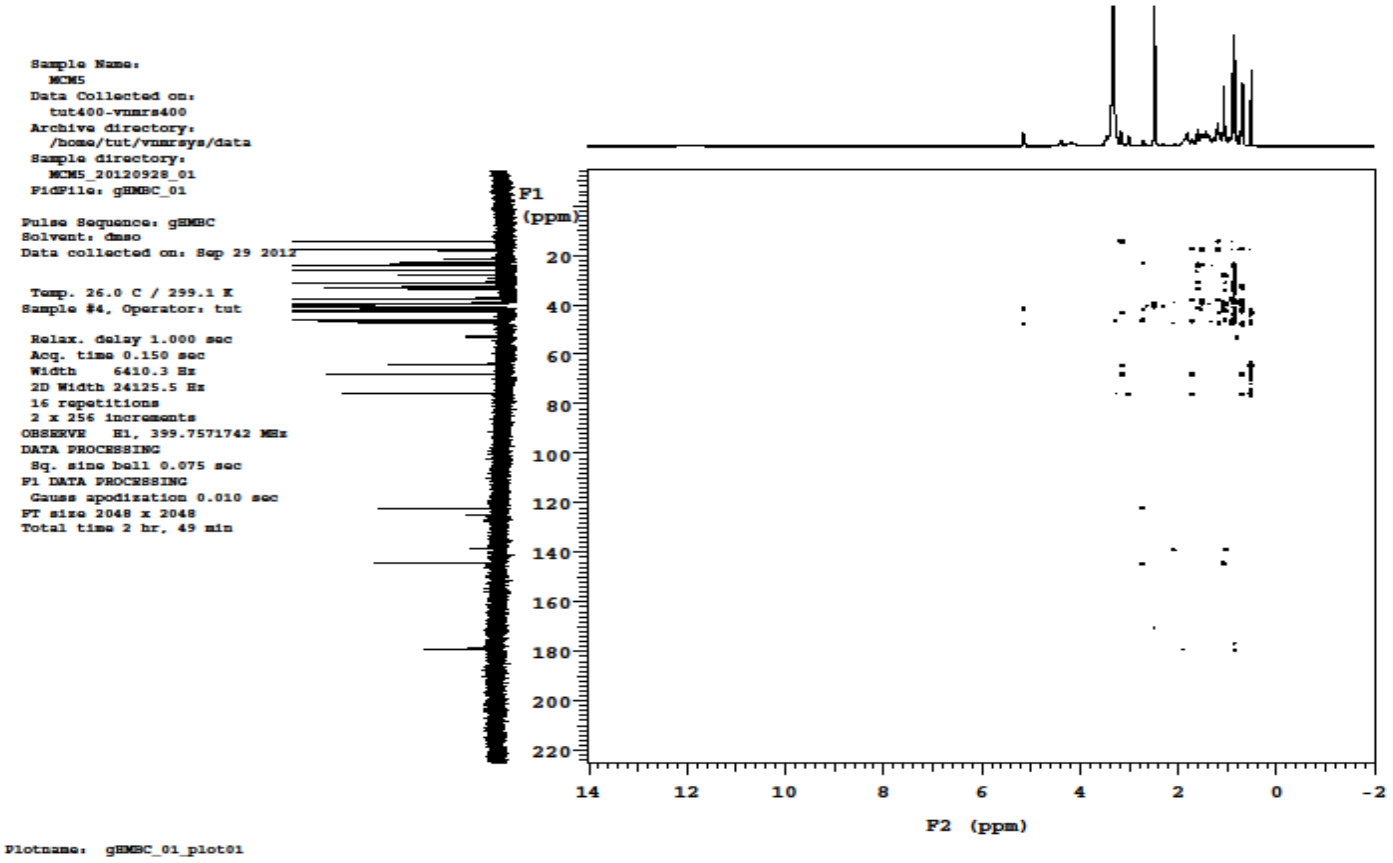
12. ^{13}C-NMR spectrum of C3



13. ASAP-HMQC-NMR spectrum of C3



14. COSY-NMR spectrum of C3



15. HMBC-NMR spectrum of C3