Isolation and characterization of antioxidant compounds from *Combretum apiculatum* (Sond.) subsp *apiculatum* leaf extracts.

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October 2007

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Dissertation submitted to the Department of Paraclinical Sciences (Phytomedicine Programme), University of Pretoria for the award of an M.Sc. (Veterinary Science)

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Co-Promoter	: Dr. M.A. Aderogba

Date of submission: December 2007

PREFACE

The research was carried out at the Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, under the supervision of Prof. J.N. Eloff and Dr. M.A. Aderogba.

This represents original work that has not been submitted to any other institution. I have consulted many publications in the course of this work and they are all acknowledged.

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Mr DT Kgatle

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Prof J N Eloff (Supervisor)

ACKNOWLEDGEMENTS

I am grateful to my supervisor Prof, J.N. Eloff and co-supervisor Dr. M.A. Aderogba for their assistance and all the members of Phytomedicine laboratory for their support. I also thank Mr Freddy Makhubele of University of Limpopo (Medunsa campus) for generating NMR data

A special thanks goes to my mother for all her efforts towards my studies. Lastly to the model of oneness God for everything He is doing in my life.

Funding for this research was provided through a grant from National Research Foundation, South Africa.

LIST OF ABBREVIATIONS USED

BuOH	n-butanol
BEA	benzene: ethanol: ammonium 18:2:0.2
C. apiculatum	*C. apiculatum* subsp. apiculatum
CEF	chloroform: ethyl acetate: formic acid 10:8:2
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DCM	dichloromethane
DMSO	dimethyl sulfoxide
EMW	ethyl acetate: methanol: water 10:1.35:1
EtOAc	ethyl acetate
HEX	hexane
IPUF	Indigenous Plant Use Forum
MeOH	methanol
MIC	minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazol)-2,5-diphenyl teetrozolium bromide
NMR	nuclear magnetic resonance
subsp	subspecies
TLC	thin layer chromatography

ABSTRACT

Combretum species are used in many cultures in folk medicine for treatment of microbial infections and several inflammatory conditions (abdominal pains, headache and toothache). There are two possible mechanisms to explain the use of plants extracts to treat microbial infections. A direct effect involves the action of active agents in the extracts on the microorganism tested and the indirect effect involves the stimulation of the host immune system to overcome the effects of microorganisms via the host immune system. Traditional healers use mainly aqueous extracts and in all Combretaceae we have studied, these extracts had hardly any *in vitro* antibacterial activity. A search of the literature confirmed our observations, aqueous plants extracts of many plant species usually have very little direct *in vitro* antimicrobial activity. One would expect that aqueous extracts would have higher anti-oxidant activities and may stimulate the immune system of patients thereby combating the infection indirectly. This possibility prompted the investigation of the antioxidant potential of more polar extracts of *Combretum* species.

Methanol extracts of leaves of ten different *Combretum* species were evaluated for qualitative antioxidant activity by spraying TLC chromatograms of leaf extracts with 2, 2-diphenyl-1-picrylhydrazyl (DPPH). Compounds with anti-oxidant activity bleached the purple colour. Leaf extracts of *Combretum apiculatum* subspp *apiculatum* had the most antioxidant compounds. This species was consequently selected for further examination.

Anti-oxidant directed fractionation of the leaf extracts of *C. apiculatum* led to the isolation of four antioxidant compounds from ethyl acetate and butanol soluble fractions. The structures of the compounds were determined by spectral analyses (^{1}H-NMR, ^{13}C-NMR and MS) and identified as cardamomin (1), pinocembrin (2), quercetrin (3) and kaempferol (4). These compounds occur commonly in plant extracts, but the anti-oxidant activities of all these compounds were not known previously.

In a quantitative antioxidant assay using DPPH with L-ascorbic acid as positive control, the more polar fractions (ethyl acetate and butanol) obtained by solvent-solvent fractionation had the highest activity among the extracts with EC_{50} values of 3.91 ± 0.02 and 2.44 ± 0.02

μg/mL respectively. Of the isolated compounds, quercetrin (3) and kaempferol (4) had strong antioxidant activity with EC_{50} values of 11.81 ± 85 and 47.36 ± 0.03 μM respectively. Cardamomin (1) and pinocembrin (2) did not have strong activity as these compounds could not scavenge 50% of the DPPH radical at the highest concentration (200 μM) tested. L-ascorbic acid was used standard antioxidant agent (EC_{50} = 13.37 ± 0.20 μM or 2.35 μg/mL). The antioxidant activity of the isolated compounds supported structure-activity relationships developed by other authors.

The cytotoxicity of cardimonin and pinocembrim was evaluated using MTT assay with, berberine as positive control and DMSO as negative control. At higher concentrations than 50 μg/ml of cardimomin or pinocembrin the cells were not viable. Cardimomin was more toxic to the cells (LC_{50} of 1.97 μg/ml) than pinocembrin (LC_{50} of 29.47 μg/ml) and even the positive control, berberine (LC_{50} of 12.35 μg/ml).

The presence of these antioxidants could provide a rationale for the ethnomedicinal use of this species for the treatment of inflammatory conditions in traditional medicine. It appears that the antimicrobial activity of aqueous plant extracts may be related to the antioxidant activity leading to a stimulated immune system rather than antimicrobial activity *per se*. Because the crude polar extract had an antioxidant activity half that of L ascorbic acid, the rationale for using these plants by traditional healers becomes clear. The toxicity of cardomomin is a warning that the safety of these extracts should be evaluated in *in vivo* assays.

CONFERENCE PRESENTATIONS

2005

Indigenous Plant Use Forum (IPUF), Rhodes University, Grahamstown, South Africa

Paper: DT Kgatle, MA Aderogba and J N Eloff. Screening of ten *Combretum* species for antioxidant activity.

2006

Indigenous Plant Use Forum (IPUF), University of Botswana, Gaborone, Botswana

Paper: DT Kgatle, MA Aderogba and J N Eloff. Isolation and characterization of antioxidant compounds from *Combretum apiculatum* subp *apiculatum.*

2006

Faculty Day, Faculty of Veterinary Science, University of Pretoria, South Africa

Paper: DT Kgatle, MA Aderogba and J N Eloff. Isolation of antioxidant compounds from *Combretum apiculatum.*

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CHAPTER 1

INTRODUCTION

1.1 Medicinal plant

Fossils date the earliest human use of plants as medicines to approximately 60 000 years ago (Fabricant and Farnsworth, 2001). One of the early written records of medicinal plants use is that of a French surgeon of note, Ambrose (1517-90). He treated gunshot wounds with a mixture of chamomile, melilot flowers, lavender, rosemary, sage, thyme and the extract from red roses boiled in white wine. Peruvian bark (from the cinchona tree), and syrup of cloves were prescribed as remedy for malaria by Thomas Sydenham (1624-89). Quinine, a widely used antimalarial, is a major component of the bark of the cinchona tree (Mann, 1995).

Today, more than 80% of the population in developing countries in the world depends on plants for their medical needs (Farnsworth, 1988: Balick *et al*., 1994). In South Africa up to 60% of the population consults one of an estimated 200 000 traditional healers, in preference to, or in addition to Western medical doctors, especially in rural areas (Van Wyk *et al*., 1997).

Traditional medicine has always been part of the cultural and religious life of African people. It is easily accessible and affordable to rural people (Steenkamp, 2003). There have been many validations of traditional remedies through scientific research (McGaw *et al*., 2000; Sparg *et al*., 2000; Rabe and Staden, 1998). In addition, the use of ethnomedical information has contributed to health care world wide through the isolation of bioactive compounds for direct use in medicine (Fabricant and Farnsworth, 2001).

In South Africa, several species of medicinal plants are used by many ethnic groups for the treatment of various ailments in both human and domestic animals. Traditional healers are known as *inyangas* (herbalist) by the Zulu people, *ixwele* and *amaquira* by the Xhosa, *nqaka* by the Sotho and *nanga* by the Vhavenda (Steenkamp, 2003). In addition to treating human ailments, the treatment of livestock diseases using traditional remedies is widely practised in many rural communities and the practice dates back some centuries (Smith, 1895). In the Eastern Cape Province, it is estimated that 75% of small-scale farmers still use herbal remedies to treat their livestock (Masika *et al*., 2000).

The use of medicinal plants in the form of crude extracts presents several difficulties. The amount of the bioactive compound(s) from plants may vary with both the locality and the season in which they are collected. Also, bioactive molecules of many plants are powerful poisons when taken in excess, and if the plant extract contains a lower content of bioactive

compound(s) than usual, suboptimal dosage may not be effective. Medicinal properties of many plants are also rapidly lost on storage, for example, foxglove leaf's bioactive molecules decompose on long storage, unless dried quickly after collection. Furthermore, crude extracts from many medicinal plants may contain, in addition to the bioactive molecules, other constituents which have harmful effects. For example aristolochic acids present in a Chinese plant, *Aristolochia fangch* are nephrotoxic and carcinogenic compounds closely associated with renal failure (Loset *et al*., 2001).

It is therefore important to isolate and identify the bioactive molecules from plant extracts. In many cases this objective has been achieved, as quinine have been isolated from the cinchona tree bark and is sold in this form.

The advantage of using pure drugs instead of crude plant extracts includes, amongst others, accurately prescribed dosage. Structural modification of isolated and identified bioactive compounds from plant extracts may allow an improvement in the efficacy and moderation of side effects. Pure bioactive molecule can frequently be synthesized economically, thus preventing dependence on plants as sources (Williams, 1947).

It is therefore not difficult to understand what prompted the early chemists in their efforts to isolate and identify bioactive molecules from medicinal plants. Indeed, it has been estimated that 25% of prescribed medicines today are substances derived from plants (Hamburger and Hostettmann, 1991) and a recent example is artemisinin obtained from *Artemisia annua* for the treatment of malaria.

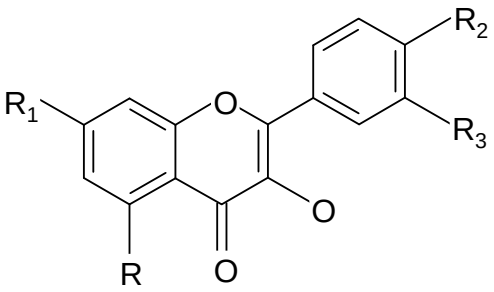
1.2 The Family Combretaceae

The Combretaceae consists of 18 genera of which the genus *Combretum* is the largest. Traditional healers throughout southern Africa employ species of the Combretaceae for the treatment of abdominal pains, backache, bilharzia, chest coughs, colds, conjunctivitis, diarrhoea, dysmenorrhoea earache, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion and snake bite, swelling caused by mumps, syphilis, toothache and general weakness (Hutching *et al*., 1996). Antimicrobial activity of many *Combretum* species has been confirmed (Eloff, 1999a) *Combretum erythrophyllum* extracts contain at least 14 antibacterial constituents (Martini and Eloff, 1998). Many of the compounds responsible for the antimicrobial activity have been isolated and characterized. Some of these compounds have higher activity than currently used antibiotics, chloramphenicol and ampicillin (Eloff and McGaw, 2006; McGaw *et al.,* 2001).

Although the efficacy of herbal remedies may be tested in the laboratory, dosage information is often lacking on plant materials that are used. This information is necessary for the scientific validation of the material and the method applied in order to properly evaluate the actual efficacy of these remedies (Masika and Afolayan, 2002).

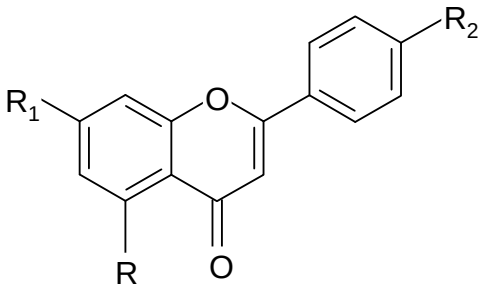
1.2.1 Compounds isolated from *Combretum* species

Phytochemical investigations of *Combretum* species have revealed diverse classes of secondary metabolites. Bioactivity directed fractionation of the leaf extracts of *C. erythrophyllum* led to the isolation of seven active compounds, four flavonols: rhamnocitrin (RC), kaempferol (KP), uercetrin-5,3'-dimethyl ether (QM), rhamnazin (RN) (Figure 1) and three flavones: apigenin (AP), genkwanin, 5-hydroxy-7,4'-dimethoxyflavone (GK) (Martini *et al* 2004b)Figure 2. All test compounds had good activity against *Vibrio cholerae* and *E. faecalis*, with MIC values in the range of 25-50 μg/ml. Rhamnocitrin and uercetrin-5,3'-dimethylether showed additional good activity (25 μg/ml) against *Micrococcus luteus* and *Shigella sonei*. Toxicity testing showed little or no toxicity towards human lymphocytes with the exception of 5-hydroxy-7,4'-dimethoxyflavone. This compound is potentially toxic to human cells and exhibited the poorest antioxidant activity. Both rhamnocitrin and rhamnazin exhibited strong antioxidant activity with potential anti-inflammatory activity. Although these flavonoids are known, it was the first report of biological activity with some of these compounds (Martini *et al*., 2004a).



Compound	R	R_1	R_2	
RC	OH	OCH_3	O	
KP	OH	OH	OH	H
QM	OCH_3	OH	OH	OCH_3

Figure 1: Flavonols isolated from *C. erythrophyllum*



Compound	R	R_1	R_2
AP	OH	OH	OH
GK	OH	OCH_3	OH
HM	OH	OCH_3	OCH_3

Figure 2: Flavones isolated from *C. erythrophyllum*

Investigation of more *Combretum* species in our laboratory yielded combretastatin B5 (2',3',4-trihydroxyl-3,5,4'-trimethoxybibenzyl) as the main antimicrobial agent in *C. woodii* leaf extracts (Eloff *et. al.*, 2005), (Figure 3). Combretastatin B5 (2',3',4-trihydroxyl-3,5,4'-trimethoxybibenzyl) had significant activity against *S. aureus* with an MIC of 16 μg/ml MIC, *P. aeruginosa (125*

μg/ml), *E. faecalis* (125 μg/ml) and slight activity against *E. coli* and an excellent anti-oxidant activity.

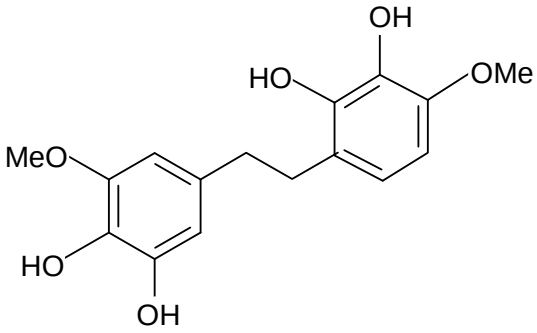
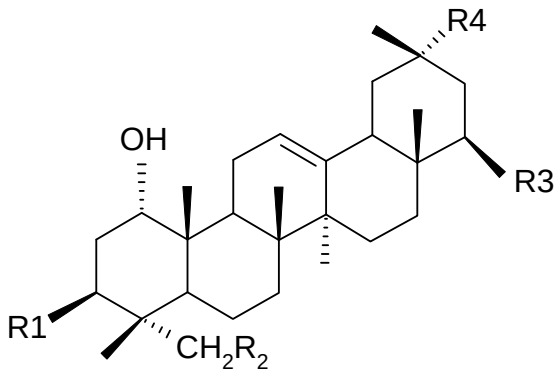


Figure 3: Combretastatin B5 isolated from *C. woodii*

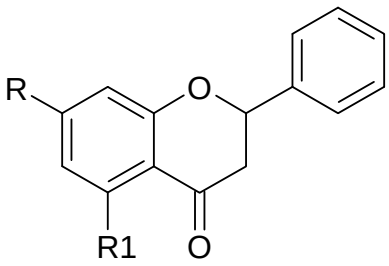
Three antimicrobial triterpenoids including new oleane type triterpenoids were isolated from *C. padoides* (Angeh, 2005). A new pentacyclic triterpenoids and four known triterpenoids were reported as the antimicrobial constituents from the leaf extracts of *C. imberbe* (Angeh, 2005)., Two triterpenoids had an MIC of 93 μg/ml compared to 63 μg/ml for pentacyclic triterpenoids against *S. aureus*. Compound 5 (1α,23-dihydroxy-12-oleanen-29-oic acid-3β-O-2,4 diacetyl-L-rhamnopyranoside) was (Fig. 4) reported for the first time (Angeh, 2005).



Compounds	R1	R2	R3	R4
1	OH	H	H	COOH
2	H	H	H	COOH
3	H	H	=O	CH_2OH
4	H	H	OH	COOH
5	2,4-Di-Ac-Rh	OH	H	COOH

Figure 4: A new pentacyclic triterpenoid and four known triterpenoids isolated from *C. imberbe*

Bibenzyls and phenanthrene derivatives have been reported from *C. apiculatum* subspp *apiculatum, C. molle* and *C. hereroense* (Letcher and Nhamo, 1971; Letcher *et al*. 1972; Letcher and Nhamo, 1973). For his M.Sc study Serage (2003) investigated the antimicrobial compounds present in the leaves of *C. apiculatum* subsp *apiculatum*. By bioassay guided fractionation he isolated and elucidated the structures of two flavanones, alpinetin, pinocembrin, and one chalcone, flavokawain (Fig. 5). All the compounds had reasonable activity with an average MIC of 268 µg/ml against *Escherichia coli* and *Pseudomonas aeruginosa* and an average MIC of 100 µg/ml against *Staphylococcus aureus* and *Enterococcus faecalis*.



Compounds	R	R1
1	OH	OH
2	OMe	OH

Figure 5: The structure of two flavonones isolated from *C. apiculatum*

Many antimicrobial, antifungal and anti-inflammatory compounds (terpenoids and their glycosides) have been reported from several *Combretum* species (Pettit *et al*., 1988; Rogers, 1989a, b).

1.3 Antioxidant activity

A biological antioxidant is a substance that, when present at a low concentration compared to that of an oxidisable substrate, prevents or delays oxidation of that substrate (Benzie and Strian, 1999). Safety concerns have prompted considerable interest in synthetic antioxidant substances such as BHT (2,6-di-*tert*-butyl-4-methylphenol) and BHA (2-*tert*-butyl-4-methoxyphenol) that are supplied to humans and animals as food components or as specific pharmaceuticals.

Natural antioxidants from plants have become prominent area of scientific research presently, as plants offer a wide range of secondary metabolites with high antioxidant potential (Demo *et al*, 1998; Sanchez-Moreno *et al*, 1999). Prevention of cancer and cardiovascular diseases has been linked to the intake of vegetables, fruits and teas rich in natural antioxidants (Johnson, 2001). It has also been demonstrated that a higher intake of antioxidants is linked to a lower

risk of death from diseases like diabetes, acute hypertension and arteriosclerosis (Lim *et al*., 2002, McCune and John 2002, Ajith and Jamardhanan 2002 and Tziveleka *et al.,* 2002). Antioxidant activity in higher plants has been associated with the presence of polyphenolic compounds present (Thabrew *et al*., 1998). Many herbal infusions frequently used in domestic medicine have antioxidative and pharmacological properties connected with the presence of phenolic compounds, especially flavonoids.

1.3.1 Evaluation of antioxidant activity

Antioxidant activity can be evaluated using several methods such as: the Trolox equivalent antioxidant capacity assay (TEAC assay), the total radical-trapping antioxidant parameter assay, (TRAP assay) and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH assay). In this study, a DPPH free radical scavenging assay described by Mensor *et al*. (2001) and modified by Aderogba *et al*. (2006) was used. The antioxidant effects of crude plant extract, solvent fractions and isolated compounds from selected plants were analysed using the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The reduction of DPPH (reaction 1) was monitored by the absorbance of its radical at 515 nm, but upon reduction by an antioxidant (AH), the absorption disappeared (Parejo *et al*., 2000).

$$DPPH + AH \rightarrow DPPH - H + A^{\cdot}$$

(Purple colour) (Yellow colour)

1.4 Rationale for this study

The use of *Combretum* species in many cultures in folk medicine for the treatment of several diseases and their established antimicrobial activities (Martini and Eloff, 1998; Eloff 1999b) prompted us to investigate the antioxidant potential of this plant genus. In many cases aqueous plant extracts have been used to combat infections in traditional medicine. When aqueous extracts were evaluated in our laboratory they had hardly any *in vivo* antimicrobial activity (Kotze and Eloff, 2002). It is possible that these extracts may have an indirect effect on microorganisms if antioxidants present in the extract stimulate the immune system of the host and the host can then withstand microbial infections.

There are two proposed mechanisms to explain the antimicrobial activity of plant extracts. The direct effect involves the action of an antimicrobial compound and the indirect effect involves the stimulation of the host immune system to overcome the microbial infection via the host's immune response to the microbes. Antioxidants protect the body by their free radical scavenging activity, inhibition of oxidative stress processes and maintenance of the immune system. Antioxidants have also been incorporated in a number of veterinary products designed to treat of inflammatory diseases.

Because aqueous plant extracts available to poor rural people do not contain the non-polar antimicrobial compounds present in the plants an explanation for the purported efficacy could relate to immune stimulation by antioxidant compounds in these extracts This motivated the investigation of the antioxidant activity of *Combretum* leaf extracts.

1.5 Objectives of this study

The objectives of this study are to:

1. Screen leaf extracts of ten *Combretum* species for qualitative antioxidant activity.
2. Identify and select the most active species for phytochemical investigation.
3. Isolate the antioxidant compounds from the selected *Combretum* species using bioassay-guided fractionation.
4. Elucidate the structures of the isolated compounds exhibiting antioxidant activity.
5. Evaluate the antioxidant potential of the leaf extracts and isolated compounds from the selected *Combretum* species as a traditional medicine.
6. Determine the safety of the isolated compounds and extracts.

CHAPTER TWO

MATERIAL AND METHODS

2.1 Plant collection

Leaves of 10 *Combretum* species namely *C. celastroides, C. orientale, C. erythrophyllum, C. taborense, C .zeyheri, C. apiculatum* subspp *apiculatum, C. moggii, C. microphyllum, C. paniculatum,* and *C. mossambicense* were collected from the Lowveld National Botanical Garden at Nelspruit. Trees were identified from the tree labels. Voucher specimens were deposited at Herbarium, Phytomedicine Laboratory, and University of Pretoria, South Africa. The collected plant materials were air dried at room temperature.

2.1.1 Preparation of plant material

Plant material was ground to a fine powder in a Macsalab 200LABmill. The ground sample of each plant was stored in closed amber glass containers in the dark at room temperature until needed.

2.1.2 Extraction procedure

Initially, 1.0 g of finely ground plant material of each *Combretum* species was extracted with 10 mL of methanol (MeOH) with occasional shaking for 24 hrs. Each extract was filtered and concentrated using a rotary evaporator at 40˚C. This afforded crude extracts of each of the selected ten plants.

2.1.3 Solvent partitioning of the crude extracts

Solvent-solvent extraction is one of the most popular methods for partial purification (group separation according to polarity) of crude plant material (Eloff 1998c). The crude extract of each *Combretum* species was separately suspended in distilled water in separatory funnel (100 ml) and successively partitioned with hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (BuOH) in this order. These solvents with varying polarities will

theoretically extract different plant constituents. This afforded four partition fractions of each plant extract.

2.1.4 TLC screening for phytochemical analysis and antioxidant activity

Qualitative screening of the constituents in each of the crude extracts of the selected ten *Combretum* species for antioxidant activity was by thin layer chromatography (TLC) analysis. For about 10µL of each sample was loaded on the TLC. The TLC chromatograms were developed in the following solvent systems.

1. Ethyl acetate/ methanol/water (EMW) 10:1.35:1
2. Chloroform/ethyl acetate/formic acid (CEF) 10:8:2
3. Benzene/ ethanol/ ammonium (BEA) 18:2:0.2

For detection of chemical compounds and antioxidants in the extracts respectively, two spray reagents were separately used.

1. Vanillin in sulphuric acid
2. DPPH 0.2% in MeOH

2.2 Column chromatography

Column chromatography using Silica gel and Sephadex LH 20 as stationary phases was used to fractionate the active compounds present in the extracts by assay-guided fractionation.

2.3 Spectroscopic analysis of isolated compounds

Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR and ^{13}C NMR) at the University of Limpopo (MEDUNSA Campus) using Varian Unity Inova 300 and 600 MHz NMR system was performed with appropriate deuterated solvent to obtain the spectroscopic data and elucidate the structures of the isolated compounds.

2.4 Mass Spectrometry (MS)

Mass spectra of each of the isolated compounds were obtained using a VG70-SEQ mass spectrophotometer (University of Johannesburg) for molecular formula elucidation.

CHAPTER THREE

ISOLATION OF ANTIOXIDANT COMPOUNDS

To determine the most active *Combretum* species for further investigation, 10 *Combretum* species were screened for antioxidant activity using DPPH as spray reagent after separating compounds by thin layer chromatography (Section 2.). The TLC chromatograms are presented in Figure 6 (DPPH) and Figure 7 (Vanillin in sulphuric acid).

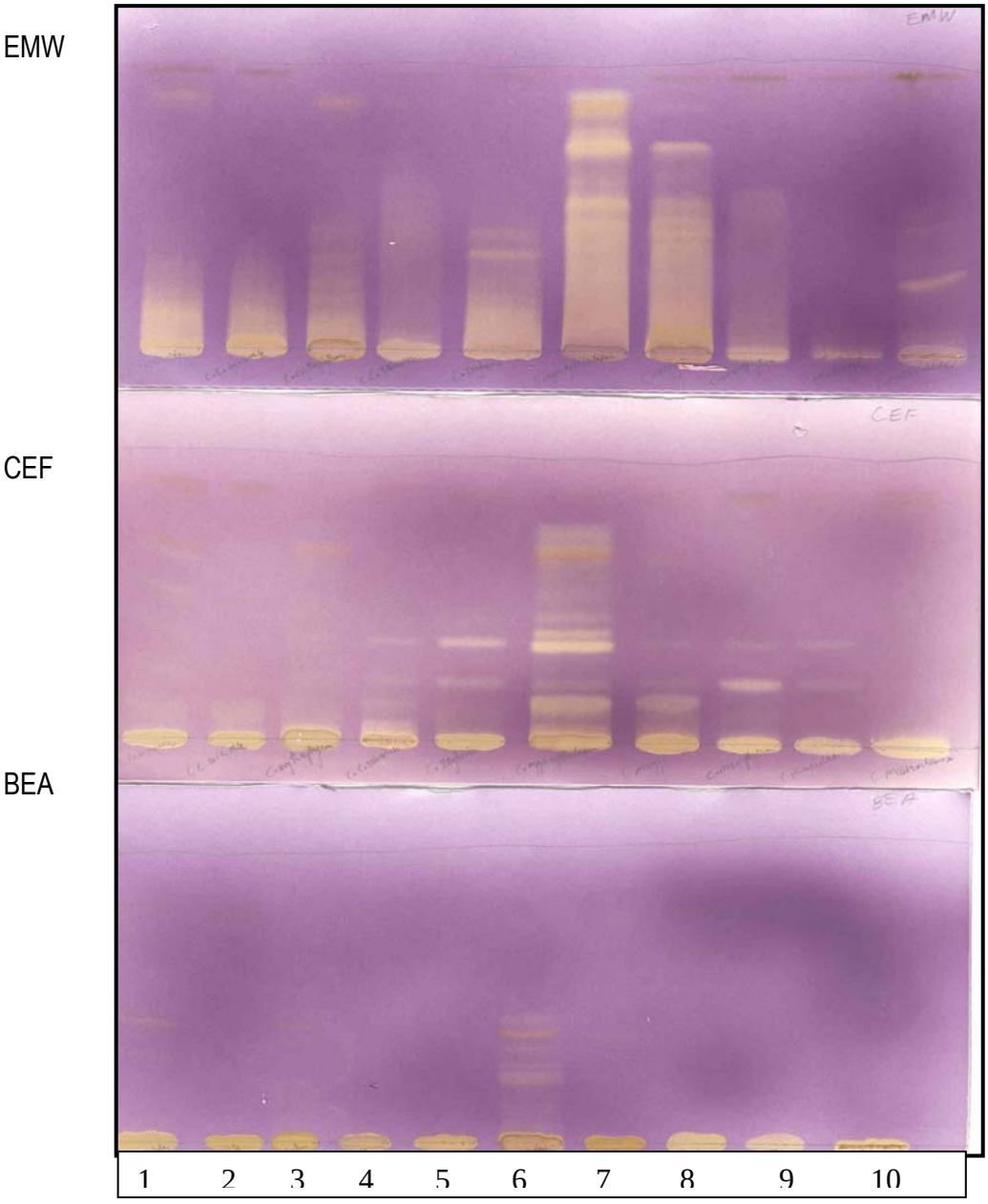


Figure 6: Chromatogram of crude methanol extracts of the 10 *Combretum* species sprayed with DPPH [See Figure 7 for identity of the species].

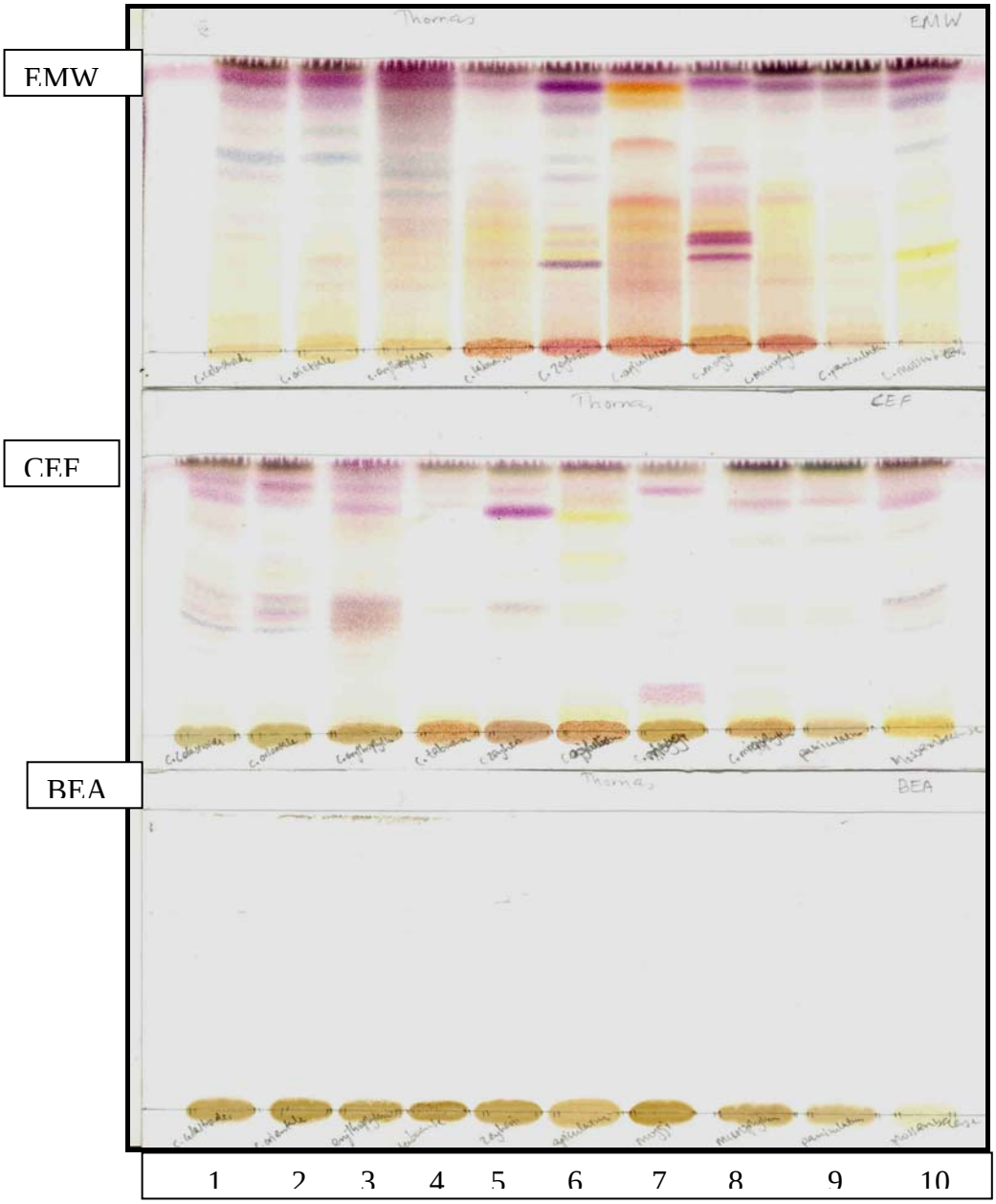


Figure 7: Chromatogram of crude methanol extracts of the 10 *Combretum* species sprayed with vanillin sulphuric acid. *C. celastroides (1), C. orientale (2), C. erythrophyllum (3), C. taborense (4), C.zeyheri (5), C. apiculatum* subspp *apiculatum (6), C. moggi (7), C. microphylum (8), C. paniculatum (9), C. mossambicense (10)*

The thin layer chromatograms (Figs 6 and 7) show the crude methanol extracts of the 10 *Combretum* species developed in three different solvent systems of varying polarity. The chromatogram in Figure 6 was sprayed with DPPH (Sect 2.) while Figure 7 shows the chromatograms sprayed with vanillin in sulphuric acid (Sect 2.). The ten *Combretum* species

developed on the plates were *C. celastroides (1), C. orientale (2), C. erythrophyllum (3), C. taborense (4), C.zeyheri (5), C. apiculatum* subspp *apiculatum (6), C. moggi (7), C.microphylum (8), C. paniculatum (9), C. mossambicense (10)* in this order.

From the chromatograms (Figures 6) Combretum *apiculatum subsp apiculatum* appeared to contain more antioxidant compounds than the other species investigated, hence *C. apiculatum* subspp *apiculatum* was selected for phytochemical investigation.

3.1 Background on *Combretum apiculatum* subsp. *apiculatum*

Figure 8: *C. apiculatum* subsp *apiculatum (from www.zimbabweflora.ca.zw)*

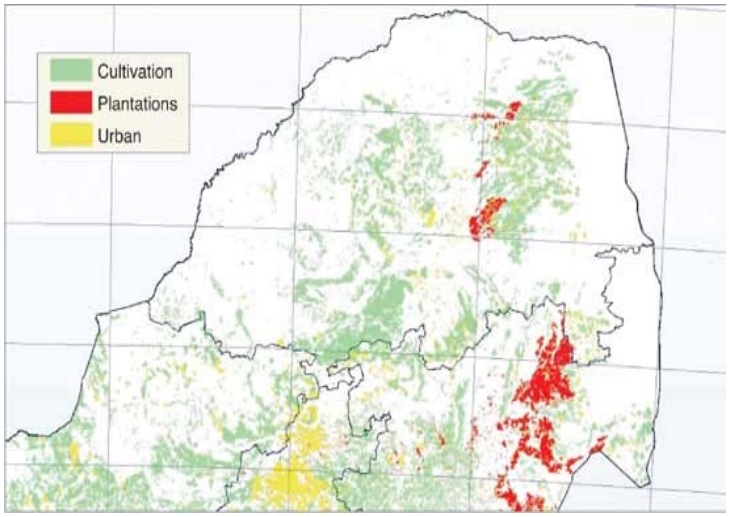


Figure 9: Distribution of *C. apiculatum* subsp *apiculatum* (from www.fao.org) in northern parts of South Africa

C. apiculatum is a small to medium sied tree 3-10 m high There are two subspecies of *C. apiculatum*. Subspecies apiculatum occurs in dry deciduous woorlands and subspecies leutweinii occurs mainly in mopane woodland (Palgrave, 2002). Common names are (red) bush willow, rooiblaar, and Zulu names: umbondo, umbondwe, umbondwe omnyama. It is used by Zulu people for abdominal disorders as a steam bath from leave decoctions and as an enema to relieve stomach disorders. The leaves are used for the weakness of the body, inserted vaginally by Zimbabwean (Hutchings *et al*., 1996)

3.2 Extraction of *C. apiculatum* subsp *apiculatum* leaf material

Powdered leaves of *Combretum apiculatum* ssp. *apiculatum* (650 g) were extracted with methanol (5.0 L) with occasional shaking for 24 hours. The methanol was removed by drying with a rotevaporator (Büchi) under vacuum. The crude extract was suspended in distilled water and successively partitioned with hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (BuOH) (sections 2.1.2 and 2.1.3). This afforded four partition fractions of the plant material. The fractions were developed on TLC plates and subsequently sprayed with vanillin in sulphuric acid (Figure 10) and DPPH solution (Figure 11) to determine the nature of the chemical compounds and the fraction(s) that contained the antioxidant compounds

From these TLC chromatograms (Figures 10 and 11), it is clear that the ethyl acetate and butanol fractions contained the antioxidant compounds. There were more antioxidant compounds in the ethyl acetate fraction than the butanol fraction. All of the compounds separated in the butanol fraction were also present in ethyl acetate fraction. Because it was easier to remove ethyl acetate than butanol this fraction was selected for further fractionation and purification to isolate and identify the antioxidant compounds.

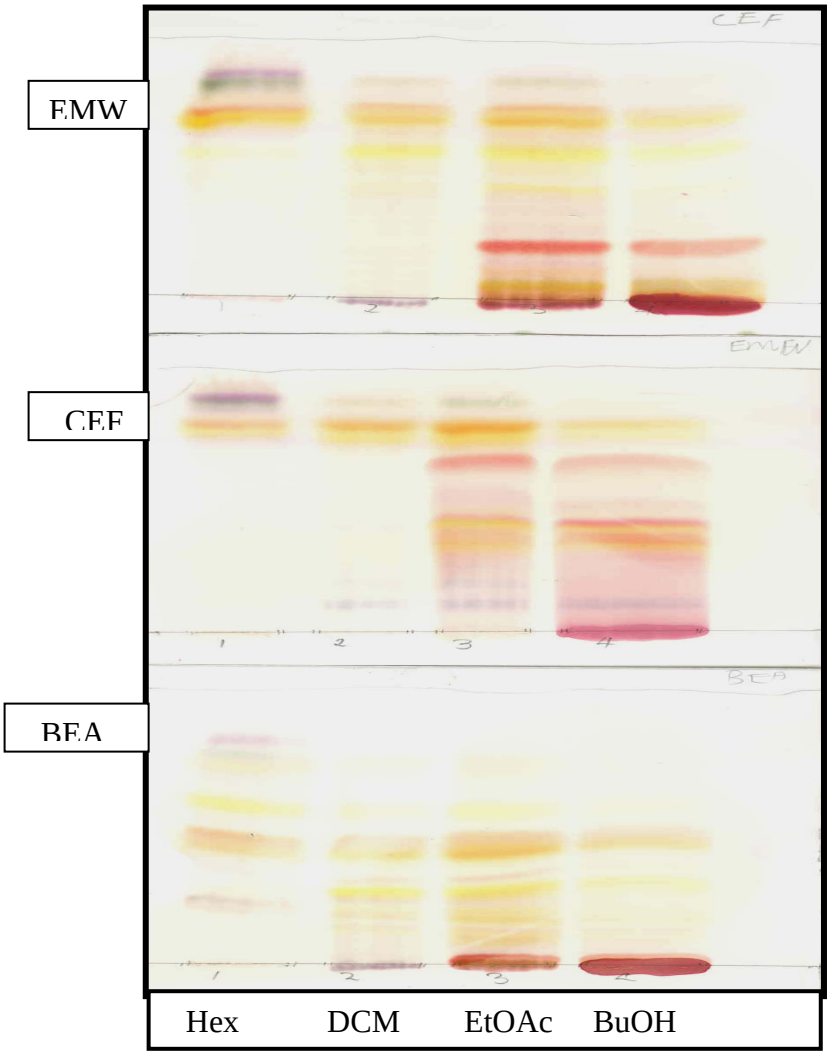


Figure 10: Solvent fractions of *C. apiculatum* subspp *apiculatum* crude extract developed in EMW, CEF and BEA solvent systems and sprayed with vanillin in sulphuric acid.

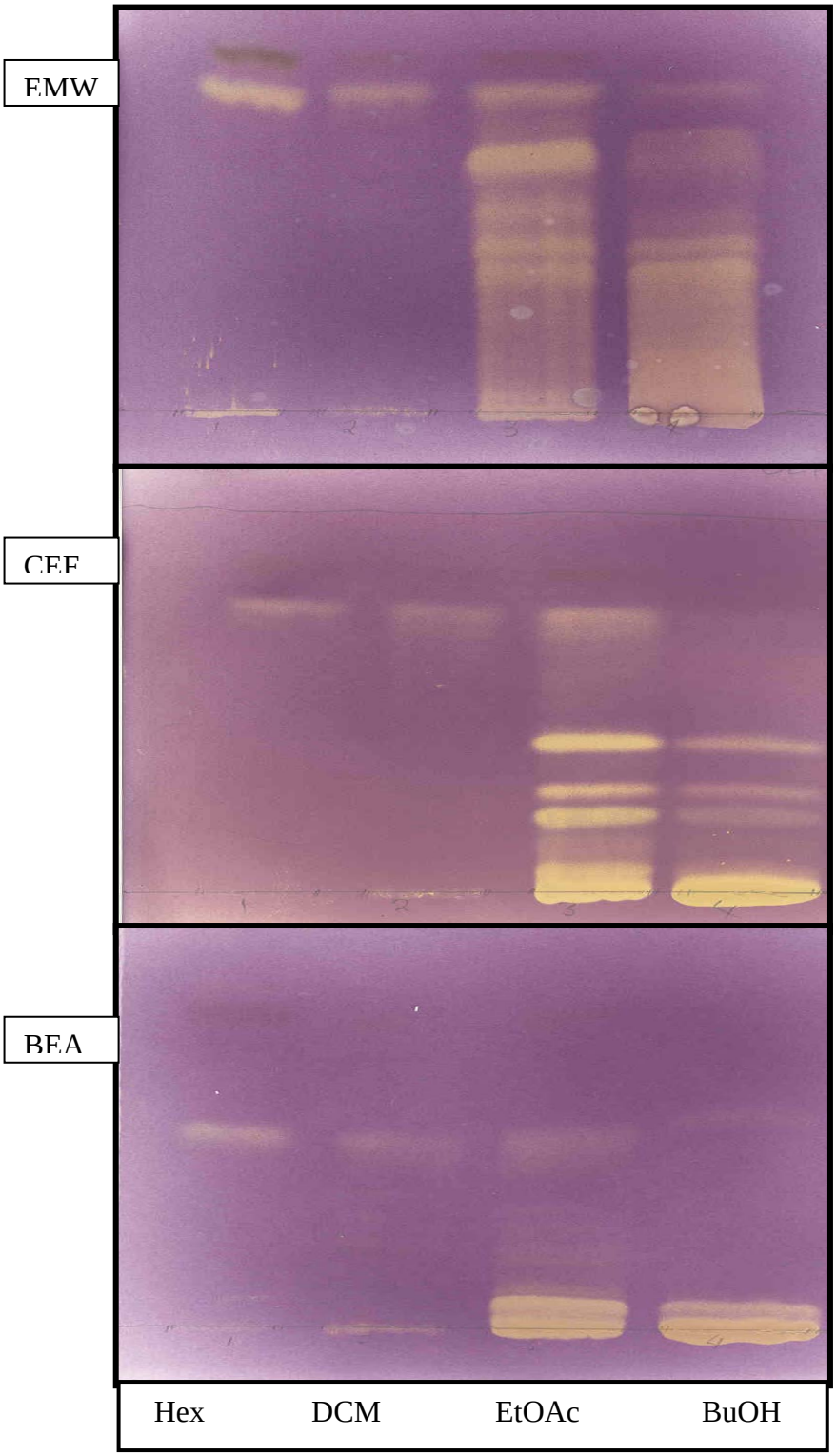


Figure 11: Solvent fractions of *C. apiculatum* subspp *apiculatum* crude extract developed in EMW, CEF and BEA solvent systems and sprayed with DPPH solution

3.3 Isolation of antioxidants from ethyl acetate fraction

Column chromatography using various solid phases was used to fractionate the ethyl acetate fraction. Firstly, 30 g of the extract was dissolved in methanol and adsorbed in silica gel (40g). The mixture was allowed to dry under a stream of air. A glass column (100 x 2.5 cm) was packed with silica gel slurry and the extract (adsorbed in silica gel) was loaded onto the column (270g). The column was eluted with hexane, followed by an increasing gradient of dichloromethane (DCM) in hexane, starting with 10% up to 100% DCM (400ml). The eluant was collected into 50 mL test tubes. DCM (100%) was followed with an increasing gradient of ethyl acetate (EtOAc) in dichloromethane starting with 5% up to 100%. EtOAc (100%) was similarly followed with 10% methanol (MeOH) up to 20%. A total of 287 tubes were collected.

3.3.1 Bulking of fractions

Test tubes 1- 50 were developed on TLC plates using 100% DCM, 51 – 150 were developed using DCM: MeOH (9:1) and 151 – 240 were developed using DCM: MeOH 4:1.The tubes with similar TLC profiles were pooled together to give seven fractions. The seven fractions were subsequently analyzed by TLC in duplicate using CEF as solvent system and separately sprayed with vanillin in sulphuric acid and DPPH solution to determine the nature of the constituents and the fractions containing the antioxidant compounds (Figures 12 and 13 respectively).

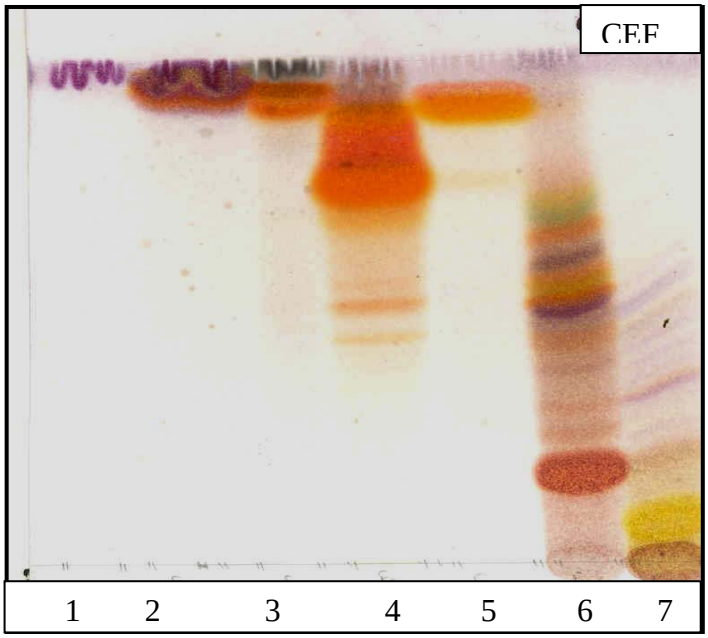


Figure 12: TLC chromatogram of fractions 1-7 sprayed with vanillin in sulphuric acid

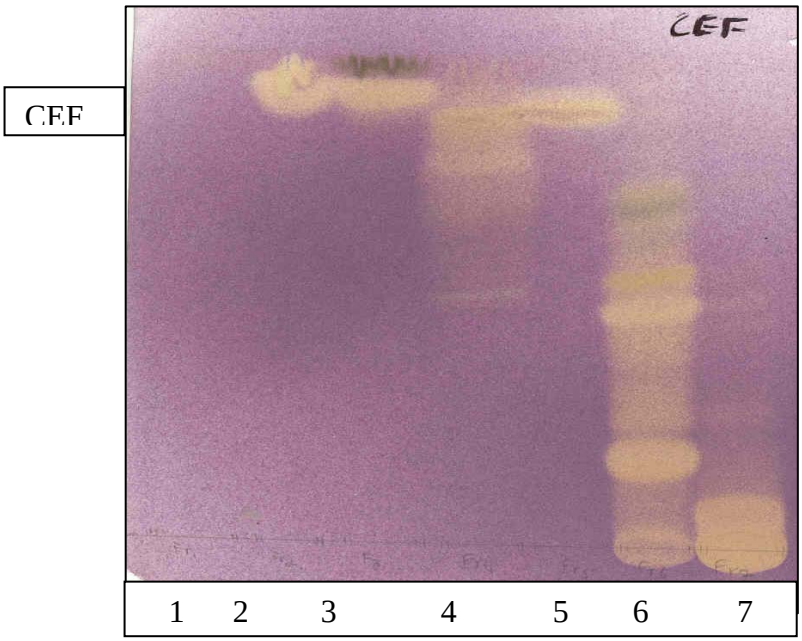


Figure 13: TLC chromatogram of fractions 1-7 developed with CEF and sprayed with DPPH solution

3.3.2 Column chromatography of fraction 4

Fraction 4 (2.1 g) was dissolved in minimal amount of MeOH and adsorbed in silica gel. The mixture was allowed to dry. A glass column (35 x 2.0 cm) was packed with silica gel slurry and the extract (adsorbed in silica gel) was loaded onto the column. The column was eluted with hexane, followed by an increasing gradient of EtOAc up to 100%. The fractions were collected in 25 mL test tubes and a total of 76 tubes were collected. Compound B crystallised out from tubes 2 -14, and the crystals (white in colour) were combined and washed with hexane: DCM 4:1. Compound A was obtained as yellow crystals from tubes 36 -76. Tubes 17 - 30 contained one major compound, and the fractions were pooled together and purified by washing with a mixture of hexane: ethyl acetate (5:1) to afforded a yellow powder of compound C (8.0 mg)

3.3.3 Column chromatography of fraction 6

A glass column (35 x 2.0 cm) was packed with slurry of pre-soaked Sephadex LH-20 in toluene/ethanol (4:1). The fraction (1.6 g) was mixed with toluene/ ethanol (4:1) and dissolved under sonication. The mixture was loaded unto the column and eluted with an increasing gradient of ethanol in toluene up to 100%. Eluant was collected in 25 mL test tubes and a total of 50 tubes were collected. TLC analysis showed that tubes 5-18 contained a single spot. These tubes were pooled together and solvent evaporated on rotary evaporator. This gave compound D, yellow powder (20.0 mg)

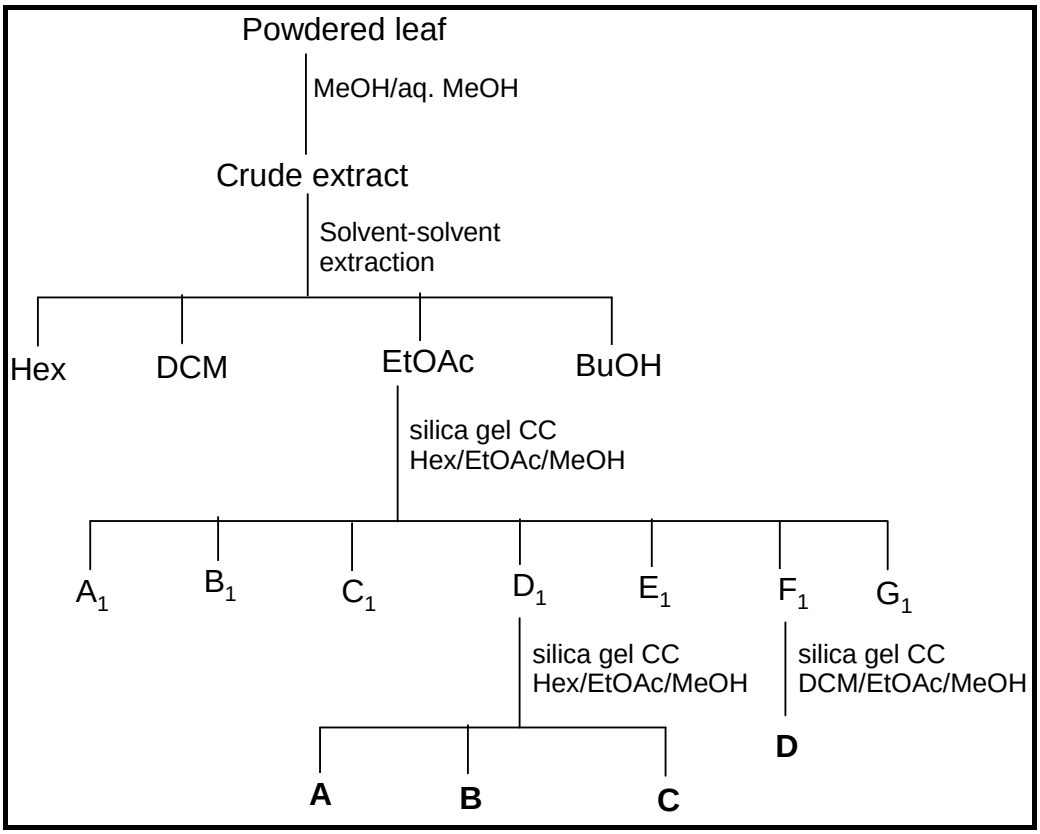


Figure 14: Isolation scheme of the antioxidants from *C. apicutatum*

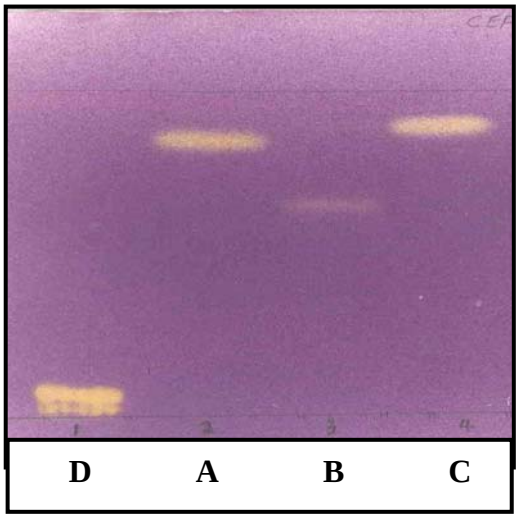


Figure 15: TLC chromatogram of compounds A-D developed in CEF solvent system and sprayed with DPPH solution

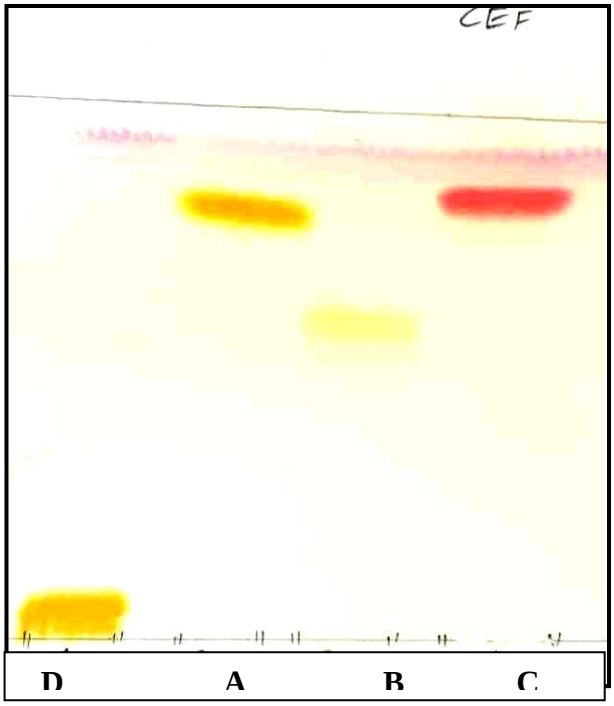


Figure 16: TLC chromatogram of compounds A-D developed in CEF solvent system and sprayed with vanillin in sulphuric acid

3.3.4 Discussion

An analysis was made of approaches to be followed towards selecting plants for research and gene banking. Plants used as phytomedicines in Africa and were also analyzed and the Combretaceae made up a major group (Eloff 1998a).Several extractants were tested and evaluated on many different parameters. Acetone was found to be the best extractant. (Eloff, 1998b). Masoko and Eloff, (2007) found that acetone and methanol extracts exhihibitted the presence of antioxidant activity after spraying the TLC chromatogram with DPPH. In this study methanol was used for screening 10 *Combretum species* for the antioxidant activity because most antioxidant compounds are known to be polar. All the species showed the qualitative antioxidant activity after been sprayed with DPPH; however the number of constituents that exhibited antioxidant activity varied amongst all the C*ombretum* species. Many antioxidant constituents were present in *Combretum apiculatum* subsp *apiculatum* followed by *Combretum moggii*.

Combretum apiculatum subsp *apiculatum* was selected for fractionation because it had more antioxidant constituents on the TLC plates than the other species screened. The solvent solvent fractionation procedure used by the USA National Cancer Institute was tested and refined and several TLC separation procedures were also developed (Eloff 1998c).

Four solvent fractions were obtained from the crude methanol extract using solvent-solvent fractionation. The four fractions contained several antioxidant compounds. These antioxidant compounds were present in higher concentrations in the ethyl acetate extract. Therefore this fraction was selected for further fractionation and purification and the isolation of the antioxidant compounds. Bioactivity directed fractionation of the ethyl acetate fraction of the leaf extract of *C. apiculatum* subspp *apiculatum* led to the isolation of four antioxidant compounds (Figure 15 and 16)

CHAPTER FOUR

STRUCTURE ELUCIDATION OF THE ANTIOXIDANT COMPOUNDS

4.1 Introduction

The structures were elucidated from the spectroscopic data in collaboration with Dr. M.A. Aderogba, my co-supervisor

4.2 Structure elucidation of compound A

Compound A (59mg) was isolated as yellow crystals. The mass spectra showed the molecular ion M^+ peak at m/z =270.08 corresponding to the molecular formula $C_{16}H_{14}O_4$. The ^1H NMR (DMSO-d_6) showed a singlet peak at δ 3.87 (3H, s, 2'-OCH_3). Meta coupling related two doublets at δ 5.93 (1H, d, J=1.8 Hz) and δ 6.02 (1H, d, J=2.1 Hz). Another two sets of doublets a δ 7.67(1H, d, J=15.3 Hz, H-7) an δ 7.85 (1H, d, J=15.9 Hz, H-8) belonging to trans olefinic group. A complex multiple appeared between δ 7.43 – 7.72 (5H, multiplet) due to monosubstituted ring B of chalcone and singlet peak at δ 13.71 (1H, s, H-6'-OH). The ^{13}C NMR data are presented in Table 1. The compound was identified as 4, 6-dihydroxyl-2'-methoxychalcone (cardamomin). The spectra data is in agreement with the literature (Itokawa *et al*. 1981; Krishna and Chaganty 1973).Figure 17.

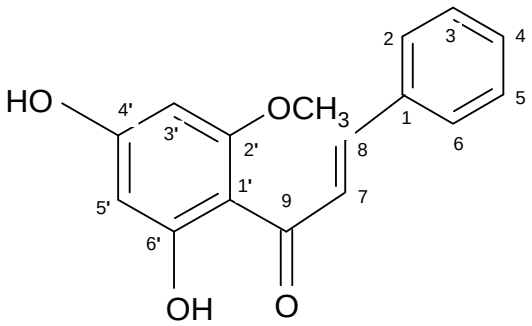


Figure 17: Structure of cardamomin

Table 1: ^{13}C NMR assignment of compound A (cardamomin)

Carbon assignment	Compound 2(A) (DMSO-d_6) δ ppm	Literature (Itokawa *et al*. 1981)
1	134.9	136.5
2	128.4	129.0
3	129.1	129.7
4	130.4	130.7
5	129.1	129.7
6	128.4	129.0
7	127.5	128.6
8	141.9	142.4
9	191.8	193.0
1'	105.2	106.4
2'	166.3	168.3
3'	91.7	92.3
4'	165.1	165.8
5'	95.9	97.0
6'	162.7	164.3
2'-OMe	56.1	56.3

4.3 Structure elucidation of compound B

Compound B (36mg) was obtained as a cream powder. The mass spectra gave a molecular ion peak at m/z = 256 corresponding to the molecular formula $C_{15}H_{12}O_4$. The ^{1}HNMR (acetone-d_6) showed two sets of doublet at δ 2.72 - 2.80 (1H, J=3.0 Hz each, H-3 cis) and a quartet at δ 3.07 – 3.17 (1H, J = 12.6 Hz each, trans). Another double doublet appeared at δ 5.47 – 5.52 (1H, J=3.0 Hz each, H-2), this was caused by coupling with 2H at position 3. Meta coupling related H-6 and H-8 appeared at δ 6.00 and 6.02 (1H each, J = 2.1 Hz) respectively. A multiplet appeared at δ 7.34 – 7.55 (5H, multiplet) indicating mono substituted ring-B and a singlet appeared at δ 12.19 (1H, s, 5 -OH). The ^{13}C NMR data are presented in Table 2. The compound was identified as 5, 7-dihydroxy-2-phenyl flavanone (pinocembrin). The spectral data is in agreement with the literature (Harborne and Mabry 1982). See figure 18.

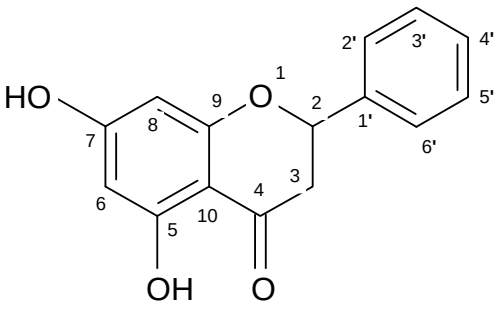


Figure 18: Structure of pinocembrin

Table 2: ^{13}C NMR assignment of compound B (pinocembrin)

Carbon assignment	Compound B (acetone-d_6)δ ppm	pinocembrin-DMSO-d_6 (Harborne and Mabry 1982)
2	79.7	78.4
3	43.4	42.2
4	196.6	195.8
5	165.1	163.6
6	96.9	96.1
7	167.2	166.6
8	95.8	95.1
9	163.9	162.7
10	103.1	101.9
1'	139.8	138.0
2'	127.1	126.5
3'	129.3	128.4
4'	129.3	128.4
5'	129.3	128.4
6'	127.1	126.5

4.4 Structure elucidation of compound C

The mass spectrum had a molecular ion peak at m/z = 286.03 [$M^{+\cdot}$ 100%] base peak, corresponding to the molecular formula $C_{15}H_{10}O_6$. The ^1H NMR (acetone-d_6) spectrum showed AA'BB' system due to ring B ortho related protons at δ 7.01 (2H, d, J= 9.0 Hz, H-3', H-5') and δ 8.14 (2H, d, J=9.0, H-2', H-6'). The presence of free 5-OH group was confirmed by signal at δ 12.13. Also there was presence of meta related two set of doublets at 6.25 and 6.50 (1H each, J = 1.80 and 2.1 Hz respectively H-6, H-8). ^{13}C NMR data are presented in Table 3. The

spectral data are in close agreement with those reported in the literature, Table 3, the compound is therefore 3, 4', 5, 7-tetrahydroxy flavone (kaempferol) (8mg) see figure 19.

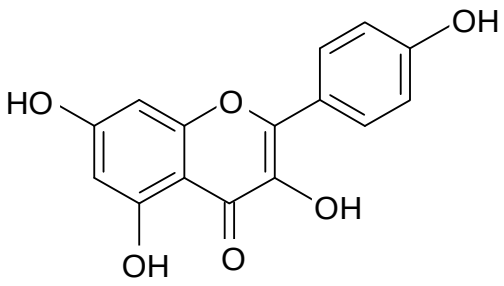


Figure 19: Structure of kaempferol

Table 3: ^{13}C NMR data of compound C (kaempferol)

^{13}C assignment	compound C (acetone-d_6)	kaempferol (DMSO-d_6) (Markham, 1982)
2	147.0	146.8
3	136.0	135.6
4	176.0	175.9
5	161.6	160.7
6	98.5	98.2
7	164.5	163.9
8	93.9	93.5
9	157.1	156.2
10	103.5	103.1
1'	122.6	121.7
2'	129.8	129.5
3'	115.7	115.4
4'	159.4	159.2
5'	115.7	115.4
6'	129.8	129.5

4.5 Structure elucidation of compound D

The mass spectrum had a molecular ion peak at m/z = 447.89 corresponding to the molecular formula $C_{21}H_{20}O_{11}$. Another prominent peak appeared at m/z = 300.95 corresponding to $C_{15}H_9O_7$, [M^+- rhamnosyl]. The ^1H NMR (300 MHz, acetone-d_6): δ 0.91 (3H, d, rhamnose-CH_3), δ 3.370 - 4.21 multiplet (rhamnose Hs), δ 5.5 (1H, rhamnose H-1), meta coupling related

protons at δ 6.25 (1H, d, J = 2.1Hz, H-6) and 6.35 (1H, d, J = 1.5 Hz, H-8). Ring B protons appeared at 6.99 (1H, d, J=8.4 Hz, H-5'), 7.49(2H, d, J = 2.1 Hz, H-2' and6'). The presence of 5-OH was evident from the peak at δ12.71. ^{13}C NMR data are presented in Table 4. The compound was identified as quercetin-3-O-rhamnoside (quercetrin) (17mg). The spectra data are in agreement with the literature (Harborne and Mabry 1982) figure 20.

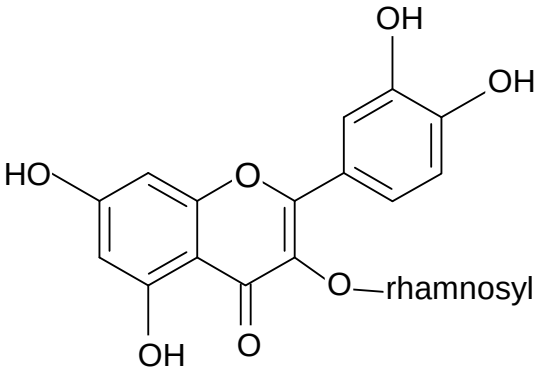


Figure 20: Structure of quercetrin (17 mg).

Table 4: ^{13}C NMR data of compound D (quercetrin)

Carbon	compound D (acetone-d6)	quercetin-3-O-rhamnoside (DMSO-d6) (Harborne and Mabry 1982)
2	157.9	156.7
3	135.7	134.6
4	179.3	178.0
5	163.1	161.6
6	99.5	99.0
7	165.0	164.4
8	94.4	93.9
9	158.3	157.5
10	105.0	104.5
1'	122.6	121.4
2'	116.1	115.9
3'	145.8	145.4
4'	149.0	148.7
5'	116.7	116.1
6'	122.5	121.2
1"	102.7	102.2
2"	71.4	70.4
3"	71.3	70.4
4"	73.0	71.7
5"	72.1	70.8
6"	17.7	17.8

4.6 Discussion

Serage (2003) investigated the antibacterial compounds in the same species and after bioassay guided fractionation using only Silica gel chromatography managed to isolate and elucidate the structures of two antibacterial flavanones alpinetin and pinocembrin as well as one antibacterial chalcone flavokawain from the leaves of *C. apiculatum* subsp *apiculatum*. All the compounds had substantial activity against the bacterial pathogens tested.

After bioassay guided fractionation for antibacterial compounds, kaempferol was also isolated from C.*erythrophyllum* in our laboratory (Martini *et al*, 2004a).

In this study antioxidant guided assay of the ethyl acetate soluble fraction of the leaf extract of *C. apiculatum* subspp *apiculatum* has led to the isolation of four antioxidant compounds. The structures of the compounds were determined on the basis of spectral studies (^{1}H-NMR, ^{13}C-NMR and MS) and identified as: 2', 4'- dihydroxy-6'-methoxy chalcone, cardamomin (A), 5, 7–dihydroxy-2-phenyl flavanone, pinocembrin (B), kaempferol (C) and quercetrin (D). All the isolated compounds, cardamomin, pinocembrin, kaempferol and quercetrin are known and have been isolated before from different plant species.

CHAPTER FIVE

EVALUATION OF ANTIOXIDANT ACTIVITY OF THE EXTRACTS AND ISOLATED COMPOUNDS.

5.1 Quantitative evaluation of antioxidant activity

Quantitative antioxidant activity was determined spectrophotometrically as described by Mensor *et al.* (2001) and modified by Aderogba *et al*. (2006). Briefly, reactions were carried out in 96-well microtitre plates and each of the solvent fractions and crude extract was tested at varying concentrations. Initial stock solutions of 300 µg/ml of the various extracts were prepared. Final concentration of 150.0, 75.0, 37.5, 18.75, 9.74, 4.69, 2.34 and 1.17 µg/ml were made from the stock solution. Isolated compounds were also evaluated at different concentrations (µM). From the initial stock solutions of 200 µM, final concentrations of 100.0, 50.0, 25.0, 12.5, 6.25, 3.13 and 1.56 µM were made. Twenty micro litres of 0.25 mM DPPH in methanol was added to 50 µl of each concentration of sample tested and allowed to react at room temperature in the dark for 30 minutes.

Blank solutions were prepared with sample solution (50 µl) and 20 µl of methanol only while the negative control was DPPH solution, 20 µl plus 50 µl methanol. Methanol served as blank for the microplate reader and the decrease in absorbance measured at 515 nm. Percentage antioxidant activity (AA%) values were calculated from the absorbance values using the formula:

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

(Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control). L-ascorbic acid (vitamin C) was used as a positive control (antioxidant agent).

The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the separate linear regression of plots of the

mean percentage of the antioxidant activity against concentration of the test extracts (µg /ml) and compounds (µM) against obtained from the three replicate assays. The results were expressed as mean ± SEM and the EC_{50} values were obtained from the regression plots using Sigma Plots^{R} 2001, SPSS Science.

5.2 Data handling

Table 5: Absorbance of the crude methanol extract.

Conc. of sample (µg/ml)	Absorbance of sample			Absorbance of solvent blank			Absorbance of DPPH control
	1	2	3	1	2	3	
18.75	0.0129	0.0129	0.0127	0.0000	0.0000	0.0022	0.1940
9.74	0.0323	0.0230	0.0331	0.0012	0.0013	0.0009	0.1799
4.69	0.0735	0.0936	0.0811	0.0000	0.0004	0.0000	0.1903
2.34	0.1368	0.1369	0.1373	0.0001	0.0000	0.0000	0.1950
1.17	0.1595	0.1751	0.1735	0.0017	0.0011	0.0000	0.2264

Table 6: Mean absorbance of the crude methanol extract

Conc. of sample (µg/ml)	Sample's mean absorbance	Solvent blank's mean absorbance
18.75	0.0128	0.0007
9.74	0.0295	0.0011
4.69	0.0827	0.0001
2.34	0.1370	0.000
1.17	0.1694	0.0009

Mean absorbance of control used = 0.1971

Values obtained were converted to percentage antioxidant activity (AA %) using the formula:

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

(Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control).

Table 7: Percentage antioxidant activity of the crude methanol extract

Conc. (µg/ml)	Mean absorbance%	% Exp1	% Exp2	% Exp3
18.75	93.86	93.81	93.81	93.91
9.74	85.59	84.17	88.89	83.76
4.69	58.09	62.76	52.56	58.90
2.34	30.49	30.59	30.54	30.34
1.17	14.51	19.53	11.61	12.43

EC_{50} = 11.81 ± SEM (Standard Error of Mean)

SEM = $\frac{\text{Standard deviation of the } EC_{50} \text{ values of Exp 1- 3 (Table 35)}}{\sqrt{n = 3}}$

EC_{50}= 14.51 ± 0.12 µg/ml (Correlation coefficient r^2 = 0.953)

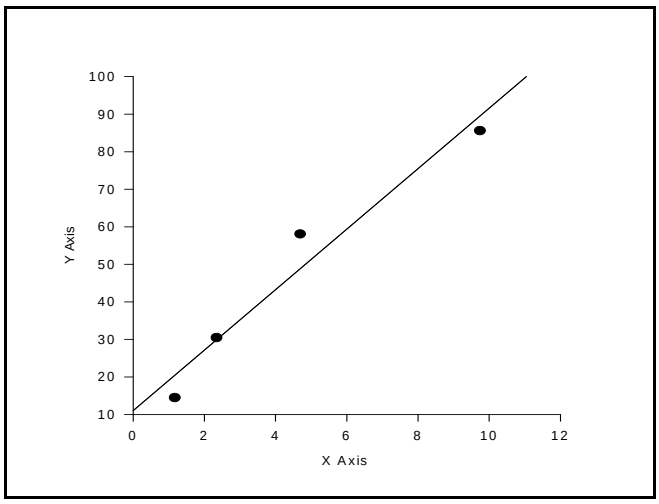


Figure 21: Absorbance of the crude methanol extract (X axis represents percentage absorption and y axis represents concentration of extract in µg/ml)

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Table 8: Absorbance of the hexane fraction.

Conc. Of sample (µg/ml)	Absorbance of sample			Absorbance of solvent blank			Absorbance of control
	1	2	3	1	2	3	
75.00	0.0199	0.0201	0.0271	0.0086	0.0086	0.0112	0.1758
37.50	0.0619	0.0469	0.0419	0.0059	0.0094	0.0093	0.1875
18.75	0.1116	0.1078	0.1132	0.0035	0.0054	0.0088	0.1941
9.74	0.1471	0.1449	0.1456	0.0000	0.0014	0.0031	0.1939
4.69	0.1627	0.1642	0.1652	0.0017	0.0008	0.0000	0.2011
2.34	0.1751	0.1740	0.1744	0.0001	0.0000	0.0000	0.2023

Table 9: Mean absorbance of the hexane fraction

Conc. of sample (µg/ml)	Sample mean absorbance	Solvent Blank mean absorbance
75.00	0.0224	0.0095
37.50	0.0502	0.0081
18.75	0.1109	0.0059
9.74	0.1459	0.0015
4.69	0.1640	0.0008
2.34	0.1745	0.0000

Mean absorbance of control used =0.2309

Values obtained were converted to percentage antioxidant activity (AA%).

Table 10: Percentage antioxidant activity of the hexane fraction

Conc. (µg/ml)	Mean absorbance%	% Exp1	% Exp2	% Exp3
75.00	94.41	95.50	95.41	92.38
37.50	81.77	76.70	83.20	85.36
18.75	54.53	54.22	55.87	53.53
9.74	37.46	36.94	37.90	37.59
4.69	29.32	29.88	29.23	28.76
2.34	24.43	24.17	24.64	24.47

EC_{50}= 17.31 ± SEM (Standard Error of Mean)

SEM = $\frac{\text{Standard deviation of the } EC_{50} \text{ values of Exp 1- 3 (Table 35)}}{\sqrt{n = 3}}$

EC_{50}= 17.31 ± 0.50 µg/ml (Correlation coefficient r^2 = 0.997)

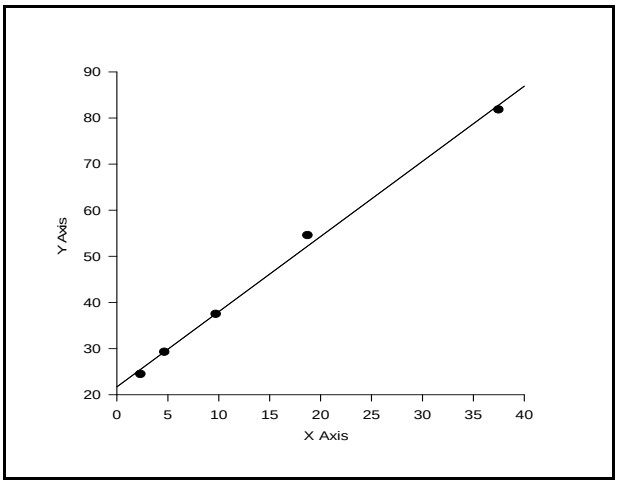

Figure 22: Absorbance of the hexane fraction(X axis represents percentage absorption and y axis represents concentration of extract in µg/ml)

Table 11: Absorbance of the dichloromethane fraction

Conc. Of sample (µg/ml)	Absorbance of sample			Absorbance of solvent blank			Absorbance of control
	1	2	3	1	2	3	
75.00	0.0189	0.0177	0.0184	0.0072	0.0065	0.0084	0.1888
37.50	0.0597	0.0317	0.0290	0.0043	0.0041	0.0039	0.1863
18.75	0.1035	0.0874	0.0855	0.0047	0.0026	0.0023	0.1877
9.74	0.1387	0.1282	0.1304	0.0019	0.0017	0.0016	0.1968
4.69	0.1636	0.1587	0.1684	0.0007	0.0008	0.0006	0.1911
2.34	0.1744	0.1750	0.1745	0.0002	0.0005	0.0008	0.1947

Table 12: Mean absorbance of the dichloromethane fraction

Conc. of sample (µg/ml)	Sample mean absorbance	Solvent blank mean absorbance
75.00	0.0183	0.0074
37.50	0.0401	0.0041
18.75	0.0921	0.0032
9.74	0.1324	0.0017
4.69	0.1602	0.0007
2.34	0.1746	0.0005

Mean absorbance of control used = 0.1909

Values obtained were converted to percentage antioxidant activity (AA%).

Table 13: Percentage antioxidant activity of the dichloromethane fraction

Conc. (µg/ml)	Mean absorbance%	% Exp1	% Exp2	% Exp3
75.00	94.29	93.98	94.60	94.24
37.50	81.14	70.87	85.54	86.96
18.75	53.43	47.46	55.89	56.89
9.74	31.53	28.23	33.73	32.58
4.69	16.45	14.67	17.23	17.39
2.34	8.80	8.91	8.59	8.85

EC_{50}= 20.33 ± SEM (Standard Error of Mean)

SEM = $\frac{\text{Standard deviation of the } EC_{50} \text{ values of Exp 1- 3 (Table 35)}}{\sqrt{n = 3}}$

EC_{50}= 20.33 ± 1.56 µg/ml (Correlation coefficient r^2 = 0.973)

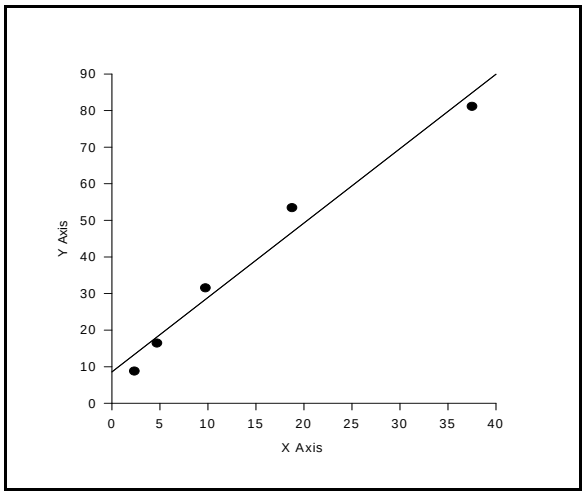

Figure 23: Absorbance of the dichloromethane fraction (X axis represents percentage absorption and y axis represents concentration of extract in µg/ml)

Table 14: Absorbance of the ethyl acetate fraction

Conc. of sample (µg/ml)	Absorbance of sample			Absorbance of solvent blank			Absorbance of control
	1	2	3	1	2	3	
9.74	0.0194	0.0228	0.0242	0.0095	0.0008	0.0002	0.2074
4.69	0.0823	0.1008	0.1005	0.0003	0.0006	0.0017	0.2085
2.34	0.1260	0.1396	0.1381	0.0000	0.0000	0.0000	0.2153
1.17	0.1692	0.1410	0.1429	0.0020	0.0012	0.0000	0.2313

Table 15: Mean absorbance of the ethyl acetate fraction

Conc. of Sample (µg/ml)	Sample mean absorbance	Solvent Blank mean absorbance
9.74	0.0222	0.0011
4.69	0.0945	0.0035
2.34	0.1346	0.0000
1.17	0.1510	0.0011

Mean absorbance of control used = 0.2156

Values obtained were converted to percentage antioxidant activity (AA%).

Table 16: Percentage antioxidant activity of the ethyl acetate fraction

Conc. (µg/ml)	Mean absorbance%	% Exp1	% Exp2	% Exp3
9.74	91.33	92.63	91.05	90.35
4.69	56.59	62.24	53.66	53.80
2.34	37.57	41.56	35.25	35.95
1.17	30.47	22.03	34.60	34.23

EC_{50}= 3.91 ± SEM (Standard Error of Mean)

SEM = $\frac{\text{Standard deviation of the } EC_{50} \text{ values of Exp 1- 3 (Table 35)}}{\sqrt{n = 3}}$

EC_{50}= 3.91 ± 0.02 µg/ml (Correlation coefficient r^2 = 0.999)

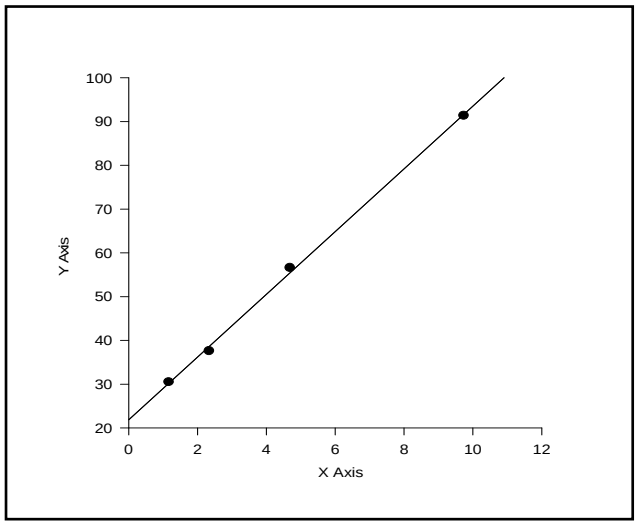

Figure 24: Absorbance of the ethyl acetate fraction (X axis represents percentage absorption and y axis represents concentration of extract in µg/ml)

Table17: Absorbance of the butanol fraction)

Conc. Of sample (µg/ml)	Absorbance of sample			Absorbance of solvent blank			Absorbance of control
	1	2	3	1	2	3	
9.74	0.0125	0.0129	0.0123	0.0000	0.0000	0.0000	0.2131
4.69	0.0257	0.0199	0.0217	0.0000	0.0000	0.0000	0.2127
2.34	0.1040	0.1006	0.1092	0.0000	0.0000	0.0000	0.2105
1.17	0.1647	0.1636	0.1565	0.0011	0.0000	0.0000	0.2242

Table 18: Mean absorbance of the butanol fraction

Conc. Of Sample (µg/ml)	Sample mean absorbance	Solvent Blank mean absorbance
9.74	0.0125	0.000
4.69	0.0224	0.0000
2.34	0.1046	0.0000
1.17	0.1616	0.0004

Mean absorbance of control used = 0.2151

Values obtained were converted to percentage antioxidant activity (AA%).

Table 19: Percentage antioxidant activity of the butanol fraction

Conc. (µg/ml)	Mean absorbance%	% Exp1	% Exp2	% Exp3
9.74	94.19	94.19	94.00	94.28
4.69	89.59	88.05	90.75	89.91
2.34	51.37	51.56	53.18	49.23
1.17	25.06	23.62	24.13	27.43

EC_{50}= 2.44 ± SEM (Standard Error of Mean)

SEM = $\frac{\text{Standard deviation of the } EC_{50} \text{ values of Exp 1- 3 (Table 35)}}{\sqrt{n = 3}}$

EC_{50}= 2.44 ± 0.02 µg/ml (Correlation coefficient r^2 = 0.993)

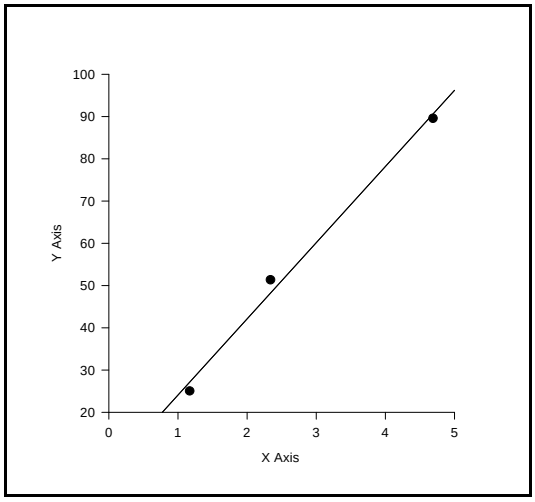

Figure 25: Absorbance of the butanol fraction (X axis represents percentage absorption and y axis represents concentration of extract in µg/ml)

Table 20: Absorbance of cardamomin

Conc. Of sample (µM)	Absorbance of sample			Absorbance of solvent blank			Absorbance of control
	1	2	3	1	2	3	
200.0	0.1613	0.1791	0.1810	0.0000	0.0000	0.0000	0.1916
100.0	0.1859	0.1942	0.1960	0.0053	.0048	0.0028	0.2012
50.0	0.1940	0.1979	0.1995	0.0013	0.0000	0.0004	0.1979

Table 21: Mean absorbance of cardamomin

Conc. Of Sample (µM)	Sample mean absorbance	Solvent Blank mean absorbance
200.0	0.1738	0.0000
100.0	0.1920	0.0043
50.0	0.1971	0.0006

Mean absorbance of control used = 0.1987

Values obtained were converted to percentage antioxidant activity (AA%).

Table 22: Percentage antioxidant activity of cardamomin

Conc. (µM)	Mean Absorbance%	% Exp1	% Exp2	% Exp3
200.0	11.73	18.08	9.04	8.08
100.0	4.67	7.77	3.56	2.64
50.0	0.20	-		

Note: Cardamomin did not inhibit 50% of DPPH radical at 200 µM concentration

Table 23: Absorbance of pinocembrin

Conc. Of sample (µM)	Absorbance of sample			Absorbance of solvent blank			Absorbance of control
	1	2	3	1	2	3	
200.0	0.1831	0.1908	0.1874	0.0002	0.0039	0.0026	0.1925
100.0	0.1922	0.2013	0.1998	0.0032	0.0049	0.0007	0.2019

Table 24: Mean absorbance of pinocembrin

Conc. Of Sample (µM)	Sample mean absorbance	Blank mean absorbance
200.0	0.1871	0.0022
100.0	0.1978	0.0029

Mean absorbance of control used = 0.1972

Values obtained were converted to percentage antioxidant activity (AA%).

Table 25: Percentage antioxidant activity of pinocembrin

Conc. (µM)	Mean Absorbance%	% Exp1	% Exp2	% Exp3
200.0	6.24	8.27	4.36	6.09
100.0	1.17	-		

Note: Pinocembrin did not inhibit 50% of DPPH radical at 200 µM concentration

Table 26: Absorbance of kaempferol

Conc. Of sample (µM)	Absorbance of sample			Absorbance of solvent blank			Absorbance of control
	1	2	3	1	2	3	
200.0	0.0086	0.0096	0.0094	0.0008	0.0006	0.0009	0.1889
100.0	0.0152	0.0103	0.0127	0.0011	0.0013	0.0009	0.1937
50.0	0.0874	0.0891	0.0780	0.0074	0.0051	0.0041	0.1882
25.0	0.1324	0.1395	0.1336	0.0061	0.0032	0.0074	0.1931
12.5	0.1624	0.1653	0.1750	0.0042	0.0008	0.0000	0.1907

Table 27: Mean absorbance of kaempferol

Conc. Of Sample (µM)	Sample mean absorbance	Solvent Blank mean absorbance
200.0	0.0092	0.0008
100.0	0.0127	0.0011
50.0	0.0848	0.0055
25.0	0.1352	0.0056
12.5	0.1676	0.0017

Mean absorbance of control used = 0.1909

Values obtained were converted to percentage antioxidant activity (AA%).

Table 28: Percentage antioxidant activity of kaempferol

Conc. (µM)	Mean absorbance%	% Exp1	% Exp2	% Exp3
200.0	95.60	95.91	95.39	95.50
100.0	93.92	92.61	95.18	93.92
50.0	58.46	57.10	56.21	62.02
25.0	32.11	33.58	29.86	32.95
12.5	13.10	15.82	14.30	9.22

EC_{50} = 47.36 ± SEM (µM)
SEM = $\frac{\text{Standard deviation of the } EC_{50} \text{ values of Exp 1- 3 (Table 36)}}{\sqrt{n = 3}}$

EC_{50} = 47.36 ± 0.03 µM (Correlation coefficient, r^2 =0.977)

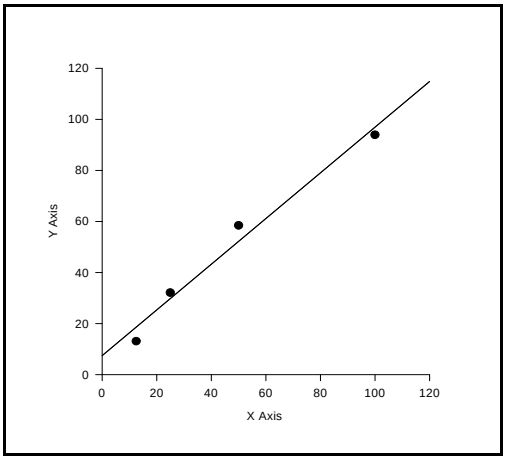

Figure 26: Absorbance of the kaempferol (X axis represents percentage absorption and y axis represents concentration in µM)

Table 29: Absorbance of quercetrin

Conc. Of sample (µM)	Absorbance of sample			Absorbance of Solvent blank			Absorbance of control
	1	2	3	1	2	3	
50.0	0.0102	0.0107	0.0117	0.0000	0.0000	0.0000	0.2056
25.0	0.0223	0.0302	0.0320	0.0000	0.0000	0.0000	0.2139
12.5	0.0795	0.0816	0.0885	0.0000	0.0000	0.0000	0.2104
6.25	0.1447	0.1506	0.1503	0.0000	0.0000	0.0000	0.2189
3.125	0.1634	0.1634	0.1712	0.0000	0.0000	0.0000	0.2119
1.56	0.1816	0.1846	0.1829	0.0000	0.0000	0.0000	0.1850

Table 30: Mean absorbance of quercetrin

Conc. Of Sample (µM)	Sample mean absorbance	Solvent Blank mean absorbance
50.0	0.0109	0.000
25.0	0.0282	0.000
12.5	0.0832	0.000
6.25	0.1485	0.000
3.125	0.1660	0.000
1.56	0.1830	0.000

Mean absorbance of control used =0.2076

Values obtained were converted to percentage antioxidant activity (AA%).

Table 31: Percentage antioxidant activity of quercetrin

Conc. (µM)	Mean absorbance%	% Exp1	% Exp2	% Exp3
50.0	94.75	95.09	94.85	94.36
25.0	86.42	89.26	85.45	84.01
12.5	59.92	61.71	60.69	57.37
6.25	28.47	30.30	27.46	27.60
3.125	20.04	21.29	21.29	17.53
1.56	11.85	12.52	11.08	11.90

EC_{50}= 11.81 ± SEM (µM)

SEM = $\frac{\text{Standard deviation of the } EC_{50} \text{ values of Exp 1- 3 (Table 36)}}{\sqrt{n = 3}}$

EC_{50}= 11.81 ± 0.32 µM (Correlation coefficient r^2 = 0.970)

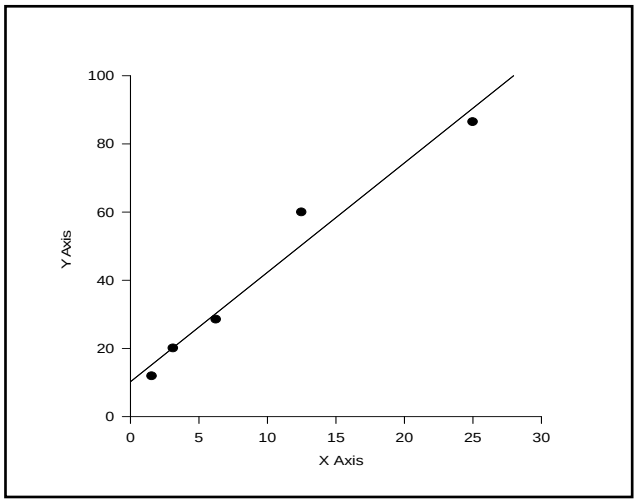

Figure 27: Absorbance of the quercetrin (X axis represents percentage absorption and y axis represents concentration in µM)

Table 32: Absorbance of L-ascorbic acid

Conc. Of sample (µM)	Absorbance of sample			Absorbance of Solvent blank			Absorbance of control
	1	2	3	1	2	3	
25.0	0.0233	0.0205	0.0172	0.0049	0.0000	0.0003	0.1902
12.5	0.1055	0.1071	0.0998	0.0064	0.0022	0.0026	0.1957
6.25	0.1493	0.1579	0.1493	0.0080	0.0012	0.0004	0.1936
3.125	0.1667	0.1689	0.1636	0.0004	0.0004	0.0001	0.1886
1.56	0.1788	0.1744	0.1766	0.0006	0.0071	0.0000	0.1961`

Table 33: Mean absorbance of L-ascorbic acid

Conc. Of Sample (µM)	Sample mean absorbance	Solvent Blank mean absorbance
25.0	0.0204	0.0017
12.5	0.1041	0.0037
6.25	0.1522	0.0032
3.125	0.1664	0.0003
1.56	0.1766	0.0026

Mean absorbance of control used = 0.1928

Values obtained were converted to percentage antioxidant activity (AA%).

Table 34: Percentage antioxidant activity of L-ascorbic acid

Conc. (µM)	Mean absorbance%	% Exp1	% Exp2	% Exp3
25.0	90.30	88.80	90.25	91.91
12.5	47.93	47.20	46.37	49.84
6.25	22.72	24.22	19.76	24.22
3.125	13.85	13.69	12.55	15.30
1.56	9.75	8.61	10.89	9.75

EC_{50} = 13.37 ± SEM

SEM = $\frac{\text{Standard deviation of the } EC_{50} \text{ values of Exp 1- 3 (Table 36)}}{\sqrt{n = 3}}$

EC_{50} = 13.37 ± 0.20 µM or 2.35 ± 0.04 µg/ml (Correlation coefficient, r^2 =0.998)

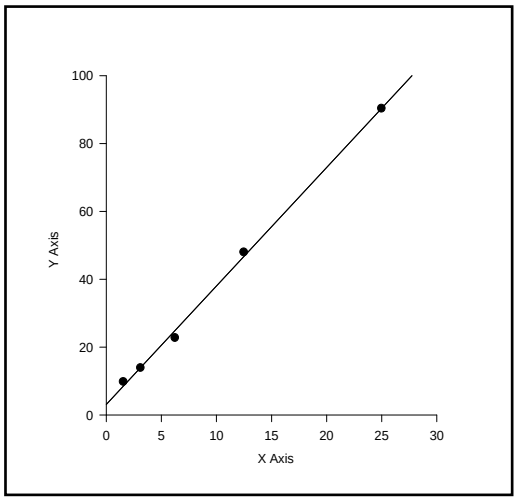

Figure 28: Absorbance of the L-ascorbic acid (X axis represents percentage absorption and y axis represents concentration in µM

Table 35: EC_{50} values from the regression plots of Experiments 1 – 3 of the extracts

Extract	EC_{50} Values (µg/mL)			
	Mean %	Exp 1	Exp. 2	Exp. 3
Crude	4.81 ± 0.12	4.58	4.95	4.92
Hexane	17.31 ± 0.50	18.41	16.90	16.90
Dichloromethane	20.33 ± 1.56	23.63	19.10	18.82
Ethyl acetate	3.91 ± 0.02	3.88	3.95	3.95
Butanol	2.44 ± 0.02	2.48	2.41	2.41
L-ascorbic acid	2.35 ± 0.04 13.37 ±0.02 (µM)	13.55 (µM)	13.65(µM)	13.01(µM)

Table 36: EC_{50} values from the regression plots of Experiments 1 – 3 of the isolated compounds

Compound	EC_{50} Values (µM)			
	Mean %	Exp 1	Exp. 2	Exp. 3
Cardamomin	>200	>200	>200	>200
Pinocembrin	>200	>200	>200	>200
Kaempferol	47.39	47.02	48.05	47.36
Quercetrin	11.85	11.82	12.46	12.91
L-ascorbic acid	13.37 ± 0.20	13.55	13.65	13.01

5.2 Discussion

In the quantitative assay, the more polar fractions (ethyl acetate and butanol) had the highest antioxidant activity among the extracts with EC_{50} values of 3.91 ± 0.02 and 2.44 ± 0.02 µg/ml respectively. The butanol extract had nearly the same activity as L-ascorbic acid. Even the crude extract had an activity approximately a half of ascorbic acid. Higher activity demonstrated by butanol fraction could be due to higher concentration of the few antioxidant compounds expressed in the extract.

Of the isolated compounds, kaempferol (C) and quercetrin (D) had strong antioxidant activity with EC_{50} values of 11.81 ± 0.32 and 47.36 ± 0.03 µM respectively. On a micromolar basis quercetrin was even more active than L-ascorbic acid,the positive control. Conditions for strong antioxidant activity found by structural activity studies in flavonoids: are the presence of a catechol group (3', 4' -OH) on ring B, the presence of 2, 3 unsaturation along with 3-OH and a keto group in position 4 (Op de Beck *et al.,* 2003 and Saskia *et al*., 1996). Only compound quercetrin (D) fulfilled all these requirement and had the highest activity comparable to L-ascorbic acid. Cardamomin (A) and pinocembrin (B) did not have strong activity as these compounds could not scavenge 50% of the DPPH radical at the highest concentration (200 µM) tested. These results may due to the absence of the conditions necessary for effective free radical scavenging activity stated for flavonoids. L-ascorbic acid was used standard antioxidant agent (EC_{50} = 13.37 ± 0.20 µM).

It is very important to determine the toxicity of the isolated compounds to determine their potential use as antioxidant agents, this was examined next.

CHAPTER SIX

CYTOTOXICITY ASSAY

6.1 Tetrazolium-based colorimetric assay (MTT)

The procedure described by McGaw *et al* (2007) was used to investigate cytotoxicity of the two isolated compounds. The compounds were tested for cytotoxicity against Vero monkey kidney cells. The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5 × 103 cells into each well of a 96-well microtitre plate. Plates were incubated overnight at 37 ºC in a 5% CO_2 incubator and the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the isolated compounds (20 mg/ml) were prepared by dissolving in DMSO. Serial 10-fold dilutions of each isolated compounds were prepared in growth medium. The viable cell growth after 120 hours incubation with isolated compounds was determined using the tetrazolium-based colorimetric assay (MTT assay) described by Mosmann (1983). The absorbance was measured on a Titertek Multiscan MCC/340 microplate reader at 540 nm test wavelength and reference wavelength of 690 nm. Berberine chloride (Sigma Chemical Company) was used as a positive control. Tests were carried out in quadruplicate and each experiment was repeated three times.

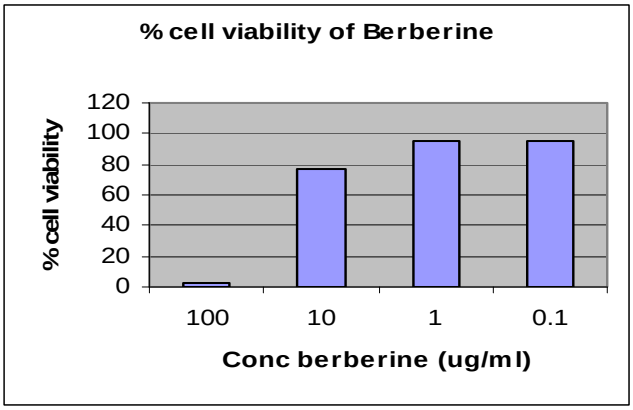

Figure 29: Percentage cell viability of berberine

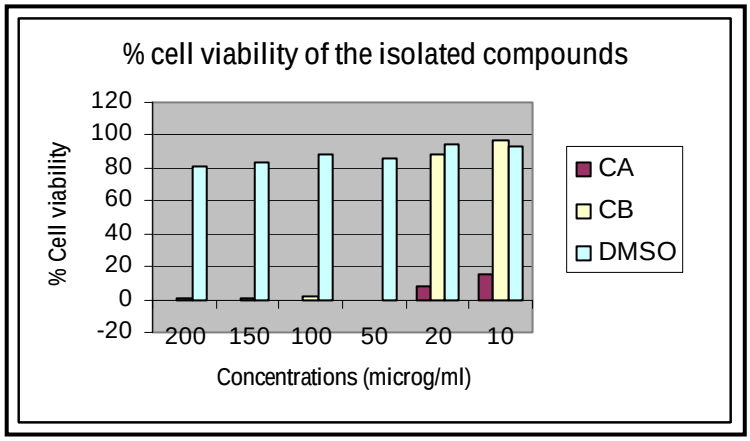

Figure 30: Percentage cell viability of the isolated compounds, pinocembrin (CB) and cardamomin (CA)

The use of effective and safe plant extracts or compounds needs to be encouraged. Toxicity testing of the isolated compound using cell line assay is important to determine the safety of these compounds (McGaw *et al* 2007). Her PhD study Martini (2002) isolated and characterized seven antibacterial compounds. Four were flavanols: kaemferol, rhamnocitrin, rhamnazin, quercitin 5,3'-dimethylether] and three flavones apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone. There was hardly any toxicity towards human lymphocytes with the exception of 5-hydroxy-7,4'-dimethoxyflavone. This compound is potentially toxic to human cells and had the lowest antioxidant activity. Both rhamnocitrin and rhamnazin exhibited strong

antioxidant activity with potential anti-inflammatory activity. Although these flavonoids are known, this was the first report of biological activity with some of these compounds.

In our study cardimomin had poor quantitative antioxidant activity and was much more toxic to the cells LC_{50} of 1.97 µg/ml than pinocembrin with an LC_{50} of 29.47µg/ml. Berberine the positive control had an LC_{50} of 12.35 µg/ml. This means that cardimomin and pinocembrin are not good candidates to be used to stimulate the immune system of animals through its antioxidant activity. Unfortunately insufficient material was available to determine the toxicity of quercetrin and kaempferol.

It is important to recognise that cellular toxicity does not necessarily equate to toxicity for humans or animals. If the toxic compound is not taken up or is rapidly excreted or metabolised to a non-toxic metabolite it may not have any deleterious effect on the animals or humans taking an extract.. Nevertheless, it would make sense to evaluate the potential toxicity of cardomomin and aqueous extracts in a animal model.

CHAPTER SEVEN

DISCUSSION AND CONCLUSION

The use of *Combretum* species in many cultures in folk medicine for treatment of several inflammatory conditions (abdominal pains, headache and toothache) (Eloff *et al*., 2001) and their established antimicrobial activities (Martini and Eloff, 1998; Eloff 1999b) prompted us to investigate the antioxidant potential of this plant genus. There are two possible mechanisms to explain the use of plants extracts to treat microbial infections. A direct effect involves the action of active agents (antimicrobial) and the indirect effect may involve the stimulation of the host immune system to overcome the effects of microorganisms via the host immune system. Traditional healers use mainly aqueous extracts and in all Combretaceae we have studied, these extracts had hardly any *in vitro* antibacterial activity. (Kotze and Eloff, 2002). One would expect that aqueous extracts would have high anti-oxidant activities and may stimulate the immune system of patients thereby combating the infection indirectly.

Leaves of ten *Combretum* species were extracted with methanol for screening because most antioxidant constituents are polar secondary metabolites phenolic compounds especially flavonoids. All the species examined contained antioxidant compounds based on TLC chromatograms sprayed with DPPH (2, 2-diphenyl-1-picrylhydrazyl), however the number of constituents with antioxidant activity varied amongst the C*ombretum* species. The highest numbers of antioxidant constituents were present in *Combretum apiculatum* subsp *apiculatum* followed by *Combretum moggii*.

Combretum apiculatum subsp *apiculatum* was therefore selected for fractionation. Four solvent fractions were obtained from the crude methanol extract using solvent-solvent fractionation. All four fractions had antioxidant compounds but the highest numbers were present in the ethyl acetate fraction based on spraying chromatograms with DPPH. Therefore

ethyl acetate was selected for further fractionation and purification that led to the isolation of the antioxidants.

Antioxidant activity directed fractionation of the ethyl acetate soluble fraction of the leaf extract of *C. apiculatum* subsp *apiculatum* led to the isolation of four antioxidant compounds. The structures of the compounds were determined by spectral analysis (^{1}H-NMR, ^{13}C-NMR and MS) and identified as: 2', 4'- dihydroxy-6'-methoxy chalcone, cardamomin (A), 5, 7–dihydroxy-2-phenyl flavanone, pinocembrin (B), kaempferol (C) and quercetrin (D).

All the isolated compounds bleached the DPPH purple colour when sprayed with 0.2% DPPH in methanol after TLC. In the quantitative DPPH antioxidant assay, the more polar fractions (ethyl acetate and butanol) had the highest activity among the extracts with EC_{50} values of 3.91 ± 0.02 and 2.44 ± 0.02 µM respectively. This compared well with the value of ascorbic acid the positive control 2.35 ± 0.04.

Of the isolated compounds, kaempferol (C) and quercetrin (D) had good antioxidant activity with EC_{50} values of 11.81 ± 85 and 47.36 ± 0.03 µM respectively compared to that of ascorbic acid (13.37 ± 0.20 µM).. Based on structural activity studies it appears that good antioxidant acitivity requires the presence of a catechol group (3', 4' -OH) on ring B, the presence of 2, 3 unsaturation along with 3-OH and a keto group in position 4 (Op de Beck *et al*., 2003 and Saskia *et al*., 1996). Only compound quercetrin (D) fulfilled these entire requirements and as expected had the highest activity comparable to L-ascorbic acid. Cardamomin (A) and pinocembrin (B) did not have as strong activity as these compounds could not scavenge 50% of the DPPH radical at the highest concentration (200 µM) tested apparently. The lower antioxidant activity confirms the relationship between structure and anti-oxidant activity.

The presence of these antioxidant compounds in a high concentration provides a rationale for the ethnomedicinal use of this plant for the treatment of inflammatory conditions in traditional

medicine. Many flavonoids possess both antimicrobial and antioxidant activities as well as nutritional supplement, as both sweetener and flavorings agent, for an example pinocembrin (Serage 2003). Pinocembrin, flavokawain-A and alpinetin were first isolated as antibacterial compounds from C.*apiculatum* subspp *apiculatum* by (Serage 2003). It is surprising that although the four major antioxidant compounds present in this species were successfully isolated, the anti-oxidant activity of the crude extract was not that much less than the anti-oxidant activity of the main antioxidant compounds. This may mean that there are synergistic antioxidant activities between different components of the crude extract.

The cytotoxicity of cardimonin and pinocembrim was evaluated using MTT assay with, berberine as positive control and DMSO as negative control. There was not enough material available to determine the cytotoxicity of kaempferol (C) and quercetrin (D). At higher concentrations than 50µg/ml of cardimomin or pinocembrin the cells were not viable. Cardimomin was more toxic to the cells with an LC_{50} of 1.97µg/ml than pinocembrin with an LC_{50} of 29.47µg/ml and for the positive control with an LC_{50} of 12.35 µg/ml.

Because the crude polar extract had an antioxidant activity half that of L ascorbic acid, it may explain the reason why plant extracts of *Combretum* species are used by traditional healers. It appears that the antimicrobial activities of aqueous plant extracts are related to the antioxidant activity leading to a stimulated immune system rather than antimicrobial activity *per se*.

The high toxicity of cardominin in C.*apiculatum* subspp *apiculatum* indicates that the safety of crude extracts may be a concern. It may be worthwhile to evaluate the anti-oxidant activity and cardominin content of aqueous extracts for use in animals and man.

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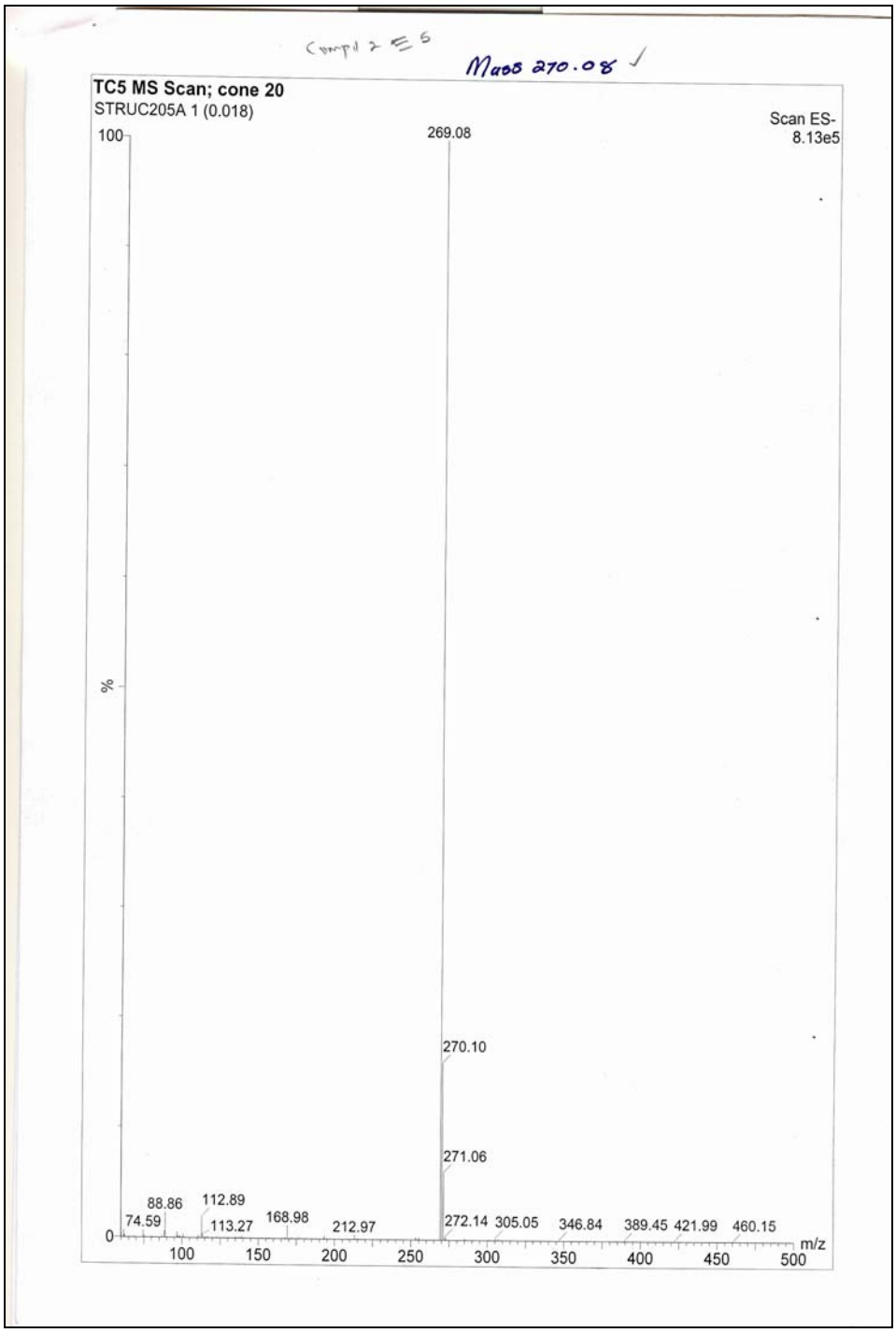
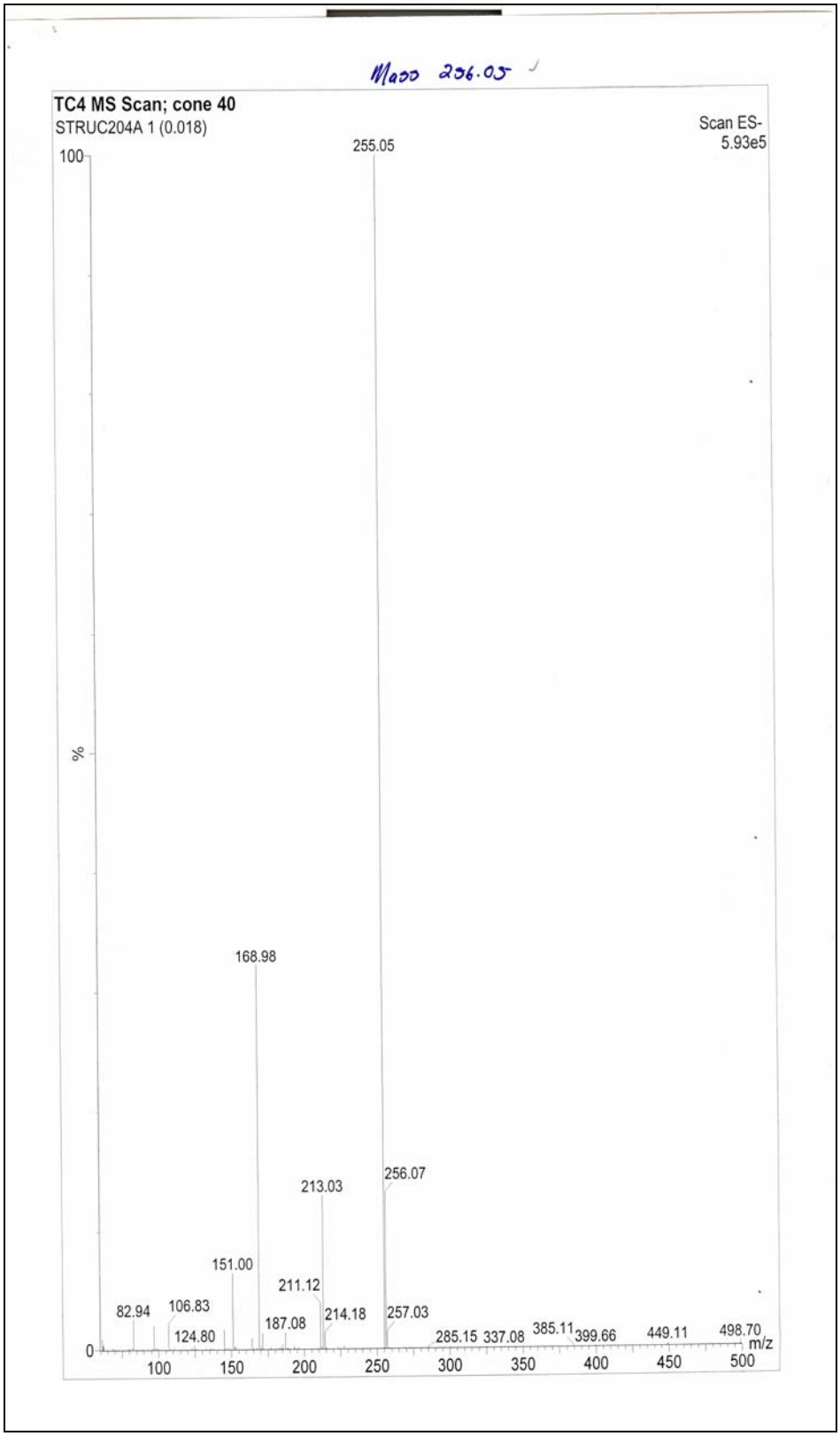
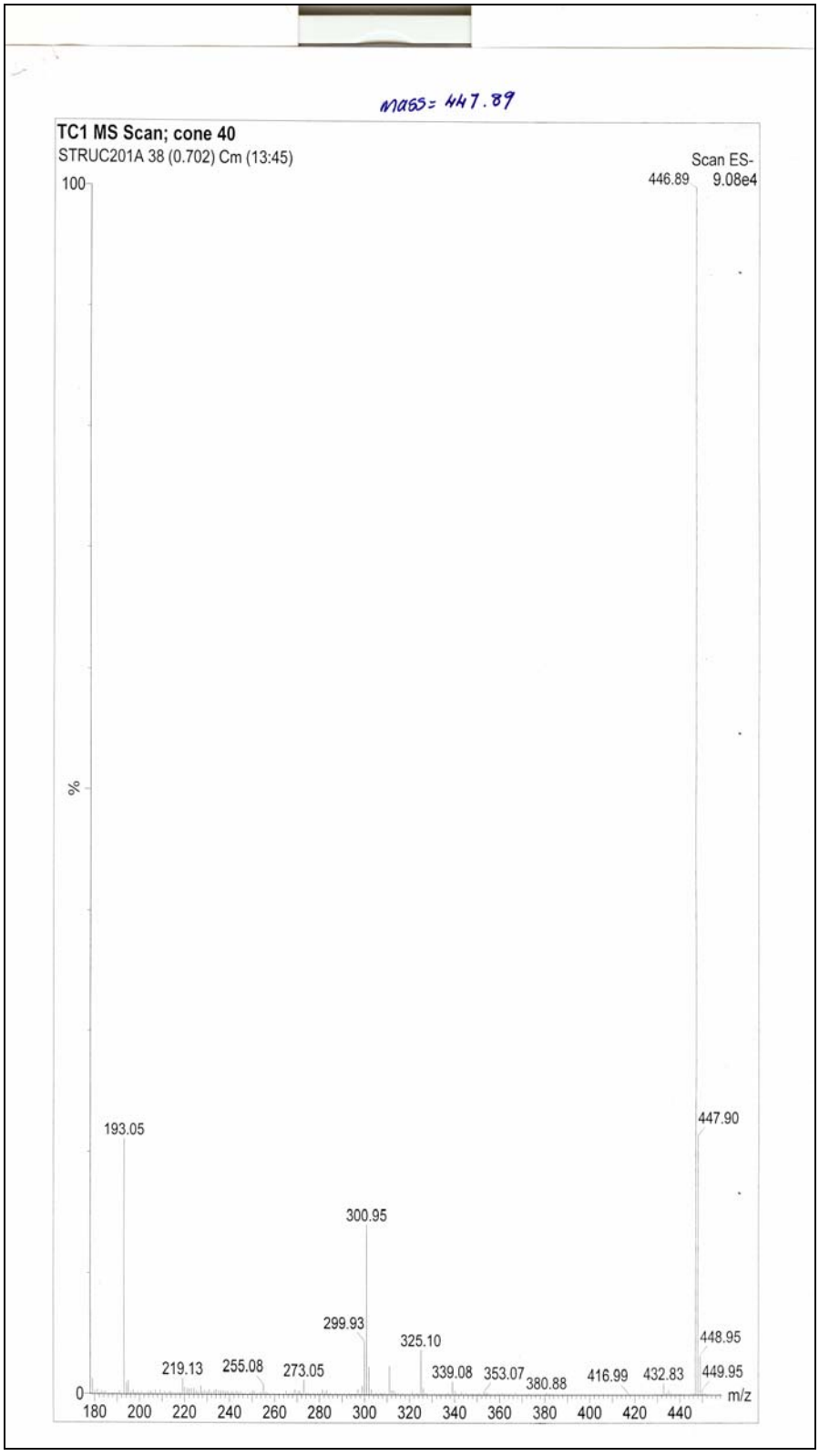
APPENDIX



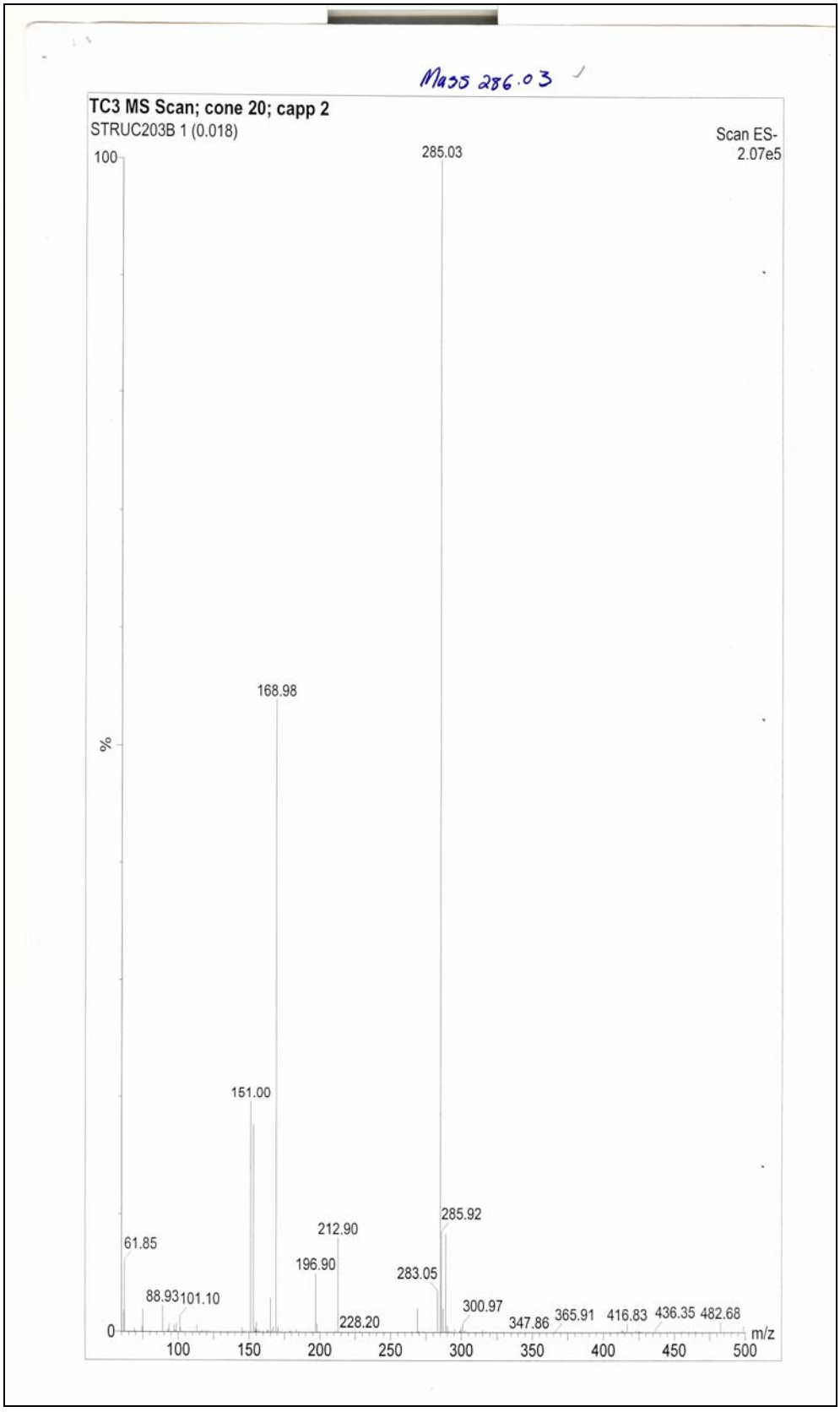
Mass spectrum of cardamomin



Mass spectrum of pinocembrin



Mass spectrum quercetrin



Mass spectrum of Kaempferol