Modulation of the redox status, phase 2 drug metabolizing enzymes and fumonisin-induced cancer promotion in rat liver by selected southern African medicinal plants.

by

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Co-supervisor: Prof WCA Gelderblom

Bellville
February 2014

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DECLARATION

I, Willem Christoph Hikuam, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed _______________________________ Date _______________________________

ii
Abstract

According to the World Health Organization, cancer is the leading cause of death in the developed world, while it is the second leading cause of death in the developing world. In particular, liver cancer is the fifth most commonly diagnosed cancer in men, however, it is the second most frequent cause of death, responsible for an estimated 700,000 deaths annually. General limited access to health services, including treatment and the overall management of cancer in developing countries often contribute to the increased mortality rates when compared to developed countries. For centuries, medicinal plants have been used to prevent, and to a certain extent, treat cancer as a readily available and affordable alternative. In many instances, the curative or preventative claims still remain anecdotal. However, increasing evidence suggest that polyphenolic components of plants possess antioxidant activities, which are credited with curative/beneficial properties of medicinal plants. The curative properties could either be related to the primary compounds present in the plant itself, or the bio-activation products of plant components affecting hepatic drug metabolising and antioxidant enzymes systems related to carcinogen metabolism and maintaining oxidative homeostasis, respectively. Similarly, chronic consumption of medicinal plants could also result in hepatotoxicity, either caused by the primary plant components or bio-activation products. Due to these observations it is paramount to understand the mechanisms involved in the metabolism of plant components to critically assess beneficial versus potential harmful properties associated with chronic consumption.

The focus of the current study was aimed at elucidating the bio-activity of four multipurpose indigenous plants to Southern Africa, i.e. *Adansonia digitata*, *Agathosma betulina*, *Siphonochilus aethiopicus* and *Myrothamnus flabellifolius*. Traditionally, *A. digitata* has been used as an immunostimulant, anti-inflammatory and analgesic agent, while also as an antipyretic agent in the treatment of diarrhoea and dysentery. Similarly, traditional medicinal uses of *A. betulina* include treatment cholera, haematuria, calculus, kidney diseases, as well as infections of the bladder, urethra, and prostate among others. *S. aethiopicus* was traditionally employed to treat infections associated with pains and fevers, whereas *M. flabellifolius* served as treatment of conditions ranging from respiratory ailments, backache, kidney problems, haemorrhoids, chest pain, and asthma.
In the first part of this study, the polyphenolic contents and antioxidant capacities of the four plants were characterised. The emphasis was placed on using different solvents, namely water, ethanol and acetone for the extraction of the plant material and different methodologies to assess the antioxidant contents and capacities of the various extracts as both these factors can influence the outcome. When considering the antioxidant contents, total polyphenols, flavanols, and flavonols of the different solvent extracts prepared from the four plants were determined, whereas three different assays were used for the antioxidant capacities, i.e. oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC) and ferric-reducing antioxidant power (FRAP) assays. The A. digitata acetone extract had the highest (7.121 mg gallic acid equivalent (GAE)/milligram (mg) soluble solids), whereas the water extract of the same plant had the lowest total phenolic content (0.008 mg GAE/mg soluble solids). In general, the acetone extracts demonstrated the highest total polyphenol, flavanol, and flavonol contents, followed by the ethanol extracts, with the water extracts having the lowest contents. M. flabellifolius was the only distinct deviation from this rule, where the water extract demonstrated the highest total polyphenol content. Considering antioxidant capacities, the acetone extracts provided the highest antioxidant capacities for all plants when assessed using the TEAC (8.56-32.68 milimole (mmole) trolox equivalent (TE)/mg soluble solids) and FRAP (5.69-37.39 mmole ascorbic acid equivalent/mg soluble solids) antioxidant assays, with the exception of M. flabellifolius where the water extract demonstrated the highest activity (22.73 mmole ascorbic acid equivalent/mg soluble solids). Antioxidant capacity determinations with TEAC and FRAP assays followed similar patterns, which were different from capacities determined by the ORAC (0.46-533.54 mmoleTE/mg of soluble solids) assay. Corroborating the antioxidant content findings, the acetone extracts also demonstrated the highest antioxidant capacities (140.41-533.54 mmoleTE/mg of soluble solids), followed by ethanol (94.62-151.29 mmoleTE/mg of soluble solids) and water (0.46-134.02 mmoleTE/mg of soluble solids). Only M. flabellifolius (TEAC and FRAP) and S. aethiopicus (FRAP) deviated from this trend. Correlations between the polyphenolic contents and antioxidant capacities indicated that acetone and ethanol were more effective in extracting polyphenolic compounds than water, while also providing extracts with superior antioxidant activities. Furthermore, ORAC assay was the antioxidant capacity determining assay of choice for the aqueous plant extracts, whereas the TEAC and FRAP assays were more suitable when determining the antioxidant capacities of the acetone and ethanol plant extracts. These results confirm the notion that no single assay can comprehensively determine the antioxidant activities of plant extracts and that a battery of assays should be used, as the various antioxidant capacity determination techniques use different substrates with different targets for measurement.
The second part of this study comprised an in vivo experimental animal model to assess the potential toxicity, antioxidant status and modulation of the hepatic phase 2 drug metabolising enzymes following chronic consumption of the various plant extracts in male Fisher rats. Rats consumed aqueous extracts of the various plants (2% and 5% (w/v)) as the sole source of drinking fluid for 90 days, and the serum chemical pathology parameters for monitoring liver and kidney function conducted. These included alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), total iron (Fe), and creatinine (CREA). Parameters for blood and hepatic redox status included total polyphenols, ORAC, reduced glutathione (GSH), oxidised glutathione (GSSG), their ratio (GSH:GSSG), conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS). Assessment of the phase 2 hepatic xenobiotic metabolising enzymes included glutathione S-transferase (GST) α and µ activity in the cytosolic fraction and, UDP-glucuronosyltransferase (UDP-GT) activity in liver microsomes. When considering the liver and kidney function none of the plant extracts induced any significant toxicity, while 2% A. digitata significantly increased serum Fe. When considering the redox status, the whole blood and liver samples yielded similar results, with significant decreases in oxidised glutathione (GSSG) in rats consuming the 2% M. flabellifolius (82.76 µmole/L) and 5% A. digitata (90.42 µmole/L) with a resultant significant increase in the glutathione redox status (GSH:GSSG ratio of 5.69 and 5.64, respectively) when compared to rats consuming water (4.77). The GSH:GSSG ratio was also significantly increased by consumption of 2% A. betulina (8.45) and 5% S. aethiopicus (5.99). The consumption of all plant extracts, except 5% A. betulina and M. flabellifolius, significantly increased lipid peroxidation in the plasma CDs assay. These results indicated an increased antioxidant capacity in the liver with/without an associated reduced cellular oxidative stress status, which could be interpreted as a reduced susceptibility to oxidative damage. When considering the phase 2 hepatic enzymes, none of the plant extracts caused any significant changes in GSTα, GSTµ or UDP-GT activities.

The third part investigated the chemoprotective properties against cancer promotion in the liver utilising diethylnitrosamine (DEN) as cancer initiator and maize culture material of Fusarium verticillioides, containing the fumonisin B mycotoxins, as promoters in male Fischer rats. The rats consumed 2% (w/v) aqueous extracts of A. digitata, A. betulina, and S. aethiopicus over 28 days after cancer initiation and liver sections subjected to glutathione-S-transferase placental form positive GSTP1 staining and pre-cancerous liver foci categorised according to size. In addition, blood and liver analyses were done as described in the chronic feeding study above. Consumption of the A. digitata and, to a certain extent,
S. aethiopicus extracts, altered the oxidative stress status in the liver as indicated by the increased lipid peroxidation, as determined by significantly increased liver CDs and the decreased GSH:GSSG ratio in the blood. This can be related to a subchronic toxicity due to the high total polyphenol intake as mentioned above. These underlying sub chronic toxic effects of A. digitata and S. aethiopicus are likely to be responsible for the observed inhibitory effect on the proliferation of GSTP⁺ minifoci in the liver. Hepatic phase 2 metabolising enzyme activities were not significantly altered by A. digitata and S. aethiopicus consumption, while GSTμ activity was significantly increased by A. betulina treatment.

Based on the findings of the current study, aqueous extracts of A. digitata, A. betulina, and S. aethiopicus may serve as hepatoprotectors with a potential to modulate liver carcinogenesis, specifically cancer promotion. To our knowledge, no other studies have attempted to describe the possible chemoprevention mechanisms of these indigenous medicinal plants. Assessments of phase 1 hepatic enzymes and other antioxidant enzymes are suggested for future studies to further describe biochemical and molecular mechanisms associated with consumption of these extracts. Additionally, identifying main compounds present in the plant extracts could culminate in development of drugs and novel nutraceuticals. It is also recommended that increasing concentrations of the plant extracts and/or the ethanol extracts to be used in future studies to better describe dose-responses of the different plants in liver carcinogenesis.
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DEDICATION

To my wife, Felicita.
# Table of Contents

Declaration  

Abstract  

Acknowledgments  

Dedication  

Abbreviations and Acronyms  

Lists of Figures, Tables, and Appendices  

Chapter One: Introduction  

1.1 Introduction  
1.2 Liver cancer  
1.3 Aims  
1.4 Objectives  

Chapter Two: Literature Review  

2.1 Medicinal plants  
2.1.1 Introduction  
2.1.2 Traditional medicines  
2.1.3 Drug discovery  


<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.4 Biological activities of medicinal plants</td>
<td>12</td>
</tr>
<tr>
<td>2.2 Secondary metabolites of plants</td>
<td>12</td>
</tr>
<tr>
<td>2.2.1 Introduction to secondary metabolites</td>
<td>12</td>
</tr>
<tr>
<td>2.2.2 Classification of secondary metabolites</td>
<td>14</td>
</tr>
<tr>
<td>2.2.2.1 Terpenes</td>
<td>15</td>
</tr>
<tr>
<td>2.2.2.2 Phenolic compounds</td>
<td>16</td>
</tr>
<tr>
<td>2.2.2.3 Alkaloids</td>
<td>16</td>
</tr>
<tr>
<td>2.2.3 Polyphenols in nutrition</td>
<td>17</td>
</tr>
<tr>
<td>2.2.4 Metabolism of secondary metabolites</td>
<td>20</td>
</tr>
<tr>
<td>2.2.4.1 Introduction</td>
<td>20</td>
</tr>
<tr>
<td>2.2.4.2 Xenobiotic metabolising enzymes</td>
<td>22</td>
</tr>
<tr>
<td>2.2.4.2.1 Phase 1 metabolising enzymes</td>
<td>23</td>
</tr>
<tr>
<td>2.2.4.2.2 Phase 2 metabolising enzymes</td>
<td>24</td>
</tr>
<tr>
<td>2.2.4.2.2.1 Sulfotransferases</td>
<td>25</td>
</tr>
<tr>
<td>2.2.4.2.2.2 UDP-glucuronosyltransferases</td>
<td>25</td>
</tr>
<tr>
<td>2.2.4.2.2.3 Glutathione-S-transferases</td>
<td>27</td>
</tr>
<tr>
<td>2.2.4.2.3 Pharmacogenetics of xenobiotic metabolising enzymes</td>
<td>27</td>
</tr>
<tr>
<td>2.2.4.2.4 Role of xenobiotic metabolising enzymes in bioactivation</td>
<td>28</td>
</tr>
<tr>
<td>2.3 Chemical Carcinogenesis</td>
<td>29</td>
</tr>
<tr>
<td>2.3.1 Mutations and Cancer</td>
<td>29</td>
</tr>
<tr>
<td>2.3.2 Multi-step carcinogenesis</td>
<td>30</td>
</tr>
<tr>
<td>2.3.3 Carcinogens</td>
<td>32</td>
</tr>
<tr>
<td>2.3.4 Sources of carcinogens</td>
<td>32</td>
</tr>
<tr>
<td>2.3.4.1 Exogenous sources</td>
<td>32</td>
</tr>
</tbody>
</table>
2.3.4.1.1 Viruses and other microorganisms 33
2.3.4.1.2 Radiation 34
2.3.4.1.3 Xenochemicals 36
2.3.4.1.4 Pharmaceuticals 36
2.3.4.1.5 Occupational cancers 37
2.3.4.1.6 Metals, arsenic, dust and fibres 37
2.3.4.1.7 Chemically-induced occupational cancer 39
2.3.4.1.8 Tobacco, areca nut, alcohol, coal smoke, and salted fish 41
2.3.4.2 Endogenous sources 42
2.3.4.2.1 Free Radicals 43
2.3.4.2.2 Antioxidants 44
2.3.4.2.3 Oxidative Stress 46
2.3.4.2.3.1 Oxidative DNA damage 46
2.3.4.2.3.2 Lipid peroxidation 47
2.4 Chemical constituents and antioxidant properties of study plants 48
2.4.1 Adansonia digitata 48
2.4.1.1 General 48
2.4.1.2 Antioxidant activity 49
2.4.1.3 Anti-mutagenic activity and toxicity 49
2.4.2 Agathosma betulina 50
2.4.2.1 General 50
2.4.2.2 Antioxidant activity 51
2.4.2.3 Anti-mutagenic activity and toxicity 51
2.4.3 *Siphonocilus aethiopicus* 52

2.4.3.1 General 52

2.4.3.2 Antioxidant activity 52

2.4.3.3 Anti-mutagenic activity and toxicity 52

2.4.4. *Myrothamnus flabellifolius* 53

2.4.4.1 General 53

2.4.4.2 Antioxidant activity 53

2.4.4.3 Anti-mutagenic activity and toxicity 54

Chapter Three: Methods and Materials 55

3.1 Study design 55

3.2 Chemicals 57

3.3 Part 1: *In vitro* antioxidant content and -capacity determination of the four medicinal plants 57

3.3.1 Plant materials and extraction procedure 57

3.3.2 Soluble solids determination 58

3.3.3 Antioxidant content 58

3.3.3.1 Total polyphenol content 59

3.3.3.2 Flavanol content 59

3.3.3.3 Flavonol content 60

3.3.4 Antioxidant capacity 60

3.3.4.1 Ferric-reducing antioxidant power (FRAP) 60

3.3.4.2 Trolox equivalent antioxidant capacity (TEAC) assay 61

3.3.4.3 Oxygen radical absorbance capacity (ORAC) assay 61
| 3.3.5 | High pressure liquid chromatography (HPLC) profiling | 61 |
| 3.4 | Part 2: Modulation of hepatic phase 2 drug metabolising enzymes and oxidative stress status using a 90-day chronic exposure rat model | 62 |
| 3.4.1 | Preparation of plant extracts | 62 |
| 3.4.2 | Experimental animals and diet | 62 |
| 3.4.2.1 | Design of the 90-day chronic consumption model: Part 2 | 63 |
| 3.4.2.2 | Design of the DEN-initiated/fumonisin-promoted liver carcinogenesis model: Part 3 | 63 |
| 3.4.3 | Analysis of blood for *in vivo* studies: Part 2 and 3 | 65 |
| 3.4.3.1 | Preparation of blood | 66 |
| 3.4.3.2 | Chemical Pathology | 66 |
| 3.4.3.3 | Plasma total polyphenols | 66 |
| 3.4.3.4 | Oxygen radical absorbance capacity assay | 66 |
| 3.4.3.5 | Conjugated dienes | 66 |
| 3.4.3.6 | Glutathione analyses | 67 |
| 3.4.4 | Analysis of liver tissue for *in vivo* studies: Part 2 and 3 | 68 |
| 3.4.4.1 | Oxygen radical absorbance capacity assay | 68 |
| 3.4.4.2 | Conjugated dienes and thiobarbituric acid reactive substances | 69 |
| 3.4.4.3 | Glutathione analyses | 70 |
| 3.4.4.4 | Preparation of microsomal and cytosolic liver fractions | 70 |
| 3.4.4.5 | Glutathione S-transferase α and μ activity | 71 |
| 3.4.4.6 | UDP-glucuronosyltransferase activity | 72 |
| 3.4.4.7 | GST-Pi immunohistochemistry of liver sections | 72 |
| 3.4.4.8 | Statistical analyses | 73 |
Chapter Four: Results

4.1 Part 1: Antioxidant characterization of selected South African indigenous medicinal plant extracts

4.1.1 Soluble solids

4.1.2 Antioxidant contents: Total polyphenols, flavanols and flavonols

4.1.3 Antioxidant capacities: TEAC, FRAP, ORAC

4.1.4 Antioxidant content and capacity correlation coefficients

4.2 Part 2 and 3: 90-day chronic feeding and liver initiation/promotion carcinogenesis studies

4.2.1 Antioxidant content and activity of plant extracts used in the 90-day chronic feeding and liver initiation/promotion carcinogenesis studies

4.2.1.1 Soluble solids

4.2.1.2 The total polyphenol content and antioxidant capacity of plant extracts utilised in the 90-day chronic feeding and liver carcinogenesis studies

4.2.2 Part 2: Modulation of phase 2 drug metabolising enzymes due to chronic consumption of the various plant extracts

4.2.2.1 Rat body weight gain, relative liver weight, and fluid and total polyphenol intake: 90-day chronic feeding study

4.2.2.2 Blood parameters: 90-day chronic feeding study model

4.2.2.2.1 Serum chemical pathology parameters

4.2.2.2.2 Total polyphenol levels and oxidative stress markers in whole blood and serum

4.2.2.3 Hepatic biochemistry parameters 90-day chronic feeding study

4.2.2.3.1 Hepatic redox and lipid peroxidation status

4.2.2.3.2 Hepatic phase 2 drug metabolising enzymes

4.2.3 DEN-initiated, FB-promoted liver carcinogenesis study

4.2.3.1 Rat body weight gained, relative liver weight, and fluid, polyphenol and FB1 intakes
4.2.3.2 Blood parameters: liver carcinogenesis study 92
4.2.3.2.1 Chemical pathology parameters 92
4.2.3.2.2 Blood redox status and lipid peroxidation parameters 94
4.2.3.3 Hepatic parameters: liver carcinogenesis study 95
4.2.3.3.1 Hepatic redox and lipid peroxidation status 95
4.2.3.3.2 Hepatic phase 2 drug metabolising enzymes 99
4.2.3.3.3 Liver Immunohistochemistry: GST-P* staining 100

Chapter Five: Discussion 104

5.1 Part 1: Antioxidant characterization of selected African indigenous medicinal plant extracts 104

5.2 In vivo studies (part 2, 3) assessing the modulation of phase 2 hepatic drug metabolising enzymes and cancer promotion utilising two experimental rat models 110

5.2.1 Modulation of phase 2 hepatic xenobiotic metabolising enzymes and antioxidant status by African medicinal plants 111

5.2.2 Modulation of hepatic phase 2 drug metabolising enzymes in DEN- initiated and fumonisin- promoted liver carcinogenesis in male Fischer rats 115

Chapter Six: Conclusion, limitations and recommendations 123

Bibliography 126

Appendices 180
Abbreviations and Acronyms

ε
Extinction coefficient
°C
Degrees Celsius
%
Percentage
4-HNE
4-hydroxy-2-nonenal
8OHdG
8-hydroxy-2′-deoxyguanosine
A
Absorbance
AAPH
2,2’-azobis (2-methylpropionamidine) dihydrochloride
ABTS
2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
AFB₁
Aflatoxin B₁
AHR
Aryl hydrocarbon receptor
ALP
Alkaline Phosphatase
ALT
Alanine Transaminase
ANOVA
Analysis of Variation
AST
Aspartate Transaminase
BC
Before Christ
BCC
Basal-cell carcinoma
BHA
Butylated hydroxyanisol
BHT
Butylated hydroxytoluene
Bw
Body weight
BWG
Body weight gain
C₁₀H₁₆
Monoterpenoids
C₁₅H₂₄
Sesquiterpenoids
C₂₀H₃₂
Diterpenoids
C₂₅H₄₀
Sesterpenoids
C₃₀H₄₈
Triterpenoids
C₃₅H₅₆
Tetraterpenoids
C₅H₈
Isoprenoid
CAR
Constitutive androstane receptor
CAT
Catalase
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<thead>
<tr>
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<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>Conjugated Dienes</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>CM</td>
<td>Cultured material</td>
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<tr>
<td>CMPM</td>
<td>Cultured material/pellet mix</td>
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<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
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<td>CREA</td>
<td>Creatinine</td>
</tr>
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<td>CPUT</td>
<td>Cape Peninsula University of Technology</td>
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<td>CVD</td>
<td>Cardiovascular diseases</td>
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<td>CYP 450</td>
<td>Cytochrome P450</td>
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<td>DCNB</td>
<td>1,2-dichloro-4-nitrobenzene</td>
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<td>DEN</td>
<td>Diethylnitrosamine</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl radical</td>
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<tr>
<td>DTNB</td>
<td>5,5'-Dithiobis (2-nitrobenzoic acid)</td>
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<td>DXP</td>
<td>1-deoxy-D-xylulose 5-phosphate</td>
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<tr>
<td>EBV</td>
<td>Epstein–Barr virus</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>et al.</td>
<td>And others</td>
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<tr>
<td>etc</td>
<td>Et cetera</td>
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<tr>
<td>FB</td>
<td>Fumonisin</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>Iron (III) chloride hexahydrate</td>
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<td>FRAP</td>
<td>Ferric-reducing antioxidant power</td>
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<tr>
<td>g</td>
<td>Centrifugal force</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>GA</td>
<td>Gallic</td>
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<td>GAE</td>
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<td>GCMS</td>
<td>Gas chromatography mass spectrometry</td>
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<td>GGT</td>
<td>γ-glutamyl transpeptidase</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>---------</td>
<td>------------------------------------------------</td>
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<tr>
<td>MD</td>
<td>Medical doctor</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
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<td>Magnesium chloride</td>
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<td>min</td>
<td>Minute</td>
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<tr>
<td>MPA</td>
<td>Metaphosphoric acid</td>
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<td>MXP</td>
<td>2-C-methyl-D-erythritol 4-phosphate</td>
</tr>
<tr>
<td>n</td>
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<td>NA</td>
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<td>Sodium hydroxide</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NAT</td>
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<td>NTP</td>
<td>National Toxicology Programme</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxide</td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>P value</td>
<td>Probability value</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PAPS</td>
<td>3'-phosphoadenosine 5'-phosphosulfate</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>PCL</td>
<td>Photochemiluminescence</td>
</tr>
<tr>
<td>PDQ</td>
<td>Physician data query</td>
</tr>
<tr>
<td>P-gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>pH</td>
<td>Power of hydrogen</td>
</tr>
<tr>
<td>pKa</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>pmole</td>
<td>Picomoles</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptors</td>
</tr>
<tr>
<td>R</td>
<td>Rand</td>
</tr>
<tr>
<td>RCS</td>
<td>Reactive chloride species</td>
</tr>
<tr>
<td>RLW</td>
<td>Relative liver weight</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous-cell carcinoma</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SO₃</td>
<td>Sulfonate</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulotransferases</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric Acid Reactive Substances</td>
</tr>
<tr>
<td>TBHQ</td>
<td>Tertiary butylhydroquinone</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzopara-dioxin</td>
</tr>
<tr>
<td>TE</td>
<td>Trolox Equivalent</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox-equivalent antioxidant capacity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TMP</td>
<td>Traditional medicine practitioner</td>
</tr>
<tr>
<td>TP</td>
<td>Total polyphenols</td>
</tr>
<tr>
<td>TPTZ</td>
<td>2,4,6-tri[2-pyridyl]-s-triazine</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile organic compounds</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UDPGA</td>
<td>UDP-glucuronic acid</td>
</tr>
<tr>
<td>UDP-GT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UNSCEAR</td>
<td>United Nations Scientific Committee on the Effects of Atomic Radiation</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>US$</td>
<td>US dollars</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>μg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitres</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>μmole</td>
<td>Micromoles</td>
</tr>
</tbody>
</table>
Lists of Figures, Tables, and Appendices

Figures

Figure 2.1  The traditional medicine use in some developing countries  8
Figure 2.2  The systematic approach of drug discovery and development from medicinal plants  11
Figure 2.3  Overview of the main pathways leading to secondary metabolites biosynthesis  13
Figure 2.4  Major secondary metabolite classes are represented in a pie chart  14
Figure 2.5  Examples of terpenoid structures: monoterpenoids (1,8-cineole and geraniol), sesquiterpenoid (valeric acid), diterpenoid (ginkgolide A), and triterpenoid (ginsenoside Rg1)  15
Figure 2.6  Chemical structures of major classes of polyphenols  18
Figure 2.7  Schematic diagramme of UDP-GT reactions  26
Figure 2.8  Multistep carcinogenesis process  31
Figure 3.1  An overview of the experimental methodology of the study  56
Figure 3.2  Design of the initiation/promotion liver carcinogenesis model  64
Figure 3.3  A 96 well microtitre plate  68
Figure 5.1  Summary of probable events leading to inhibition of cancer promotion in rat livers  122

Tables

Table 1.1  Five cancers with the highest approximate incidence rates and their estimated mortality rates worldwide  2
Table 2.1  Ratios of traditional medicinal practitioners (TMP’s) and medical doctors (MD) to total populations in selected African countries in the 1980’s  9
Table 2.2  Drug development from natural products in 2008  11
Table 2.3  Summary of polyphenol classes, main representatives and sources of food  19
Table 2.4  Human oncogenic viruses  33
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>Radiation type and associated tumours or anatomical sites</td>
<td>35</td>
</tr>
<tr>
<td>2.6</td>
<td>Summary of carcinogenic metals, arsenic, dust and fibres, and the tumours/sites of cancers caused by their exposure in humans</td>
<td>38</td>
</tr>
<tr>
<td>2.7</td>
<td>Occupation cancer inducing chemicals and processes and tumour sites and types</td>
<td>40</td>
</tr>
<tr>
<td>2.8</td>
<td>Activities that are classified as carcinogenic to humans with the affected sites and/or tumours</td>
<td>42</td>
</tr>
<tr>
<td>2.9</td>
<td>Integral antioxidant capacity of <em>A. digitata</em></td>
<td>49</td>
</tr>
<tr>
<td>2.10</td>
<td>ORAC results of different <em>A. betulina</em> extracts</td>
<td>51</td>
</tr>
<tr>
<td>3.1</td>
<td>Working dilutions for the antioxidant content determinations in the different solvents (extract dry weight: solvent)</td>
<td>59</td>
</tr>
<tr>
<td>3.2</td>
<td>Working dilutions for the antioxidant capacity assays in the different solvents (extract dry weight: solvent)</td>
<td>60</td>
</tr>
<tr>
<td>3.3</td>
<td>The random division of rats into groups for the 90-day chronic feeding model</td>
<td>63</td>
</tr>
<tr>
<td>3.4</td>
<td>The random division of rats into groups for initiation/promotion liver carcinogenesis model</td>
<td>65</td>
</tr>
<tr>
<td>4.1</td>
<td>Soluble solids (mg/mL) of the various plant extracts</td>
<td>74</td>
</tr>
<tr>
<td>4.2</td>
<td>Antioxidant concentrations of the different plant extracts</td>
<td>75</td>
</tr>
<tr>
<td>4.3</td>
<td>Antioxidant capacity of the various plant extracts</td>
<td>77</td>
</tr>
<tr>
<td>4.4</td>
<td>Spearman correlation values comparing antioxidant contents and capacities of aqueous extracts</td>
<td>78</td>
</tr>
<tr>
<td>4.5</td>
<td>Spearman correlation values comparing antioxidant contents and capacities of acetone extracts</td>
<td>79</td>
</tr>
<tr>
<td>4.6</td>
<td>Spearman correlation values comparing antioxidant contents and capacities of ethanol extracts</td>
<td>79</td>
</tr>
<tr>
<td>4.7</td>
<td>Soluble solids (mg/mL) for 2% and 5% (w/v) plant extracts for <em>in vivo</em> models</td>
<td>80</td>
</tr>
<tr>
<td>4.8</td>
<td>Total polyphenol, flavanol, flavonol content and antioxidant activity (ORAC) of the 2% and 5% plants extracts utilized in the <em>in vivo</em> rat models</td>
<td>82</td>
</tr>
<tr>
<td>4.9</td>
<td>Body weight gains, relative liver weights, and daily fluid and polyphenol intakes of rats in the 90-day chronic feeding study</td>
<td>84</td>
</tr>
</tbody>
</table>
Table 4.10  Serum chemical pathology parameters of rats in the 90-day chronic feeding study 85
Table 4.11  Whole blood and plasma redox status and lipid peroxidation: 90-day chronic feeding model 87
Table 4.12  Liver redox status: 90-day chronic feeding study 89
Table 4.13  Hepatic phase 2 drug metabolizing enzymes in rats consuming the various plant extracts for 90days 91
Table 4.14  Body weight gain, relative liver weight and daily fluid, polyphenol and FB intake in the liver carcinogenesis study 93
Table 4.15  Chemical pathology: liver carcinogenesis study 94
Table 4.16  Blood redox status and lipid peroxidation: liver carcinogenesis study 96
Table 4.17  Liver redox status and lipid peroxidation: liver carcinogenesis study 98
Table 4.18  Phase 2 drug metabolizing enzymes: liver carcinogenesis study 99
Table 4.19  Effects of various plant extracts on the induction of the number and relative incidence of GST-P+ foci after DEN initiation and CM promotion 102
Table 4.20  Effects of various plant extracts on the induction of the number and relative incidence of GST-P+ foci after DEN initiation and CM promotion excluding the mini foci 103

Appendices

Appendix 1  HPLC profile of A. digitata water extract (280nm) 180
Appendix 2  HPLC profile of A. digitata water extract (320nm) 181
Appendix 3  HPLC profile of A. digitata water extract (350nm) 182
Appendix 4  HPLC profile of A. digitata acetone extract (280nm) 183
Appendix 5  HPLC profile of A. digitata acetone extract (320nm) 184
Appendix 6  HPLC profile of A. digitata acetone extract (350nm) 185
Appendix 7  HPLC profile of A. digitata ethanol extract (280nm) 186
Appendix 8  HPLC profile of A. digitata ethanol extract (320nm) 187
Appendix 9  HPLC profile of A. digitata ethanol extract (350nm) 188
Appendix 10  HPLC profile of S. aethiopicus water extract (280nm) 189
Appendix 11  HPLC profile of S. aethiopicus water extract (320nm) 190
Appendix 12  HPLC profile of S. aethiopicus water extract (350nm) 191
Appendix 13  HPLC profile of *S. aethiopicus* acetone extract (280nm)  192
Appendix 14  HPLC profile of *S. aethiopicus* acetone extract (320nm)  193
Appendix 15  HPLC profile of *S. aethiopicus* acetone extract (350nm)  194
Appendix 16  HPLC profile of *S. aethiopicus* ethanol extract (280nm)  195
Appendix 17  HPLC profile of *S. aethiopicus* ethanol extract (320nm)  196
Appendix 18  HPLC profile of *S. aethiopicus* ethanol extract (350nm)  197
Appendix 19  HPLC profile of *A. betulina* water extract (280nm)  198
Appendix 20  HPLC profile of *A. betulina* water extract (320nm)  199
Appendix 21  HPLC profile of *A. betulina* water extract (350nm)  200
Appendix 22  HPLC profile of *A. betulina* acetone extract (280nm)  201
Appendix 23  HPLC profile of *A. betulina* acetone extract (320nm)  202
Appendix 24  HPLC profile of *A. betulina* acetone extract (350nm)  203
Appendix 25  HPLC profile of *A. betulina* ethanol extract (280nm)  204
Appendix 26  HPLC profile of *A. betulina* ethanol extract (320nm)  205
Appendix 27  HPLC profile of *A. betulina* ethanol extract (350nm)  206
Appendix 28  HPLC profile of *M. flabellifolius* water extract (280nm)  207
Appendix 29  HPLC profile of *M. flabellifolius* water extract (320nm)  208
Appendix 30  HPLC profile of *M. flabellifolius* water extract (350nm)  209
Appendix 31  HPLC profile of *M. flabellifolius* acetone extract (280nm)  210
Appendix 32  HPLC profile of *M. flabellifolius* acetone extract (320nm)  211
Appendix 33  HPLC profile of *M. flabellifolius* acetone extract (350nm)  212
Appendix 34  HPLC profile of *M. flabellifolius* ethanol extract (280nm)  213
Appendix 35  HPLC profile of *M. flabellifolius* ethanol extract (320nm)  214
Appendix 36  HPLC profile of *M. flabellifolius* ethanol extract (350nm)  215
1.1 Introduction

According to World Health Statistics 2012, a World Health Organization annual compilation of health-related data for its 194 member states, 63% of the estimated 57 million global deaths in 2008 were due to non-communicable diseases (NCD), by definition diseases that are non-infectious and non-transmissible among people (WHO, 2012). The WHO estimates the burden of NCDs in South Africa to be 2-3 times higher than in developed countries (WHO, 2008). The four main types of NCD include cancers, cardiovascular diseases (CVD), chronic respiratory disease and diabetes, and they contribute to the most deaths caused by NCD, with CVD contributing 48%, cancers 21% and chronic respiratory diseases 12% (WHO, 2012).

Cancer is the second leading cause of death in the developing world, whereas it is the leading cause of death in the developed world. Population ageing, growth and the adoption of cancer-associated lifestyle choices, amongst others, are responsible for the increasing incidence of cancer, especially in the developed world (WHO, 2008). The Global Cancer Statistics report of 2011 reported an estimated 12.7 million cases of cancer with 7.6 million deaths thereof worldwide in 2008 (Jemal et al., 2011). According to this report, the combined incidence of cancers is nearly double in the developed countries when compared to their developing counterparts, both in males and females. However, on average, the mortality rates of combined cancers in developed countries are, comparatively, only 21% higher in males and 2% higher in females than in developing countries. Table 1.1 represents a snapshot of the estimated new cases of cancer worldwide, in males and females, clearly indicating the numbers affected by cancer, as well as the types of cancers affecting both sexes. The International Agency for Research on Cancer (IARC) projected that by 2030 there will be 26 million new annual cases of cancer with 17 million deaths (IARC, 2008). Global population growth and ageing were suggested to be the major contributors.
Table 1.1 Five cancers with the highest approximate incidence rates and their estimated mortality rates worldwide (Adapted from Jemal et al., 2011)

<table>
<thead>
<tr>
<th></th>
<th>Estimated New Cases</th>
<th>Estimated Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Lung and bronchus</td>
<td>1 095 200</td>
<td>513 600</td>
</tr>
<tr>
<td>Prostate (Male)</td>
<td>N/A</td>
<td>903 500</td>
</tr>
<tr>
<td>Cervix Uteri (Female)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Colon and rectum</td>
<td>663 600</td>
<td>570 100</td>
</tr>
<tr>
<td>Stomach</td>
<td>640 600</td>
<td>349 000</td>
</tr>
<tr>
<td>Liver</td>
<td>522 400</td>
<td>225 900</td>
</tr>
</tbody>
</table>

Even though some cancers reportedly have lower estimated new cases, their mortality rates are higher than more prevalent cancers. For example, in males, liver cancer is ranked the 5th most common cancer, yet is ranked as the cancer with the second highest mortality rate (Jemal et al., 2011). Factors such as the regional differences in prevalence and distribution patterns of major risk factors, as well as the detection and treatment access, contribute to this inconsistency between incidence and mortality rates of developing and developed world countries (Jemal et al., 2011). In developing countries, cancers are only detected at a later stage, making prognosis very poor, while the treatment is not readily available and affordable for the general population.

Manifestation of cancer is as multidimensional as the complex organization of the human body. Corresponding to the number of different types of cells inside an organism, many different variations of cancers also exist. More than 100 different types of cancers have already been described with the heterogeneity of the disease further compounded by diverse variants, due to the level of organization within a cell, tissue or organ at which the cancer manifestation occurs (Brafford and Herlyn, 2005). These variations in the types of cancers contribute to the diversity in cancer treatment and management approaches.

The two main approaches towards cancer include that of treatment and that of prevention, be it primary or secondary prevention. The principal treatment approaches of the National Cancer Institute (NCI) of the National Institutes of Health are chemotherapy, radiation therapy, surgery, transplantation, and other, mostly targeted specifically at one particular type of cancer, while taking the characteristics of that cancer into consideration (NCI,
2012a). An evidence-based approach is taken in cancer treatment, and depends on the type and stage of cancer. NCI has set up a comprehensive cancer database, referred to as a Physician Data Query (PDQ), where the choice of a particular cancer treatment is recommended, based on information from clinical trials (NCI, 2012b). Of note is the difference between the choices of treatment for adults and paediatrics. This is generally based on the higher doses of chemo- and radiation therapy that can be tolerated by paediatric patients compared to adults.

The other approach includes prevention, already recognised in 1736 by founding father of the USA and scientist, although not in the current context. He quoted: “an ounce of prevention is worth a pound of cure”. In 2011, South African Health Minister Aaron Motsoaledi remarked that “South Africa’s healthcare model needs to shift from being hospice-centric to more preventative if the country is to win the fight against the burden of diseases” when addressing the first Global Ministerial Conference on healthy lifestyles and non-communicable disease in Moscow (www.mg.co.za/article/2011-04-29-motsoaledi-calls-for-more-emphasis-on-disease-prevention). Avoiding exposure to biological, chemical, or physical mediators for carcinogenesis, in addition to adopting a healthy lifestyle, are the cornerstones of cancer prevention (Glade, 1999). Chemoprevention is the process of, retardation or reversal of cancer progression by naturally occurring, or synthetic agents. These agents possess the ability to universally suppress the risk of cancer development by suppressing oxidative stress through a variety of mechanisms, as will be discussed in chapter 2 (literature review). Oxidative stress is the imbalance between the production and elimination of reactive oxygen and nitrogen species, commonly referred to as ROS and RNS, respectively (Kowluru and Chan, 2007). The excess ROS and RNS are generally associated with carcinogenesis, in addition to a magnitude of other diseases (Valko et al., 2007). Under normal circumstances, the human antioxidant defence system protects the body against oxidative stress (Franco et al., 2008). However, persistent exposure to both exogenous and endogenous agents which cause oxidative stress, can lead to an insufficiency of the physiological defence system, leading to diseases like certain cancers. Therefore, to avoid this, exogenous assistance to the body’s antioxidant defence has increasingly been regarded as a potential chemopreventive approach. Antioxidants in natural agents, such as plants, contain secondary metabolites which have previously been suggested to possess the ability to suppress/modulate oxidative stress (Khan et al., 2008).
In view of the above, indigenous medicinal plants can be explored to address the aspect of availability and affordability of cancer prevention and treatment, since they already serve in the treatment of different ailments as a readily accessible and inexpensive means of medication (WHO, 2002). Having contributed already to promising anticancer drug candidates such as taxol, combrestatin, camptothecin, epipodophyllotoxin and vinca alkaloids, medicinal plants play a promising role in the fight against cancer (Johnson et al., 1963; Wall, 1998; Canel et al., 2000; Cirla and Mann, 2003).

1.2 Liver cancer

Hepatocellular carcinoma (HCC) or liver cancer is the fifth most commonly diagnosed cancer in men and the seventh in women worldwide. However, HCC is the second most frequent cause of death in men, while being the sixth leading cause of death in women (Jemal, et al., 2011) and is responsible for an estimated 700,000 deaths annually. The incidence of HCC varies with geographical location, with the highest prevalence reported in East and South-East Asia (Jemal et al., 2011).

Elevated prevalence of chronic hepatitis B virus (HBV) infection has been implicated for the high liver cancer prevalence in Asia and sub-Saharan Africa (London and McGlynn, 2006). HBV infection was reported to account for an estimated 60% and 23% of total liver cancer in developing and developed countries, respectively, whereas hepatitis C virus (HCV) infection accounted for 33% in developing countries, and 20% in developed countries (Parkin, 2006). In parts of Africa and Asia, interactions of chronic HBV infection with aflatoxin B1 (AFB1) exposure further increased liver cancer (London and McGlynn, 2006). In other low-risk, mostly Western countries, alcohol-related cirrhosis and non-alcoholic fatty liver disease, associated with obesity, are suggested to be responsible for the majority of liver cancer occurrences (El-Serag, 2007).

Due to the limitations encountered in the treatment of liver cancer, mortality associated with this cancer still remain high, making it the third most common cause of death from cancer worldwide (Jemal et al., 2011). Surgical removal of liver cancerous cells offers the best chance of cure, however, less than 20% of the patients meet the criteria for this procedure at the time of diagnosis (Bismuth et al., 1999). Early diagnosis is thus critical to successful
treatment of liver cancer. Preventing liver cancer remains the most viable for survival. Most countries have embarked on introducing HBV vaccines during national infant vaccination programmes (WHO, 2009). Since no HCV vaccine is currently available, adopted preventative strategies against HCV include screening of donor blood, instituting adequate infection control practices for medical procedures, and encouraging needle exchange programmes among injection drug users, while crop substitution and safer grain storage were introduced to control AFB1 contamination in sub-Saharan African countries (Turner et al., 2005).

1.3 Aims

This study was aimed at investigating the chemopreventive potential and certain of the associated mechanisms of selected multipurpose South African indigenous medicinal plants, *Adansonia digitata*, *Agathosma betulina*, *Siphonochilus aethiopicus* and *Myrothamnus flabellifolius*.

1.4 Objectives:

The major objectives of this study were to:

1. Determine antioxidant contents of *A. digitata*, *A. betulina*, *S. aethiopicus*, and *M. flabellifolius* extracts
   a. in terms of total polyphenols, flavanols, flavonols
   b. comparing effects of using different extraction solvents, namely water, ethanol, and acetone
2. Determine antioxidant capacities of *A. digitata*, *A. betulina*, *S. aethiopicus*, and *M. flabellifolius* extracts
   a. using ferric reducing antioxidant power (FRAP), trolox-equivalent antioxidant capacity (TEAC), and oxygen radical absorbance capacity (ORAC) assays
   b. comparing effects of using different extraction solvents, namely water, ethanol, and acetone
3. Assess correlations between various antioxidant parameters and antioxidant capacity measurements
4. Investigate impacts of chronic *A. digitata*, *A. betulina*, *S. aethiopics*, and *M. flabellifolius* extract consumption on:
   a. Liver function
   b. Blood redox status and lipid peroxidation
   c. Hepatic redox status and lipid peroxidation
   d. Hepatic phase 2 metabolising enzymes

5. Investigate potential chemoprotective mechanisms due to consumption of *A. digitata*, *A. betulina*, *S. aethiopics*, and *M. flabellifolius* by assessing:
   a. Liver function
   b. Blood redox status and lipid peroxidation
   c. Hepatic redox status and lipid peroxidation
   d. Hepatic phase 2 metabolising enzymes
   e. Liver immunochemistry: GSTP* staining
Chapter 2

2. Literature Review

2.1 Medicinal plants

2.1.1 Introduction

For centuries, mankind has relied on nature for food, shelter, clothing, transportation, flavours, fertilizers, fragrances and medicine for survival (Cragg and Newman, 2005). Driven by fear of disease and death, human civilizations of different cultural backgrounds developed sophisticated traditional medicine systems which were predominantly made up of medicinal plants (Ellis, 1986). The oldest accounts of medicinal plant use, written on clay tablets of cuneiform, were recorded from Mesopotamia around 2600 BC, where oils of Cedrus species, as well as Cupressus sempervirens, Glycyrrhiza glabra, Commiphora species and Papaver somniferum were used to treat ailments ranging from coughs, colds, parasitic infections and inflammation, amongst other uses (Heinrich et al., 2004).

Fortunately, the information on the specific plants and the methods of application for the treatment of diseases, were passed down through oral history, making it possible for the continued use of medicinal plants in traditional medicine, as well as in the search for new drugs by pharmaceutical companies (Kinghorn, 2001; Samuelsson, 2004). A famous example comes from the last century BC, when Cleopatra was using an atropine-containing extract of Hyoscyamus muticus to dilate her pupils, thereby making her more attractive. Today, synthetic derivatives of atropine are routinely used in eye examinations to dilate patients’ pupils (Scinto et al., 1994).

From an estimated 422 000 flowering plant species globally, more than 50 000 are utilized today for medicinal purposes with approximately 2500 species being traded worldwide (Schippmann et al., 2003). It was estimated that 25% of the total higher plants are found in sub-Saharan Africa (Klopper et al., 2006). Africa hosts more than 5400 medicinal plant taxa with over 16300 medicinal uses (Neuwinger, 2000), from which approximately 3000 plants are regularly used for medicinal purposes in southern Africa (Van Wyk and Gericke, 2000).
2.1.2 Traditional medicines

Indigenous plants and herbs have been used for centuries in Africa and the rest of the world as traditional medicine (Hostettmann et al., 1995). A World Health Organisation (WHO) study indicated that, in African countries such as Benin, Rwanda, Uganda, Ethiopia and Tanzania, traditional medicines, derived from indigenous medicinal plants, are employed to meet the health care needs of between 60% and 90% of the populations (Fig 2.1). Similarly, 70% of the Indian population make use of traditional medicine as a readily accessible and inexpensive means of treatment (WHO, 2005).

![Figure 2.1 The traditional medicine use in some developing countries (Adapted from WHO, 2005)](image)

The increased usage of medicinal plants, especially in the developing countries, reportedly stems from the relative inaccessibility of western medicine in rural areas, where healthcare practitioners are more readily practicing in urban areas compared to the rural setting (Wibulpolprasert and Pengpaibon, 2003). Already in the 1980’s a negatively skewed ratio of professional healthcare practitioner to the general population in the developing world was observed (Table 2.1). This situation is further aggravated by the emigration of skilled practitioners from the developing countries to Europe, North America or Australia (Hagopian et al., 2004).

Consequently, WHO policies were drafted to encourage the expansion of the use of traditional medicine as an inexpensive and effective treatment of diseases and to promote further research into the efficacy and quality of such methods and practices (WHO, 2002).
As a result of the importance and exposure that medicinal plants were receiving in terms of primary health care treatment, major pharmaceutical companies started showing a renewed interest in investigating and developing medicinal plants into effective, safe and quality phytotherapeutic agents (Brevoort, 1995; De Smet, 1997; Blumenthal, 1999a; Blumenthal, 1999b).

Table 2.1 Ratios of traditional medicinal practitioners (TMP’s) and medical doctors (MD) to total populations in selected African countries in the 1980’s (adapted from Cunningham, 1993)

<table>
<thead>
<tr>
<th>Country (City/Area)</th>
<th>TMP : Total population</th>
<th>MD : Total population</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nigeria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benin City</td>
<td>1:110</td>
<td>1:16400</td>
<td>Oyenye and Orubuloye, 1983</td>
</tr>
<tr>
<td>National average</td>
<td>NA</td>
<td>1:15740</td>
<td>Gestler, 1984</td>
</tr>
<tr>
<td>Ghana</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kwahu district</td>
<td>1:224</td>
<td>1:20625</td>
<td>Anyinam, 1984</td>
</tr>
<tr>
<td>Kenya</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban (Mathare)</td>
<td>1:833</td>
<td>1:987</td>
<td>Good, 1987</td>
</tr>
<tr>
<td>Rural (Kilungu)</td>
<td>1:146-345</td>
<td>1:70000</td>
<td>Good, 1987</td>
</tr>
<tr>
<td>Tanzania</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dar es Salaam</td>
<td>1:350-450</td>
<td>NA</td>
<td>Swantz, 1984</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban Areas</td>
<td>1:234</td>
<td>NA</td>
<td>Gelfand et al., 1985</td>
</tr>
<tr>
<td>Rural Areas</td>
<td>1:956</td>
<td>NA</td>
<td>Gelfand et al., 1985</td>
</tr>
<tr>
<td>Swaziland</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>National</td>
<td>1:110</td>
<td>1:10000</td>
<td>Green, 1985</td>
</tr>
<tr>
<td>South Africa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venda Area</td>
<td>1:700-1200</td>
<td>1:17400*</td>
<td>Savage, 1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arnold and Gulumian, 1987</td>
</tr>
</tbody>
</table>

*so-called homeland areas only. Abbreviations: NA = data not available;

Despite current popular use of medicinal plants and herbs for traditional medicine practices, there was a decline in its usage with the advent of modern medicine in most Western countries, during the industrialisation and urbanisation period, where a general rejection of traditional values and systems was observed. However, in the 1970’s, medicinal herbs became more popular in Europe, particularly in Germany and France, followed by North America, coinciding with the “greening” of society (Bannerman et al., 1983).
The financial benefits of medicinal plant trade are also visible in developing countries. In 1998, the indigenous plant trade in South Africa was nearly 20,000 tonnes and estimated to be worth 110 million US$ with 27 million South Africans being indigenous medicine consumers. During that time, 20–30,000 Kwazulu-Natal residents, mostly of rural habitation, gained an income from the trading of indigenous plants with the industry worth 10 million US$ at that time (Mander, 1998). In 2005, the global market share for plant-derived drugs was estimated to be worth 18 billion US$. By 2011, it has been projected to be more than 26 billion US$ (McWilliam, 2006). The US accounts for 50% of this market share (McWilliam, 2003).

2.1.3 Drug discovery

Despite the exploration of alternative techniques to drug discovery in the early 1980’s, such as molecular modelling, synthetic chemistry approaches, as well as combinatory chemistry applications, natural products, especially of medicinal plant origin, still contributed significantly towards the discovery of new drugs (Newman et al., 2003). In 2008, more than 100 natural product-derived compounds were undergoing clinical trials, as shown in Table 2.2 with at least another 100 estimated to be at a pre-clinical stage of development (Butler, 2008). From the data presented in Table 2.2, it is evident that more medicinal plants entered the pre-clinical developmental stage with only 2 reaching the pre-clinical stage. This comparatively lower progression rate into the pre-registration stage than semi-synthetic products, could mainly be attributed to the difficulties encountered with the access to medicinal plants, the relatively complex chemistry of natural products, and readily occurring intellectual property rights legal battles (Barker et al., 1995: Butler, 2004; Koehn and Carter, 2005), considerably slowing the process of working with natural products.

For pharmaceutical companies to invest in more drug discovery research and development using natural products, a continuous improvement of quality and quantity of natural products entering the developmental stage of drug discovery also needs to be addressed (Butler, 2004). This process of drug discovery has been estimated to be longer than 10 years (Reichert, 2003) with investments of approximately 800 million US$ required (Dickson and Gagnon, 2004).
Table 2.2 Drug development from natural products in 2008 (Butler, 2008)

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Plant</th>
<th>Bacterial</th>
<th>Fungal</th>
<th>Animal</th>
<th>Semi-Synthetic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-clinical</td>
<td>46</td>
<td>12</td>
<td>7</td>
<td>7</td>
<td>27</td>
<td>99</td>
</tr>
<tr>
<td>Phase I</td>
<td>14</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>Phase II</td>
<td>41</td>
<td>4</td>
<td>0</td>
<td>10</td>
<td>11</td>
<td>66</td>
</tr>
<tr>
<td>Phase III</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>Pre-registration</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
<td>25</td>
<td>7</td>
<td>24</td>
<td>61</td>
<td>225</td>
</tr>
</tbody>
</table>

Generally, the process of drug discovery from medicinal plants, as seen in Figure 2.2, commences by taking leads from traditional knowledge of medicinal plants (Cox and Balick, 1994; Balunas and Kinghorn, 2005), followed by various in vitro screening procedures, before more specific in vivo investigations are performed on more promising compounds. The optimization of the lead compound is then conducted using combinational chemistry and/or synthetic medical chemistry approaches and principles before the compound is further developed to address issues relating to toxicology, pharmacology, pharmacokinetics, drug delivery, as well as absorption, distribution, metabolism and excretion of drugs. Finally, human trials are employed to assess the effectiveness of the compound in the body (Balunas and Kinghorn, 2005).

Figure 2.2 The systematic approach of drug discovery and development from medicinal plants (Source: Balunas and Kinghorn, 2005).
2.1.4 Biological activities of medicinal plants

The biological activity of medicinal plants has attracted the research interest in recent years as a reasonably cost-effective and readily accessible means of treating various disease conditions (Mander, 1998) with possibly, longer lasting availability, depending on proper conservation mechanisms in place (Asfaw, 1998). This interest has resulted in assessing the scientific rational of what was previously seen as anecdotal claims of medicinal plants, in addition to the discovery of novel pharmaceutical drugs that have subsequently followed (Cox and Balick, 1994).

Common biological activities of medicinal plants range from antioxidant (Kahkonen et al., 1999), antibacterial (Eloff et al., 2005), antifungal (Masoko et al., 2005), antimutagenic (Jo et al., 2005), anthelmintic (Nahar et al., 2010) antimalarial (Onaku et al., 2011), anti-inflammatory (Manjamalai et al., 2011), anti-amoebic, antischistosomal, antiviral and anticancer (Akbar et al., 2011), as well as antidiabetic activities (Motlhanka and Mathapa, 2012), amongst a whole range of possible activities that medicinal plants are known to exhibit. These biological activities of plants are generally attributed to their secondary metabolites.

2.2 Secondary metabolites of plants

2.2.1 Introduction to secondary metabolites

The secondary metabolites from plants, often referred to as phytochemicals and xenobiotics, were initially considered to be of no significance. In fact, they were even considered to be merely waste or end products of primary metabolism. In recent years, however, their function and structure have been receiving the much deserved attention as a result of increasing interest in investigating and developing plants for medicinal, as well as a whole range of purposes for which they were utilised. Consequently, this perception changed and the metabolites which are actually generated through the secondary metabolic pathway, in particular, shikimate, acetate-malonate and acetate-mevalonate pathways (simplified in Figure 2.3) (Kuc and Rush, 1985; Tyler et al., 1988), were viewed as possessing the essential properties for the survival of the plants, amongst other vital cellular functions (Bennet and Wallsgrove, 1994).
For survival in nature, plants need protection from their hostile environment. Each metabolite, based on their chemical and physical structure, plays a pivotal role to shield the plant from a host of external attacks. Protection is constantly required against herbivores, insects (Ehrlich and Raven, 1967; Bennett and Wallsgrove, 1994), microbial infestation, ultra-violet exposure, and the consequential oxidative stress (Jansen et al., 1998; Wink and Schimmer, 1999). Additionally, the plant needs secondary metabolites to attract favourable insects for pollination (Langenhein, 1994), and even suppress neighbouring plant growth, in order to compete for shared resources (Brown, 1995; Inderjit, 1996). Furthermore, secondary metabolites play important roles in protecting plants against abiotic stresses, associated with changes in temperature, UV exposure, light levels, water status and levels of mineral nutrients, in addition to suggested roles in cell growth regulation, modulation of gene expression and signal transduction (Kaufman et al., 1999). Upon herbivory consumption, the secondary metabolite functions are commonly expressed in the consumer. For instance, if a plant possesses cytotoxicity towards a particular microorganism for survival, certain
secondary metabolites of this plant will have a similar response to pathogens that their consumers are exposed to. This remarkable feature provides the basis of how medicinal plants work (Wink and Schimmer, 1999).

All plants comprise carbohydrates, lipids, proteins, heme, chlorophyll, and nucleic acids which are involved in the primary metabolic processes of building and maintaining plant cells (Kaufman et al., 1999; Wink, 1999). These components are considered to be of primary essence to the existence of plants. They can thus not be used concretely to differentiate species or groups of plants from one another, whereas the combinations and presence of certain secondary metabolites are used to characterize plants taxonomically into species. This can be demonstrated by the similarities in medicinal or biological properties between plants of the same species (Wink, 1999). Microorganisms also possess secondary metabolites, employed in the same fashion as plant phytochemicals. Overall, more than 100,000 secondary metabolites have been characterized with approximately 50% contributed by microbes (Fenical and Jensen 1993; Berdy 1995). Their contribution to the development of antibiotics was a major highlight in the 1970's (Berdy 1995).

### 2.2.2 Classification of secondary metabolites

Secondary metabolites from plants are classified into three major groups based on their biosynthetic origins as seen in Figure 2.3. These groups are the terpenoids, phenolics and nitrogen containing compounds, referred to as alkaloids [Oxylipins] (Schmidt et al., 2005). Figure 2.4 graphically depicts the proportions of different classes of secondary metabolites, demonstrating that terpenoids make up more than half of secondary metabolites, followed by alkaloids, then phenolics (Zwenger and Basu, 2008). For the focus of this study, phenolics, including flavonoids, will be further discussed in section 2.2.3.

![Pie chart showing proportions of terpenes, alkaloids, and phenolics](image)

**Figure 2.4** Major secondary metabolite classes are represented in a pie chart (Zwenger and Basu, 2008).
2.2.2.1 Terpenes

Terpenes, also referred to as terpenoids or isoprenoids, are a large group of more than 40,000 lipid-soluble compounds. They originate from the repetitive fusion of branched 5-carbon isoprene units, through two possible pathways, namely the mevalonate and the deoxy-d-xylulose pathways (Rohmer, 1999; Withers and Keasling, 2007). Their classification is based on the number of isoprenoid units that they contain. Isoprenoid \((C_5H_8)\) is the building block unit of terpenoids and is classified as a hemiterpenoid. Two incorporated units of isoprenoid form monoterpenoids \((C_{10}H_{16})\), while sesquiterpenoids \((C_{15}H_{24})\) are made up of 3 units, diterpenoids \((C_{20}H_{32})\) by 4 units, sesterpenoids \((C_{25}H_{40})\) by 5 units, triterpenoids \((C_{30}H_{48})\) by 6 units and tetraterpenoids \((C_{35}H_{56})\) comprise of 8 units. Even higher order terpenoids, termed polyterpenoids, are possible through the condensation of more than 40 isoprenoid units (Rohmer, 1999). Figure 2.5 illustrates structures of selected terpenoids.

![Examples of terpenoid structures](image)

Figure 2.5 Examples of terpenoid structures: monoterpenoids (1,8-cineole and geraniol), sesquiterpenoid (valerenic acid), diterpenoid (ginkgolide A), and triterpenoid (ginsenoside Rg1) (Kennedy and Wightman, 2011).
Terpenoids, having enormous structural diversity, similarly demonstrate diverse functions in the cells of plants. So diverse are the functions that terpenoids double-up as primary metabolites, as well as secondary metabolites. Their primary metabolic functions range from the vital roles they play in the growth, development, reproduction and defence of plants (Wink and Van Wyk, 2008). A very important terpenoid, gibberellins which is a diterpenoid plant hormone, is involved in the control of seed germination, the elongation of the stem, as well as the induction of flowers (Thomas et al., 2005).

By far, the best known and most widely used terpenoid is rubber which is a polyterpenoid. With the addition of sulphur to rubber, Charles Goodyear created vulcanised rubber with varying degrees of flexibility, depending on the mixture ratio (Stiehler and Wakelin, 1947). Vulcanised rubber has since been extensively utilised in the making of tyres.

2.2.2.2 Phenolic compounds

Phenolics are a group of structurally diverse secondary metabolites (Wong, 1973) which are chemically classified as substances which possess aromatic rings with one or more hydroxyl groups, including their functional derivatives (Shahidi and Naczk., 2004). The majority of phenolics in plants originate from phenylalanine and, to a lesser extent in some plants, also from tyrosine (van Sumere, 1989; Shahidi, 2000, 2002). Phenylalanine and tyrosine are deaminated to cinnamic acids by the action of phenylalanine ammonia lyase. The cinnamic acids enter the phenylpropanoid pathway where, through the actions of various enzymes, coenzymes, and/or the introduction of one or more hydroxyl groups, phenolic compounds, such as, benzoic acids (C₆-C₁), flavonoids (C₆-C₃-C₆), proanthocyanidins [(C₆-C₃-C₆)n], coumarins (C₆-C₃), stilbenes (C₆-C₂-C₆), lignans (C₆-C₃-C₃-C₆) and lignins [(C₆-C₃)n] are formed (Seabra et al., 2006).

2.2.2.3 Alkaloids

Alkaloids are a group of nitrogenous compounds which are predominantly, but not exclusively, from flowering plants. The nitrogen is mainly derived from amino acids. Linguistically, the name “alkaloid” is derived from the Arabic word “al-qali” which was the plant from which soda as first obtained (Southon and Buckingham, 1989). Plants which had high concentrations of alkaloids were generally known to be highly toxic and were noticeably avoided by herbivores. These plants were universally bitter in taste, owing the property to their alkaloid contents (Kingsbury, 1964; Van Beek et al., 1990). Historical records of alkaloid
toxicity dates back as far as 399 BC when Socrates died after consuming a coniine-containing hemlock (*Conium maculatum*) (Scinto *et al*., 1994).

After the identification of the first alkaloid, morphine, from the opium poppy, *Papaver somniferum*, by Sertürner in 1806, considered by many as the determining point to pursue phytochemical screening for pharmaceutically important metabolites in plants, more than 12,000 alkaloids have been isolated and their structures elucidated (Caporale, 1995). At present, there is no common classification of alkaloids, although they have previously been categorised based on structural similarities or common precursors (Kennedy and Wightman, 2011). Their classification based on common precursors group alkaloids into terpenoid indole alkaloids, benzylisoquinoline alkaloids, tropane alkaloids and nicotine, and purine alkaloids.

2.2.3 Polyphenols in nutrition

The term polyphenols is widely used when discussing beneficial effects of food and beverages, therefore mentioning secondary metabolites of plants without featuring polyphenols, will be unreasonable. They are generally a diverse class of secondary metabolites (Goldberg, 2003; Stevenson and Hurst, 2007), named polyphenols because of their structures, comprising of several hydroxyl groups on two or more six-carbon aromatic rings (D'Archivio *et al*., 2007). Phenolic acids and phenolic alcohols are not polyphenols, but because of similar properties that they share with other polyphenols (Stevenson and Hurst, 2007), they have been included into the class of polyphenols (D'Archivio *et al*., 2007). Polyphenols are categorised in different classes depending on their structures and numbers of aromatic rings. The main groups are flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans (D'Archivio *et al*., 2007). Figure 2.6 illustrates the chemical structures of the main polyphenol classes and Table 2.3 summarizes the classes of polyphenols with nutritional contributions.
Flavonoids, the most abundant polyphenols are further subdivided, according to the degree of oxidation of the oxygen heterocycle, into subclasses of flavones, flavanones, flavanols, flavonols, anthocyanidins and isoflavones (D’Archivio et al., 2007). Quercetin, the major flavonol in human diets, is readily present in many fruits, vegetables and beverages. Particularly high concentrations were observed in onions (Hertog et al., 1992) and tea (Hertog et al., 1993). Often significant differences in concentrations of flavonols of the same fruits from different trees or even different sides of a single fruit have been observed. This is mostly attributed to the location of the flavonols inside the fruits, as flavonols accumulate closer to the surface of a fruit because their biosynthesis is highly dependent on sunlight (Price et al., 1995; D’Archivio et al., 2007).
Table 2.3 Summary of polyphenol classes, main representatives and sources of food (Adapted from Weichselbaum and Buttriss, 2010)

<table>
<thead>
<tr>
<th>Group</th>
<th>Sub-class</th>
<th>Main representative</th>
<th>Major sources of sub-class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Flavonols</td>
<td>Quercetin, myricetin, kaempferol</td>
<td>Onions, leeks, broccoli, celery, buckwheat, apples, apricots, variety of berries, tea, red wine</td>
</tr>
<tr>
<td></td>
<td>Flavones</td>
<td>Apigenin, luteolin</td>
<td>Parsley, celery, hot peppers, rosemary</td>
</tr>
<tr>
<td></td>
<td>Flavanones</td>
<td>Naringenin, hesperetin, eriodictyol</td>
<td>Citrus fruits: grapefruit, oranges, lemons, tomatoes, aromatic plants</td>
</tr>
<tr>
<td></td>
<td>Anthocyanidins</td>
<td>Cyanidin, malvidin, delphinidin, pelargonidin</td>
<td>Blackberries, blueberries, black grapes, strawberries, cherries, black currant, red wine, cabbage, beans, onions</td>
</tr>
<tr>
<td></td>
<td>Flavanols</td>
<td>Catechin, epicatechin, gallo catechin, epigallocatechin, epigallocatechin gallate</td>
<td>Apples, apricots, peaches, plums, raspberries, blackberries, cherries, cranberries, tea, chocolate, red wine</td>
</tr>
<tr>
<td></td>
<td>Isoflavones</td>
<td>Genistein, daidzein</td>
<td>Soya.</td>
</tr>
<tr>
<td>Phenolic acids</td>
<td>Hydroxylbenzoic acids</td>
<td>Gallic acid, protocatechuic acid</td>
<td>Blackberries, raspberries, tea</td>
</tr>
<tr>
<td></td>
<td>Hydroxycinnamic acids</td>
<td>Caffeic acid, ferulic acid, coumaric acid, sinapic acid</td>
<td>Coffee, blueberries, apples, cider, cereal brans, spinach, broccoli, citrus juices</td>
</tr>
<tr>
<td>Stilbenes</td>
<td>Resveratrol</td>
<td>Grapes, berries, peanuts, red wine</td>
<td></td>
</tr>
<tr>
<td>Lignans</td>
<td>Secoisolariciresinol, matairesinol, lariciresinol, pinoresinol</td>
<td>Linseed, grains, soybeans, beans, broccoli</td>
<td></td>
</tr>
<tr>
<td>Phenolic alcohols</td>
<td>Tyrosol, hydroxytyrosol</td>
<td>Virgin olive oil, red and white wines, beer</td>
<td></td>
</tr>
</tbody>
</table>

The main flavanols are catechins which are mostly abundant in teas (Hara et al., 1995). Other major sources of catechins are wines (Frankel et al., 1995) and chocolate (Arts et al., 1999). Anthocyanins are pigments of red fruits such as strawberries, cherries, raspberries and black currants. Their concentrations have been determined in these fruits (Clifford, 1996), as well as red wines (Frankel et al., 1995). Isoflavonones, reported to be present in soy (Reinli and Block, 1996), and flavanones, present in citrus fruits such as oranges (Rousseff et al., 1987), are restricted to very few foodstuffs.
The two most important phenolic acid subclasses in human diets are the hydroxybenzoic acids and hydroxycinnamic acids. The former are restricted to only a few edible plants, whereas the hydroxycinnamic acids have been reported to be the most abundant phenolic compound in typical (United Kingdom) UK diets, accounting for approximately half of the total polyphenol intake there (Stevenson and Hurst, 2007). Hydroxycinnamic acids are found in all parts of fruits with the highest concentrations in the outer parts of ripe fruits. The riper the fruits become, the lower the concentrations of hydroxycinnamic acids, but the total content increases again when the fruit increases in size (Manach et al., 2004). Hydroxycinnamic acids are further subdivided in major subclasses of p-coumaric, caffeic, ferulic, and sinapic acids. These acids are only found in free form in processed food that has been frozen, sterilized or fermented. Otherwise, they are present as glycosylated derivatives or esters of quinic acid, shikimic acid, and tartaric acid (Clifford, 1999). Caffeic acid is the most frequently encountered phenolic acid, representing 75-100% of hydroxycinnamic acids in most fruits (Manach et al., 2004), whereas ferulic acids are the most abundant phenolic acid in cereals, and accountable for up to 90% of total polyphenols in wheat grains (Sosulski et al., 1982; Lempereur et al., 1997).

Only a very small amount of edible plants contain lignans, stilbenes and phenolic alcohols (D’Archivio et al., 2007). Lignans which are made up of 2 phenylpropane units, are readily present in linseed, but trace amounts have also been reported in cereals, grains, as well as some fruits and vegetables. It must be noted that the contents reported in linseed were about 1000 times as high as the concentrations reported in the other sources (Adlercreutz and Mazur, 1997). Low concentrations of stilbenes, in the form of resveratrol, were reported in wine. Resveratrol is a subject of anticarcinogenic screening as activities were observed in medicinal plants (Bertelli et al., 1998; Bhat and Pezzuto, 2002; Vitrac et al., 2002). However, the small amounts in normal nutritional intake will most probably not be enough for making significant protective contributions (Manach et al., 2004).

2.2.4 Metabolism of secondary metabolites

2.2.4.1 Introduction

Once the secondary metabolites are ingested, considerable interactions take place with various tissues, enzymes and compounds within the body. The interactions can occur during absorption, deposition, metabolism, or excretion stages of its journey through the body. Sometimes, after ingestion, the secondary metabolites can pass through the body without any interactions, and are excreted in the same form, through urine, or after interaction with
bile salts, in the faeces. Other times, they are excreted, even though interactions took place with tissues and compounds within the gastrointestinal tract. The metabolites can, at instances, be absorbed directly or after transformation within the gastrointestinal tract and be absorbed then deposited for future usage. If absorbed, the secondary metabolites can be transformed into more hydrophilic compounds before being excreted though urine. The different modalities presented are dependent on the physico-chemical properties of the compounds, as well as the susceptibility of the compounds to transformation (Timbrell, 1992).

The physico-chemical properties of secondary metabolites are very critical in the fate of the original compound, and factors, such as the molecular size and architecture, pH of the environment, hydrophilicity, lipophilicity, charge and polarity, ability to form micelles, and solubility of the compound play an influential role (Timbrell, 1992; Cheeke, 1998; Harborne, 2001). It is worth noting the inter-dependence of these factors. For instance, smaller molecules have greater hydrophilicity, increasing their chances to be absorbed from the gastrointestinal tract (Acamovic and Brooker, 2005). Xenobiotic or drug metabolising enzymes, playing an essential role in the fate of secondary metabolites which enter the body, will be discussed later.

To a certain degree, many plant secondary metabolites may cause liver dysfunction or liver damage, a condition commonly referred to as hepatotoxicity. Hepatotoxicity is widely associated with an overload of drugs or xenobiotics (Navarro and Senior, 2006). Drugs or xenobiotics, responsible for hepatotoxicity are called hepatotoxins or hepatotoxicants (Willett et al., 2004). In some instances, hepatotoxicity may be due to the primary compound, while in other cases, as a result of a reactive metabolite or from immunologically-mediated responses affecting the liver (Deng et al., 2009). Hepatotoxicity could result in acute liver failure, or even death (Navarro and Senior, 2006). However, since most xenobiotic-induced liver injuries are reversible, a prompt detection of the hepatotoxic state, followed by the identification and removal of the offending agent or hepatotoxin is critical (Lee, 1995; Yang et al., 2012).

Hepatotoxicity can be clearly established and classified on the basis of clinical and biochemical evidence (Benichou, 1990), however, definitive diagnosis of the type of injury could only be provided through histological examinations of liver biopsies (Friedman et al., 1996). Establishing clinical symptoms, however, increases the risk of mortality, as hepatotoxicity symptoms often present at severe stages of injury (Lee, 1993). In contrast, the diagnosis and classification using biochemical analyses of serum alanine aminotransferase
(ALT), aspartate aminotransferase, alkaline phosphatase (ALP), and \(\gamma\)-glutamyl transpeptidase (GGT) activity, as well as total bilirubin and bile acids provide an earlier indication of liver injury (Yang et al., 2012). These markers are not essentially specific for liver injury, while some are more sensitive than others. ALT, primarily localised to the liver, is commonly used to evaluate hepatocellular injury. The enzyme activity is elevated due to liver necrosis; however, elevated ALT levels are not specific to liver injury. Heart and skeletal muscle injury also presents with elevated serum ALT. AST is known to be less specific than ALT. This enzyme is localised in the heart, brain, skeletal muscle, and liver tissue, and can be elevated due to liver or other extrahepatic tissue injury. ALP and GGT are markers of hepatobiliary injury, but being present in more tissues than ALT and AST, they are less liver injury-specific enzymes (Yang et al., 2012). Due to the low specificities of the serum liver indicators, more research is being conducted to discover novel serum markers. Approaches being pursued include potential markers based on genomics, proteomics, and metabolomics methodologies that have been identified, but currently pending qualification for intended use in the detection of liver injury (Yang et al., 2012).

2.2.4.2 Xenobiotic metabolising enzymes

Innate systems are in place for the control of toxins into the body. Studies were performed on cage animals which are dependent on the feed provided by scientist. According to Provenza et al. (1998), when animals ingest high amounts of toxins, their stomach lining cells are damaged and result in the release of serotonin, ultimately stimulating the nausea centre in the brain (Provenza et al., 1998). With nausea being a powerful stimulus, the animals learn to avoid the tastes and flavours associated with that compound. Additionally, other studies implicated the roles played by taste receptors to bitter tasting compounds, such as most alkaloids, where through evolutionary experiences, the animals developed a recognition mechanism, allowing them to link species with that specific bitter compound. This is also evident from herbivores which possess inhibition taste thresholds for tannins that are considerably greater than those for alkaloids (Margolskee, 2002; Shi et al., 2003). In another study by Wiggins et al. (2003), captive animals offered high concentrations of secondary metabolites as feed, eat very small amounts over a longer period (Wiggins et al., 2003). This suggested that the animals were innately lowering the pressure on their detoxification systems. A membrane-bound transport protein, permeability glycoprotein (P-gp), on the lining of the small intestine was observed to actively exclude certain drugs and secondary metabolites from being absorbed by rats (Sorensen et al., 2004; Green et al., 2004). These findings suggested that the P-gp proteins of the rats could selectively decide on which compounds to absorb and others to excrete directly.
There are two essential types of enzymes responsible for the metabolism of secondary metabolites, or any drug that enters the body. These enzymes are necessary for the detoxification of the metabolites and allow the body to excrete the foreign compound (Timbrell, 1992). Once the body is exposed, certain responses are triggered, resulting in an increase in gene expression of xenobiotic metabolising enzymes. This assists the body to “neutralise” the possible chemical assault associated with exposure to the xenobiotics (Rushmore and Kong, 2002). Nuclear receptors such as pregnane X receptors (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AHR) play an essential role in the transcriptional regulation of xenobiotic metabolising enzymes which will act either as Phase 1 or Phase 2 enzymes in the detoxification process.

The actions of xenobiotic metabolising enzymes, although in existence to protect the body from toxins, are also implicated in the biotransformation of exogenous metabolites into more toxic compounds than the original compound. The process is known as metabolic activation (Nebert 1997), and will be addressed later.

2.2.4.2.1 Phase 1 metabolising enzymes

Phase 1 metabolising enzymes oxidize, reduce or hydrolyze xenobiotics, introducing a polar functional group or unmasking of a polar functionality, making the compounds reactive for ultimate interactions with the Phase 2 enzymes. Phase 1 enzymes, either alone or in collaboration with Phase 2 enzymes, are primarily responsible for the conjugation of secondary metabolites and the generation of readily excretable metabolites (Low and Castagnoli, 1982). The major Phase 1 enzymes are the cytochrome P450 (CYP450) isoenzymes (Rendic and Di Carlo, 1997), comprising 70-80% of all Phase 1 xenobiotic metabolising enzymes (Evans and Relling, 1999). CYP450 enzymes are primarily responsible for monooxidising or reducing substrates, while epoxide hydrolases are responsible for the hydrolysis function. Hydroxylases, flavin-containing monooxygenases and monoaminoxidases are other phase 1 enzymes which add small molecules (e.g., OH- or O-groups) to their substrates (Zeigler, 1988; Daly et al. 1993; Nebert 1994).

CYP proteins were discovered in 1958 by Klingenberg and colleagues (Klingenberg et al., 1958). The proteins were further designated P450, due to the distinct spectrophotometric properties demonstrated by their pigments at 450 nm when reduced and bound to carbon monoxide (Omura and Sato, 1962). With different subtypes of these enzymes being purified and identified over the years, CYP450 has developed into a superfamily of microsomal
enzymes which are abundantly present in the liver, gastrointestinal tract, kidneys and lungs. The different families and subfamilies of these enzymes are classified according to their amino acid sequence identities and similarities (Nerbert and Gonzalez, 1987).

Molecular biology has been employed to systematically classify CYP450 enzymes, and a standard naming method has been developed (Nelson et al., 1993). Using the example of CYP3A4, “CYP”, is the abbreviation of cytochrome P450, followed by the number “3”, indicating the gene family name, a letter “A” representing the subfamily, then another number “4” referring to the gene (Nelson et al., 1996). Enzymes with 40% sequence identity or more are placed in the same family. Within this family, enzymes with more than 55% sequence identity will be included in the same subfamily. Within this subfamily, enzymes are given numbers at random. In order to have the same gene number the genes must have the same function and exhibit high conservation (Nelson et al., 1993; 1996).

In humans, CYP1, CYP2, CYP3 and CYP4 are suggested to be the major role players in hepatic, as well as extra-hepatic metabolism, and biotransformation of secondary metabolites and drugs, whereas CYP5-51 are more applicable to the metabolism of endogenous metabolites (Nelson et al., 1993). Studies suggest CYP1-3 is responsible for 70-80% of all Phase 1 dependent metabolisms of clinically relevant drugs (Bertz and Granneman, 1991; Evans and Relling, 1999) with CYP2D6 and CYP3A4 being involved in the metabolism of the majority of commonly used drugs. The CYP3A family accounts for approximately 30% of all microsomal species in the human livers with the widest substrate specificity of all CYP450 enzymes (Shimada et al., 1994). CYP3A4 is suggested to be involved in the metabolism of approximately half of all marketed drugs, in most part, due to its strategic location in the liver and small intestine where it serves in the barrier function of xenobiotics entering the circulation (Guengerich, 1999).

2.2.4.2.2 Phase 2 metabolising enzymes

Phase 2 metabolising enzymes usually detoxify secondary metabolites, but at times, also activate both endogenous and exogenous metabolites. Their main function is converting the metabolites into a more hydrophilic form by conjugating with, for instance, glutathione. If a compound is very lipophilic, it will first be metabolised by Phase 1 enzymes before the Phase 2 enzymes can act on them, but less lipophilic compounds are directly metabolised by Phase 2 enzymes. The main Phase 2 enzymes are categorised into superfamilies of sulfotransferases (SULTs) (Falany 1991; Matsui and Homma, 1994), UDP-glucuronosyltransferases (UDP-GTs) (Burchell et al., 1995; Mackenzie et al., 1997),
glutathione-S-transferases (GSTs) (Awasthi et al., 1994; Armstrong 1997), N-acetyltransferases (NATs) (Weber and Hein, 1985; Kadlubar, 1994) and various methyltransferases (Weinshilboum, 1989; Daly et al., 1993). Each of these enzyme superfamilies consists of families and subfamilies of genes which are encoding the different isoforms in terms of substrate specificity, tissue and developmental expression, as well as inducibility and inhibition by secondary metabolites (Rushmore and Kong, 2002).

### 2.2.4.2.2.1 Sulfotransferases

SULTs are a superfamily of Phase 2 enzymes which catalyse the transfer of sulfonate ($SO_3^-$) from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a hydroxyl or amino- group of compounds. The process of sulfonate conjugation was first reported in 1876 (Bauman, 1876). Initially, sulfonate conjugation by SULTs was reported as a detoxification pathway of xenobiotics and drugs, leading to the formation of water-soluble products which can be easily excreted. Since then, their involvement in other pathways has been reported, leading to metabolic activation of other xenobiotics producing highly reactive electrophiles that are both mutagenic and carcinogenic (Falany, 1997; Weinshilboum et al., 1997).

The SULTs superfamily is divided into two broad classes depending on their location in the cell, i.e. membrane-bound and cytosolic SULTs. The membrane-bound SULTs are located in the Golgi apparatus of the cell and are responsible for the sulfonation of peptides, proteins, lipids, and glycosaminoglycans, whereas their cytosolic counterparts are found in the cytosol, and responsible for the metabolism of xenobiotics and other small endogenous substrates (Falany, 1997; Negishi et al., 2001). Blanchard et al. further categorised mammalian SULTs in five families, i.e. SULT1, SULT2, SULT3, SULT4, and SULT5, where members of a family share at least 45% amino acid sequence identity, and subfamily members 60% and more (Blanchard et al., 2004).

### 2.2.4.2.2.2 UDP-glucuronosyltransferases

UDP-GTs are a family of membrane-bound multifunctional isoenzymes, catalysing the transfer of UDP-glucuronic acid (UDPGA) to countless structurally unrelated substances possessing hydroxyl-, amino-, carboxyl-, or sulfhydryl groups, converting them to water-soluble b-(D)-glucuronides (Fig 2.7) which can be readily excreted through the kidneys and/or bile (Meech and Mackenzie, 1997). Additionally, these isoenzymes have also been implicated in an important cytoprotective role where they either prevent the accumulation of toxic metabolites in the body or avoid the biotransformation of xenobiotics to more toxic reactive products (Vienneau et al., 1995; Green and Tephly, 1996). Therefore, it is apparent
that using UDP-GT inhibitors in experimental models will most probably result in the accumulation of toxins in the body. Another role was suggested in the control of endogenous signalling compounds such as hormones, when the direct inhibition of UDP-GTs by xenobiotics affected steroid glucuronide production. Consequently, the expectant tissues were deprived of the pharmacological activities of the impaired hormone production (Burchell and Coughtrie, 1997).

![Figure 2.7 Schematic diagramme of UDP-GT reactions (Ritter, 2000).](image)

UDP-GT activities of different tissue origins are inhibited by various endogenous and exogenous compounds. Endogenous inhibitors range from hormones, i.e. testosterone and ethynyl estradiol (Herber et al., 1992), long-chain acyl-CoAs, i.e. myristoyl-CoA to arachidonoyl-CoA (Yamashita et al., 1997), to nucleotides such as UDP and UTP (Yokota et al., 1998).

UDP-GTs are predominantly located in the endoplasmic reticulum of liver cells but can also be found in a few other mammalian tissues (Battaglia and Radominska-Pandya, 1998). Their concentrations are always highest in microsomal preparations of the liver, kidneys and intestines, but the exact amounts of UDP-GT in any tissue at any time depends on factors such as age, gender, hormonal status, genetic factors, and environmental exposures (Mackenzie et al., 1997). Using molecular cloning approaches, UDP-GTs are categorised into two large families with less than 50% amino acid identity between them. They are designated UDP-GT1 and UDP-GT2. Nine isoenzymes of UDP-GT1 origin are known in humans (UDP-GT1, UDP-GT3-10) (Ritter et al., 1992; Mackenzie et al., 1997), whereas seven isoenzymes have been characterised in rats (UDP-GT1A1-1A3, UDP-GT1A5-1A8) (Emi et al., 1995).
2.2.4.2.3 Glutathione-S-transferases

GSTs belong to the family of Phase 2 enzymes responsible for the detoxification of xenobiotics by catalysing the conjugation of glutathione (GSH) to these electrophilic or alkylating compounds through thioester linkages, thereby rendering the compounds hydrophilic for excretion in the bile and/or urine (Boyland and Chasseaud, 1967). Additionally, a number of endogenous molecules such as prostaglandins (Bogaards et al., 1997), steroids (Barycki and Colman, 1997), urocaric acid (Shimizu and Kinuta, 1998) [a histidine metabolite] are known to be metabolised via glutathione conjugation and also excreted from the body. Under normal circumstances, the glutathione conjugate is excreted immediately through the bile or transported to the kidneys where gamma-glutamyltranspeptidases are employed to cleave the gamma-glutamyl moiety, dipeptidases split the glycine off, and the remaining cysteine is N-acetylated to be excreted as a mercapturic acid (Chasseaud, 1979). Sometimes, instead of N-acetylation, the cysteine conjugate can undergo other metabolic pathways which may lead to bioactivation.

In humans, GSTs are categorized into 3 main families, namely, cytosolic, mitochondrial and membrane-bound microsomal GSTs. The microsomal family of GSTs is designated as “membrane associated proteins in eicosanoid and glutathione metabolism” (MAPEGs). They are structurally different to the cytosolic GSTs, but are functionally similar in their ability to catalyse the conjugation of GSH to electrophilic compounds (Hayes et al., 2005).

GSTs are further divided into 8 classes, based on the difference in amino acid sequence identities, where the differences from one class to another is determined by more than 40% difference in amino acid sequence identity. The classes are designated as: alpha, kappa, mu, omega, pi, sigma, theta and zeta (Parl FF, 2005). GST subfamilies identified in mammalian species are alpha (Addya et al., 1994), mu (Raza et al., 2002), kappa (Harris et al., 1991) and theta (Harris et al., 1991).

2.2.4.2.3 Pharmacogenetics of xenobiotic metabolising enzymes

The study of genes started early in the 20th century, first prompted by the observation that a variation of an inherited deficient gene caused an inability to taste phenylthiourea (Snyder, 1932). This episode was followed by the observation of adverse reactions caused by the antimalarial drug primaquine, especially in black soldiers during the Second World War, as a consequence of a deficiency in glucose-6-phosphate dehydrogenase enzymes (Carson et al., 1956). While investigating cases similar to the two mentioned above, the concept of
pharmacogenetics was “born” and the term was coined by Friedrich Vogel as the role of genetics in drug response (Vogel, 1959). Today, pharmacogenetics is widely employed to understand the expression of xenobiotic metabolism enzymes’ genes in individuals, as well as the process following the induction of the enzymes when the body is exposed to endogenous or exogenous metabolites.

Variability in response to any xenobiotic or drug that enters the body can be caused at any point, from the time the metabolite enters the body to the excretion in the bile or urine. During the different steps, transport proteins, pumps, carrier molecules and other enzymes, which are all participating in the process from start to finish, can demonstrate pharmacogenetic variability (Pelkonen and Ruskoaho 1998). Additionally, more pharmacogenetic alterations in xenobiotic metabolising enzymes have been observed since the detection of polymorphic acetylation (Nebert 1994; Nebert 2000). Perhaps the breakthrough in pharmacogenetic alterations of xenobiotic metabolising enzymes was observed during the discovery of defective debrisoquine 4'-hydroxylation which was later demonstrated to be due to polymorphisms of CYP2D6, responsible for the metabolism of more than 30 drugs (Daly et al., 1996).

The factors influencing the expression and activity of xenobiotic metabolising enzymes are, for purposes of easier understanding, divided into three major groups consisting of genetic factors, non-genetic host factors (such as age, sex, physical condition, stress, obesity etc) and environmental factors (Pelkonen, 1992). Often, it is observed that there is a significant amount of interplay between these factors. The extent to which any one of the factors affect the activity of xenobiotic metabolising enzymes depends on the particular enzymes, the specific individual and the specific factor. This makes the regulation of xenobiotic metabolising enzymes extremely complex.

2.2.4.2.4 Role of xenobiotic metabolising enzymes in bioactivation

Xenobiotic metabolising enzymes, both Phase 1 and Phase 2, are primarily responsible for the conversion of reactive electrophilic compounds to non-reactive hydrophilic compounds that can be excreted through bile and/or urine. Under normal circumstances, the conversion of a xenobiotic from a lipid to water soluble form, results in the loss of biological activity, meaning “neutralization” has occurred. This is generally the principle of the detoxification process (Gonzalez, 1990; Nelson et al., 1996, Rendic and Di Carlo, 1997; Nebert and Russell, 2002;; Rendic, 2002). However, in other instances, foreign compounds are
converted to more cytotoxic, mutagenic and carcinogenic products (Green and Tephly, 1996), and are commonly referred to as bioactivation or metabolic activation.

Bioactivation is normally coupled with bioinactivation, where xenobiotic metabolising enzymes, whether of Phase 1 or Phase 2 origin, can cause the bioactivation. Either, in most cases Phase 2 (if Phase 1 was the cause), can inactivate the metabolite. However, if the process is uncoupled, the reactive metabolites react with cellular proteins, lipids or nucleic acids, consequently leading to protein dysfunction, lipid peroxidation, DNA lesions and oxidative stress. Additionally, disruptions of the ionic gradient and intracellular calcium stores, due to reactions of reactive metabolites, may result in loss of mitochondrial function and energy production which can possibly result in apoptosis and organ failure (Park et al., 2005). The electrophilic metabolites also readily bind to glutathione, which is an antioxidant in the circulation. This process occurs with or without the assistance from GSTs, and is regarded as one of the first line defences in case bioinactivation is not following the bioactivation (Coles et al., 1988). The cells can also sense reactive metabolites through transcriptional factors and signalling proteins such as AP-1, NF-κB, Nrf2, PPAR-gamma and other nuclear receptors.

In Phase 1 metabolism, enzymes such as CYP1A1, 1A2, 1B1, 2A6, 2B6 and 3A4 have been identified to regularly catalyse the metabolic activation of procarcinogens (Guengerich and Shimada, 1991; Green and Tephly, 1996; Guengerich, 2001). CYP1A and 1B enzymes have previously been observed to bioactivate polycyclic arylamines, polynuclear aromatic hydrocarbons and aflatoxin B1 (Gonzalez and Gelboin, 1994). These enzymes are also considered to be targets for blocking of tumour initiation. Therefore, it has been suggested that selecting the correct inhibitors or inactivators for these enzymes could be beneficial for preventing tumour formation (Guengerich and Shimada, 1991; Shimada et al., 1996). Thus the role played by xenobiotic metabolising enzymes in chemoprevention will next be discussed.

2.3 Chemical Carcinogenesis

2.3.1 Mutations and Cancer

Mutations are alterations in the DNA structure, leading to permanent changes in the genetic information encoded in the DNA (Nelson and Cox, 2000). Individuals differ in their susceptibility to cancer and birth defects. Genetic polymorphisms such as oncogenes, tumour suppressor genes and mutations in the xenobiotic metabolising enzymes are often implicated in this case (Vainio and Husgafvel-Pursiainen, 1996; Spivack et al., 1997).
Inherited genetic factors are implicated to be responsible for only 15% of human cancers (Lichtenstein et al., 2000). Variability on the genome can occur at a genetic level, as mutations, deletions, insertions and DNA adducts, or chromosomes, as sister chromatid exchanges. In fact, three levels of mutational changes have been identified, (1) gene mutations often referred to as point mutations are generally either single base modifications, or an insertion or deletion of bases, (2) chromosomal mutations characterised by large deletions or rearrangements of DNA with these two levels of mutations being a result of unrepaired or erroneously repaired DNA damage and (3) gaining or losing of complete chromosomes (Wallace et al., 1996; Mortelmans and Zeiger, 2000). Gene and chromosomal mutations are often implicated in carcinogenesis with an estimated 83% correlation between mutagenicity and carcinogenicity (Ramel, et al., 1986).

Some of these variabilities may be associated with interindividual differences in susceptibility to toxicity and tumour initiation (Pelkonen and Ruskoaho, 1998). However, cancer induction by environmental factors has been indentified as the major route of cancer initiation in humans. Epidemiological studies suggest that environmental factors contribute to more than 65% of all human cancers (Doll and Peto, 1981; Ames et al., 1987). Therefore, both endogenous and exogenous sources of carcinogens contribute to carcinogenesis.

2.3.2 Multi-step carcinogenesis

Carcinogenesis is both a multi-step and multi-stage process where multiple molecular and cellular events transform normal cells to malignant neoplastic cells. Experimental data suggest that the carcinogenesis process comprise of three steps, i.e. initiation, promotion and progression as illustrated in Figure 2.8 (Armitage and Doll, 1954; Pitot and Dragan, 1994; Surh, 1999; Klaunig and Kamendulis, 2004).

During the initiation step, the normal cells undergo unrepaired DNA damage and synthesis, producing mutated cells (Klaunig and Kamendulis, 2004). These cells are also referred to as initiated cells, and can be formed through either interaction with physical or chemical carcinogens. Physical carcinogens include UV light and radiation, while chemical carcinogens are genotoxic agents that possess DNA damaging or mutagenic properties. The resultant mutations are passed on to a clone of daughter cells, through repeated cell divisions. Changes during the initiation step remain at the genetic level, as they are dependent on cell division, and are irreversible (Klaunig and Kamendulis, 2004).
After the cells are initiated, the genotoxic agents, as well as other endogenous physiological compounds determine the clonal growth of the initiated cell through tumour promotion. The promotion stage is a reversible process, leading to clonal expansion of initiated cells and the formation of actively proliferating preneoplastic cells (Klaunig and Kamendulis, 2004). These cells are less responsive to cellular signals of homeostasis, including normal growth. More mutations occur to proto-oncogenes and tumour suppressor genes, resulting in progressive loss of differentiation and regulated growth. Proto-oncogenes are normally expressed to facilitate increased cellular proliferation at critical stages in the development or function of tissues. However, when inappropriately activated within the mammalian genome, they transform to oncogenes, leading to increased cellular proliferation. For instance, mutations of p53, a tumour suppressor gene, regulating cell proliferation, as well as mediating programmed cell death (apoptosis) in response to unreppaired DNA damage, can lead to uncontrolled proliferation of cells containing DNA damage, whilst not undergoing apoptosis (Klaunig and Kamendulis, 2004).
The final step in carcinogenesis is the progression phase where the formation of neoplastic cells or neoplasms is observed. These neoplasms are known by their increased growth rates, invasiveness, metastatic properties, as well as hormonal responsiveness. This step is irreversible with demonstrable changes in the cell genome (Klaunig and Kamendulis, 2004).

2.3.3 Carcinogens

Various approaches have been taken to classify carcinogens, whether according to chemical structures, origins or mutagenic potentials in humans. Two of the most widely used carcinogen classification bodies are the National Toxicology Programme (NTP) and the International Agency for Research on Cancer (IARC). These two programmes are complementing each other, and classify carcinogens based on whether their exposures pose carcinogenicity in humans, and even the degree of carcinogenicity. Classification can also be done based on the origins of carcinogens, or the type of exposure that culminates in carcinogenesis. A series of IARC reports grouped carcinogens for discussions under ‘Part A: pharmaceutical agents’, ‘Part B: biological agents’, ‘Part C: metals, arsenic, dust, fibres’, ‘Part D: radiation’, ‘Part E: tobacco, areca nut, alcohol, coal smoke, and salted fish’, and ‘Part F: chemical agents and related occupations’.

2.3.4 Sources of carcinogens

The prevalence and incidence of cancer varies significantly from one geographical region to another. This is more noteworthy when looking at the differences in the incidence of cancers between developing and developed world countries (Jemal et al., 2011). These differences indicate that environmental factors are to a great extent responsible for the pathophysiology of cancer. A multitude of exogenous factors from our environments, including biological, chemical and physical agents have been implicated to be responsible for cancer development. Endogenous risk factors, in particular, oxidative stress which has received a great deal of interest in past 30 years, has also been identified to be a cause of cancer (Jemal et al., 2011).

2.3.4.1 Exogenous sources

The concept of exogenous carcinogenesis was first observed in 1775 when a London surgeon, Percivall Pott, commented on the occurrence of scrotal skin cancer in chimney sweeps. At that time, the idea was novel. However, his recommendations that chimney sweeps bathe on a daily basis to avoid scrotal cancer development was implemented 3 years later by Danish sweeps (Macdonald, 1957). The results were evident a century later
as studies on scrotum cancer in chimney sweeps revealed that protective clothing and regular bathing prevented scrotal cancer in chimney sweeps (Butlin, 1892). Today, Potts’ comments are regarded as pioneering to the understanding of chemical carcinogenesis. Since then, a range of human carcinogens have been identified.

2.3.4.1.1 Viruses and other microorganisms

It is estimated that infectious agents of biological origin are responsible for 20% of human cancers (Zur Hausen, 2006) with 16% of cancers worldwide due to oncogenic viruses (Pisani et al., 1997). Less than 10% of cancers due to oncogenic viruses are observed in high income countries, whereas up to 25% incidences of oncogenic virus-associated cancers have been observed in African countries (Parkin et al., 2001; Talbot and Crawford, 2004). Table 2.4 shows three groups of double stranded DNA viruses and two groups of diploid RNA viruses which are associated with human cancers. Globally, hepatitis B virus (HBV) and hepatitis C virus (HCV) infect 300 million and 170 million people, respectively, mainly in Africa and Asia. Chronic infections with these viruses lead to hepatocellular carcinoma (IARC, 1994a). Sufficient evidence further indicates that chronic HCV infections can subsequently lead to non-Hodgkin lymphomas, in particular B-cell lymphomas (Hermine et al., 2002). In the developing world, human papilloma virus (HPV), especially HPV type 16, associated with cervical cancer, and HBV are regarded as the most frequently occurring oncogenic viruses (IARC, 1995a; IARC, 1997).

Table 2.4 Human oncogenic viruses (Adapted from Belpomme et al., 2007)

<table>
<thead>
<tr>
<th>Virus family</th>
<th>Virus</th>
<th>Human tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papaviridae</td>
<td>Human papilloma virus</td>
<td>Cervical cancer, Anogenital cancer, Skin cancer</td>
</tr>
<tr>
<td>Herpesviridae</td>
<td>Epstein-Barr virus</td>
<td>Nasopharyngeal carcinoma, Burkitt’s lymphoma, Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>Retroviridae</td>
<td>Kaposi’s sarcoma-associated herpes virus</td>
<td>Kaposi’s sarcoma, primary effusion lymphoma</td>
</tr>
<tr>
<td></td>
<td>Human T-cell lymphotropic virus type 1</td>
<td>Adult T-cell Leukaemia</td>
</tr>
<tr>
<td></td>
<td>Human Immunodeficiency Virus</td>
<td>B-cell lymphoma, Kaposi’s sarcoma</td>
</tr>
<tr>
<td>Hepadnaviridae</td>
<td>Hepatitis B virus</td>
<td>Liver cancer</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Hepatitis C virus</td>
<td>Liver cancer</td>
</tr>
</tbody>
</table>

Epstein–Barr virus (EBV) is another prominently occurring oncogenic virus capable of causing several types of cancer, including nasopharyngeal carcinoma (one of the most common cancers in southeastern Asia) and Burkitt’s lymphoma in African children. Furthermore, EBV infection is linked to 5-10% of gastric carcinomas worldwide (Zur Hausen,
2006). It has also been reported that EBV is associated with Hodgkin’s disease, as well as non-Hodgkin lymphoma in immuno-compromised patients (IARC, 1997; Griffin, 2000). Human immunodeficiency virus (HIV) also has oncogenic roles in humans. It was established that in the presence of the human herpes virus type 8 (HHV-8), HIV is associated with Kaposi sarcoma (De The, 1995; IARC 1996; IARC 1997; Pagano et al., 2004). Epidemiological studies have strongly linked the occurrences of various cancers with HIV. Even though HIV is not an oncogenic virus on its own, it is able to promote the initiating effect of oncogenic viruses through immunosuppression induction (IARC, 1996). It has been estimated that HIV-infected children have a 10-40-fold increased risk of cancer (Pollock et al., 2003).

Non-viral biological agents are also associated with cancer incidences, but not as much as viral agents. *Helicobacter pylori* infections are associated with gastric cancer (IARC, 1994b), while the prevalence of infections with *Opisthorchis viverrini* and *Clonorchis sinensis*, two endemic liver flukes in north-eastern Thailand and south-eastern Asia, respectively, correlates with the incidence of cholangiocarcinoma (Honjo et al., 2005; Choi et al., 2006). *Schistosoma haematobium*, an endemic worm in most African countries and the eastern Mediterranean region, has been linked with urinary bladder cancer, and thus also classified as carcinogenic to humans (IARC, 1994b).

**2.3.4.1.2 Radiation**

Generally, it is speculated that radiation contributes to 10% of all human cancers. However, it is worth mentioning that this estimation needs refining, as there is no clear assessment of this population attributable risk. Most of the ionising radiation assessments are based on incidences of cancer following medical irradiations or the exposure to radiation following the atomic bombs at Hiroshima and Nagasaki (UNSCEAR, 2000). Investigations to determine radiation exposure were initially limited to workers, occupationally at risk. These included radiologists, underground miners and radium dial painters. Thereafter, investigations were targeted towards survivors of the atomic bombs during the last world war where, an increase in malignancies was observed (Preston et al., 2004). Furthermore, investigations continue to be carried out more frequently in patients receiving radiotherapy (Belpomme et al., 1974), as well as radiation technologists, uranium miners and nuclear industry workers (UNSCEAR, 2000).

Ionising and non-ionising radiation are the two types primarily associated with human cancers, including some leukemias and lymphomas, thyroid cancers, skin cancers, some
sarcomas and lung and breast carcinomas (Wakeford, 2004). Table 2.5 summarises the different radiation types and the associated tumour and/or sites.

Ionising radiation comprises of alpha and beta particles. Alpha particles consist of two protons and two neutrons and are considered a densely ionising type of radiation, even though they have a low capacity to penetrate living tissues. On the other hand, the beta particles are composed of electrons or positrons that are less ionising, but have a greater penetration capacity (ICRP, 1991). The health hazards associated with exposure to these particles largely occur after internal depositions. Evidence from epidemiological investigations demonstrated that alpha and beta particles which are emitted by some radionuclides increase cancer at several anatomical sides (Cardis et al., 2006). X- and gamma-rays, as well as neutron radiations are also classified as ionising radiation, and penetrating tissues in a similar fashion as the alpha and beta particles (Preston et al., 2004; Wakeford, 2004).

Table 2.5 Radiation type and associated tumours or anatomical sites (Adapted from IARC, 2009d)

<table>
<thead>
<tr>
<th>Radiation type</th>
<th>Tumour/Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha particles and beta particle emitters</td>
<td></td>
</tr>
<tr>
<td>Radon-222 and decay products</td>
<td>Lung</td>
</tr>
<tr>
<td>Radium-224 and decay products</td>
<td>Bone</td>
</tr>
<tr>
<td>Radium-226 and -228 and decay products</td>
<td>Bone and paranasal sinus</td>
</tr>
<tr>
<td>Thorium-232 and decay products</td>
<td>Liver, extrahepatic bile ducts, gall bladder and leukaemia (excl. CLL)</td>
</tr>
<tr>
<td>Plutonium</td>
<td>Lung, liver and bone</td>
</tr>
<tr>
<td>Phosphorus-32</td>
<td>Acute leukaemia</td>
</tr>
<tr>
<td>Fission products, including strontium-90</td>
<td>Solid cancers and leukaemia</td>
</tr>
<tr>
<td>Radioiodine, including iodine-131</td>
<td>Thyroid</td>
</tr>
<tr>
<td>X-radiation or gamma-radiation</td>
<td>Salivary gland, oesophagus, stomach, colon, lung, bone, skin (BCC), female breast, urinary bladder, brain and CNS, leukaemia (excluding CLL), thyroid, kidney (atomic-bomb survivors, medical patients); multiple sites (in-utero exposure)</td>
</tr>
<tr>
<td>Solar radiation</td>
<td>Skin (BCC, SCC, melanoma)</td>
</tr>
<tr>
<td>UV-emitting tanning devices</td>
<td>Skin (melanoma), eye (melanoma, particularly choroid and ciliary body)</td>
</tr>
</tbody>
</table>

Abbreviations: CLL=chronic lymphocytic leukaemia. BCC=basal-cell carcinoma. SCC=squamous-cell carcinoma

All types of ionising radiation transfer energy in the form of highly structured ionisation and excitation processes that can produce several molecular lesions and DNA damage.
Thereafter, the affected cells respond to the damage through processes such as apoptosis, chromosomal aberrations, mutations, genomic instabilities and cell transformations amongst others which all contribute to carcinogenesis (Wakeford, 2004). Based on these characteristic mechanisms that all types of ionising radiation have in common, they have been classified as “carcinogenic to humans” by a special working group on radiation associated carcinogenesis (IARC, 2009d).

Exposure to radon and its decay products have been identified to be the major sources of ionising radiations in homes and workplaces alike (Akerblom, 1999). Studies before and after this finding determined a correlation between radon exposure and lung cancers in humans, even suggesting that radon exposure at low levels is responsible for approximately 10% of lung cancers (Lubin and Boice, 1997; Darby et al., 2005).

2.3.4.1.3 Xenochemicals

During the second half of the last century, an industrial revolution occurred, requiring aggressive considerations to cope with increased needs for transport, energy, food, agriculture and health. While addressing these needs, millions of synthetic chemicals were produced and introduced into the environment. Subsequently, according to the European commission, more than 100,000 chemicals were marketed without enough toxicological control. This persistently exposed our immediate environments with pollutants and toxins, through the air, food, water and soil. Many of these chemicals are considered to be mutagenic and/or carcinogenic (Clapp et al., 2005).

2.3.4.1.4 Pharmaceuticals

Chemicals utilised for the production of oral contraceptives and hormone replacement therapies, as well as anti-estrogens, are the most widely studied human carcinogens of pharmaceutical origin (Beral, 2003). IARC reports from 1999 found no evidence of any association between oestrogen-only therapy and human cancers (IARC, 1999), however, in the 2009 Work Group meeting, the IARC scientists declared oestrogen-only therapy as carcinogenic to humans (IARC, 2009a). This was based on reports of increased risks of ovarian cancer in women who are on oestrogen-only treatments (Zhou et al., 2008), as well as a meta-analysis study showed a dose-dependent increase in the risk of ovarian cancer when using oestrogen-only therapy (Greiser et al., 2007).
Other investigated carcinogens in the pharmaceutical industry are anticancer chemotherapeutic agents. Due to their mutagenic nature, these agents can cause the late onset of secondary cancers in apparently cured cancer patients (Etiemble et al., 1979; Zahm and Devesa, 1995). The benefit to toxicity ratio for these agents is usually high, justifying their use within the oncology settings (Belpomme et al., 2007).

### 2.3.4.1.5 Occupational cancers

Occupational cancers represent 2-10% of all cancer incidences, however, this estimate can increase to 15-20% in men. These estimates were suggested by The Harvard Centre for Cancer Prevention (HCCP) when they classified 32 industries or substances to be carcinogenic to humans (HCCP, 1996). Subsequent studies by other groups classified 28 agents as definite occupational carcinogens, 27 as probable occupational carcinogens and 113 as possible occupational carcinogens (Siemiatycki et al., 2004; Clapp et al., 2005). Occupational cancers are often discussed as only being induced by chemicals in the workplace, but cancers caused by radiation and biological agents, as well as other physical agents such as metals, dusts, and fibres at the workplace, strictly speaking, should also be included in this category.

### 2.3.4.1.6 Metals, arsenic, dust and fibres

Several types of exposure to arsenic oxide, resulting in various types of cancer, have been reported. Inhalation of this compound has been linked to lung cancer, whereas the ingestion has been associated with bladder, kidney, liver and lung cancers (Szymanska-Chabowska et al., 2002). Exposure to arsenic could be in the workplace through processes such as arsenic production, glass manufacturing, wood preservation, and the production and use of arsenic-based pesticides (IARC, 2009c). Non-occupational exposures, through food and water, are also possible (IARC, 2004).

Other metals such as lead, nickel, beryllium, cadmium and chromium have also been associated with lung cancer, with nasal cavity and paranasal sinus cancers linked to nickel compounds (IARC, 2009c). The mechanisms of action for metals and metalloids are not adequately addressed. Metals and metalloids could act as co-carcinogens by either activating the procarcinogens in the liver (Hayes, 1997; Cantor et al., 2006), or by accentuating the promoting effect of estrogens (Gurpide et al., 1984). Even though several possible mechanisms to carcinogenicity exist, it has been proposed that the metals’
inhibition of the zinc finger in DNA repair proteins can culminate into carcinogenesis and is regarded as a novel mechanism in carcinogenesis (Witkiewicz-Kucharczyk and Bal, 2006).

Some cancers are regulated by removing the causative agents from the workplace, such as the classical case with asbestos which was estimated to be responsible for 10% of lung cancers. Asbestos was recognised as a carcinogen about a century ago in the UK, but was only legally abolished in Europe less than 30 years ago (IARC, 1977; IARC, 2002). Yet, it has been reported that globally, 125 million people are still exposed to asbestos in the workplace (WHO, 2006) with an increase in asbestos use in Asia, South America and Eastern Europe (LaDou, 2004). Other factors increasing asbestos levels in the environment include, naturally occurring sources of asbestos, its use in brake linings and the deterioration of asbestos-containing products (Anderson et al., 1979). While some occupational cancers are well declared and classified, others like the wood-related cancers are still insufficiently (ethmoid cancers) or not yet declared (sinus cancers). This could be due to the limited incidence of these cancers in carpenters (Hayes et al., 1986; Blot et al., 1997). However, IARC has acknowledged wood-related cancers as occupation cancers (IARC, 1995b). The tumour sites and types of cancers caused by the exposure to carcinogenic metals, arsenic, fibres and dusts such as silica, leather and wood, are summarized in Table 2.6.

Table 2.6 Summary of carcinogenic metals, arsenic, dust and fibres, and the tumours / sites of cancers caused by their exposure in humans (Adapted from IARC, 2009c)

<table>
<thead>
<tr>
<th>Carcinogenic metals, arsenic, dust and fibres</th>
<th>Tumours / sites of cancers with sufficient evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic and inorganic arsenic compounds</td>
<td>Lung, skin and urinary bladder</td>
</tr>
<tr>
<td>Beryllium and beryllium compounds</td>
<td>Lung</td>
</tr>
<tr>
<td>Cadmium and cadmium compounds</td>
<td>Lung</td>
</tr>
<tr>
<td>Chromium (VI) compounds</td>
<td>Lung</td>
</tr>
<tr>
<td>Nickel compounds</td>
<td>Lung, nasal cavity, and paranasal sinuses</td>
</tr>
<tr>
<td>Asbestos fibres</td>
<td>Lung, mesothelioma, larynx and ovary</td>
</tr>
<tr>
<td>Erionite</td>
<td>Mesothelioma</td>
</tr>
<tr>
<td>Silica dust</td>
<td>Lung</td>
</tr>
<tr>
<td>Leather dust</td>
<td>Nasal cavity and paranasal sinuses</td>
</tr>
<tr>
<td>Wood dust</td>
<td>Nasal cavity and paranasal sinuses and nasopharynx</td>
</tr>
</tbody>
</table>
2.3.4.1.7 Chemically-induced occupational cancer

Other well declared occupational cancers are caused by solvents, paints, dyes and other petroleum products. Since the leukemogenic effect of benzene was first described (Goguel et al., 1967; Surralles et al., 1997), mutagenic effects of other solvents such as trichloroethylene and perchloroethylene have been observed. Trichloroethylene has been strongly associated with kidney, liver and oesophageal cancers, as well as non-Hodgkin’s lymphoma (IARC, 1995c; ASTDR, 1997; Wartenberg et al., 2000; Hansen et al., 2001; Wartenberg and Siegel Scott, 2002; Raaschou-Nielsen et al., 2003). Perchloroethylene has been linked with oesophageal cancers (Ruder et al., 1994; Weiss, 1995).

In 2009, the IARC workgroup reviewing carcinogenic chemicals of occupational interest, classified human carcinogenic chemicals which have genotoxicity as the main mechanism. The carcinogenic chemicals were broadly divided into aromatic amines, polycyclic aromatic hydrocarbons (PAHs), other chemicals and complex exposures (IARC, 2009f). Generally, exposure to aromatic amines was prominently linked to urinary bladder cancers. In this case, benzidine and dyes that are metabolised to benzidine, 4-aminobiphenyl, 4,4’-methylenebis(2-chloroaniline) and 2-naphthylamine, providing strong evidence of genotoxicity as the main mechanism in this carcinogenesis process. Ortho-toluidine and two industrial related processes of auramine and magenda production demonstrated moderate to weak mechanistic evidence of genotoxicity in workers (Cantor et al., 2006; Baan et al., 2008). The exposure to PAHs causes skin and lung cancers in humans. PAH-related industries and chemicals were confirmed as being carcinogenic in humans, often based on strong evidence in various animal species and sufficient mechanistic evidence. In some instances, due to the diversity and complexity of these exposures such as aluminium production, other mechanisms than genotoxicity may also be relevant in the classification as human carcinogens (Xue and Warshawsky, 2005).

Dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) was classified as carcinogenic to humans in 1997, supported by limited evidence of carcinogenicity in humans (IARC, 2009f). However, mechanisms for strong evidence in humans and animals through initial binding to the aryl hydrocarbon receptor, leading to changes in gene expression, cell replication and programmed cell death, justified the classification as human carcinogen (Nebert et al., 2000). Currently enough epidemiological proof has surfaced for all cancers combined. This makes TCDD the first agent to be initially classified as a human carcinogen, based on sufficient evidence in animals and mechanism, and only confirmed at a later stage in humans (IARC, 2009f). Mechanistic information therefore is regarded as robust evidence of
carcinogenicity. Similarly, 3,3′,4,4′,5-pentachlorobiphenyl and 2,3,4,7,8-pentachlorodibenzofuran are observed to be carcinogens in experimental animals (NTP, 2006a; NTP, 2006b) with widespread evidence that they act through the same AhR-mediated mechanism and as a result, these two chemicals are also classified as carcinogenic in humans (IARC, 2009f).

Table 2.7 Occupation cancer inducing chemicals and processes and tumour sites and types (Adapted from IARC, 2009f)

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Tumour sites or types</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Other chemicals</strong></td>
<td></td>
</tr>
<tr>
<td>Aflatoxins</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>Benzene</td>
<td>Acute non-lymphocytic leukaemia</td>
</tr>
<tr>
<td>Bis(chloromethyl)ether/chloromethyl methylether</td>
<td>Lung</td>
</tr>
<tr>
<td>1,3-Butadiene</td>
<td>Haematolymphatic organs</td>
</tr>
<tr>
<td>Dioxin (2,3,7,8-TCDD)</td>
<td>All cancers combined</td>
</tr>
<tr>
<td>2,3,4,7,8-Pentachlorodibenzofuran</td>
<td>See text</td>
</tr>
<tr>
<td>3,3′,4,4′,5-Pentachlorobiphenyl (PCB-126)</td>
<td>See text</td>
</tr>
<tr>
<td>Ethylene oxide</td>
<td>See text</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Nasopharynx and Leukaemia</td>
</tr>
<tr>
<td>Sulfur mustard</td>
<td>Lung</td>
</tr>
<tr>
<td>Vinyl chloride</td>
<td>Hepatic angiosarcoma and hepatocellular carcinoma</td>
</tr>
<tr>
<td><strong>Other complex exposures</strong></td>
<td></td>
</tr>
<tr>
<td>Iron and steel founding</td>
<td>Lung</td>
</tr>
<tr>
<td>Isopropyl alcohol manufacture using strong acids</td>
<td>Nasal cavity</td>
</tr>
<tr>
<td>Mineral oils</td>
<td>Skin</td>
</tr>
<tr>
<td>Occupational exposure as a painter</td>
<td>Lung, urinary bladder, pleural and Mesothelioma</td>
</tr>
<tr>
<td>Rubber-manufacturing industry</td>
<td>Leukaemia, lymphoma, urinary bladder, lung, stomach</td>
</tr>
<tr>
<td>Shale oils</td>
<td>Skin</td>
</tr>
<tr>
<td>Strong inorganic acid mists</td>
<td>Larynx</td>
</tr>
</tbody>
</table>

Ethylene oxide is another special inclusion in the group of human carcinogens, as there is limited epidemiological evidence associating it to the incidence of any cancers. There is nevertheless enough proof for its carcinogenicity in rodents, supported by *in vitro* and *in vivo* data, demonstrating the genotoxicity and mutagenicity in animals, as well as cytogenetic effects in lymphocytes of workers of exposed workers (IARC, 2008). Another study
described workers in the rubber-manufacturing industry as having increased leukaemia, lymphoma, and cancers of the bladder, lung and stomach. Since the exposures in the industry are diverse and complex, it is difficult to identify the causative agents (Somorovská et al., 1999). Taking all the above-mentioned evidence into consideration, ethylene oxide was also classified as carcinogenic to humans (IARC, 2009f). Table 2.7 lists all chemicals under “other chemicals” and “other complex exposures” that are carcinogenic in humans, indicating the tumour sites or types of cancer that they cause in humans.

2.3.4.1.8 Tobacco, areca nut, alcohol, coal smoke, and salted fish

Tobacco smoke is by far the largest cause of cancer worldwide with more than 1 billion smokers around the world (IARC, 2009e). Growing numbers of new tobacco-related cancers are being added to the already extensive list, which is summarised in Table 2.8. Sufficient evidence has surfaced yet again, linking tobacco use to colon and ovarian cancers (Kurian et al., 2005; Liang et al., 2009). The number of tobacco-related cancers is increased as tobacco smoke and tars contain more than 40 known tumour mutagens and/or promoters (Hecht, 1999). PAHs, N-nitroso compounds, aromatic amines, heterocyclic aromatic amines and ethylene oxide are just a few known tumour mutagens and/or promoters. In a single cigarette smoke, there are several compounds associated with tumour-initiation and -promotion properties, which will readily induce and generate the process of carcinogenesis almost spontaneously (Severson et al., 1976; Rubin, 2006). Additionally, there are different types of cancers, due to the various carcinogens within the cigarette itself. For instance, exposure to PAHs has been classified to be carcinogenic in humans, causing skin and lung cancer (IARC, 2009f), while 1,3-butadiene, also a known human carcinogen, is associated with haematolymphatic cancers (IARC, 2009f), rendering all carcinogens within a single cigarette smoke to contribute to the overall carcinogenic nature of the smoke.

Cancers associated with second-hand and parental smoking, as well as smokeless tobacco, were also reviewed and established (Table 2.8). Second-hand smoking was strongly linked to lung cancer (IARC, 2004), but limited evidence associates it with cancers of the larynx and pharynx (Lee et al., 2008). Studies investigating parental smoking, whether from the expectant mother or the father, observed that the risk of hepatoblastoma, a rare embryonic cancer, is increased (Pang et al., 2003; Lee et al., 2009). The use of smokeless tobacco, generally containing nicotine and nitrosamines, was associated with cancers of the oral cavity and pancreas (IARC, 2004a) with cancers of the oesophagus as the latest addition with substantial evidence as human carcinogen (Boffetta et al., 2008).
Betel quid, comprised of areca nut, betel leaf slaked lime, catechu and frequently tobacco, is chewed by approximately 600 million people globally (Gupta and Warnakulasuriya, 2002). During chewing, carcinogenic nitrosamines are formed, generally known to induce oral preneoplastic disorders, regularly leading to cancers of the oral cavity, pharynx and oesophagus (IARC, 2004b).

Alcohol intake, inhalation of coal smoke and ingestion of Chinese-style salted fish, were also associated with cancers ranging from lung, oral cavity, pharynx, larynx, oesophagus, liver, colorectum, female breast and nasopharynx (Takezaki et al., 2001; Yokoyama et al., 2005; Straif et al., 2006; Baan et al., 2007) (Table 2.8).

Table 2.8 Activities that are classified as carcinogenic to humans with the affected sites and/or tumours (Adapted from IARC, 2009e)

<table>
<thead>
<tr>
<th>Carcinogenic activity</th>
<th>Tumour/site of cancer (with substantial evidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco smoking</td>
<td>Oral cavity, oropharynx, nasopharynx, and hypopharynx, oesophagus stomach, colorectum, liver, pancreas, nasal cavity and paranasal sinuses, larynx, lung, uterine cervix, ovary, urinary bladder, kidney, ureter and bone marrow</td>
</tr>
<tr>
<td>Parental smoking (cancer in the off spring)</td>
<td>Hepatoblastoma</td>
</tr>
<tr>
<td>Second-hand smoke</td>
<td>Lung</td>
</tr>
<tr>
<td>Smokeless tobacco</td>
<td>Oral cavity, oesophagus and pancreas</td>
</tr>
<tr>
<td>Areca nut</td>
<td>Oral cavity, pharynx and oesophagus</td>
</tr>
<tr>
<td>Betel quid with added tobacco</td>
<td>Oral cavity and oesophagus</td>
</tr>
<tr>
<td>Betel quid without added tobacco</td>
<td>Oral cavity and oesophagus</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>Oral cavity, pharynx, larynx, oesophagus, liver, colorectum, female breast</td>
</tr>
<tr>
<td>Acetaldehyde associated with alcohol consumption</td>
<td>Oesophagus, head and neck</td>
</tr>
<tr>
<td>Chinese-style salted fish</td>
<td>Nasopharynx</td>
</tr>
<tr>
<td>Indoor emissions from household combustion of coal</td>
<td>Lung</td>
</tr>
</tbody>
</table>

2.3.4.2 Endogenous sources

The role of oxidative stress and subsequent damage has received great interest in recent years, pertaining to its role in disease development. Free radicals are largely responsible for causing oxidative stress, while antioxidant systems attempt to counter the effects of free
radicals in the body. Under normal circumstances, there is a balance between free radicals and antioxidants in the body. The imbalance between free radicals/oxidants and antioxidants, favouring the former, generally result in oxidative stress (Sies, 1985). Both the free radicals and antioxidants have endogenous as well as exogenous sources. Free radicals, antioxidants and oxidative stress cannot be discussed in isolation without mentioning the one, or the other.

2.3.4.2.1 Free Radicals

In 1954, Gerschman and Gilbert proposed that Superoxide radicals (O$_2^-$) were responsible for the damaging effect of oxygen (Gerschman et al., 1954). Subsequently, the superoxide theory of oxygen toxicity was developed, suggesting that in vivo superoxide radical formation is largely responsible for the toxic effects of oxygen (Halliwell and Gutteridge, 1999).

Free radicals are defined as “any species containing one or more unpaired electrons” (electrons singly occupying an atomic or molecular orbital) (Halliwell and Gutteridge, 2007). They are commonly known to be very unstable and readily reactive with proteins, carbohydrates, lipids, RNA and DNA, as well as with other inter-and intracellular organelles (Halliwell and Gutteridge, 1999). Exogenous free radicals, including environmental agents, such as xenobiotics (Klaunig et al., 1997), as well as endogenous free radicals with origins including the electron transfer chain reactions in the mitochondria, cytochrome P450 metabolism reactions, liver peroxisomes, and inflammatory cell activation processes (Conner and Grisham, 1996; Inoue et al., 2003), were previously reported. The endogenous free radicals are known to be of greater oxidative threat than the ones of exogenous origin (Babalan et al., 2005).

Free radicals and their derived species also have essential functions in the body, ranging from the roles they play in the regulation of energy metabolism (Meng et al., 2011), ion transport (Bolotina et al., 1994; Dong et al., 1995) and signal transduction (Ignarro, 1991; Lander, 1997; Zheng and Storz, 2000; Poli, 2004). Additional roles of reactive oxygen species (ROS) in cancer treatment have also been reported where sufficient ROS levels were generated by radiation (Moeller et al., 2004) and the use of an anticancer agent (Serrano et al., 1999) to kill cancer cells through their oxidative actions.

Free radicals are generally divided into ROS and reactive nitrogen species (RNS). The most common ROS include the O$_2^-$, a hydroxyl radical (OH') and hydrogen peroxide (H$_2$O$_2$),
whereas RNS which are often classified under ROS, primarily consists of nitric acid (NO), nitrous oxide (NO$_2^-$), nitroxyl anion (HNO$^-$) and peroxynitrous acid (HNO$_3$). Even though O$_2^-$ and H$_2$O$_2$ radicals are known to be far less reactive than OH radicals, they cause direct injury when in excess (Fridovich, 1975; 1978; 1983; 1989). Superoxide, hydroxyl and nitric acid are the most unstable species, frequently present in the circulation for nanoseconds, and are ranked amongst the most potent reactive species. Hydrogen peroxide, hypochlorous acid and singlet oxygen are regarded as the main non-radical species (Surai, 2002). Reactive chloride species (RCS) also exist (Halliwell and Gutteridge, 2007).

2.3.4.2.2 Antioxidants

Antioxidants are defined as any substance, delaying or preventing oxidative damage to a targeted molecule (Halliwell and Gutteridge, 2007). They are responsible for controlling the levels of free radicals in the body. In addition to the cellular or endogenous antioxidant systems in the body, exogenous antioxidants, primarily obtained through the diet, are important in humans for protection against free radical-induced damage (Pietta, 2000).

Under normal circumstances, humans have adequate an adequate antioxidant system which can function on different levels of free radical damage by initially preventing radical formation, but if already formed, they can also intercept the radicals. Furthermore, this antioxidant system can reduce oxidative damage, increase the elimination of damaged molecules, and minimise the introduction of mutations by enhancing the repair of markedly damaged molecules (Gutteridge, 1995; Sies, 1997). These preventative and corrective actions are jointly executed by intracellular, membrane and extracellular agents through both enzymatic and non-enzymatic reactions.

The principle antioxidant defence enzymes in the human body include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), which are all actively participating in metabolic processes. SOD forms the first line of defence against superoxide radicals, by dismutating superoxide radicals to H$_2$O$_2$ and oxygen (O$_2$), in the presence of redox active transitional metals (McCord and Fridovich, 1969; Culotta et al., 2006). GPx further catalyses the reduction of H$_2$O$_2$ to water, while also catalysing the reduction of organic hydroperoxides to alcohol. Most GPx reactions are predominantly facilitated by reduced glutathione (GSH) as electron donor, where GSH is oxidised to glutathione disulfide (GSSG) (Bjornstedt et al., 1997). Glutaredoxin and thioredoxin are also electron donors in GPx reduction reactions (Bjornstedt et al., 1994; Bjornstedt et al., 1997). GSH, being a principal component in most
reduction reactions, will be completely depleted, if not generated continuously. Therefore, glutathione reductase, a flavin containing enzyme, facilitates the reduction of GSSG to GSH as a pathway of GSH regeneration (Meister and Anderson, 1983). Similarly to GPx, CAT catalyses the reduction of H₂O₂ to water and O₂. Even though CAT and GPx use the same substrates, CAT has a considerably lower affinity for H₂O₂ at lower levels compared to GPx (Sies, 1985; Kirkman and Gaetani, 2007). Additionally to the three primary antioxidant enzymes, three other accessory proteins with antioxidant properties can act directly or indirectly to the maintenance of a balanced redox state. These are peroxiredoxin (Kim et al., 1988), thioredoxin (Arner and Holmgren, 2000), and glutaredoxin (Berndt et al., 2007).

Of the non-enzymatic antioxidants, GSH probably has the most diverse antioxidant contributions in vivo. Apart from the above-mentioned roles of facilitating GPx and GSH reduction reactions, GSH can also act directly with various radicals by donating a hydrogen atom (Meister and Anderson, 1983). Moreover, GSH also play significant roles through a variety of interventions in the reduction of other antioxidants such as vitamins C and E, therefore preserving these limited resources in reduced form, at their own expense (Ji and La, 2000). Other non-enzymatic endogenous compounds with antioxidant potential include bilirubin, uric acid and coenzyme Q10 (Baranano et al., 2002; Halliwell and Gutteridge, 2007). Vitamins C and E, as well as α-lipoic acid are found inside the body in very small concentrations and primarily obtained through the diet (Janero, 1991; Kagan et al., 1992; Car and Frei, 1999).

There are many more exogenous antioxidant compounds. In food science, antioxidants have widely been used as additives in fats and oils during food processing to prevent or slow down the oxidative deterioration of food. Synthetic antioxidants have enjoyed the preferential use initially until about 1980, when they were systematically replaced by natural antioxidants (Pokorny et al., 2001). This change was based on the emergence of new toxicological data suggesting a cautious use of synthetic compounds (Thompson and Moldeus, 1988). Natural products are reported to be healthier and safer alternatives to synthetic antioxidants (Valenzuela and Nieto, 1996). Phenolic compounds such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and esters of gallic acid are some of the most popular synthetic antioxidants (Hudson, 1990). BHA and BHT are known to be relatively stable in heat, consequently being used in the stabilisation of fats in baked and fried products, while also usually used in combination, due to their combined synergistic property (Omura, 1995). TBHQ is the most suitable antioxidant for vegetable oils (Sherwin, 1972).
Natural antioxidants, on the other hand, are present in almost all plants, micro-organisms, fungi and animal tissues (Pratt and Hudson 1990; Pokorny, 1999). The majority of the natural antioxidants are phenolic compounds which act as reducing agents, free radical terminators, metal chelators, and singlet oxygen quenchers (Mathew and Abraham, 2006). Still, vitamins E and C present in higher concentrations than phenolic compounds in vivo, in plasma and tissues, play a more significant role as dietary antioxidants than polyphenols (Spencer et al., 2004). Vitamin E is the most widely distributed natural antioxidant, with α-tocopherol the best known vitamin E structural isomer exhibiting the highest antioxidant potential (Janero, 1991). Vitamin C is readily present in the form of ascorbate and due to its low pKa value is widely distributed in mammalian tissues (Yu, 1994). Vitamin C contributes to the antioxidant potential by directly scavenging superoxide, lipid hydroperoxide and hydroxyl radicals (Car and Frei, 1999), in addition to its role in the recycling of vitamin E (Packer et al., 1979).

2.3.4.2.3 Oxidative Stress

Under normal conditions, the actions of the extremely reactive and stable free radicals are tightly controlled by antioxidant systems of the body. However, if due to various endogenous and/or exogenous stimuli, there is either an overproduction of free radicals or a deficiency in the antioxidant system, the actions of the free radicals will overpower the antioxidant contributions. This imposes oxidative stress on the system (Gutteridge, 1995; Sies, 1997). Oxidative stress was initially defined as “a disturbance in the pro-oxidant-antioxidant balance in favour of the former” (Sies, 1985), “leading to potential damage” (Sies, 1991). As the field of redox biology is advancing, the term oxidative stress has been redefined as “a disruption of redox signalling and control” (Jones, 2006). The unstable and reactive free radicals in the absence of adequate antioxidant control, readily interact with vulnerable biological macromolecules causing the different pathologies, referred to as oxidative damage (Halliwell and Gutteridge, 1999). Important cellular components such as DNA, RNA, lipids, proteins and carbohydrates are all targets of free radical interactions.

2.3.4.2.3.1 Oxidative DNA damage

Oxidative DNA damage has been identified as one of the major sources of mutations in living organisms with the frequency of oxidative damage estimated at $10^4$ lesions / cell / day in humans (Lu et al., 2001). Hydroxyl radicals are the major ROS implicated in oxidative DNA damage, interacting with DNA bases, deoxyribose and even free nucleotides (Lu et al., 2001). Several other ROS, as well as products of lipid and protein oxidation, have been
demonstrated to be responsible for oxidative DNA damage (Klaunig et al., 1998; Marnet 2000; Tuma, 2002). Interaction of the different causative agents with DNA can generate a wide variety of DNA damage, ranging from small adducts to cross-linked lesion and double strand breaks. Often, the reactions form covalent bonds with the DNA bases, causing very stable and irreversible DNA adducts (Hemminki, 1993) which, in most cases, cannot be properly repaired by the cell repair systems (Sancar, 1994).

At least 24 DNA base modifications due to DNA ROS attacks have been identified (Wilson et al., 2003) with the most widespread DNA base modification resulting in the formation of 8-hydroxy-2′-deoxyguanosine (8OHdG) through the oxidation of guanine at the C8 position of the base (Kasai et al., 1984). 8OHdG has since been adopted as a biomarker for DNA damage and oxidative stress assessment tools, both in vitro and in vivo (Kasprzak, 2002).

2.3.4.3.2 Lipid peroxidation

Fatty acids which contain two or more double bonds are commonly susceptible to oxidation by ROS, consequently producing lipid peroxyl radicals and lipid hydroxides. This process is referred to as lipid peroxidation or auto-oxidation of lipids, and may be initiated enzymatically or non-enzymatically (Ratty and Das, 1988; Rice-Evans and Burdon, 1993). Lipid peroxidation is a multi-step free radical chain process involving initiation, propagation, and termination (Porter et al., 1995; Gago-Dominguez et al., 2005). Any free radical, possessing ability to extract a hydrogen atom from a reactive methylene group of unsaturated fatty acids, initiates the process (Gago-Dominguez et al., 2005). Once lipid radicals are formed during initiation, they will react with adjacent lipid molecules and generate new radicals in the process referred to as propagation (Porter et al., 1995; Gago-Dominguez et al., 2005). This step will naturally repeat itself until terminated by reactions between radicals, which produce dimers and higher polymers (Wheatley, 2000), or by the actions of chain breaking antioxidants (Rice-Evans and Burdon, 1993; Niki et al., 2005). Reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) are formed from the breakdown of lipid peroxidation products in biological systems (Esterbauer and Cheeseman, 1990; Tuma, 2002). MDA and 4-HNE commonly interact with protein, DNA and RNA molecules to form adducts (Esterbauer and Cheeseman, 1990; Uchida and Stadtman, 1993), whilst MDA, as well as MDA-MDA dimers, have been reported to be mutagenic (Spalding, 1988).

The determination of MDA in tissue samples using the thiobarbituric acid (TBA) test has traditionally been used to assess the lipid peroxidation status (Moore and Roberts, 1998). This test is based on the reaction of one molecule of MDA with two molecules of TBA,
forming a pink pigment with an absorption maximum at 532-535 nm (Esterbauer and Cheeseman, 1990). However, this spectrophotometric analysis has been criticised for low specificity and artefact formation, as only a small fraction of the MDA measured was generated in vivo (Draper and Hadley, 1990; Moore and Roberts, 1998). Recently, several HPLC-based TBA assays with increased specificities have emerged (Fukunaga et al., 1998; Volpi and Tarugi, 1998; Suttnar et al., 2001). A study investigating the validity of different methodologies of MDA determination suggested that the spectrophotometric assessments of MDA concentrations should only be regarded as rough estimations due to the low specificity observed against HPLC-based measurements. It was further recommended that spectrophotometric-based measurement only be utilised for tissue samples, principally in the comparison of groups of samples obtained from the same species (Lykkesfeldt, 2001).

2.4 Chemical constituents and antioxidant properties of study plants

2.4.1 Adansonia digitata

2.4.1.1 General

*Adansonia digitata* (Bombacaceae family), a common feature on the African savannahs, is a multipurpose tree, also referred to as “the tree of life” due to its ability to sustain life as it possesses a high capacity to hold water. More commonly referred to as ‘baobab’, this plant has traditionally been used as immunostimulant (El-Rawy et al., 1997), anti-inflammatory and analgesic agent (Gruenwald and Galizia, 2005). It is also known to be antipyretic in the treatment of diarrhoea and dysentery (Ramadan et al., 1994).

Glew et al. conducted protein content and amino acid analysis, mineral analysis, as well as lipid extraction and fatty acid investigation of *A. digitata*, among 24 other African plants (Glew et al., 1997). Generally, *A. digitata* contains 10-20% protein and lipid, while it has relatively high amounts of iron, calcium and copper (Glew et al., 1997). The active components of this plant have been identified as ursolic acid (Sipra-Dan and Dan, 1986), β-sitosterol (Ramesh et al., 1992), β-amyrin palmitate (Ramesh et al., 1992), and triterpenoids (Ramadan et al., 1994)). However, *A. digitata* is mostly characterised by its high vitamin C content of 124mg/100g of the plant (Affo and Akande, 2011). The nutritional value of the different components from this tree is highly regarded internationally, being accepted for import into the European Union as a novel food (Buchmann et al., 2010), while also being approved by the Food and Drug Administration of the US as an important food ingredient for the US markets (Addy, 2009). More recently, it had been described for larvacidal and

### 2.4.1.2 Antioxidant activity

The total antioxidant capacity was measured as 7.7 μmole Trolox equivalent per gram dry weight of *A. digitata* fresh leaves using the Trolox assay (Cook *et al.*, 1998). During a subsequent experiment, the integral antioxidant capacity of aqueous and methanol extracts of its fresh fruit pulp, fruit shell and dry leaves was determined using a photochemiluminescence (PCL) method (Vertuani *et al.*, 2002). The results are as shown in Table 2.9.

<table>
<thead>
<tr>
<th>Baobab products</th>
<th>Trolox equivalents (mmole/g)</th>
<th>Water extract</th>
<th>Methanol extract</th>
<th>Integral antioxidant capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit pulp</td>
<td>6.96</td>
<td>4.148</td>
<td>11.11</td>
<td></td>
</tr>
<tr>
<td>Dry leaves</td>
<td>6.39</td>
<td>2.35</td>
<td>8.74</td>
<td></td>
</tr>
<tr>
<td>Fruit shell grounded</td>
<td>9.35</td>
<td>0.46</td>
<td>9.81</td>
<td></td>
</tr>
<tr>
<td>Fruit glycolic extract</td>
<td>0.93</td>
<td>0.092</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>Leave glycolic extract</td>
<td>4.39</td>
<td>0.025</td>
<td>4.41</td>
<td></td>
</tr>
</tbody>
</table>

The PCL experiment was then improved by using an ascorbic acid standard solution for the antioxidant capacity of a water soluble substance, thus expressing this capacity in ascorbic acid equivalents (μmol/g), whereas the antioxidant capacity of the lipid-soluble substances was expressed as trolox equivalents as before by Vertuani and colleagues (2002). *A. digitata* red fibre demonstrated the highest antioxidant capacity, followed by the fruit pulp, leaves, seeds, radix cuticle and radix (Besco *et al.*, 2007).

### 2.4.1.3 Anti-mutagenic activity and toxicity

An aqueous extract of *A. digitata* demonstrated hepatoprotective activity against carbon tetrachloride-induced toxicity in rats indicating the importance of further research on toxicity and chemo-preventive activity. No report on anti-mutagenicity is available (Al-Qarawi *et al.*, 2003).
2.4.2 Agathosma betulina

2.4.2.1 General

Belonging to the Agathosma species, this indigenous plant is distributed throughout the Western Cape Province of South Africa (Goldblatt and Manning, 2000). The three most important species are *Agathosma betulina* Pillans (round-leaf buchu), *A. crenulata* Pillans (oval-leaf buchu) and *A. serratifolia* Spreeth (long-leaf buchu), and were initially wild-harvested for medicinal use (Spreeth, 1976). However, *A. crenulata* and *A. betulina* were considered to be more important by the industry (Roberts 1990), resulting in calls for their cultivation for use in medicine, flavour and fragrance industries (Turpie *et al*., 2003).

Historically, the San and Khoi tribes chewed *A. betulina* leaves to cure stomach ailments (Rust, 2003). Then they introduced the *A. betulina* leaves to European settlers, who took the leaves back to Europe and used them as a diuretic (Lis-Balchin *et al*., 2001). Since then, these leaves have been exported to Europe. Records from 1821 showed Reece and Company imported *A. betulina* leaves to Germany (Simpson, 1998). During the Crimean and First World Wars, vinegar produced from this plant was used as a powerful antiseptic (Rust, 2003). Current uses include the treatments of cholera, kidney diseases, haematuria, calculus and infections of the bladder, urethra and prostate. *A. betulina* leaves are also used as a digestive tonic as well as to treat rheumatism and gout (Lis-Balchin *et al*., 2001). Fever, coughs, flu and colds are also treated with this multi-purpose indigenous plant (Simpson, 1998; Moola, 2006).

Since the *A. betulina* export industry is estimated to be worth R100 million per annum (Rust, 2003), a renewed interest has resulted in the increased exploitation of the wild species. Additionally, a hybridisation between the two of these species (*A. betulina* and *A. crenulata*) has complicated the identification of these two species. This identification is important as the international buyers prefer *A. betulina*, which is naturally identified by its round leaves and also contain higher camphor (diosphenol) concentrations (Blommaert and Bartel, 1976).

To date, most of the studies conducted on *A. betulina* have been directed at the corrected classification of the two commercially important species with Linnaeus performing phenotypic analysis as early as 1755 (Bean, 1993). Further analyses include the chemical composition of the plant oils, which are mainly made up of 1-pulegone and diospenol (Fluck *et al*., 1961; Kaiser *et al*., 1973), and the analytical examination of the essential oils in order
to differentiate the species from each other (Blommaert and Bartel, 1976; Posthumus et al., 1996).

2.4.2.2 Antioxidant activity

Investigating antioxidant activity of *A. betulina*, Steenkamp and colleagues observed that the hydroxyl (OH) radical scavenging ability was reduced between 80% and 90% for ethanol and water extracts of *A. betulina*, respectively (Steenkamp et al., 2006). When an oxygen radical absorbance capacity assay (ORAC) was performed on different extracts of *A. betulina*, the following results were obtained, expressed in μmole trolox equivalent per g dry weight (Table 2.10) (Wojcikowski et al., 2007).

<table>
<thead>
<tr>
<th><em>A. betulina</em> extracts</th>
<th>ORAC Results (μmole Trolox equivalent / dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>22.72</td>
</tr>
<tr>
<td>Methanol</td>
<td>61.60</td>
</tr>
<tr>
<td>Aqueous / Methanol</td>
<td>174.92</td>
</tr>
<tr>
<td><strong>Total ORAC</strong></td>
<td><strong>259.25</strong></td>
</tr>
</tbody>
</table>

Subsequently, another group observed that most *Agathosma* species possess moderate to poor antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. Contrary to the findings of the DPPH assay, the 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay demonstrated good activity in most of the species in that study (Moolla et al., 2007). The ABTS and DPPH assay results do not always correlate with each other, as they use different reagents which could possibly exercise their protective properties at different stages of the oxidation process (Parejo et al., 2002; Arts et al., 2003).

2.4.2.3 Anti-mutagenic activity and toxicity

An *in vitro* anti-mutagenic activity assessment of *A. betulina* demonstrated no activity when using *Salmonella typhimurum* TA 98 and TA 100 strains with ethyl methanesulphonate and amino anthracene-induced mutagenicity (Badria, 1994). The water extract of *A. betulina* showed no toxicity to mononuclear cells in the Comet assay, but demonstrated substantial DNA damage to these cells at the same concentrations (Steenkamp et al., 2005).

Generally, using the 3-[4,5-dimethyl-2-thiazol-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) cellular viability assay to determine toxicity of the *Agathosma* species, it was observed that
essential species such as *A. betulina* and *A. crenulata* were not toxic at concentrations up to 100 μg/mL, whereas two of the species proved very toxic with IC$_{50}$ values ranging from $25.20 \pm 6.30$ μg/mL to $26.17 \pm 9.58$ μg/mL (Moolla *et al.*, 2007).

2.4.3 *Siphonocilus aethiopicus*

2.4.3.1 General

This plant is also commonly known as African ginger. *S. aethiopicus* is restricted to southern Africa, most commonly to South Africa, Zambia, Malawi and Zimbabwe. The rhizomes and roots are traditionally used for the treatments of colds, coughs and influenza (Van Wyk *et al.*, 1997). Other common uses include the management of hysteria and pain (Hutchings, 1962; Watt and Breyer-Brandwijk, 1962; Van Wyk *et al.*, 1997). Due to its reputation as a reliable treatment of infections associated with pains and fevers, African ginger is the ninth most frequently bought plant in the Durban muthi market (Mander, 1997). The plant also exhibits anti-allergic and anti-inflammatory properties, in both *in vitro* and *in vivo* models (Fouche *et al.*, 2011). Very little is known about the chemical constituents of the plant.

2.4.3.2 Antioxidant activity

The hydroxyl radical scavenging activity for the water and methanol *S. aethiopicus* extracts was determined around 84% and 70%, respectively (Steenkamp *et al.*, 2005), whereas the water extract showed substantial potentiation of lipid peroxidation in the membranes of normal peripheral blood mononuclear cells (Steenkamp *et al.*, 2005).

2.4.3.3 Anti-mutagenic activity and toxicity

The Comet assay was used to assess the toxicity of *S. aethiopicus*, demonstrating the non-toxic nature of the plant to mononuclear cells. However, when the same non-toxic concentrations were used to examine DNA damage in these cells, it was observed that the methanol extract of *S. aethiopicus* produced a very high amount of damage to the DNA (Steenkamp *et al.*, 2005). No information on anti-mutagenicity was previously reported.
2.4.4. Myrothamnus flabellifolius

2.4.4.1 General

The leaves of this plant fold up when desiccated, but during rainy seasons, the seemingly dead plants are revived from their dry state. This remarkable feature of the plant is reflected in English (resurrection bush) and Zulu (uvukwabafile – meaning wake from the dead) (Hutchings, 1996; Van Wyk et al., 1997).

The traditional medicinal uses of this plant are remarkably multifaceted, and accordingly summarised below:

- Infusions are drunk for colds and respiratory ailments, nosebleeds and fainting.
- Different extracts are taken orally to relieve backache, kidney problems, haemorrhoids and menstrual pains.
- Dried powdered leaves are used in dressings for burns and wounds.
- The smoke from the burnt plants is inhaled to treat chest pains and asthma. It can also be used to treat pains in the uterus.
- Other medicinal uses across Africa include treatments of breast complaints, epilepsy, "madness" and coughs (Hutchings, 1996; Van Wyk et al., 1997).

The major compounds in the essential oil of *M. flabellifolius* are carvone and perillic acid (Da Cunha, 1974), but another study focussing on the major constituents of the essential oil, identified 43 compounds with trans-pinocarveol, pinocarvone, α-pinene and β-selinene as the major ingredients (Chagonda et al., 1999). The composition of the essential oil of this plant was further re-examined using gas chromatography mass spectrometry (GCMS) analysis, identifying 85 compounds with pinocarvone, trans-pinocarveol limonene, trans-p-menth-1-(7)-8-diene-2-ol and cis-p-menth-1-(7)-8-diene-2-ol being the major compounds (Viljoen et al., 2002).

A known phenolic acid, 3,4,5 tri-O-galloylquinic acid, was identified as the major polyphenol from the *M. flabellifolius* bush using $^1$H and $^{13}$C one-and two-dimensional NMR spectroscopy. This compound was present at a concentration of 44% (by weight) in hydrated leaves and 74% (by weight) in dehydrated leaves (Moore et al., 2005).

2.4.4.2 Antioxidant activity

No information on antioxidant activity of this plant is available.
2.4.4.3 Anti-mutagenic activity and toxicity

No research literature could be retrieved regarding any chemo-preventive properties and toxicity of *M. flabellifolius*, creating the interest to further investigate this property.

Seemingly, not much research has been published on the antioxidant activity/capacity, toxicity and anti-mutagenic activity of these plants. This prompted conducting this study.
Chapter 3

3. Methods and Materials

3.1 Study design

This study was divided into three parts which were as follows:

1. Part 1: *In vitro* antioxidant content and capacity determination of the four medicinal plants.

   Three solvents were used for extraction of the various plant materials. Antioxidant content, in terms of total polyphenols, flavanol and flavonol contents were determined. This was followed by the determination of the various extracts’ antioxidant capacity which was expressed by means of the ferric-reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays. The section was completed by performing a high pressure liquid chromatography (HPLC) profile of the various plant extracts.


   Male Fischer 344 rats were given 2% (w/v) and 5% (w/v) aqueous plant extracts *ad libitum* over 90 days. During the feeding period, the body weights, plant extract consumption and overall condition of the experimental rats were monitored. At termination of the study, blood was drawn and liver tissue harvested for clinical chemistry, glutathione redox status and lipid peroxidation analyses. Additionally, phase 2 drug metabolising enzymes were measured in the cytosolic and microsomal fractions of the liver.

3. Part 3: Modulation of hepatic phase 2 drug metabolising enzymes and pre-cancer foci using a diethylnitrosamine (DEN)-initiated and fumonisin-promoted liver carcinogenesis model.

   Liver cancer was initiated using DEN in male Fischer 344 rats, whereafter the animals consumed the various plant extracts for two weeks, before promotion was effected with fumonisin. Three weeks after consuming the 2% (w/v) plant extracts as sole source of drinking water, the rats were sacrificed. Blood was drawn and liver
tissue harvested for clinical chemistry, redox status, lipid peroxidation, phase 2 drug metabolising enzymes and liver tissue immunohistochemistry analyses. An overview of the experimental methodologies used in the 3 parts of this study is diagrammatically described in Figure 3.1 below.

**1. Part 1**
**Antioxidant characterisation**
- **Antioxidant content:**
  - Total polyphenols, flavanols, flavonols
- **Antioxidant activity:**
  - FRAP, TEAC, ORAC
  - HPLC profiling

**2. Part 2**
**Modulation of hepatic phase 2 drug metabolising enzymes and oxidative status using a chronic feeding model**
- **Animal experimental parameters:**
  - Body weights, extract volumes, relative liver weights
- **Blood parameters:**
  - Clinical chemistry (ALT, AST, ALP, Fe, creatinine)
  - REDOX status (total polyphenols, ORAC, GSH: GSSG ratio)
  - Lipid peroxidation (CD, TBARS)
- **Liver parameters:**
  - Redox status (ORAC, GSH ratio)
  - Lipid peroxidation (CD, TBARS)
  - Phase 2 drug metabolising enzymes (GSTα, GSTμ, UDP-GT)

**3. Part 3**
**Chemoprotective properties using a rat liver cancer**
- **Animal experimental parameters:**
  - Body weights, extract volumes, relative liver weights
- **Blood parameters:**
  - Clinical chemistry (ALT, AST, ALP, Fe, CREA)
  - REDOX status (Total polyphenols, ORAC, GSH ratio)
  - Lipid peroxidation (CD, TBARS)
- **Liver parameters:**
  - Redox status (ORAC, GSH ratio)
  - Lipid peroxidation (CD, TBARS)
  - Phase 2 drug metabolising enzymes (GSTα, GSTμ, UDP-GT)
  - Liver immunohistochemistry

Figure 3.1 An overview of the experimental methodology of the study

Abbreviations: FRAP=ferric-reducing antioxidant power; TEAC=trolox equivalent antioxidant capacity; ORAC=oxygen radical absorbance capacity; HPLC=high pressure liquid chromatography; ALT=alanine transaminase; AST=aspartate transaminase; ALP=alkaline phosphatase; Fe=iron; CREA=Creatinine; GSH=reduced glutathione; GSSG=oxidized glutathione; CD=conjugated dienes; TBARS=thiobarbituric acid reactive substances; GST=glutathione s-transferase; UDP-GT=UDP-glucuronosyltransferase;
3.2 Chemicals

Butylated hydroxytoluene, 1,2-dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 2,2'-azobis (2-methylpropionamidine) dihydrochloride, 2,4,6-tri[2-pyridyl]-s-triazine, 4-(Ddimethylamino)cinnamaldehyde, 5,5'-Dithiobis (2-nitrobenzoic acid), catechin hydrate, di-sodium hydrogen orthophosphate dehydrate, ethylenediaminetetraacetic acid, fluorescein sodium salt, gallic acid, iron (III) chloride hexahydrate, L-ascorbic acid, L-glutathion-Reduced, magnesium chloride, potassium chloride, potassium phosphate, potassium-peroxodisulphate, quercetin, sodium azide, sodium carbonate, sodium chloride, sodium di-hydrogen orthophosphate monohydrate, sodium hydroxide, and trolox were purchased from Sigma (Sigma-Aldrich, Cape Town, South Africa). Acetone, 1-methyl -2-vinylpyridinium trifluoromethanesulphonate, chloroform, cyclohexane, diethylnitrosamine, ethanol, folin-Ciocalteu reagent, formalin, glutathione reductase, glutathione standard, hydrochloric acid, metaphosphoric acid, methanol, nicotinamide adenine dinucleotide phosphate, n-butanol, o-phosphoric acid, perchloric acid, petroleum benzene, p-nitrophenol, sodium pentobarbital, thiobarbituric acid, trifluoroacetic acid, triton x-100 and udp-glucuronic acid were purchased from Merck (Cape Town, South Africa). All reagents used were of analytical grade.

3.3 Part 1: In vitro antioxidant content and –capacity determination of the four medicinal plants

3.3.1 Plant materials and extraction procedure

Adansonia digitata fruits (BN11432), Agathosma betulina leaves (BN11574) and Siphonochilus aethiopicus rhizomes (BN11538) were purchased, in powder form, from Afriplex (Paarl, South Africa) with indicated batch numbers in brackets. Sufficient amounts of plant material were purchased from a reputable company to ensure availability of material (from same batch) for all three studies, as well as maintaining the same quality of plant material. Wild collection of plants is also protected under the Biodiversity Act of South Africa, therefore purchasing was the better option at the time. Myrothamnus flabellifolius branches with leaves were a gift obtained from the Clanwilliam area (Western Cape, South Africa). Subsequently, this plant material was also milled into a powder. M. flabellifolius material was only sufficient for the first two parts of this study, therefore was not continued into the DEN-initiation/CM-promotion model.
All aqueous extracts of the various plants materials, except *A. betulina*, were prepared by infusing 200 g of the powdered plant materials with 2 L of freshly boiled water for 30 min, resulting in 10% w/v extracts. Due to the viscosity of the *A. betulina*/water mixture, only 100 g of this plant material was used in 2 L of freshly boiled water for the aqueous extract preparation. The extracts were filtered using double-folded cheese-cloth, and the resultant filtrate was centrifuged at 2750 \( g \) at 4\( ^\circ \)C for 10 min. The supernatant was removed, soluble solids determined, and the remainder freeze-dried (Virtis, USA) overnight. Dried aqueous extracts were stored in glass, wrapped with aluminium foil at room temperature in the dark.

The acetone and ethanol extracts were prepared by extracting 200 g of the various plant materials with 2 L of the relevant solvent overnight while stirring, followed by filtering the extracts using double-folded cheese-cloth. Thereafter, the filtrates were centrifuged at 2750 \( g \) for 10 min at 4\( ^\circ \)C, and soluble solids determined, before the solvents were removed from the remaining filtrates using a rotavap (Heidolph, Germany) with settings adjusted to 40\( ^\circ \)C and 120 rpm. The resultant supernatants recovered from the various extraction processes were used in part 1 of the study for the determination of antioxidant contents; total polyphenols, flavanols, flavonols, and antioxidant activities; ORAC, TEAC and FRAP assays.

3.3.2 Soluble solids determination

The soluble solids for each plant extract were determined by allowing 1 mL of the filtered and centrifuged material to dry in an open-top tube with a pre-measured weight, at 40\( ^\circ \)C until all the water, ethanol, or acetone evaporated. The tubes containing the dried extract material were weighed and soluble solids were determined as the difference in weight before filtrates were added and after the drying process. The determination was performed in quintuplicate and the results were expressed in mg soluble solids per mL extract.

3.3.3 Antioxidant content

Since plants with different chemical compositions were studied, optimisation experiments were conducted to determine the working dilutions for each plant extract needed for the determination of total polyphenols, flavanols, and flavonols (Table 3.1).
Table 3.1 Working dilutions for the antioxidant content determinations in the different solvents (extract dry weight: solvent). Dilutions were determined using the total polyphenol assay.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Water</th>
<th>Acetone</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. digitata</em></td>
<td>1:5</td>
<td>1:50</td>
<td>1:50</td>
</tr>
<tr>
<td><em>A. betulina</em></td>
<td>1:15</td>
<td>1:40</td>
<td>1:40</td>
</tr>
<tr>
<td><em>S. aethiopicus</em></td>
<td>1:5</td>
<td>1:40</td>
<td>1:20</td>
</tr>
<tr>
<td><em>M. flabellifolius</em></td>
<td>1:80</td>
<td>1:80</td>
<td>1:100</td>
</tr>
</tbody>
</table>

3.3.3.1 Total polyphenol content

The total polyphenol content of the various plant material extracts was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). The working Folin-Ciocalteu reagent was prepared by combining 1 mL of the reagent with 9 mL of distilled water. Hundred and twenty-five microliters of the Folin reagent was added to the wells of a 96 well microplate, followed by a series of 25 µL gallic (GA) standards (0, 20, 50, 100, 250, 500 mg/L), made up in 10% ethanol and plant extracts. After 5 min, 100 µL of 7.5% sodium carbonate solution was added to each microwell, the plates were incubated at room temperature for 2 h, and the absorbance read at 765 nm using a Multiskan Spectrum plate reader (Thermo Scientific, USA) at room temperature. All specimens were analyzed twice in triplicate. The three values providing the lowest standard deviation (SD) were used as the measurement. The results were expressed in mg Gallic equivalents (GAE)/L.

3.3.3.2 Flavanol content

The flavanols were measured according to a modified method by Treutter (Treutter, 1989). Using a multi-channel pipette, 250 µL of freshly prepared 4-(dimethylamino)-cinnamaldehyde (0.25 g/500 mL hydrochloric acid (HCl)-methanol mixture) was added to all the wells of a 96-well plate. The standard solution was freshly made by dissolving 0.0145 g catechin hydrate in 50 mL methanol. Thereafter, 50 µL of catechin standards (0-27 mg/L) and plant extracts (in triplicate) were added to the micowells, followed by 30 min incubation at room temperature. The absorbance was read at 640 nm using the Multiskan Spectrum plate reader, and the results, made up of two assay runs, in triplicate, were expressed in mg catechins/L after the three values with the lowest SD were used as measurement.
3.3.3.3 Flavonol content

The method for flavonol determination was previously described by Mazza and colleagues (1999). Briefly, 100 μL of a premix solution (225 μL of 2% HCl and 12.5 μL of 0.1% HCl in 95% ethanol was added to all the wells of a 96-well plate, followed by 12.5 μL of the various plants extracts (in triplicate), and quercetin standards (0-80 mg/L). Thereafter, 137.5 μL of the above-mentioned premix solution was again added to the wells. The plate was incubated for 30 min at room temperature, and the absorbance measured at 360 nm on a Multiskan Spectrum plate reader with the results given in mg quercetin equivalents/L. All samples were analysed twice in triplicate. The three values with the lowest SD were used as the measurement.

3.3.4 Antioxidant capacity

Similarly for the antioxidant content, working dilutions were also optimised for determination of the antioxidant capacity parameters (Table 3.2).

Table 3.2 Working dilutions for the antioxidant capacity assays in the different solvents (extract dry weight: solvent). Dilutions were determined using the ORAC assay

<table>
<thead>
<tr>
<th>Solvent</th>
<th>A. digitata</th>
<th>A. betulina</th>
<th>S. aethiopicus</th>
<th>M. flabellifolius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (ORAC)</td>
<td>1:5 (1:250)</td>
<td>1:15 (1:750)</td>
<td>1:5 (1:250)</td>
<td>1:80 (1:4000)</td>
</tr>
<tr>
<td>Ethanol (ORAC)</td>
<td>1:50 (1:2500)</td>
<td>1:20 (1:1000)</td>
<td>1:20 (1:1000)</td>
<td>1:100 (1:4000)</td>
</tr>
</tbody>
</table>

Abbreviation: ORAC = Oxygen radical absorbance capacity

3.3.4.1 Ferric-reducing antioxidant power (FRAP)

The FRAP assay was done according to Benzie and Strain (1996). Briefly, 10 μL of the plant extracts, in triplicate, as well as L-ascorbic acid controls and standards (0-100 μM) were added to the wells, followed by 300 μL of freshly prepared FRAP reagent (prepared by mixing 30 mL of 300 mM acetate buffer at pH 3.6, 3 mL of 10 mM 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), 3 mL of 20 mM Iron (III) chloride hexahydrate (FeCl₃) solution, and 6.6 mL distilled water). The mixture was incubated at room temperature and the absorbance read at 593 nm using the Multiskan Spectrum plate reader. The results were expressed in mmole ascorbic acid equivalents/mg soluble solids. All samples were repeated on a second run, and the three values with the lowest SD were accepted as measurements.
3.3.4.2 Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay, known as the 2,2’-azino-di-3-ethylbenzthialozine sulphonate radical cation scavenging (ABTS) assay, was performed as described by Miller et al. (1993). An ABTS mix was prepared 24 h before analysis, by adding 88 μL of a 140 mM potassium-peroxodisulphate solution to 5 mL of a 7 mM 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution, and stored at room temperature in the dark. Just before analysis, the ABTS solution was diluted to a ratio of 1:200 with ethanol, giving an absorbance of approximately 2 on a Multiskan Spectrum plate reader, set at 734 nm and 25°C. Three hundred microliters of the ABTS-ethanol solution was added to each well, followed by 25 μL of trolox controls, standards (0-500 μM) and plant extracts (in triplicate). After a 30 min incubation period, the absorbance was read on a Multiskan Spectrum plate reader at 734 nm, and the results were expressed in mmole trolox equivalent (TE)/mg soluble solids. All samples were analysed twice in triplicate. The three values with the lowest SD were used as the measurement.

3.3.4.3 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was used as previously described by Prior et al. (2003). Twelve μL of the various plant extracts as well as trolox controls and standards (0-417 μM) were added to the appropriate wells on a 96-well microplate in triplicate, followed by the addition of 138 μL of freshly prepared ORAC buffer (8350-fold dilution of 120 μM fluorescein sodium salt solution in 75 mM phosphate buffer, pH7.4) to all the wells. Fifty microliters of a freshly prepared 9.22 mM 2,2’-azobis (2-methylpropionamidine) dihydrochloride (AAPH) was then added, and using the Fluoroskan plate reader, preset at 37°C, fluorescence was recorded every 5 min for 2 h at excitation and emission wavelengths of 485 nm and 530 nm, respectively. The results were expressed in mmole TE/mg of soluble solids. All samples were repeated on a second run, and the three values with the lowest SD were accepted as measurements.

3.3.5 High pressure liquid chromatography (HPLC) profiling

Plant extracts were prepared by dissolving 30 mg of dried extracts in 10 mL of the respective solvents, i.e. water or ethanol. One millilitre of acetone, followed by 9 mL of methanol was added to the acetone extracts (30 mg). The samples were subsequently vortexed for 1 min each, and heated in the waterbath at 50°C for 1 h to dissolve. Undissolved particulate matter of extracts was homogenized using the Yellowline D1 25 Basic homogenizer. Trifluoroacetic acid (500 mL) was added to both solvents, namely 500 mL of water and 500 mL of methanol. The solvent gradient was set at 100:0 (water:methanol) to 0:100 (water:methanol)
in 25 min. After filtering the samples through a 0.45 μm syringe filter, the samples (20 μL) were injected into the Agilent Technologies 1200 series HPLC autoanalyzer (SpectraLab, USA) using a YMC Pack Pro C18 (150 x 4.6 mm ID) column. The resultant peaks were detected at 250 nm, 280 nm, and 320 nm and the chromatograms with retention times of the peaks are presented in Appendix 1.

3.4 Part 2: Modulation of hepatic phase 2 drug metabolising enzymes and oxidative stress status using a 90-day chronic exposure rat model

3.4.1 Preparation of plant extracts

Powdered plant material, as described in section 3.3.1 was used for this second part of the study.

For part 2 of the study, two concentrations of each plant extract were prepared, namely 2% (w/v) and 5% (w/v), by infusing 200 g or 500 g of powdered plant material from each of the 4 species, with 10 L of freshly boiled water, respectively. After 30 min, the extracts were filtered through double-folded cheese cloth and the resulting filtrate was centrifuged at 2750 g for 10 min at 4°C to avoid particulate matter. The resultant supernatant was used to determine soluble solids (3.1.2), total polyphenols (3.1.3.1), flavanols (3.1.3.2), flavonols (3.1.3.3), and ORAC (3.1.4.3) as previously described, while the remainder was decanted into clean plastic bottles and frozen at -20°C until used for the experimental animal studies (Part 1, 2). The extracts for both parts were freshly prepared on a 2-week basis.

3.4.2 Experimental animals and diet

Male Fischer 344 rats (150-170g) were obtained from the Primate Unit of the Medical Research Council (Tygerberg, South Africa), and randomly divided into treatment groups of 10 rats per group. The rats were accommodated individually in stainless steel wire-bottomed cages, fitted with Perspex houses, in a closed environment at 24-25°C and 50% humidity with a 12 hour light-dark cycle. The cages were cleaned on a weekly basis. The fluid intake was monitored daily and the usage was logged on every second day, whereas rat pellets were made available ad libitum to all the rats. The general physical conditions of the rats were monitored daily, while the body weights were checked and recorded on a weekly basis. Both experimental animal studies were approved by Faculty of Health and Wellness Science Research Ethics Committee of Cape Peninsula University of Technology (CPUT) (CPUT/HW-REC 2008/012).
3.4.2.1 Design of the 90-day chronic consumption model: Part 2

The experimental rats were divided into nine groups (Table 3.3) and offered free access to either the 2% or 5% of the various aqueous extracts as their sole source of drinking water for the duration of the study, whereas the control group received tap water.

<table>
<thead>
<tr>
<th>Table 3.3 The random division of rats into groups for the 90-day chronic feeding model</th>
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<tr>
<td><strong>Fluid intake</strong></td>
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<td>Group 9 (Negative Control)</td>
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n=10 rats per treatment group

After 10 weeks, at termination of the experiment, the rats were euthanized by intra peritoneal injection (i.p) of 0.15 mL/100 g body weight sodium pentobarbital. Blood from the abdominal aorta was collected in vacutainer tubes containing either ethylenediaminetetraacetic acid (EDTA) or no anticoagulant, and immediately placed on ice, aliquoted and stored at -80°C until analysed for the various chemical pathology, plasma polyphenols, ORAC, and oxidative stress indicators. Livers were also excised at termination, weighed and immediately frozen in liquid nitrogen, followed by storage at -80°C until determination of antioxidant capacity, oxidative stress status and activities of phase 2 metabolising enzymes.

3.4.2.2 Design of the DEN-initiated/fumonisin-promoted liver carcinogenesis model: Part 3

Male Fischer rats were randomly divided into seven groups of 10 rats per group (Table 3.4). A single dose (200 mg/kg body weight, i.p.) of DEN was used to affect cancer initiation. Thereafter, for one week, the rats were provided tap water and rat cubes at *libitum*, followed by the introduction of the 2% aqueous extracts of the various plants, where applicable, until the end of the experiment. Tap water was made available for the control groups during this time (Fig. 3.2).
Figure 3.2 Design of the initiation/promotion liver carcinogenesis model

Abbreviations: DMSO = dimethyl sulfoxide; DEN = diethylnitrosamine; CM = cultured material; mg = milligrams; kg = kilograms; bw = body weight

Promotion was attained by offering the rats a diet consisting a 120 g of cultured material (CM) of Fusarium vertilliodes (Batch #: P131, PROMEC Unit, Tygerberg, Western Cape), mixed with 6 kg of milled standard rat chow pellets (mash), thoroughly mixed for 20 min and stored at 4°C under nitrogen prior to use. This diet was offered for three weeks after initiation, as indicated in Table 3.4. The total fumonisin (FB) concentration of the culture material (CM) was previously determined to be 14.8 g/kg, comprising of FB$_1$, FB$_2$, and FB$_3$ at 10.4 g/kg, 3.2 g/kg, and 1.2 g/kg, respectively.

The average feed intake for the positive control was calculated, by using the mean daily rat feed per body weight, and all the rats were provided the same amount of feed, according to this average intake of the positive control. The body weights of the rats were recorded on a weekly basis, as well as the daily fluid intake. At the end of the study period, the rats were fasted overnight and the next morning euthanised intra peritoneally with a sodium pentobarbital (0.15 mL/100 g body weight). Blood was then collected from the abdominal aorta in vacutainer tubes containing either EDTA or no anticoagulant, immediately placed on ice and processed the same day before storage at -80°C. Blood and/or plasma were used for the various chemical pathology analyses, plasma polyphenols and ORAC determinations, as well as indicators of oxidative stress. At termination, the livers were excised and weighed, whereafter sections of the livers, representing the three lobes, were processed in buffered...
formalin for histological examination, while the remaining liver tissue was frozen in liquid nitrogen for, and stored subsequently at -80°C for the various biochemical analyses.

| Table 3.4 The random division of rats into groups for initiation/promotion liver carcinogenesis model |
|-----------------------------------------------|------------|--------------|
| Group 1 (A. digitata)                        | DEN        | CMPM         |
| Group 2 (S. aethiopicus)                     | DEN        | CMPM         |
| Group 3 (A. betulina)                        | DEN        | CMPM         |
| Group 4 (Positive control)                   | DEN        | CMPM         | Water         |
| Group 5 (Negative control)                   | DMSO       | Normal pellets | Water         |
| Group 6 (CM control)                         | DMSO       | CMPM         | Water         |
| Group 7 (DEN control)                        | DEN        | Normal diet  | Water         |

Abbreviations: DMSO= dimethyl sulfoxide ; DEN= diethylnitrosamine; CM = Cultured material of Fusarium vertillioides; *CMPM = Cultured Material /Pellet Mx. n=10 rats per group

3.4.3 Analysis of blood for in vivo studies: Part 2 and 3

3.4.3.1 Preparation of blood

Blood samples, collected in no- anticoagulant-containing tubes, were centrifuged at 1500 g for 10 min at 4°C. The resultant plasma or serum was removed, aliquoted in 1.5 mL freezer vials and stored at -80°C until analysis. For glutathione (GSH) analyses, whole blood samples (100 μL) were directly aliquoted into freezer vials, while samples for oxidised glutathione (GSSG) were prepared by the addition of 10μL of 1-methyl -2-vinylpyridinium trifluoromethanesulphonate (M2VP) to 100 μL of whole blood. All samples were stored at -80°C until analysed.
3.4.3.2 Chemical Pathology

Clinical chemistry parameters, namely alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), total iron (Fe), and creatinine (CREA) were determined in all serum samples using an automated Medica Easy RA analyser (Medica Corporation, USA) with Medica Easy RA kits (USA).

3.4.3.3 Plasma total polyphenols

The plasma total polyphenols were determined using a modified Folin-Ciocalteu method by Serafini et al., (1998). Conjugated polyphenols were first hydrolysed, followed by the precipitation of proteins, before spectrophotometric measurements were conducted. The hydrolysis of conjugated polyphenols was achieved by incubating 100 μL of thawed plasma with 200 μL 1 M hydrochloric acid (HCl) for 30 min at 37°C. Two hundred microliters of 2 M sodium hydroxide (NaOH) in 75% methanol was then added, and the mixture was incubated for another 30 min at 37°C. This process enabled the release of polyphenols, linked to lipids. To precipitate proteins from the plasma, 300 μL of a 0.75 M metaphosphoric acid was added, before the mixture was centrifuged at 1500 g for 5 min. The supernatant, containing the extracted polyphenols was removed, and the Folin-Ciocalteu method (3.1.3.1) was used to determine the total polyphenols in the plasma, in triplicate, expressed in mg GAE/L of plasma.

3.4.3.4 Oxygen radical absorbance capacity assay

Briefly, the plasma was thawed at 4°C, and the plasma proteins subsequently precipitated by adding 400 μL of 0.5 M perchloric acid (PCA) to 400 μL of plasma. The mixture was vortexed for 30 s, followed by centrifugation at 2750 g for 5 min at 4°C. One hundred microliters of the supernatant was removed and diluted with 400 μL phosphate buffer, and ORAC determination was completed, according to the methodology in section 3.1.4.3 with the results being expressed in micromole trolox equivalents per litre of plasma (μmoleTE/L). All samples were analysed in triplicate.

3.4.3.5 Conjugated dienes

A 2:1 ratio of chloroform to methanol was prepared and 202.5 μL of this mixture was added to 50 μL of each thawed plasma sample in an eppendorf tube. This mixture was vortexed for 60 s, and centrifuged at 8000 g for 15 min at 4°C. The top aqueous layer was removed, and lipid layer was carefully collected by gently plunging a glass pipette along the wall of the
tube, through the protein layer. The lipid layer was then transferred to a clean eppendorf tube, and completely dried under liquid nitrogen, before 1 mL of cyclohexane was added. The mixture was vortexed for 60 s, and 300 μL was added to the wells of a clear 96-well plate, and the absorbance was immediately read at 234 nm using the Multiskan spectrophotometer. Cyclohexane damages the clear micro-well plates and therefore it is vital that the reading be done immediately. The concentrations of conjugated dienes (CD) were calculated by using the formula: 
\[ \text{plasma of CD/mL nmol} = \frac{A_{234} \text{(Sample)} - A_{234} \text{(Blank)}}{\varepsilon} \times 10 \text{nmol CD/mL of plasma} \]
representing the absorbance readings, and \( \varepsilon \) is the extinction coefficient \( 2.95 \times 10^4 \) for a 1 cm cuvette. Adjustments were made to accommodate the 300 μL that were used in this study, representing 0.9 cm in the plate wells. All samples were analysed in duplicate.

3.4.3.6 Glutathione analyses

Whole blood was used to measure the level of reduced glutathione (GSH), whereas whole blood with added M2VP was used to determine the level of oxidized glutathione (GSSG). The whole blood for GSH analyses was thawed and mixed well. Three hundred and fifty microliters of ice-cold MPA (5%) was added to the 100 μL GSH sample and vortexed for 15 s, before centrifugation for 10 min at 1000 g. Twenty five microliters of the supernatant was removed and added to 1.5 mL of the assay buffer (pH 7.4) which was prepared with EDTA, sodium di-hydrogen orthophosphate monohydrate, and di-sodium hydrogen orthophosphate dehydrate. Since the GSH and GSSG analyses were conducted in the same 96 well plates, the diluted GSH sample was stored on ice until the analysis, while preparing the GSSG samples. This allowed for a reliable determination of the GSH:GSSG ratio, taking the conditions of all complementary analyses into consideration. The GSSG sample was prepared from the M2VP-treated whole blood. The samples were thawed at room temperature, followed by the addition of 290 μL of ice-cold 5% MPA. The mixture was vortexed for 15 s, before being centrifuged at 1000 g for 10 min. Fifty microliters of the supernatant was added to 700 μL of the assay buffer, and the diluted sample was stored on ice, until analysis.

In the rows A and B on the 96 well plate, 50 μL of a series of GSH standard dilutions (0 (blank), 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM) were loaded (Fig 3.3). Rows C to E accommodated 50 μL of GSH samples, whereas rows F to H were used for the corresponding GSSG samples. All samples, blanks and standards were loaded in triplicate. Fifty microliters of a chromogenic compound, 0.3 mM 5,5'-dithiobis (2-nitrobenzoic acid)
(DTNB), and another 50 µL of the assay enzyme (16 µL of glutathione reductase -168 U/mg, diluted with 984 µL of the assay buffer) were then added to the samples, standards and blanks. The plates were incubated for 5 min at room temperature, before 50 µl of 1 mM Nicotinamide adenine dinucleotide phosphate (NADPH) was added to all wells to initiate the reactions. The absorbance of the reaction products were read at 412 nm, every 30 s for 5 min. The formula for the concentration of GSH from the calibration curve is:

\[ \text{GSHt} = \frac{\text{Net rate} - \text{intercept}}{\text{Slope}} \times \text{dilution factor} \]

GSHt represents the total GSH. The GSH:

\[ \frac{GSH}{GSSG} = \frac{GSHt - GSSG}{GSSG} \]

Figure 3.3 A 96 well microtitre plate (indicating the positions of reaction wells as explained in 3.4.3.6 above)

3.4.4 Analysis of liver tissue for in vivo studies: Part 2 and 3

3.4.4.1 Oxygen radical absorbance capacity assay

Frozen liver samples were homogenized using the Yellowline D1 25 Basic homogenizer (Merck, South Africa) in 75 mM phosphate buffer (pH 7.0) at a ratio of liver: phosphate buffer of 1:4 for 1 min. The homogenate was then centrifuged at 12000 g for 10 min at 4°C, followed by the addition of 0.25 M PCA to the resultant supernatant. PCA was used here to precipitate the proteins in the supernatant. The mixture of supernatant and PCA was then centrifuged at 12000 g for 15 min at 4°C, and the resulting supernatant removed and stored at -80°C until analyses. The ORAC assay was conducted according to Cao and Prior (1990) (section 3.4.3.4) and the results expressed in µmole TE/g of wet liver. All samples were analysed in triplicate.
3.4.4.2 Conjugated dienes and thiobarbituric acid reactive substances

Multi extraction steps were used to extract lipids from the liver tissues for, both, the CD and TBARS determinations. Hence, the extraction process for both assays was performed simultaneously. Liver tissue was defrosted at 4°C, and 0.2 g of the livers was placed in a measuring cylinder, containing 0.8 mL SEAP buffer (physiological saline, EDTA at 1 mg/mL of final concentration, 23 mM sodium azide in a standard phosphate buffer). When homogenizing the liver tissues, using this buffer, the sodium azide is known to act as an antibacterial agent, whereas EDTA is a metal chelating agent, inhibiting metals interfering with lipid peroxidation. After 1 min of homogenizing, using the Yellowline D1 25 Basic homogenizer, the first of three extractions started. One millilitre of chloroform and 2 mL of methanol were added to the homogenate, the mixture was vortexed for 20 s and centrifuged at 1500 g for 10 min at 4°C. The monolayer of supernatant, containing the lipids, was removed and stored on ice. This process was repeated another 2 times. After 3 extractions, the three supernatants were pooled and 3 mL chloroform and 3 mL SEAP buffer were added. The mixture was centrifuged at 1500 g for 10 min at 4°C, yielding a supernatant which consisted of 2 phases. The aqueous, which was found to be on top of the organic phase, was removed and discarded. Using a Pasteur pipette, the clear organic phase at the bottom was carefully removed and dried under nitrogen. Once completely dry, 100 μL of chloroform was added to the dried lipids and aliquoted in volumes of 20 μL each (x 4 tubes). Two of the four tubes were dried under nitrogen and used for CD determinations. Cyclohexane (1 mL) was added to these two tubes and vortexed for 15 s. Three hundred microliters were transferred to a 96-well microplate and the absorbance read at 234 nm on the Multiskan spectrophotometer. The concentration of CD was determined using the formula: 

\[
\frac{A_{234}(\text{sample}) - A_{234}(\text{blank})}{\varepsilon} \times 2.5 \ \mu\text{mole CD/g of liver},
\]

where \( A_{234} \) represents the absorbance readings and \( \varepsilon \) stands for the extinction coefficient which is equal to \( 1.54 \times 10^5 \) for 1 cm in a cuvette. Since our reading volume was only 300 μL, adjustments to the extinction coefficient were made to compensate for 300 μL volume in the micro well.

The two remaining tubes containing the 20 μL chloroform and dried lipid extraction were used for the determination of TBARS. Two and a half microliters of a solution, prepared from 9.17 mg of butylated hydroxytoluene in 10.42 mL of ethanol, was added to the 20 μL sample and 20 μL of o-phosphoric acid. The mixture was vortexed for 10 s before 2.5 mL of 0.11 M thiobarbituric acid (TBA) in 0.1 NaOH was added, followed by more mixing of 10 s. The mixture was subsequently heated in a water bath at 90°C for 45 min, before transferred to
ice for 2 min. This step was performed to stop the reaction. The tubes were then transferred to room temperature for 5 min, followed by the addition of 200 μL of n-butanol and 20 μL of saturated NaCl for better separation of liquid phases. After a brief vortex of 10 s, the samples were centrifuged at 12000 g for 2 min at 4°C. Hundred and fifty microliters of the butanol phase were then added to a clear 96-well plate, and the absorbance was read at 532 and 572 nm on the Multiskan spectrophotometer. The TBARS concentration was calculated using the formula: 

\[
\frac{A_{532} - A_{572}}{\varepsilon} \times 33.4 \mu\text{mol/L of sample}
\]

where \(A_{532}\) and \(A_{572}\) are the two absorbance values, and \(\varepsilon\) is the extinction coefficient (1.54 \times 10^5), based on a 1 cm cuvette. Adjustments were made for 150 μL which has a length of 0.45 cm in a plate well. All samples were analysed in duplicate. The total protein concentration of the liver samples was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, South Africa). TBARS results were expressed in nmol/mg protein.

3.4.4.3 Glutathione analyses

Total glutathione (GSH and GSSG) determinations were conducted according to the modified method of Tietz (1969). For GSH analysis, 0.07 g of the liver was homogenized in 0.7 mL of a 15% (w/v) Trichloroacetic acid (TCA) solution, containing 1 mM EDTA, whereas 0.07 g of livers for GSSG determination were homogenized in 0.7 mL 6% PCA solution, containing 3 mM of freshly prepared M2VP and 1 mM EDTA. The homogenizing was performed on ice using the Yellowline D1 25 Basic homogenizer for 1 min. The homogenates of both GSH and GSSG samples were centrifuged at 10000 g for 10 min at 4°C, and 50 μL of the resulting supernatants were used to perform the spectrophotometric determination, as described in section 3.2.3.5.

3.4.4.4 Preparation of microsomal and cytosolic liver fractions

Three hundred micrograms of frozen liver tissue were homogenized in 900 μL of 0.15 M ice-cold potassium chloride (KCl) solution for 10 s using a Yellowline D1 25 Basic homogenizer. The homogenates were then filtered through a double layer cheese-cloth and further homogenized using a glass dounce (10 strokes) with a loose pestle, followed by centrifugation at 9000 g for 10 min at 4°C. The resultant supernatant was removed and further ultra-centrifuged at 100000 g for 60 min at 4°C. The supernatant was collected as the cytosolic fraction, whereas the remaining microsomal pellet was washed 10 times in ice-cold 0.15 M KCl, before resuspension in 1 mL of the KCl, and centrifuged again at 100000 g for 60 min at 4°C. The microsomal pellet was resuspended in 1 mL KCl solution, and
represented the microsomal fraction. The entire process of harvesting the cytosolic and microsomal fractions was conducted on ice, and the two fractions were stored at -80°C until analysis.

3.4.4.5 Glutathione S-transferase α and μ activity

The GST-α activity was determined according to Habig et al. (1974). A 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) was prepared by dissolving 0.02 g in 1 mL ethanol and the solution was kept on ice. Eighteen microliters of 0.1 M potassium phosphate buffer (pH 6.5) was added to each well of a 96 well microplate, followed by 2 μL of thawed (on ice) hepatic cytosol fraction. A freshly prepared assay premix (180 μL), comprised of the 0.1 M potassium phosphate buffer 200 mM L-glutathione-reduced and 100 mM CDNB was added to each well, and the absorbance read at 340 nm (UV plate) for 3 min at 25°C. The GST-α activity was calculated using the formula:

\[
\frac{(A_{340})/\text{min} \times \text{V} (\text{mL}) \times \text{dil}}{\varepsilon_{\text{mM}} \times \text{V}_{\text{enzyme}} (\text{mL})}
\]

where \(A_{340}\) is the absorbance reading, min is the minutes, and dil, \(\varepsilon_{\text{mM}}\) and \(V_{\text{enzyme}}\), represents dilution of original sample, extinction coefficient (9.6 mM⁻¹) and volume of the enzyme sample tested, respectively. The extinction coefficient is given for a spectrophotometric reading in a 1 mL cuvette. Adjustments were made to compensate for the assay volumes in a 96-well microplate. Using the above-mentioned formula, the activity was expressed as μmole/mL/min. However, for more specific comparability of results, the protein concentrations of the different samples were performed using the Pierce® BCA Protein Assay Kit, and the GSTα activities were expressed in pmole/min/mg protein. All specimens were analysed in triplicate.

The GSTμ activity was determined according to Habig et al. (1974). For this assay, 60 mM 1,2-dichloro-4-nitrobenzene (DCNB) was prepared in 2 mL ethanol, and 86 mM of L-glutathione in 3 mL distilled water. Twenty four microliters of a 0.1 M potassium phosphate buffer (pH 7.0) was added to each of the 96 wells on a microplate. This was followed by the adding of 6 μL of cytosol, in triplicate, and 235 μL of the premix to each well. The premix was prepared with 0.1 M potassium phosphate buffer, 86 mM L-glutathione, and 60 mM DCNB. The absorbance was read at 344 nm for 3 min at 25°C on a Multiskan spectrophotometer with the activity calculated using the formula:

\[
\frac{(A_{344})/\text{min} \times \text{V} (\text{mL}) \times \text{dil}}{\varepsilon_{\text{mM}} \times \text{V}_{\text{enzyme}} (\text{mL})}
\]

where \(A_{344}\), min, \(V\), dil, \(\varepsilon_{\text{mM}}\), and \(V_{\text{enzyme}}\) representing absorbance value, minutes of reading (3 min), volume of
sample, dilution factor of original sample, extinction coefficient (8.5mM⁻¹) and the volume of the enzyme sample tested, respectively. This extinction coefficient is for analysis in 1 mL cuvettes. Adjustments were made to calculate the extinction coefficient on the 96-well microplate. The activity, in this case, will be expressed in μmole/mL/min, but similarly to the GSTα assay, the cytosolic protein content was determined using the Pierce ® BCA Protein Assay Kit, and the GST-μ activities were expressed in pmole/min/mg protein. All specimens were analysed in triplicate.

3.4.4.6 UDP-glucuronosyltransferase activity

The microsomal UDP-GT activity was determined spectrophotometrically using p-nitrophenol and UDP-glucuronic acid as substrates (Bock et al., 1983). Total protein concentrations were determined for the liver microsomes using the Pierce ® BCA Protein Assay Kit. One hundred microliters of a 1 mg/mL microsome solution was added to an eppendorf tube, followed by 20 μL of (25% w/v) of Triton X-100, 50 μL of 1M Tris-HCl (pH 7.0), 50 μL of a 50 mM magnesium chloride (MgCl₂) solution, 50 μL of 5 mM p-nitrophenol, and 180 μL of distilled water. The tubes were pre-incubated for 2 min at 37°c, followed by the initiation of the reaction by adding 50 μL of 30 mM UDP-glucuronic acid. The tubes were then incubated for a further 10 min at 37°C, followed by the addition of 500 μL ice-cold 0.5 M TCA, and another incubation cycle of 5 min on ice before centrifugation at 3000 g for 10 min. The resultant supernatants (400 μL) were collected, added to 400 μL of 2 M NaOH, and 300 μL of this dilution was added to the wells of a 96 well microplate. The absorbance was read at 405 nm on a Multiskan spectrophotometer. The activity was calculated using the formula: \( A_{405} \times (\varepsilon \times 0.9) \), where \( A_{405} \) represented the absorbance reading and \( \varepsilon (18.1) \) was the millimolar coefficient for a reading in 1mL cuvettes. Adjustments (x 0.9) were made for using a 96-well microplate when reading the absorbance. The results were expressed in nmole/min/mg protein. All microsomes were analysed in triplicate.

3.4.4.7 GST-Pi immunohistochemistry of liver sections

Formalin-preserved livers were embedded in paraffin wax. The resultant wax sections (5 mm) were washed with petroleum benzene and graded alcohol series. GST-P⁺ staining was performed using avidin-biotin-peroxidase complex (Vector Laboratories, CA) and affinity-purified biotin-labelled goat anti-rabbit IgG serum (Vector Laboratories, CA), according to the method of Ogawa and colleagues (1980). A 1:800 dilution of GST-P⁺-antiserum was briefly added to the dewaxed liver section, followed by counterstaining with Carazzi’s haematoxylin to provide blue stained nuclei, while the GST-P⁺ cells showed a
reddish-brown pigmentation. As a negative control, liver section with no primary GST-P* antiserum were included. The enzyme altered foci were quantified microscopically (10X magnification), using NIS-Elements (Nikon) image analysis software, according to their number and size (internal diameter). The foci were categorized into groups of <5\(\mu\)m, 5<10\(\mu\)m, 10<20\(\mu\)m, 20<30\(\mu\)m, and >30\(\mu\)m. The total numbers of foci were also determined of which the value was used to calculate the relative amount of foci which was expressed as a % of the total GST-P* foci. The GST-Pi immunohistochemistry staining of the liver sections was conducted at the Diabetes Discovery Platform, Medical Research Council of South Africa, Cape Town.

3.4.4.8 Statistical analyses

All samples were analysed either in triplicate, or in duplicate, and presented as means ± standard deviation (SD). Differences between means were determined using Analysis of Variation (ANOVA), and considered positive or significant, if \(P<0.05\). However, prior to ANOVA, the Levene’s Test for Equality of Variances was performed, and if \(P<0.05\), meaning that the data demonstrated an abnormal distribution, the data was log-transformed and the \(P\) value was again observed to see if >0.05 (ANOVA could only be performed if \(P>0.05\) using the Levene’s Test). If \(P\) was still lower than 0.05, a non-parametric analysis (Kruskal-Wallis test) was conducted, where a \(P<0.05\) indicated the differences between groups in a post-hoc analysis table.
Chapter 4

4. Results

4.1 Part 1: Antioxidant characterization of selected South African indigenous medicinal plant extracts

4.1.1 Soluble solids

The soluble solid content was significantly (P<0.05) different for the various plant extracts at 10% (w/v) concentration, except for A. betulina that was used at 5% (w/v) (Table 4.1). Significant differences were also observed when using the different solvents (i.e. water, acetone, and ethanol). Similarly, significant differences existed amongst each of the plants, using the same solvents for extraction. The soluble solid content was the highest in the water extracts, followed by the ethanol extracts, while acetone extracts yield the lowest soluble solids. The water extract of A. digitata yielded the highest soluble solids, whereas the acetone extract demonstrated the lowest. In contrast, the water extracts of M. flabellifolius, has the lowest soluble solid content with the acetone and ethanol extracts exhibit the highest yields. Similar yields for the acetone and ethanol soluble solids were noticed for S. aethiopicus and A. betulina while the aqueous extract of the latter was about 50 % lower.

Table 4.1 Soluble solids (mg/mL) of the various plant extracts

<table>
<thead>
<tr>
<th>Plants</th>
<th>Water*</th>
<th>Ethanol</th>
<th>Acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. digitata</td>
<td>159.85 ± 49.10&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>5.13 ± 0.22&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>1.54 ± 0.23&lt;sup&gt;b1&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. aethiopicus</td>
<td>27.73 ± 0.51&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>3.81 ± 0.31&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>3.49 ± 0.34&lt;sup&gt;b2&lt;/sup&gt;</td>
</tr>
<tr>
<td>A. betulina</td>
<td>11.20 ± 0.13&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>4.69 ± 0.14&lt;sup&gt;c3&lt;/sup&gt;</td>
<td>2.68 ± 0.20&lt;sup&gt;b3&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. flabellifolius</td>
<td>5.19 ± 0.15&lt;sup&gt;a4&lt;/sup&gt;</td>
<td>11.62 ± 0.31&lt;sup&gt;c4&lt;/sup&gt;</td>
<td>7.97 ± 0.27&lt;sup&gt;b4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=3). Different letters in the above rows show significant differences (P<0.05) between different solvents of the same plants. Different numbers in the different columns indicate significant differences (P<0.05) between the plants.*10% extracts were prepared except for A. betulina (5%).
4.1.2 Antioxidant contents: Total polyphenols, flavanols and flavonols

The total polyphenols, flavanols and flavonols differed significantly (P<0.05) between the various plants when extracted using the same solvent (Table 4.2). As discussed for the soluble solid content above, the polyphenolic content also differed for individual plants, when using the different solvents for extraction.

Table 4.2 Antioxidant concentrations of the different plant extracts

<table>
<thead>
<tr>
<th>Plants</th>
<th>Water*</th>
<th>Acetone</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. digitata</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total polyphenols</td>
<td>0.008 ± 0.00012&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>7.121 ± 0.098&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>2.072 ± 0.039&lt;sup&gt;c1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavanols</td>
<td>0.001 ± 0.00002&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>1.568 ± 0.017&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>0.468 ± 0.004&lt;sup&gt;c1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols</td>
<td>0.0004 ± 0.00001&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>3.431 ± 0.022&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>0.348 ± 0.004&lt;sup&gt;c1&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>S. aethiopicus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total polyphenols</td>
<td>0.033 ± 0.0026&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>1.600 ± 0.047&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>1.432 ± 0.020&lt;sup&gt;c2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavanols</td>
<td>0.002 ± 0.0001&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.051 ± 0.002&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>0.084 ± 0.0008&lt;sup&gt;c2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols</td>
<td>0.010 ± 0.0005&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>2.208 ± 0.13&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>2.327 ± 0.031&lt;sup&gt;c2&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>A. betulina</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total polyphenols</td>
<td>0.264 ± 0.0043&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>2.456 ± 0.103&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>1.337 ± 0.010&lt;sup&gt;c3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavanols</td>
<td>0.005 ± 0.0002&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>0.264 ± 0.024&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>0.069 ± 0.002&lt;sup&gt;c3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols</td>
<td>0.156 ± 0.003&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>4.024 ± 0.092&lt;sup&gt;b3&lt;/sup&gt;</td>
<td>1.975 ± 0.044&lt;sup&gt;c3&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>M. flabellifolius</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total polyphenols</td>
<td>4.059 ± 0.135&lt;sup&gt;a4&lt;/sup&gt;</td>
<td>2.774 ± 0.051&lt;sup&gt;b4&lt;/sup&gt;</td>
<td>2.255 ±0.023&lt;sup&gt;c4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavanols</td>
<td>0.222 ± 0.006&lt;sup&gt;a4&lt;/sup&gt;</td>
<td>0.243 ± 0.007&lt;sup&gt;b4&lt;/sup&gt;</td>
<td>0.176 ± 0.003&lt;sup&gt;c4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols</td>
<td>0.231 ± 0.031&lt;sup&gt;a4&lt;/sup&gt;</td>
<td>1.230 ± 0.022&lt;sup&gt;b4&lt;/sup&gt;</td>
<td>0.411 ± 0.003&lt;sup&gt;c4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=3). Different letters in the above rows show significant differences (P<0.05) between different solvents of the same plants. Different numbers in the different columns indicate significant differences (P<0.05) between the plants. The results were expressed as follows: total polyphenols (mg GAE/mg soluble solids), flavanols (mg catechins /mg soluble solids) and flavonols (mg quercetin/mg soluble solids). *10% extracts except for A. betulina (5%).

**A. digitata, S. aethiopicus and A. betulina** water extracts significantly (P<0.05) demonstrated the lowest amount of total polyphenols, flavanols, and flavonols, whereas the acetone extracts of these plants comprised of the highest contents. However, similarly to the soluble solids, **M. flabellifolius** water extracts yielded the highest total polyphenolic content, followed by the acetone and ethanol extracts, respectively. The flavanol and flavonol contents were the lowest in the water extract of **M. flabellifolius**, with the acetone extracts having the highest levels followed by the ethanol extract. The highest total phenolic content was observed in the **A. digitata** acetone extract, whereas the lowest was demonstrated by the water extract of the same plant. Similarly, the acetone extract of **A. digitata** demonstrated the highest flavanol content of all the plants and the water extract yielded the lowest flavanol
content. The *A. betulina* water extract also provided the lowest flavonol content amongst the various plants, while the acetone extract of *A. betulina* demonstrated the highest flavonols.

Overall, the sum of flavanols and flavonols was less than the total polyphenolic content of the different extracts, except for *S. aethiopicus* and *A. betulina*, where the flavanol and flavonol contents of the acetone and ethanolic extracts, exceeded the total polyphenols. In general the flavonol content was higher than the flavanols except for the aqueous and ethanol extracts of *A. digitata*.

### 4.1.3 Antioxidant capacities: TEAC, FRAP, ORAC

All extracts significantly (P<0.05) differed from each other using TEAC and FRAP assays for antioxidant capacity determinations (Table 4.3). The antioxidant activities differed significantly (P<0.05) between different plants using the same solvents for extraction, as well as between different solvents for extraction of the same plant. When considering the TEAC and FRAP assays, the *A. digitata* acetone extract demonstrated the highest antioxidant activity while the water extract of the same plant showed the lowest activity in these assays. The acetone extracts provided the highest antioxidant capacities for all plants using the TEAC and FRAP assays, except in *M. flabellifolius* where the water extract demonstrated the highest activity, followed by acetone and ethanolic extracts, respectively.

Determining antioxidant activities using the ORAC assay a different pattern is revealed. No significant differences were observed between ethanolic extracts of *A. digitata* and *A. betulina* and the water and acetone extracts of *M. flabellifolius*. On the other hand, *A. digitata*, *S. aethiopicus* and *A. betulina* all followed the same trend as in the TEAC and FRAP assays where the antioxidant activity was highest in the acetone extracts, followed by ethanolic and water extracts, respectively. The *A. digitata* acetone and water extracts also demonstrated the highest and lowest antioxidant activities using the ORAC assay, concurring with the TEAC and FRAP assays.
Table 4.3 Antioxidant capacity of the various plant extracts

<table>
<thead>
<tr>
<th>Plants</th>
<th>Water</th>
<th>Acetone</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. digitata</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEAC</td>
<td>0.01 ± 0.00(^{a1})</td>
<td>32.68 ± 0.36(^{b1})</td>
<td>10.46 ± 0.12(^{c1})</td>
</tr>
<tr>
<td>FRAP</td>
<td>0.05 ± 0.00(^{a1})</td>
<td>37.39 ± 0.98(^{b1})</td>
<td>11.59 ± 0.20(^{c1})</td>
</tr>
<tr>
<td>ORAC</td>
<td>0.46 ± 0.02(^{a1})</td>
<td>533.54 ±39.33(^{b1})</td>
<td>150.33 ± 5.24(^{c1})</td>
</tr>
<tr>
<td>S. aethiopicus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEAC</td>
<td>0.11 ± 0.01(^{a2})</td>
<td>8.56 ± 0.10(^{b2})</td>
<td>5.90 ± 0.11(^{c2})</td>
</tr>
<tr>
<td>FRAP</td>
<td>0.16 ± 0.01(^{a2})</td>
<td>5.69 ± 0.18(^{b2})</td>
<td>7.48 ± 0.02(^{c2})</td>
</tr>
<tr>
<td>ORAC</td>
<td>2.83 ± 0.04(^{a2})</td>
<td>189.06 ± 11.52(^{b2})</td>
<td>109.86 ± 2.43(^{c2})</td>
</tr>
<tr>
<td>A. betulina</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEAC</td>
<td>0.60 ± 0.00(^{a3})</td>
<td>9.18 ± 0.15(^{b3})</td>
<td>5.64 ± 0.13(^{c3})</td>
</tr>
<tr>
<td>FRAP</td>
<td>1.48 ± 0.01(^{a3})</td>
<td>9.43 ± 0.23(^{b3})</td>
<td>6.24 ± 0.02(^{c3})</td>
</tr>
<tr>
<td>ORAC</td>
<td>18.50 ± 0.14(^{a3})</td>
<td>227.27 ± 8.11(^{b3})</td>
<td>151.29 ± 0.65(^{c3})</td>
</tr>
<tr>
<td>M. flabellifolius</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEAC</td>
<td>17.43 ± 0.15(^{a4})</td>
<td>10.93 ± 0.03(^{b4})</td>
<td>8.63 ± 0.22(^{c4})</td>
</tr>
<tr>
<td>FRAP</td>
<td>22.73 ± 0.80(^{a4})</td>
<td>14.72 ± 0.30(^{b4})</td>
<td>13.06 ± 0.39(^{c4})</td>
</tr>
<tr>
<td>ORAC</td>
<td>134.02 ± 6.56(^{a4})</td>
<td>140.41 ± 7.28(^{b4})</td>
<td>94.62 ± 1.14(^{c4})</td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=3). Different letters in the above rows show significant differences (P<0.05) between different solvents of the same plants. The numbers in the different columns indicate the significant difference (P<0.05) between the plants. Abbreviations: TEAC= trolox equivalent antioxidant capacity; FRAP= ferric-reducing antioxidant power; ORAC= oxygen radical absorbance capacity. The results were expressed as follows: TEAC (mmoleTE/mg of soluble solids), FRAP (mmole ascorbic acid equivalents/mg soluble solids) and ORAC (mmoleTE/mg of soluble solids).

4.1.4 Antioxidant content and capacity correlation coefficients

Spearman correlations revealed that the relationships between the various antioxidant contents and -capacities were influenced by the solvent used for extractions. Correlation coefficients above 0.92 (P<0.0001) were observed for the aqueous extracts when comparing total polyphenols, flavanols, flavonols, TEAC, FRAP and ORAC with each other (Table 4.4). Total polyphenols demonstrated the strongest relationship with flavonols (0.9492) when comparing correlations between contents, while also having the strongest antioxidant capacity correlation with TEAC (0.9650). Flavanols shared the highest antioxidant capacity correlation with FRAP (0.9581), whereas TEAC was observed to have the strongest correlation with flavonols (0.9913). TEAC and ORAC shared the strongest relationship between the various antioxidant capacities of aqueous extracts.
Table 4.4 Spearman correlation values comparing antioxidant contents and capacities of aqueous extracts

<table>
<thead>
<tr>
<th>Chemical/antioxidant parameters</th>
<th>Total Polyphenols</th>
<th>Flavanols</th>
<th>Flavonols</th>
<th>TEAC</th>
<th>FRAP</th>
<th>ORAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Polyphenols</td>
<td>1.000</td>
<td>0.9371</td>
<td>0.9492</td>
<td>0.9650</td>
<td>0.9371</td>
<td>0.9301</td>
</tr>
<tr>
<td></td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
</tr>
<tr>
<td>Flavanols</td>
<td>-</td>
<td>1.000</td>
<td>0.9282</td>
<td>0.9231</td>
<td>0.9581</td>
<td>0.9301</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
</tr>
<tr>
<td>Flavonols</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>0.9913</td>
<td>0.9282</td>
<td>0.9877</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
</tr>
<tr>
<td>TEAC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>0.9231</td>
<td>0.9720</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
</tr>
<tr>
<td>FRAP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>0.9301</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
</tr>
</tbody>
</table>

Abbreviations: TEAC= trolox equivalent antioxidant capacity; FRAP= ferric-reducing antioxidant power; ORAC= oxygen radical absorbance capacity; P=probability value/level of significance

Considering the correlations between antioxidant constituents and -capacities in acetone extracts, the correlation coefficients ranged from 0.035 (P<0.9914) to 0.9650 (P<0.0001) (Table 4.5). Here, total polyphenols shared the strongest correlation with flavanols (0.8182) and FRAP (0.9650) for antioxidant contents and -capacity, respectively. No correlation was noticed with ORAC. Flavanols demonstrated the highest correlation with FRAP (0.8112) and TEAC, while only marginal with flavonols (0.5044) and weaker correlation with ORAC (0.6853), respectively. The flavonols shared the strongest correlation only with ORAC (0.7916). When comparing the three assays for antioxidant capacity of the different plant extracts, TEAC correlated with FRAP (0.9091; P<0.0001).

The correlations coefficients between antioxidant constituents and capacities in ethanol extracts ranged from 0.0949 (P<0.7366) to 0.9741 (P<0.0001) (Table 4.6). As with the aqueous extract, total polyphenols shared the strongest antioxidant content correlation with flavanols (0.8722), while having the best antioxidant capacity correlation with FRAP (0.9598), TEAC (0.8669), and lacking an association with ORAC. Flavanols correlated with the TEAC (0.9741) and FRAP (0.596) assays, whereas flavonols showed no correlation with any of the antioxidant parameters. Similar to the acetone extractions, TEAC and FRAP were more closely correlated to each other (0.8524), while no correlation was shown with ORAC.
Table 4.5 Spearman correlation values comparing antioxidant contents and capacities of acetone extracts

<table>
<thead>
<tr>
<th>Chemical/antioxidant parameters</th>
<th>Total Polyphenols</th>
<th>Flavanols</th>
<th>Flavonols</th>
<th>TEAC</th>
<th>FRAP</th>
<th>ORAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Polyphenols</td>
<td>1.000</td>
<td>0.8182</td>
<td>0.0210</td>
<td>0.9161</td>
<td>0.9650</td>
<td>0.3706</td>
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<tr>
<td></td>
<td></td>
<td>(P&lt;0.001)</td>
<td>(P=0.9483)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P=0.2356)</td>
</tr>
<tr>
<td>Flavanols</td>
<td>-</td>
<td>1.000</td>
<td>0.5044</td>
<td>0.7413</td>
<td>0.8112</td>
<td>0.6853</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P=0.0945)</td>
<td>(P=0.0058)</td>
<td>(P=0.0014)</td>
<td>(P=0.0139)</td>
</tr>
<tr>
<td>Flavonols</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>0.0035</td>
<td>0.0035</td>
<td>0.7916</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(P=0.9914)</td>
<td>(P=0.9914)</td>
<td>(P=0.0022)</td>
</tr>
<tr>
<td>TEAC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>0.9091</td>
<td>0.3776</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P=0.2262)</td>
</tr>
<tr>
<td>FRAP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>0.3916</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>(P=0.2081)</td>
</tr>
</tbody>
</table>

Abbreviations: TEAC = trolox equivalent antioxidant capacity; FRAP = ferric-reducing antioxidant power; ORAC = oxygen radical absorbance capacity; P = probability value/level of significance

Table 4.6 Spearman correlation values comparing antioxidant contents and capacities of ethanol extracts

<table>
<thead>
<tr>
<th>Chemical/antioxidant parameters</th>
<th>Total Polyphenols</th>
<th>Flavanols</th>
<th>Flavonols</th>
<th>TEAC</th>
<th>FRAP</th>
<th>ORAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Polyphenols</td>
<td>1.000</td>
<td>0.8722</td>
<td>0.1896</td>
<td>0.8669</td>
<td>0.9598</td>
<td>0.1214</td>
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<td></td>
<td></td>
<td>(P&lt;0.001)</td>
<td>(P=0.4985)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P=0.6664)</td>
</tr>
<tr>
<td>Flavanols</td>
<td>-</td>
<td>1.000</td>
<td>0.0949</td>
<td>0.9741</td>
<td>0.8596</td>
<td>0.4021</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P=0.7366)</td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P=0.1373)</td>
</tr>
<tr>
<td>Flavonols</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>0.1021</td>
<td>0.1988</td>
<td>0.4115</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(P=0.7174)</td>
<td>(P=0.4776)</td>
<td>(P=0.1276)</td>
</tr>
<tr>
<td>TEAC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>0.8524</td>
<td>0.3896</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(P&lt;0.0001)</td>
<td>(P&lt;0.0001)</td>
<td>(P=0.1511)</td>
</tr>
<tr>
<td>FRAP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1591</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(P=0.5712)</td>
</tr>
</tbody>
</table>

Abbreviations: TEAC = trolox equivalent antioxidant capacity; FRAP = ferric-reducing antioxidant power; ORAC = oxygen radical absorbance capacity; P = probability value/level of significance
4.2 Part 2 and 3: 90-day chronic feeding and liver initiation/promotion carcinogenesis studies

4.2.1 Antioxidant content and activity of plant extracts used in the 90-day chronic feeding and liver initiation/promotion carcinogenesis studies

4.2.1.1 Soluble solids

Significant (P<0.05) differences in the soluble solids of the aqueous extracts of the plants utilised were observed (Table 4.7). The 2 and 5% (w/v) concentrations of each plant, differed significantly (P<0.05). The 5% extracts demonstrated higher soluble solid contents than their 2% counterparts. The soluble solid yields between the various plants also differed significantly (P<0.05) for each plant extract. Comparatively, A. betulina demonstrated the highest percentage increase from 2% to 5% (65%), followed by A. digitata (58%) S. aethiopicus (55%) and M. flabellifolius (54%), respectively. A. digitata yielded the highest soluble solids amongst all plants, while M. flabellifolius provided the lowest soluble solid measurements for both for the 2% and 5% extractions.

<table>
<thead>
<tr>
<th>Plants</th>
<th>2% extracts</th>
<th>5% extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. digitata</td>
<td>17.60 ± 0.436&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>41.80 ± 1.000&lt;sup&gt;b1&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. aethiopicus</td>
<td>7.00 ± 0.346&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>15.53 ± 0.379&lt;sup&gt;b2&lt;/sup&gt;</td>
</tr>
<tr>
<td>A. betulina</td>
<td>4.20 ± 0.361&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>12.13 ± 0.252&lt;sup&gt;b3&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. flabellifolius</td>
<td>2.30 ± 0.100&lt;sup&gt;a4&lt;/sup&gt;</td>
<td>5.00 ± 0.000&lt;sup&gt;b4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=3). Different letters in the above rows show significant differences (P<0.05) between different solvents of the same plants. Different numbers in the different columns indicate significant difference (P<0.05) between the plants.

4.2.1.2 The total polyphenol content and antioxidant capacity of plant extracts utilised in the 90-day chronic feeding and liver carcinogenesis studies

No significant differences were observed between the total polyphenols of the 2% and 5% A. digitata extracts (Table 4.8). The other plant extracts showed a significant (P<0.05) increase between the yield of the 2% and 5% extractions. Comparing the total polyphenols of the various plants, there were no significant differences between the yields of the 2% A. digitata and 2% A. betulina extracts. However, there were significant (P<0.05) differences in the
yields between the 5% extractions of these two plants with *A. betulina* demonstrating a significantly (P<0.05) higher total polyphenols yield than *A. digitata*. No significant differences in total polyphenols were observed between the 5% extraction of *A. betulina* and *S. aethiopicus*, even though their 2% extracts were significantly (P<0.05) different. *M. flabellifolius* demonstrated the highest yield in total polyphenols, in both the 2% and 5% extract, compared to the other plants with a 54.73% increase in total polyphenols from 2% to 5% concentrations. *A. betulina* demonstrated the second highest total polyphenol increase from 2% to 5% (43%), followed by *S. aethiopicus* and *A. digitata* with 40% and 5%, respectively.

A significant (P<0.05) decrease in the flavanol content was noticed from 2% and 5% concentrations for *A. digitata*, *A. betulina* and *M. flabellifolius*, whereas *S. aethiopicus* showed no significant differences. The flavanols of the 5% *A. betulina* decreased by 62% as compared to the 2% extract. Similarly, 30% and 12% decreases in flavanols were recorded for *A. digitata* and *M. flabellifolius*, respectively. Significant differences (P<0.05) were observed when comparing the flavanol content of the 2% and 5% extracts of the various plants except for *A betulina* and *S. aethiopicus*. In this regard, *A. betulina* demonstrated a 38% decrease in flavonols.

Using the ORAC assay, the antioxidant capacity of *A. digitata* (36.60%), *A. betulina* (36.76%), and *M. flabellifolius* (26.09%) decreased significantly (P<0.05) between 2% and 5% extractions. However, for *S. aethiopicus* no significant difference was noticed between the 2% and 5% concentrations. Significant (P<0.05) differences were observed between the different plants, when considering the 2% and 5% extract concentrations. *M. flabellifolius* exhibited the highest ORAC activity for both 2% and 5% concentrations, whereas *S. aethiopicus* exhibited the lowest activity for the 2% concentration and *A. digitata* for the 5% extractions.
Table 4.8 Total polyphenol, flavanol, flavonol content and antioxidant activity (ORAC) of the 2% and 5% plants extracts utilized in the in vivo rat models

<table>
<thead>
<tr>
<th>Plants</th>
<th>2%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. digitata</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total polyphenols (mg GAE/mg soluble solids)</td>
<td>0.020 ± 0.0004&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.021 ± 0.002&lt;sup&gt;a1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols (mg catechins/mg soluble solids)</td>
<td>0.0023 ± 0.0001&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.0016 ± 0.0000&lt;sup&gt;b1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols (mg quercetin/mg soluble solids)</td>
<td>0.0028 ± 0.0001&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.00266 ± 0.0006&lt;sup&gt;a1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ORAC (mmol TE/mg of soluble solids)</td>
<td>39.67 ± 3.05&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>25.15 ± 1.19&lt;sup&gt;b1&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>S. aethiopicus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total polyphenols (mg GAE/mg soluble solids)</td>
<td>0.026 ± 0.001&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.043 ± 0.005&lt;sup&gt;b2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols (mg catechins/mg soluble solids)</td>
<td>0.00108 ± 0.00005&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.0011 ± 0.00003&lt;sup&gt;a2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols (mg quercetin/mg soluble solids)</td>
<td>0.0054 ± 0.0002&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.007 ± 0.0005&lt;sup&gt;a2&lt;/sup&gt;</td>
</tr>
<tr>
<td>ORAC (mmol TE/mg of soluble solids)</td>
<td>35.30 ± 3.24&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>33.22 ± 2.32&lt;sup&gt;a2&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>A. betulina</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total polyphenols (mg GAE/mg soluble solids)</td>
<td>0.020 ± 0.002&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.035 ± 0.002&lt;sup&gt;b2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols (mg catechins/mg soluble solids)</td>
<td>0.00029 ± 0.00003&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>0.00011 ± 0.00002&lt;sup&gt;b3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols (mg quercetin/mg soluble solids)</td>
<td>0.021 ± 0.008&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>0.013 ± 0.001&lt;sup&gt;b3&lt;/sup&gt;</td>
</tr>
<tr>
<td>ORAC (mmol TE/mg of soluble solids)</td>
<td>112.08 ± 2.04&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>70.88 ± 3.74&lt;sup&gt;a3&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>M. flabellifolius</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total polyphenols (mg GAE/mg soluble solids)</td>
<td>0.158 ± 0.018&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>0.349 ± 0.025&lt;sup&gt;b3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols (mg catechins/mg soluble solids)</td>
<td>0.026 ± 0.001&lt;sup&gt;a4&lt;/sup&gt;</td>
<td>0.023 ± 0.002&lt;sup&gt;b4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonols (mg quercetin/mg soluble solids)</td>
<td>0.022 ± 0.000&lt;sup&gt;a3&lt;/sup&gt;</td>
<td>0.019 ± 0.0012&lt;sup&gt;a4&lt;/sup&gt;</td>
</tr>
<tr>
<td>ORAC (mmol TE/mg of soluble solids)</td>
<td>273.46 ± 19.41&lt;sup&gt;a4&lt;/sup&gt;</td>
<td>202.11 ± 6.85&lt;sup&gt;a4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=3). Different letters in the above rows show significant differences (P<0.05) between different solvents of the same plants. Different numbers in the different columns indicate significant differences (P<0.05) between the plants. * denotes that groups are different (P<0.05). Abbreviations: ORAC=oxygen radical absorbance capacity
4.2.2. Part 2: Modulation of phase 2 drug metabolising enzymes due to chronic consumption of the various plant extracts

4.2.2.1 Rat body weight gain, relative liver weight, and fluid and total polyphenol intake: 90-day chronic feeding study

No significant differences were observed in the body weight gain (BWG) for all the groups (Table 4.9). However, rats consuming the 2% *M. flabellifolius* extract demonstrated the highest mean BWG as compared to the rats receiving the tap water, but this was not significant. The 2% and 5% *S. aethiopicus* and *A. betulina*, and 5% *A. digitata* treated rats showed significantly (P<0.05) increased relative liver weights as compared to the control rats and those receiving the 5% *M. flabellifolius* extracts. No significant differences were observed between 2% *A. digitata* and *M. flabellifolius* treated rats and the other treatment groups.

Rats consuming the 5% *A. digitata* had a significantly (P<0.05) higher fluid intake, while no significant differences were observed between the tap water control and the other treatment groups. Furthermore, there was a significant (P<0.05) difference in the fluid intake between rats drinking the 2% and 5% *M. flabellifolius* extracts. Integral to the experimental design the tap water contained no total polyphenols. Consumption of 2% *A. betulina* extract, resulted into a significant (P<0.05) lower daily total polyphenols intake as compared to the other plants (Table 4.9). No significant differences in daily total polyphenol intake were noted between 2% extracts of *A. digitata* and *M. flabellifolius*. Otherwise, all remaining groups differed significantly (P<0.05) from each other. Clear dose effects were noticed with the 5% *M. flabellifolius* extract which yielded (P<0.05) the highest total polyphenol intake followed by *A. digitata*. 
Table 4.9 Body weight gains, relative liver weights, and daily fluid and polyphenol intakes of rats in the 90-day chronic feeding study

<table>
<thead>
<tr>
<th>Plants</th>
<th>Body weight gained (g)</th>
<th>Relative liver weight (g)</th>
<th>Average daily fluid intake (mL/day)</th>
<th>Daily total polyphenol intake (mg GAE/mg soluble solids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td>112.36 ± 15.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.55 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.76 ± 1.86&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>2% &lt;i&gt;A. digitata&lt;/i&gt;</td>
<td>117.70 ± 21.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.75 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22.74 ± 1.73&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.64 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% &lt;i&gt;A. digitata&lt;/i&gt;</td>
<td>131.90 ± 23.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.84 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.57 ± 3.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.77 ± 0.42&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2% &lt;i&gt;S. aethiopicus&lt;/i&gt;</td>
<td>117.75 ± 14.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.94 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.67 ± 2.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.36 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% &lt;i&gt;S. aethiopicus&lt;/i&gt;</td>
<td>120.90 ± 23.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.86 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.89 ± 2.27&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.52 ± 0.32&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2% &lt;i&gt;A. betulina&lt;/i&gt;</td>
<td>124.25 ± 17.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.87 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.08 ± 2.37&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.62 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% &lt;i&gt;A. betulina&lt;/i&gt;</td>
<td>126.15 ± 22.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.82 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.19 ± 1.87&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.33 ± 0.28&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2% &lt;i&gt;M. flabellifolius&lt;/i&gt;</td>
<td>136.10 ± 12.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.72 ± 0.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>22.41 ± 2.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.64 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% &lt;i&gt;M. flabellifolius&lt;/i&gt;</td>
<td>118.65 ± 15.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.61 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.54 ± 1.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.94 ± 0.84&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. ND = not determined. Abbreviations: GAE=gallic equivalent.

4.2.2.2 Blood parameters: 90-day chronic feeding study model

4.2.2.2.1 Serum chemical pathology parameters

No significant differences in liver function as measured by ALP, AST and ALT levels were observed between the different treatment groups and the tap water control group (Table 4.10). The consumption of 2% <i>A. digitata</i> significantly (P<0.05) increased the ALP levels when compared to the rats receiving the 5% <i>M. flabellifolius</i> extract with the former having the highest ALP levels, and the latter the lowest among all the treated groups. Although the results were not significantly different when comparing the AST and ALT parameters, the rats receiving the 2% <i>M. flabellifolius</i> extract demonstrated the lowest levels. In addition the 2% <i>A. digitata</i> treatment resulted in the highest ALT levels with the consumption of the 5% <i>A. digitata</i> extract having the lowest. Only the consumption of the 2% <i>A. digitata</i> extract demonstrated significantly (P<0.05) higher serum iron concentration than the tap water control. Although significantly (P<0.05) different than the rats receiving the 2% plant extracts, the consumption of 5% <i>A. digitata</i> extract was associated with lowest mean serum iron levels among all groups, except for <i>M. flabellifolius</i>. Consumption of 2% extracts of
A. *digitata* and A. *betulina* significantly (P<0.05) lowered serum creatinine (CREA) concentrations, when compared to the tap water control, 2% *S. aethiopicus*, and 5% extracts of *A. digitata*, *A. betulina*, and *M. flabellifolius*.

4.2.2.2 Total polyphenol levels and oxidative stress markers in whole blood and serum

The 2% *S. aethiopicus* extract significantly (P<0.05) increased plasma total polyphenol levels, compared to the tap water control, as well as the other treatment groups (Table 4.11). The remaining groups demonstrated no significant differences in total plasma polyphenols, except the groups receiving 5% *S. aethiopicus* and *M. flabellifolius* extracts. Consumption of the latter extract exhibited a higher (P<0.05) level than the former. Consumption of the 2% *S. aethiopicus* extract demonstrated the highest plasma total polyphenol concentrations with the rats receiving the 5% having the lowest levels. No significant differences in plasma
ORAC activity was observed between the rats receiving tap water and the different treatment groups. When comparing the various plant extracts, rats consuming the 5% *A. betulina* extract demonstrated the lowest mean plasma ORAC activity, while the rats treated with the 5% *S. aethiopicus* extracts exhibited the highest activity.

No significant differences in whole blood GSH levels were found between the consumption of tap water, and the different plant treatment groups (Table 4.11). When comparing the different plant extracts, 5% *A. betulina* extract consumption significantly (P<0.05) increased blood GSH levels compared to 2% *A. digitata* and *M. flabellifolius* treatments. Although consumption of the 2% *A. betulina* extract demonstrated the lowest mean blood GSSG levels, no statistically significant differences were observed when compared to the tap water control group. Consumption of 2% *M. flabellifolius* and 5% *A. digitata* extracts, however, exhibited a significant (P<0.05) lower blood GSSG levels than the tap water control, the 2% extracts of *A. digitata* and *S. aethiopicus*, as well as the 5% extracts of *A. betulina* and *M. flabellifolius*. Interesting dose response effects is noticed regarding the *A. digitata* and *S. aethiopicus* where the 5% treatments increased the GSSG levels while the opposite was noted with the treatments of *M. flabellifolius* and *A. betulina*.

Treatments with 2% extracts of *A. betulina* and *M. flabellifolius*, as well as 5% extracts of *A. digitata* and *S. aethiopicus*, significantly (P<0.05) increased the GSH:GSSG ratio, compared to the tap water control. Furthermore, the rats receiving 2% *A. betulina* recorded the highest mean GSH:GSSG ratio value, whereas the 2% *A. digitata* treated rats demonstrated the lowest. No significantly different GSH:GSSG ratios were observed between consumption of the tap water and the 2% extracts of *A. digitata* and *S. aethiopicus*, as well as the 5% extracts of *A. betulina* and *M. flabellifolius*.

Overall, consumption of the various plant extracts increased the serum CD levels, an indication of increased lipid peroxidation when compared to the tap water control rats, except for the 5% extracts of *A. betulina* and *M. flabellifolius* (Table 4.11). The treatment with the 5% *A. digitata* extract recorded the highest mean CD levels, while the consumption of the 5% *A. betulina* extract demonstrated the lowest values.
Table 4.11 Whole blood and plasma redox status and lipid peroxidation: 90-day chronic feeding model

<table>
<thead>
<tr>
<th>Plants</th>
<th>Plasma total Polyphenols (mg/L)</th>
<th>Plasma ORAC (μmoleTE/L)</th>
<th>Whole blood GSH (μmole/L)</th>
<th>Whole blood GSSG (μmole/L)</th>
<th>GSH:GSSG</th>
<th>Plasma CD (nmCD/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td>157.05&lt;sup&gt;ac&lt;/sup&gt; (18.80)</td>
<td>1684.71&lt;sup&gt;a&lt;/sup&gt; (191.81)</td>
<td>486.10&lt;sup&gt;abc&lt;/sup&gt; (103.74)</td>
<td>102.36&lt;sup&gt;abcd&lt;/sup&gt; (5.54)</td>
<td>4.77&lt;sup&gt;ad&lt;/sup&gt; (1.07)</td>
<td>109.46&lt;sup&gt;d&lt;/sup&gt; (6.68)</td>
</tr>
<tr>
<td>2% A. digitata</td>
<td>147.73&lt;sup&gt;ac&lt;/sup&gt; (16.49)</td>
<td>1674.13&lt;sup&gt;a&lt;/sup&gt; (313.56)</td>
<td>413.45&lt;sup&gt;a&lt;/sup&gt; (124.36)</td>
<td>107.93&lt;sup&gt;a&lt;/sup&gt; (18.38)</td>
<td>3.92&lt;sup&gt;a&lt;/sup&gt; (1.29)</td>
<td>157.63&lt;sup&gt;abc&lt;/sup&gt; (19.48)</td>
</tr>
<tr>
<td>5% A. digitata</td>
<td>148.06&lt;sup&gt;abc&lt;/sup&gt; (26.65)</td>
<td>1704.53&lt;sup&gt;a&lt;/sup&gt; (213.06)</td>
<td>506.74&lt;sup&gt;a&lt;/sup&gt; (57.95)</td>
<td>90.42&lt;sup&gt;c&lt;/sup&gt; (5.92)</td>
<td>5.64&lt;sup&gt;bc&lt;/sup&gt; (0.81)</td>
<td>172.64&lt;sup&gt;a&lt;/sup&gt; (19.52)</td>
</tr>
<tr>
<td>2% S. aethiopicus</td>
<td>203.99&lt;sup&gt;b&lt;/sup&gt; (53.23)</td>
<td>1666.54&lt;sup&gt;a&lt;/sup&gt; (259.52)</td>
<td>472.42&lt;sup&gt;abc&lt;/sup&gt; (88.34)</td>
<td>101.18&lt;sup&gt;ab&lt;/sup&gt; (35.66)</td>
<td>5.82&lt;sup&gt;ab&lt;/sup&gt; (3.73)</td>
<td>135.32&lt;sup&gt;b&lt;/sup&gt; (12.23)</td>
</tr>
<tr>
<td>5% S. aethiopicus</td>
<td>119.14&lt;sup&gt;a&lt;/sup&gt; (16.10)</td>
<td>1749.80&lt;sup&gt;a&lt;/sup&gt; (250.13)</td>
<td>545.50&lt;sup&gt;bc&lt;/sup&gt; (41.22)</td>
<td>91.44&lt;sup&gt;d&lt;/sup&gt; (8.07)</td>
<td>5.99&lt;sup&gt;c&lt;/sup&gt; (0.43)</td>
<td>151.31&lt;sup&gt;c&lt;/sup&gt; (30.18)</td>
</tr>
<tr>
<td>2% A. betulina</td>
<td>159.11&lt;sup&gt;ac&lt;/sup&gt; (23.17)</td>
<td>1634.75&lt;sup&gt;a&lt;/sup&gt; (136.56)</td>
<td>536.09&lt;sup&gt;abc&lt;/sup&gt; (100.47)</td>
<td>78.92&lt;sup&gt;ac&lt;/sup&gt; (31.64)</td>
<td>8.45&lt;sup&gt;c&lt;/sup&gt; (4.62)</td>
<td>143.73&lt;sup&gt;bc&lt;/sup&gt; (9.59)</td>
</tr>
<tr>
<td>5% A. betulina</td>
<td>134.11&lt;sup&gt;ac&lt;/sup&gt; (13.67)</td>
<td>1388.00&lt;sup&gt;a&lt;/sup&gt; (352.36)</td>
<td>583.86&lt;sup&gt;c&lt;/sup&gt; (72.44)</td>
<td>107.80&lt;sup&gt;a&lt;/sup&gt; (9.11)</td>
<td>5.42&lt;sup&gt;bc&lt;/sup&gt; (0.51)</td>
<td>102.38&lt;sup&gt;d&lt;/sup&gt; (10.59)</td>
</tr>
<tr>
<td>2% M. flabellifolius</td>
<td>145.82&lt;sup&gt;abc&lt;/sup&gt; (22.77)</td>
<td>1525.77&lt;sup&gt;a&lt;/sup&gt; (268.46)</td>
<td>451.65&lt;sup&gt;ab&lt;/sup&gt; (83.99)</td>
<td>82.76&lt;sup&gt;c&lt;/sup&gt; (12.66)</td>
<td>5.69&lt;sup&gt;bc&lt;/sup&gt; (1.78)</td>
<td>156.40&lt;sup&gt;abc&lt;/sup&gt; (23.58)</td>
</tr>
<tr>
<td>5% M. flabellifolius</td>
<td>160.81&lt;sup&gt;c&lt;/sup&gt; (28.08)</td>
<td>1500.28&lt;sup&gt;a&lt;/sup&gt; (472.47)</td>
<td>501.20&lt;sup&gt;abc&lt;/sup&gt; (72.50)</td>
<td>113.70&lt;sup&gt;a&lt;/sup&gt; (6.67)</td>
<td>4.44&lt;sup&gt;ad&lt;/sup&gt; (0.76)</td>
<td>118.74&lt;sup&gt;d&lt;/sup&gt; (16.61)</td>
</tr>
</tbody>
</table>

The results are mean values ± SD in brackets under the means (n=7-10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Abbreviations: ORAC=oxygen radical absorbance capacity; GSH=reduced glutathione; GSSG=oxidised glutathione; CD=conjugated dienes. The results were expressed as follows: Total polyphenols (mg/L); ORAC (μmoleTE/L); GSH, GSSG - (μmole/L); CD (nmCD/mL Plasma)
4.2.2.3 Hepatic biochemistry parameters 90-day chronic feeding study

4.2.2.3.1 Hepatic redox and lipid peroxidation status

No significant differences in ORAC activity were noted between treatment with the various plant extracts and the tap water control (Table 4.12). When considering the various plant extracts alone, treatment with 2% and 5% *S. aethiopicus* extracts, exhibited significantly (P<0.05) lower ORAC activities when compared to 2% and 5% *A. digitata as well as the 2% *A. betulina* extract treatments. A significant (P<0.05) decrease in ORAC activity was observed following the 2% and 5% *A. betulina* and, to a certain extent, also the consumption of the corresponding *M. flabellifolius*. The liver GSH levels did not differ significantly between the different plant treatments and the tap water control groups. However, when considering differences between the various plant extracts, a slight but not significant increase in the GSH levels was observed in the rats drinking 2% compared to the rats consuming 5% of the plant extracts. Consumption of 2% extracts of *A. betulina* and *M. flabellifolius*, as well as the 5% *A. digitata* extracts exhibited significantly (P<0.05) lower liver GSSG levels when compared to the tap water control. Treatment with *A. betulina* and *M. flabellifolius* extracts induced a significant (P<0.05) dose response effect in liver GSSG levels that increased with a corresponding increase in the extract concentration from 2 to 5%. However, the GSSG level was significantly (P<0.05) decreased with the consumption of the 2% *A. digitata* extract as compared to the 5% extract. Although not significant, a decrease in the GSSG level was also noticed with the consumption of the 5% *S. aethiopicus*. Only a slight, but not significant GSSG decrease was noted with *S. aethiopicus* extract consumption, from 2% to 5% concentration. Subsequently, as a result of the decreased levels of GSSG, the liver GSH:GSSG ratio was significantly (P<0.05) increased in rats consuming the 2% extracts of *A. betulina* and *M. flabellifolius*, as well as 5% *A. digitata*, compared to the tap water control. When comparing the plant extract alone, the GSH:GSSG ratio in the liver of rats treated with *A. betulina* and *S. aethiopicus* extracts significantly (P<0.05) increased from 2% to 5%, indicating a dose response effect. In contrast, the GSH:GSSG ratio was significantly decrease when using the 2% to the 5% *A. betulina* and *M. flabellifolius* extract.
Table 4.12 Liver redox status: 90-day chronic feeding study

<table>
<thead>
<tr>
<th>Plants</th>
<th>ORAC</th>
<th>GSH</th>
<th>GSSG</th>
<th>GSH:GSSG</th>
<th>CD</th>
<th>TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% <em>A. digitata</em></td>
<td>20.99&lt;sup&gt;b&lt;/sup&gt; (1.90)</td>
<td>9.67&lt;sup&gt;a&lt;/sup&gt; (1.41)</td>
<td>0.33&lt;sup&gt;ab&lt;/sup&gt; (0.06)</td>
<td>29.39&lt;sup&gt;a&lt;/sup&gt; (4.33)</td>
<td>0.24&lt;sup&gt;a&lt;/sup&gt; (0.06)</td>
<td>0.09&lt;sup&gt;ab&lt;/sup&gt; (0.02)</td>
</tr>
<tr>
<td>5% <em>A. digitata</em></td>
<td>20.42&lt;sup&gt;b&lt;/sup&gt; (2.90)</td>
<td>11.14&lt;sup&gt;a&lt;/sup&gt; (1.40)</td>
<td>0.15&lt;sup&gt;d&lt;/sup&gt; (0.05)</td>
<td>76.34&lt;sup&gt;d&lt;/sup&gt; (18.18)</td>
<td>0.35&lt;sup&gt;b&lt;/sup&gt; (0.03)</td>
<td>0.12&lt;sup&gt;ab&lt;/sup&gt; (0.03)</td>
</tr>
<tr>
<td>2% <em>S. aethiopicus</em></td>
<td>15.69&lt;sup&gt;a&lt;/sup&gt; (3.84)</td>
<td>9.43&lt;sup&gt;a&lt;/sup&gt; (1.50)</td>
<td>0.34&lt;sup&gt;ab&lt;/sup&gt; (0.06)</td>
<td>28.08&lt;sup&gt;a&lt;/sup&gt; (4.69)</td>
<td>0.22&lt;sup&gt;a&lt;/sup&gt; (0.04)</td>
<td>0.09&lt;sup&gt;ab&lt;/sup&gt; (0.01)</td>
</tr>
<tr>
<td>5% <em>S. aethiopicus</em></td>
<td>15.33&lt;sup&gt;a&lt;/sup&gt; (3.04)</td>
<td>9.57&lt;sup&gt;a&lt;/sup&gt; (1.39)</td>
<td>0.27&lt;sup&gt;bc&lt;/sup&gt; (0.04)</td>
<td>35.62&lt;sup&gt;ace&lt;/sup&gt; (4.46)</td>
<td>0.31&lt;sup&gt;b&lt;/sup&gt; (0.02)</td>
<td>0.22&lt;sup&gt;d&lt;/sup&gt; (0.05)</td>
</tr>
<tr>
<td>2% <em>A. betulina</em></td>
<td>24.33&lt;sup&gt;c&lt;/sup&gt; (2.18)</td>
<td>11.13&lt;sup&gt;a&lt;/sup&gt; (1.95)</td>
<td>0.23&lt;sup&gt;c&lt;/sup&gt; (0.07)</td>
<td>53.51&lt;sup&gt;bc&lt;/sup&gt; (21.67)</td>
<td>0.33&lt;sup&gt;b&lt;/sup&gt; (0.05)</td>
<td>0.13&lt;sup&gt;abc&lt;/sup&gt; (0.06)</td>
</tr>
<tr>
<td>5% <em>A. betulina</em></td>
<td>17.67&lt;sup&gt;ab&lt;/sup&gt; (2.13)</td>
<td>11.56&lt;sup&gt;a&lt;/sup&gt; (3.97)</td>
<td>0.37&lt;sup&gt;ab&lt;/sup&gt; (0.12)</td>
<td>30.88&lt;sup&gt;af&lt;/sup&gt; (3.87)</td>
<td>0.35&lt;sup&gt;b&lt;/sup&gt; (0.05)</td>
<td>0.12&lt;sup&gt;abc&lt;/sup&gt; (0.04)</td>
</tr>
<tr>
<td>2% <em>M. flabellifolius</em></td>
<td>20.76&lt;sup&gt;ab&lt;/sup&gt; (5.08)</td>
<td>9.92&lt;sup&gt;a&lt;/sup&gt; (2.64)</td>
<td>0.16&lt;sup&gt;d&lt;/sup&gt; (0.04)</td>
<td>65.14&lt;sup&gt;bd&lt;/sup&gt; (18.74)</td>
<td>0.33&lt;sup&gt;b&lt;/sup&gt; (0.04)</td>
<td>0.15&lt;sup&gt;abcd&lt;/sup&gt; (0.06)</td>
</tr>
<tr>
<td>5% <em>M. flabellifolius</em></td>
<td>16.14&lt;sup&gt;ab&lt;/sup&gt; (3.60)</td>
<td>12.40&lt;sup&gt;a&lt;/sup&gt; (2.06)</td>
<td>0.41&lt;sup&gt;a&lt;/sup&gt; (0.06)</td>
<td>31.07&lt;sup&gt;af&lt;/sup&gt; (5.82)</td>
<td>0.37&lt;sup&gt;b&lt;/sup&gt; (0.03)</td>
<td>0.31&lt;sup&gt;e&lt;/sup&gt; (0.07)</td>
</tr>
<tr>
<td>Tap water</td>
<td>19.52&lt;sup&gt;abc&lt;/sup&gt; (3.23)</td>
<td>11.04&lt;sup&gt;a&lt;/sup&gt; (1.83)</td>
<td>0.34&lt;sup&gt;ab&lt;/sup&gt; (0.09)</td>
<td>33.75&lt;sup&gt;ae&lt;/sup&gt; (6.32)</td>
<td>0.33&lt;sup&gt;b&lt;/sup&gt; (0.02)</td>
<td>0.18&lt;sup&gt;ad&lt;/sup&gt; (0.05)</td>
</tr>
</tbody>
</table>

The results are mean values ± SD in brackets under the means (n=7-10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Abbreviations: ORAC=oxygen radical absorbance capacity; GSH=reduced glutathione; GSSG=oxidised glutathione; CD=conjugated dienes; TBARS=thiobarbituric acid reactive substances. The results were expressed as follows: ORAC (µmoleTE/g wet liver); GSH, GSSG – (µmole/g wet liver); CD (mM CD/100g liver); TBARS (nmole/mg protein).
When considering biomarkers of lipid peroxidation, rats consuming the 2% extracts of *A. digitata* and *S. aethiopicus* showed significantly (P<0.05) reduced levels of hepatic CD when compared to the tap water control and the groups receiving other plants extracts (Table 4.12). No significant differences in CD were noted between groups consuming the 2% and 5% concentrations of the same plants, except for *A. digitata* and *S. aethiopicus*, where the 2% extract consumption yielded significantly (P<0.05) higher CD levels. Similarly, rats treated with 2% *A. digitata* and *S. aethiopicus* extracts showed significantly (P<0.05) reduced hepatic TBARS levels when compared to the tap water control rats (Table 4.12). While no significant differences in TBARS were observed between rats consuming 2% and 5% extractions of *A. digitata* and *A. betulina*, significant (P<0.05) dose response increases were demonstrated from 2% to 5% *S. aethiopus* and *M. flabellifolius* extract consumption. Treatment with 5% *M. flabellifolius* extract significantly increased the TBARS levels when compared to the tap water control and the remaining treatment groups.

4.2.2.3.2 Hepatic phase 2 drug metabolising enzymes

The GST-α activity was significantly (P<0.05) decreased by treatment with the 2% *A. betulina* extract, compared to the tap water control. Rats consuming the 2% and 5% extracts of *A. digitata*, *S. aethiopicus*, and *M. flabellifolius*, as well as the 5% *A. digitata* extract, showed markedly (not significantly) reduced GSTα activities when compared to the tap water control. No significant dose response effects on the GSTα activity differences were observed when considering the 2% to 5% plant extracts.

Similarly, as mentioned for the GSTα activity, consumption of all the plant extracts lowered the mean GSTβ activities when compared to the tap water control treatment, although not significantly. When comparing the plant extracts with each other, no significant differences were noted for all treatment groups. Consumption of the plant extracts demonstrated no significant differences in UDP-GT activity either, when compared to the tap water control. The treatment with 2% *A. digitata* extract exhibited, although not significant, the lowest UDP-GT activity when compared to the other plant extracts, including its 5% counterpart. With *S. aethiopicus* treatment, a similar pattern was followed, where the 2% extract demonstrated a markedly (not significantly) lower UDP-GT activity compared to the 5% extraction. No significant differences were observed between the treatment with the 2% and 5% extractions of *A. betulina* and *M. flabellifolius*. 

90
Table 4.13 Hepatic phase 2 drug metabolizing enzymes in rats consuming the various plant extracts for 90days

<table>
<thead>
<tr>
<th>Plants</th>
<th>GST(\alpha)</th>
<th>GST(\mu)</th>
<th>UDP-GT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% A. digitata</td>
<td>1.14(^{ab})</td>
<td>0.11(^{ab})</td>
<td>34.98(^{ab})</td>
</tr>
<tr>
<td></td>
<td>(0.340)</td>
<td>(0.025)</td>
<td>(1.737)</td>
</tr>
<tr>
<td>5% A. digitata</td>
<td>1.39(^{ab})</td>
<td>0.15(^{ac})</td>
<td>37.33(^{abcd})</td>
</tr>
<tr>
<td></td>
<td>(0.564)</td>
<td>(0.046)</td>
<td>(0.666)</td>
</tr>
<tr>
<td>2% S. aethiopicus</td>
<td>1.35(^{ab})</td>
<td>0.18(^{ac})</td>
<td>36.30(^{a})</td>
</tr>
<tr>
<td></td>
<td>(0.499)</td>
<td>(0.052)</td>
<td>(1.807)</td>
</tr>
<tr>
<td>5% S. aethiopicus</td>
<td>1.43(^{ab})</td>
<td>0.13(^{abcd})</td>
<td>37.54(^{ace})</td>
</tr>
<tr>
<td></td>
<td>(0.246)</td>
<td>(0.028)</td>
<td>(0.697)</td>
</tr>
<tr>
<td>2% A. betulina</td>
<td>0.97(^{a})</td>
<td>0.12(^{abcd})</td>
<td>36.61(^{ad})</td>
</tr>
<tr>
<td></td>
<td>(0.126)</td>
<td>(0.028)</td>
<td>(1.086)</td>
</tr>
<tr>
<td>5% A. betulina</td>
<td>1.04(^{ab})</td>
<td>0.09(^{abd})</td>
<td>36.85(^{ad})</td>
</tr>
<tr>
<td></td>
<td>(0.279)</td>
<td>(0.013)</td>
<td>(0.774)</td>
</tr>
<tr>
<td>2% M. flabellifolius</td>
<td>1.31(^{ab})</td>
<td>0.14(^{abcd})</td>
<td>37.22(^{ac})</td>
</tr>
<tr>
<td></td>
<td>(0.515)</td>
<td>(0.049)</td>
<td>(0.861)</td>
</tr>
<tr>
<td>5% M. flabellifolius</td>
<td>1.51(^{ab})</td>
<td>0.14(^{ac})</td>
<td>37.06(^{ace})</td>
</tr>
<tr>
<td></td>
<td>(0.488)</td>
<td>(0.013)</td>
<td>(0.463)</td>
</tr>
<tr>
<td>Tap water</td>
<td>2.19(^{b})</td>
<td>0.20(^{ac})</td>
<td>37.13(^{ace})</td>
</tr>
<tr>
<td></td>
<td>(1.040)</td>
<td>(0.103)</td>
<td>(0.578)</td>
</tr>
</tbody>
</table>

The results are mean values ± SD in brackets under the means (n=7-10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Abbreviations: GST= glutathione s-transferase; UDP-GT= UDP-glucuronosyltransferase. The results were expressed as follows: GST\(\alpha\), GST\(\mu\) – (pmole/min/mg protein); UDP-GT (nmole/min/mg protein)

4.2.3. DEN-initiated, FB-promoted liver carcinogenesis study

4.2.3.1 Rat body weight gained, relative liver weight, and fluid, polyphenol and FB\(_1\) intakes

No significant differences in the BWG were noted between the different treatment groups of rats consuming the fumonisin (FB)-containing cultured material (CM), namely the positive and CM controls, and the various plant extracts, i.e. 2% A. digitata, S. aethiopicus and A. betulina (Table 4.14). However, the rats treated with the different plants exhibited a mean negative BWG. The remaining two control groups, i.e. negative and DEN controls, showed significant (P<0.05) increases in the BWG, as expected. The 2% A. digitata extract-treated rats showed the highest loss in BWG while the negative control, although not significantly different from the DEN control, demonstrated the highest BWG. The relative liver weights were not significantly different between the different treatment and control groups, except the culture material control group, where the liver weight was significantly (P<0.05) reduced. The positive control group (DEN, FB) demonstrated the highest mean average fluid intake. However, the average fluid intake volume was not significantly higher than the culture...
material control and the 2% *A. digitata* extract treatment. The consumption of 2% extracts of *S. aethiopicus* and *A. betulina* significantly (P<0.05) lowered volume intakes compared to the positive control, CM control and 2% *A. digitata* extract treatments. The negative control consumed the lowest mean amount of fluid, although not significantly different from the DEN control. Assuming that tap water contains no polyphenols, a zero value was allocated. The 2% *A. digitata*-treated rats ingested the highest amount of total polyphenols over the course of the study, followed by the 2% *S. aethiopicus* treatment rats. The consumption of 2% *A. betulina* yielded the lowest daily total polyphenol content among the three groups. The positive control rats ingested the highest mean FB content, although it did not differ significantly from the 2% extracts of *A. digitata* and *S. aethiopicus* treatments. The cultured material control rats and 2% *A. betulina* extract treated rats ingested the lowest amount (P<0.05) of FB, while the negative and DEN controls, inherently to the conditions of the experiment, did not receive any FB in their diets.

4.2.3.2 Blood parameters: liver carcinogenesis study

4.2.3.2.1 Chemical pathology parameters

No significant differences in ALP levels were observed among the different groups that were consuming the CM containing FB in their diets. The CM control treatment demonstrated a significantly (P<0.05) higher ALP level than the rats consuming the CM as well as the 2% extracts of *S. aethiopicus* and *A. betulina*. The different plant and CM treatment groups significantly (P<0.05) increased ALP levels in comparison to the negative and DEN control. AST and ALT levels followed a similar pattern as the ALP levels. All groups that received CM as part of their diet demonstrated increased AST and ALT levels compared to the negative and DEN control groups. No significant differences in the AST and ALT levels were observed between the groups on the diet containing CM and the different plant extracts.

The CM treated control rats recorded the highest mean serum iron levels, although not significantly different from the other groups, except for the DEN control group. Furthermore, the DEN control demonstrated significantly (P<0.05) lower serum iron levels when compared to the negative control group and the rats provided with CM in their diets. The groups that ingested CM demonstrated no significant differences in the CREA level, however, they had significantly (P<0.05) higher levels than the negative and DEN control groups.
Table 4.14 Body weight gain, relative liver weight and daily fluid, polyphenol and FB intake in the liver carcinogenesis study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight gain (g)</th>
<th>Relative liver weight (g)</th>
<th>Average fluid Intake (mL/100g Body Weight/day)</th>
<th>Daily total polyphenol intake (mg GAE/mg soluble solids)</th>
<th>Average FB intake (mg/100g BW/day)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>51.1 ± 20.42b</td>
<td>3.36 ± 0.25a</td>
<td>10.29 ± 0.77d</td>
<td>0 ± 0.00</td>
<td>0 ± 0.00c</td>
</tr>
<tr>
<td>DMSO+ND+water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN control</td>
<td>35.5 ± 19.11b</td>
<td>3.28 ± 0.29a</td>
<td>10.76 ± 1.23cd</td>
<td>0 ± 0.00</td>
<td>0 ± 0.00c</td>
</tr>
<tr>
<td>DMSO+ND+water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM control</td>
<td>9.3 ± 23.00a</td>
<td>2.87 ± 0.23b</td>
<td>13.26 ± 1.49a</td>
<td>0 ± 0.00</td>
<td>0.080 ± 0.00a</td>
</tr>
<tr>
<td>DMSO+CM+water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>4 ± 14.17a</td>
<td>3.71 ± 0.42a</td>
<td>14.03 ± 1.08a</td>
<td>0 ± 0.00</td>
<td>0.098 ± 0.01b</td>
</tr>
<tr>
<td>DEN+CM+water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% A. digitata</td>
<td>-8.6 ± 14.28a</td>
<td>3.64 ± 0.37a</td>
<td>13.33 ± 0.85a</td>
<td>4.64 ± 0.29a</td>
<td>0.091 ± 0.01ab</td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% S. aethiopicus</td>
<td>-3 ± 15.70a</td>
<td>3.50 ± 0.43a</td>
<td>11.98 ± 0.89b</td>
<td>2.19 ± 0.16b</td>
<td>0.088 ± 0.01ab</td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% A. betulina</td>
<td>-0.1 ± 14.65a</td>
<td>3.65 ± 0.44a</td>
<td>11.46 ± 0.88bc</td>
<td>0.94 ± 0.07c</td>
<td>0.081 ± 0.01a</td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Based on the total FBs concentration of the culture material of *F. verticillioides* utilised. Abbreviations: DMSO=dimethyl sulfoxide; ND=normal diet; DEN= diethylnitrosamine; CM= culture material of *F. verticillioides*; FB=fumonisin; GAE=gallic equivalents.
Table 4.15 Chemical pathology: liver carcinogenesis study

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALP</th>
<th>ALT</th>
<th>AST</th>
<th>Fe</th>
<th>CREA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(U/L)</td>
<td>(U/L)</td>
<td>(U/L)</td>
<td>(µmole/L)</td>
<td>(µmole/L)</td>
</tr>
<tr>
<td>Negative control</td>
<td>129.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>19.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMSO+ND+water</td>
<td>(22.47)</td>
<td>(20.24)</td>
<td>(18.03)</td>
<td>(6.59)</td>
<td>(3.34)</td>
</tr>
<tr>
<td>DEN control</td>
<td>136.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>91.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN+ND+water</td>
<td>(20.04)</td>
<td>(27.91)</td>
<td>(22.83)</td>
<td>(2.68)</td>
<td>(3.42)</td>
</tr>
<tr>
<td>CM control</td>
<td>362.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>288.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>236.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMSO+CM+water</td>
<td>(127.63)</td>
<td>(112.18)</td>
<td>(101.09)</td>
<td>(5.80)</td>
<td>(4.90)</td>
</tr>
<tr>
<td>Positive control</td>
<td>298.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>276.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>226.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>39.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN+CM+water</td>
<td>(93.70)</td>
<td>(89.68)</td>
<td>(72.55)</td>
<td>(4.44)</td>
<td>(6.14)</td>
</tr>
<tr>
<td>2% A. digitata</td>
<td>294.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>318.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>256.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>39.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td>(57.83)</td>
<td>(47.79)</td>
<td>(74.02)</td>
<td>(4.06)</td>
<td>(5.65)</td>
</tr>
<tr>
<td>2% S. aethiopicus</td>
<td>259.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>302.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>270.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>36.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td>(54.67)</td>
<td>(66.06)</td>
<td>(75.44)</td>
<td>(5.16)</td>
<td>(6.84)</td>
</tr>
<tr>
<td>2% A. betulina</td>
<td>260.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>259.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>220.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>34.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td>(67.29)</td>
<td>(67.20)</td>
<td>(98.50)</td>
<td>(5.48)</td>
<td>(4.88)</td>
</tr>
</tbody>
</table>

The results are mean values ± SD in brackets under the means (n=7-10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Abbreviations: DMSO=dimethyl sulfoxide; ND=normal diet; DEN=diethylnitrosamine; culture material of F. verticillioides; FB=fumonisin; GAE=gallic equivalents; ALT=alanine transaminase; AST=aspartate transaminase; ALP=alkaline phosphatase; Fe=iron; CREA=creatinine

4.2.3.2.2 Blood redox status and lipid peroxidation parameters

Although no significant differences were observed in plasma total polyphenols between all the groups in the study, the serum total polyphenol content of the positive control was the highest among all the groups, while the negative control contributed to the lowest serum total polyphenol value (Table 4.16). The rats consuming the various plant extracts had significantly (P<0.05) higher ORAC activities when compared to the control groups, including the positive control group. The negative, DEN and CM control groups exhibited significantly (P<0.05) lower ORAC activities in the plasma when compared to the plant extract-treated groups and the positive control.

No significant differences were observed in the blood GSH levels between the positive control group, and the plant extract treatment groups and control groups, except for the DEN control group, which was significantly (P<0.05) increased than the CM and positive control
groups. The CM treated group exhibited the lowest blood GSH level among all groups. For the whole blood GSSG, the positive control group was not significantly different from all the treatment groups. The whole blood GSSG levels was significantly (P<0.05) reduced by the plant extract treated groups and the CM control when compared to the DEN control, which provided the highest GSSG level. The 2% *A. digitata* extract treated rats demonstrated significantly (P<0.05) lower GSSG levels than the negative control groups. However, no significant differences were noted between the GSH:GSSG ratios when comparing the different groups, although the CM and positively control rats exhibited markedly (not significantly) lower levels.

When considering the serum lipid peroxidation marker, rats consuming the 2% *A. digitata* and *S. aethiopicus* extracts showed, significantly lowered (P<0.05) serum CD levels when compared to the positive control rats (DEN, FB) (Table 4.16). No significant CD differences were observed between the positive control and the 2% *A. betulina* extract treated group, as well as the negative, DEN, and CM treated control groups. The lowest CD levels were observed with 2% *S. aethiopicus* extract treatment although it was not significantly different from treatment with 2% *A. digitata*, the CM and negative control groups.

4.2.3.3 Hepatic parameters: liver carcinogenesis study

4.2.3.3.1 Hepatic redox and lipid peroxidation status

The hepatic ORAC activity of the positive control (DEN, FB) group was not significantly different from the various groups fed with CM as part of their diet (Table 4.17). The negative control group was also not significantly different from the positive control group. The DEN control group demonstrated the lowest mean ORAC activity, although not significantly different from the negative control group. The CM and 2% *A. betulina* extract treated groups exhibited significantly (P<0.05) higher ORAC activities than the DEN and negative control groups.
Table 4.16 Blood redox status and lipid peroxidation: liver carcinogenesis study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma total polyphenols</th>
<th>ORAC (μmoleTE/L)</th>
<th>GSH (μmole/L)</th>
<th>GSSG (μmole/L)</th>
<th>GSH:GSSG</th>
<th>CD (nm CD/mL Plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Negative control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO+ND+water</td>
<td>195.58&lt;sup&gt;a&lt;/sup&gt; (44.22)</td>
<td>2199.93&lt;sup&gt;b&lt;/sup&gt; (378.70)</td>
<td>552.27&lt;sup&gt;abc&lt;/sup&gt; (52.78)</td>
<td>90.55&lt;sup&gt;bc&lt;/sup&gt; (11.07)</td>
<td>6.20&lt;sup&gt;a&lt;/sup&gt; (1.10)</td>
<td>182.84&lt;sup&gt;abc&lt;/sup&gt; (30.30)</td>
</tr>
<tr>
<td><strong>DEN control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN+ND+water</td>
<td>213.92&lt;sup&gt;a&lt;/sup&gt; (53.56)</td>
<td>2097.24&lt;sup&gt;a&lt;/sup&gt; (359.04)</td>
<td>571.73&lt;sup&gt;a&lt;/sup&gt; (65.64)</td>
<td>100.79&lt;sup&gt;c&lt;/sup&gt; (8.70)</td>
<td>5.76&lt;sup&gt;a&lt;/sup&gt; (1.14)</td>
<td>194.48&lt;sup&gt;bc&lt;/sup&gt; (50.32)</td>
</tr>
<tr>
<td><strong>CM control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO+CM+water</td>
<td>211.18&lt;sup&gt;a&lt;/sup&gt; (37.74)</td>
<td>2326.01&lt;sup&gt;b&lt;/sup&gt; (295.18)</td>
<td>401.92&lt;sup&gt;b&lt;/sup&gt; (55.62)</td>
<td>78.28&lt;sup&gt;ab&lt;/sup&gt; (10.30)</td>
<td>5.26&lt;sup&gt;a&lt;/sup&gt; (1.22)</td>
<td>170.92&lt;sup&gt;abc&lt;/sup&gt; (42.27)</td>
</tr>
<tr>
<td><strong>Positive control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN+CM+water</td>
<td>249.39&lt;sup&gt;a&lt;/sup&gt; (30.38)</td>
<td>2787.42&lt;sup&gt;c&lt;/sup&gt; (402.82)</td>
<td>411.75&lt;sup&gt;bc&lt;/sup&gt; (156.90)</td>
<td>85.15&lt;sup&gt;bc&lt;/sup&gt; (16.51)</td>
<td>5.10&lt;sup&gt;a&lt;/sup&gt; (1.96)</td>
<td>210.89&lt;sup&gt;c&lt;/sup&gt; (26.53)</td>
</tr>
<tr>
<td><strong>2% A. digitata</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DEN+CM+extract</td>
<td>243.58&lt;sup&gt;a&lt;/sup&gt; (33.72)</td>
<td>3883.42&lt;sup&gt;a&lt;/sup&gt; (582.06)</td>
<td>465.18&lt;sup&gt;abc&lt;/sup&gt; (120.40)</td>
<td>65.00&lt;sup&gt;a&lt;/sup&gt; (12.56)</td>
<td>7.44&lt;sup&gt;a&lt;/sup&gt; (2.47)</td>
<td>158.42&lt;sup&gt;ab&lt;/sup&gt; (28.47)</td>
</tr>
<tr>
<td><strong>2% S. aethiopicus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td>212.51&lt;sup&gt;a&lt;/sup&gt; (42.98)</td>
<td>3611.06&lt;sup&gt;a&lt;/sup&gt; (425.78)</td>
<td>479.25&lt;sup&gt;abc&lt;/sup&gt; (48.56)</td>
<td>72.38&lt;sup&gt;ab&lt;/sup&gt; (14.12)</td>
<td>6.94&lt;sup&gt;a&lt;/sup&gt; (1.90)</td>
<td>139.09&lt;sup&gt;a&lt;/sup&gt; (40.29)</td>
</tr>
<tr>
<td><strong>2% A. betulina</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td>212.49&lt;sup&gt;a&lt;/sup&gt; (30.35)</td>
<td>3473.86&lt;sup&gt;a&lt;/sup&gt; (416.47)</td>
<td>518.68&lt;sup&gt;abc&lt;/sup&gt; (77.23)</td>
<td>75.37&lt;sup&gt;ab&lt;/sup&gt; (8.21)</td>
<td>6.90&lt;sup&gt;a&lt;/sup&gt; (0.82)</td>
<td>209.69&lt;sup&gt;bc&lt;/sup&gt; (39.93)</td>
</tr>
</tbody>
</table>

The results are mean values ± SD in brackets under the means (n=7-10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Abbreviations: DMSO=dimethyl sulfoxide; ND=normal diet; DEN=diethylnitrosamine; culture material of F. verticillioides; ORAC=oxygender radical absorbance capacity; GSH=reduced glutathione; GSSG=oxidised glutathione; CD=conjugated dienes. The results were expressed as follows: Total polyphenols (mg/L); ORAC (μmoleTE/L); GSH, GSSG - (μmole/L); CD (nm CD/mL Plasma).
When considering the hepatic glutathione status, no significant differences were observed for liver GSH levels between the groups fed with CM and the different plant extracts although the plant extract tended to counter the effect of the CM treatment. The DEN and negative control groups had significantly (P<0.05) higher liver GSH levels as compared to the rats receiving the CM and plant extract diets. The liver GSSG levels also followed the same trend, where no significant differences were observed between the rats fed the CM and different plant extracts. The GSSG level was however, significantly (P<0.05) lower as compared to the DEN and negative control groups. Significantly lower GSH:GSSG ratios were recorded in the positive control and those rats treated with 2% *A. digitata* and *S. aethiopicus* plant extracts when compared to the DEN control group. A marked (not significant) reduction in the ratio was also recorded in the liver of rats treated with the *S. aethiopicus*. The positive control group only had a significantly (P<0.05) lower GSH:GSSG ratio when compared than the DEN control group (Table 4.17).

The hepatic lipid peroxidation marker, CD, was significantly (P<0.05) elevated in the 2% *A. digitata* and *S. aethiopicus* treated groups when compared to the negative, positive, the DEN control and the 2% *A. betulina*-treated groups (Table 4.17). The CM control group exhibited a significant higher hepatic CD level when compared to the other treatments and controls. When considering the other lipid peroxidation marker, TBARS, rats consuming the 2% *S. aethiopicus* extracts had significantly lower levels than the positive (DEN, FB) control rats, with no significant changes induced by *A. digitata* and *A. betulina*. The TBARS levels were also significantly (P<0.05) increased by the CM when compared to the negative and DEN control groups.
### Table 4.17 Liver redox status and lipid peroxidation: liver carcinogenesis study

<table>
<thead>
<tr>
<th>Groups</th>
<th>ORAC</th>
<th>GSH</th>
<th>GSSG</th>
<th>GSH:GSSG</th>
<th>CD</th>
<th>TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Negative control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO+ND+water</td>
<td>15.83&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>9.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.49&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.28&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>DEN control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN+ND+water</td>
<td>13.91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>CM control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO+CM+water</td>
<td>20.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.34&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.62&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Positive control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN+CM+water</td>
<td>18.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.17&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>2% A. digitata</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td>18.44&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>2% S. aethiopicus</strong></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td>18.42&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>2% A. betulina</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN+CM+extract</td>
<td>20.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.53&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.20&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The results are mean values ± SD in brackets under the means (n=7-10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Abbreviations: DMSO=dimethyl sulfoxide; ND=normal diet; DEN=diethylnitrosamine; CM= culture material of *F. verticillioides*; ORAC=oxygen radical absorbance capacity; GSH= reduced glutathione; GSSG=oxidised glutathione; CD=conjugated dienes; TBARS=thiobarbituric acid reactive substances. The results were expressed as follows: ORAC (µmoleTE/g wet liver); GSH, GSSG – (µmole/g wet liver); CD (mM CD/100g liver); TBARS (nmole/mg protein).
4.2.3.3.2 Hepatic phase 2 drug metabolising enzymes

No significant GST\(\alpha\) differences were noticed between the groups receiving the various 2% plant extracts. The positive control group demonstrated significantly (\(P<0.0.5\)) higher GST\(\alpha\) activity than the DEN and CM control groups (Table 4.18). Considering the GST\(\mu\) activities, the A. betulina-treated rats and the positive control group demonstrated significantly (\(P<0.05\)) enhanced GST\(\mu\) activity when compared to the DEN control group. However, they did not differ significantly from the remaining groups. The S. aethiopicus-treated rats showed markedly (not significantly) decreased activity of UDP-GT, when compared to the positive control and the rats treated with A. betulina and A. digitata.

| Table 4.18 Phase 2 drug metabolizing enzymes: liver carcinogenesis study |
|-------------------------------|------------------|------------------|
| **Groups**                     | **GST\(\alpha\)** | **GST\(\mu\)**  | **UDP-GT** |
| Negative control DMSO+ND+water | 5.83\(^{ab}\)     | 0.30\(^{bc}\)    | 38.96\(^{c}\) |
| (2.37)                         | (0.12)            | (0.59)           |
| DEN control                    | 4.41\(^{a}\)     | 0.25\(^{bc}\)    | 38.97\(^{bc}\) |
| DEN+ND+water                  | (1.11)            | (0.08)           | (0.60)     |
| CM control                     | 5.14\(^{a}\)     | 0.36\(^{ab}\)    | 39.44\(^{c}\) |
| DMSO+CM+water                 | (2.00)            | (0.18)           | (0.81)     |
| Positive control               | 8.47\(^{b}\)     | 0.50\(^{a}\)     | 38.82\(^{bc}\) |
| DEN+CM+water                  | (1.71)            | (0.17)           | (0.60)     |
| 2% A. digitata                 | 6.64\(^{ab}\)    | 0.42\(^{ab}\)    | 38.69\(^{bc}\) |
| DEN+CM+extract                | (1.76)            | (0.17)           | (0.62)     |
| 2% S. aethiopicus             | 6.46\(^{ab}\)    | 0.37\(^{ab}\)    | 37.89\(^{ab}\) |
| DEN+CM+extract                | (1.41)            | (0.08)           | (0.45)     |
| 2% A. betulina                 | 6.99\(^{ab}\)    | 0.50\(^{a}\)     | 38.22\(^{ab}\) |
| DEN+CM+extract                | (1.19)            | (0.06)           | (0.60)     |

The results are mean values ± SD in brackets under the means (\(n=7-10\)). Different letters in the above columns show significant differences (\(P<0.05\)) between different experimental groups. Abbreviations: DMSO=dimethyl sulfoxide; ND=normal diet; DEN=diethylnitrosamine; CM= culture material of F. verticillioides; GST= glutathione s-transferase; UDP-GT= UDP-glucuronosyltransferase. The results were expressed as follows: GST\(\alpha\), GST\(\mu\) – (pmole/min/mg protein); UDP-GT (nmole/min/mg protein)
4.2.3.3 Liver Immunohistochemistry: GST-P\(^+\) staining

The mean total number and area of GSTP\(^+\) foci did not differ significantly between the positive control and the different plant treatment groups (Table 4.19). All the rats exposed to both initiation (DEN) and CM promotion as well as the various plant extracts demonstrated significantly (P<0.05) higher numbers of foci when compared to the negative, CM and DEN control groups. A. digitata treatment significantly (P<0.05) decreased the mean total foci area as compared to A. betulina, although no significant differences were noticed from the positive control. When compared to the rats receiving A. betulina and the positive control, the number of mini foci (<0.01 mm\(^2\)) were significantly (P<0.05) increased by the A. digitata and S. aethiopicus extracts. Considering the relative number of foci, which is the percentage number of the total number of foci, the positive control and the rats receiving A. betulina followed a similar pattern whereas A. digitata and S. aethiopicus treatment resulted in significant (P<0.05) increase. When considering the 0.01 to <0.1 mm\(^2\) category size S. aethiopicus treatment significantly decreased the relative foci number when compared to the positive control, while a marginal effect was noticed with A. digitata. No significant differences were observed in the absolute and relative number of foci between the A. betulina-treated rats and the positive control group. Rats treated with A. digitata had significantly (P<0.05) less absolute and relative numbers of foci in the category size 0.1 mm\(^2\) to 0.2 mm\(^2\) when compared to the positive control and A. betulina treatment. A marked (not significant) reduction in the relative number of foci was also noticed for S. aethiopicus treatment. For the 0.2 to <0.4 mm\(^2\) foci size category, A. digitata treatment significantly (P<0.05) decreased both the absolute and relative number of foci when compared to the rats treated with A. betulina. Of interest was that A. betulina treatment significantly (P<0.05) increased the number of 0.4 to <0.8 mm\(^2\) foci when compared to the positive control and rats treated with A. digitata groups and to a certain extent also in the S. aethiopicus treated rats. However, when considering the relative number of foci in this category, no significant differences were noted between the rats treated with the various plant extracts and the positive control. Foci above 0.8 mm\(^2\) did not differ significantly between the various plant extract groups and the positive control group. Overall, A. digitata- and to a certain extent, S. aethiopicus-treated rats, showed a significantly (P<0.05) increased number of mini foci and decreased number of foci in the <0.8 mm\(^2\) size categories when compared to the A. betulina-treated and positive rats. No significant differences in the total number of foci were noted between the rats receiving the different plants extracts and the positive control group.
When considering the total number of foci after excluding the mini foci (<0.01 mm²), *A. digitata* significantly (P<0.05) increased the relative number of foci between 0.01 mm² and 0.1mm² when compared to the rats treated with the *A. betulina* extract (Table 4.20). When considering the foci size category 0.1 to <0.2 mm², *A. digitata*-consuming rats showed significantly (P<0.05) decreased relative number of foci when compared to the *A. betulina*-treated rats and positive control group. In the 0.2 to <0.4 mm² foci size category, the *A. digitata*-treated rats showed significantly reduced relative number of foci when compared to the *A. betulina*-treated rats. In this case, the *A. digitata* treated rats did not significantly differ from the positive control group. In summary, when considering all three size categories, the rats consuming the *A. digitata* extracts had significantly increased smaller foci in their livers, while rats treated with *S. aethiopicus* and *A. betulina* extracts, lacked any effects as compared to the positive control group. For relative foci numbers above 0.4 mm², no significant differences were noticed between the rats treated with the various plant extracts and the positive control group. Similarly, the total numbers of foci also did not differ significantly between the different treated groups.
Table 4.19 Effects of various plant extracts on the induction of the number and relative incidence of GST-P⁺ foci after DEN initiation and CM promotion

<table>
<thead>
<tr>
<th>Groups</th>
<th>No of foci &lt;0.01 mm²</th>
<th>% of total</th>
<th>No of foci 0.01-0.1 mm²</th>
<th>% of total</th>
<th>No of foci 0.1-0.2 mm²</th>
<th>% of total</th>
<th>No of foci 0.2-0.4 mm²</th>
<th>% of total</th>
<th>No of foci 0.4-0.8 mm²</th>
<th>% of total</th>
<th>No of foci &gt;0.8 mm²</th>
<th>% of total</th>
<th>Total foci number</th>
<th>Total Area of foci (mm²)</th>
<th>Total Area of Liver section (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.00c (0.00)</td>
<td>0.00c (0.00)</td>
<td>1.40b (1.65)</td>
<td>35.00ab (41.16)</td>
<td>1.00d (1.25)</td>
<td>35.00ab (41.16)</td>
<td>10.00c (31.62)</td>
<td>0.00c (0.00)</td>
<td>0.00b (0.00)</td>
<td>0.00b (0.00)</td>
<td>0.00b (0.00)</td>
<td>2.50cd (2.22)</td>
<td>0.22c (0.18)</td>
<td>360.89cd (58.60)</td>
<td></td>
</tr>
<tr>
<td>CM control</td>
<td>2.00d (2.35)</td>
<td>7.55bc (8.52)</td>
<td>21.44c (13.27)</td>
<td>90.63c (8.58)</td>
<td>0.22e (0.44)</td>
<td>0.66e (1.30)</td>
<td>1.17f (3.51)</td>
<td>0.00c (0.00)</td>
<td>0.00b (0.00)</td>
<td>0.00b (0.00)</td>
<td>0.00b (0.00)</td>
<td>23.89c (14.51)</td>
<td>0.66d (0.37)</td>
<td>321.03cd (43.35)</td>
<td></td>
</tr>
<tr>
<td>DEN control</td>
<td>0.50cd (0.85)</td>
<td>2.84c (5.18)</td>
<td>14.70c (5.46)</td>
<td>84.44c (9.44)</td>
<td>1.70d (1.34)</td>
<td>9.54d (7.26)</td>
<td>2.20c (4.16)</td>
<td>0.10c (0.32)</td>
<td>0.63b (1.98)</td>
<td>0.36b (1.13)</td>
<td>17.70d (6.50)</td>
<td>1.09d (0.67)</td>
<td>277.02cd (54.68)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>48.40b (53.84)</td>
<td>12.69b (13.74)</td>
<td>214.80a (66.92)</td>
<td>58.92b (13.80)</td>
<td>59.60bc (16.57)</td>
<td>16.38b (3.71)</td>
<td>33.30ab (20.20)</td>
<td>9.00b (4.82)</td>
<td>8.70a (6.78)</td>
<td>2.35a (1.61)</td>
<td>2.30a (2.06)</td>
<td>0.66a (0.61)</td>
<td>367.50ab (72.33)</td>
<td>33.85ab (13.49)</td>
<td>322.14ab (43.89)</td>
</tr>
<tr>
<td>2% A. digitata</td>
<td>163.56a (129.88)</td>
<td>31.92a (20.95)</td>
<td>213.11a (66.00)</td>
<td>49.58ab (14.62)</td>
<td>41.67a (16.48)</td>
<td>9.88a (3.85)</td>
<td>22.89a (9.44)</td>
<td>5.46a (2.18)</td>
<td>8.78a (5.59)</td>
<td>2.38a (2.00)</td>
<td>2.67a (2.60)</td>
<td>0.79a (1.05)</td>
<td>452.78a (161.31)</td>
<td>30.54a (10.35)</td>
<td>318.86a (45.03)</td>
</tr>
<tr>
<td>2% S. aethiopicus</td>
<td>142.10a (95.45)</td>
<td>31.24a (17.60)</td>
<td>182.20a (40.08)</td>
<td>44.99a (12.35)</td>
<td>49.40ab (8.58)</td>
<td>12.33ab (3.25)</td>
<td>28.60ab (10.79)</td>
<td>7.26ab (3.63)</td>
<td>11.80ab (8.01)</td>
<td>3.05a (2.53)</td>
<td>4.40a (4.86)</td>
<td>1.14a (1.36)</td>
<td>418.50a (95.33)</td>
<td>34.65ab (12.59)</td>
<td>330.34a (43.15)</td>
</tr>
<tr>
<td>2% A. betulina</td>
<td>62.25b (104.56)</td>
<td>10.93b (16.30)</td>
<td>208.50a (52.67)</td>
<td>55.95ab (9.78)</td>
<td>66.38c (25.31)</td>
<td>17.42b (4.45)</td>
<td>40.50b (16.59)</td>
<td>10.85b (3.37)</td>
<td>15.50b (8.19)</td>
<td>4.18b (2.26)</td>
<td>2.25b (2.05)</td>
<td>0.66b (0.58)</td>
<td>395.50b (163.41)</td>
<td>41.05b (13.60)</td>
<td>340.02ab (89.91)</td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=7-10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Abbreviations: CM=culture material of F. verticillioides; DEN=diethylnitrosamine; ND=normal diet
Table 4.20 Effects of various plant extracts on the induction of the number and relative incidence of GST-P+ foci after DEN initiation and CM promotion excluding the mini foci.

<table>
<thead>
<tr>
<th>Groups</th>
<th>0.01&lt;0.1 mm²</th>
<th>0.1&lt;0.2 mm²</th>
<th>0.2&lt;0.4 mm²</th>
<th>0.4&lt;0.8 mm²</th>
<th>&gt;0.8 mm²</th>
<th>Total foci number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>1.40±</td>
<td>35.00±</td>
<td>1.00±</td>
<td>10.00±</td>
<td>0.00±</td>
<td>2.50±</td>
</tr>
<tr>
<td>CM control</td>
<td>21.44±</td>
<td>98.08±</td>
<td>0.22±</td>
<td>0.22±</td>
<td>0.00±</td>
<td>21.89±</td>
</tr>
<tr>
<td>DEN control</td>
<td>14.70±</td>
<td>87.13±</td>
<td>9.60±</td>
<td>2.26±</td>
<td>0.63±</td>
<td>17.00±</td>
</tr>
<tr>
<td>Positive control</td>
<td>214.80±</td>
<td>67.32±</td>
<td>18.82±</td>
<td>33.30±</td>
<td>0.32±</td>
<td>318.70±</td>
</tr>
<tr>
<td>2% A. digitata</td>
<td>213.11±</td>
<td>73.61±</td>
<td>14.14±</td>
<td>22.89±</td>
<td>3.41±</td>
<td>289.11±</td>
</tr>
<tr>
<td>2% S. aethiopicus</td>
<td>182.20±</td>
<td>49.40±</td>
<td>18.06±</td>
<td>28.60±</td>
<td>11.80±</td>
<td>276.40±</td>
</tr>
<tr>
<td>2% A. betulina</td>
<td>208.50±</td>
<td>66.38±</td>
<td>12.02±</td>
<td>15.50±</td>
<td>4.65±</td>
<td>333.13±</td>
</tr>
</tbody>
</table>

The results are mean values ± SD (n=7-10). Different letters in the above columns show significant differences (P<0.05) between different experimental groups. Abbreviations: CM=culture material of *F. verticillioides*; DEN=diethylnitrosamine; ND=normal diet.
Chapter 5

5. Discussion

Medicinal plants are considered to be important sources of alternative medicine on the African continent. *A. digitata*, *S. aethiopicus*, *A. betulina* and *M. flabellifolius* are multipurpose medicinal plants, and although based on anecdotal evidence of efficacy, they are commonly used as traditional medicine due to their availability in isolated rural areas of Africa (El-Rawy *et al.*, 1997; Gruenwald and Galizia, 2005; Moolla, 2006; Van Wyk, 2011). Van Wyk (2011) reviewed and described some common traditional uses for different parts of the various study plants. Here, the traditional medicinal uses of *A. digitata* were described to include treatment of fever, diarrhoea, and urinary disorders, whereas *A. betulina* was historically used as a bitter tonic, as well as digestive and diuretic agents. *A. betulina* was also used as an antiseptic agent for wounds (Van Wyk, 2011). Similarly, *S. aethiopicus* was traditionally used to treat colds, influenza, cough, hysteria, asthma and pain (Van Wyk, 2011). Traditional medicinal uses of *M. flabellifolius* ranged from providing oral hygiene and treatment of gingivitis to an agent used as topical antiseptic and for aromatherapy (Van Wyk, 2011). Additional reasons why these plants were selected for this study include their varying levels of total polyphenol content and that information on their antioxidant capacity and other possible biological activities were limited.

5.1 Part 1: Antioxidant characterization of selected African indigenous medicinal plant extracts

During part 1 of the present study, the conventional heat-assisted water extraction method of the different plant materials, as used in traditional settings, was compared to direct conventional extractions utilising the organic solvents, acetone and ethanol. The focus was predominantly on the quantification of the total polyphenolic compounds, flavonols and flavanols, as well as the antioxidant activities, measured by the TEAC, FRAP, and ORAC assays, in order to advise on the optimum conditions for polyphenolic-rich extractions. Polyphenolic compounds have been shown to be highly bioactive, normally attributed to their antioxidant activities (Skerget *et al.*, 2005; Einbond *et al.*, 2008). Furthermore, polyphenolic constituents have previously been characterised for *A. digitata*, *S. aethiopicus*, *A. betulina*...
and *M. flabellifolius*. These studies mostly focused on determining phenolic compounds, such as ursolic acid (Sipra-Dan and Dan, 1986), β-sitosterol (Ramesh et al., 1992), β-amyрин palmitate (Ramesh et al., 1992) and triterpenoids (Ramadan et al., 1994) isolated from *A. digitata*. For *A. betulina*, 1-pulegone and diospenol were previously isolated (Fluck et al., 1961; Kaiser et al., 1973), while carvone and perflic acid were indicated to be the major polyphenolic constituents of *M. flabellifolius* (Da Cunha, 1974). Almost 30 years later, another study identified pinocarvone, trans-pinocarveol limonene, trans-p-menth-1-(7)-8-diene-2-ol and cis-p-menth-1-(7)-8-diene-2-ol as the major compounds of *M. flabellifolius* (Viljoen et al., 2002). A separate study by Moore and colleagues (2005) identified a polyphenol called 3,4,5 tri-O-galloylquinic acid which was reported to protect the plants’ membranes against desiccation, which is a state of extreme dryness, and free radical-induced oxidation. Furanoterpenoid derivatives, namely 4αH-3,5ox,8ab-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8-one and 2-hydroxy-4αH-3,5ox,8ab-trimethyl-4,4a,9-tetrahydro-naphtho[2,3-b]-furan-8-one, were isolated from *S. aethiopicus* (Holzapfel et al., 2002). To date, according to our knowledge, no study has been published describing the total polyphenolic, flavanol, and flavonol contents, in addition to assessing the antioxidant capacities of these plants.

Similarly to results yielded by the antioxidant content and activity measurements, the amount of soluble solids yielded were also influenced by the extraction solvent. Whereas increased amounts of soluble solids were extracted with water compared to acetone and ethanol, the antioxidant content and activity were decreased when water was used as a solvent. Water could only extract water-soluble bioactive compounds from the plants, whereas the organic solvents, acetone and ethanol, were more successful at extracting polyphenolic-like compounds which were predominantly of lipophilic nature. Previously, it has been established that the choice of solvent used and the extraction method influenced the yield of components for investigation (Goli et al., 2004; Mohammadi and Atik, 2011). Mohammadi and Atik (2011) further established that water combined with organic solvents provided better yields of polyphenolic compounds. Other studies on *A. digitata* expressed the need to consider both water-and lipid-soluble fractions of plants during extraction (Vertuani et al., 2002; Besco et al., 2007). This was required to give a more comprehensive determination of antioxidant capacities of plants as more diverse compounds were extracted. The effectiveness of solvents was deemed to generally depend on the ability to extract the components of interest, simultaneously, avoiding the modifications of the investigated compounds (Zuo et al., 2002). The differences in extracted solids between various plants
could be attributed to the ecological, in particular, genetic and environmental differences between them. Confirmatory to the findings of the present study, earlier studies also indicated that water was found to be less effective for extraction of polyphenols (Yao et al., 2004; Zhou and Yu, 2004). In one study, water extracted less tea polyphenols when compared to absolute methanol (Yao et al., 2004), while acetone extracted superior polyphenols than water from wheat (Zhou and Yu, 2004). Polyphenols are made up of compounds with varying structures due to the polymerization with sugars and proteins, in addition to the changes attributed to high temperatures, alkaline environments and effects of oxidation. These factors were suggested to contribute to the disparities in the quality of extracted polyphenols using different solvents and conditions of extraction (Tura and Robards, 2002). The conditions in the plants’ natural growing environments, storage before analysis and processing at analysis could potentially also play roles in the final structures of polyphenols and other bioactive compounds of plants when extracted (Amarowicz et al., 2009).

In the current study the acetone and ethanol extracts of S. aethiopicus and A. betulina yielded higher flavonol content than total polyphenol content. This finding is contrary to the norm, where TP represents a group of many different phytochemicals, including flavanols and flavonols, and thus one would expect TP to be highest. An earlier study by Escarpa and Gonzalez (2001) reported similar results, where abnormally high flavonols were measured using the spectrophotometric method. However, when comparing this method to the referenced chromatographic method, it was concluded that the spectrophotometric readings were non-specific. This uncharacteristic finding was suggested to be due to interferences from cellular compounds in the extract which also absorbed at the same wavelength of 360 nm (Escarpa and Gonzalez, 2001). In the present study, acetone and ethanol could possibly have extracted cellular compounds that absorbed at the same wavelength as the flavonols. Flavonol determination using the chromatography method will most probably provide more reliable measurements for acetone and ethanol extracts of S. aethiopicus and A. betulina.

The plants investigated in the present study consisted of a wide variety of phytochemicals (as indicated above), most of which have not been fully characterized to date. These secondary metabolites serve a variety of essential functions in the survival of plants (Ehrlich and Raven, 1967; Williams et al., 1989; Bennett and Wallsgroove, 1994; Jansen et al., 1998;
Naturally, plants were evolutionary equipped with antioxidant systems to maintain internal growth and metabolism. The antioxidant systems comprise networks of phytochemicals and enzymes working cooperatively to deal with various stress conditions confronting the plants. Plants with higher antioxidant capacity are known to be more tolerant to herbicide-induced oxidative stresses than the ones with low antioxidant capacities (Arora et al., 2002). The three antioxidant capacity assays used in the current study differ in principle. The FRAP assay, a versatile method which is readily applied to both water and alcohol extracts of plants, measures antioxidant capacity on the basis of reducing ferric (III) ions to ferrous (II) ions (Benzie and Strain, 1996). The second technique, the TEAC assay, is also highly recommended for plant extracts, and measures the ability of a compound to scavenge ABTS\(^{•+}\) radicals (Awika et al., 2003). The ORAC assay is a fluorescence-based technique, where radicals generated by 2,2’ azobis (2-amidinopropane) dihydrochloride (AAPH) are scavenged by fluorescein salt until completion. The antioxidant capacity is established by calculating the area-under-the-curve, combining simultaneously, time and degree of radical generation inhibition (Cao et al., 1995). Hence variations exist in the results using these antioxidant assays due to ecological, geographic and time of harvest, amongst other differences between the plants and the universal complex chemical nature of polyphenols and their antioxidant properties. Considering the above, no single assay can comprehensively determine antioxidant activities of plant extracts (Prior and Cao, 1999; Buenger et al., 2006). Therefore, a combination of FRAP, TEAC and ORAC were used in the present study. Earlier, the ORAC assay was suggested to mimic antioxidant activity of phenolic compounds more closely than other available methods of antioxidant activity determination. This characteristic made the ORAC assay the method of choice for antioxidant activity measurement of polyphenols in that study (Ou et al., 2002). However, in the present study, the ORAC assay was more effective in determining antioxidant capacities of TP, flavanols and flavonols in aqueous plant extracts than in acetone and ethanol extracts. In the acetone and ethanol extracts of the various plants, the ORAC assay was significantly less reliable when compared to the TEAC and FRAP assays. The TEAC and FRAP assays were equally effective in demonstrating antioxidant capacity in aqueous plant extracts. In summary, thus the latter two assays proved to be more effective indicators of TP and flavanols antioxidant capacity in acetone and ethanol extracts while ORAC was more effective in an aqueous environment. This is an indication that the TEAC and FRAP assays provided more effective determinations of both hydrophilic and lipophilic antioxidant capacities (Thaipong et al., 2006).
Various studies have described the polyphenol content and/or antioxidant capacity of *A. digitata* (Vertuani *et al*., 2002; Besco *et al*., 2007; Lamien-Meda *et al*., 2008), *S. aethiopicus* (Steenkamp *et al*., 2005), *A. betulina* (Moolla and Viljoen, 2008), and *M. flabellifolius* (Moore *et al*., 2005). However, the approaches and techniques used in those studies were all different, again emphasizing the importance to standardise measurements to allow for universal interpretation thereof.

The current study established the antioxidant contents and activities by using spectrophotometric techniques. Where Moore and colleagues (2005) used NMR spectroscopy to quantify a purified polyphenolic compound in *M. flabellifolius*, the present study quantified the overall phenolic content and antioxidant capacity of extracts, important when considering the extract as a whole. The antioxidant activity determinations for *A. digitata* (Vertuani *et al*., 2002; Besco *et al*., 2007; Lamien-Meda *et al*., 2008) resembled our approach more closely, but they compared different parts of the plant, as well as how to maximize the antioxidant activity measurement, extracting both water- and lipid-soluble fractions for the quantification. Additionally, different antioxidant capacity determination techniques were used in the previous studies, with results also being expressed in dissimilar units from each other and the present study. In 1998, Cook and colleagues used the trolox assay to estimate the antioxidant activity of 17 traditional medicinal plants of Niger, with *A. digitata* as one of the screened plants. An activity of 7.7 μmole TE/g dry weight of the plant was reported, which indicated poor antioxidant capacity when compared to the other tested plants (Cook *et al*., 1998). A study by Vertuani *et al*., (2002) using the photochemiluminescence (PCL) method, utilising both water and methanol extractions of different parts of the *A. digitata* plant, calculated the integral antioxidant capacity as the sum of the water and methanol extracts (Vertuani *et al*., 2002). The *A. digitata* leaves, reported a combined activity of 8.74 mmole TE/g dry weight. Furthermore, the water extract (6.39 mmole TE/g dry weight) demonstrated a higher antioxidant capacity than the methanol (2.35 mmole TE/g dry weight) extract for dried leaves. Similarly, the antioxidant capacities of the water extracts of the fruit pulp, grounded fruit shell, fruit glycolic extract and leave glycolic samples were also higher than the methanol counterparts (Vertuani *et al*., 2002). These finding contradicted the present study, where water extracts of the studied plants demonstrated the lowest antioxidant capacities when compared to ethanol and acetone extracts. *M. flabellifolius*, when measuring TEAC and FRAP, was the only exception to the above trend, where the water extracts demonstrated higher antioxidant capacity than ethanol and acetone extracts.
Methanol and acetone extracts of *A. digitata* fruit were previously assessed for total polyphenol and flavonoid content, while antioxidant capacity was determined using the DPPH, FRAP, and TEAC assays (Lamien-Meda *et al.*, 2008). The acetone extract had a higher total polyphenol (4057.50 mg GAE/100 g of fruit) and total flavonoids (42.73 mg GAE/100 g of fruit) contents as compared to the methanol extract of 3518.33 mg GAE/100 g of fruit and 31.70 mg GAE/100 g of fruit extract, respectively. When considering the antioxidant capacities, using the DPPH assay, no significant difference between acetone and methanol extracts was observed. However, the acetone extract demonstrated significantly higher antioxidant capacity than the methanol extract using the FRAP assay, whereas these results were reversed for the TEAC assay (Lamien-Meda *et al.*, 2008).

Steenkamp and colleagues (2006) used the hydroxyl (OH•) radical scavenging ability assay to assess the antioxidant activity of *A. betulina*. The group observed that the hydroxyl (OH•) radical scavenging ability was reduced between 80% and 90% for ethanol and water extracts of *A. betulina*, respectively. Another study using the DPPH assay reported that *A. betulina* possess moderate to poor antioxidant activity (>100 µg/mL), while also demonstrating a good radical scavenging ability using the ABTS assay (Moolla *et al.*, 2007). Once again, differences in correlation between the various assays are evident, as the various assays operate on different principles (Parejo *et al.*, 2002; Arts *et al.*, 2003). The ORAC assay was used to investigate the antioxidant activity of *A. betulina*, utilising different extraction solvents (Wojcikowski and colleagues, 2007). The sum of ethyl acetate (22.72 µmole TE/dry weight), methanol (61.60 µmole TE/dry weight) and water/methanol mixture (174.92 µmole TE/dry weight) ORAC measurements of *A. betulina* were used to calculate the total ORAC value of 259.25 µmole TE/dry weight (Wojcikowski *et al.*, 2007). The water/methanol extraction is likely to extract components with both water- and lipid-soluble characteristics, however, ethyl acetate and separate methanol extractions were also included in the calculation for total ORAC. This serves as further evidence that the different antioxidant assays respond differently and that more than one technique should be used for reliable assessments. Nevertheless, all above-mentioned studies confirmed, to some degree, that the plants in the present study contain polyphenolic compounds while also exhibiting varying antioxidant capacities.

The current study, therefore, established total polyphenol, flavanol and flavonol contents of *A. digitata*, *S. aethiopicus*, *A. betulina* and *M. flabellifolius* in whole plant extracts.
Additionally, antioxidant activities and the effects of different solvents used for extraction were also described for the first time. Corresponding to the theory of medicinal plants, where the internal protective properties of plants can be acquired upon herbivore consumption of plants (Wink and Schimmer, 1999), more in vitro, in vivo and epidemiological evidence surfaced of associations between polyphenols in plants and their resulting antioxidant activities. Often, the antioxidant capacity has been implicated to be responsible for suggested protective properties against human diseases (Visioli and Galli, 2002; Ruzaidi et al., 2005; Marnewick et al., 2011).

5.2 In vivo studies (part 2, 3) assessing the modulation of phase 2 hepatic drug metabolising enzymes and cancer promotion utilising two experimental rat models

In the first in vivo study (part 2), 2% and 5% extracts of A. digitata, S. aethiopicus, A. betulina and M. flabellifolius were used to investigate the possible modulation of hepatic phase 2 drug metabolising enzymes after consuming these extracts as drinking fluid for 90 days. In the second in vivo study (part 3) liver cancer was initiated using DEN, while promotion was effected by feeding the experimental rats with fumonisin-containing cultured material (CM; 5%) of F. verticillioides for 3 weeks. For this study, only the 2% extracts of A. digitata, S. aethiopicus and A. betulina were included as drinking fluid to assess the possible modulation of the cancer promotion. Results from the chronic study (part 2), showed a dose dependent increase in the soluble solid content as an increased amount of plant material was used for extraction. The increase in the soluble solid content of the different plant extracts translated into significantly higher TP, except for A. digitata. The general significant lowering of flavanols and flavonols from 2% to 5% concentrations, except for A. digitata and S. aethiopicus, respectively, could possibly be due to the decrease in solvent-to-solid ratios in cases of higher concentrated extracts which is in agreement with findings described elsewhere (Cacace and Mazza, 2003; Pinelo et al., 2005). The overall significant decrease in antioxidant capacity obtained from 2% to 5% concentrations, when using the ORAC assay, except for S. aethiopicus, is probably related to the decreased flavanol content. For A. digitata, A. betulina, and M. flabellifolius, significant decreases in flavanols and flavonols also resulted in corresponding significant decreases in antioxidant activity, which could be related to the interference of non-flavonoid-like compounds, counteracting the antioxidant properties of the flavonoid-like constituents. It is unclear at present why the antioxidant activity and TP content was lower in the freeze dried aqueous extract prepared from the same dried plant material.
5.2.1 Modulation of phase 2 hepatic xenobiotic metabolising enzymes and antioxidant status by African medicinal plants

The in vitro antioxidant contents and capacities of *A. digitata*, *S. aethiopicus*, *A. betulina* and *M. flabellifolius*, at 2% and 5% concentrations, are described in Section 5.1. This prompted further investigations into possible bio-activity, in this case the possible modulation of phase 2 xenobiotic metabolising enzymes and the hepatic oxidative status in rats chronically consuming aqueous extracts of these plants. Furthermore, this part of the study also evaluated and compared the effects of the two concentrations used, i.e. 2% and 5%. Dietary phytochemicals possess the ability to modulate endogenous enzyme systems responsible for the metabolic activation or detoxification of xenobiotics in cells (Marnewick *et al.*, 2003; Keyser, 2012). Hepatic phase 1 and 2 enzymes play this essential role and often lead to either protective or adverse effects in cells. Marnewick and colleagues (2003) demonstrated that polyphenolic compounds of two South African herbal teas provided protection against DNA damage in rats by inducing phase 2 hepatic drug metabolising enzymes. More recently, Keyser (2012) suggested that modulation of phase 2 xenobiotic metabolising enzymes in male Fischer rats, following the consumption of phenolic and organosulphur compounds in garlic extracts, could provide protective properties against oxidative stress and mutagenesis. *A. digitata*, *S. aethiopicus*, *A. digitata* and *M. flabellifolius* are multipurpose indigenous medicinal plants and could provide important therapeutic approaches in modulating xenobiotic metabolising enzymes to protect against oxidative stress (Hutchings, 1962; Watt and Breyer-Brandwijk, 1962; Ramadan *et al.*, 1994; Hutchings, 1996; El-Rawy *et al.*, 1997; Van Wyk *et al.*, 1997; Simpson, 1998; ; Lis-Balchin *et al.*, 2001; Rust, 2003; Moolla, 2006; Moolla and Viljoen, 2008).

In general the chronic intake (90 days) of aqueous extracts of *A. digitata*, *S. aethiopicus*, *A. betulina* and *M. flabellifolius* presented no adverse effects on the body weight gain and liver function of the experimental rats. Although the relative liver weights were significantly increased by the 2% and 5% *S. aethiopicus*, and *A. betulina*, as well as the 5% *A. digitata* extracts, the normal liver function tests (ALP, AST and ALT) suggested an intact structural integrity of the liver tissues. However, extracts of *A. digitata* and *A. betulina* significantly increased serum creatinine, indicating some adverse effects in the kidney of the rats, which raised some concern for the chronic consumption of these extracts. Apart from these changes, there was no preliminary evidence of overt toxicity associated with ingestion of the extracts. According to Provenza (1998), ingestion of toxic substances by an animal damages
the stomach lining, resulting in the release of serotonin, which induces nausea, with a subsequent reduction in feed consumption, which was not the case in the present study. The daily average feed intakes resulted in an increased daily total polyphenol intake for the different treatment groups. The significantly increased serum iron levels caused by the 2% *A. digitata*, compared to the tap water control, could possibly be due to an enhancing effect on iron absorption from the gastrointestinal tract. Of interest is that the 5% extract did not cause this increase, as iron levels were similar to that of the control animals. Some *in vivo* studies have indicated no effect on iron absorption due to the intake of polyphenol-rich plant extracts (Hesseling *et al.*, 1979; Marnewick *et al.*, 2003; Keyser, 2012), whereas another study reported a reduced iron absorption by black and green tea consumption in humans (Temme and Van Hoydonck, 2002). Since polyphenols can act as antioxidants by chelating metals (Brown *et al.*, 1998), the significantly decreasing serum iron concentration from 2% to 5% extracts can possibly be related to the increasing polyphenol concentration as well as the resultant increase in TP consumed during the course of the study. Rats consuming the 5% *A. digitata* demonstrated a significant decrease in serum iron levels when compared to rats consuming the 2% *A. digitata* extract, although this reduction in iron absorption was not significantly below the levels of the control rats. Of interest was that the far higher level and intake of *M. flabellifolius* polyphenols did not reduce the serum iron levels which may be related to its unique polyphenolic composition compared to that of *A. digitata*, *S. aethiopicus* and *A. betulina*.

The 2% *S. aethiopicus* consumption caused a significant increase in the serum TP levels when compared to rats that consumed tap water as well as all the other plant extracts which co-incident with an increased plasma CD level. In this instance, interference from the increased plasma polyphenol level in the CD assay could explain this phenomenon, as the increase in plasma TP level was not reflected in the plasma ORAC activity nor in the whole blood GSH and GSSG levels and GSH:GSSG ratio. In the current study, plasma ORAC activity and whole blood GSH levels were not affected by the consumption of the various plant extracts. The whole blood GSSG levels were decreased in rats consuming the *M. flabellifolius* (2%) and *A. digitata* (5%) extracts and it tends to coincided with the increased plasma CD levels, which is an early indication of lipid peroxidation. A dose-dependent increase in the whole blood GSH:GSSG ratio was observed with *A. digitata* consumption, but not for the other plant extracts. Rats consuming the 2% extracts of *A. betulina* and *M. flabellifolius* also showed an increased glutathione ratio, while no differences were noticed for *S. aethiopicus* (2% and 5%). It is not known whether these changes could be related to the altered creatinine levels and the recorded adverse effects in
the kidneys. Previous studies have reported that blood biomarkers of oxidative status were not indicative of organ/tissue condition (Arguelles et al., 2004; Tong et al., 2005). Some blood biomarkers of lipid peroxidation, such as TBARS have additionally been reported to be later indicators of liver damage due to valproic acid treatment, where liver necrosis occurred before markers were detected in the peripheral blood (Tong et al., 2005). Therefore, there was a need to measure the oxidative status parameters in the liver.

Regardless of the polyphenolic intake in the form of plant extracts and their associated ORAC activities, no significant hepatic antioxidant activity differences were observed between consumption of the various aqueous plant extracts and the tap water control in the liver. Comparison between the plant extracts indicated that the 5% A. betulina extract significantly reduced the hepatic ORAC activity compared to the 2% A. betulina extract, despite the higher total polyphenol intake. This also became evident as no significant relationship between the amount of polyphenol intake and the hepatic ORAC activity could be established. Presumably it could be related to pro-oxidant properties of the increased polyphenol constituents of the 5% A. betulina extract. Other studies on different plants were also inconclusive on this matter (Marnewick et al., 2003; Keyser, 2012). When considering the hepatic GSH levels, no significant differences were noticed in the liver of rats consuming the various plant extracts and those consuming the tap water, a finding that concur with that in whole blood. However, similar to the whole blood, significant decreases in GSSG, the oxidised glutathione, were observed with consumption of 2% extracts of A. betulina and M. flabellifolius, as well as 5% A. digitata, when compared to the tap water consumption. As a result, the GSH:GSSG ratio was significantly increased for 2% A. betulina, 2% M. flabellifolius, and 5% A. digitata. Increased GSH:GSSG ratios may result in an increased antioxidant capacity in the cells, associated with a reduced cellular oxidative stress. Either scenario could be interpreted as reduced susceptibility to oxidative damage. Similar, effects of unchanged GSH levels, compared to decreased GSSG levels, were demonstrated by rooibos and honeybush teas, resulting into increased GSH:GSSG ratios. The authors ascribed the effects to possible stabilization of GSH levels in the liver (Marnewick et al., 2003). The 2% extracts of A. digitata and S. aethiopicus significantly reduced the GSH:GSSG ratios, compared to the tap water control, although their individual hepatic GSH and GSSG levels were not significantly different from the tap water control. This effect could be due to decreases in GSH and increases in GSSG levels in rats consuming these extracts, although changes were not significantly. This could be indicative of an increasing susceptibility to oxidative damage. When considering lipid peroxidation, the 2% extracts of A. digitata and S. aethiopicus significantly reduced hepatic lipid peroxidation both in the CD
and TBARS assays. The 5% *M. flabellifolius* was the only extract demonstrating increased hepatic lipid peroxidation using the TBARS assay. In the case of *A. digitata* (2%), hepatic lipid peroxidation (CD and TBARS) was significantly reduced, regardless of the polyphenolic intake, which coincided with the reduction in the GSH:GSSG ratio compared to the 5% extract. In this case, the superior GSH:GSSG of the 5% *A. digitata* when compared to the 2% counterpart resulted from a significant decrease in GSSG and marked increased level of GSH indicative of oxidative stress. Reduced glutathione (GSH) is known to be an important intracellular antioxidant, protecting cells against oxidative damage (Awad and Bradford 2006). Glutathione was also suggested to play a vital role in stabilizing many enzymes (Wang and Jiao, 2000) and is generally considered to be a good indicator of redox status in tissues (Kidd, 1997). Presence of excess *in vivo* GSH levels in tissues was shown to offer protection against toxic metabolites where GSH conjugated with toxic metabolites, resulting in less toxic products (Younes and Siegers, 1980). Decreased *in vivo* GSH levels translated into detrimental effects, such as an increase in lipid peroxidation as a result of the lowered cellular redox potential (Exner *et al.*, 2000). It would appear that in the present study, the utilised plant extracts altered the GSH:GSSG ratio and hence the oxidative status in the liver and, depending on the level of exposure, could either protect against or increase the susceptibility for oxidative stress.

When considering the phase 2 hepatic enzymes, polyphenols are known to alter these xenobiotic metabolising enzymes in the liver that are predominantly responsible for detoxification of reactive electrophilic compounds to non-reactive hydrophilic ones, which can subsequently be excreted through the bile and/or urine (Rendic, 2002; Nerbert and Russell, 2002). However, in some cases, the xenobiotics are converted to even more toxic, mutagenic and/or carcinogenic products in a process referred to as bio-activation (Zhou, 2003). The induction of phase 2 metabolising enzymes expression, including the glutathione-S-transferases (GST-α and GST-μ) and UDP-glucuronosyltransferase have been suggested as major mechanisms of plant polyphenol chemopreventive properties (Sies *et al.*, 2000). In certain instances, phase 2 metabolising enzymes are inhibited. Compounds demonstrating GST inhibition can be beneficially used to inhibit drug resistance to tumours (Mukanganyama *et al.*, 2002), whereas a reduced GST activity may result in decreased detoxification of electrophilic xenobiotic compounds with a resultant increased cellular toxicity (Capela *et al.*, 2007). The expressions of different isoforms of GST are inhibited by a variety of compounds, often of polyphenolic nature, i.e. flavonoids have previously inhibited expression of GSTs in platelets of humans (Ghaemali and Waring, 1999), while quercetin, genistein, kaempferol and caffeic acid also inhibited the expression of various subtypes of
GSTs (Das et al., 1984; Burg and Mulder, 2002). In the present study, no induction of the hepatic phase 2 metabolising enzymes, GST\(\alpha\), GST\(\mu\) and UDP-GT occurred. In fact, the consumption of 2% A. betulina significantly (P<0.05) inhibited the GST\(\alpha\) activity when compared to the tap water control. This could possibly be due to compounds in A. betulina affecting gene expression of the hepatic GST activity by the polyphenols as described elsewhere (Hayeshi et al., 2004). More information on the composition and associated concentration of polyphenols of the various plant extracts used in the present study is required. This could determine the presence of potential xenobiotic metabolising enzyme inhibitors and/or enzyme inducers.

Another consideration is that GSH stabilization could possibly have occurred upon administration of the A. digitata, S. aethiopicus, and A. betulina extracts. However, protection/detoxification could potentially be afforded by other GST subtypes, not affected as well as other phase 1 and phase 2 metabolising enzymes. Therefore, the induction or inhibition of phase 1 hepatic drug metabolising enzymes, namely CYP 450, could also provide more inside to fully characterizing the protective/damaging effects of the plant extract consumption in the current study. It is important to note that, the reduced or increased expression of xenobiotic metabolising enzymes has been shown to be tissue and species specific (Krajka-Kuźniak et al., 2008). Therefore, similar findings might not occur during human consumption of the plant extracts utilised in the present study.

5.2.2 Modulation of hepatic phase 2 drug metabolising enzymes in DEN- initiated and fumonisin- promoted liver carcinogenesis in male Fischer rats

To date, no studies have reported on the possible chemopreventive properties of A. digitata, S. aethiopicus, and A. betulina, which are popular indigenous plants, readily used for their multipurpose medicinal properties in herbal and traditional medicinal settings (Hutchings, 1962; Watt and Breyer-Brandwijk, 1962; Ramadan et al., 1994; Hutchings, 1996; El-Rawy et al., 1997; Van Wyk et al., 1997; Simpson, 1998; Lis-Balchin et al., 2001; Rust, 2003; Moolla, 2006; Moolla and Viljoen, 2008). Epidemiological evidence suggests that the consumption of a polyphenolic rich diet is inversely associated with chronic human diseases, including cancer (Khan and Mukhtar, 2008) and cardiovascular diseases (Dubick and Omaye, 2001). This epidemiological evidence is followed up with in vitro and in vivo research to provide evidence of the existing or absent relationships. One of the more established models for
investigating the relationship between polyphenols and chronic human diseases related to oxidative stress, in particular, depends on the initiation of cancer in male Fischer rats with dietylnitrosamine (DEN) followed by the promotion using maize culture material (CM) of *F. verticillioides*. The rats are offered polyphenolic rich diets or fluids during the course of the study and serum and livers harvested at sacrifice to investigate different oxidative stress and chemopreventive properties related to cancer initiation and promotion (Gelderblom *et al*., 1991; Marnewick *et al*., 2009; Keyser, 2012).

In the present study, cultured material contaminated with a combination of various types of fumonisins, fumonisins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>, (FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub>) was incorporated into the diets (standard rat chow) of rats receiving the various plant extracts (2%) in their drinking water following DEN initiation. Fumonisins are secondary metabolites produced by moulds and fungi which are generally considered to be risk factors for causing different types of cancers in some parts of the world, more notably in Asia and sub-Saharan Africa (Shephard *et al*., 1996; Gelderblom *et al*., 1997; Hui-Chen *et al*., 2009). They are produced by several species of the genus *Fusarium* with *F. verticillioides* and *F. proliferatum* amongst the most frequently encountered species which commonly contaminating maize-containing food products (Shephard *et al*., 1996). Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is the most abundant amongst the fumonisins, accounting for approximately 70% of the total concentration of detected fumonisins (Merril *et al*., 1996). Various experimental animal studies demonstrated hepatotoxicity when chronically ingesting diets containing small amounts of FB<sub>1</sub> (Jaskiewicz *et al*., 1987; Ledoux *et al*., 1992; Voss *et al*., 1993; Dilkin *et al*., 2003; Marnewick *et al*., 2003; Orsi *et al*., 2007; Marnewick *et al*., 2009). In humans, FB<sub>1</sub> ingestion was predominantly, epidemiologically associated with the development of oesophageal cancer (Rheeder *et al*., 1992).

In the present study, a significant decrease in body weight gain (BWG) of groups that received fumonisin-containing CM as part of their diet, coupled with significant increases in liver function tests (ALP, AST, ALT) and serum creatinine, suggestive of initial experimental indicators of liver and kidney damage. No significant differences in BWG and relative liver weight (RLW), as well as liver function tests, iron, and creatinine were observed between the positive control- and plant extract-treated rats. This finding indicated that the TP intake associated with the plant extract consumption could not inhibit the fumonisin-induced toxicity. No significant differences in FB intake were observed between *A. digitata* and *S. aethiopicus* consuming groups and the positive control. The 2% *A. betulina* consuming group ingested significantly less FB than the positive control. According to Gelderblom and
CHAPTER 5

colleagues (2002), FB₁ possesses weak initiation potential even at high concentrations, while bearing more effect on cancer-promotion (Gelderblom et al., 1991) and organ-specific toxicities (Gelderblom et al., 1988; Voss et al., 1995).

Serum TP was not altered by intake of the various plant extracts. However, the plasma ORAC was enhanced when compared to the negative and positive control groups. In addition, the synergistic effects of DEN-initiation and fumonisin-promotion in the positive control presented increased plasma ORAC when compared to the negative control group, as well as DEN and CM control groups. Although earlier reports (Chap 4; Table 4.11) suggested unaltered plasma ORAC with consumption of A. digitata, S. aethiopicus, and A. betulina, the findings in the present study suggest that the plasma ORAC was increased by the fumonisin-induced toxicity under the current experimental conditions. Furthermore, fumonisin and DEN/fumonisin treatments reduced whole blood GSH levels, however, it appeared as if the plant extracts, specifically A. betulina countered this effect to an extent. The lowered whole blood GSSG levels that were associated with A. digitata, and to a certain extend the other extracts as well, contributed to the maintenance of the GSH:GSSG ratios that were not significantly different from the positive and fumonisin control treated rats. Simultaneously, plasma CDs, were significantly decreased by A. digitata and S. aethiopicus consumption, implying protection against lipid peroxidation. Not enough data is available to determine the actual bioavailability of plasma polyphenols (Manach et al., 2004). Manach and colleagues (2004) suggested that the actual bioavailability of the different plant polyphenols have to be determined. Often, there is an interference with polyphenol measurement in the blood, as polyphenols are conjugated to plasma proteins, such as albumin (Boulton et al., 1998). Furthermore, blood biomarkers of oxidative status were previously reported to have no indication of the situation in the organ/tissue (Arguelles et al., 2004; Tong et al., 2005). Therefore, in the current study, determinations of oxidative stress were also conducted in livers of the study rats.

The ORAC activity and GSH:GSSG ratios are commonly considered to be important antioxidant capacity indicators in vivo. The hepatic ORAC activity was not significantly altered by TP intake in fumonisin-fed rats, whereas hepatic GSH depletion was observed with fumonisin ingestion. Earlier, in the absence of DEN/fumonisin toxicity, no GSH depletion was observed (Table 4.12). Glutathione, is an important intracellular antioxidant, yet also responsible for the detoxification of electrophilic metabolites (Younes and Siegers, 1980; Awad and Bradford 2006). As the hepatic GSSG levels were also significantly reduced in
groups fed with fumonisin, the hepatic GSH, although in a stabilized form, could have become depleted by the increased oxidative stress in the liver. The reduced levels of hepatic GSH led to a reduced GSH:GSSG ratio known to be associated with oxidative stress. Reduced GSH:GSSG ratios were previously established to result in an increased susceptibility to oxidative damage (Marnewick et al., 2003; Keyser, 2012). In the present study, hepatic lipid peroxidation was determined by the CD's and TBARS assays. Overall, no distinct relationship was observed between hepatic lipid peroxidation and the hepatic ORAC activity and/or hepatic GSH: GSSG ratios. While no significant differences in hepatic ORAC activity and the GSH status were observed between the positive control and plant extract consuming rats, a significant difference in CD and TBARS levels were noticed, as A. digitata and S. aethiopicus consumption increased the CD levels as compared to the positive control and A. betulina consumption. When considering TBARS, the extract of S. aethiopicus significantly decreased the level compared to the other extracts and positive control. The TBARS assay was earlier suggested to be an indicator of later stage lipid peroxidation (Tong et al., 2005). A. digitata consumption demonstrated no significantly different TBARS levels compared to S. aethiopicus, the positive control and A. betulina.

The activities of hepatic phase 2 drug metabolising enzymes were also determined to assess the metabolism of the plant extracts components. Phase 2 drug metabolising enzymes are instrumental in the detoxification of potentially harmful xenobiotics to hydrophilic metabolites that are readily excreted through the bile and/or urine (Rendic, 2002; Nerbert and Russell, 2002). An earlier study demonstrated that up-regulating phase 2 metabolising enzymes resulted in protection against potentially harmful environmental insults (Kong et al., 2001). Further subsequent studies, using the DEN-initiation and FB1-promotion model in male Fischer rats demonstrated similar protective properties due to induction of phase 2 metabolising enzymes (Marnewick et al., 2009; Keyser 2012). GST (α and μ) and UDP-GT are phase 2 metabolising enzymes. GSTs detoxify xenobiotics by catalysing the conjugation of GSH to electrophilic or alkylating compounds, ultimately rendering these compounds hydrophilic for excretion (Boyland and Chesseaud, 1967). In similar fashion, UDP-GT which is a membrane-bound enzyme was suggested to catalyse the transfer of UDP-glucuronic acid to potentially harmful substances. This mechanism was also suggested to provide a route of converting electrophilic compounds to hydrophilic ones which are easily excreted (Meech and Mackenzie, 1997). In the present study, fumonisins in the presence of DEN initiation significantly increased the cytosolic GSTα and GSTμ activity. DEN initiation followed by fumonisin promotion was previously described to induce hepatic GSTs.
(Marnewick et al., 2009; Keyser, 2012). Although not significantly different from the positive control, A. digitata and S. aethiopicus extract consumption generally decreased GST activity. This indicated that A. digitata and S. aethiopicus countered the damaging effects of the fumonsins. An earlier experiment (Section 5.2.1) described the inhibition of phase 2 metabolising enzymes due to consumption of plant extracts in this study. There, GSTα and μ activities were markedly decreased with 90-day consumption of A. digitata, S. aethiopicus, while the consumption of A. betulina significantly (P<0.05) inhibited the GSTα activity. Inhibition of GST enzyme expression was previously observed with polyphenolic compound consumption (Hayeshi et al., 2004). This condition which is generally associated with decreased GST activity can consequently result in decreased detoxification of electrophilic compounds, increasing the toxicity inside cells (Capela et al., 2007).

The GSTP+ immunohistochemistry staining confirmed the fumonisin-induced cancer promotion in the current model. In the CM group which received the fumonisin-containing diet for promotion, without cancer initiation affected by DEN, the total numbers of GSTP+-stained hepatic foci were significantly lower than the other groups that received only DEN initiation. This finding indicated that following DEN initiation, promotion occurred, affected by the fumonisin-containing CM diet. Previous studies reported similar findings where hepatotoxicity and nephrotoxicity were induced in the presence of cancer-promoting properties due to CM consumption using male Fischer rats (Gelderblom et al., 1991; Marnewick et al., 2009). However, Gelderblom and colleagues (1996) also described the cancer-promotion properties of FB1 in the absence of adverse hepatotoxicity. FB1 was also documented to act both in cancer initiation and promotion of rat livers in earlier studies (Gelderblom et al., 1994, 1996, 2008), whereas it acted predominantly as a cancer promoter in the current study. Various studies reported an association between FB1 intake with DEN administration, and an increase in the number and sizes of GSTP+ foci in male F344 rats (Gelderblom et al., 1996; Marnewick et al., 2009). In the present study, consumption of A. digitata and S. aethiopicus extracts significantly increased the number of mini foci (<0.01 mm²) when compared to the positive control group with the A. betulina treated rats did not have any effect on the number of foci in any of the size categories. For foci size categories between 0.1 mm² and 0.4 mm², consumption of A. digitata and S. aethiopicus extracts significantly lowered the absolute and/or relative numbers of foci when compared to the positive control. The increase in mini foci, coupled with a decrease in foci sizes below 0.4 mm² was indicative that A. digitata and S. aethiopicus consumption counteracted the proliferation of GSTP+ hepatocytes, in the presence of FB. The reduction of total number of
foci above 0.1 mm² by *A. digitata* and to an extent *S. aethiopicus*, could be related to the increased oxidative stress due to CM consumption. Oxidative stress is known to have a dual role that could either stimulate or inhibit cell proliferation, increasing cell depletion via the induction of apoptosis (Klaunig and Kamendulis, 2004). FB₁-induced oxidative stress has been implicated to enhance apoptosis by delaying cancer induction through removal of initiated cells from the liver (Gelderblom *et al*., 1992, 1994; Lemmer *et al*., 1999; Marnewick *et al*., 2009). Since DEN initiation was also established to contribute to GSTP⁺ stained mini foci (<0.01 mm²), these smaller background foci sizes were removed from the total number of GSTP⁺ foci to better contextualize a more realistic GSTP⁺ quantification. Consumption of *A. digitata*, *S. aethiopicus* and *A. betulina* extracts as the sole source of drinking fluid did not significantly alter the total number of GSTP⁺ in the presence of fumonisn cancer promotion when compared to the positive control. *A. digitata* again significantly arrested the proliferation of foci of both 0.1-0.2 mm² and 0.2-0.4 mm² sizes in comparison to *A. betulina* and the positive control. In both size categories, *S. aethiopicus* was not significantly different from the positive control or *A. digitata* and *A. betulina* extracts. This finding suggested that *A. digitata* and to some extent *S. aethiopicus*, interfered with or impaired the fumonisn-induced cancer promotion. Polyphenols have previously been reported to demonstrate similar activities in the same DEN-initiation/CM-promotion model in male F344 rats over 28 days, in some instances, even reducing the total number and sizes of foci (Marnewick *et al*., 2009; Keyser 2012).

The arrest of proliferation in GSTP⁺ stained cells associated with *A. digitata* and *S. aethiopicus* consumption could possibly be related to the higher average daily intake of TP during the course of the study, compared to *A. betulina* (in decreasing order *A. digitata* > *S. aethiopicus* > *A. betulina*). Simultaneously *A. digitata* consumption provided the highest protective effect when considering the serum chemistry markers for hepatotoxicity, followed by *S. aethiopicus* and *A. betulina*. Chronic consumption of 2% *A. digitata* and *S. aethiopicus* extracts were earlier reported to significantly inhibit hepatic lipid peroxidation, using both CDs and TBARS assays (Section 5.2.1). In the present study, fumonisn-induced oxidative stress resulted in early hepatic lipid peroxidation while consuming *A. digitata* and *S. aethiopicus*. However, the lipid peroxidation was countered, and even reduced by *S. aethiopicus* consumption (decreased TBARS – an indication of latter lipid peroxidation). An earlier study by Al-Qarawi and colleagues (2003) reported that consumption of an aqueous extract of *A. digitata* provided hepatoprotection against carbon tetrachloride-induced damage in mature Wistar rats. The mechanism of liver protection was not described. Similar to their suggestion, the arrest in proliferation was ascribed to the diverse
polyphenolic compounds of *A. digitata*. Additionally, significantly more TP were consumed by groups that were provided with *A. digitata* as their sole source of drinking fluid.

To our knowledge, no studies attempted to describe chemoprevention mechanisms of *A. digitata*, *S. aethiopicus* and *A. betulina*. In the present study, consumption of *A. digitata* and to some extent, *S. aethiopicus*, significantly arrested the proliferation of pre-neoplastic lesions below 0.4 mm² in size. In the presence of fumonisin-induced oxidative stress, no induction of phase 2 metabolising enzymes (GSTα and μ) occurred, consequently not increasing hepatic GSH levels. In fact, it appeared as if the prolonged oxidative stress (Section 5.2.1) resulted in GSH depletion. Regardless of the decreased GSH levels, *A. digitata* and *S. aethiopicus* consumption maintained a redox status that was similar to the one without fumonisins-induced oxidative stress (Section 5.2.1). Although GSH depletion was generally observed, it appeared as if *A. digitata* and *S. aethiopicus* consumption decreased the oxidation of GSH to form GSSG.

In summary, 2% *A. digitata* and *S. aethiopicus* consumption was associated with high soluble solid content and flavanols in part 1 of the present study (Fig 5.1). This could attribute to the high serum TP in both parts 2 and 3 of the current study. As a good correlation was shown between TP and flavanols (0.9371) in aqueous extracts, the high TP intake could most probably be due to the high flavanol contents of the *A. digitata* and *S. aethiopicus* extracts. Consumption of the latter two extracts, also created toxicity in the liver, as demonstrated by slightly increased ALT and AST activities, coupled with an increased RLW and plasma CDs in the 90-day feeding study. A decrease in blood GSSG and associated lower blood GSH:GSSG ratio, as well as slightly increased ALT and AST activities and increased liver CDs levels were also agreeing with toxicity due to consumption of *A. digitata* and *S. aethiopicus* extracts. This underlying toxicity, as indicated by increases in ALT and AST, may be due to a low level of necrosis, but may also involve the induction of apoptosis which is likely to delay the development of GSTP+ foci below 0.01mm². In this case, the inhibition of growth in these pre-neoplastic lesions was most probably related to apoptosis.
Figure 5.1 Summary of probable events leading to inhibition of cancer promotion in rat livers

**Abbreviations:** TP=total polyphenols; ALT=alkaline transferase; AST=aspartate transferase; RLW=relative liver weight; CD=conjugated diene; GSH=reduced glutathione; GSSG=oxidised glutathione

### Inhibition of Cancer Promotion in Rat Liver

**Part 1:**
- High Soluble Solids
- High Flavanols
  - Increased Blood GSH:GSSG
  - Increased (Slightly) ALT/AST
  - Increased RLW

**Part 2:**
- High Serum TP Intake
  - Increased Blood GSH:GSSG
  - Increased (Slightly) ALT/AST
  - Increased RLW
  - Increased Plasma CDs

**Part 3:**
- High TP Intake
  - Increased (Slightly) ALT/AST
  - Increased liver CDs
  - Decreased Blood GSSG
  - Decreased Blood GSH:GSSG ratio;
  - Increased GSTP* foci (<0.01mm²)
Chapter 6

6. Conclusion, limitations and recommendations

The first part of this study provides the first antioxidant characterization, in terms of content and associated antioxidant capacities of *A. digitata*, *S. aethiopicus*, *A. betulina*, and *M. flabellifolius*. The choice of solvent for optimal phenolic extraction, which was ascribed to the unique chemical composition and ecological nature of the various plants, was also determined. Furthermore, the correlations between antioxidant contents and capacities were affected by the choice of solvent. In this case, the ORAC assay was more applicable in determining antioxidant capacities of aqueous extracts than acetone and ethanol extracts. With acetone and ethanol extracts, the FRAP and TEAC assays were more suitable for antioxidant determination.

In the second part of this study, the modulatory effects of chronic consumption of 2% and 5% (w/v) extracts of *A. digitata*, *S. aethiopicus*, *A. betulina*, and *M. flabellifolius* in male Fischer rats were reported. Initial screening of liver and kidney function indicated no adverse effects associated with consumption of both 2% and 5% extracts, with 2% extracts of *A. digitata* and *S. aethiopicus* further reducing early hepatic lipid peroxidation. Chronic consumption of 5% *M. flabellifolius* was observed to cause hepatic lipid peroxidation. The hepatic glutathione redox status was significantly improved by consumptions of the 2% extracts of *A. betulina* and *M. flabellifolius*, as well as the 5% extract of *A. digitata*. Generally, tendencies demonstrating inhibitory effects of GSTs were observed with chronic consumption of all extracts, although the inhibition was not significant with consumption of all extracts. Aspects which were not addressed, but are of value to elucidate the various mechanisms of action in the future will be to investigate the modulation of the hepatic phase 1 xenobiotic metabolizing enzymes, such as the cytochrome P450 enzymes and isoenzymes by the of plant polyphenolic constituents. The roles of endogenous antioxidant enzymes such as superoxide dismutase and catalase could provide further clarification on the relationships between polyphenolic components of the indigenous medicinal plants and the associated redox status, *in vivo*. 
Finally, effects of consuming the various plant extracts in the presence of DEN-initiation with culture material (CM) of *Fusarium verticillioides* promotion were investigated in third part of this study. *A. digitata* and *S. aethiopicus* inhibited the proliferation of mini-foci, indicating their potential as liver cancer promotion inhibitors. The mechanisms of modulation related to the inhibition of cell proliferation and the induction of apoptosis still need to be fully elucidated. The answers addressing the possible mechanisms of modulation might potentially be found in additional investigations of the study plants. These additional investigations could be regarded as limitations of this study and the inclusion of both 2% and 5% extracts of the different plant extracts would have allowed for description of dose-responses in the DEN-initiation/CM promotion model. For the purpose of this experiment, only the 2% plant extracts were used due to insufficient amount of plant material to utilising both 2% and 5% feeding regimens. The 2% dose was merely selected based on previous work using this model. Higher concentrations can also bring about other complications such as enhancing toxicity, as more total polyphenols do not always result in superior protection. Additional studies regarding the modulating role of the drug metabolising and antioxidant enzymes should entail gene expression analyses to determine the exact role they may play in the protection against liver cancer promotion.

Additionally, the compositions of the different aqueous extracts, as indicated in the HPLC analysis (Appendices1 – 36), could be explored to determine the impact of the various components, preferably, individually as well as in the whole extract. Studying of both the isolated components of the plants and the impacts due to whole plant consumption will provide clarity on inhibition mechanisms of hepatic phase 2 metabolizing enzymes. Elucidating the individual compound structures did not fall within the scope of this study, however, using purified and structurally well-defined phytochemicals would have aided in better description of the underlying biochemical and molecular mechanism associated with consumption of study plants.

As this was the first documentation of antioxidant characterization, modulation of hepatic phase 2 metabolizing enzymes, and their possible chemo preventative mechanisms in rats, it provides the basis for a point of departure for follow-up studies using *A. digitata*, *S. aethiopicus*, *A. betulina*, and *M. flabellifolius*. The above-mentioned limitations and shortcomings of this study will further aid follow-up studies on aspects such as choice of concentration to be used in *in vivo* studies, selection of enzymes to analyse, and systematic
experimental designs to allow for purification of lead plant compounds to better describe biochemical and molecular mechanisms associated with consumption thereof. This approach would also promote the development of drugs and novel foods to be used for further pre-clinical and clinical trials that could be beneficial to mankind.


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167


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Appendix 1 HPLC profile of *A. digitata* water extract (280nm)
Appendix 2 HPLC profile of *A. digitata* water extract (320nm)
Appendix 3 HPLC profile of *A. digitata* water extract (350nm)
Appendix 4 HPLC profile of *A. digitata* acetone extract (280nm)
Appendix 5 HPLC profile of *A. digitata* acetone (320nm)
Appendix 6 HPLC profile *A. digitata* acetone extract (350nm)
Appendix 7 HPLC profile of *A. digitata* ethanol extract (280nm)
Appendix 8 HPLC profile of *A. digitata* ethanol extract (320nm)
Appendix 9 HPLC profile of *A. digitata* ethanol extract (350nm)
Appendix 10 HPLC profile of *S. aethiopicus* water extract (280nm)
Appendix 11 HPLC profile of *S. aethiopicus* water extract (320nm)
Appendix 12 HPLC profile of *S. aethiopicus* water extract (350nm)
Appendix 13 HPLC profile of *S. aethiopicus* acetone extract (280nm)
Appendix 14 HPLC profile of *S. aethiopicus* acetone extract (320nm)
Appendix 15 HPLC profile of *S. aethiopicus* acetone extract (350nm)
Appendix 16 HPLC profile of *S. aethiopicus* ethanol extract (280nm)
Appendix 17 HPLC profile of *S. aethiopicus* ethanol extract (320nm)
Appendix 18 HPLC profile of *S. aethiopicus* ethanol extract (350nm)
Appendix 19 HPLC profile of A. betulina water extract (280nm)
Appendix 20 HPLC profile of *A. betulina* water extract (320nm)
Appendix 21 HPLC profile of *A. betulina* water extract (350nm)
Appendix 22 HPLC profile of *A. betulina* acetone extract (280nm)
Appendix 23 HPLC profile of *A. betulina* acetone extract (320nm)
Appendix 24 HPLC profile of *A. betulina* acetone extract (350nm)
Appendix 25 HPLC profile of *A. betulina* ethanol extract (280nm)
Appendix 26 HPLC profile of *A. betulina* ethanol extract (320nm)
Appendix 27 HPLC profile of *A. betulina* ethanol extract (350nm)
Appendix 28 HPLC profile of *M. flabellifolius* water extract (280nm)
Appendix 29 HPLC profile of *M. flabellifolius* water extract (320nm)
Appendix 30 HPLC profile of *M. flabellifolius* water extract (350nm)
Appendix 31 HPLC profile of *M. flabellifolius* acetone extract (280nm)
Appendix 32 HPLC profile of *M. flabellifolius* acetone extract (320nm)
Appendix 33 HPLC profile of *M. flabellifolius* acetone extract (350nm)
Appendix 34 HPLC profile of *M. flabellifolius* ethanol extract (280nm)
Appendix 35 HPLC profile of *M. flabellifolius* ethanol extract (320nm)
Appendix 36 HPLC profile of *M. flabellifolius* ethanol extract (350nm)