Isolation and characterization of antibacterial and antioxidant compounds present in Combretum collinum subspecie suluense

TE Ramurafhi

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M.Sc. (Chemistry)

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Isolation and characterization of antibacterial and

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collinum subspecie suluense

by

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DECLARATION

I declare that the chemistry dissertation hereby submitted to the University of limpopo, for the degree of Master of chemistry in phytochemistry has not previously been submitted by me for a degree at this of any other university; that is my work in design and in execution, and that all material contained herein has been duly acknowledged.

_____ _____

Initials & Surname(Title) **Date**

Student Number: 210437860

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List of abbreviation used

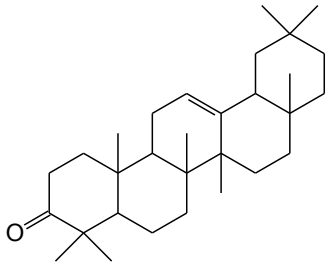
WHO	:	World Health Organization
UV	:	Ultraviolet light
^{13}CNMR	:	Carbon-13 nuclei magnetic resonance
^{1}HNMR	:	Hydrogen nuclei magnetic resonance
Ms	:	Mass spectrometry
TLC	:	Thin layer chromatography
E. coli	:	*Escherichia coli*
CEF	:	Chloroform Ethyl acetate Formic acid
INT	:	*P*-iodonitrotetrazolium violet
TDH	:	Threonine dehydrogenase
PLC	:	Preparative layer chromatography
IR	:	Infrared spectroscopy
TMS	:	Tetramethylsilane
MIC	:	Minimum inhibitory concentration
DPPH	:	1,1-diphenyl-2-picrylhydrazyl
FTIR	:	Fourier transformation infrared spectroscopy
Hex	:	Hexane
Chloro	:	Chloroform
DCM	:	Dichloromethane
Am	:	*Adina microcephala*

Fm	:	*Ficus morecae*
Cb	:	Combretum bracteosum
Cc	:	Combretum collinum
Fd	:	Filicium decipiens
Ca	:	Calpurnia aurea
Ppm	:	Parts per million
MHz	:	Mega Hertz
NADH dinucleotide	:	Reduced form of nicotinamide adenine
NAD	:	Nicotinamide adenine dinucleotide
Ssp	:	Sub specie

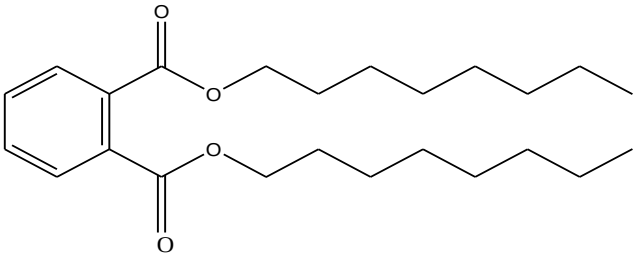
Abstract

Isolation and characterization of antibacterial and antioxidant compounds present in *Combretum collinum* Sub specie *Suluense*

Leaf extracts of six different plants, many of which are used by traditional healers, were screened for antibacterial activity. Ethyl acetate and Methanol extracts of *Adina microcephaha, Combretum aurea, Combretum bracteosam, Combretum collinum, Filicium decipiens* and *Ficus moraceae* were screened. Of these acetone extracted the highest mass of 118 mg from *Filicium decipiens* as compared to the extracts from the *Adina microcephala, Combretum aurea, Combretum bracteosum, Combretum collinum* and *Ficus decipiens*. Two unknown compounds were isolated, characterized and their minimum inhibitory concentrations were determined; compound A = 0.5689 mg/ml and compound E = 1.479 mg/ml. The chemical structure proposed for unknown compound A from *Combretum collinum* subsp *suluense* was determined by correlation of HNMR, FT-IR and GC-MS data. The chemical structure of compound E was not proposed because it was masked by a phthalate pollutant, the structure of which was confirmed by correlation of GC-MS, FT-IR and ^{1}HNMR data.



Proposed structure of compound from vial A



Proposed structure of phthalate pollutant

CHAPTER 1

Introduction

1.1 Overview

Traditional medicine based on plant-derived remedies is the primary source of relief from a variety of diseases in Southern African cultures, it should be promoted, investigated and its potential developed for wider use and benefit to mankind (WHO, 1978). Primary health care has been adopted by all WHO member states, including those in the African continent, as the appropriate strategy, for developing national health systems (Akerele, 1988). In South Africa, about 60-80% of the population relies on medicinal plants to treat various illnesses (Hutchings &Van Staden, 1994).

In South Africa, traditional medicine is not yet fully accepted in the mainstream primary health care system. For its full acceptance, the chemical contents in the medicines, the safety of the preparations and their quality must be investigated and thoroughly validated to comply with the Medicines and Related Substances (MRS) Act 101 of 1965.

1.2 Informant Consensus Factor (ICF) for category of ailments and fidelity level of medicinal plants

The Informant Consensus Factor (ICF) is a formula used to estimate the use of various medicinal plants and to determine plants which are to be further investigated in the search for bioactive compounds. The Informant Consensus Factor is calculated using the following formula:

$$ICF = n_{ur} - n_t / n_{ur} - 1 \qquad \text{Equation 1}$$

where n_{ur} = number of use-reports in each category and n_t = number of taxa. The product of this factor ranges from 0 to 1. A high value (close to 1) indicates that relatively few taxa, usually species, are used by more people. A small value (close to zero) indicates that the informants disagree on the taxa to be used in the treatment in the group of illness (Heinrich *et al.*, 1998a).

The Informant Consensus Factor (ICF) and the Fidelity Level (FL) of medicinal plants were determined by Teklehaymanot *et al*. (2007). Sixty-seven medicinal plants used as cures for 52 ailments were documented; they are distributed across 42 families and 64 genera. The most frequently utilized plant part was the underground part (root/rhizome/bulb) (42%). The largest number of remedies was used to treat gastrointestinal disorders and parasitic infections (22, 8%) followed by external injuries (22, 1%). The administration routes were orally (51, 4%), externally (38, 6%), nasal (7, 9%) and in the ear (2, 1%) (Teklehaymanot *et al*., 2007).

Several plants e.g. *Acokanthera schimperi* and *Calpurnia aurea* have so far been tested for anti-bacterial properties(Asfaw *et al*., 1993). Hydro alcoholic extracts of eight species of medicinal plants, namely *Acokanthera schimperi* (Apocynaceae), *Combretum aurea* (Leguminosae), *Lippia adoensis* (Verbenaceae), *Malva parviflora* (Malvaceae), *Olinia rochetiana* (Oliniaceae), *Phytolacca dodecandra* (Phytolaceaceae) and *Verbascum sinaiticum* (Scrophulariaceae), traditionally used in the treatment of various skin disorders were screened for antimicrobial activity against different strains of bacteria and fungi, known to cause different types of skin infections (Asfaw *et al*., 1993).

1.3 Secondary plant metabolites

Primary metabolites are present in every living cell capable of mitosis. Secondary metabolites are present only incidentally and are not for plant life. They are used by humans for medicinal, nutritive and cosmetic purposes (Kossel; 1891). Plants release secondary metabolites depending on the environmental conditions they are exposed to. It has been postulated that African plants, *which* are exposed to harsh conditions such as infections, herbivory foraging and competition are the best candidates for potential anti-infection drugs (Geissman *et al*., 1963). Secondary metabolites use the availability of hydroxyl (–OH), amino (-NH_2) and thiol (–SH) groups, as well as aromatic nuclei and unsaturated aliphatic chains for antioxidant and antiradical activity (Edreva *et al*., 2008). Secondary metabolites such as flavonoids, terpenoids and other volatile compounds give colour and scent for attraction or repulsion of

herbivores to encourage or prevent them from eating those plants (Hadacek, 2002) (table 1). Carotenoids are the best candidates to protect lipophilic surfaces such as membranes (Dixon *et al.*, 2002) (table 9). An ortho-dihydroxy substitution in the B-ring of anthocyanins, pontentiating its antioxidant capacity was proposed as a protective mechanism in a physiological leaf reddening disorder of cotton due to oxidative stress caused by Na^+/K^+ imbalance (Edreva, 2005).

Table 1: Diverse chemical types of secondary metabolites (Edreva *et al.*, 1998)

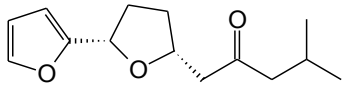
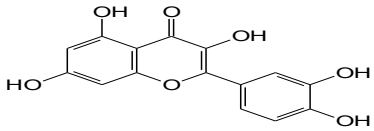
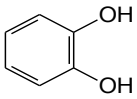
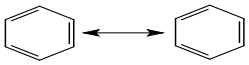
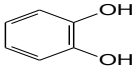
#	Chemical types	Formulae	Representatives
1	Hydroaromatic		Terpenoids
2	Heterocyclic		Flavonoids

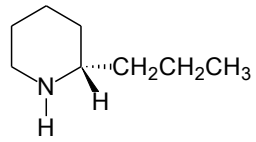
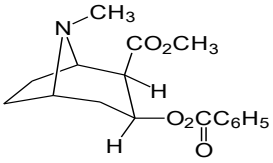
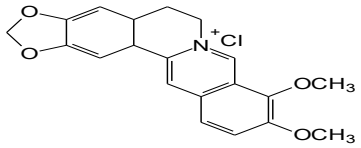
Table 2: Chemical characteristics of secondary metabolites (Edreva *et al.*, 1998)

characteristics	**Interactions**	**Functions**
Availability of electrical charge	Electrostatic	Structure-stabilizing
Presence of –COOH, -OH, -NH_2 	Covalent bonding; Polymerisation, Condensation, Complexation	Structure-stabilizing (Cellwall-strenghtening) Antimicrobial (blocking of active sites) Hypersensitive response (HR)
Availability of conjugated double bonds (delocalized π-electrons) 	Light and UV-absorbance. Energy dessipation	Photoprotective
Availability of –NH_2, –SH, –OH  -CH=CH-CH=CH- (Unsaturated carbon chains)	H and electron transfers	Antioxidant. Antiradical

1.4 Alkaloids

Alkaloids have been of interest to chemists for nearly two centuries, and in that time thousands of alkaloids have been isolated. Extracts from the bark, root, leaves, berries, and fruits often produce nitrogen containing bases called alkaloids. These compounds are 'alkali-like", hence the name alkaloids. Hence alkaloids are mostly amines. They can react with acids to yield soluble salts (Solomons, 1996). The nitrogen atoms of most alkaloids are present in heterocyclic rings. In a few instances, however, nitrogen may be present as a primary or secondary amine or as a quaternary ammonium group. In animals, most alkaloids produce striking physiological effects that vary greatly. Some alkaloids stimulate the central nervous system; others cause paralysis; while some modulate blood pressure (Southon & Buckingham, 2003). Others act as pain relievers, and antimicrobials (Goodman & Morehouse, 1973). For instance, berberine, an important representative of the alkaloid group, is effective against Trypanosomes and plasmodia bacteria. Berberine was isolated from *Alchornea cordifora* (Omulokoli *et al*., 1997) and structure of berberine is shown on table 3. Most alkaloids are toxic at high doses, although with some this toxic dosage is very low. Despite the toxicity, many alkaloids are used in medicine. Most of these have had their structures determined through the application of chemical and physical methods, and in many instances these structures have been confirmed by independent synthesis. Compound 3 from table 3 was isolated from *Conium maculatum*, a member of the carrot family. Compound 4 from table 3 was isolated from *Erythroxylon coca* (Solomons, 1996). Both of these compounds are alkaloids well known for their effects in stimulating the central nervous system. Cocaine is a banned substance the world over.

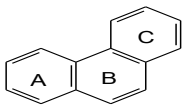
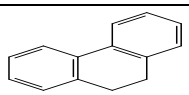
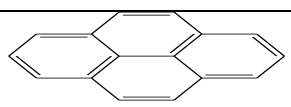
Table 3: Structure of alkaloids which have been previously isolated from different plants (Solomons, 1996; Cho, 1990).

#	Structure	Name	Reference
3		Coniine	Solomons, 1996
4		Cocaine	Solomons, 1996
5		Berberine	Cho, 1990

1.5 Stilbenes, Phenanthrenes, Terpenoids and Steroids

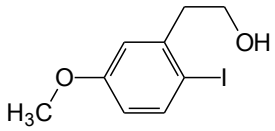
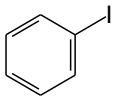
Phenanthrenes, 9, 10-dihydrophenanthrenes and pyrenes (Table 4) have been isolated from the wetland plant *Juncus acutus*, and many of these compounds were tested against *Selenastrum capricornutum,* and showed phytotoxicity in *in vitro* tests (Dellagreca *et al;* 1993). The same compounds were also isolated from *Combretum appiculum* (Letcher & Nhamo, 1971), *Combretum molle* (Letcher *et al*., 1972), and *Combretum hereroense* (Letcher & Nhamo, 1973).

Table 4: Chemical structure of phenanthrene and dihydrophenanthrene (Wendland & Lalondse, 1963; Majumder & Pal, 1992).

#	Structure	Name	References
6		Phenanthrene	Wendland & Lalondse, 1963
7		9,10-Dihydrophenanthrene	Majumder & Pal, 1992
8		Pyrene	Fetzer, 2000

The extensive use of medicinal plants by phytomedical scientists and traditional healers, who rely on plants for medicines and research material, has caused fear among botanists that the plants might be utilized to extinction. These days however, chemists and pharmacists rely on the synthesis of the drugs, they use chemical components from the plants to identify the drug that should be synthesized. For example 9,10-dihydrophenanthrenes and phenanthrenes were isolated from various plants, which are already mentioned as natural compounds but now they can be synthesized through cross-coupling of 1-(2-iodo-5-methoxy)-phenyl ethanol with variously substituted iodobenzenes (Dellagraca *et al*, 2000).

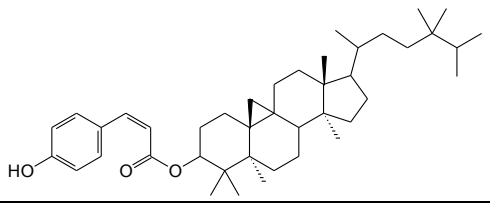
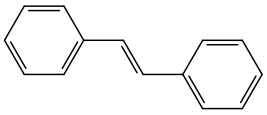
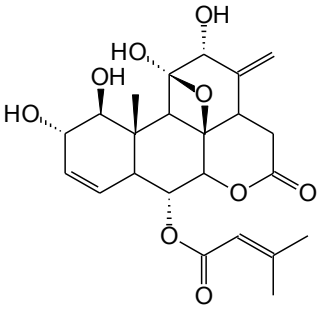
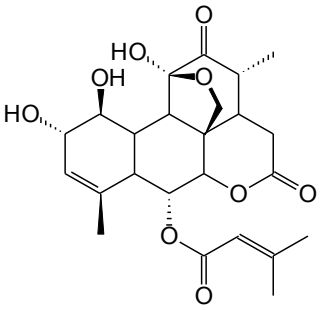
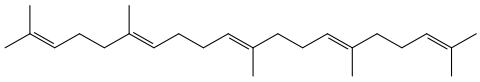
Table 5: Chemical structure of 1-(2-iodo-5-methoxy)-phenyl ethanol and iodobenzene (Hirt *et al.*, 1998; Lucas & Kennedy, 1939).

#	Structure	Name	Reference
8		1-(2-iodo-5-methoxy)-phenyl ethanol	(Hirt *et al.,* 1998)
9		Iodobenzene	(Lucas & Kennedy, 1939)

The synthetic derivatives of phenathrene, bearing a hydroxy or a methoxy group at position 2 and a methyl group in the C ring, were tested against green algae *Selenastrum capricornutum* (table 4). All compounds except 2-methoxy-7-methylphenanthrene, caused inhibition of algal growth by more than 70% at the concentration of 10^{-4} M, and many of them retained this strong activity even at the lower concentration of 10^{-5} M (Dellagraca *et al*, 2000).

Steroids are a class of fat soluble compounds with a basic 17-carbon atom structure, arranged into 4 rings (table 8) (Skoda-foldes & Takaes, 2008). According to Majumder and Pal (1990) a steroidal compound of biogenetic importance was isolated from the orchid *Coelogyne uniflora* and identified to be 24-ethyl-4α,14 α,24-trimethyl-9β,19-cyclo-cholest-25-en-3 βyl cis-*p*-hydroxycinnamate.

Table 6: Structures of compounds 10 – 15 (Majumder & Pal, 1990; *Langkilde et al*., 1994; Moretti *et al*., 1986; Rogers & Verota, 1996)

#	Structure	Name	Reference
10		24-ethyl-4α,14α,24-trimethyl-9β,19-cyclo-cholest-25-en-3-βyl-cis-*p*-hydroxycinnamate	(Majumder & Pal, 1990)
11		stilbene	(Langkilde *et al*., 1994)
12		The quassinoid 13,18-dehydro-6α-senecioyloxychaparrin	(Moretti *et al*., 1986)
13		quassinoid 12-dehydro-6α-senecioyloxychaparrin	(Moretti *et al*., 1986)
14		The triterpenoid squalene	(Rogers & Verota, 1996)

Carr and Rogers (1987) confirmed the presence of triterpenoids (compound 14, table 6) and flavanoids in the leaf extract of several *Combretum* species. More than 30 quassinoids for example (compound 12-13) Table 6 have been isolated from the seeds of *Brucea javanica* and are used in Chinese traditional medicine for the treatment of dysentery, malaria and cancer (Long-ze Lin *et al*., 1990).

Eloff *et al* (2005) found stilbene (compound 11, table 6) to have antibacterial activities and most stilbenes were isolated from the seeds of *Combretum kraussii* and *Combretum woodii.* 2, 3, 4-trihydroxyl-3, 5, 4-trimethoxybibenzyl is an example of a stilbene that was isolated from the seeds of *Combretum kraussii.* A 2,3,4-trihydroxyl-3,5,4-trimethoxybibenzyl was tested against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and against *Escherichia coli* and showed activity against *Staphylococcus aureus* and lower activity against *Escherichia coli* and *Pseudomonas aeruginosa* (Eloff *et al*., 2005). Several stilbenes with cytotoxic activity as well as acidic triterpenoids and their glycosides with molluscicidal, antifungal, antimicrobial and anti-inflammatory activity have also been isolated from species of *Combretum* (Rogers & Verotta, 1996).

Table 7: Diseases treated by traditional healers using *Combretum* species (Hutching *et al*., 1996; Gelfand *et al*.,1985; Van *et al*., 1997; Watt, 1996).

Species	**Traditionally used to cure**	**References**
Combretum appiculam	Snake and scorpion bite, bloody diarrhoea, leprosy, abdominal disorders, conjunctivitis and weak body.	Hutching *et al*., 1996
Combretum erythrophyllum	Fattening tonic for dogs, to reduce the size of the vaginal orifice.	Gelfand *et al*., 1985
Combretum hereroense	Bilharzia, headache, infertility in women.	Van *et al.,* 1997
Combretum molle	Hookworm, stomachache, snakebite, leprosy, fever, dysentery, chest complaints and as an antihemintic.	Watt, 1996
Combretum zeyheri	Toothache, cough, scorpion bite, bloody diarrhoea, arrest menstrual flow, eye lotion, embrocating and abdominal disorders.	Hutching *et al*., 1996

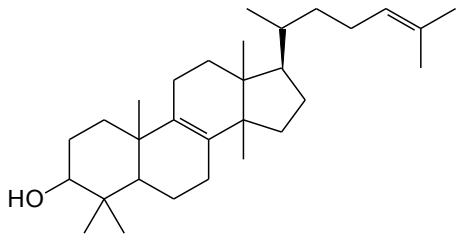
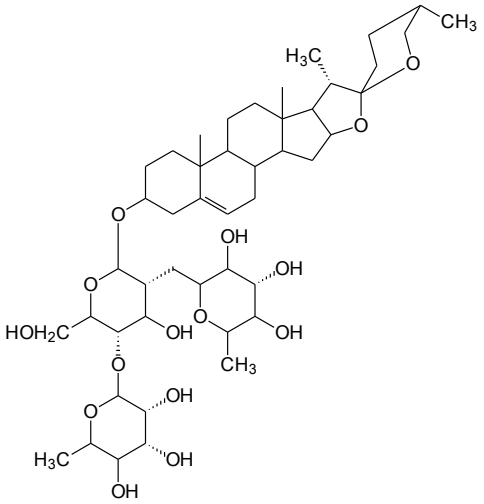
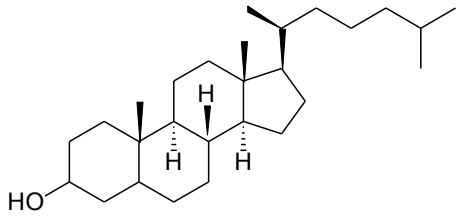
1.6 Saponins

Saponins are a class of chemical derivatives of many secondary metabolites found in many plant species. They are amphipathic glycosides characterised by soap-like foaming produced when shaken in aqueous solutions and structurally by their hydrophilic glycoside moieties combined with a lipophilic residue (Baker, 2009).

They are contained in numerous herbal remedies and are anticancer agents and ion channel-blockers (Luisella Verotta & Nadia, A.E, 2001). Saponins have a range of biological and pharmacological activities such as immunomodulation and anticancer

activities (Hostettmann& Marston., 1995). A plant glycoside saponin dioscin (compound 16, table 8) was isolated from the roots of *Polygonatum zanlanscianense* and was shown to induce apoptosis in a number of human cell lines (Liu *et al.*, 2004; Cai *et al.*, 2005).

Table 8: Structure of compounds 15 - 18.

#	Structure	Name	Reference
15		The triterpenoid lanosterol	(Jossang, 1996;Cáceres-Castillo *et al.*, 2008)
16		The saponin dioscin	(Wang et al., 2007)
18		cholesterol	(Wilczura-wachnik *et al.*, 2008)

Potter *et al* (1979) found that saponins from soya beans have the ability to lower cholesterol by forming emulsion or micelles with bile salt in the intestine. Bile salts form small micelles with cholesterol facilitating its absorption and some saponins form insoluble complexes with cholesterol resulting in inhibition of the absorption of cholesterol.

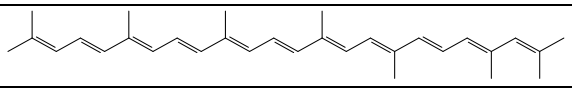
Colon cancer is known to display a histologic range from normal colonic mucosa through increasingly forceful adenomas to envelop carcinoma (Kinzler & Vogelstein,1996). Even though hyperplasia is observed through carcinoma diseases in the breast cancer, the pattern of molecular change and biological behavior suggests a more complex etiology than colon cancer. Specific genetic unsteadiness is seen in some breast laceration at early stages (Dairkee & Smith, 1996).

Fresh garlic has antioxidants (compounds 24-26; Table 9 & 10) that can protect against cardiovascular diseases reducing risk factors for heart attacks, stroke and blood pressure (Borek, 2001). It also increases circulation in capillaries and prevent clot-forming platelet activity and inflammation. The antioxidant compounds from fresh garlic have anti-cancer activity that protects against free radical and carcinogen induced DNA damage and increases carcinogen detoxification and also prevents the growth of a wide variety of human cancer cells including breast; colon and prostate colon cancer (Pinto *et al.*, 2000).

Table 9: Antioxidant and anticancer compounds from vegetables and fruits (Cancerproject.org, 2009)

Colours	**Foods**	**Protective substances**	**Structures**	**Possible action**
Red	Tomatoes and tomato product as well as water melon and guava	Lycopene	19	Antioxidant and also cuts prostate cancer risk
Orange	Carrots, yams, sweet potatos, mangos and pumpkins	Beta-carotene	20	Support immune system and has power antioxidant
Green	Spinach, kale, collard and other greens	Folate	21	Builds healthy cells and genetic material
Yellow-orange	Orange, lemons, grapefruits, papayas and peaches	Vitamin C and flavonoids	22	Tumor cell growth inhibitor and detoxify harmful substances
Green-white	Broccoli, brussels sprout, cabbage and cauliflower	Indoles	23	Eliminate excess estrogen and carcinogens
White-green	Garlic, onions,chives and asparagus	Allyl sulfides	24 - 26	Destroy cancer cells
Blue	Blueberries, purplegrapes, plums	Anthocyanins	27	Destroy free radicals
Red-purple	Grapes, berries, plums	Reservatrol	28	May decrease estrogen production

Table 10: The structures of compounds that have antioxidant and anticancer activity

#	Structure	Name	References
19		Lycopene	Rozzi *et al.,* 2002

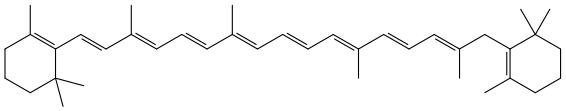
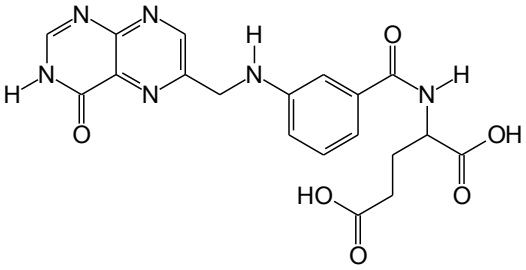
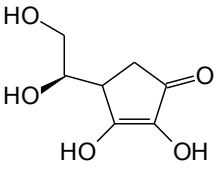
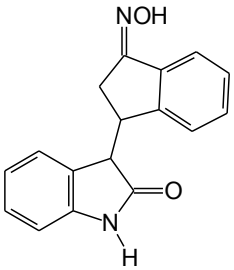
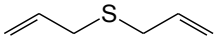
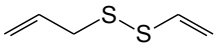
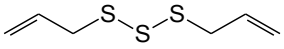
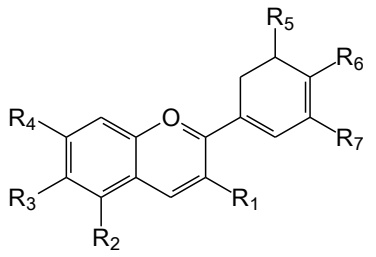
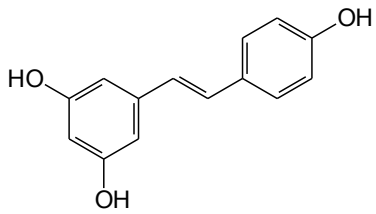
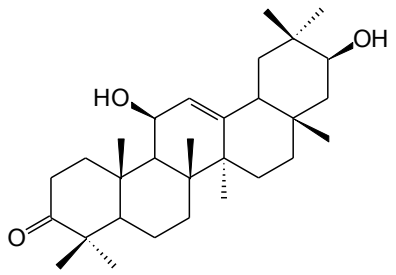
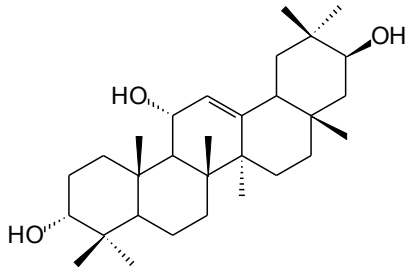
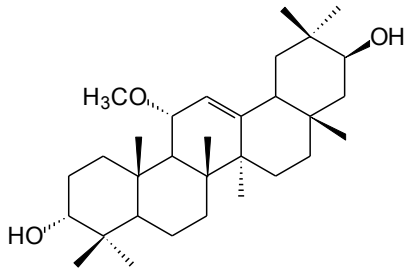
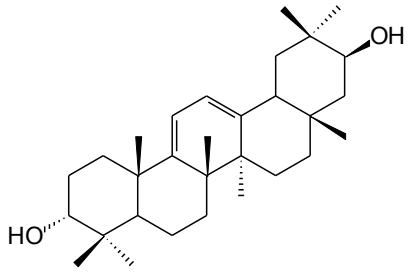
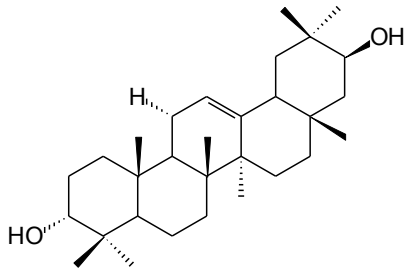
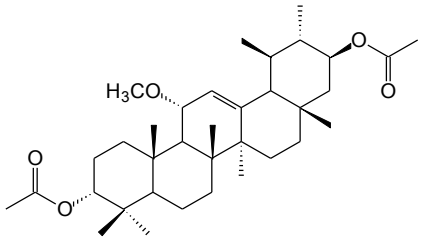
20		β-carotene	Karrier et al., 1930
21		Folate	He, Y.Y *et al.*, 2008
22		L-ascorbic acid	Bender, 2003
23		Indirubin-3`-monoxime (Indole derivatives)	Wang et al., 2009
24		Diallyl sulfide (DAS)	Koizumi, K et al., 2009
25		Diallyl disulfide(DADS)	Koizumi, K et al., 2009
26		Diallyl trisulfide (DATS)	Koizumi, K et al., 2009

Table 11: The structures of anthocyanins and reservatrol compounds (Castanẽda-ovando et al., 2009; Bishayee *etal.*, 2009)

#	Structure	Name	References
27		General structure of anthocyanines. Where R_1 to R_7 can be either H or OH and different anthocyanine derivatives is obtained depending in the position of H or OH	Castanẽda-ovando *et al.*, 2009
28		Trans-resveratrol	Bishayee *etal.*, 2009

Epidemiology and clinical laboratory studies have shown that crushed garlic and their active compounds such as diallyl sulfide (DAS) and diallyl disulfide (DADS) as well as diallyl trisulfide (compounds 24-26; Table 10) give biological activities including antitumorigenesis, antiatherosclerosis and also blood sugar modulation and antibiotic activity (Milner, 2001; Siege *et al,* 2004).

Table 12: Saponins isolated from extracts of the dried bark of *Hippocratea excels* (Cáceres-Castillo *et al.*, 2008)

#	Structure	Name	References
29		11-β, 21-β-dihydroxy-olean-12-ene-3-one	Cáceres-Castillo *et al.*, 2008
30		3α,11α,21-β-trihydroxy-olean-12-ene	Cáceres-Castillo *et al.*, 2008
31		3α,21β-dihydroxy-11α-methoxy-olean-12-ene	Cáceres-Castillo *et al.*, 2008
32		3α,21β-dihydroxy-olean-9(11)-1,2-diene	Cáceres-Castillo *et al.*, 2008
33		3α,21β-dihydroxy-olean-12-ene	Cáceres-Castillo *et al.*, 2008
34		3α,21β-dihydroxy-11- α-methoxy-urs-12-ene	Cáceres-Castillo *et al.*, 2008

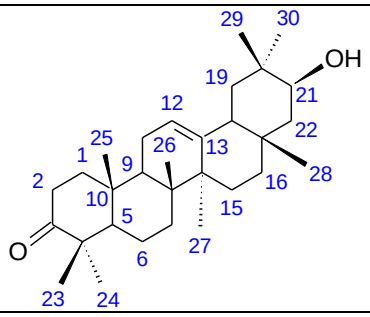
35		11-β, 21-β-dihydroxy-olean-12-ene-3-one	Cáceres-Castillo *et al.*, 2008

Table 13: ^{1}HNMR (400 MHz) spectroscopic data for compounds (33-34) in $CDCl_3$ (Cáceres-Castillo *et al.*, 2008).

H	29[a]	30	31	32	33	34	35
1	1.41 *m*, 1.90 *m*	1.65, 1.85 *m*	1.60, 1.77 *m*	1.54, 1.67 *m*	1.60, 1.80 *m*	1.90, 1.95 *m*	1.53, 1.66 *m*
2	2.36 *ddd* (16, 6.8, 3.7)	2.40, 2.55 *m*	1.60, 1.98 *m*	1.53, 1.97 *m*	1.66, 2.00 *m*	1.52, 1.95 *m*	1.58, 1.90 *m*
	2.55 *ddd* (16, 11, 7.3)						
3			3.42 *brs*	3.40 *brs*	3.42 *brs*	3.41 *t* (2.4)	4.61 *brs*
5	1.33 *m*	1.37 *m*	1.30 *m*	1.27 *m*	1.32 *m*	1.32 *m*	1.27 *m*
6	1.48, 1.55 *m*	1.05, 1.52 *m*	1.40, 1.47 *m*	1.40, 1.47 *m*	~1.52 *m*	1.40, 1.47 *m*	1.40, 1.47 *m*
7	1.35, 1.52 *m*	1.34, 1.52 *m*	1.27, 1.53 *m*	1.25, 1.53 *m*	1.36, 1.70 *m*	1.30, 1.53 *m*	1.30, 1.56 *m*
9	1.65 *dd* (11.5, 6.3)	1.96 *m*	1.68 *m*	1.83 *m*		1.69 *m*	1.78 *m*
11	1.88, 1.97 *m*	4.54 *dd* (9.6, 3.2)	4.21 *dd* (8.2, 3.8)	3.88 *dd* (9, 3.5)	5.61 *d* (5.8)	1.86, 1.87 *m*	3.77 *dd* (8.8, 3.2)
12	5.24 *t* (3.5)	5.50 *d* (4)	5.28 *d* (3.4)	5.37 *d* (3.4)	5.54 *d* (5.8)	5.21 *t* (3.6)	5.43 *d* (3.6)
15	1.00, 1.76 *m*	1.05, 1.69 *m*	0.98, 1.68 *m*	0.97, 1.68 *m*	1.03, 1.85 *m*	0.95, 1.12 *m*	1.02, 1.74 *m*
16	1.00, 1.96 *m*	1.04, 1.96 *m*	1.03, 1.96 *m*	1.00, 1.96 *m*	1.06, 1.95 *m*	1.53, 1.93 *m*	1.15, 2.00 *m*
18	2.00 *dd* (14.8, 3.5)	2.10 *dd* (13.6, 3.6)	2.02 *m*	2.04 *m*	2.18 *m*	2.00 *m*	1.44 *m*
19	1.15, 1.75 *m*	1.22. 1.77 *m*	1.21, 1.75 *m*	1.22, 1.76 *m*	1.20, 1.75 *m*	1.14, 1.76 *m*	1.55 *m*
20							1.12 *m*
21	3.53 *dd* (12, 4.7)	3.52 *dd* (12, 4.8)	3.53 *dd* (11.9, 4.6)	3.53 *dd* (12, 4.6)	3.53 *dd* (12, 4.5)	3.53 *dd* (12.0, 4.8)	4.73 *td* (11.2, 3.8)
22	1.37, 1.50 *m*	1.36, 1.52 *m*	1.37, 1.50 *m*	1.40, 1.50 *m*	1.42, 1.55 *m*	1.35, 1.46 *m*	1.28, 1.80 *m*
23	1.10 *s*	1.09 *s*	0.97 *s*	0.97 *s*	1.00 *s*	0.96 *s*	0.92 *s*
24	1.06 *s*	1.05 *s*	0.87 *s*	0.87 *s*	0.87 *s*	0.85 *s*	0.86 *s*
25	1.07 *s*	1.15 *s*	1.08 *s*	1.06 *s*	1.20 *s*	0.94 *s*	1.09 *s*
26	1.01 *s*	1.05 *s*	0.99 *s*	0.99 *s*	1.12 *s*	0.94 *s*	1.03 *s*
27	1.14 *s*	1.21 *s*	1.23 *s*	1.22 *s*	1.01 *s*	1.13 *s*	1.18 *s*
28	0.88 *s*	0.87 *s*	0.87 *s*	0.87 *s*	0.93 *s*	0.86 *s*	0.86 *s*
29	0.97 *s*	0.97 *s*	0.98 *s*	0.97 *s*	0.95 *s*	0.96 *s*	0.93 *d* (6)
30	0.86 *s*	0.87 *s*	0.87 *s*	0.88 *s*	0.89 *s*	0.86 *s*	0.96 *d* (6)

The positions 1-30 are shown in structure 35 in table 13.

1.7 Combretaceae

The family Combretaceae consists of 18 genera with the largest genus, *Combretum* composed of about 370 species whereas *Terminalia* has about 200 species (Lawrence, 1951). *Terminalia* are commonly and widely distributed throughout western and southern Africa (Rogers and Verotta, 1996). The Combretaceae which has the greatest variations with as many as 11 subspecies in the taxon presents a major taxonomic challenge (Okafor, 1967). According to Carr (1988) only three subspecies are currently recognized in South Africa namely *gazense*, *suluense*, and *taborense*.

1.7.1 *Combretum collinum* subspecie *suluense*

Figure 1: A fresh twig of *Combretum collinum* ssp *suluense* (Neilspruit National botanical garden)

Traditional healers throughout southern Africa employ species of Combretaceae and use them for many medicinal purposes, which include the treatment of abdominal pains, backache, chest coughs, colds, conjunctivitis, diarrhoea, dysmenorrheal, earache, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion and snake bite, swelling caused by mumps, syphilis and general weakness (Watt & Breyer-Brandwijk, 1962). According to Njoroge and Bussmann (2006), Traditional healers use *Combretum collinum* to manage throat and nose diseases in central Kenya.

According to Katende (1995) *Combretum collinum* subspecies *suluense* is a medium-sized, thickly leafy deciduous tree, about 6-15 m high, is found mostly in Bushveld on granite. It has a stem or multi-stem which is rough, the bark is pale and grayish-brown, and young branches are covered with brown hairs (Neilspruit National botanical garden).

Schmidt *et al* (2002) reported that leaves are opposite and often elliptic to obovate (70–250 x 30-100 mm), they are hairless except in the pockets below the axils of the vein and along the midrib. New leaves are hairy and densely covered in scales. Below the leaf, there is a petiole which is 25 mm long.

Figure 2: Trunk of *Combretum collinum* ssp *suluense* (Neilspruit National botanical garden)

Flowers are small, yellow-white, borne on spikes and 50-80 mm long from axils of leaves and they grow during August to November. Its fruit is 4-winged about 35-45 x 30-50 mm large, light to dark reddish-brown. The wings are grayish or plum-coloured and covered in hairs and borne in hanging clusters from January to April (Schmidt et al., 2002). Fruit can be stored for only a short period, but seed storage practice is orthodox, and they can be stored up to 3 years (Hong *et al.*, 1996).

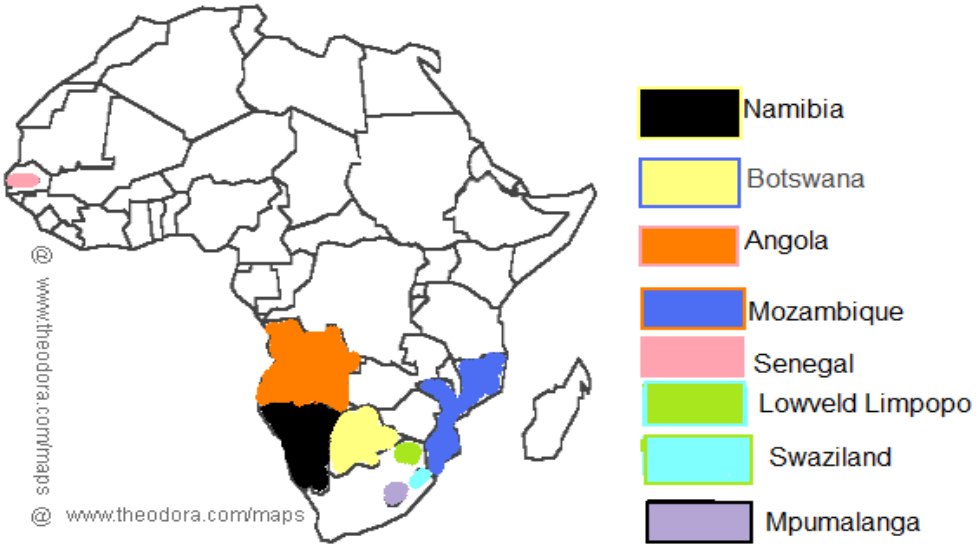


Figure 3: A map of the distribution of *Combretum collinum* (Theodora.maps, 2009).

The latin word *collinus* meaning growing on a hill (Katende, 1995). *Combretum collinum* subspecie *suluense* is distributed tropically, from South Africa to Namibia, Botswana, Zimbabwe, Mozambique, Lowveld of Limpopo Province, Mpumalanga, Swaziland and into northern Kwazulu-Natal (Katende, 1995).

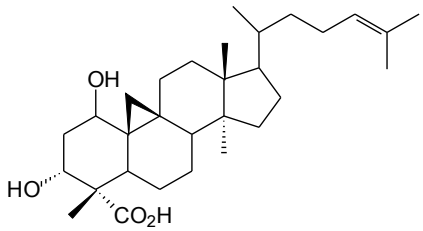
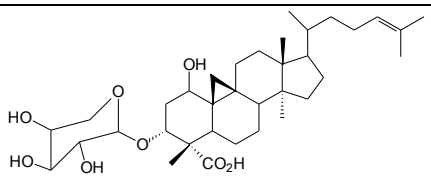
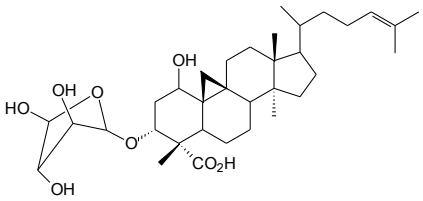
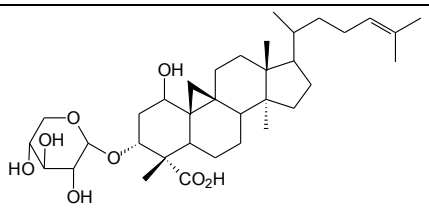
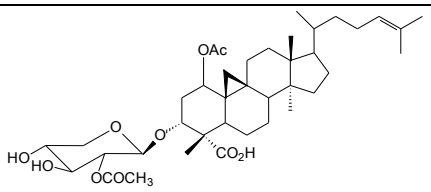
According to Storrs (1995), *Combretum collinum* occurs at medium to low altitudes in open woodlands; it is found throughout Zambia and is locally a common tree in Baikiaea forest margins, deciduous thickets, and Kalahari and Lake Basin Chipya woodland. It is occasional on anthills, in Miombo woodlands, and in the dry, evergreen forests, outside Zambia, it is widespread, extending from Senegal to east Africa and south to Natal and also westwards to Angola. They are dominant wherever they occur.

According to Hedberg *et al* (1982), *Combretum collinum* has various medicinal uses in African traditional medicine; the roots are used together with bark and roots of *Kigelia Africana* for the treatment of excessive menstrual bleeding. The hot water extract from the same plant is used for the treatment of diarrhoea and anal bleeding. Haerdi (1964) found that Traditional healers prescribed the hot water extract from leaves and roots to the patient to drink, as the treatment of malaria. Investigation by Kokwaro (1976) showed that patients who suffer from snakebites chew the roots of *Combretum collinum* as treatment. Adjanouhoun *et al* (1986) reported that three spoons of decoction vapour of leaves are used for the treatment of malaria; however no antimicrobial activity has been reported so far. Some compounds from *Combretum* species have shown anti-inflammatory, antihelmintic, antibilharzia and anti-DNA damaging. Significant activity in more than one bioassay was exhibited by *Combretum apiculatum*, *Combretum hereroense*, *Combretum molle* and *Combretum mossambicense* (MacGaw *et al*., 2001).

Combretum collinum is one of the trees that produce a mean charcoal kiln efficiency of 23%. Most of the industrial wood energy is consumed by small-scale industries which include food processing industries and service sectors such as brewing, fish smoking, salt production, baking, restaurants, schools, hospitals, vending, agro-processing industries such as tobacco curing, tea drying and bees wax processing and production of building materials such as burnt bricks, lime, pottery and ceramics. These industry and domestic activities which rely upon wood energy

provide employment and income for rural people particularly during off-season in agricultural production (Monela & Kihiyo., 1998).

Table 14: Some chemical compounds isolated from Combretum species (Pegel & Rogers, 1985).

#	Structure	Name	References
36		1α-hydroxycycloartenoid (mollic acid)	(Pegel & Rogers, 1985).
37		3β-D-Xyloside	(Pegel & Rogers, 1985).
38		α-arabinoside	(Pegel & Rogers, 1985).
39		glucoside	(Pegel & Rogers, 1985).
40		β-D-xyloside tetraacetate	(Pegel & Rogers, 1985).

The cycloartenoid 30-carboxy-1α-hydroxycycloartenol (Mollic acid) and its 3β-o-arabinoside, glucoside, xyloside derivatives have been isolated from *Combretum molle*, *Combretum moggii*, *Combretum edwardsii* and the South American species *Combretum leprosum* (Pegel *et al.,* 1985; *Rogers et al.,*1986).

These were secreted from the epidermal trichomes which were present in all species of the subgenus *Combretum* (Stace, 1969). The triterpenoid and trichome have been found to be species specific (Carr *et al*., 1987; Stace, 1969). Recent evidence suggests that there is a bifurcation in triterpenoid biosynthesis within the genus

Combretum with species producing compounds with a 30-carboxy-1α-hydroxycycloartane skeleton (Rogers *et al*., 1996). So far, no compounds from *Combretum collinum* sub species *suluense* have been reported.

1.7.2 *Combretum bracteosum*

Figure 4: Leaves and flowers of *Combretum bracteosum* (Pooley, 1993)

The common names of *Combretum bracteosum* are hiccough-nut, hiccupnut, hiccough creeper in English and in Xhosa it is called uQotha (Coates-Palgrave, 2002). It is usually a small tree; it grows up to 2 to 4 m high. It depends on other trees for support; it spreads to 4 and 5 m wide. The shrub is multi-stemmed. The leaves are simple and opposite. The side veins of the leaves do not reach the margin, leaves are green in colour, but in autumn the colour changes to reddish purple (Carr, 1988).

Aubrey (2004) reported that *Combretum bracteosum* is found naturally along the coast in Kwazulu-Natal and Eastern Cape where it grows in sand close to the sea. It favours hill forest and sandbank forest. It is commonly found inland, away from the sea, usually at low altitude growing on sand. It does not grow in icy or frost places and it tends to grow in high annual rainfall areas.

According to Watt and Breyer-Brandwijk (1932), the roasted nuts are eaten, but there is a debate, some researchers say it cures hiccups and others say it causes the above mentioned disease.

1.7.3 *Combretum apiculatum*

Figure 5: A branch of *Combretum apiculatum* (Hyde & Wursten, 2009)

Wickens (1973) found that the bark of *Combretum apiculum* is grey to greyish black, smooth or reticulated and leaf buds are black or dark brown. The leaves are opposite and the lamina is sticky when young, however broadly to narrowly obovate-elliptic or oblong-elliptic or ovate to subcircular, 3-14 cm long and 1.5-7.5 cm wide.

According to Wickens (1973), Van Wyk and Van Wyk (1997) it occurs on granitic, rhyolitic and basaltic soils in Miombo woodland, wooded grassland and in Acacia-Commiphora bushland, and is also found on rocky hill slopes, mostly on well-drained soils.

Traditional healers use the hot water extracts from roots for the treatment of mental illness. They give the patient the extract to drink and also they can bend the root and give the patient to wear in the form of necklace as a cure of the same disease instead of using the extract (Chhabra *et al*., 1989). According to Kokwaro (1976), the roots are chewed and the sap is swallowed for the treatment of snakebite and the water extract from roots is drunk for the treatment of scorpion sting, whereas root decoctions are used for the treatment of leprosy and bloody diarrhoea. The leaves are used for abdominal disorders and the stem bark is used for the treatment of conjunctivitis (Watt & Breyer-Brandwijk, 1962).

1.7.4 *Combretum zeyheri*

Figure 6: Small branches and leaves of *Combretum zeyheri* (Dubinovsky & Tatiana, 2003)

This is a slender, deciduous tree reaching heights of 10-13 m, or rarely a shrub. The bark is brown or grayish-brown. The leaves are arranged oppositely on broadly elliptic to obovate elliptic. The leaf apex is usually rounded to obtuse. The leaves are tomentose-pubescent. Inflorescences are unbranched axillary spikes, up to 8 cm long. The flowers are greenish-yellow. The large fruit is sub circular to elliptic, 4-winged, usually 6.5 cm long and 5.5-6 cm wide. It is wide, straw coloured to light brown. The apical peg is very short or does not appear. The wings of the fruit are up to 4 cm wide (Drummond & Coates-Palgrave, 1973).

According to Drummond and Coates-Palgrave (1973) *Combretum zeyheri* occurs from Congo and Tanzania, South West Africa, Botswana, Transvaal to Natal. It occurs in woodland, wooded grassland and *Acacia-Commiphora* bushland and it prefers sandy soils, and also on termite mounds, and tolerant to high metal soil.

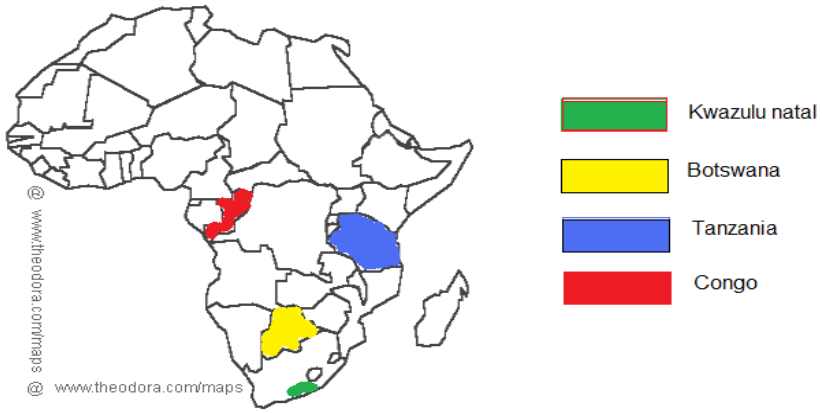


Figure 7: Distribution map of *Combretum zeyheri* (CIA, 2009)

Drummond and Coates-Palgrave (1973) reported that leaves of *Combretum zeyheri* are burnt and its smoke is inhaled for the treatment of coughs. The water extract of powdered leaves is drunk for the treatment Colic, and also mixed with crushed melon seed for the treatment of rheumatism and joint pain. Decoctions of finely ground leaves are used as the treatment of eye inflammation and conjunctivitis (Kremniz *et al*., 1988). According to Kokwaro (1976), Hedberg *et al* (1982), Gelfland *et al* (1985) and Neuwinger (2000) leaves are powdered and mixed with oil; the mixture is applied on the area that has pain for the treatment of back pain. Moreover the hot water extract from *Combretum zeyheri* is mixed with porridge to treat diarrhoea, dysentery, and vomiting. According to Watt and Breyer-Brandwijk (1962), powdered stem bark is placed in the vagina to arrest menstrual flow.

1.8 *Calpurnia aurea*

Calpurnia aurea is a small slender evergreen tree (2-4 m tall) belonging to the family Fabaceae (Germishuizen & Meyer, 2003). According to the same author, the leaves are compound, up to 20 cm long, each having 5-15 pairs of leaflets and a terminal leaflet. *Calpurnia aurea* is one of the smaller genera of approximately 16 species, eight of which occur in Southern Africa where they are found in the eastern and north – eastern parts of the country.

Figure 8: A small *Calpurnia aurea* leaf (Germishuizen and Meyer, 2003).

Figure 9: A *Calpurnia aurea* tree (Germishuizen and Meyer, 2003).

Calpurnia is easily raised from seed sown in spring or early summer. Soaking in hand-hot water and treating with a fungicide that combats pre- and post-emergence damping, although not essential for germination, will increase the number of successful seedlings. Seed germinates in 10-14 days. They must be pricked out soon after the first pair of true leaves have developed, taking care not to damage the taproot (Leisner, 2000). The flowers of *Calpurnia aurea* are bright yellow, each about 2.5 cm long, in showy hanging bunches of 8 to 30 flowers. In summer rainfall areas the peak flowering period is mid-late summer (Dec-Feb), but in the winter-rainfall Western Cape, particularly during hot dry summers, they don't flower well in summer but start up again in the autumn. The fruit is a thin pod

drying light brown with a papery texture, 5-12 cm long and 0.8-1.9 cm wide, narrowly winged on one side containing up to 8 brownish seeds (Coates, 2002).

Figure 10: A fruit of *Calpurnia aurea* (Germishuizen and Meyer, 2003).

Figure 11: Flowers of *Calpurnia aurea* (Germishuizen and Meyer, 2003).

Calpurnia aurea is the most widespread of the genus. It is found growing in the forest, on forest margins and clearings, on hillsides or on the edge of woody kloofs and in the bushveld. In the forest it can be a 9 to 15m tree while in the open it is more often a shrub or small tree 2-4m tall. In southern Africa it occurs from the Eastern Cape through Kwazulu-Natal and Swaziland to Mpumalanga, Gauteng and Limpopo. It also occurs northwards into tropical Africa as far as Ethiopia (Palmer and Pitman, 1972).

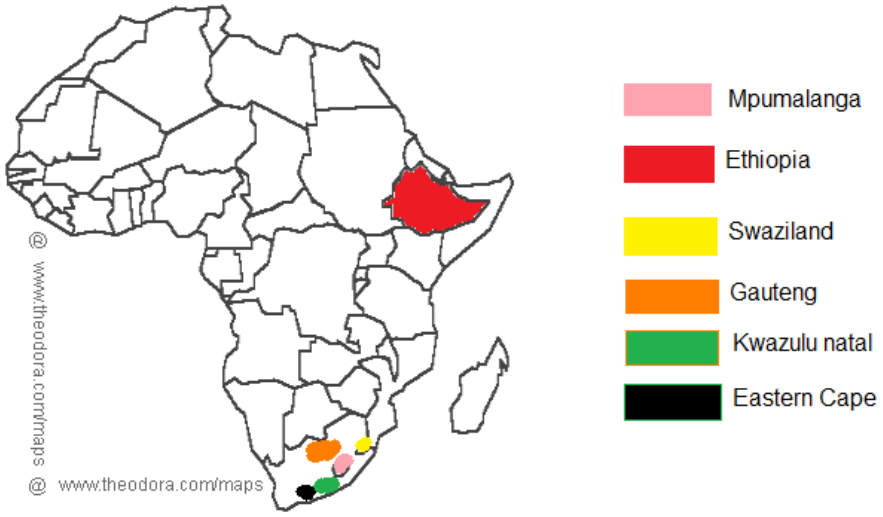
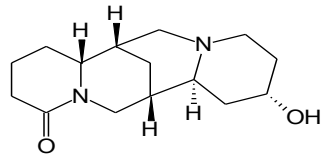
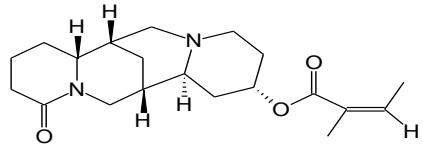
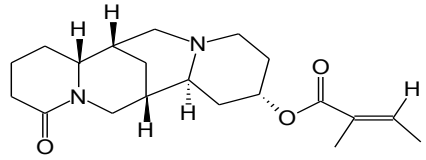
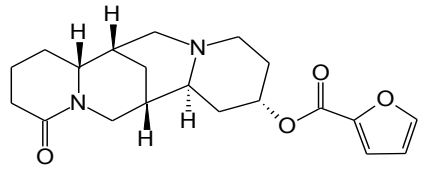
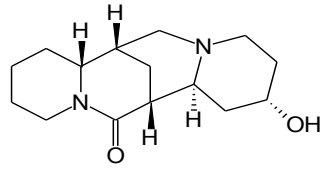
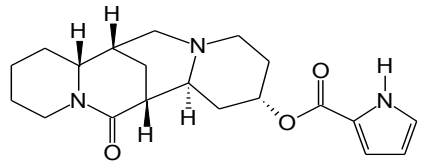
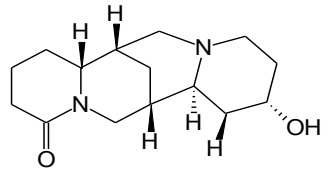
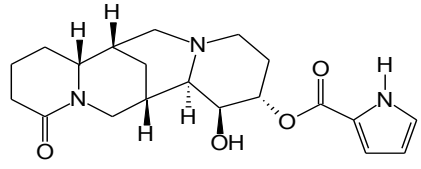
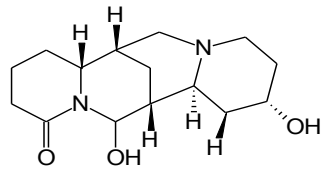


Figure 12: Distribution map of *Calpurnia aurea* (CIA, 2009)

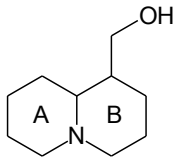
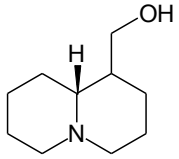
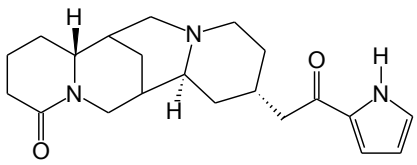
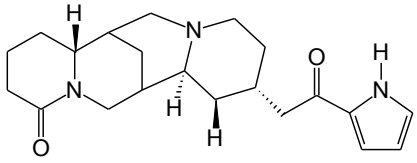
Calpurnia leaves are used to treat allergic rashes (Asres *et al.*, 1989). In East Africa, the leaf sap is used to destroy maggots in wounds (Abebe, 2001). This plant is fast growing and it does best in fertile, well – drained soil with plenty of water in summer, although it is tolerant of summer drought (Coates, 2002). In South Africa, *Calpurnia* leaves and powdered roots are used to destroy lice and to relieve itches. Unspecified parts are used to destroy maggots and the leaves are used to treat allergic rashes, particularly those caused by caterpillars (Hutchings, 1996). In Nigeria, the seeds are used to treat abscesses. In Ethiopia it is used to treat stomach complaints, headache, eye disease, amoebic dysentery, scabies and as an insecticide (Asres *et al.*, 2001). Many compounds from *Calpurnia areus* have been reported and have been tested against bacteria and fungi (Asres *et al.*, 1989). Compounds 41, 44, 45, and 46 (Table 5) has been isolated from Ethiopian *Calpurnia aurea* (Asres et al., 1986).

Table 15: Compounds previously isolated from *Calpurnia aurea*

#	Structure	Name	References
41		13-hydroxylupanine	(Asres et al., 1986)
42		Angelate	(Asres et al., 1986)
43		Tiglate	(Asres et al., 1986)
44		-Pyrrolecarboxylic acid ester (Calpurmine)	(Asres et al., 1986)
45		Virgilline	(Asres et al., 1986)
46		Virgilline pyrroledecarboxylic acid ester	(Asres et al., 1986)
47		12β,13α-dihydroxylupanine (Calpurminine)	(Asres et al., 1986)
48		13α-Pyrrolecarboxylic acid ester	(Asres et al., 1986)
49		10,13-dihydroxylupanine	(Asres et al., 1986)

The structures of these alkaloids were determined by chemical transformation and spectroscopic techniques including UV, CD, MS, H-NMR, ^{13}C-NMR and 2-dimensional NMR (Asres, 1986). The chemical components of a close relative *Calpurnia sylvatica* have been isolated. These include Lupinine, Epilupinine, Calpurnenine and Calpurmenine pyrrolecarboxylic acid ester(compound 50-53; Table 16) (Asres *et al*.,1989). The other species are *Calpurnia capensis, Calpurnia glabrata, Calpurnia floribunda, Calpurnia intrusa*, *Calpurnia reflexa, Calpurnia sericea and Calpurnia woodii* (Leistner, 2000).

Table 16: Compounds previously isolated from *Calpurnia syvatica* (Asres et al., 1989)

#	Structure	Name	References
50		Lupinine	Koziol *et al*., 1978
51		Epilupinine	Sparatore *et al*., 2005
52		Calpurnine	Cordell, 1995
53		Calpurmenine pyrrolecarboxylic acid ester	Cordell, 1995

1.9 *Ficus ingens*

Burkill (1985) reported that *Ficus ingens* is from Moraceae family. It grows up to 13 m tall, with a short bole, spreading crown and aerial roots.

Figure 13: Branch and fruit of *Ficus ingens* (Jordaan, 2005)

Ficus ingens is found in savanna, in rocky places from Senegal to north and south Nigeria, and in drier parts of tropical Africa and South Africa (Burkill, 1985). *Ficus ingens* is used to treat fever, gastrointestinal problems (Grosvenor *et al.*,1995). According to Burkill (1985) barks, leaves, and roots of *Ficus ingens* are used by Traditional healers to treat diarrhoea, dysentery, diuretics and kidney problems.

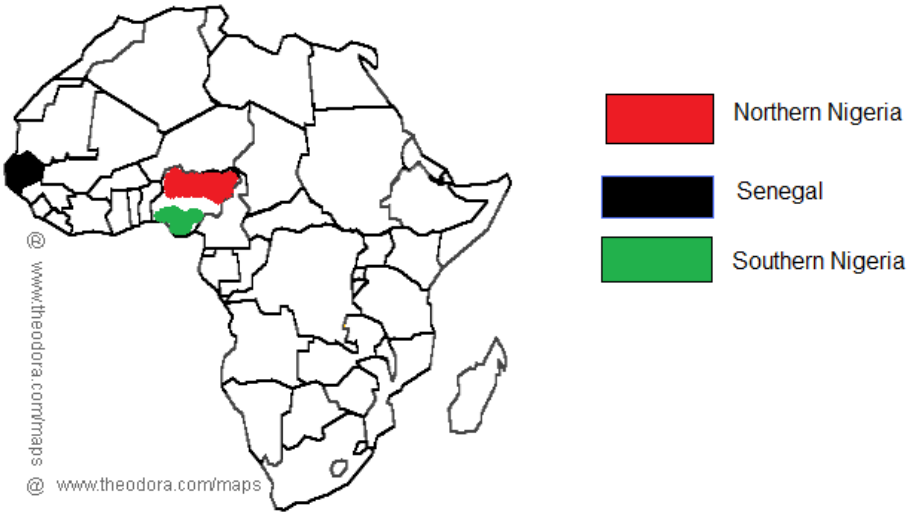
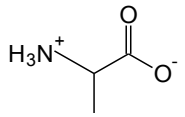
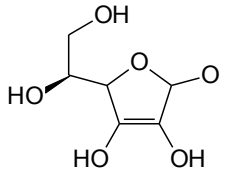
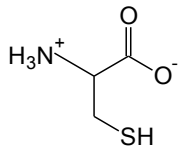
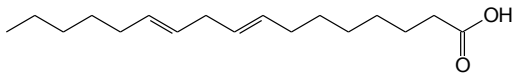
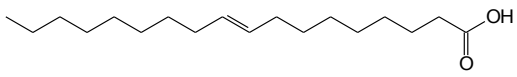
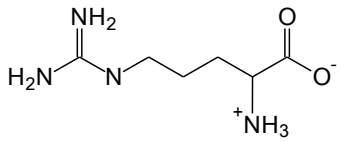
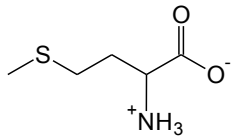
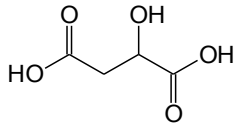
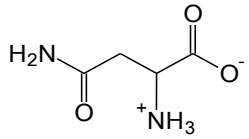
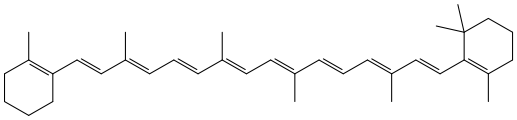


Figure 14: Distribution map of *Ficus ingens* (CIA, 2009)

Chemical components isolated from *Ficus moraceae* are alanine, asparatine, arginine, cystine, beta-carotene, ascorbic acid, lioleic-acid, niacin, methionine, malic-acid, and oleic-acid (Berg & Corner, 2005) and their structures are shown in table 17.

Table 17: Structure of chemical compounds isolated from *Ficus moraceae*

#	Structure	Name	References
54		alanine	(Brady & Senese, 2004)
55		L-ascorbic acid	(Bender, 2003)
56		cysteine	(Brady & Senese, 2004)
57		Linoleic acid	(Brady & Senese, 2004)
58		Oleic acid	(Brady & Senese, 2004)
59		L-arginine	(Helmenstine, 2001)
60		L-methionine	(Helmenstine, 2001)
61		Malic-acid	(Dobbin, 1931)
62		L-asparagine	(Helmenstine, 2001)
63		βeta-Carotene	(Karrier et al., 1930)

1.10 ***Filicium decipiens*** (Fern tree)

Filicium decipiens belongs to Sapindaceae family, it grows up to 6-9 m. Flowers are white or near white in colour. It flowers mid spring, late spring or early summer and

leaves or foliage are evergreen, It prefers a soil with ₚH from 8,6 to 9,0 which is strongly alkaline (Godfrey et al, 1981). *Filicium decipiens* are found in tropical lowland to submontane environments, as well as subtropical forests (Tryon & Gastony, 1975; Moran, 1995a). *Filicium decipiens* have antioxidant activities (Lo *et al*, 2004)

Figure 15: Fern tree (Van den bos, 2004)

1.11 *Adina microcephala* var*. galpinii*

Adina microcephala is from family Rubiciaceae and it grows up to 15 – 50 feet high. It has grey scaly bark, with shiny green leaves and yellow flowers (Coates-Palgrave, 1957). *Adina microcephala* is found in Angola, Cameroon, Kenya, Mozambique, Sudan and Mtoko in Southern Zimbabwe, mostly near streams in savanna forest (Coates, 1957).

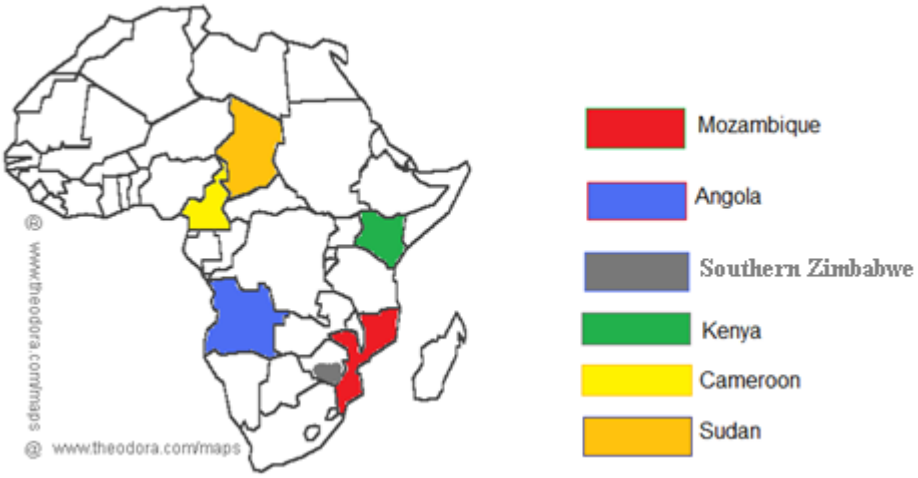
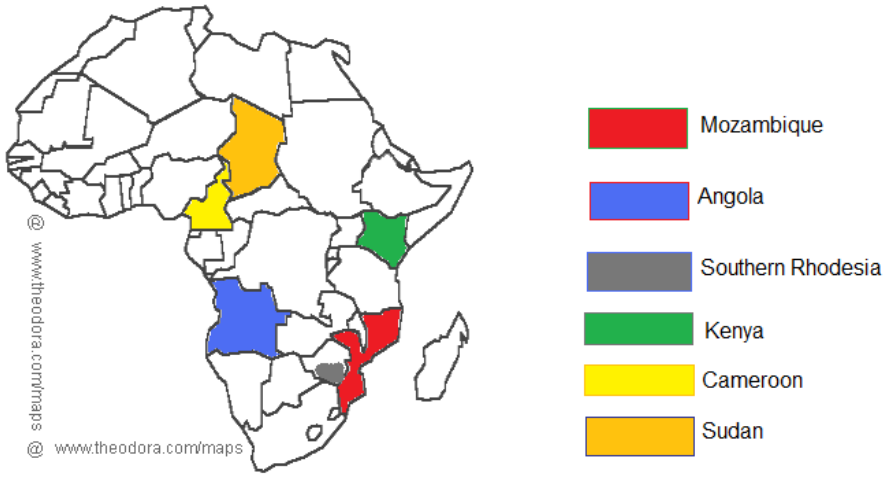


Figure 16: Distribution of *A. microcephaha* (CIA, 2009)

Figure 17: A painted *Adina microcephala* (Coate, 1957)

1.12 Introduction of bacteria used.

Staphylococcus aureus is one of the most commonly identified pathogens in human medicine and is a major cause of nosocomial infections (Boyce *et al.*, 1983) and community-acquired infections (Naimi et al., 2003). Enterotoxigenic *Escherichia coli* (ETEC) has been reported as the major cause of diarrheoa in humans throughout the world (Sack, 1975), especially in developing countries (Black *et al.*, 1984). It is a cause of morbidity and mortality in children up to 5 years of age in developing countries (Black, 1993).



Baille *et al* (2004) *Escherichia coli* can generally cause several intestinal and extra intestinal infections such as urinary tract infection, meningitis, peritonitis, mastitis, septicemia and pneumonia, it is often the causative agent of Traveler's diarrhoea, and the primary source of infection is by ingestion of fecally contaminated food or water. Certain strains of *Staphylococcus aureus* are also the causative agent for septicemia infections, pneumonia, meningitis, endocardis, and food poisoning occurs after eating food containing toxins produced by the organism.

Campylobacter jejuni is a gram-negative bacteria; the name of the illness caused by *campylobacter jejuni* is Campylobateriosis. The infection can cause diarrhoea, fever, abdominal pain, nausea, headache and muscle pain. The illness usually occurs 2-5 days after ingestion of the contaminated food or water. *Campylobacter jejuni* contaminates raw chicken, raw milk and non-chlorinated water (Baille *et al*., 2004).

1.13 Isolation of compounds with preparative thin-layer chromatography.

The invention of chromatography can be traced to the milestone report published in 1906 by the Russian botanist and plant physiologist Mikhali Semyonovitch Tswett; he managed to separate chloroplast pigments from leaves in a column of precipitated chalk washed with carbon disulfide mobile phase. During the 20th century and in the new millennium, chromatography has become an indispensable separation tool that is very widely used in natural and life science laboratories throughout the world. Tswett`s column liquid chromatography method was developed and applied in two modes, liquid–solid adsorption and liquid–liquid partition (Kowalska & Shema, 2006). Adsorption chromatography based on partition with mechanically coated stationary phase has become the most important liquid chromatographic method and this remains true today in thin layer chromatography, for which silica gel as chemically bonded stationary phases is the most popular. Preparative thin layer chromatography (PTLC) was apparently reported by Ritter and Meyer in 1962. They used layers of 1-mm thickness. Earlier preparative work, reported by Miller *et al*

(1963) the inventors of TLC is performed today by development in a closed tank (Kowalska & Shema, 2006).

PTLC may be defined as the TLC of relatively large amounts of material used in order to prepare and isolate quantities of separated compounds for further investigations such as chemical derivatization, structure elucidation and chromatographic standards or biological activity determination. Preparative layer chromatography is an excellent method for cleaning up synthetic reactions with mixtures (Miller *et al*, 1962), for natural products, plant extracts, and biotechnological products. The classical preparative layer plate involves migration of a mobile phase by capillary action through 0,5 to 2mm layer of adsorbent for separating compounds in amounts of 10 to 1000mg. The analytical TLC methods are tested first for quick judgment of the sample, to try to identify unknown compounds or to optimize a separation before starting with large amounts of samples. The main differences between TLC and PLC are due to large layer thickness and particle size of the stationary phase and the amount of sample applied to the plate (Kowalska & Shema, 2006).

1.14 Column chromatography

Column chromatography is a physical method of separation in which the components to be separated are distributed between two phases one of which is a stationary phase whereas the other one is a mobile phase (Ettre, 1981).

The stationary phase is one of the two phases forming a chromatographic system and it may be a solid, a gel or a liquid and may be distributed on a solid. This solid may or may not contribute to the separation process. The liquid may also be chemically bonded to the solid (Bonded phase) or immobilized onto it (Ettre, 1993); a stationary phase is covalently bonded to support particles inside or to the inside wall of the column tubing and used for separation. The most popular support is micro particulate silica gel (Stahl, 1968). A stationary phase which is stationary on the support particles or on the inner wall of the column tubing by polymerization

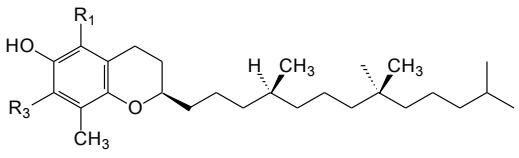
after coating (Ettre, 1981). Sulfonated resins were prepared as an immobilized phase transfer catalyst for the hydrolysis of benzophenone azine to hydrazine (Hayashi *et al*., 1991)

1.15 Antioxidant activities

Emerging evidence suggests that during pregnancy oxidative damage to DNA, protein, and lipids may be associated with reduced birth weight and increased risks of outcomes such as low birth weight, preterm delivery, and preeclampsia. The risk may depend on the mother's antioxidant status which potentially protects the maternal-fetal unit, thus increasing intra-uterine growth and infant weight at birth. Antioxidants scavenge free radicals and buffer the effects of pro oxidants by reducing oxidative stress preventing oxidative damage. Antioxidants are produced endogenously by the body or are consumed in the diet (Rimm *et al*, 1993).

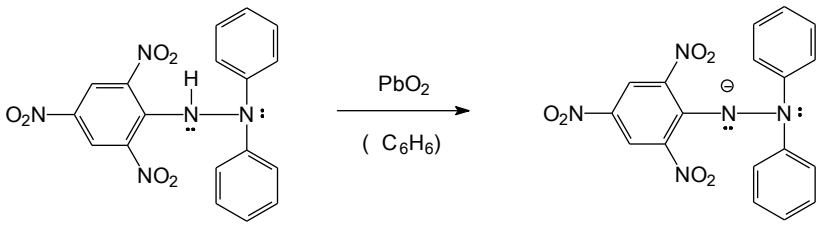
The DPPH method was used alongside with dose-response curve to refer to the titration plot (Sanchez-Moreno *et al*., 1999). The DPPH method gives a validation to the use of substrate as an antioxidant in a biological system (Sanchez-Moreno *et al*., 1999). The higher the antioxidant activity, the lower the EC_{50} value and this is a disadvantage when the results are presented graphically as a bar chart (Ames *et al*., 2001). EC_{50} means concentration of substrate that causes 50% loss of DPPH activity (colour) and that should be noted from any residual yellow from reduced form (Sanchez-Moreno *et al*., 1999). Vitamin E is a lipid soluble chain breaking antioxidant that is dietary in origin (Ames *et al*., 2001). Of the 8 isomers of vitamin E that occur naturally, α-tocopherol is the most abundant in plasma and other human tissues, whereas -tocopherol is the primary form found in the human diet (Knekt *et al*, 2000).

Table 18: Structure of α and -tocopherol

#	Structure	Name	References
64		a. R_I = CH_3, R_2 = CH_3 (α-tocopherol) b. R_I = H, R_2 = CH_3 (-tocopherol)	(Swanson *et al*., 1999)

1.16 Antioxidant due to free radical scavenging

DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable purple coloured compound (if it is in liquid form) used in free radical scavenging which does not dimerize and can be prepared in crystalline form. The DPPH method measures hydrogen atom or electron donating activity and provides an evaluation of antioxidant activity due to free radical scavenging (Ames *et al.*, 2001). DPPH is formed by the oxidation reaction of 1,1-diphenyl-2-picrylhydrazine with lead peroxide (PbO_2).



1,1dipheny-2-picrylhydrazine 1,1dipheny-2-picrylhydrazyl

Equation 2

Figure18: Conversion reaction of 1, 1-diphenyl-2-picrylhydrazine to 1,1-diphenyl-2-picrylhydrazyl (Poirier *et al.*, 1952; Molyneux, 2004).

DPPH is a violet-black solid showing paramagnetic properties of a molecule containing one unpaired electron (Poirier et al., 1952). DPPH is characterized as a stable free radical by the delocalization of the spare electron over the molecule as a whole so that the molecules do not react with one another as it happens with many free radical molecules. The delocalization also gives rise to the deep violet colour characterized by an absorption band in ethanol solution centred at about 520nm. When a solution of DPPH is mixed with that of a substance that transfers a hydrogen atom, this gives rise to the reduced 1,1-diphenyl-2-picrylhydrazine (Nonradical) with the loss of this violet colour to the residual pale yellow colour from the picryl group (Molyneux, 2004). A method of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging with high antioxidant-potential effectively traps the radical thereby preventing its propagation and the resultant chain reaction (Brand-Williams *et al.*,

1995). The free radical-scavenging activity of the extracts is attributed to their hydrogen-donating capabilities (Shimada *et al*., 1992).

Ethanolic and ethylacetate extracts of bee pollen of *Melipona submitida* are free radical inhibitors and primary antioxidants that react with free radicals and DPPH screening for free radical scavenging capability showed that extracts of yellow or brown pollen posses the most impressive free radical scavenging capability consistent with their contents of phenolic constituents especially in flavonoids (Silva *et al*, 2006).

1.17 Structural elucidation by mass spectrometry.

Mass spectrometry (*Ms*) is an analytical tool used for measuring the molecular mass and fragmentation pattern of a sample (Ashcraft, 1997). It helps by determining the purity of a sample (Ashcraft, 1997). Mass spectrometry is also useful for determining the chemical structure of unknown natural products. The mass spectrometer is divided into three main parts namely; ionization source, analyzer and detector.

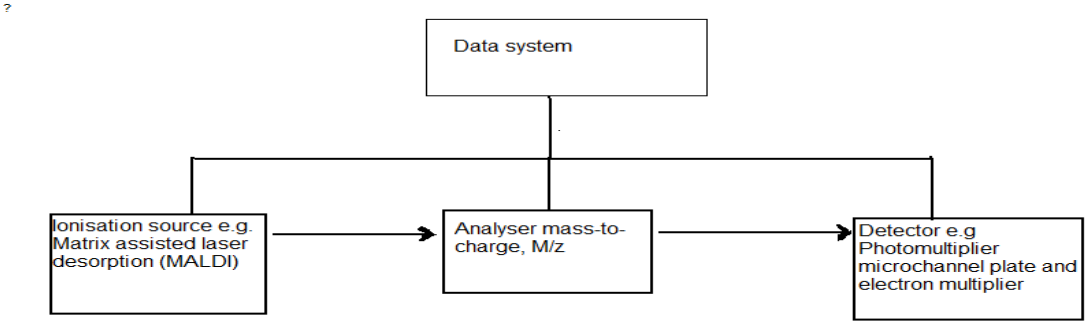


Figure 19: Schematic representation of mass spectrometer (Ashcraft, 1997).

The sample is introduced into the ionization source of the instrument and gets ionized. They rush into the analyzer region of the mass spectrometer where they are separated according to their mass-to-charge ratio (*M/z*). The separated ions are detected and the signal is sent to a data system where the *M/z* ratios are stored

together with their relative abundance for presentation in the form of a *M/z* spectrum. The *M/z* values can be expressed as follows:

$$M/z = (MW + H^{+})/n \qquad \text{Equation 3}$$

Where M/z = mass-to-charge ratio marked on the x-coordinate of the spectrum, MW = molecular mass of the sample or fragment, n = integer number of charge on the ions, H = mass of a proton = 1.00Da (Ashcraft, 1997).

The various components of the mass spectrometer are often maintained under vacuum gradient in order to give ions more chance of travelling from one end of the instrument to the other end without interferences from carrier gas molecules.

The sample in the mass spectrometer is vaporized at low pressure and bombarded with a beam of high-energy electrons. The energy of the beam of electrons is usually 70eV and this bombardment is useful for dislodging one of the electrons from the molecules of the sample and produce positively charged ions called the molecular ions.

$$M + e^{-}\,(70ev) \longrightarrow M.^{+} + 2e^{-} \qquad \text{Equation 4}$$

The molecular ion is not only cation, but because it contains an odd number of electrons, it is also a radical (McMurry, 2006). Thus it belongs to a general group of ions called radical cations. Because of the high energy the molecular ion, undergoes fragmentation. Fragmentation can take place in a variety of ways depending on the nature of the particular molecular ion and the way a molecular ion fragments can give highly useful information about the structure of a complex molecule. The mass spectrometer sorts the radical cations on the basis of their mass/charge ratio. Since for all practical purposes the charge on all of the ions is +1, this amounts to sorting them on the basis of their mass. The conventional mass spectrometer does this by accelerating the ions through a series of slits and then it passes the ion beam through a curved tube (Munson & Field, 1966).

The curved tube passes through a variable magnetic field and the magnetic field exerts an influence on the moving ions. Depending on its strength at a given moment, the magnetic field will cause ions with a particular *M/z* ratio to follow a curved path that exactly matches the curvature of the tube. These ions are said to be "in register." These ions pass through another slit and impinge on an ion collector where the intensity of the ion beam is measured electronically. The intensity of the beam is simply a measure of the relative abundance of the ions with a particular *M/z* ratio. Some mass spectrometers are so sensitive that they can detect the impact of a single ion (McMurry, 2006).

1.18 Infrared Spectroscopy

Fourier-Transform Infrared spectroscopy (FT-IR) is a commonly used structural elucidation tool. FT-IR is a technique used for identifying both organic and inorganic compounds by analysis of vibration and rotation properties of the molecules of the sample. The infrared spectrum can be used to specifically identify the material or provide generic information regarding its chemical family by the functional groups present as well as quantitatively analyze the sample (Luypaert *et al*, 2003).

A variety of sample handling accessories makes this a versatile technique having the ability to examine gases, liquids and solids. Infrared spectroscopy measures the energy difference between vibrational and rotational energy levels in molecules (Mukamel *et al*, 1997).

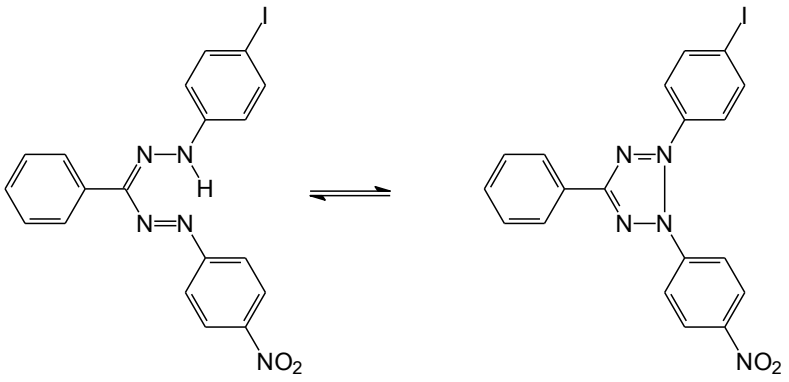
Absorption of infrared radiation is restricted to compounds with small energy differences in the possible vibration and rotational states. For a molecule to absorb infrared radiation, the rotations or vibrations within a molecule must cause a net change in the dipole moment of the molecule. The alternating electrical field of the radiation interacts with fluctuations in the dipole moment of the molecule. If the frequency of the radiation matches the vibration frequency of the molecule then radiation will be absorbed (Kirkwood *et al*, 2000), causing a change in the amplitude

of molecular vibration. In a molecule, the atoms are not held rigidly apart. Instead they move, as if they are held together by a spring, and the chemical bonds can either bend or stretch. If the bond is subjected to infrared radiation of a specific frequency (400-4000 cm^{-1}), it will absorb the energy, and the bond will move from the lowest vibration state, to the next higher vibration state. In a simple diatomic molecule, there is only one direction of vibrating, stretching. This means there is only one band of infrared absorption. If there are more atoms, there will be more bonds, and therefore more modes of vibration. This will produce a more complicated spectrum. In general, a polyatomic non-linear molecule with N atoms has $3N^6$ distinct vibrations, but for linear molecules it will have $3N^5$ vibration modes (Tretiak *et al*, 2002).

There is one restriction; the molecule will only absorb radiation if the vibration is accompanied by a change in the dipole moment of the molecule. A dipole occurs when there is a difference of charge across a bond. If the two oppositely charged atoms get closer or further apart as the bond bends or stretches, the dipole moment will change. Infrared is normally determined from 4000-400 cm^{-1}, but a wider range can be utilized down to a few cm^{-1}, but for the purpose of this study the above range was used on a Perkin Elmer Spectrum RXI FT-IR Spectrometer (Bruker IFS 113v, DTGS detector, and resolution 2 cm^{-1}) (Mukamel, 2000).

1.19 *p*-Iodonitrotetrazolium violet (INT) reaction

The *p*-iodonitrotetrazolium violet (INT) reaction is based on the transfer of electrons from NADH in a reaction catalyzed by dehydrogenases. This converts INT into its respective formazan. Viable cells are identified by the red formazan colour. Dead cells are unable to form the formazan colour (Nachlas, 1960).



Equation 5

Figure 20: Equilibrium reaction of *p*-iodonitrotetrazolium violet red (ITVr) and *p-iodonitrotetrazolium* violiox (ITVox) (Engeh *et al.*, 2006).

Consider the example of threonine dehydrogenase catalyzed reaction with *p*-iodonitrotetrazolium violet (INT) reaction. Threonine dehydrogenase (TDH) from bacteria catalyses the NAD-dependent oxidation of threonine to form 2-amino-3-ketobutyrate and NADH (Engeh *et al,* 2006).

In the course of active growth of bacteria an electron is transferred from the NADH which is transparent in the visible range to *p*-iodonitrotetrazolium violet and formazan dye which is purple-red in colour (Engeh *et al*, 2006). The clear zones on the chromatogram represent areas of inhibition or zones where no active growth of bacteria (Engeh *et al,* 2006).

Chapter 2

Materials and Methods

2.1. Plant collection

Leaves of six plants, namely [*Calpurnia aurea* (437,948g), *Combretum collinum* (174,324g), *Combretum bracteosam* (116,085g), *Adina mcrocephaha* (238,674g), *Ficus ingens* (53, 995g), *and Filicium decipiens* (246, 57g) were collected from the National Botanical garden in Nelspruit, Mpumalanga during summer in 2007.

2.2 Plant drying and Storage

The leaves of the six plant species were air-dried in the dark for three weeks. The dried leaves were ground to fine powder using a mortar and pestle and placed in glass containers until further use (Rogers & Verotta, 1996; Eloff, 1998a).

2.3 Extraction

The ground leaves (1g) of each of the six different plant species were suspended in 10 ml of acetone, dichloromethane, and hexane for preliminary screening (Masoko & Eloff, 2006). The mixtures were shaken for 30 minutes and filtered using Whatman no.1 filter paper. The extracts were air-dried and the mass of the extracts was measured and recorded (Asfaw & Lwande, 1993; Masoko & Eloff, 2006)

In the antimicrobial activity results from preliminary screening (Eloff, 2004), *C. collinum* was shown to have many compounds with antibacterial activity, hence *C. collinum* (171, 5g) was further extracted with hexane, chloroform, ethyl acetate and methanol respectively (Shai *et al*, 2008). Since the acetone extract showed many compounds with antibacterial activity from preliminary screening, the acetone extract (4, 1g), was further investigated.

2.4 Antimicrobial activity

2.4.1 Chromatogram development.

The TLC plate (20 x 20 cm) loaded with 100 µg of hexane, acetone, and dichloromethane extract were developed in a tank saturated with mobile phase of chloroform, ethyl acetate and formic acid (5:4:1) (CEF). The chemical components were visualized by spraying the plates with a vanillin/methanolic mixture [vanillin (0,1g): methanol (28 ml): sulphuric acid (1 ml)] and heating at 110°C for 5 min, for adequate colour development (Kotze & Eloff, 2002).

2.4.2 Bacterial cultures

Bacterial cells were cultured in a sterile Schott Duran bottle (500 ml) in Luria Bertani (LB) broth at 37°C (Reynolds, 2005). The bacterial cultures were prepared 14 hrs, prior antibacterial activity assays. *Staphylococcus aureus*, *Escherichia coli, Enterococcus faecalis* and *Pseudomonas aeruginosa* were kindly donated by Dr. L.J. McGaw of the Phytomedicine Programme at the University of Pretoria, South Africa (Miller, 1972).

2.4.3 Bioautographic method

The TLC plates (20 x 20 cm) were loaded with each extract and developed as described in section 2.4.1. The chromatograms were dried up at room temperature under a stream of air for at least a day to remove the remaining solvent. Cultures were grown in LB broth solution overnight. The plates were sprayed with cultures of selected bacteria until wet. The plates were incubated overnight at 37°C in an incubator. The plates were then sprayed with 2mg/ml of INT. The plates were incubated at 37°C for adequate colour development (Begue & Kline, 1972), due to the formazan reaction.

2.4.4 Minimum inhibitory concentration determination

The microplate dilution method was used to determine the Minimum inhibitory concentration (MIC) of the extracts against each test bacterial species (Eloff, 1998). The plant fractions or compounds were constituted to 10 mg/ml with acetone and 100 µl of fractions or compounds were serially diluted to 50% with water in a 96-wel microplates. Muller Hinton (MH) broth culture was inoculated (1%) with the test bacteria (*S. aureus* or *E. coli*) and was incubated at 37°C overnight and 100 µl of the resulting culture were added to each well (Eloff, 2004).The negative growth control contained both MH broth as well as test organism. The microplates were sealed and incubated at 37°c at 100% relative humidity for 18 hours. 40 µl of a 0.2 mg/ml of *P*-iodonitrotetrazolium violet (INT) dissolved in water was used as an indicator for bacterial growth and the solution was added to the microplate wells and incubated at 37°c for a further 30 minutes. The MIC was recorded as the lowest concentration of fraction or compounds at which bacterial growth was inhibited (Eloff, 2004). A lack of colour in the well shows that the bacteria were inhibited from growth and a pink colour indicated that there was bacterial growth (Begue and Kline, 1972).

2.5 Screening of antioxidant compounds

TLC plates were loaded with the same amount of extracts and developed as described in sections 2.4.1 and 2.4.4.The plates were allowed to dry and then sprayed with red DPPH. Antioxidant activity was regarded as the ability to reduce red DPPH into colourless product (Brand-Williams *et al*., 1995). White zones on the plates therefore indicated components of the extracts with antioxidant activity.

2.6 The instruments used

The instrument used for GC-MS spectra was a Hewlett Packard 5973 mass selective detector, connected to a HP 6890 series GC system from Council for Scientific and Industrial Research Pretoria (CSIR), Agilent Technologies Automatic Liquid Injection system no.7683 series, with split less injection mode. The column used was HP-5MS (30m x 0, 25mmid x 0, 25um df). The column temperature was programmed from 120°C with a 1min ramp to 320 at 15°C/min thereafter held for 5 min at 320°c. The

carrier gas was helium, electron energy was 700eV, emission current 34,6Ua, injector temperature was 250°c and the transfer line temperature was 25°C and the quadrupole temperature was 150°c. The total flow rate was 14ml/min, with a purge flow at a split vent of 0,1ml/min and the acquisition mode was full scan and the scan range was 35-750amu.

The instrument used for NMR spectra was mercury-300BB from Limpopo University (Medunsa campus) at an ambient temperature. The solvent used to dissolve compound in NMR tube was acetone. The compound was relaxed and delayed by 1.000 second in the NMR instrument. The acquisition time was 1.997 seconds. The observe H1 was 300.0689246 MHz. The total time spent by compound in the NMR instrument was 59min and 1 second.

The instrument used for Fourier transform infrared spectroscopy was Perkin Elmer spectrum RX I FTIR from University of Pretoria (Acadia campus). Compound was mixed with potassium bromide to remove scattering effects from large crystals. The mixture was pressed in a mechanical die press to form a translucent pellet through which the beam of the spectrometer can pass. This pellet was placed between two small plates and inserted to Perkin Elmer spectrum RXI FTIR for analysis.

Chapter 3

Purpose of the research

3.1 Problem statement

According to Warrier *et al* (2001) medicinal plants provide accessible and largely safe sources of primary health care to the majority of the population in India. Poor People who are unable financially to afford formal health care systems are dependent on herbal medicine. Government is always experiencing problems with pharmaceutical companies that are regularly increasing prices of their medicines. There is a growing interest in the value and efficacy of medicinal plants based on local health systems as a means of meeting the current and future health care needs of the people (Warrier *et al*, 2001). Many investigators found that *combretum collinum* sub specie *suluense* has antibacterial activities. However its chemical components have not been identified (Pegel *et al.,* 1985; Rogers *et al.,* 1986).

Many Traditional healers use water extracts of *Combretum collinum* which means there is a need to characterize its chemical components. Many Traditional healers use water extract to heal people without the knowledge of concentration measurement which means there is a need to test plant against different organisms and measure the minimum effective concentration.

3.2 Aim

The aim of this study is to isolate and characterize the antibacterial and antioxidant compounds from *Combretum collinum* sub specie *suluense*, and to evaluate their properties and propose their chemical structures.

3.3 Objectives

Extract compounds from *Combretum collinum* subspecie *suluense* using sequential solvent extraction.

Determine the antibacterial activity of the extracts. This will help in the selection of extracts from which to isolate active compounds.

Isolate compounds from the extracts using chromatographic techniques.

Determine the minimum inhibitory concentration of chemical components that have antibacterial activities.

Determine the chemical structure of the antibacterial compounds using spectroscopic techniques.

Chapter 4

Results and discussion

4.1 Extraction for preliminary screening.

In this work the following plant species *Adina mcrocephaha*, *Calpurnia aurea*, *Combretum bracteosam*, *Combretum collinum*, *Filicium decipiens*, and *Ficus moraceae* were used.

Table 19: Mass of extracts from a gram of each of six different plants and the % yield of each extract.

Name of plants	Mass of extracts (mg)			% yield		
	Acetone	DCM	Hexane	Acetone	DCM	Hexane
Adina microcephaha	74	86	37	7.4	8.6	3.7
Combretum aurea	35	20	63	3.5	2.0	6.3
Combretum bracteosam	27	47	33	2.7	4.7	3.3
Combretum collinum	40	30	30	4.0	3.0	3.0
Filicium decipiens	118	75	52	11.8	7.5	5.2
Ficus moraceae	44	30	18	4.4	3.0	1.8

Table 19 shows that 1g of ground plant material, acetone extracted 118 mg of extract from *Filicium decipiens* and was found to be the highest extracted mass as compared to the extracts from the other five plants and hexane extracted the least mass of 18 mg from *Ficus moraceae.*

4.2 Preliminary screening of medicinal plants.

The preliminary screening of the plant extracts was carried out as described in section 2.4.3 and 2.5, for antibacterial activity and antioxidant activity respectively. The two chromatograms shown in figure 21 & 26 showed the results of antibacterial screening of all six dichloromethane plant extracts against *Staphylococcus aureus* and antibacterial screening against *Staphylococcus aureus* of hexane extracts in figure 22. Antibacterial activity of the extracts of *Combretum collinum* subsp *suluense* and *Filicium decipiens* are shown in figure 21 & 24.

The chromatograms in figure 23 and 24 show antibacterial activity results only from *Combretum collinum* subsp *suluense* and *filicium decipiens* extracted with dichloromethane and acetone against *Escherichia coli* and the chromatogram in figure 25 shows the antibacterial activity against *Staphylococcus aureus* from *Combretum collinum* subspecies *suluense* extracted by acetone. In figure 21, 23,24 and 25 at *Cc* chromatogram, the white zones indicate the area of bacterial growth inhibition and pink zones indicate the bacteria growth. On *Fd* chromatogram there is colourless chromatogram which is not as active as the one in *Cc* chromatogram. This indicates that the chemical components of that plant extract have antibacterial activity.

Preliminary screening of plant extract was performed by spraying the chromatogram on the thin layer chromatography in figure 21-25 with *Escherichia coli* or *Staphylococcus aureus* followed by *p*-iodonitrotetrazolium violet. The TLC plates were incubated overnight, and taken out for observation. The pink background shows that there was no bacterial growth. White zones in figure 20, 23, 24 and 25 indicate that there was no bacterial growths.

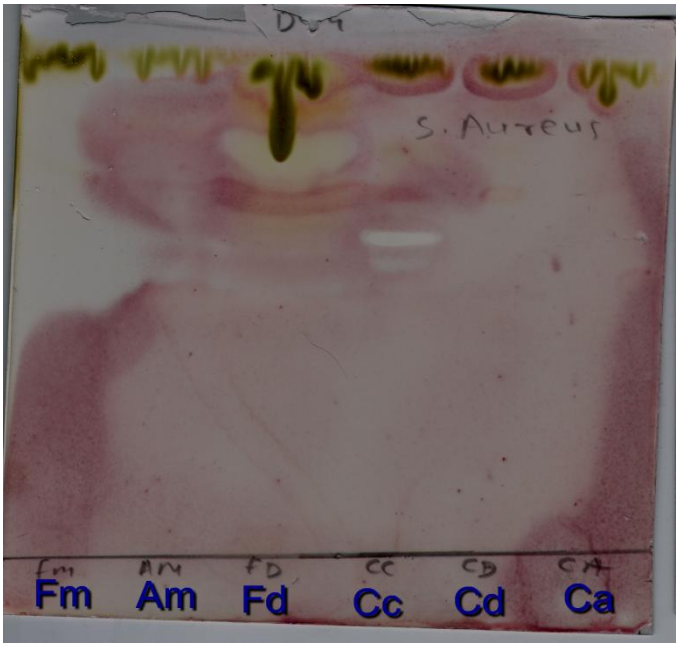


Figure 21: Chromatogram of dichloromethane; extract against *S. aureus.* Fm: *Ficus ingens* (Morecea); Am*: Adina microcephala*; Fd: *Filicium decipiens;* Cc: *Combretum collinum*; Cb: *Combretum bracteosam*; Ca: *Calpurnia aurea*

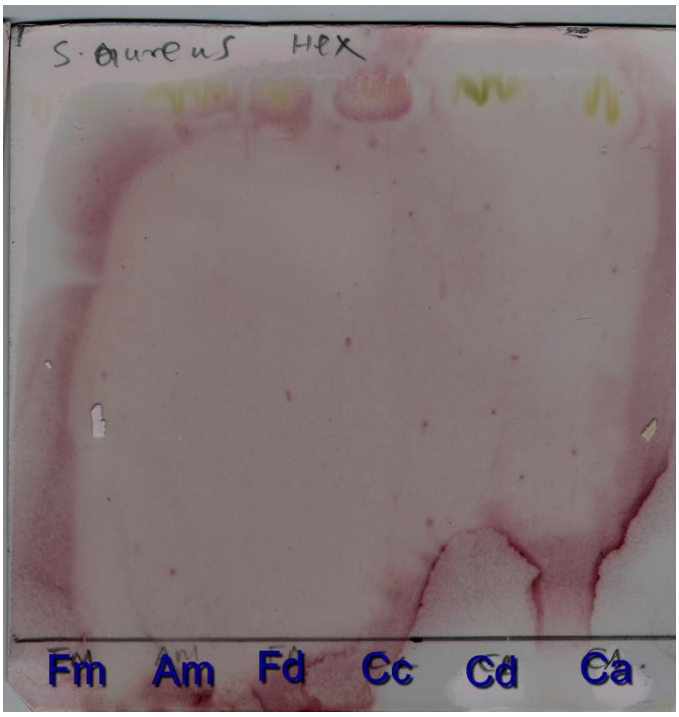


Figure 22: Hexane extract against *S. aureus.* Fm: *Ficus ingens* (Morecea); Am*: Adina microcephala*; Fd: *Filicium decipiens;* Cc: *Combretum collinum*; Cb: *Combretum bracteosam*; Ca: *Calpurnia aurea*

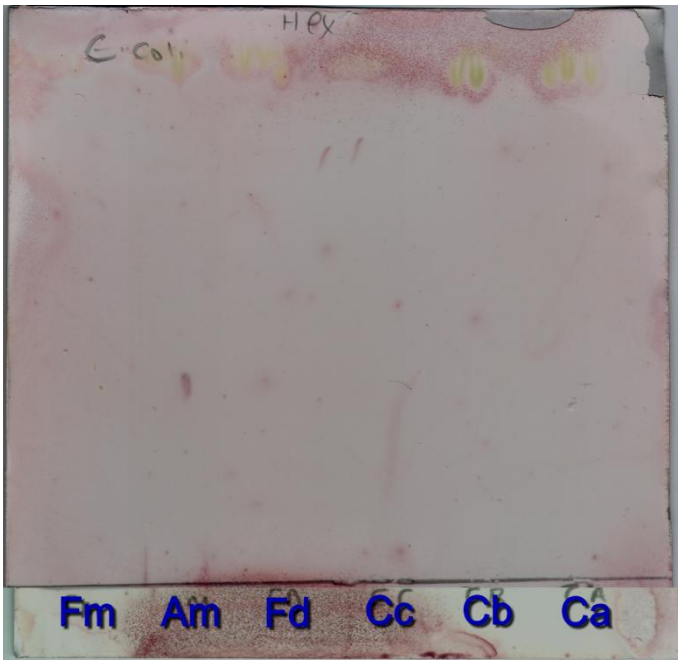


Figure 23: Hexane extract against *Escherichia coli*

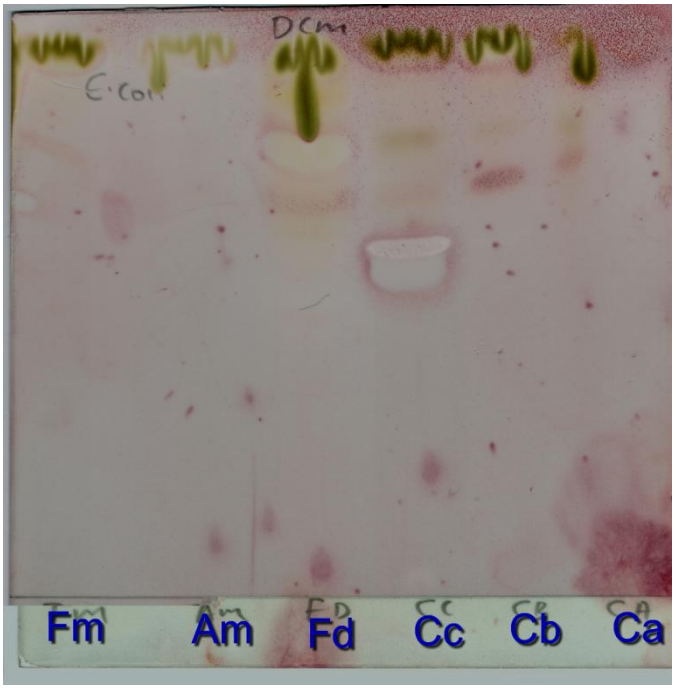


Figure 24: Dichloromethane extract against *Escherichia coli*

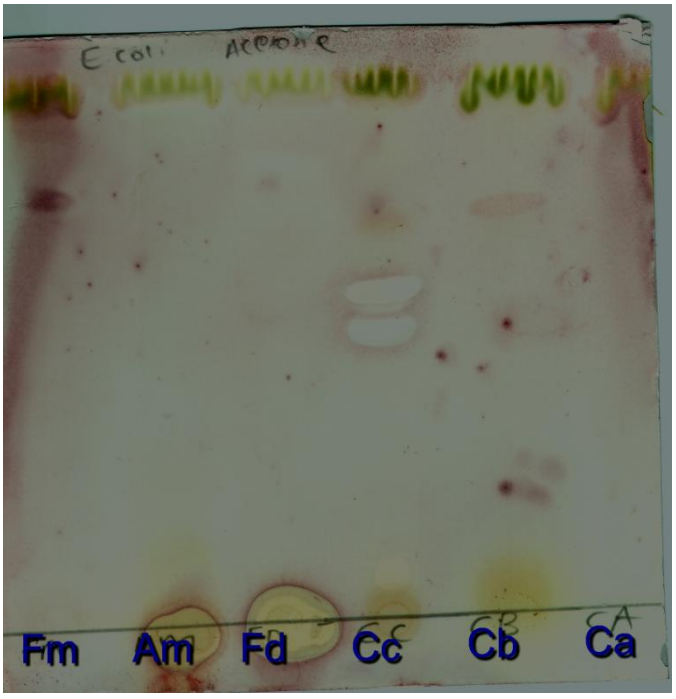


Figure 25: Acetone extract against *Escherichia coli*

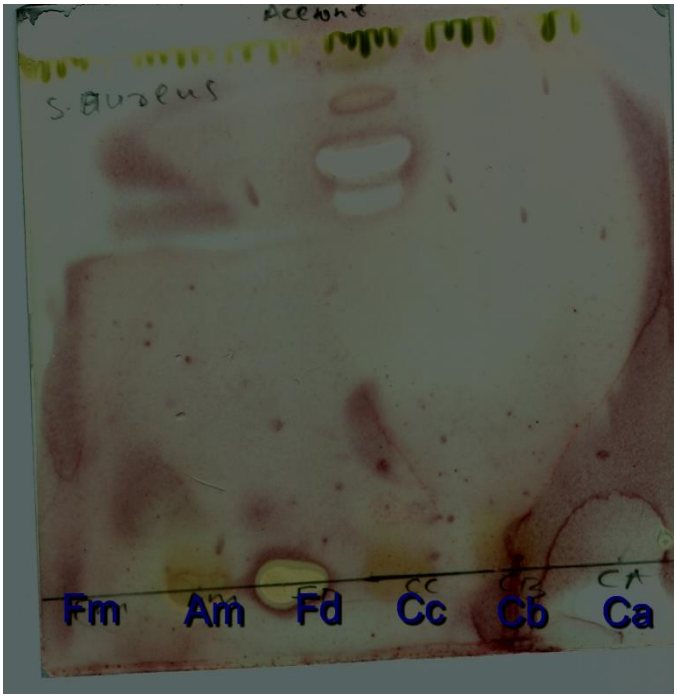


Figure 26: Acetone extract against *S. aureus.* White zones on chromatogram indicate active compounds against that bacteria. *Adina microcephaha (Am), Filicium decipiens (Fd), Combretum collinum (Cc), Combretum bracteosum (Cb) and Calpurnia aurea (Ca). Ficus ingens*

4.3 Serial extraction

The quantity in grams extracted from each extract (hexane, chloroform, ethyl acetate and methanol) in a serial exhaustive extraction from *Combretum collinum* ssp *suluense* was 174. 3 g

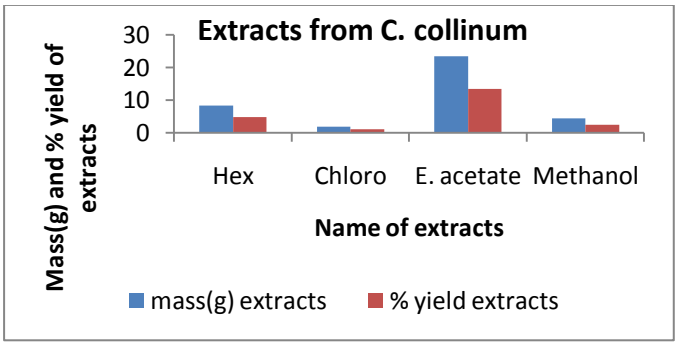


Figure 27: Graph of percentage yield extracted from *Combretum collinum ssp suluense*

In figure 27 ethyl acetate extract (23,3 g) was th*e* extract that had the highest mass followed by hexane extract (8,4 g), methanol extract (4,43 g) and chloroform extract (1,9 g). This indicates that *C.collinum* subsp *suluense* has more moderately polar compounds than non polar compounds and mostly polar compounds since ethyl acetate is a moderate polar solvent and therefore can extract moderately polar and polar compounds.

4.4 Preparative Thin layer Chromatography (PTLC)

The acetone extract (1.0 g) was dissolved in acetone (15 ml), and loaded on a line drawn 2mm from the bottom of the preparative plate as shown in figure 28. The mobile phase used to separate the compounds was 2:1 ethyl acetate and hexane. The PTLC plate used was 20 cm x 20 cm in size.

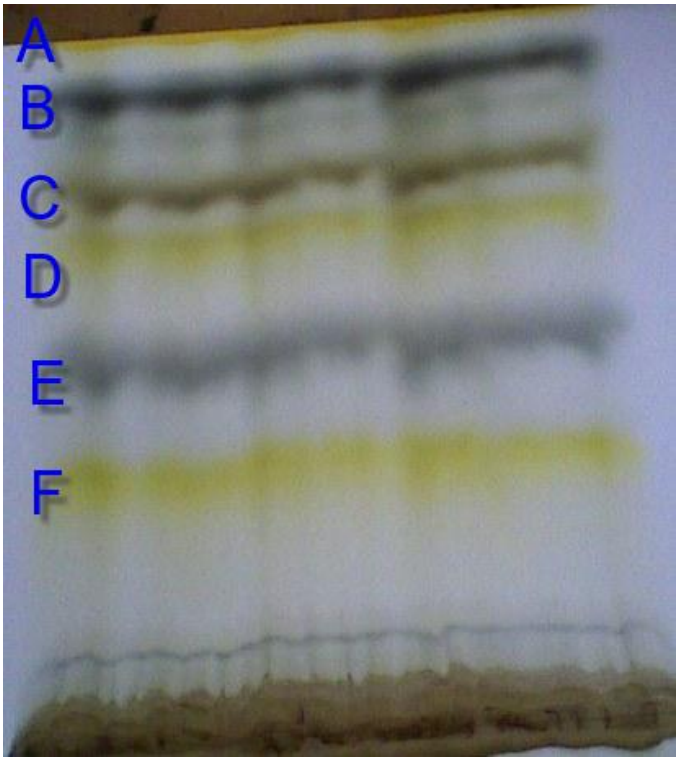


Figure 28: The preparative TLC plate of 2 mm thickness was developed using hexane and ethyl acetate in 2:1 ratio.

The bands shown in figure 28 were scrapped from the PTLC plate. The support material with adsorbed components from the scrapped bands was suspended in ethyl acetate to dissolve the adsorbed components. The adsorbed components were

isolated by filtration washing several times with the ethyl acetate. The ethyl acetate was reduced by evaporation on the rotary evaporator. The components separated in this procedure were examined by spotting on an analytical TLC plate and developing with a mixture of chloroform ethyl acetate and formic acid (5:4:1) on section 3.1.8.

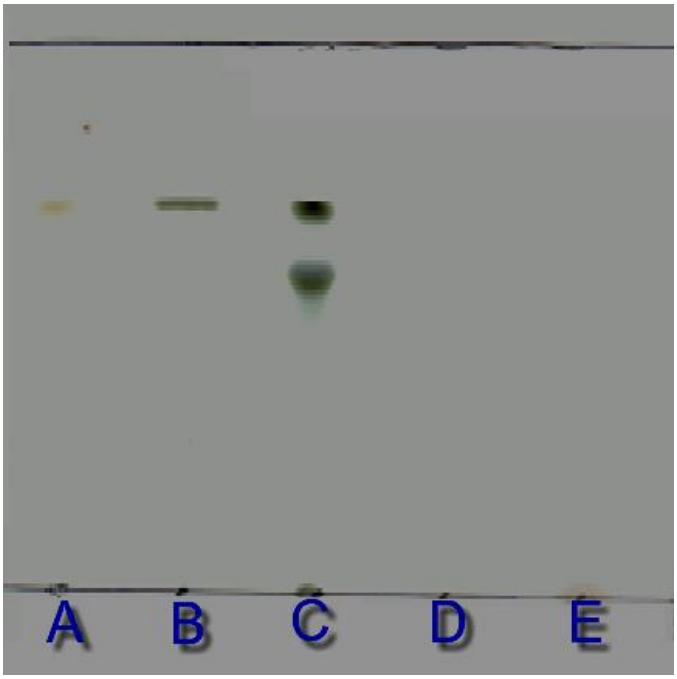


Figure 29: Compounds separated by thin layer chromatography from *Combretum collinum* using chloroform, ethyl acetate and formic acid (5:4:1).

The chromatogram A,and B in figure 29 shown only one compound, whereas in chromatogram C and D shown more than one compound. Further investigation was performed on compound from chromatograms A, B and E in figure 29.

4.5 Scrapped layers

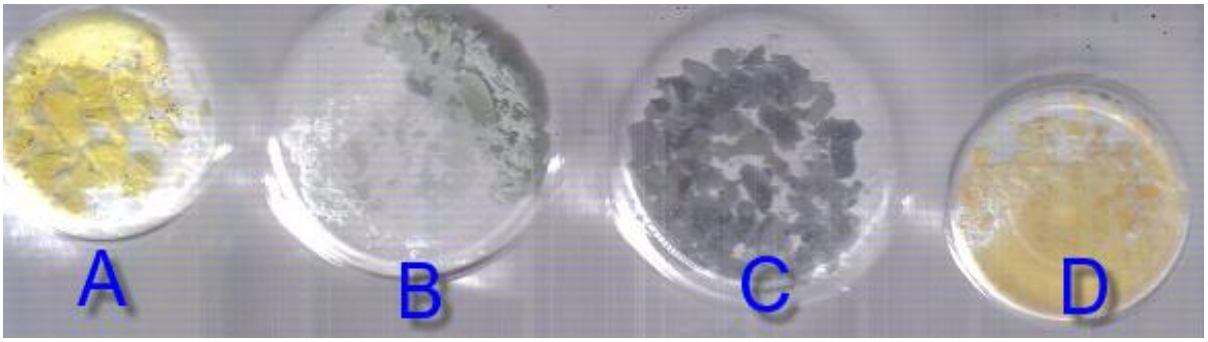


Figure 30: shows the layers that were scrapped from the preparative layer plate. The scrapped layers are indicated from layer A-D

The bands on the PTLC plate shown in figure 28 were scrapped and transferred into separate beakers. The picture of the beakers that contained scrapped material from band E and F are not shown in figure 30.

4.6 Thin Layer Chromatography.

In figure 29 chromatogram A, B and E show that they only have one compound for each chromatography and C has more than one compound and it was not further investigated because the yield was not enough to be re-separated.

Only compounds in band B, E and F were re-dissolved and spotted on a separate plate which was sprayed with vanillin and placed under UV light to check how many compounds were present on the spots found on the TLC plate in figure 30. The remaining filtrate was filtered into pre-weighed beakers and the solvent was removed by evaporation on a rotary evaporator.

The following are the yields for each compound; compound in band A (0.152%), in band E (0.048%) and in band F (0.0299%), band B was not counted because more one compounds were found. The compounds isolated from the bands A, E and F was named as unknown compound A, compound E and compound F respectively.

4.7 Chromatogram investigation

The TLC plate was placed under UV light to display invisible compounds on the plate. The same compounds separated on the TLC plate in figure 29 were respotted on the TLC shown in figure 31, and developed,then placed under UV light to investigate if there were invisible compound without spraying with vanillin.

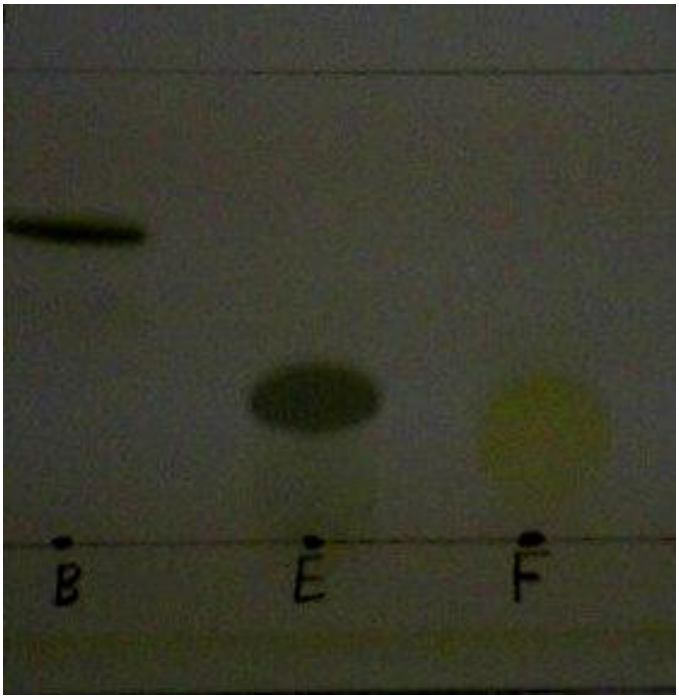


Figure 31: The compounds which were on the thin layer chromatography plate were placed under the UV light at 254nm to display even the invisible compounds.

4.8 Bioautography

TLC was used to confirm if there was one compound, from the band which was scrapped from each preparative layer plate in figure 28. The scrapped band was transferred into a beaker and ethyl acetate (20 ml) was used to dissolve the compound from silica which was then filtered by Whatman no.1 filter paper and the filtrate were collected into clean beakers as described in section 2.4.1. The solvent was evaporated by a rotary evaporator. The thin layer chromatography plate in figure 30 shown that chromatogram B, E and F have one compound after revealed by ultraviolet light and compounds in vial labeled B, E and F were dissolved and spotted on the TLC plate in figure 32 for bioautography. The TLC was developed in hexane and ethyl acetate in a ratio of 2:1 and the TLC plate was then dried and sprayed with active *Escherichia coli* cultures and later sprayed with INT. White areas indicate zones of growth inhibition as described in section 2.4.4.

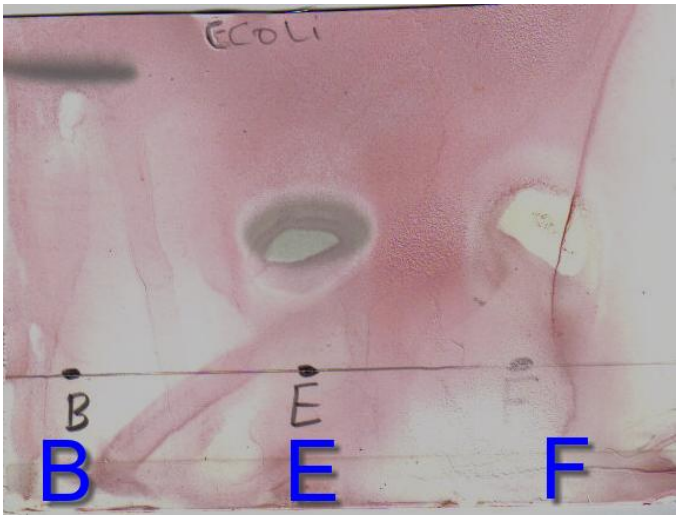


Figure 32: Bioautograms of acetone extract of *Combretum collinum* sub specie *suluense*.

Clear zones in the chromatogram shown in figure 32 represent bacterial growth inhibition as described in section 2.4.3. Bacterial growth was observed on compound B in figure 31. Bacterial growth inhibition was observed on compound E and F in figure 32.

4.9 Antioxidant activity

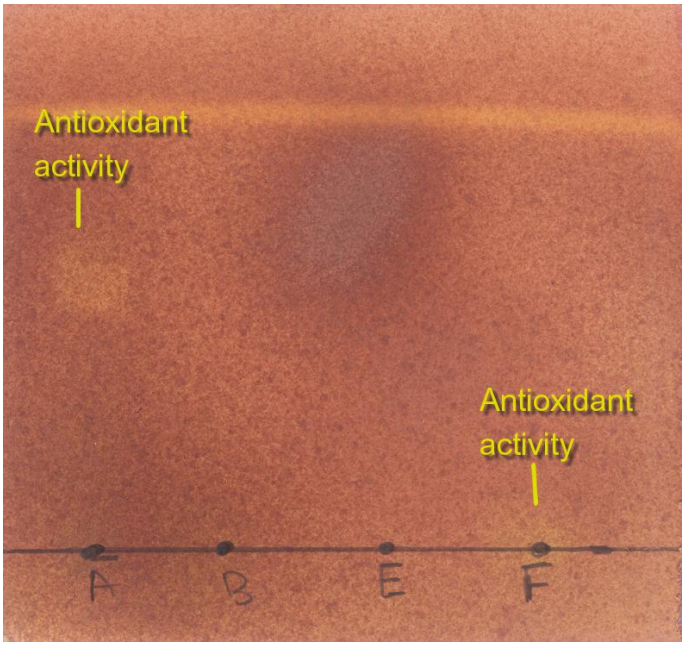


Figure 33: DPPH sprayed on compounds or fractions isolated from acetone extract.

TLC analysis of compounds A, B, E and F were performed by spotting each compound on the TLC plate followed by developing the plate with hexane and ethyl acetate 2:1. The TLC plate was dried by a stream of air for 24hrs. A purple-red DPPH solution was sprayed on the chromatograms. A yellow colour developed on compound A and F as shown in figure 33, represents the antioxidant activity as it is explained by Sánchez-Moreno et al (1999) in section 2.5.

4.10 Summary of the extraction process

After placing thin layer chromatography plate under ultraviolet light the compounds in vials labeled B, C and D from acetone extracts were more than one and not separable because the yield was very low. Only compounds in vials labeled A, E and F in figure 33, were further investigated.

Figure 34 shows the summary of the extraction process of ground leaves of *Combretum collinum* (174.3 g) extracted by five solvents namely hexane, chloroform, ethyl acetate, acetone and methanol. All extracts from *Combretum collinum* leaves were tested against *Escherichia coli* or *Staphylococcus aureus* as shown in figure 21-26. The compounds from the acetone extract that were isolated using preparative TLC were found to be active against both bacteria (*Escherichia coli* and *S. aureus*). These were percentage yields for each compound, compound A (15.2 %), compound E (4.8 %) and compound F (9.9 %), isolated using preparative thin layer chromatography.

4.11 Retardation factor (rf) value.

According to IUPAC Compendium of Chemical Terminology (1997), the retardation factor is a ratio of the distance travelled by the centre of the spot to the distance travelled by the mobile phase. rf = b/a, where b is distance travelled by analyte and a is a distance travelled by mobile phase (IUPAC Compendium of Chemical Terminology, 1997).

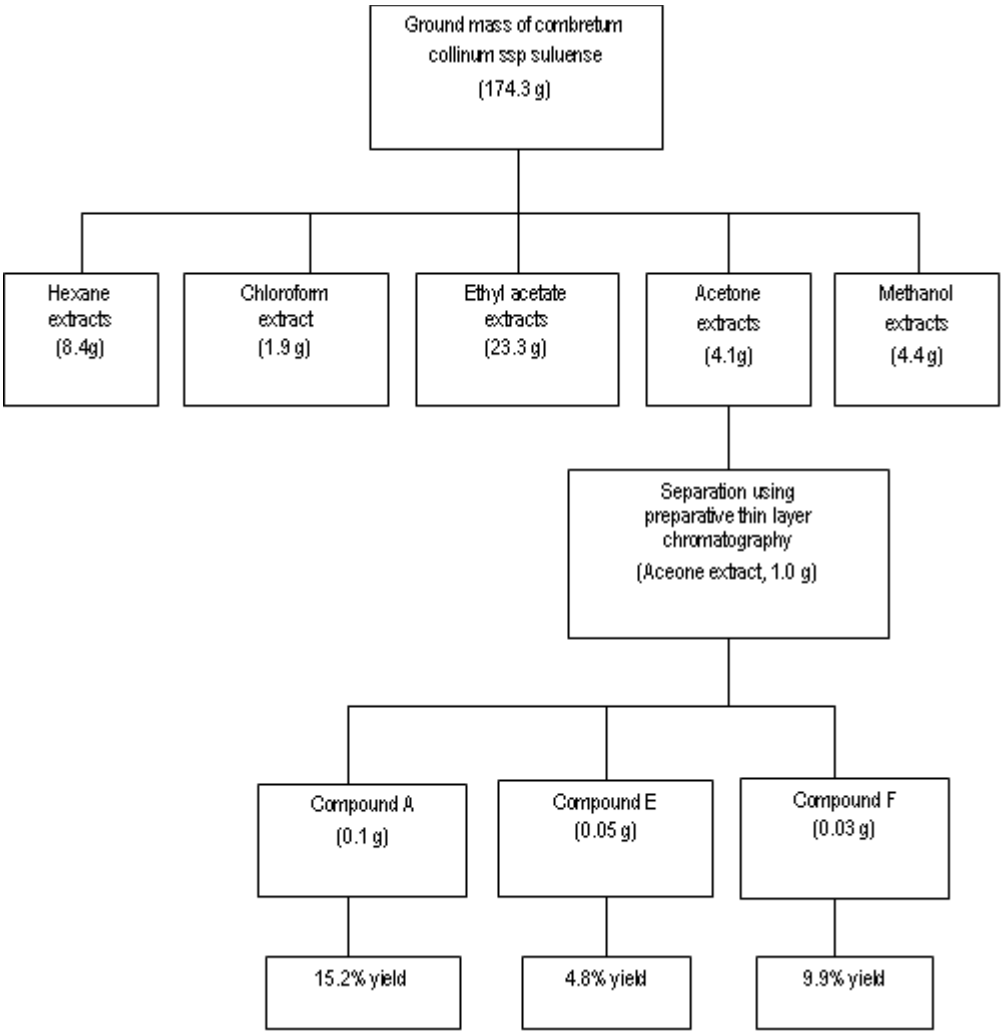


Figure 34: Schematic flow chart of extraction procedure of *Combretum collinum* ssp *suluense.*

The distance travelled by a mobile phase and compound A and B in figure 29 was measured and their retardation factor values were calculated and reported in table 20 and the same procedure for measuring distance travelled by compound F and mobile phase in figure 31 was applied. The mobile phase was prepared by hexane and ethyl acetate (2:1).

Table 20: Represents the isolated active compounds and their retardation factor values (rf).

Name of compounds	rf values	%yield
Compound A	0.7	15
Compound E	0.3	4.8
Compound F	0.5	2.9

4.12 Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after an overnight incubation (Andrews, 2001). Compound A (100 mg) and compound E (50 mg) were dissolved in acetone (1.0 ml and 0.5 ml respectively) to make concentration of 10 mg/ml. Distilled water (10 µl) was pipetted into 49 of the 96 wells in micro-plate. The solution of compound A (10 µl) was pipetted into the first column of wells of the micro-plate and the solution was stirred by micropipette. A sample of the solution (5 µl) in the first well of the first column was transferred into the second well of the first column and the mixture was stirred in the same way. The same amount was transferred to the third well of the same column and the same procedure was followed until the seventh well of the same column. A culture of *Escherichia coli* (1 µl) was added to the each well of this column until the seventh well. Finally *p*-iodonitrotetrazolium violet (10 µl), was added to each of these wells.

In the second column, the same procedure was followed as in the first column except that the test organism used was *Staphylococcus aureus.* In column 3, the same procedure as in the first column was followed except that fraction B was used.

In column 4, the same procedure was applied as in the third column except that the test organism used was *Staphylococcus aureus.* In column 5, the same procedure as in column 1 was followed except that compound E was used. Column 6 was prepared the same way as in column 5 and *Staphylococcus aureus* was used as the test organism.

Lastly the same procedure as in column 1 was followed except that there was no test organism used and this column was the control. The micro-plate was incubated at 37°C for overnight. The formula which was used to calculate the concentration of the diluted compounds is given in equation number 6.

The micro plate was sealed and incubated in 37°C for overnight (Eloff, 2004). The white colour was developed in compound A (0.5689 mg/ml) and in compound E (1.479 mg/ml) and a pink colour developed in the rest of the wells. Therefore compound A only inhibits growth of *Staphylococcus aureus* at a concentration of 0.5689 mg/ml and compound E inhibits growth of *Staphylococcus aureus* at a concentration of 1.479 mg/ml. The pink colour in the wells indicates that there was bacteria growth (Begue and Kline, 1972).

$$\mathbf{C_{concd} \times V_{concd} = C_{dil} \times V_{dil}} \qquad \text{Equation 6}$$

Where $\mathbf{C_{concd}}$ = concentration of a compound, $\mathbf{V_{concd}}$ = volume of a compound, $\mathbf{C_{dil}}$ = concentration of diluted compound and $\mathbf{V_{dil}}$ = volume of the solution. V_{dil} = (volume of solution of compound + volume of distilled water + volume of bacteria mixture + volume of *p*-iodonitrotetrazolium violet solution) - (volume of solution transferred). V_{dil} = (10 µl distilled water + 10µl solution of compound + 1µl solution bacteria + 10 µl solution p-iodonitrotetrazolium violet)-(Solution of a 5 µl solution reabsorbed) = 26 µl.

Table 21: Summary of the diluted solutions of the compounds tested against *Staphylococcus aureus*.

Diluted concentration of compounds	**Concentration compound A in mg/ml against *Staphylococcus aureus***	**Concentration compound E in mg/ml against *S. aureus***
Initial conc	10.00	10.00
1st dilution	3.846	3.846
2nd dilution	1.479	1.479
3rd dilution	0.5689	0.5689
4th dilution	0.2188	0.2188
5th dilution	0.0842	0.0842
6th dilution	0.0324	0.0324
7th dilution	0.01245	0.01245

The micro-plate was sealed and incubated at 37°c overnight (Eloff, 2004), A white colour developed in compound A solution (3.846 mg/ml) and in compound E solution (0.5689 mg/ml) and therefore pink colour developed in the rest of the wells. Compound A can only inhibit growth of *Escherichia coli* at a concentration of 3. 846

mg/ml and compound E can inhibit growth of *Escherichia coli* (0.5689 mg/ml). The pink colour in the wells indicates that there was bacteria growth (Begue and Kline, 1972).

Table 22: Summary of diluted solutions of compounds tested against *Escherichia coli*

Diluted concentration of compounds	**Concentration compound A in mg/ml against *Staphylococcus aureus***	**Concentration compound E in mg/ml against *S. aureus***
Initial conc	10.00	10.00
1st dilution	3.846	3.846
2nd dilution	1.479	1.479
3rd dilution	0.5689	0.5689
4th dilution	0.2188	0.2188
5th dilution	0.0842	0.0842
6th dilution	0.0324	0.0324
7th dilution	0.01245	0.01245

4.13 Proposed structure of the isolated unknown compound from vial A

The structure proposed for unknown compound from vial A shown in figure 34, was proposed following the correlation of HNMR, FT-IR and GC-MS spectroscopy. The unknown compound from vial A was isolated from the leaves of *Combretum collinum* subsp *suluense,* extracted using acetone and its retardation factor (rf) value is 0.7 on TLC which was developed using mobile phase of hexane and ethyl acetate 2:1.

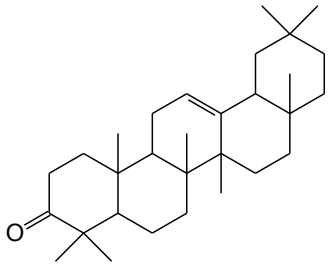


Figure 35: Structure of unknown compound from vial A.

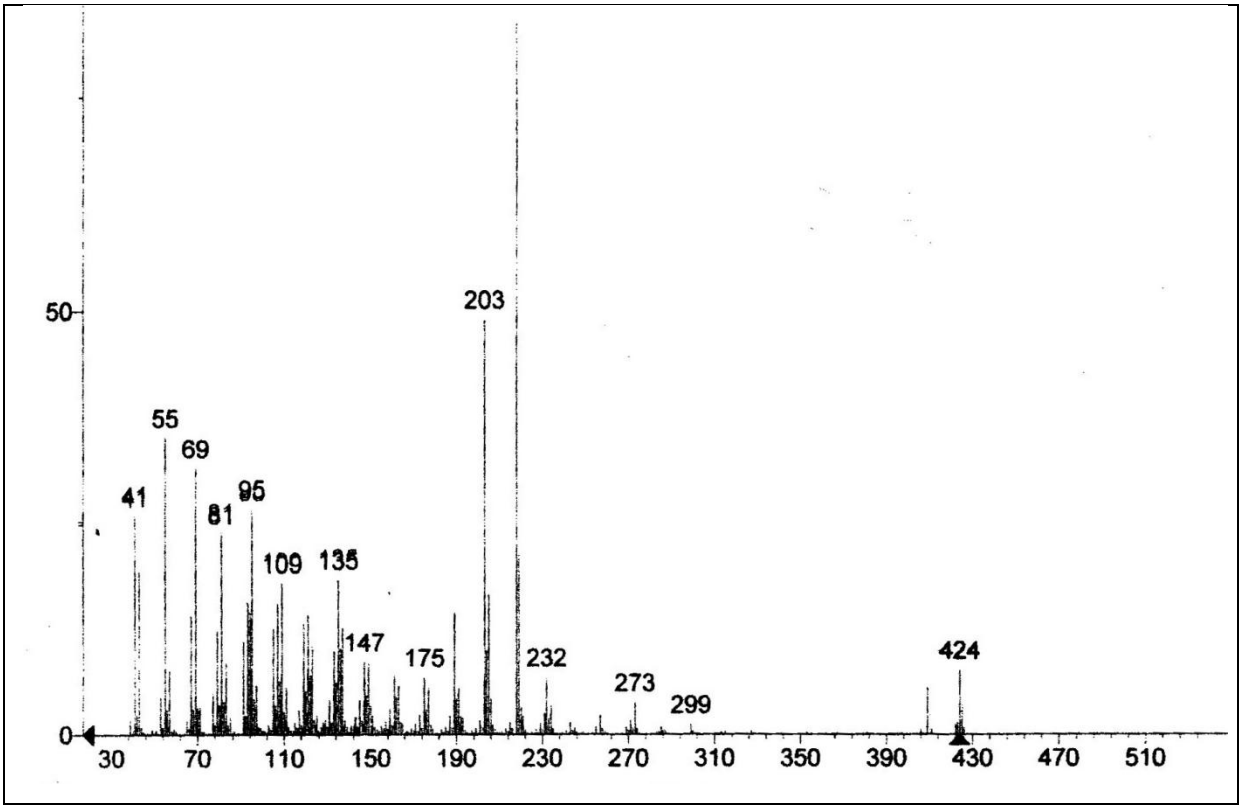


Figure 36: Mass spectrum of unknown compound from vial A.

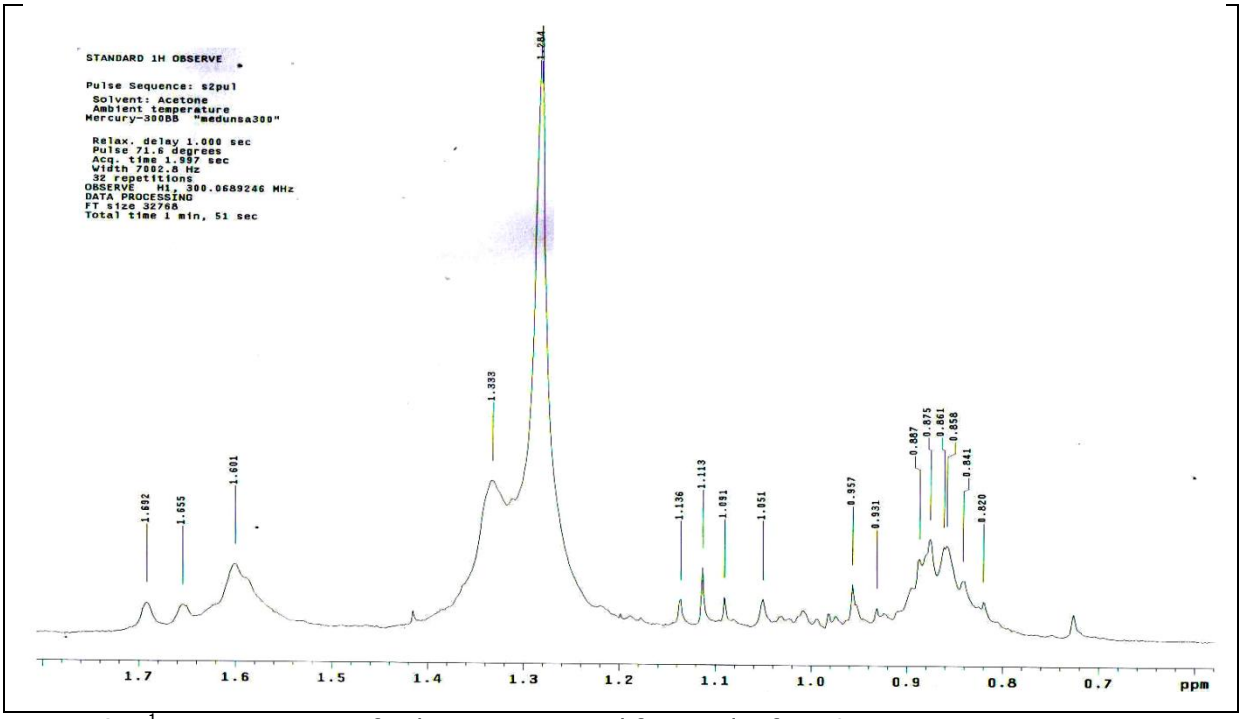


Figure 37: ^{1}HNMR spectrum of unknown compound from vial A from 0.7 to 1.7 ppm

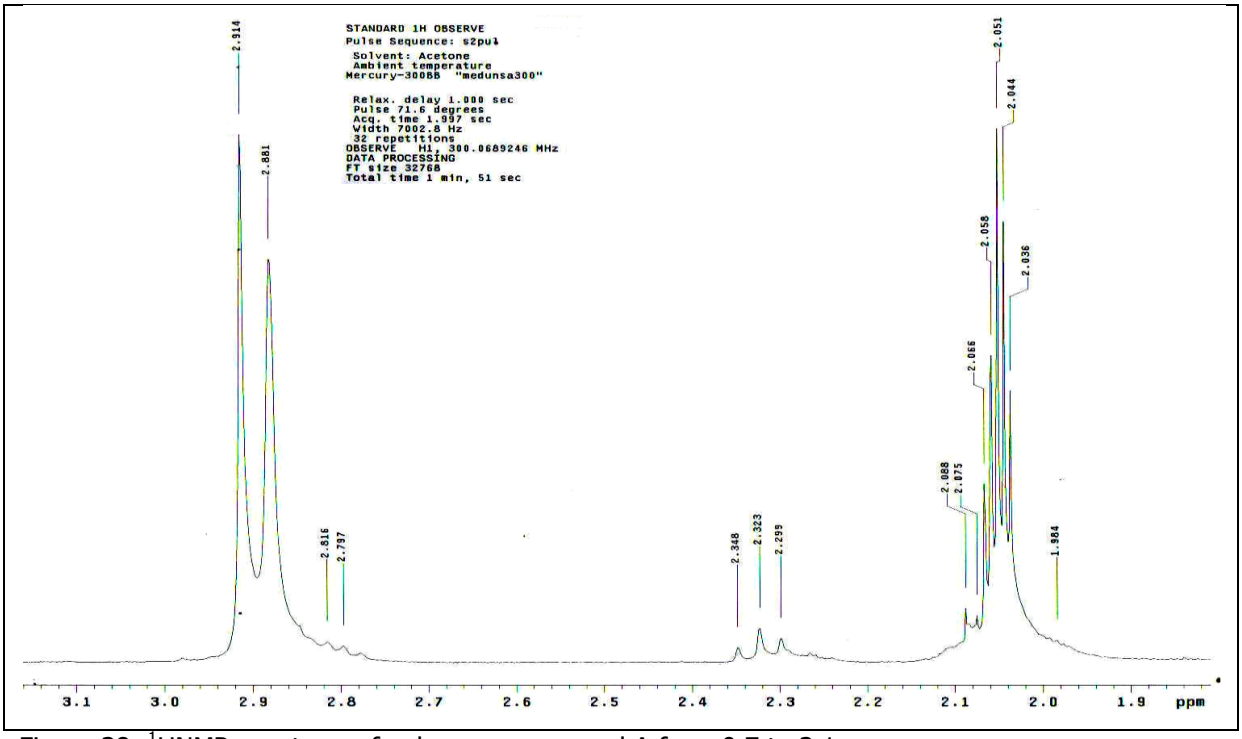


Figure 38: [1]HNMR spectrum of unknown compound A from 0.7 to 3.1 ppm.

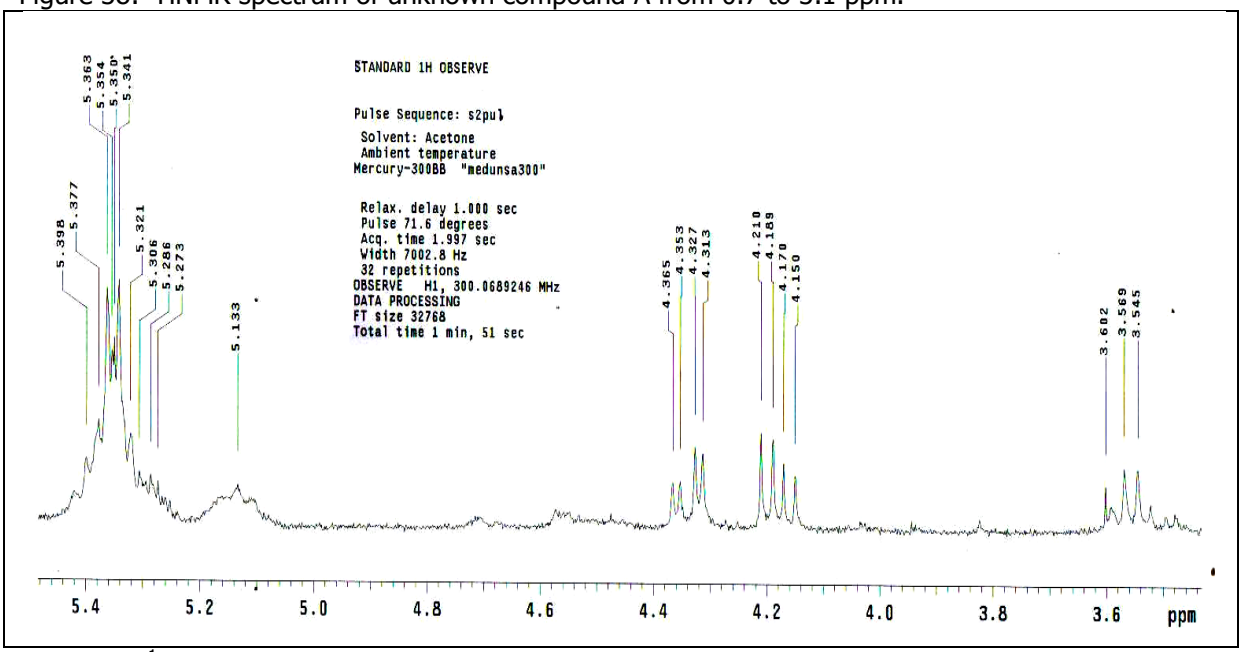


Figure 39: [1]HNMR spectrum of unknown compound A from 3.6 to 5.4 ppm.

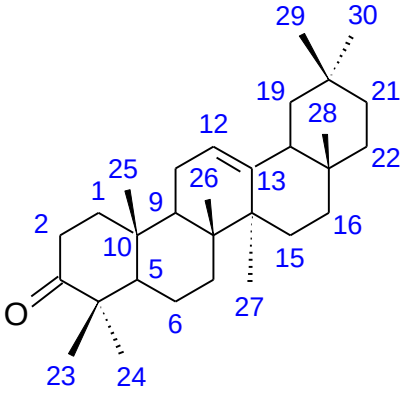
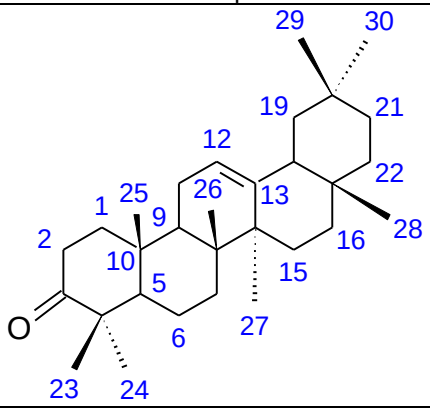
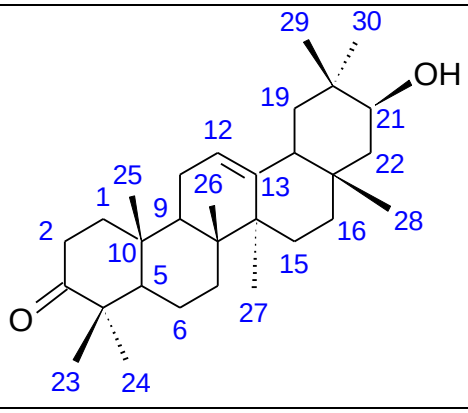


Figure 40: Numbered structure of unknown compound from vial A

4.14 ^{1}HNMR analysis of unknown compound from vial A

The unknown compound A was re-crystallized from 98% ethanol, the yield was 15% and the compound was analyzed using ^{1}HNMR (300 MHz), using tetramethylsilane (TMS) as a reference and chloroform as solvent: 0.858 (3H, s, 30), 0.861 (3H, s, 28), 0.875 (3H, s, 29), 0.887 (3H, s, 27), 0.887 (1H, m, 1), 1.981 (1H, m, 1), 0.931 (3H, s, 26), 0.997 (3H, s, 23), 1.051 (1H, m, 15) 1.091 (1H, m, 15), 1.091 (3H, s, 24), 1.113 (1H, m, 16) 1,136 (1H, m, 16), 1.113 (3H, s, 25), 1.284 (1H, m, 5), 1.333 (1H, m, 7) 1.601 (1H, m, 7), 1.37 (1H, m, 22) 1.50 (1H, m, 22), 1.601 (2H, m, 6), 1.655 (1H, m, 19) 1.672 (1H, m, 19), 1.984 (1H, m, 11) 2.036 (1H, m, 11), 2, 881 (1H, dd, 2) 2.914 (1H, dd, 2), 2.797 (1H, dd, 18), 2.816 (1H, dd, 9), 2.914 (1H, dd, 21),5.133 (1H, t, 12). The data compares well with literature as shown in table 23.

Table 23: ^{1}HNMR results of compound A

		
H #	**Isolated compound**	**29[a] (Liturature compound) (Mena-Rejón et al., 2008)**
1	0.887 m, 1.984 m	1.41 m, 190 m
2	2, 881 dd, 2.914 dd	2.36 ddd (16, 6.8,3.7), 2.55 ddd (16, 11, 7.3)
3	-	-
4	-	-
5	1.284 m	1.33 m

6	1.588 m, 1.601 m	1.48, 1.55 m
7	1.312 m, 1.333 m	1.35, 1.52 m
8		-
9	1.655 dd	1.65 dd (11.5, 6.3)
10	-	-
11	2.044 dd, 2.058 dd	1.88, 1.97 m
12	5.133 t	5.24 t (3.5)
13	-	-
14	-	-
15	1.051 m, 1.692 m	1.00, 1.76 m
16	1.113 m, 1,136 m	1.00, 1.96m
17	-	-
18	2.082 dd	2.00 dd (14.8, 3.5)
19	1.655, 1.672 m	1.15, 1.75 m
20	-	-
21	2.914, 2.323 dd	3.53 dd (12, 4.7)
22	1.42 m	1.37, 1.50 m
23	0.997 s	1.10 s
24	1.091 s	1.06 s
25	1.113 s	1.07 s
26	0.931 s	1.01 s
27	0.887 s	1.14 s
28	0.861 s	0.88 s
29	0.875 s	0.97 s
30	0.858 s	0.86 s

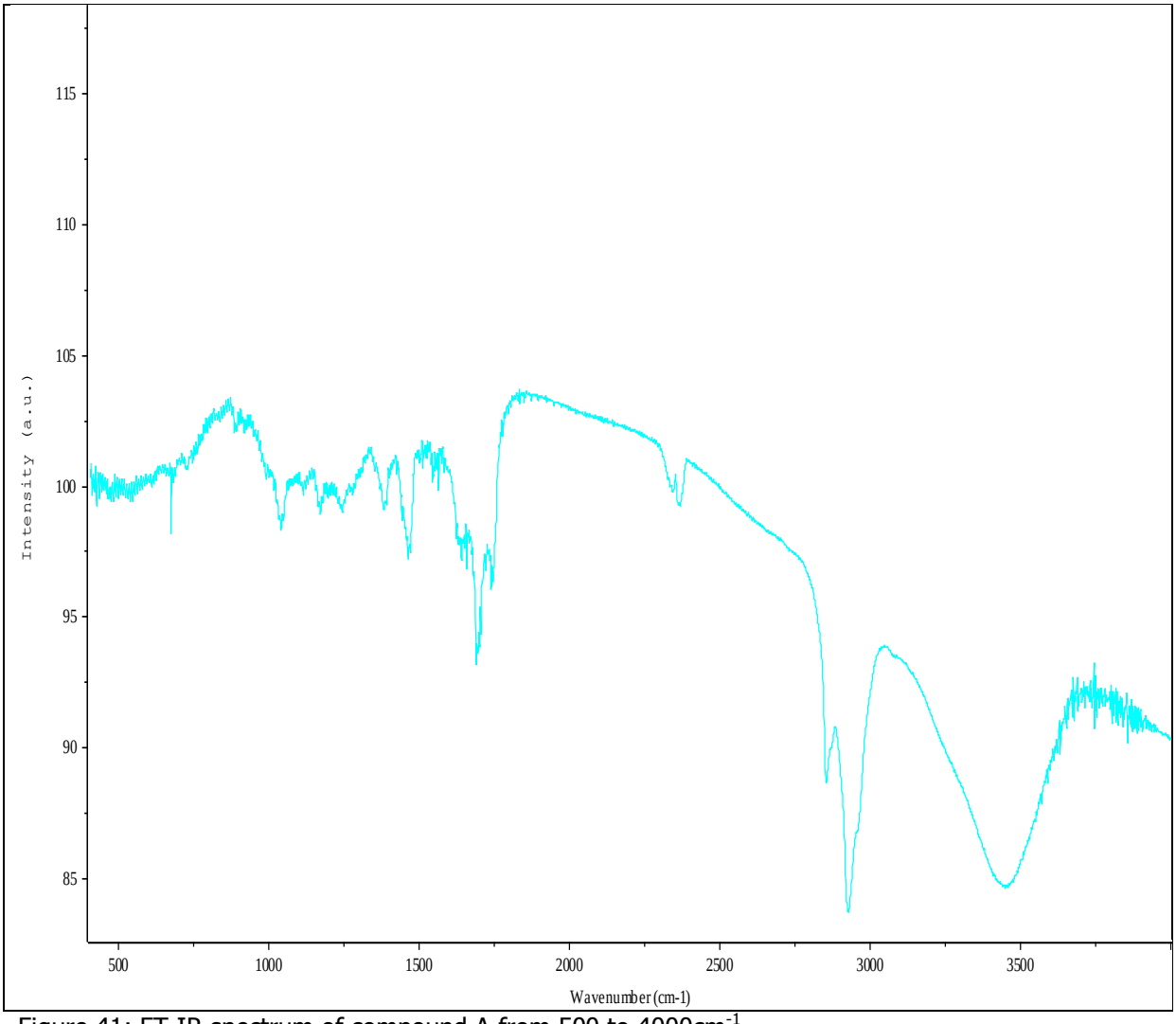


Figure 41: FT-IR spectrum of compound A from 500 to 4000cm^{-1}

4.15 FTIR analysis of unknown compound from vial A

The unknown compound which was yellow in color was analyzed using fourier transform infra-red spectrometer (FTIR). The functional groups of methyl (-CH_3) at position 23-30 in figure 39 were measured at the wavelength of 700 cm^{-1} on figure 40. All the carbon-carbon single bonds in the structure in figure 39 were measured at 1150, 1180, 1200 and 1300 cm^{-1} wavelength on figure 40. A double bond at position 13 on figure 39 was measured at 1500 cm^{-1} wavelength on figure 40. Ketone group on position 3 of figure 39 was measured at 1750 cm^{-1} wavelength in figure 40. All the single bonds between carbon and hydrogen atoms of the chemical structure in figure 39 were measured at the wavelength of 2300 cm^{-1} in figure 40.

Table 24: FTIR spectrum in (cm^{-1}) of unkown compound from vial A (see figure 40)

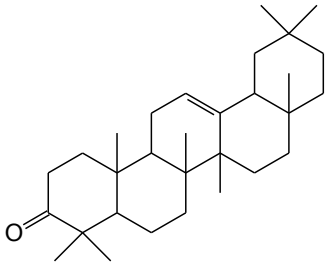
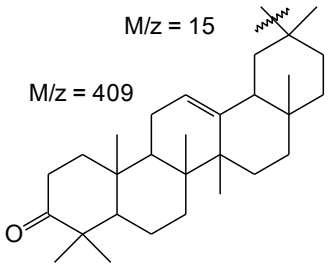
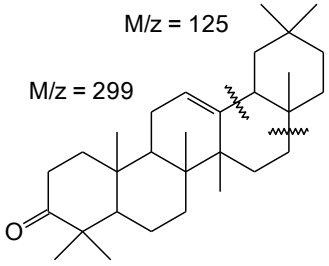
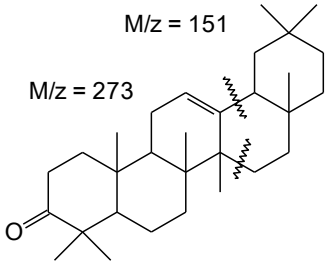
Infra-red spectrum of unknown compound from vial A		
Bond	**cm^{-1}**	**Notes**
R-C-H	700	Saturated alkane and alkyl groups
R-R	1150	Single bonds (Other than hydrogen)
R-R	1180	Single bonds (Other than hydrogen)
R-R	1200	Single bonds (Other than hydrogen)
R-R	1300	Single bonds (Other than hydrogen)
R-R	1400	Single bonds (Other than hydrogen)
C=C	1650	Double bond (often weak)
C=O	1750	Ketone
C-H	2300	Saturated alkane, single bonds to hydrogen

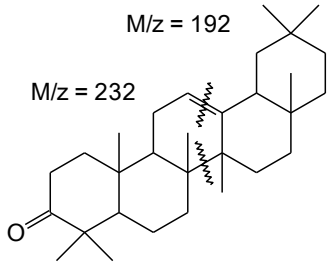
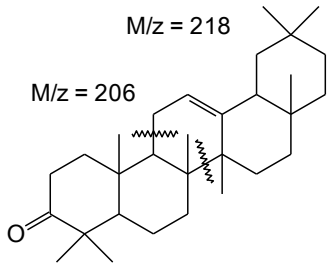
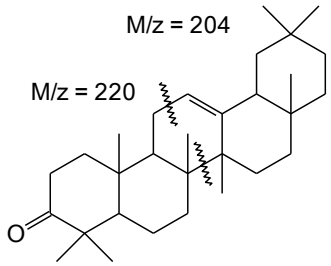
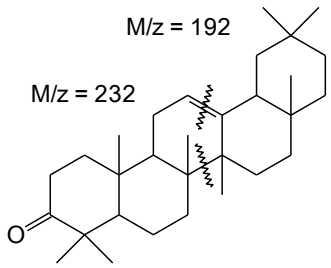
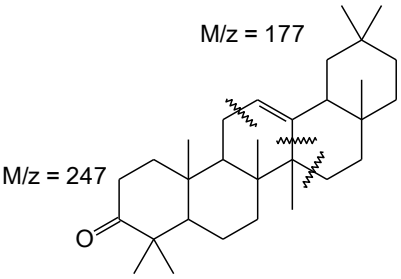
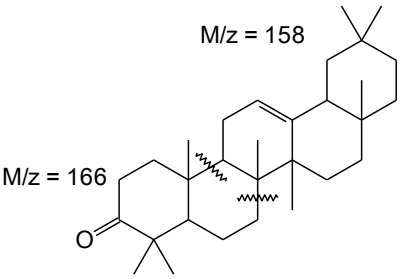
4.16 GC-MS fragmentation results of unknown compound A

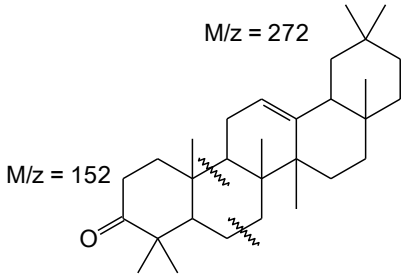
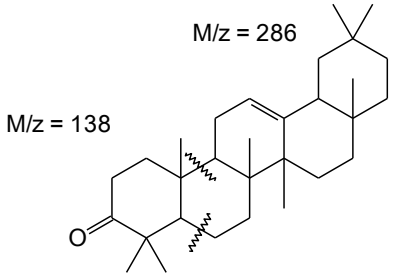
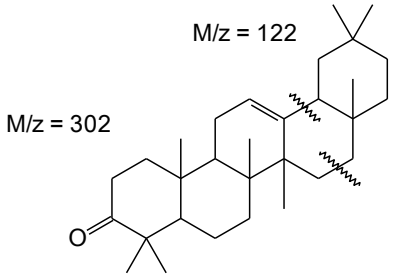
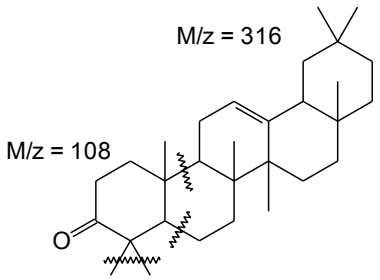
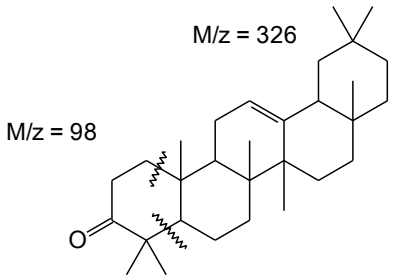
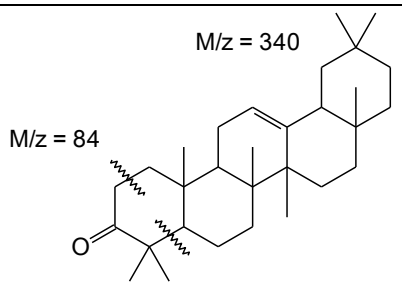
The unknown compound was analyzed with GC-MS. The calculated molecular ion of unknown compound was 424 amu. The molecular mass of compound A was 424 amu; this ion went through several fragmentation reactions involving the breaking of one or two carbon-carbon bonds: 424, calc: 424.37 (100.0%), 425.37 (33.4%), 426.38 (5.6%); 409, calc: 409.35 (100.0%), 410.35 (33.0%), 411.35 (5.2%); 299, calc: 300.2 (100.0%), 274.2 (21.6%), 275.2 (2.4%); 273, calc: 273.2 (100.0%), 274.4 (21.6%), 275.2 (2.4%); 232, calc: 233 (100.0%), 234.2 (18.2%), 235.2 (1.8%); 218, 218.2 (100.0%), 219.2 (17. 1%), 220.2 (1.6%); 203, 203.1 (100.0%), 204.1 (15.9%), 205.2 (1.2%); 190, calc: 190.1 (100.0%), 191.1 (14.8%), 192, 1

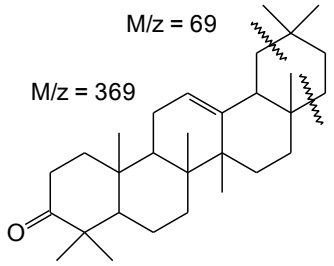
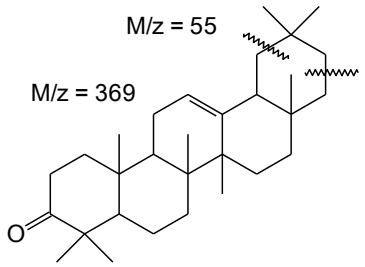
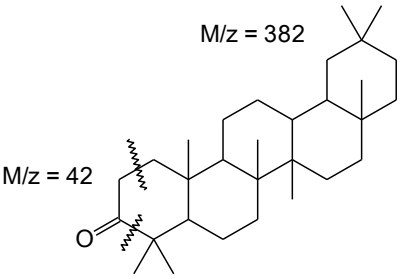
(1.2%); 175, calc: 175.1 (100.0%), 176.1 (13.6%), 177.1 (1.1%); 161, calc: 161.1 (100.0%), 162.1 (12.5%); 147, calc: 147.1 (100.0%), 148.1 (11.3%); 135, calc: 135.1 (100.0%), 136.1 (10.2%); 122, calc: 122.1 (100.0%), 123.1 (9.1%); 109, calc: 109.1 (100.0%), 110.1 (8.0%); 95, calc: 95.0 (100.0%), 96.1 (6.8%); 81, calc: 81.0 (100.0%), 82.0 (5.7%); 69, 68.0 (100.0%), 69.0 (4.5); 55, 55.0 (100.0%), 56.0 (3.4); 41, 41.0 (100.0%), 42.0 (2.3%). The fragmentation reactions are illustrated in table 26.

Table 25: GC-MS fragmentation of unknown compound from vial A (see figure 35)

Fragment (M/z)	Found (M/z)	Calculated (M/z)	Fragmentation structure
1 (M^+)	424	424.37 (100.0%), 425.37 (33.4%), 426.38 (5.6%)	
2	409	409.35 (100.0%), 410.35 (33.0%), 411.35 (5.2%)	
3	299	300.2 (100.0%), 274.2 (21.6%), 275.2 (2.4%)	
4	273	273.2 (100.0%), 274.4 (21.6%), 275.2 (2.4%)	

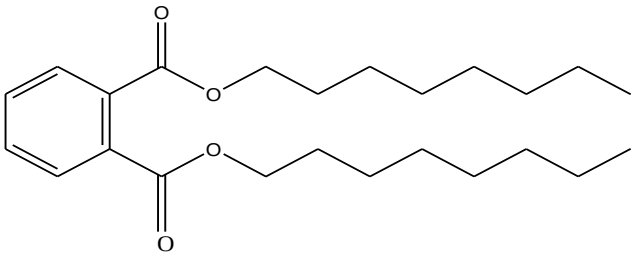
5	232	233 (100.0%), 234.2 (18.2%), 235.2 (1.8%)	
6	218	218.2 (100.0%), 219.2 (17. 1%), 220.2 (1.6%)	
7	203	203.1 (100.0%), 204.1 (15.9%), 205.2 (1.2%)	
8	190	190.1 (100.0%), 191.1 (14.8%), 192, 1 (1.2%)	
9	175	175.1 (100.0%), 176.1 (13.6%), 177.1 (1.1%)	
10	161	161.1 (100.0%), 162.1 (12.5%)	

11	147	147.1 (100.0%), 148.1 (11.3%)	
12	135	135.1 (100.0%), 136.1 (10.2%)	
13	122	122.1 (100.0%), 123.1 (9.1%)	
14	109	109.1 (100.0%), 110.1 (8.0%)	
15	95	95.0 (100.0%), 96.1 (6.8%)	
16	81	81.0 (100.0%), 82.0 (5.7%)	

17	69	68.0 (100.0%), 69.0 (4.5)	
18	55	55.0 (100.0%), 56.0 (3.4)	
19	41	41.0 (100.0%), 42.0 (2.3%)	

4.17 Proposed structure of unknown compound from vial E

The chemical structure of compound from vial E was not proposed because it was masked by a pollutant which interfered during the analytical process. This pollutant may have contaminated the leaves since they were not washed before extraction. According to Kirchmann *et al* (1991), phthalates released to environment can be deposited on or taken up by crops, meaning that there is a possibility that *Combretum collinum* subsp *suluense* may have absorbed di-n-octylphthalate from soil. The structures of compound B and F were not elucidated because their yields were not enough for spectroscopic analysis.



M/z = 391.31

Figure 42: Structure of pollutant that masked compound from vial E

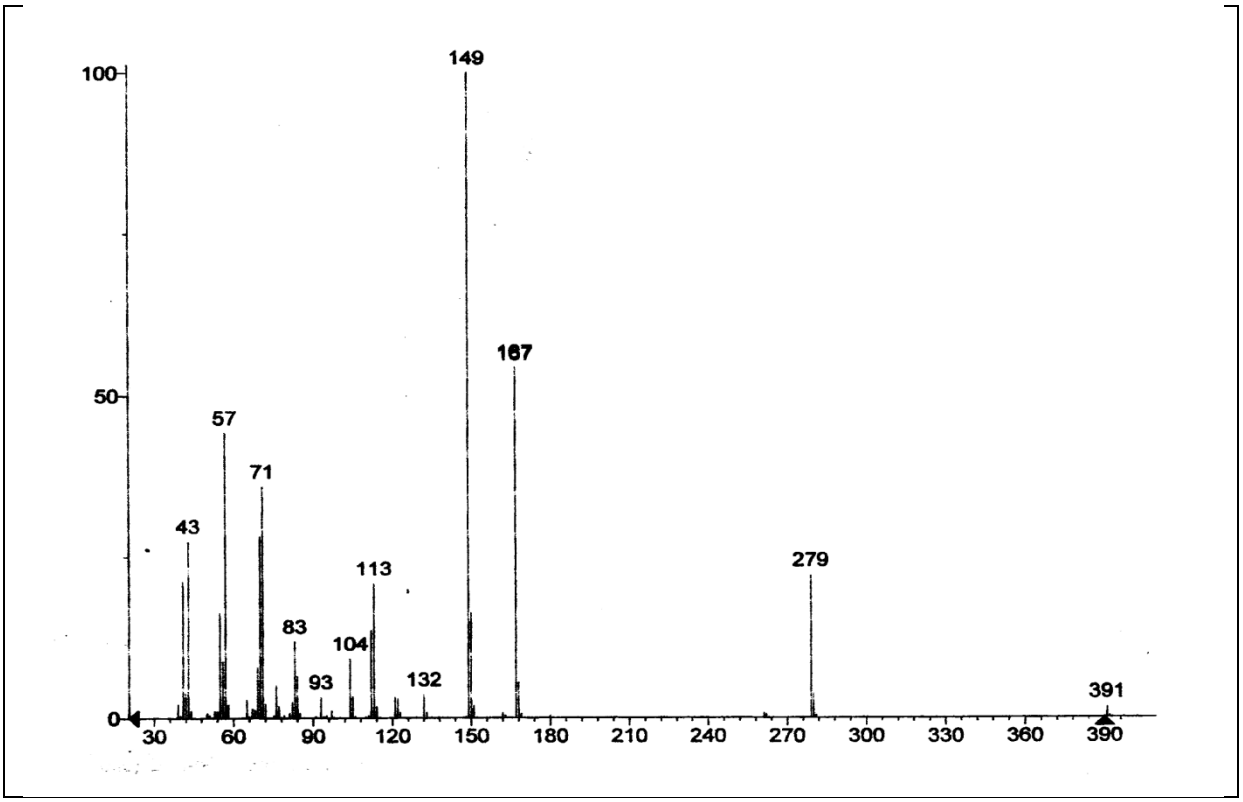


Figure 43: Mass spectrum of second unknown compound from vial E

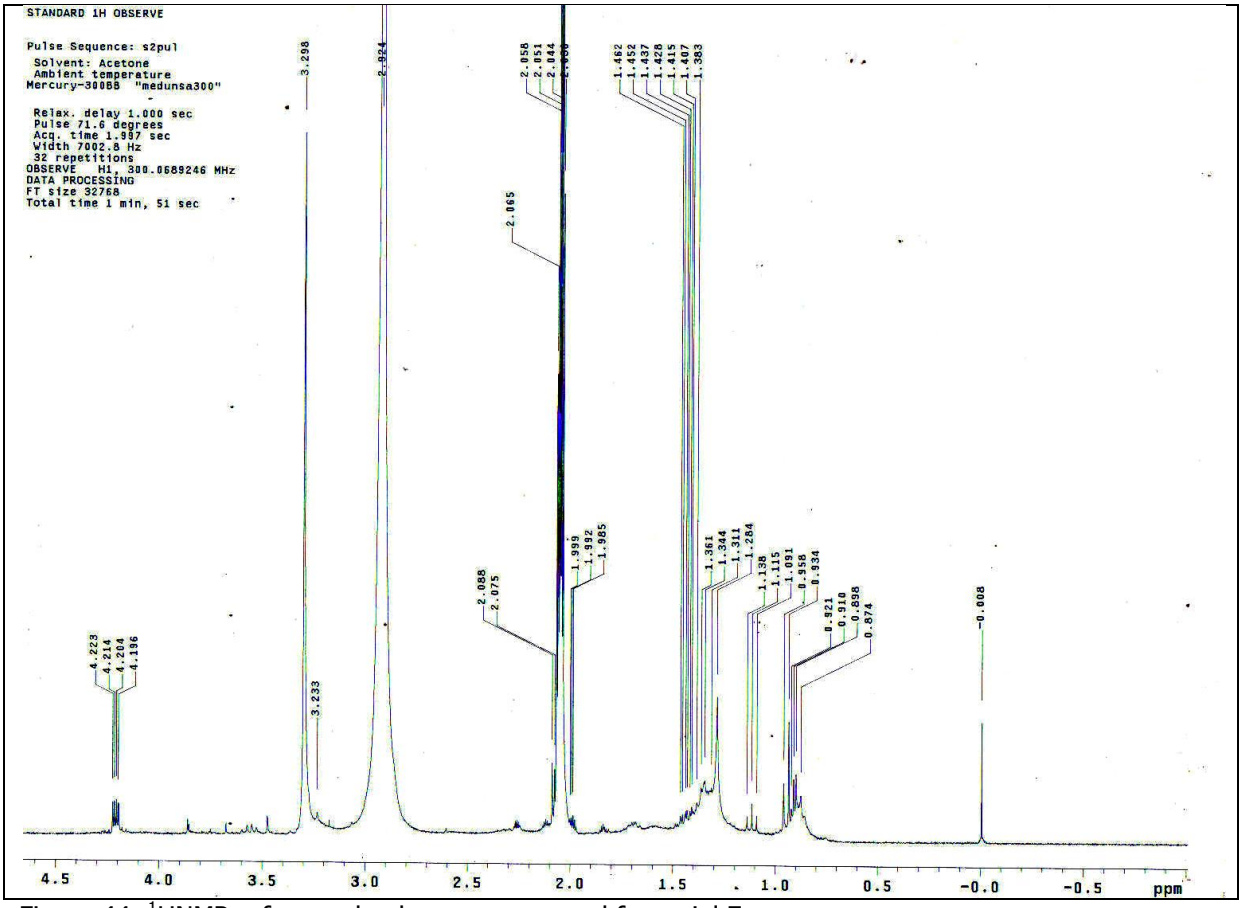


Figure 44: [1]HNMR of second unknown compound from vial E

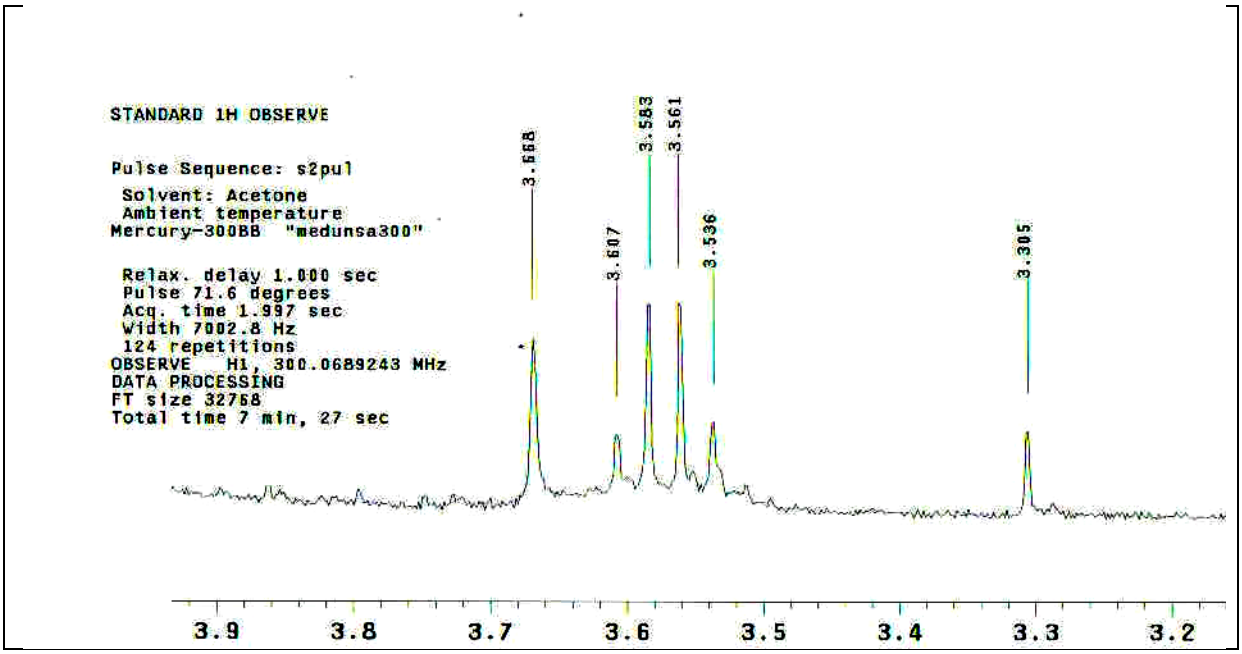


Figure 45: 1HNMR of second unknown compound from vial E from chemical shift 3.2 to 3.9 ppm.

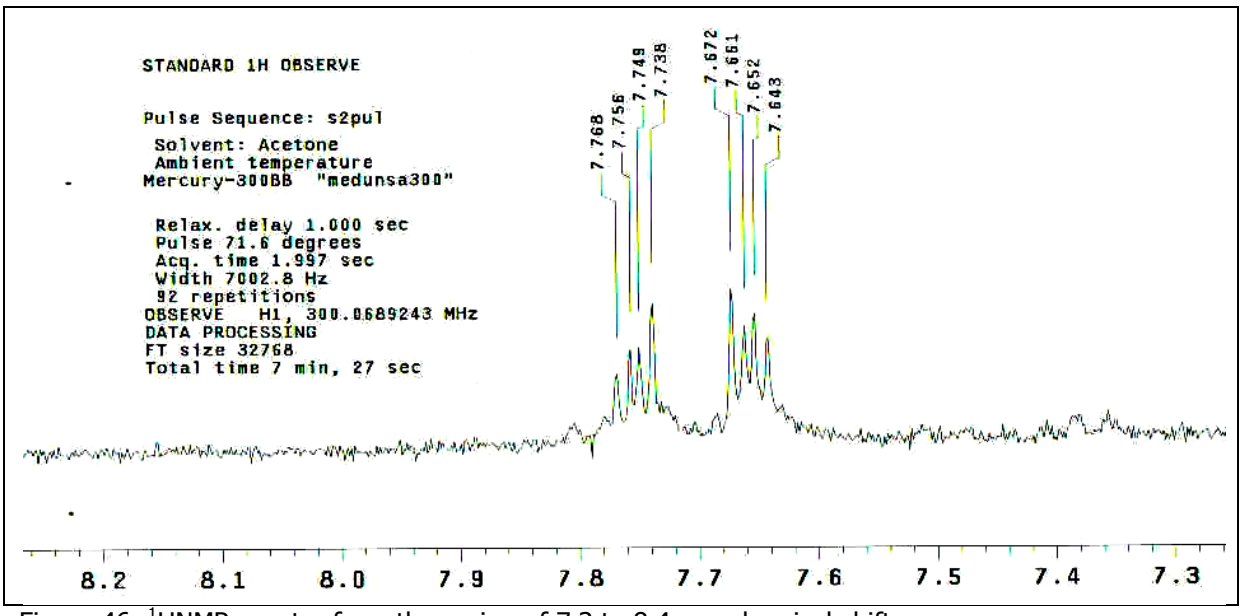


Figure 46: [1]HNMR spectra from the region of 7.3 to 8.4ppm chemical shift.

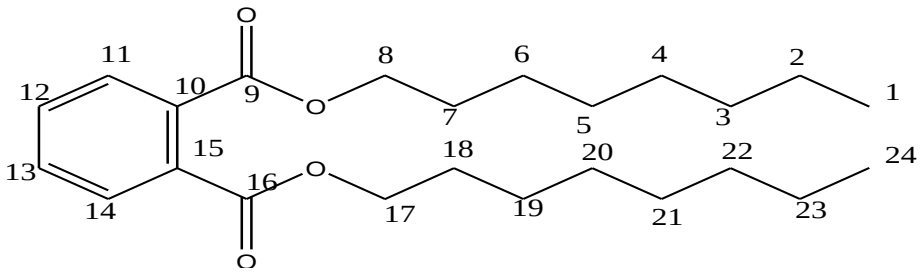


Figure 47: Numbered structure of compound E.

4.18 [1]HNMR analysis of second unknown compound E

The yield of unknown compound was 4.8% and it was yellow in colour. This compound analyzed using [1]HNMR (300 MHz), using tetramethylsilane (TMS) as a reference and chloroform as solvent. The data are listed in table 26.

Table 26: Interpretation of ^{1}HNMR results of second unknown compound

Position	Integration	Multiplicity	Chemical shift
1	3H	Triplet	1.091-1.138
2	2H	Sextet	2.044-2.088
3	2H	Quintet	3.305
4	2H	Quintet	3.536-3.668
5	2H	Quintet	3.305
6	2H	Quintet	3.536-3.668
7	2H	Quintet	3.305
8	2H	Triplets	1.985-1.999
9	-	-	
10	-	-	
11	1H	Doublets	4.196& 4.204
12	1H	Doublet of doublets	7.738-7.768
13	1H	Doublet of doublets	7.643 - 7.672
14	1H	Doublets	4.214& 4.223
15	-		
16	-		
17	2H	Triplets	1.985-1.999
18	2H	Quintet	3.536-3.668
19	2H	Quintet	3.305
20	2H	Quintet	3.536-3.668
21	2H	Quintet	3.305
22	2H	Quintet	3.536-3.668
23	2H	Quintet	-
24	3H	Triplets	1.091-1.138

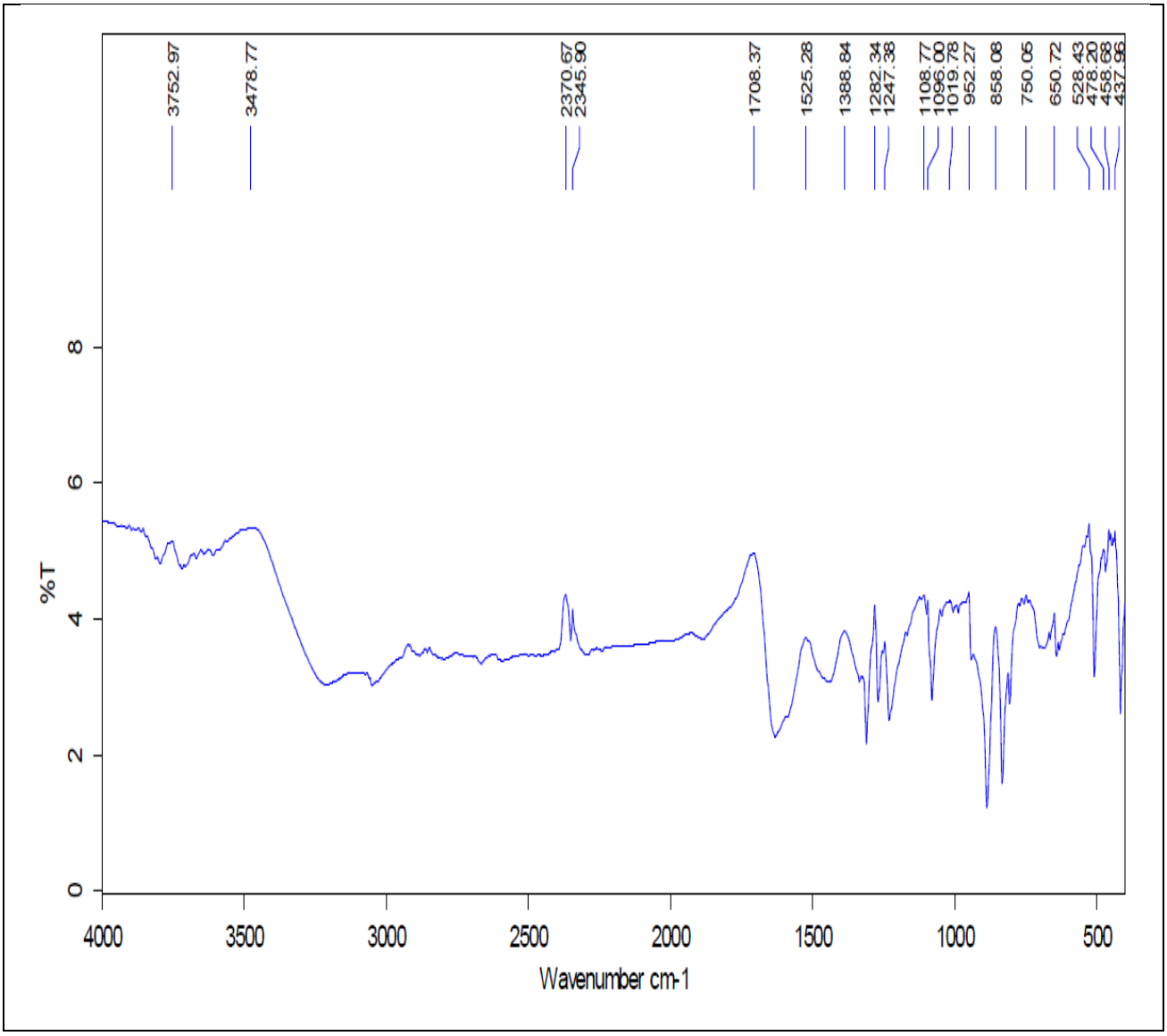


Figure 48: FTIR spectrum of second unknown compound

4.19 FT-IR analysis of second unknown compound E

This compound analyzed using Fourier transform infrared spectroscopy. The carbon-carbon single bonds on unknown compound in figure 46 were measured at 437.96 cm^{-1} to 650.72cm^{-1} wavelength in figure 48. The carbon-carbon and carbon-hydrogen bonds in benzene ring of compound E in figure 47were measured at the wavelength of 750.06 cm^{-1}, 858.08cm^{-1}, 952.27 cm^{-1}, 1019.78 cm^{-1}, 1096.00 cm^{-1} and 1108.77 cm^{-1} in figure 48. The carbon oxygen single bonds from ester groups at compound E in figure 46 were measured at 1247.38 cm^{-1} and 1282.34 cm^{-1} in figure 48. The two functional groups C=O of esters in figure 48 were measured at 1708

cm^{-1} and 1525.28 cm^{-1} in figure 48. The carbon-hydrogen single bonds of saturated alkane were measured at 2370.67 cm^{-1} wavelength in figure 48. The data are listed in table 27.

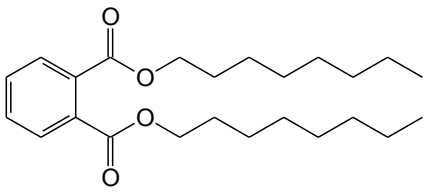
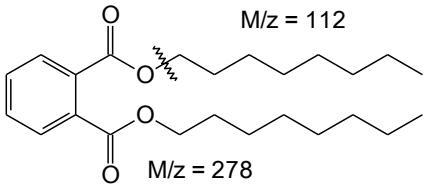
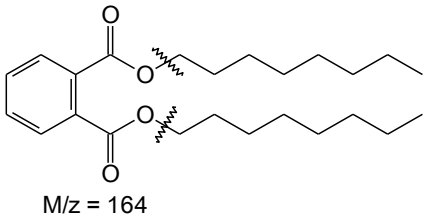
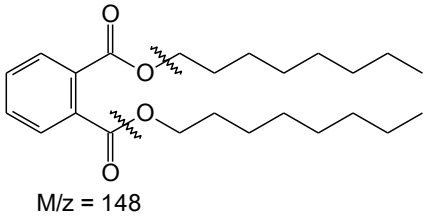
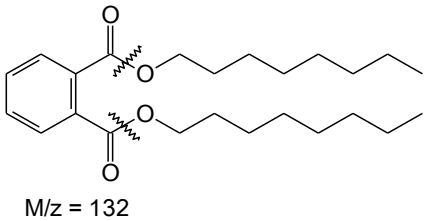
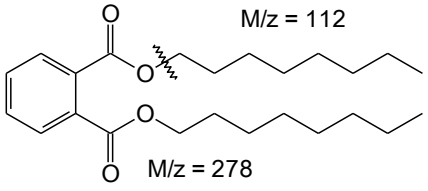
Table 27: FTIR spectrum results of compound E wave number in (cm^{-1}) (See figure 47)

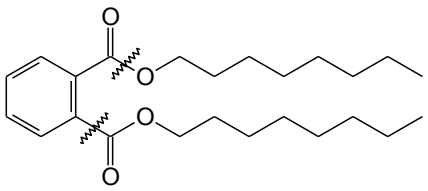
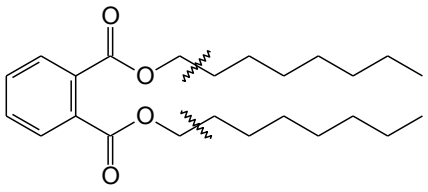
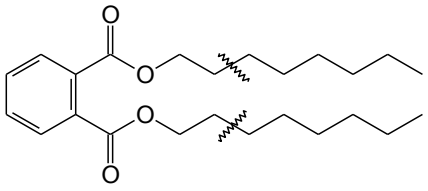
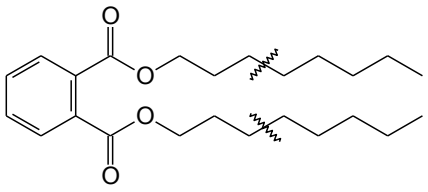
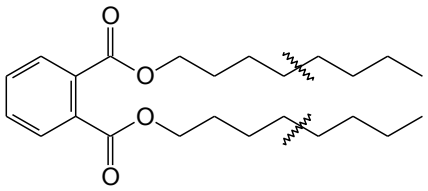
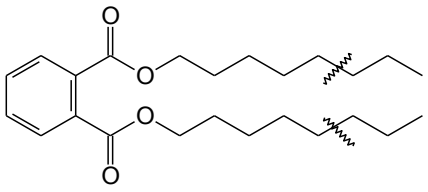
Infrared spectrum of Compound E		
Bond	**cm^{-1}**	**Notes**
C-C	437.96	No diagnostic value
C-C	458.68	No diagnostic value
C-C	528.43	No diagnostic value
C-C	650.72	No diagnostic value
R-C-H	750.06	Aromatics
R-C-H	858.08	Aromatics
R-C-H	952.27	Aromatics
R-C-H	1019.78	Aromatics
R-C-H	1096.00	Aromatics
R-C-H	1108.77	Aromatics
C-O	1247.38	Difficult to assign
C-O	1282.34	Difficult to assign
R-C-H	1525.28	Alkyl groups
C=O	1708.37	Esters
C-H	2370.67	Saturated alkane, single bond to hydrogen

4.20 The GC-MS analysis of second unknown compound

The compound was analyzed using GC-MS. The molecular ion of unknown compound E was 391 amu. This molecular ion went through a series of fragmentation reactions: 391, calc: 390.28 (100.0%), 391.28 (27.4%), 392.28 (4.3%); 279, calc: 279.16(100.0%), 278.15 (18.3%), 280.16 (2.4%); 167, calc: 166.03 (100.0%), 167.03 (9.1%), 168.03 (1.2%); 149, calc: 149.0 (100.0%), 150.0 (9.1%); 132, calc: 132.0 (100.0%), 133.0 (9.0%); 113, calc: 116.0 (100.0%), 117.0 (9.0%); 104, calc: 104.0 (100.0%), 105.0 (7.9%); 93, calc: 93.1 (100.0%), 94.1 (7.9%); 83, calc: 83.1(100.0%), 84.1 (6.8%); 71, 71.1 (100.0), 72.1 (5.7%); 57, 57.1 (100.0%), 58.1 (4.6%); 43, calc: 43.1 (100.0%), 44.1 (3.4%). The fragmentation reactions are illustrated in table 30.

Table 28: GC-MS fragmentation of Compound E (See figure 46)

Fragment	Found (M/z)	Calculated	Fragment structure
1 (M^+)	391	390.28 (100.0%), 391.28 (27.4%), 392.28 (4.3%)	
2	279	279.16(100.0%), 278.15 (18.3%), 280.16 (2.4%)	
3	167	166.03 (100.0%), 167.03 (9.1%), 168.03 (1.2%)	
4	149	149.0 (100.0%), 150.0 (9.1%)	
5	132	132.0 (100.0%), 133.0 (9.0%)	
6	113	116.0 (100.0%), 117.0 (9.0%)	

7	104	104.0 (100.0%), 105.0 (7.9%)	 M/z = 104
8	93	93.1 (100.0%), 94.1 (7.9%)	 M/z = 99
9	83	83.1(100.0%), 84.1 (6.8%)	 M/z = 85
10	71	71.1 (100.0), 72.1 (5.7%)	 M/z = 71
11	57	57.1 (100.0%), 58.1 (4.6%)	 M/z = 57
12	43	43.1 (100.0%), 44.1 (3.4%)	 M/z = 43

Chapter 5

Conclusion

5.1 Background

Borek (2001) found that some antioxidant compounds can protect against cardiovascular diseases and reduce risk factors for heart attacks as well as stroke and blood pressure, whereas other antioxidant compounds increase circulation in capillaries and prevent clot-forming platelet activity and inflammation. According to Pinto *et al* (2000) some antioxidant compounds have anti-cancer activity that protect against free radical and carcinogen induced DNA damage and increases carcinogen detoxification and also stop the growth of a wide variety of human cancer cells including breast, colon and prostate cancer.

5.2 Antioxidant Activity

In conclusion unknown compound A which was found to be Olean-12-ene-3-one isolated from *Combretum collinum* subspecies *suluense*, was found to have antioxidant activity and it must undergo further tests to find out if it can protect against cardiovascular diseases, heart attack and if it can stop the growth of human cancer cells which include breast; colon and prostate cancer, which are considered to be dangerous diseases.

5.3 Inhibition of bacterial strain by two isolated compounds

An olean-12-ene-3-one was found to inhibits the growth of *Escherichia coli* at 0.5689 mg/ml concentration and *Staphylococcus aureus* at 3.846 mg/ml in table 21 & 22.

Compound E which was contaminated with dioctylphthalate was found to inhibit the growth of *S. aureus* at 1.479 mg/ml and *E. coli* (0.5689 mg/ml) in table 21 & 22.

These two compounds can be modified and used as the remedy for diseases caused by *S. aureus* and *E. coli.*

Chapter 6

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