CHAPTER 1: GENERAL INTRODUCTION

The utilisation of plants in the treatment of certain human diseases is evidence of man's ingenuity. The contribution of these plants to the therapeutic arsenal in the fight against disease dates back several centuries, and has, to a certain extent, been documented by the ancient Chinese, Indian and North African civilisations. Currently, traditional medicine is widely practiced, especially in developing countries. This is a result of primary health care facilities being unable to manage the number of patients requiring aid, the high cost of Western pharmaceuticals and health care, as well as the fact that traditional health care is highly sought after in terms of certain cultural elements in the lives of these individuals within these societies (Taylor *et al.*, 2001).

In southern Africa, a large proportion of the population still uses traditional remedies. More than 700 plant species are being traded for medicinal purposes throughout South Africa, in the informal medicinal plant market (Dold and Cocks, 2002). This vast usage of and great dependence on traditional plants as the preferred form of health care is aided by the fact that most of these plants are widely available and affordable, and additionally encompasses practices based on the social-cultural norms and religious beliefs. It is evident that, even though scientific advances have been made in our quest to understand the physiology of the body, biotechnology and the treatment of disease, natural products remain a crucial component of the comprehensive health care strategy for the future (Patwardhan, 2005).

The World Health Organization (WHO) defines traditional medicine as the "diverse health practices, approaches, knowledge and beliefs incorporating plant-, animal- and/or mineral-based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose, or prevent illness". It is clear, however, that there is a need to validate the information through an organised infrastructure for it to be used as an effective therapeutic means, either in conjunction with existing therapies, or as a tool in novel drug discovery. Traditional medicine utilises biological resources and the indigenous knowledge of traditional plant groups, the latter being conveyed verbally from generation to generation. This is closely linked to the conservation of biodiversity and the related intellectual property rights of indigenous people (Timmermans, 2003).

Although it is these traditional medicines that provided the link between medicine and natural products, it was not until the 19th century that active compounds were isolated and principles of medicinal plants identified (Phillipson, 2001). The Greek physician Dioscorides (AD 70) compiled an extensive listing of medicinal herbs and their virtues. This was originally written in Greek, and later translated into Latin as *De Materia Medica,* and remained the authority in medicinal plants for over 1500 years (Mendonça-Filho, 2006). Another Greek physician, Galen (AD 129-200), devised the pharmacopoeia describing the appearance, properties and use of many plants of his time. It was the discovery of medicines such as those listed in Table 1.1 that sparked an interest in the study of plants as medicinal agents; with the isolation of morphine from opium by Serturner (1805) being the start of natural product chemistry (Patwardhan *et al*., 2004).

Table 1.1: Drugs derived from plants, their clinical uses and sources (Fabricant and Farnsworth, 2001).

Drug	**Action or clinical use**	**Plant source**
Atropine	Anticholinergic	*Atropa belladona*
Colchicine	Antitumour, antigout agent	*Colchicum autumnale*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Emetine	Emetic, amoebicide	*Cephaelis ipecacuanha*
Morphine	Analgesic	*Papaver somniferum*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Quinine	Antimalarial	*Cinchona ledgeriana*

Despite the discovery of natural products from higher plants, the interest of chemists, pharmaceutical scientists and pharmacologists turned to the production of synthetic compounds. In the late 19th century, research was focused mainly on the modification of natural products, in an attempt to enhance biological activity, to increase selectivity and to decrease toxicity and side effects. Aspirin is one such example and was the earliest of these modified natural products. In more recent years, however, industry has once again turned its interests to natural product research (Phillipson, 2001). This is as a result of the

development of drug-resistant micro-organisms, side effects of modern drugs and emerging diseases for which no medicine is available.

1.1 Ethnopharmacological research

The study of plants used in traditional medicine requires the effective integration of information on chemical composition of extracts, pharmacological activities of isolated compounds, as well as indigenous knowledge of traditional healers. The acquisition of ethnobotanical information remains an empirical aspect in any such study (Soejarto, 2005). The process of isolating and identifying lead compounds from a complex mixture requires a number of specific resources, including comprehensive knowledge, specialised equipment and skill. The urgency of the discovery of new agents is a result of impenetrable factors that come into play, including the emergence of new killer diseases, known antimicrobial drug-resistance, the inefficiency of synthetic drug discovery and the high cost of bringing to market a single drug. A shift towards natural product research, which is further driven by remarkable advances in plant extract technology, biotechnology and analytical chemistry, is therefore inevitable.

There is a great need and ethical obligation to accurately document investigative findings on plants used for health purposes. This will additionally aid in the efficient preservation and conservation of traditional knowledge, thereby preventing the further disappearance of indigenous systems of medicine, which may potentially benefit society in general.

According to the Southern African Trade Directory of Indigenous Natural Products, more than 1 000 species of plants are used traditionally in southern Africa (Izimpande, 2005) of which the genus *Commiphora* is one.

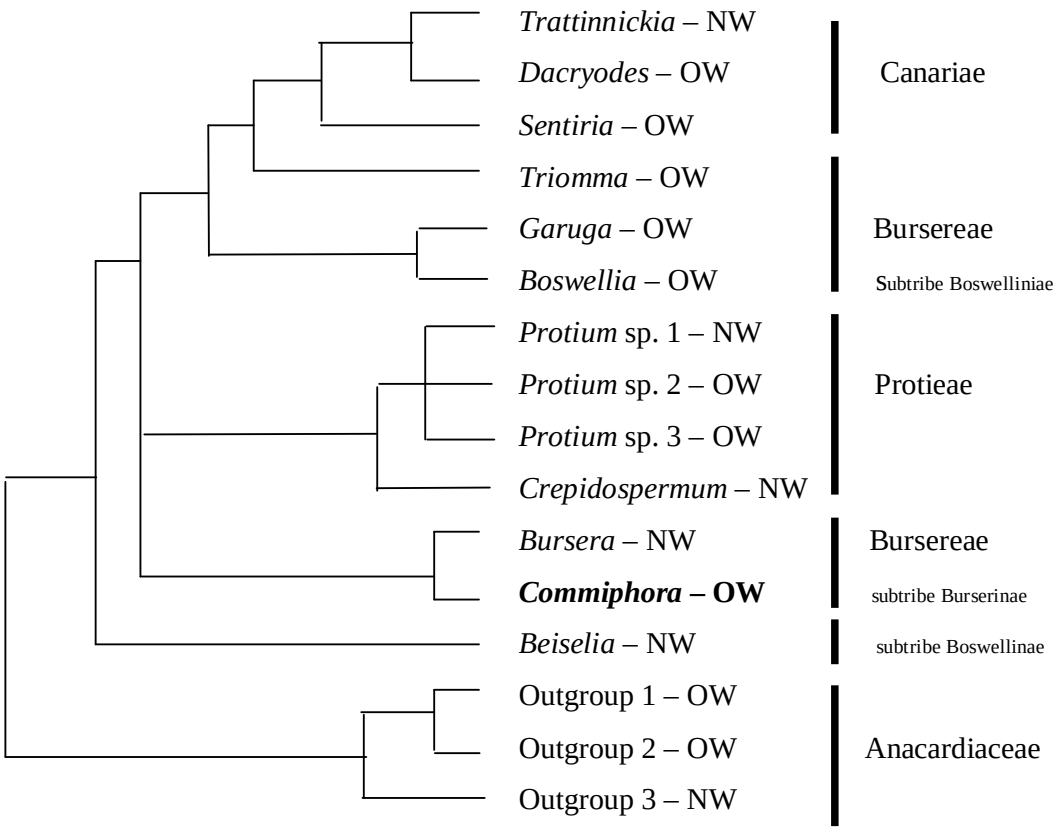
1.2 An introduction to the family Burseraceae and genus *Commiphora*

1.2.1 The family: Burseraceae

The Burseraceae is composed of both trees and shrubs of tropical and sub-tropical geographical distribution (Watson and Dallwitz, 1992). The bark of the trees are known mostly for producing fragrant resins of economic, medicinal and cultural value

(Langenheim, 2003), and for baring essential oils. The Burseraceae consists of approximately 700 species from 18 genera. This family is divided into three tribes, namely Canarieae, Protieae and Bursereae, each distributed pantropically across a broad range of low-elevation, frost-free habitats including rainforest, dry deciduous forest and desert (Weeks *et al.*, 2005). This classification is based exclusively upon their fruit structure. In a study by Clarkson *et al*. (2002), the tribal relationships within the Burseraceae were revealed by the phylogeny depicted in Figure 1.1. This was determined through the sampling of chloroplast rps 16 intron sequences (ribosomal protein genes) from 13 species of 11 genera within the Burseraceae, rooting their analysis with several genera in the Anacardiaceae, a family closely resembling the Burseraceae and with which it has been closely allied traditionally. The Burseraceae is one of nine flowering plant families belonging to the order Sapindales that comprise the monophyletic group (5,700 species), whos first known fossils appear in Europe ca. 65 million years ago (Ma). Two of the three tribes, namely Canarieae and Protieae, were determined to be monophyletic, while the Burserae were shown to be polyphyletic.

Commiphora has several species in Africa and at least one species (*Commiphora leptophloeos*) in South America. A Gondwanan origin of the family was assumed and the separation of Africa and South America (95 - 100 Ma) was used to date disjunct *Commiphoras* common ancestor and thereby calibrate the difference in times across the family (Becerra, 2005). The hypothesis of the Gondwanan origin needed to be refined. The molecular phylogenies of the Burseraceae were based on nuclear and chloroplast DNA data for 13 of the 18 genera. A map of the Early Eocene continents and their relative positions helps illustrate the expansive distribution of the Burseraceae (Figure 1.2). The Early Eocene age of the Burserinae and its broadly North American origin implicates at least one migration event to the Old World to explain the African, Madagascar, and Indian distributions of *Commiphora* species. The data obtained supported a North American Paleocene origin for the Burseraceae, followed by the dispersal of ancestral lineages to North America and the Southern Hemisphere. *Commiphora* appears to have dispersed and radiated within continental Africa approximately 44 Ma, during the Middle Eocene. The spread of *Commiphora* to India appears to have occurred in relatively recent geologic times, approximately 5.0 Ma (Weeks *et al*., 2005).



NW = New world, OW = Old world

Figure 1.1: The tribal relationships within the family Burseraceae as suggested by the phylogeny of Clarkson *et al*. (2002).

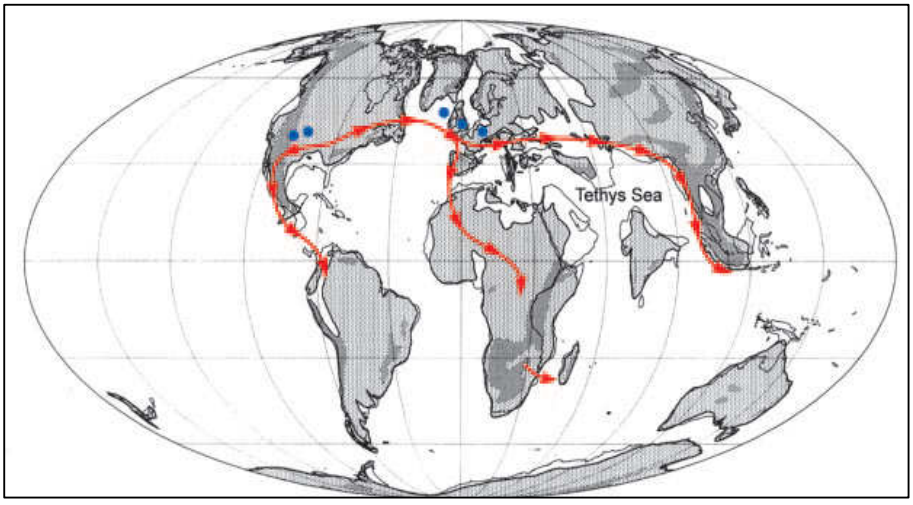


Figure 1.2: The North American origin and dispersal hypothesis for Burseraceae. The map shows Eocene shorelines (53 Ma) and early Eocene fossil locations of Burseraceae (blue circles) (Weeks *et al*., 2005).

1.2.2 The genus *Commiphora*

The name *Commiphora* originates from the Greek words *kommi* (meaning 'gum') and *phero* (meaning 'to bear'). The majority of the species yield a fragrant oleo-gum-resin following damage to the bark (Steyn, 2003). *Commiphora* has shown to dominate over 1.6 million km^2 of *Acacia-Commiphora* woodland in (sub-) tropical East Africa (Weeks and Simpson, 2006). Of the more than 200 species of *Commiphora* native to the seasonally dry tropics of Africa, Arabia and India, about 40 species occur in southern Africa (Steyn, 2003).

Although the common name for *Commiphora* species is 'corkwood' (an indication of the softness of the wood), it is suitable for use as fences, as well as for carving utensils and other ornaments. The Afrikaans name for *Commiphora* is 'kanniedood', the direct translation being 'cannot die'. This is an indication of the sustainability of the plant and also refers to the fact that the truncheons grow easily when planted. Yellowing and shedding of the leaves occurs early in autumn, and the plants are deciduous for most of the year, a feature very typical of *Commiphora*. Aspects of the life history of the species include a deciduous habit, a predominantly dioecious breeding system and a tendency to produce flowers prior to developing leaves (Weeks and Simpson, 2006).

1.2.3 Characteristic features of *Commiphora* species

Characteristic features of the species are very diverse, and thus require a combination of morphological characters for identification. The bark of most *Commiphora* species is papery and peels off into papery flakes, revealing a green bark underneath (Figure 1.3, left). The leaves are mostly compound, with only a few species bearing simple leaves.

The fruit of *Commiphora* greatly enhances the identification of the species. When ripe, the fruit splits into halves revealing a brightly coloured pseudo-aril, shown in Figure 1.3 (right). This fleshy appendage completely or partially encompasses the seed as part of an attachment around part of the seed. The shape of the pseudo-aril differs from species to species. The flowers may be uni or bisexual, with the unisexual flowers only being semi-developed with non-functional stamens (Steyn, 2003).

Figure 1.3*: Characteristic features of *Commiphora*, with its papery bark, trifoliate leaves, ripe fruit and pseudo-aril (left); pseudo-aril with exposed black seeded stone (right).

*All photographs supplied by A.M. Viljoen unless otherwise stated.

1.3 *Commiphora myrrha*

"Who is coming up from the wilderness
Like palm-trees of smoke,
Perfumed with myrrh and frankincense,
From every powder of the merchant?"

"Till the day doth break forth,
And the shadows have fled away,
I will get me unto the mountain of myrrh,
And unto the hill of frankincense."

(from: The Holy Bible, in the Old Testament as quoted by Dharmananda, 2003)

In biblical times, *Commiphora myrrha* (Figure 1.4) was valued as much as gold, and is mentioned numerous times in the Old Testament, in instructions to Moses about making incense and anointing oil, for which it has been used throughout history. Also of great

religious importance, it was presented as one of the three gifts to Christ by the Magi, and was used to anoint the body of Christ after the crucifixion (Dharmananda, 2003).

Figure 1.4: *Commiphora myrrh*, a thorny shrub or small tree about 3 m in height (http://www.naturesessences.com).

In Arabic and Hebrew the word *murr* means 'bitter'. Myrrh is an aromatic oleo-gum-resin of pale yellow colour, changing to dark red upon hardening (Figure 1.5).

Figure 1.5: Oleo-gum-resin of myrrh (http://www.aromatherapyforeveryone.com/myrrh).

The gum is obtained from the bark of a number of different species of *Commiphora*, and more specifically by bark incising, which results in gum exudation. The following *Commiphora* species are collectively considered as a source of myrrh (Hanuš *et al.*, 2005):

- *Commiphora myrrha* (Nees) Engl. - True myrrh
- *C. myrrha* (Nees) Engl. var. *molmol* Engl. (*C. molmol* Engl. ex Tschirch) - Somalian myrrh
- *C. abyssinica* (Berg.) Engl. (syn. *C. madagascarensis* Jacq.) - Arabian myrrh, Abyssinian myrrh
- *C. africana* (A. Rich.) Engl. - Myrrh, African bdelium
- *C. guidottii* Chiov. - Sweet myrrh
- *C. mukul* (Hook ex Stocks) Engl. (*C. wightii* (Arnott.) Bhanol.) - Guggul, Guggulu, False myrrh
- *C. opobalsamum* (*C. gileadensis* (L.) Christ; *Balsamodendron meccansis* Gled.) - Balm of Mecca. (*C. opobalsamum* balm is the thickened gum from the juice of the balsam tree)
- *C. erythraea* (Ehrenb.) Engl. - Opopanax (originally *Hemprichia erythraea*)
- *C. erythraea* var. *glabrascens* - Opopanax
- *C. kataf* (Forssk.) Engl. - African opopanax

Currently the chief source of true myrrh is from *C. myrrha*, however, *C. erythraea*, a variable species found in southern Arabia, northeast Africa, and as far south as Kenya; was the principle source during ancient and classical times (Hanuš *et al.*, 2005).

1.4 Medicinal uses of myrrh and guggul

It is said that Greek soldiers would not go into battle without a poultice of myrrh to apply as a wound dressing on their wounds (Hanuš *et al.*, 2005). It is also applied to abrasions as an antiseptic and anti-inflammatory agent, and for the treatment of menstrual pain. It is an astringent of the mucous membranes and the mouth. *Commiphora molmol* is used as an effective antimicrobial agent, as a herbal remedy for sore throats, canker sores and gingivitis. Myrrh is also used as a healing tonic, as a stimulant and as a hypolipidaemic agent (Urizar *et al.*, 2002; Wu *et al.*, 2002; Ulbright *et al.*, 2005). Traditionally, myrrh is used for the common cold, to relieve nasal congestion and coughing. It is also used for the

treatment of wounds and ulcers, especially infections of the mouth, gums and throat (van Wyk and Wink, 2004). Myrrh is also found in most Egyptian, Syrian and Roman recipes for drugs or ointments for beauty treatments in antiquity (Ciuffarella, 1998).

1.4.1 *In vitro* pharmacological investigations of myrrh and guggul

i. Antimicrobial activity

The antibacterial activity of some constituents of *C. mukul* oleo-gum-resin essential oil, chloroform extract and isolated sesquiterpenoids have been evaluated. A wide range of inhibitory activity against Gram-positive and Gram-negative bacteria was observed (Saeed and Sabir, 2004). The isolation and identification of muscanone from *C. wightii*, by Fatope *et al*. (2003), was found to be active against *Candida albicans*. *Commiphora* has been used in combination with other plant species in the development of a pharmaceutical formulation. One of the formulas of "The Jerusalem Balsam", found in a manuscript form in the archive of the monastery contains four plants: olibanum (*Boswellia* spp.), myrrh (*Commiphora* spp.), aloe (*Aloe* spp.) and mastic (*Pistacia lentiscus* L.). Pharmacological assays conducted on this formulation indicated antiseptic properties (Moussaieff *et al*., 2005).

ii. Antimycobacterial activity

Commiphora mukul, used traditionally for the treatment of tuberculosis, was assayed for antimycobacterial activity (Newton *et al*., 2002). The crude methanolic resin extract displayed significant antimycobacterial activity, with a minimum inhibitory concentration (MIC) of 62.5 µg/ml against *Mycobacterium aurum*. Fractionation of the resin lead to a decrease in activity, indicating that the activity displayed is as a result of synergistic interaction of the compounds present.

iii. Anti-oxidant activity

Antioxidant effects are a possible mediator in the protection against myocardial necrosis, inhibition of platelet aggregation, as well as increased fibrinolysis by guggulipid, the extract from the myrrh resin.

iv. Anti-inflammatory activity

A number of studies suggest that guggul elicits significant anti-inflammatory activity (El-Ashry *et al.*, 2003). "The Jerusalem Balsam", contains four plants, one of which is myrrh. In order to examine the anti-inflammatory effect of the Balsam, an external ear mouse model of inflammation was used. Sabra white mice were injected intraperitoneally, 1 hour prior to arachidonic acid administration on the ears (5 mg in 5 µl of ethanol). Significant inhibitory activity on arachidonic acid-induced swelling of the external mouse ear was observed, both in thickness and in redness, indicating the Balsam's anti-inflammatory properties (Moussaieff *et al.*, 2005).

v. Antitumour activity

Recently the cytotoxic and antitumor activity of myrrh has proved to be substantially significant. Hydrogen peroxide (H_2O_2) induces a number of mutations, resulting in a variety of genetic alterations. The ability of medicinal herbs, one of which being *C. molmol*, to suppress H_2O_2-induced mutant frequency in treated human fibroblast cells (GM00637) at the hypoxanthine guanine phosphoribosyl transferase (HPRT) locus, was examined. The result was a percentage inhibition greater than 60% at the HPRT locus (You and Woo, 2004).

1.4.2 *In vivo* pharmacological investigations of myrrh and guggul

i. Treatment of hyperlipidaemia

The farnesoid X receptor (FXR) has been identified as a bile acid-activated nuclear receptor (ligand-inducible transcription factors consisting of two binding domains, viz. a ligand- and a DNA-binding domain) with a regulatory role in cholesterol metabolism, controlling bile-acid synthesis, conjugation and transport, as well as lipid metabolism (Sinal and Gonzalez, 2002). It has been hypothesised that FXR senses bile acid levels and mediates the transcriptional repression of genes responsible for the conversion of excess cholesterol into bile acids. It is thought to be responsible for the induction of genes necessary for bile acid transport (Koch and Waldmann, 2005). Guggulsterone, isolated from *C. mukul*, can act as an effective antagonist to the FXR, thereby decreasing the expression of bile acid-activated genes (Sahoo *et al.*, 2003).

Guggulsterone also blocked the accumulation of hepatic cholesterol in cholesterol fed mice, while this action was not observed in those mice devoid of FXR. Any manipulation of FXR would thus affect hepatic cholesterol levels (Urizar *et al.*, 2000). The effects of natural products such as guggulsterone may thus result in the acquisition of additional agents with desired therapeutic effects on the functioning of nuclear hormone receptors.

ii. Cardioprotective effects

Chander *et al.* (2003) induced myocardial necrosis in rats with isoproterenol. Treatment with guggulsterone and both its isomers viz. *Z*-guggulsterone (*trans*) and *E*-guggulsterone (*cis*), at a concentration of 50 mg/kg, for 5 days, significantly protected cardiac damage, as assessed by the reversal of blood and heart biochemical parameters in ischaemic rats. Guggulsterone also inhibited oxidative degradation of low-density lipoprotein in humans and rat liver microsomes (induced by metal ions) *in vitro*.

iii. Parasitological studies

In a study by Haridy *et al*. (2003), the efficacy of *C. molmol* (Mirazid) was evaluated in sheep naturally infected with fascioliasis. Mirazid proved to be safe (no clinical side effects) and very effective with a total dose of 600 mg (given for two to three consecutive days) being 83.3% effective, and 900 – 1200 mg being 100% effective in sheep.

The efficacy of *C. molmol* (Mirazid) in the treatment of *Schistosomiasis haematobium* in individuals of the Tatoon village in Egypt was evaluated by El Baz *et al*. (2003). The treatment regimen for schistosomiasis consisted of 600 mgm for six successive days. This proved to be very effective and safe.

Commiphora molmol has a molluscicidal effect on infected *Bulinus truncates* and *Biomphalaria alexandrina* snails at concentrations of 10 µg/ml and 20 µg/ml, respectively, after a 24 hour exposure period, as reported by Masoud and Habib (2003). The number of dead snails increased with prolonged exposure time. Myrrh was also observed to have an ovicidal effect on one day old egg masses, at concentrations of 60 µg/ml and 80 µg/ml.

1.4.3 Preclinical and clinical investigations of myrrh and guggul

i. Osteoarthritis

Preclinical and clinical studies were conducted on guggul (an oleo-resin from *Commiphora mukul*), for the treatment of pain, stiffness and improved function of the knee in patients with osteoarthritis. Significant improvement was noted after taking 500 mg three times a day of the supplement for one month, an indication that guggul is an effective supplement to reduce symptoms of osteoarthritis (Shepard *et al.*, 2003).

1.5 The phytochemistry of myrrh

Myrrh consists of water-soluble gum (40 - 60%), alcohol-soluble resins (23 - 40%), volatile oils (2 - 8%) and a bitter principle (10 - 25%), and has a characteristic odour ascribed to the presence of furanosesquiterpenes (El-Ashry *et al*., 2003).

Numerous researchers have investigated the phytochemistry of myrrh, reporting a number of different chemical constituents within the resin, gum and oil. These results have been reviewed by Hanuš *et al.* (2005), and are summarised in Table 1.2.

1.6 *Commiphora* and its traditional uses

The use of plants in organised traditional medicine has been demonstrated in systems such as Ayurveda, Unani, Kampo and traditional Chinese medicine, all of which are still widely used and may play an important role in the search for novel medicines. Ayurveda and traditional Chinese medicine remain the most ancient, yet living, traditions (Dharmananda, 2003), and both these systems focus on the patient rather than the disease. Many successes (of botanical reference) have come from Chinese medicine, most notably quinghaosu and artemisin; and considerable research on the pharmacognosy, chemistry, pharmacology and clinical therapeutics have been conducted on ayurvedic medicinal plants (Patwardhan *et al*., 2004). Myrrh entered the formal herb books in 973 A.D. in the Kaibao Bencao, the Materia Medica of the Kaibao era (Dharmananda, 2003).

Table 1.2: The different sources of myrrh and their chemical constituents (El-Ashry *et al.*, 2003; Hanuš *et al.*, 2005).

Myrrh constituents: background and application	***Commiphora myrrha***	***Commiphora opobalsam***
Water-soluble gum • Used as incense for many years, but is also being used as an antiseptic and anti-inflammatory agent.	• D-galactose, L-arabinose, and 4-methyl D-glucuronic acid • Acidic oligosaccharides and aldobiuronic acids.	
Alcohol-soluble resins • A food additive, a fragrance. • Used in traditional medicine.	• Furanosesquiterpenoids • Terpene and terpenoid with the diterpenoids and triterpenoids being the most common, but these compounds have never been identified in a resin concomitantly. • Isolinalyl acetate 7,3-epi-lupenyl acetate, lupeone, 3-epi-α-amirin, α-amirone, acetyl β-eudesmol and a sesquiterpenoid lactone. • α-, β- and γ-commiphoric acid.	
Volatile oils • Lewinsohn (1906), von Friedrichs (1907) and Trost and Doro (1936) carried out the first investigation of myrrh oil almost 100 years ago, determining the presence of certain constituents	• α-pinene, dipentene, limonene, cuminaldehyde, cinnamic aldehyde, eugenol, m-cresol, heerabolene (probably tricyclic sesquiterpene), formic acid, acetic acid, palmitic acid, myrrholic acid. • p-cymene, α-terpineol, δ-elemene, β-elemene.	
Extract	• steroids, sterols and terpenes.	• Aqueous extract of the branch used to reduce arterial blood pressure, due to the activation of muscarinic cholinergic receptors.

Table 1.2 *continued*: The different sources of myrrh and their chemical constituents (El-Ashry *et al.*, 2003; Hanuš *et al.*, 2005).

Myrrh constituents	*Commiphora molmol*	*Commiphora mukul*
Water-soluble gum	• Composed of proteoglycans, with uronic acid polymers. • Galactose, arabinose, 4-O-methyl-glucuronic acid, arabino-3,6-galactan protein fractions and protein.	• D-galactose, L-arabinose, L-fructose, 4-*O*-methyl-D-glucuronic acid, aldobiouronic acid. • Degraded gum – branched polysaccharide.
Alcohol-soluble resins	• Sesquiterpene fractions isolated with antibacterial and antifungal properties • Compounds also have strong anaesthetic properties.	• Guggulsterone (steroid) *Z*- and *E*-isomer.
Volatile oils	• 3 new furanogermacrenes isolated $C_{16}H_{22}O_3$, $C_{18}H_{24}O_3$, $C_{16}H_{20}O_3$.	• Linoleic, oleic, steric and palmitic acids.
Extract		• Mukulol (allylcembrol) isolated from aerial parts of the plant • Flavonoid quercetin and derivatives (quercetin-3-*O*-α-L-arabinoside, quercetin-3-*O*-β-D-galactoside, quercetin-3-*O*-α-L-rhamnoside, and quercetin-3-*O*-β-D-glucuronide) isolated from the flower.

1.6.1 *Commiphora* and its role in Ayurvedic medicine

Ayurveda is one of the most ancient traditions practiced throughout India and Sri Lanka. It is 'the science of life' and aims at a holistic approach to the management of both health and disease. The *Charak, Samhita* and *Sushrut Samhita* are the main Ayurvedic classes, describing an excess of 700 plants, their pharmacological and therapeutic properties (Patwardhan *et al.*, 2005). A considerable amount of research has been conducted on Ayurvedic medicinal plants, particularly in terms of their respective properties in the fields of chemistry, pharmacognosy, pharmacology and clinical therapeutics (Patwardhan *et al.*, 2004).

Guggul-gum resins have been prescribed in Ayurvedic folk medicine as anti-obesity, anti-inflammatory, antibacterial, anticoagulant and anti-arthrosclerotic agents, to be administered in the form of mixtures with powders and other crude drug extracts (Kimura *et al.*, 2001). From guggul, the gum resin of *C. mukul* known in Ayurveda for the treatment of hypercholesterolaemia, two steroidal ketones with hypocholesterolemic and hypolipaemic activity, were isolated. These ketones are known as *Z*-guggulsterone and *E*-guggulsterone (Singh and Srikrishna 2003).

1.6.2 *Commiphora* and its role in Chinese medicine

Traditional Chinese medicine is based on two separate theories, yin and yang, and the five elements, viz. water, earth, metal, wood and fire, about the natural laws that govern good health and longevity. Treatment is not only based on symptoms, but also on patterns of imbalances, which are often detected through observation of the patients' tongue, as well as taking their pulse. Warming or hot herbs, such as ginger and cinnamon, are used to treat ailments associated with cold symptoms, such as cold hands, abdominal pain and indigestion (van Wyk and Wink, 2004).

Myrrh was first described in Chinese medical literature in A.D. 973, where it was referred to as 'moyao'. The resin of myrrh is classified as a herb and is currently still in use, specifically for stimulating blood circulation and treating painful swellings, menstrual pain due to blood stagnation, as well as other traumatic injuries (Dharmananda, 2003).

1.6.3 The African traditional uses of *Commiphora* species

The traditional uses of indigenous *Commiphora* species are summarised in Table 1.3. These species are indigenous to southern Africa and the species denoted by * have been investigated in this study.

Table 1.3: The traditional uses in all parts of Africa of *Commiphora* species indigenous to southern Africa.

Species	Plant part used	Disease	Reference
C. africana (A.Rich.) Engl. var. *africana**	Stem	Colds and fever, malaria, snake bite	Kokwaro (1976) Hutchings *et al.* (1996)
	Fruit	Typhoid	Kokwaro (1976)
	Resinous exudates	Wound healing and antiseptic	Kokwaro (1976)
	Burnt resin	Fumes serve as antiseptic, migraine, insecticide	Kokwaro (1976)
	Bark, resin, leaf	Tumour, stomache ache	Lemenih *et al.* (2003)
*C. pyracanthoides** Engl.	Bark	Diseases of the gall bladder	Steyn (2003)
*C. schimperi** (O.Berg) Engl.	Bark	Medicine	Steyn (2003)
C. serrata Engl.	Roots	Chest ailments	Steyn (2003)
*C. viminea** Burtt Davy	Resin	Skin ailments	Steyn (2003)
C. zanzibarica (Baill.) Engl.	Root	Ulcers	Steyn (2003)

* denotes species that have been investigated in this study.

1.6.4 Additional uses

The resins from the African Burseraceae are important as medicinals and tick repellents, as well as in commerce, as glues and perfumes. In the dry region of Kunene in northwestern Namibia, the Himba tribe traditionally harvests the aromatic resin of several species of *Commiphora* including *C. multijuga*. The harvesting of these fragrant plants has a very strong cultural basis, specifically among the women of the tribe. The resin exuded by the trunks is blended with fat and used as body perfume (Steyn, 2003). Extracts from the gum of the bark is also used to produce lather for washing. This tribe also places a long pole of certain species, smeared with red-pigmented fat, to attract the spirits during ceremonies such as marriages, initiations and funerals (Steyn, 2003).

1.7 A review of the phytochemistry documented for certain *Commiphora* species

Studies have been carried out on a few *Commiphora* species to identify several of the constituents of the species. Table 1.4 presents a few of the plant species and their constituents. Having extensively studied the phytochemistry of *Commiphora*, research groups identified the following chemical constituents: dammarene triterpenes (Dekebo *et al.*, 2002a; Dekebo *et al.*, 2002b; Manguro *et al.*, 2003), triterpenes (Provan and Waterman, 1988), ferulates (Zhu *et al.*, 2001), furanosesquiterpenes (Manguro *et al.*, 1996) guggultetrols (Kumar and Dev, 1987), guggulsterones (Swaminathan *et al.*, 1987), lignans (Provan and Waterman, 1985; Dekebo *et al.*, 2002c), flavanones (Fatope *et al.*, 2003), sesquiterpenes (Andersonn *et al.*, 1997) and steroids (Bajaj and Dev, 1982).

1.8 Selection of plant material

The selection of plant material for the screening of biological activity can be based on a random selection, or based on ethnopharmacology, where existing knowledge of the particular healing properties have been handed down from generation to generation, especially amongst traditional healers. An additional mechanism for the identification of plants for the study of its chemical constituents is based on chemotaxonomy, the latter being a science focusing on the correlation between related plant species and the occurrence of similar secondary metabolites.

Table 1.4: The phytoconstituents of extracts and the oleo-gum-resin documented for a few species of *Commiphora* presenting interesting chemical profiles, adapted from Hanuš *et al.* (2005).

***Commiphora* species**	**Extracts**	**Oleo-gum-resin**
Commiphora kua var. *kua* Vollesen (syn. *Commiphora flaviflora*)	• Four active compounds, namely mansumbinone, mansumbinoic acid, picropolygamain, lignan-1(methoxy-1,2,3,4-tetrahydropolygamain). • Petrol extract of stem bark yielded three labile C_{22} octanordammarene triterpenes, and their derivatives.	• α-pinene, p-cymene, α-thujene, β-pinene, limonene, sabinene, terpinene-4-ol, car-3-ene and myrcene.
Commiphora pyracanthoides Engl.		• Rich in triterpene acids, comic acid A-E.
Commiphora guidotti Chiov.		• Sesquiterpene hydrocarbons, fuaranodiene (furanosesquiterpenoid). • Car-3-ene, α- and β-santalene, epi- β–santalene, β–bergamotene, α- and β-bisabolene, β-farnesene, and furanodiene. • Ethyl acetate extract (+)-T-cardinol sesquiterpene.
Commiphora erlangeriana Engl.		• Erlangerins A-D.
Commiphora africana (A. Rich.) Engl.	• Dihydroflavonol glucoside – phellamurin.	• α-pinene, α-thujene, p-cymene.

T**able 1.4 *continued*:** The phytoconstituents of extracts and the oleo-gum-resin documented for a few species of *Commiphora* presenting interesting chemical profiles, adapted from Hanuš *et al.* (2005).

***Commiphora* species**	**Extracts**	**Oleo-gum-resin**
Commiphora confusa Vollesen		• Dammarene triterpenes, an example of which is (20S)-3β-acetoxy-12β,16β-trihydroxydammar-24-ene. • β-amyrin, β-sitosterol, 3β-amyrinacetate, 2-methoxyfuranodienone, 2-acetoxyfuranodienone. • Other dammarene triterpenes.
Commiphora angolensis Engl.	• Condensed tannins found in powdered bark.	
Balsamodendron pubescens	• Hexane extracts of the roots yielded β-sitosterol and cedrelone and siderin.	
Commiphora terebinthina Vollesen and *C. cyclophylla* Chiov.		• Liquid resin consists primarily of monoterpene hydrocarbons, limonene. • *C. terebinthina* rich in sesquiterpenoids.
Commiphora tenuis Vollesen		• Triterpenes, α-pinene, α-thujene, p-cymene, β-thujene, camphene, sabinene, 3-carene.
Commiphora dalzielli Hutch.	• Seven dammarene triterpenes from the stem bark, lupeol and β-amyrin.	
Commiphora rostrata Engl.		• Alkanone constituent, 2-decanone and 2-undecanone.
Commiphora merkeri Engl.	• Pentacyclic triterpene with anti-inflammatory activity, 2α,3β,23-trihydroxylean-12-ene.	

1.8.1 The selection of *Commiphora* species for the screening of biological activities

Despite the extensive traditional use of *Commiphora* species, as well as its extreme botanical diversity, it is remarkable that *Commiphora* species indigenous to southern Africa have neither been the subject of pharmacological nor extensive phytochemical studies. Claims of the efficacy of *Commiphora* in its traditional usage therefore requires validation and accurate documentation. For this purpose, such studies were initiated as a basis for scientific verification regarding the traditional use of *Commiphora* species. The 10 species of *Commiphora* studied were collected at random, within a selected geographical region (Table 2.1).

1.8.2 The selection of biological activity assays performed

The origin and design of a screening process incorporates knowledge attained in ethnomedicine, traditional uses of the plant species, phytochemical evaluation and correlation to specific biological targets (which may be predicted but requires confirmation), as well as the use of natural product libraries and general or targeted literature reviews.

The selection of screening assays for the evaluation of biological activity is a complex process during which a number of factors require careful consideration. Ethnobotanicals may possess a number of biological activities, all of which must be evaluated, including their respective applications. This is possible only through thorough understanding and implementation of basic assays designed to target a selected activity. An understanding of both the physiology of the processes and the chemical composition of the specific extracts is therefore imperative.

Stable standardised crude extracts are prepared and assayed for the known/claimed activities for which the particular plant species is traditionally used, as well as for activities documented for related species - both indigenous and exotic species. The present study, therefore, focused on anti-inflammatory, anti-oxidant, antimicrobial and anticancer activities.

1.9 Aim of the study

Traditional uses associated with southern African *Commiphora*, as well as properties portrayed by non-indigenous species, suggested that the *in vitro* anti-oxidant, anticancer and antimicrobial activity of the 10 selected species indigenous to southern Africa should be determined. Additionally, the cytotoxicity of each of the extracts was investigated and the compounds responsible for the selected biological activities isolated and identified.

1.10 Objectives of the study

(i) Investigation of the *in vitro* anti-oxidant activity of the crude extracts of both the leaf and stem of 10 species of *Commiphora*, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assay.

(ii) Isolation and identification of the chemical compounds responsible for the anti-oxidant activity in the most active species, through bioassay-guided fractionation using column chromatography, thin layer chromatography (TLC) and nuclear magnetic resonance (NMR).

(iii) Determination of the inhibitory effects of each of the extracts on the growth of selected bacteria and yeast and to investigate the death kinetics of the selected species against specific micro-organisms.

(iv) Evaluation of the *in vitro* anti-inflammatory activity of the crude extracts by investigating their ability to inhibit the 5-lipoxygenase (5-LOX) enzyme involved in the inflammatory process.

(v) Evaluation of the anticancer activity of both the leaf and stem extracts against three human tumour cell lines, namely the HT-29 (colon adenocarcinoma) cell line, MCF-7 (breast adenocarcinoma) cell line and the SF-268 (neuronal glioblastoma) cell line.

(vi) Reporting of the cytotoxicity of each of the species, using the 3-[4,5-dimethylthiazol-2yl]-2,5 diphenyltetrazolium bromide (MTT) cellular viability assay.

(vii) Phytochemical profiling of the plant extracts using high performance liquid chromatography (HPLC).

Figure 1.6 is a diagrammatic summary of this study undertaken on the 10 indigenous *Commiphora* species.

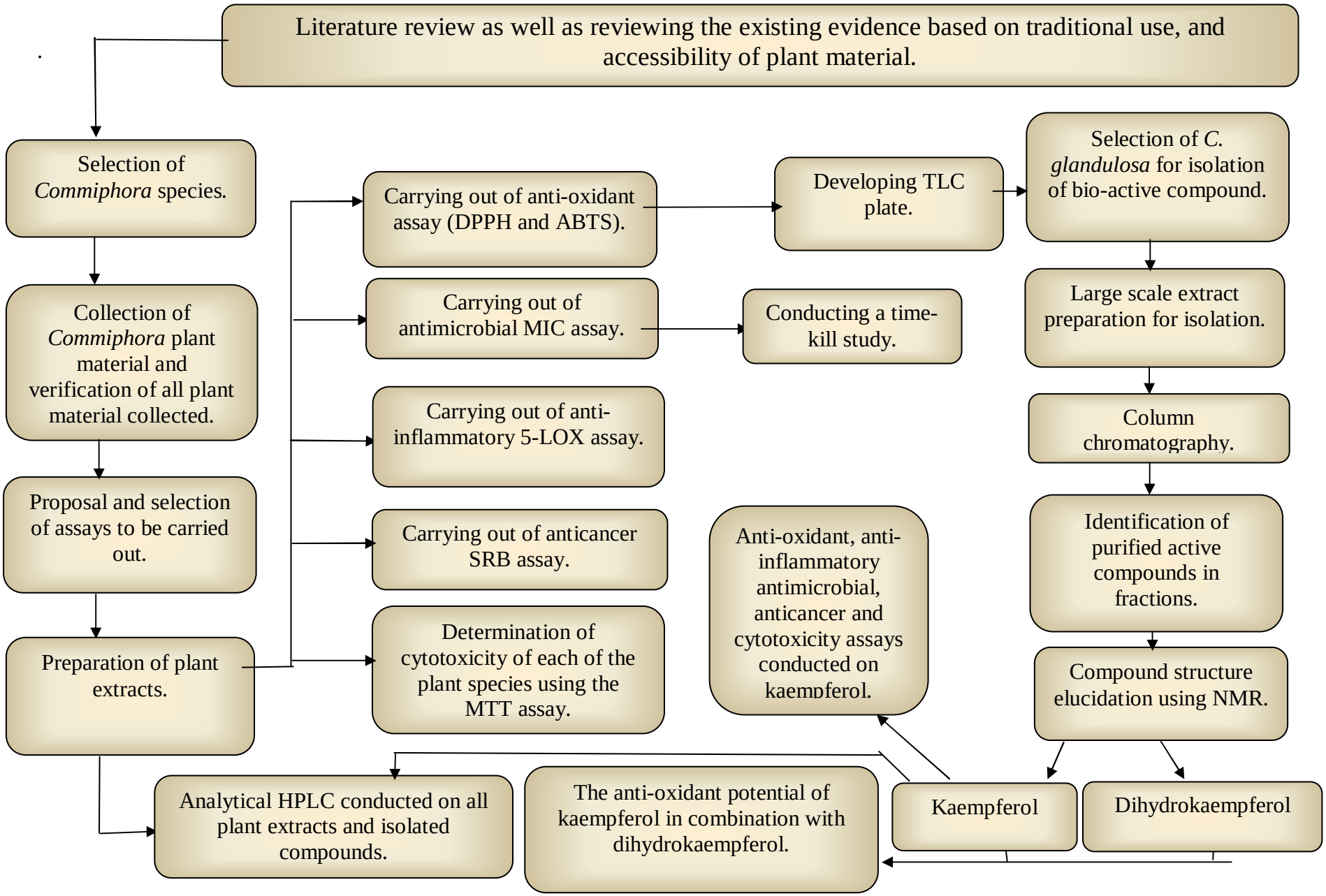


Figure 1.6: A diagrammatic summary of steps in the study of indigenous *Commiphora* species, evaluating the phytochemistry and biological activities

CHAPTER 2: SPECIES STUDIED, PLANT COLLECTION AND PREPARATION OF EXTRACTS

2.1 Brief introduction to the species under investigation

Of the 40 *Commiphora* species occurring in southern Africa (Steyn, 2003), 10 species were selected for evaluation of phytochemical constituents and pharmacological activity. Surprisingly, none of the species under investigation were observed to exude a gum-like resin, and it is for this reason that bark and leaf extracts were chosen for evaluation. It is also evident that, as depicted in Table 1.1 (Chapter 1), little research, and especially with respect to biological activity, has been conducted on extracts of the aerial parts of the plant, for both indigenous and exotic species. An identification key of *Commiphora* species indigenous to South Africa is given in Figure 2.1.

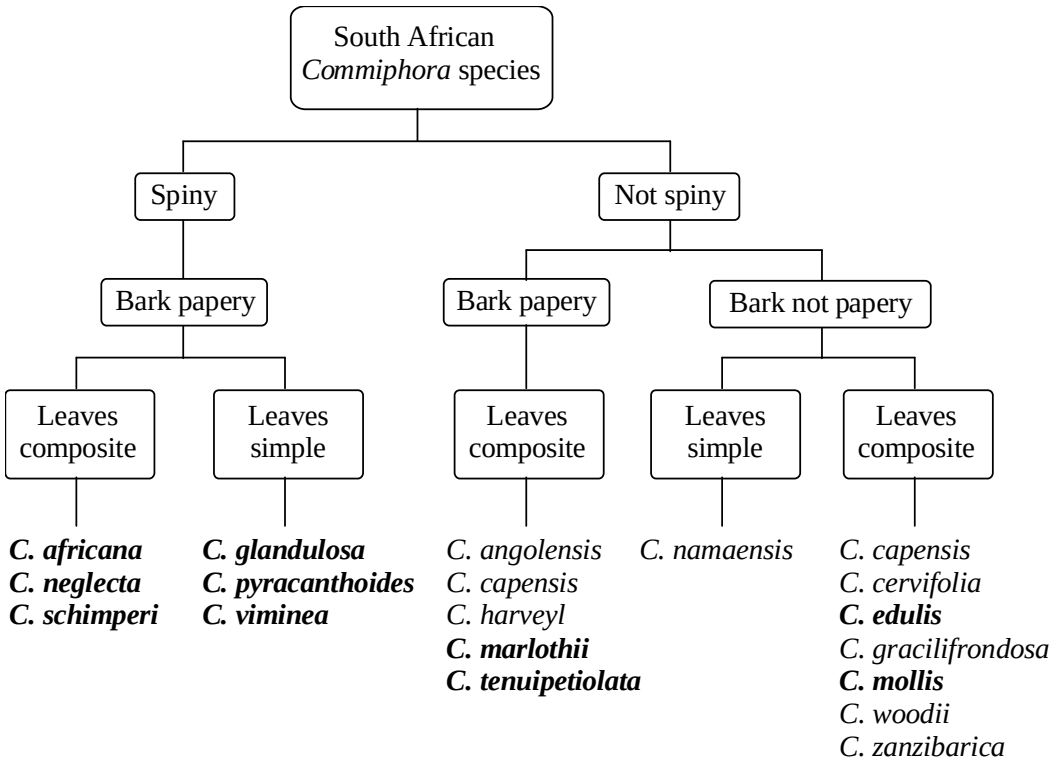


Figure 2.1: *Commiphora* species indigenous to South Africa (the species highlighted in bold were investigated in this study) [Adopted from van der Walt, 1986; Steyn, 2003].

2.2 A description of the 10 indigenous *Commiphora* species under investigation

2.2.1 *Commiphora africana* (A. Rich.) Engl. var. *africana*

The epithet of the species name of *Commiphora africana* is derived from the word 'African'. It is also known as 'hairy corkwood' and 'harige kanniedood' (the latter being in a South African native language). It grows in southern Africa, specifically in South Africa, Namibia, Botswana, Zimbabwe and Mozambique (Figure 2.2).

Commiphora africana is a many-stemmed shrub, less than one meter tall or small tree, up to four meters tall. It is spiny and has a papery bark. In arid regions, the bark is rough and fragmented while in milder regions, the bark appears grey to green with yellow papery flakes. Young branchlets are pilose to tomentose (van der Walt, 1986). The leaves (Figure 2.2) are tri-foliolate, pilose to tomentose, with a large terminal leaflet and two small side leaflets. The leaves have a velvety texture, and the margins are coarsely toothed. The flowers are small and reddish in colour, may be found singly or in clusters, and appear in early spring, before the leaves appear (Steyn, 2003). The flowers are unisexual, hypogynous and glabrous. The fruit is round and red in colour when ripe (Steyn, 2003). When split into half, the fruit reveals a red pseudo-aril with four arms of variable size and form.

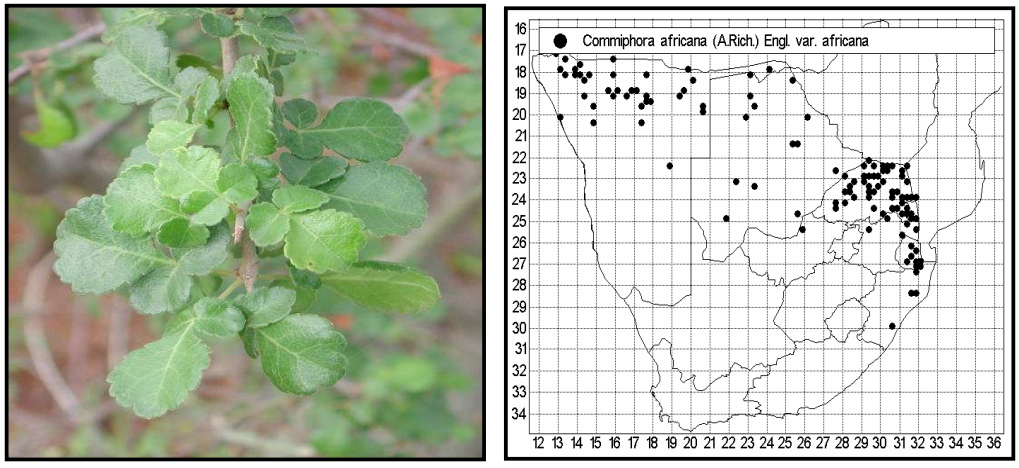


Figure 2.2*: *Commiphora africana* leaves (left) and the recorded geographical distribution of the species (right).

*Distribution maps were purchased and included with permission from the South African National Biodiversity Institute.

2.2.2 *Commiphora edulis* (Klotzsch) Engl. subsp. *edulis*

The epithet of the species name of *Commiphora edulis* is derived from the Latin word 'edulis', which means 'edible', specifically referring to the fruit. Also known as 'rough-leaved corkwood', 'skurweblaar-kanniedood' and 'mubobobo' (Steyn, 2003), it grows in South Africa, Namibia, Botswana, Zimbabwe and Mozambique (Figure 2.3).

Commiphora edulis is a tree without spines and the bark does not peel. The bark is grey in colour with black scales in places, and rough to the touch. Densely pubescent, obtuse young branchlets are present (van der Walt, 1986). The leaves have intertwining branches, which give the tree an untidy, disordered appearance (Figure 2.3). The leaves are compound, pinnate, with 3 - 7 leaflets, which are pubescent. Their margins are toothed and the leaves are hairy and rough to the touch. The flowers are green and small, with a hairy calyx. The flowers are found in clusters or singly, are unisexual and occur on different trees.The fruit is found in clusters on short stalks, has a thick fleshy part, and is red when ripe (Figure 2.3 insert). When split into half, the fruit reveals a red pseudo-aril with a black seeded stone (Steyn, 2003).

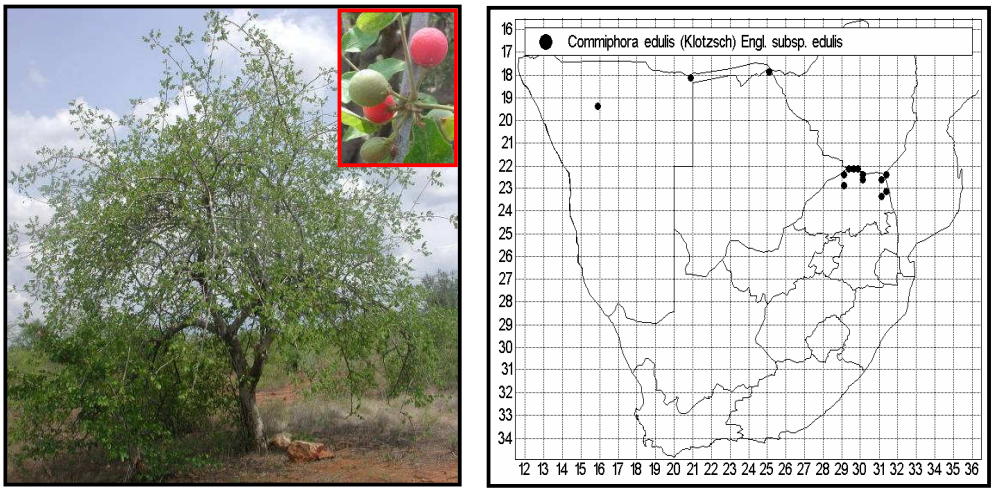


Figure 2.3: *Commiphora edulis* tree with fruit (left) and the recorded geographical distribution of the species (right).

2.2.3 *Commiphora glandulosa* Schinz

The species name, *Commiphora glandulosa,* refers to the presence of glands on the calyx. Also known as 'tall common corkwood', 'groot gewone kanniedood' and 'moroka' (Steyn, 2003), it is found in regions similar to those mentioned for *C. africana* and *C. edulis* (Figure 2.4).

Commiphora glandulosa (Figure 2.4) is a spiny tree with simple leaves, occasionally compound with three leaflets, and a bark that peels. The bark is fragmented, rough or smooth. It has yellowish papery flakes, which peel off. Young branchlets are glabrous and spine-tipped. (van der Walt, 1986). The leaves are usually simple, but may be compound with one large leaf and two small leaflets (Steyn, 2003). The margins of the leaves are toothed, and are a glossy green on the dorsal side (above) and a paler green ventrally (below). The flowers are small, may be found singly or in clusters, and can be unisexual or bisexual. The glandular hairs on the calyx form the characteristic feature of this species. The fruit is round, red when ripe, and reveals a red pseudo-aril with four arms of various thicknesses but equal length, when split into half. The seeded stone is black.

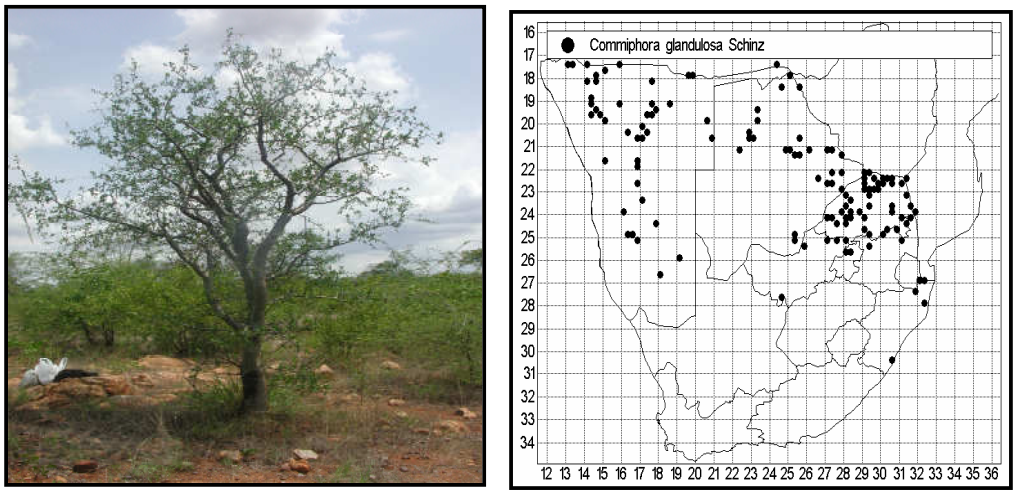


Figure 2.4: *Commiphora glandulosa* tree (left) and the recorded geographical distribution of the species (right).

2.2.4 *Commiphora marlothii* Engl.

Commiphora marlothii derives the epithet of its species name from the famous South African botanist, Rudolf Marloth. Also known as ‘paper-barked corkwood’, ‘papierbas-kanniedood’ and ‘paper tree’ (Steyn, 2003), it grows in similar regions to the other species mentioned, that being South Africa, Botswana, Zimbabwe and Mozambique (Figure 2.5).

Commiphora marlothii is a dioecious tree. It is not spiny and the bark is green and smooth (Figure 2.5). The bark peels off into yellow papery flakes. Young branchlets are obtuse, are densely pilose to pubescent (van der Walt, 1986). The leaves are compound; with 3 - 5 leaflets present (Figure 2.5). The leaves are densely hairy, the margins are toothed and the venation on the leaves sunken above (Steyn, 2003). The flowers are small and may be found individually or in groups. They are greenish-yellow and are very hairy, especially the calyx. The flowers are unisexual, occur on different trees, and are found on rocky mountain slopes in the dry bushveld. The fruit is red when ripe, and is found on short stalks. The fleshy part of the fruit is fairly thick and when cut in half reveals a yellow pseudo-aril, with four arms. The seeded stone is black.

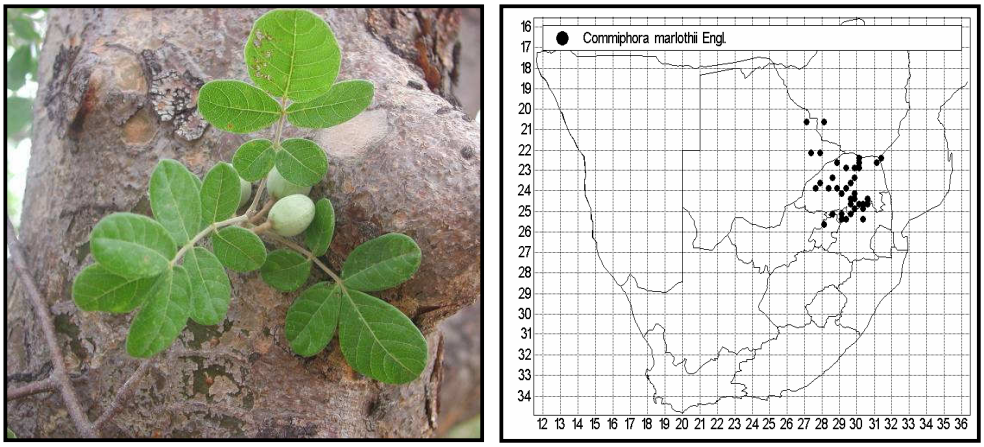


Figure 2.5: *Commiphora marlothii* tree (left) and the recorded geographical distribution of the species (right).

2.2.5 *Commiphora mollis* (Oliv.) Engl.

The species name of *Commiphora mollis* is derived from the Latin word 'mollis', meaning soft (Steyn, 2003). This is in reference to the velvety hairs present on the plant. Also known as 'velvet-leaved corkwood**',** 'fluweel-kanniedood' and 'soft-leaved', it grows in South Africa, Namibia, Botswana, Zimbabwe and Mozambique (Figure 2.6).

Commiphora mollis is a tree (Figure 2.6), which is not spiny and the bark does not peel. The bark differs in structural appearance; it may be wrinkled, smooth, or fragmented and is silvery when burnt by the sun. The trunks are sometimes knobbly or angular. The young branchlets are sparsely pilose to densely pubescent (van der Walt, 1986). The leaves are compound, pinnate, with 3 – 7 leaflets present. They are greyish-green dorsally and a paler green ventrally, and are densely covered or scattered with velvety hairs. The flowers are small and are found in groups on long, red slender stalks. The flowers are unisexual, maroon-red in colour and velvety (Steyn, 2003). The fruit is round and red in colour when ripe and a distinctive red pseudo-aril with four arms is present when the fruit is halved.

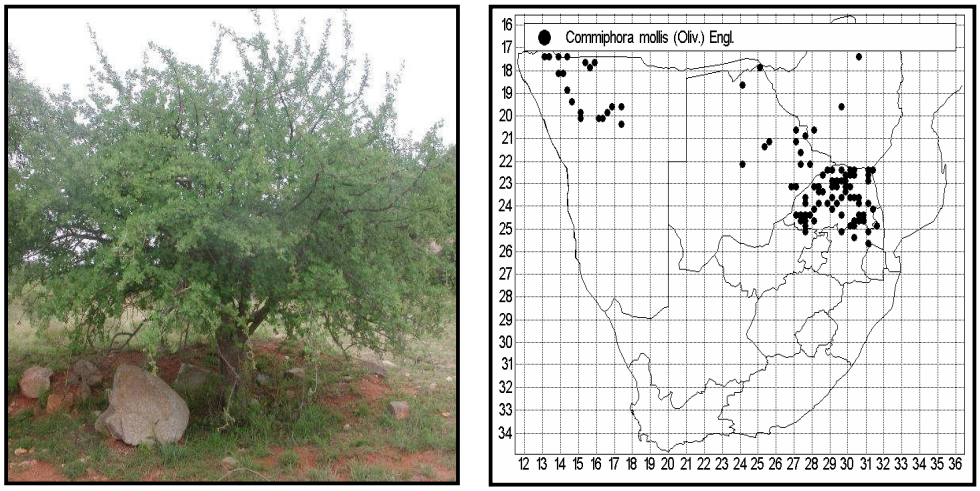


Figure 2.6: *Commiphora mollis* tree (left) and the recorded geographical distribution of the species (right).

2.2.6 *Commiphora neglecta* I.Verd

Commiphora neglecta derives its name from the word 'neglected', due to the delay in the recognition of the plant as a separate species (Steyn, 2003). Also known as 'green-stemmed corkwood', 'groenstam-kanniedood' and 'sweet-root commiphora', it grows in South Africa, Swaziland and Mozambique (Figure 2.7).

Commiphora neglecta is a many-stemmed shrub or a small spiny tree, and has a bark that peels. The bark is grey to green and peels off in brownish papery strips (Steyn, 2003). Young branchlets have few short hairs. The leaves are trifoliolate, glossy and have smooth margins. The flowers are small, and are green to yellow in colour. They are found on longish stalks, in clusters, are bisexual and hypogynous (van der Walt, 1986). The fruit is fleshy, red when ripe, and round (Figure 2.7). When split into half the fruit reveals a red pseudo-aril, and has three arms. The seeded stone is black.

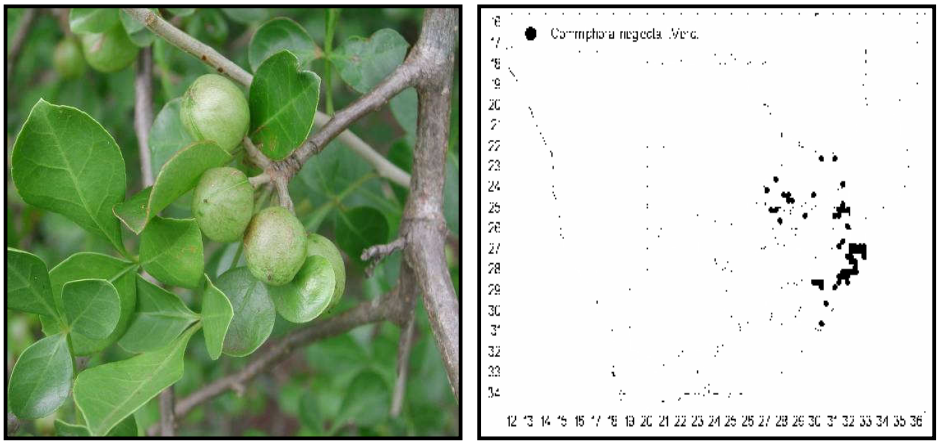


Figure 2.7: *Commiphora neglecta* twigs bearing fruit (left) and the recorded geographical distribution of the species (right).

2.2.7 *Commiphora pyracanthoides* Engl.

Commiphora pyracanthoides derives its species name from of the similarity with the genus *Pyracantha* (Rosaceae). It is also known as 'common corkwood' and 'gewone kanniedood' (Steyn, 2003). It grows in South Africa, Swaziland, Namibia, Botswana, Zimbabwe and Mozambique (Figure 2.8).

Commiphora pyracanthoides is a shrub or small tree, which is spiny and has a papery bark. The trunk is twisted and branching occurs from the base. The bark is a greyish-green colour and peels off into yellowish, papery flakes. Branchlets are glabrous (van der Walt, 1986). The leaves are simple but on long shoots, a glossy green on its dorsal side (Figure 2.8), and a paler green on its ventral side. The smaller lateral leaflets are clustered on short branches and the margins are toothed (Steyn, 2003). The flowers are small, found in groups or occur singly, and appear in early spring before the leaves appear. The flowers are unisexual or bisexual, and hypogynous. The fruit is round and red in colour when ripe. When split into half the fruit reveals a red pseudo-aril, with four arms of equal length. The seeded stone is black.

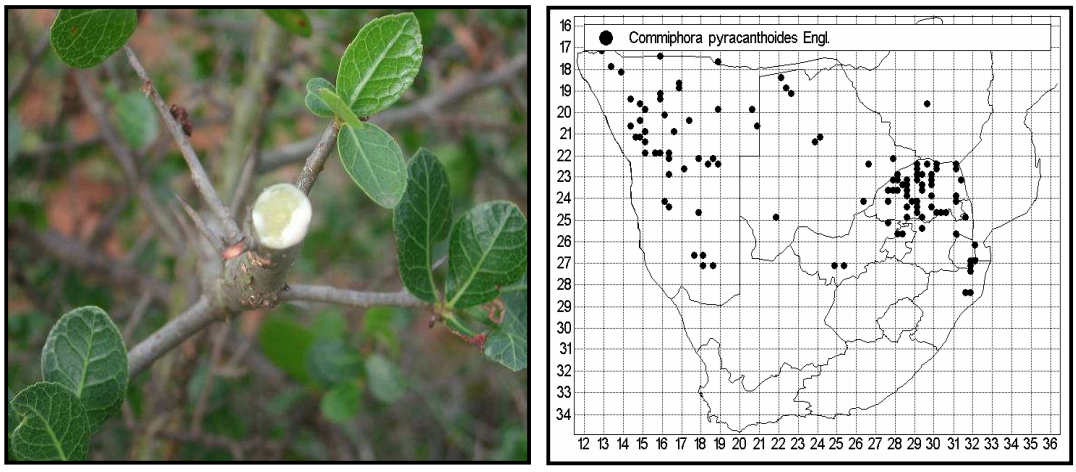


Figure 2.8: *Commiphora pyracanthoides* stem (left) and the recorded geographical distribution of the species (right).

2.2.8 *Commiphora schimperi* (O.Berg) Engl.

The species name *Commiphora schimperi* is in recognition of the author of a standard work on plant geography, Dr A.F.W. Schimper. This species is also known as 'glossy-leaved corkwood' and 'blinkblaar-kanniedood' (Steyn, 2003). It grows in South Africa, Botswana, Zimbabwe and Mozambique (Figure 2.9).

Commiphora schimperi is a shrub or small spiny tree and has a bark that peels. The bark is fragmented and peels off in small yellowish, papery flakes, revealing a green layer underneath. Young branchlets are glabrous (van der Walt, 1986). The leaves are tri-foliolate, glossy with deeply toothed margins. The flowers are small, with a red calyx and yellow petals and may be found singly or in clusters. They are found present on spiny branches and are bisexual. The flowers appear early in spring before the leaves appear and are bisexual only (Steyn, 2003). The fruit is red when ripe and is pointed. When split into half the fruit reveals a knobbly, pseudo-aril, which is scarlet in colour (Figure 2.9). The pseudo-aril is slightly bent toward one side and completely encompasses the seeded stone.

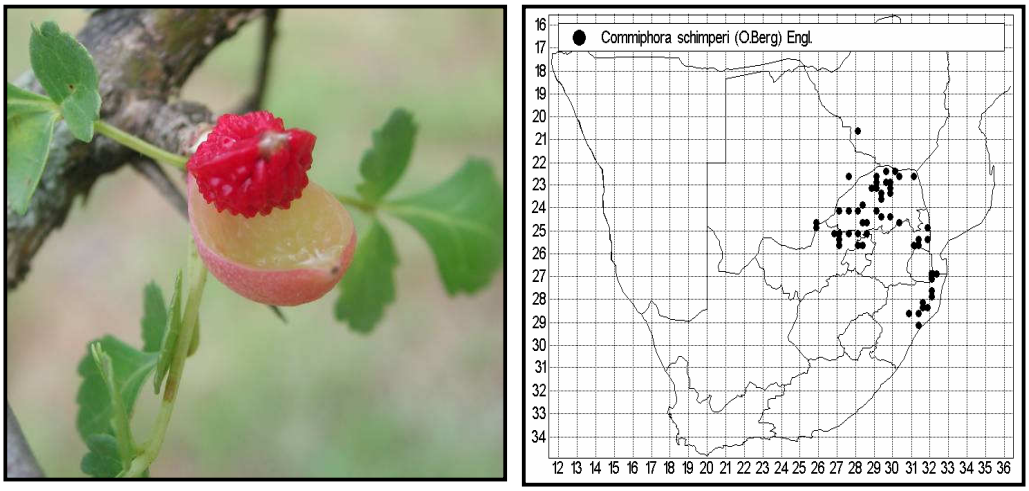


Figure 2.9: *Commiphora schimperi* bearing fruit revealing pseudo-aril (left) and the recorded geographical distribution of the species (right).

2.2.9 *Commiphora tenuipetiolata* Engl.

The species name *Commiphora tenuipetiolata* is derived from the meaning (of tenuipetiolata) 'with a thin petiole' (leaf stalk). It is also known as 'white-stemmed corkwood', and 'witstam-kanniedood' (Steyn, 2003). It grows in South Africa (northern region), Namibia, Zimbabwe and Mozambique (Figure 2.10).

Commiphora tenuipetiolata is a tree (Figure 2.10) without spines and has a single trunk with a whitish appearance and a bark that peels. The bark is a bluish-green colour (Figure 2.10 insert) and peels off in white papery flakes and may also be fragmented. Young branchlets are are glabrous, sparsely pilose (van der Walt, 1986). The compound leaves have 3 – 5 leaflets, and the margins are toothed or sometimes smooth. The surface of the leaf may be smooth to hairy, and the leaf stalk is very long (Steyn, 2003). The yellow flowers are found in clusters on long stalks and are hairy. They are unisexual and occur on different trees. The fruit is fleshy, a red colour when ripe and reveals a red cupular pseudo-aril with two facial lobes of variable length. The seeded stone is red.

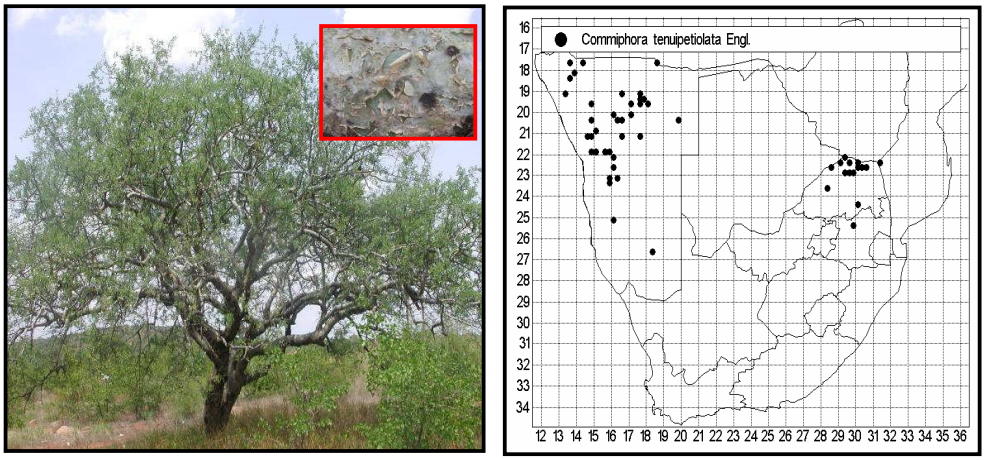


Figure 2.10: *Commiphora tenuipetiolata* tree (left) with papery bark (insert) and the recorded geographical distribution of the species (right).

2.2.10 *Commiphora viminea* Burtt Davy

Commiphora viminea derives its species name ‘viminea’ from the meaning ‘to have long flexible shoots’. It is also referred to as ‘zebra-barked corkwood’, ‘sebrabas-kanniedood’ and ‘zebra tree’ (Steyn, 2003) and it grows in South Africa and Namibia (Figure 2.11).

Commiphora viminea is a spiny tree (Figure 2.11). The bark is typically dark in colour, and peels off in yellowish papery strips leaving the characteristic bark that has dark horizontal bands (Figure 2.11 insert). The leaves are simple and occasionally compound with three leaflets. The margins of the leaf are scalloped, toothed or almost entire. The colour of the leaf dorsally (above) is a blue-green and ventrally (below) a paler green. with small, yellow flowers that are found in clusters. The flowers are unisexual, occur on different trees, and appear at the same time as the leaves appear (Steyn, 2003).The very pale red fruit is fleshy and is oval in shape with a very sharp point. When split in half, the fruit reveals a yellow pseudo-aril, which covers the seeded stone almost entirely.

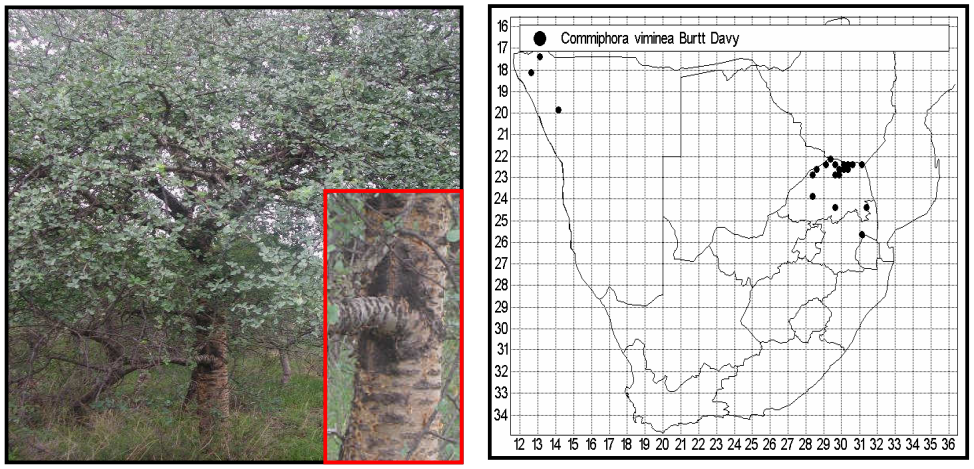


Figure 2.11: *Commiphora viminea* tree with characteristic bark that has dark horizontal bands (insert) and the recorded geographical distribution of the species (right).

The *Commiphora* species investigated in the present study are summarised in Table 2.1, specifically in terms of the collection of specimens in January 2005. The identification of the species collected were verified by Mr Marthinus Steyn (a recognised authority on the genus and author of a book titled Southern Africa *Commiphora*). Voucher specimens were pressed and deposited in a collection housed at the Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg.

2.3 Preparation of plant extracts for determination of biological activity

The presence and degree of variation of different compounds contained in the leaf and stem extracts of the different species was indicated through the use of thin layer chromatography, which justified separate extractions of leaves and stems, respectively.

After collection, fresh plant material was allowed to air dry at ambient temperature (25°C) in the laboratory for approximately 15 days, after which the plant material was sufficiently dry for further experimental use. The leaves were separated from the bark and any fruit present was removed and stored separately. The plant material was then crushed to a fine powder using a mill.

The leaves (10 g of each species) underwent extraction in a conical flask using chloroform: methanol (1:1). Approximately 200 ml of solvent was added to the flask and covered with parafilm, after which the flask was incubated in a water bath at 40°C for 2 hours, followed by filtration into a weighed vial. The extraction process was repeated to ensure maximal extraction. The extracts were reduced by overnight evaporation in a standard fume cupboard. The stems (10 g) were extracted in a similar manner to the extraction of the leaves, as described above, although dichloromethane was used as the solvent. This solvent selection was aimed at reducing the number of polar compounds extracted in the stems, such as tannins, as these compounds are non-selective in their biological activity.

After reduction of the extracts, the dried extracts were prepared in the solvents and concentrations required for each biological activity assay (Chapters 3 - 6). Dimethyl sulfoxide (DMSO) was used as the solvent of choice in numerous assays, specifically as the solubility of extracts was ensured by the presence of a highly polar domain combined with two non-polar methyl groups in this solvent. Dimethyl sulfoxide has been shown to

improve the efficiency of fungicides, to possess anti-inflammatory effects, as well as additional non-specific biological effects (Santos *et al.*, 2003), and it is for this reason that control experiments were conducted in all cases to account for additive effects, if any.

Table 2.1: Collection data for the 10 indigenous *Commiphora* species under investigation.

Species	Locality	GPS Reading	Habitat	Date of collection	Voucher specimen number
***C. africana* (A.Rich.) Engl. var. *africana* (Hairy corkwood)**	Limpopo Province (near Bethel)	23°26'37"S Elevation 3452 m 29°44'36"E	Bushveld on sandy soils and on rocky slopes.	25-01-2005	AV 1080
***C. edulis* (Klotzsch) Engl. subsp. *edulis* (Rough-leaved corkwood)**	Near the border of Musina	n.d.	Dry, hot bushveld and mopane veld in well-drained sandy soils.	25-01-2005	AV 1089
***C. glandulosa* Schinz (Tall common corkwood)**	Near the border of Musina	n.d.	Warm, dry bushveld.	25-01-2005	AV 1088
***C. marlothii* Engl. (Paper-barked corkwood)**	Limpopo Province	23°26'20"S Elevation 3456 m 29°44'48"E	Dry bushveld, usually on rocky mountain slopes.	25-01-2005	AV 1083
***C. mollis* (Oliv.) Engl. (Velvet-leaved corkwood)**	Limpopo province	23°26'22"S Elevation 3510 m 29°44'49"E	Hot, dry bushveld often on rocky outcrops.	25-01-2005	AV 1082
***C. neglecta* I.Verd. (Green-stemmed corkwood)**	Limpopo Province (near Waterpoort –R522)	23°26'32"S Elevation 3706 m 29°44'33"E	Bush and savannah sandveld, often on rocky slopes.	25-01-2005	AV 1085
***C. pyracanthoides* Engl. (Common corkwood)**	Limpopo Province (near Bethel)	23°26'37"S Elevation 3452 m 29°44'36"E	Bushveld on sandy soils.	25-01-2005	AV 1081
***C. schimperi* (O.Berg) Engl. (Glossy-leaved corkwood)**	Limpopo Province (near Capricorn Toll)	23°22'23"S Elevation 3698 m 29°46'09"E	Bushveld on sandy soils or against rocky slopes.	25-01-2005	AV 1084
***C. tenuipetiolata* Engl. (White-stemmed corkwood)**	Near the border of Musina	n.d.	Wide distribution, from sandy flatlands to rocky slopes in hot regions.	25-01-2005	AV 1087
***C. viminea* Burtt Davy (Zebra-barked corkwood)**	Limpopo Province (near Waterpoort – R523)	n.d.	Hot, dry bushveld, often associated with the mopane woodland.	25-01-2005	AV 1086

n.d. = not determined; GPS = Global Positioning System (location); AV = Voucher specimen numbers as defined by Professor Alvaro Viljoen.

CHAPTER 3: THE ANTI-OXIDANT ACTIVITY OF *COMMIPHORA* SPECIES AND THE ISOLATION OF KAEMPFEROL AND DIHYDROKAEMPFEROL

3.1 Free radicals and their scavengers

Oxygen is important for life processes to occur, however, an excess of oxygen could result in oxidative damage, which may even lead to death. The damage is not due to the presence of oxygen, but rather due to its role in the reduction of certain products to toxic free radicals. These free radicals are produced within living cells and are part of the cell's normal metabolic processes, including detoxification processes and immune system defences. It is the excessive generation of the free radicals, reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals and hydrogen peroxide that contribute to the development of various diseases such as cancer, rheumatoid arthritis, certain neurodegenerative diseases, tissue damage and also ageing, especially if free radical production exceeds the capacity of tissues to remove them (Larkins, 1999).

In aerobic organisms, the defence system against these free radicals is provided by free radical scavengers which act as anti-oxidants. Free radical scavengers function by donating an electron to the free radical, the latter of which pairs with the unpaired electron and thereby stabilising it. Anti-oxidant defence involves both enzymatic mechanisms, which utilise specific enzymes such as superoxide dismutase, catalase and glutathione peroxidase, as well as non-enzymatic mechanisms, which utilise nutrients and minerals (Aggarwal *et al.*, 2005).

These anti-oxidants can act at several different stages, by:

(i) the removal of or decrease in the local O_2 concentrations;

(ii) the removal of catalytic metal ions;

(iii) the removal of ROS such as O_2^- and H_2O_2;

(iv) scavenging initiating radicals such as $^{\bullet}OH$, $RO^{\bullet}$ and $RO_2^{\bullet}$;

(v) breaking the chain of an initiated sequence;

(vi) quenching or scavenging singlet oxygen (rearrangement of electrons that produces very rapid oxygen) (Gutteridge, 1994).

3.1.1 Natural anti-oxidants

Defence provided for by the anti-oxidant systems is crucial to survival and can operate at different levels within the cells through the prevention of radical formation, intercepting formed radicals, repairing oxidative damage, increasing the elimination of damaged molecules, and recognition of excessively damaged molecules, which are not being repaired but rather eliminated to prevent mutations from occurring during replication.

Non-enzymatic anti-oxidants are classified as being either water-soluble or lipid-soluble, depending on whether they act primarily in the aqueous phase or in the lipophilic region of the cell membranes. The hydrophilic anti-oxidants include vitamin C (ascorbic acid) and certain polyphenol flavonoid groups, while the lipophilic anti-oxidants include ubiquinols, retinoids, carotenoids, apocynin, procyanidins, certain polyphenol flavonoid groups and tocopherols (Middleton *et al*., 2000). Other non-enzymatic anti-oxidants include anti-oxidant enzyme cofactors, oxidative enzyme inhibitors and transition metal chelators such as ethylene diamine tetra-acetic acid (EDTA).

3.1.2 Flavonoids - their therapeutic potential

The establishment of an inverse correlation between the intake of fruits and vegetables and the occurrence of diseases such as inflammation, age-related disorders, cancer and cardiovascular disease is derived from clinical trials and epidemiological studies (Middleton *et al*., 2000). Polyphenolic compounds are effective in the prevention of oxidative stress related diseases. Flavonoids are a group of polyphenolic compounds with diverse characteristics and chemical structures. The therapeutic potential of these flavonoids has been determined and are known to have a number of pharmacological and biochemical properties, namely antibacterial, antiviral, anti-allergic, vasodilatory and anti-inflammatory, exhibiting activity against the enzymes cyclo-oxygenase and lipoxygenase. Flavonoids also exert the effects of anti-oxidants, free radical scavengers and are chelators of divalent cations (Cook and Samman, 1996). As discussed elsewhere, it is the excessive generation of the free radicals, reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals and hydrogen peroxide, that contribute to the causes of various diseases

such as cancer, rheumatoid arthritis, various neurodegenaritive diseases, tissue damage and also ageing, especially if their production exceeds the capacity of tissues to remove them. Flavonoids have been shown to be effective scavengers of ROS (Middleton *et al*., 2000). This activity is, however, meaningless without the context of specific reaction conditions.

Anti-oxidant properties elicited by plant species therefore have a full range of applications in human healthcare, as they protect against these radicals. Knowledge of the potential anti-oxidant compounds present within a plant species does not necessarily indicate its anti-oxidant capacity, as the total anti-oxidant effect may be greater than the individual anti-oxidant activity of one compound, due to synergism between different anti-oxidant compounds. Methods to measure the anti-oxidant activity in plant material generally involve both the generation of radicals (and their related compounds), and the addition of anti-oxidants, the latter resulting in the reduction of the radical and its consequent disappearance (Arnao *et al.*, 1999).

Synthetic anti-oxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, have been developed, but their uses are limited due to their toxicity. In search for sources of novel anti-oxidants with low toxicity, medicinal plants have over the past few years been studied extensively for their radical scavenging activity (Molyneux, 2004). As plants produce a large number of anti-oxidants to control the oxidative stress caused by sunbeams and oxygen, it is clear that plants may represent a source of new compounds with anti-oxidant activity (Scartezzini and Speroni, 2000).

3.1.3 *Commiphora* and its anti-oxidant potential

Natural resins and bio-active triterpenes have been studied for their anti-oxidant effects on vegetable oils such as olive, corn and sunflower oils and animal fats, all of which are used as oil base resins and triterpene dispersion media in cosmetic and pharmaceutical preparations. Assimopoulou and colleagues (2005) investigated the anti-oxidant properties of myrrh from *Commiphora myrrha* and other natural resins such as mastic derived from *Pistacia lentiscus* and gum exudates from *Boswellia serratax*. *Commiphora myrrh* displayed anti-oxidant activity in sunflower oil, with only slight anti-oxidant activity in lard (Assimopoulou *et al*., 2005).

3.1.4 Isolation of bio-active compounds

Medicinal plants have formed the basis of health care since earliest times of humanity and are still being widely used. The clinical, pharmaceutical and economic value continues to grow, varying between countries. Chemodiversity in plants has proven to be important in pharmacological research and drug development, not only for the isolation of bio-active compounds used directly as therapeutic agents, but also as leads to the synthesis of drugs or as models for pharmacologically active compounds (Mendonça-Filho, 2006). The rapid identification of these bio-active compounds, however, is critical if this tool of drug discovery is to compete with developments in technology.

Plant preparations are distinguished from chemical drugs due by their complexity - mixtures containing large numbers of bio-active compounds. This brings about the challenge of drug discovery from natural sources. When an active extract has been identified, the first task is the identification of the bio-active phytocompounds. Figure 3.1 provides an overview of the procedure from extraction to identification. The coupling of chromatographic methods such as high performance liquid chromatography with diode array detection, mass spectrometry and nuclear magnetic resonance spectroscopy are important, together with the bioassays, for the acquisition of biologically active compounds.

Active organic compounds in extracts are isolated using bioassay guided fractionation, in which chromatographic techniques (such as thin layer chromatography and column chromatography) are used. The successful isolation of compounds from plant materials largely depends on the type of solvent used for extraction (Lin *et al.*, 1999). The extract is separated into individual components and the biological activity of each fraction is determined until a pure active compound is obtained. The pure compound is then identified using methods such as mass spectrometry and nuclear magnetic resonance spectroscopy

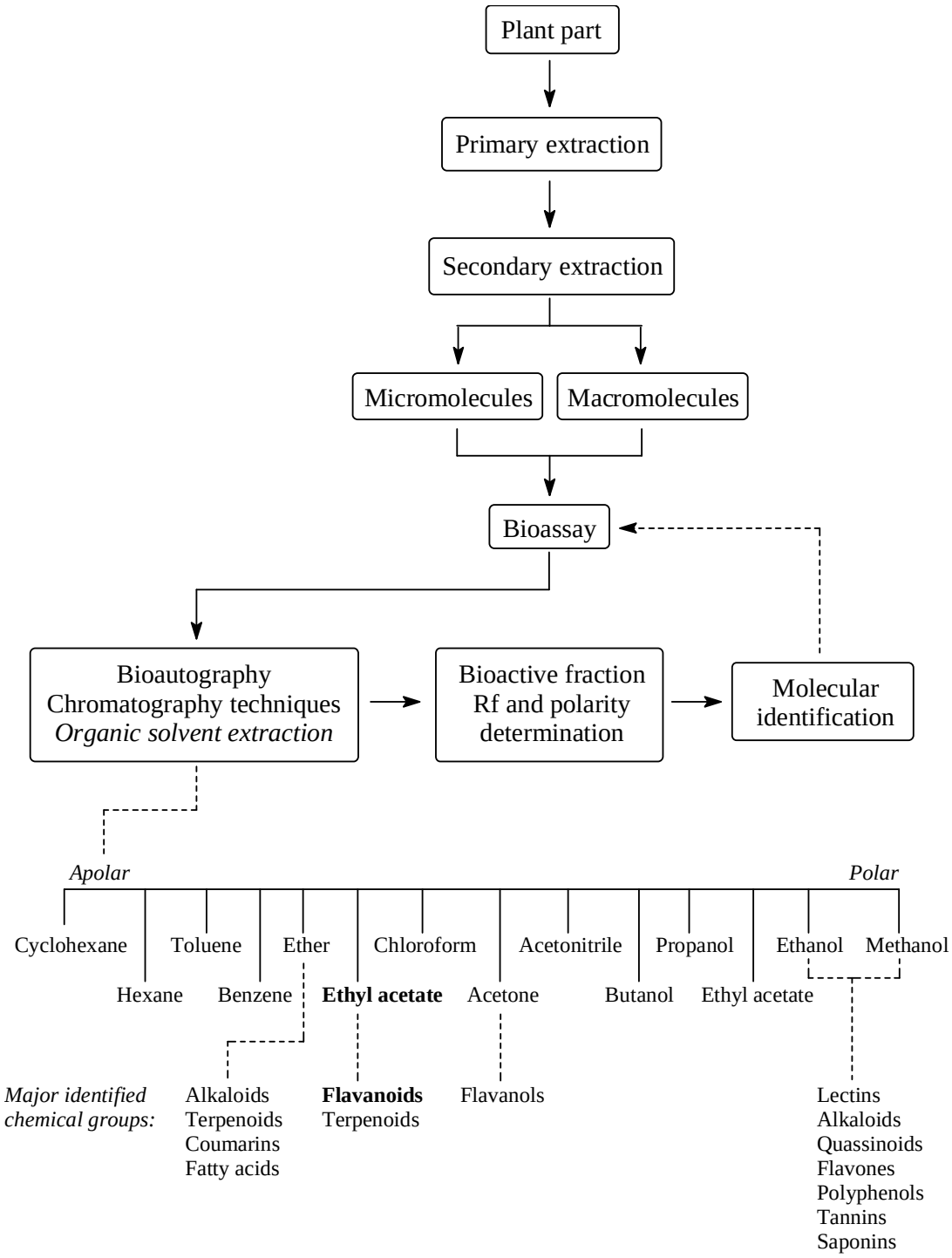


Figure 3.1: Overview of procedure from extraction to identification (modified from Mendonça-Filho, 2006).

Nuclear magnetic resonance (NMR) is a spectroscopic technique involving a magnetic field in which a sample is placed. The sample is then subjected to radiofrequency radiation at the appropriate frequency, allowing for the absorption of energy depending on the type of nucleus, whether, for example, it is a ^{1}H or ^{13}C. The frequency also depends on chemical environment of the nucleus, whether methyl or hydroxyl protons are present, molecular conformations, and dynamic processes. Both ^{1}H NMR and ^{13}C NMR are used, with the latter providing information on the carbon skeleton, and the former relating to the specifics of the hydrogen atoms, thus complementing each other (James, 1998).

Research has demonstrated that natural products represent an unparalleled reservoir of molecular diversity. The isolation and identification of bio-active phytocompounds in medicinal plant extracts generally used by local population to treat diseases would prove an immeasurable contribution to drug discovery and development (Mendonça-Filho, 2006). This would not only validate the traditional use of herbal remedies but also provide leads in the search for new active principles. Investigations surrounding the non-volatile constituents of indigenous *Commiphora* species in general, have not been the focus of any phytochemical study.

3.2 Materials and methods

3.2.1 Thin layer chromatography

The subsequent chapters provided an indication of the good pharmacological activity portrayed by the species and on this basis the stem extract of *C. glandulosa* was chosen for the isolation of bio-active compounds. Screening of the anti-oxidant activity of the stem and leaf extracts was conducted through the use of thin layer chromatography (TLC). Activity guided fractionation through the use of TLC is used to separate the biologically active compounds. Aliquots of 2 µl of the chloroform: methanol (1:1) extracts of the leaves and 5 µl of the dichloromethane extracts of the stems was applied to a silica gel (Alugram® Sil G/UV_{254}, Macherey-Nagel) TLC plate using a calibrated glass capillary tube (Hirschmann Laborgerate). The TLC plate was placed in a developing chamber and allowed to develop in a mobile phase consisting of toluene: dioxin: acetic acid (90:25:10). Once developed, the TLC plate was removed and air-dried. The plate was then viewed under UV light (Camag) of wavelength 254 nm and 356 nm. Anti-oxidant activity was

determined by spraying the TLC plate, using an atomizer, with a solution of DPPH spray reagent (0.04% in HPLC grade methanol from Ultrafine Limited). The plates were allowed to stand for approximately 5 min and the colour change (if any) was noted for compounds with the ability to scavenge the radical, reducing the DPPH, resulting in a colour change from a deep purple to a yellow-white.

Once the screening of the anti-oxidant activity was conducted using TLC, two assays, viz. the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the 2,2'-azino-*bis*(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assay were employed for the determination of potential anti-oxidant activity of 10 indigenous species of *Commiphora*. Both these methods employ a colorimetric quantification of the degree of anti-oxidant activity of the extracts.

3.2.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

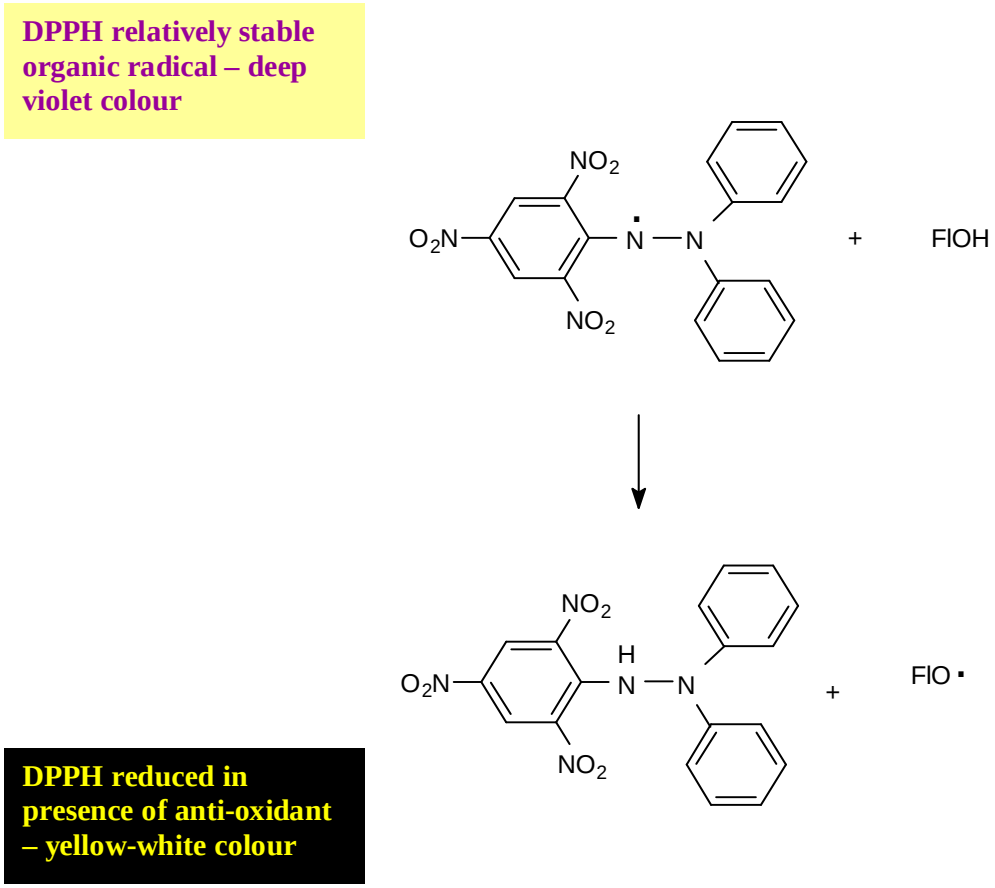
The anti-oxidant activity of each of the plant extracts was determined using the colorimetric DPPH assay, as described by Shimada *et al*. (1992), to determine the radical scavenging activity of the plant extracts.

3.2.2.1 Principle of the assay

The hydrogen donating capacity of test samples is quantified in terms of their ability to scavenge the relatively stable, organic free radical DPPH and by consequent reduction thereof (Figure 3.2). The absorption of the deep violet DPPH solution is measured at 550 nm, after which absorption decreases due to decolourisation to a yellow-white colour, in the event of reduction. This decrease in absorption is stoichiometric according to the degree of reduction. The remaining DPPH is measured at a time interval of 30 min after the addition of the DPPH, which corresponds inversely to the radical scavenging activity of the sample extract or anti-oxidant.

3.2.2.2 Screening for anti-oxidant activity using thin layer chromatography

Thin layer chromatography was employed as a preliminary screening method to determine the potential anti-oxidant activity of compounds in extracts of indigenous *Commiphora* species. Extracts were prepared in chloroform and methanol in a volumetric ratio of 1:1.



FIOH = Flavonoid compound, FIO$^{\bullet}$ = Flavonoid having donated a hydrogen

Figure 3.2: Diagrammatic representation of chemical reaction of the reduction of DPPH in the presence of an electron donating anti-oxidant (Brand-Willians *et al.,* 1995).

Approximately 4 µl of each extract was applied to the TLC plate, which were developed in a mobile phase comprising of toluene: dioxan: acetic acid (90:25:10). After drying, compounds were visualised using 0.4 mM DPPH spray reagent. After approximately 5 min, reduction of DPPH, resulting in a colour change from a deep purple to a yellow-white (Figure 3.2), was observed for compounds with radical scavenging abilities.

3.2.2.3 Colorimetric spectrophotometric assay

A 96-well microtiter plate was used to generate the quantitative measure of the radical scavenging activity of 10 indigenous species of *Commiphora*.at different concentrations, prepared as serial dilutions, ranging from 100 µg/ml to 0.78 µg/ml. The assay was conducted in triplicate. Aliquots of 50 µl of plant extract prepared in DMSO (Saarchem) were plated out, to which 200 µl of DPPH (Fluka), prepared in HPLC grade methanol (Ultrafine Limited), was added to the wells in columns 1, 3, 5, 7, 9 and 11 (Figure 3.3). The plate was shaken for 2 min, after which it was stored in the dark for 30 min. The percentage decolourisation was measured spectrophotometrically at 550 nm using the Labsystems Multiskan RC microtiter plate reader, linked to a computer equipped with GENESIS® software. The negative controls contained 50 µl of plant extract to which 200 µl of methanol was added, in the absence of DPPH (wells in columns 2, 4, 6, 8, 10 and 12) and the positive control was prepared using analytical grade ascorbic acid, to which 200 µl of DPPH was added (Figure 3.3). The percentage decolourisation was then determined for each of the test samples (using equation 3.1), as a measure of the free radical scavenging activity.

For those extracts with a percentage decolourisation of more than 50% at the starting concentration of 100 µg/ml, the IC_{50} value was determined, which relates to the concentration of test sample required to scavenge 50% of DPPH in 1 ml of the reaction mixture. Percentage decolourisation was plotted against the concentration of the sample and the IC_{50} values were determined using Enzfitter® version 1.05 software.

Equation 3.1

$$\% \text{ decolourisation} = \frac{[\text{Av controls} - (\text{Av sample}_{DPPH} - \text{Av sample}_{methanol})] \times 100}{\text{Av controls}}$$

where:

Av controls = average absorbance of all DPPH control wells – average absorbance of all methanol control wells

Av sample_{DPPH} = average absorbance of sample wells with DPPH

$\text{Av sample}_{methanol}$ = average absorbance of sample wells with methanol

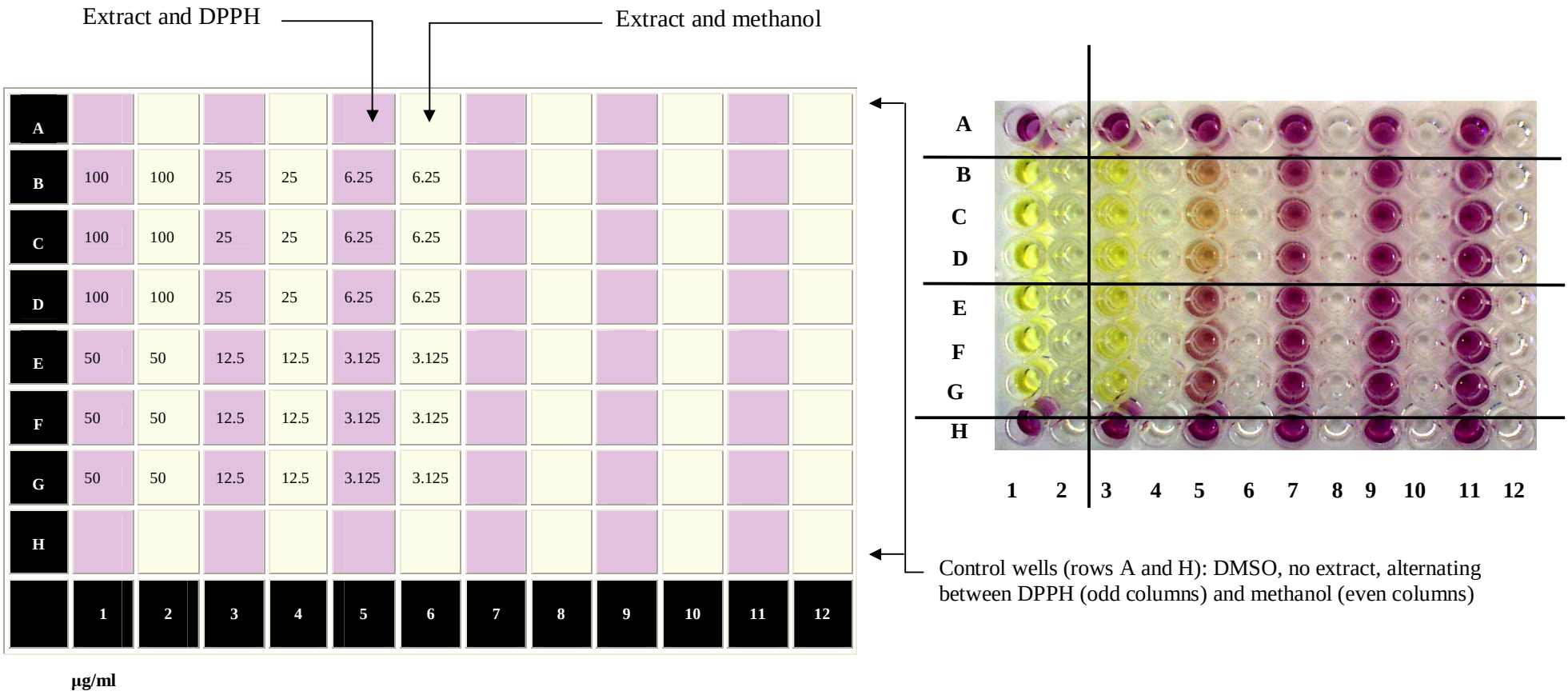


Figure 3.3: Representative 96-well microtiter plate, indicating final concentrations of plant extracts (left); A 96-well microtiter plate prepared for use in the DPPH assay. Purple wells indicate the absence of an anti-oxidant effect; yellow wells are indicative of the presence of extracts with anti-oxidant activity (right)

Vitamin C (L-ascorbic acid) and Trolox were used as positive controls. At least three independent replicates were performed for each sample and results are given as mean ± s.d.

3.2.3 2,2'-Azino-*bis*(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assay

3.2.3.1 Principle of the assay

The ABTS anti-oxidant assay, also known as the Trolox equivalent anti-oxidant capacity (TEAC) assay, assesses the total radical scavenging capacity of the plant extracts. This is determined through the ability of these extracts to scavenge the long-lived specific ABTS radical cation chromophore in relation to that of Trolox, the water-soluble analogue of vitamin E. This assay was first reported by Miller *et al*. (1993) and Rice-Evans (1994).

The generation of the $ABTS^+$ blue/green chromophore occurs through the oxidation of ABTS diammonium salt in the presence of potassium persulfate (Figure 3.4), with the absorption maxima occurring at wavelengths 645 nm, 734 nm and 815 nm. Anti-oxidants will reduce the pre-formed radical cation to ABTS, and in so doing bring about the decolourisation of $ABTS^+$ to a colourless product. The extent of this decolourisation is a measure of the $ABTS^+$ radical cation that has been scavenged, after a fixed time period, and is relative to the Trolox standard.

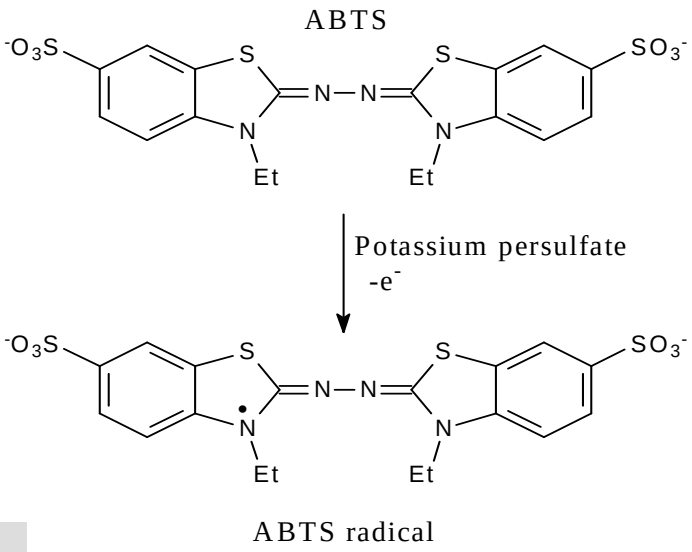
3.2.3.2 Screening for anti-oxidant activity using thin layer chromatography

As described in section 3.2.2.2 thin layer chromatography was employed, using a 7 mM $ABTS^+$ spray reagent. After approximately 5 min, a colour change, from deep turquoise to yellow-white, was observed on the TLC plate for compounds with radical scavenging abilities.

3.2.3.3 Colorimetric spectrophotometric method

The quenching of the ABTS radical cation results in the evaluation of the radical scavenging activity of each of the plant extracts. Stock solutions of concentration 10 mg/ml of each of the plant extracts were prepared in DMSO. Serial dilutions were prepared, ranging from 5 mg/ml to 0.001 mg/ml.

ABTS – transparent



ABTS radical relatively stable organic radical – deep turquoise green colour

Figure 3.4: Diagrammatic representation of the formation of the ABTS radical after the addition of potassium persulphate (Dorman *et al*., 2000).

Trolox (6-hydroxy-2,5,7,8-tetramethylchromon-2-carboxylic acid, Sigma-Aldrich) was prepared in ethanol and serial dilutions of this positive control were also prepared. Ethanol was used as the negative control. A 7 mM stock solution of ABTS (Sigma-Aldrich) was prepared in double distilled water. The ABTS radical cation was then prepared by the addition of 88 µl of 140 mM potassium persulfate ($K_2S_2O_8$) to 5 ml of ABTS. This solution was then stored in the dark for 12 - 16 hours in order to stabilise it before use. It remains stable for 2 - 3 days in the dark.

The concentrated $ABTS^+$ solution was diluted with cold ethanol shortly before conducting the assay, to a final absorbance of 0.70 ± 0.02 at 734 nm at 37 °C, in a 3 cm cuvette. The total scavenging capacity of the extracts was quantified through the addition of 1000 µl $ABTS^+$ to 50 µl of plant extract. The solutions were heated on a heating mantle to 37 °C for 4 min, after which the absorbance was read at 734 nm on a spectrophotometer (Milton Roy Spectronic GENESYS 5). All experiments were done in triplicate. The percentage decolourisation was calculated using equation 3.2 and the extent of inhibition of the absorbance of the $ABTS^+$ was plotted as a function of the concentration to determine the Trolox equivalent anti-oxidant capacity (TEAC), which can be assessed as a function of time.

Equation 3.2

$$\% \text{ decolourisation} = \frac{1 - (\text{Abs test sample at 734 nm})}{\text{Abs control at 734 nm}} \times 100$$

3.2.4 Isolation of compound 1 – column chromatography

3.2.4.1 Silica gel column chromatography

A glass column (Figure 3.5) was clamped upright and packed with silica gel (size 0.063-2 mm, Macherey-Nagel) mixed with the appropriate mobile phase (chloroform, Figure 3.6) and poured into the column as a compact even suspension. This constituted the stationary phase. The extract was then mixed with a small amount of the mobile phase, and introduced as a thin band to the silica gel. Once the extract was loaded onto the silica gel, the mobile phase was added at a constant flow rate. Gradient elution of increasing polarity was initiated consisting of successive elutions of chloroform, chloroform:ethyl acetate (9:1→1:9) and ethyl acetate (Figure 3.6).

Fractions were collected from the column and subsequently the anti-oxidant activity of each fraction obtained was determined and confirmed by TLC through the use of DPPH

spray reagent. Fractions 6-10 were then combined and further fractionation procedures were carried out using size exclusion column chromatography.

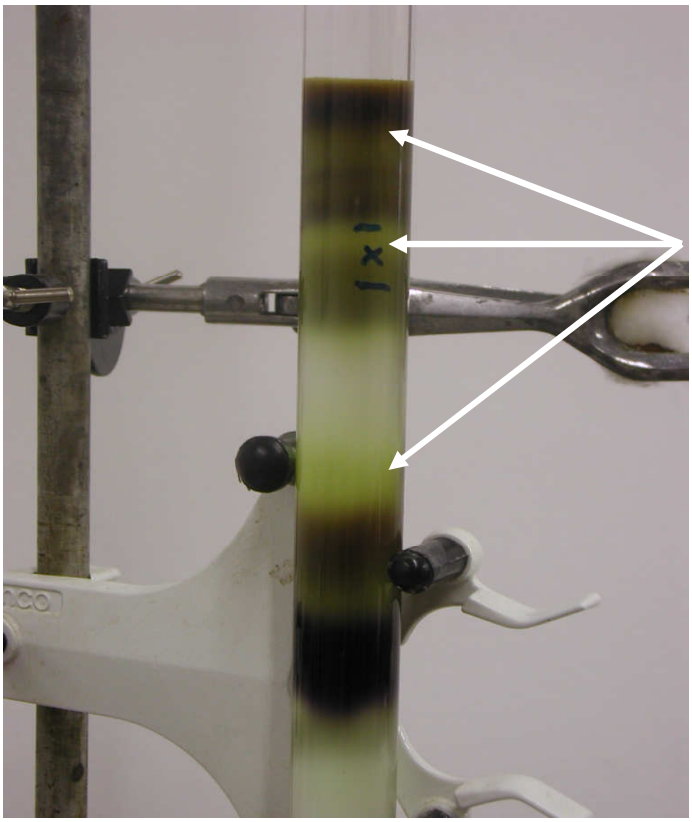


Figure 3.5: Glass column used in silica gel column chromatography for the isolation of compound 1. The extract was loaded onto the silica; the mobile phase was added at a constant flow rate.

3.2.4.2 Size-exclusion column chromatography

Subsequent to the silica gel column chromatography, size-exclusion column chromatography was utilised. The principle governing this method is based on the molecular size differences of the compounds, which result in the separation of these compounds. The stationary phase comprised of a porous three-dimensional polymeric matrix, namely Sephadex^{TM} LH-20 (Amersham Biosciences) with a fractionation range of <1.5 Daltons. This matrix was initially saturated with methanol in order to facilitate swelling before use. Once this was achieved the matrix was then introduced into a glass column and the fractions containing the active compound introduced as a thin band was then applied to the column. The mobile phase consisted of chloroform: methanol (1:1) (Figure 3.6). Fractions were collected from the column and the anti-oxidant activity was

again determined. Fractions 62-74 appeared to be the isolated active fractions and these were combined for nuclear magnetic resonance (NMR) characterisation.

3.2.5 Isolation of compound 2 – column chromatography

3.2.5.1 Silica gel column chromatography

The isolation procedure followed a similar protocol as that outlined for compound 1. Silica gel (size 0.063-2 mm, Macherey-Nagel) mixed with the appropriate mobile phase, in this case chloroform: ethyl acetate (3:7) was poured into the column. The chloroform extract was introduced to the silica gel. Once loaded, the mobile phase was added at a constant flow rate (Figure 3.6). Fractions were collected from the column and the anti-oxidant activity determined. Fractions 35 - 41 were then combined and further fractionation procedures were carried out using size exclusion column chromatography.

3.2.5.2 Size-exclusion column chromatography

Size-exclusion column chromatography was utilized using Sephadex^{TM} LH-20 (Amersham Biosciences). This matrix was initially saturated with methanol in order to facilitate swelling, and introduced into a glass column and the fractions containing the active compound combined and loaded onto the column matrix as a thin band. The mobile phase consisted of methanol. Fractions were collected from the column and the anti-oxidant activity was again determined. Fractions 5 – 11 appeared to contain isolated active compound (Figure 3.6), and these fractions were combined for NMR characterisation.

3.2.6 Nuclear magnetic resonance spectroscopy

Final chemical characterisation of the two isolated compounds was achieved by NMR, which was performed in collaboration with Professor S. Drewes (School of Chemistry, University of KwaZulu-Natal, South Africa).

Nuclear magnetic resonance spectroscopy was performed on a 500MHz Varian UNITY-INOVA spectrophotometer. All spectra were recorded at room temperature in a 1:1

mixture of deuteromethanol (CD_3OD) and deuterochloroform ($CDCl_3$). The chemical shifts were all recorded in parts per million (ppm) relative to Trimethyl silane (TMS).

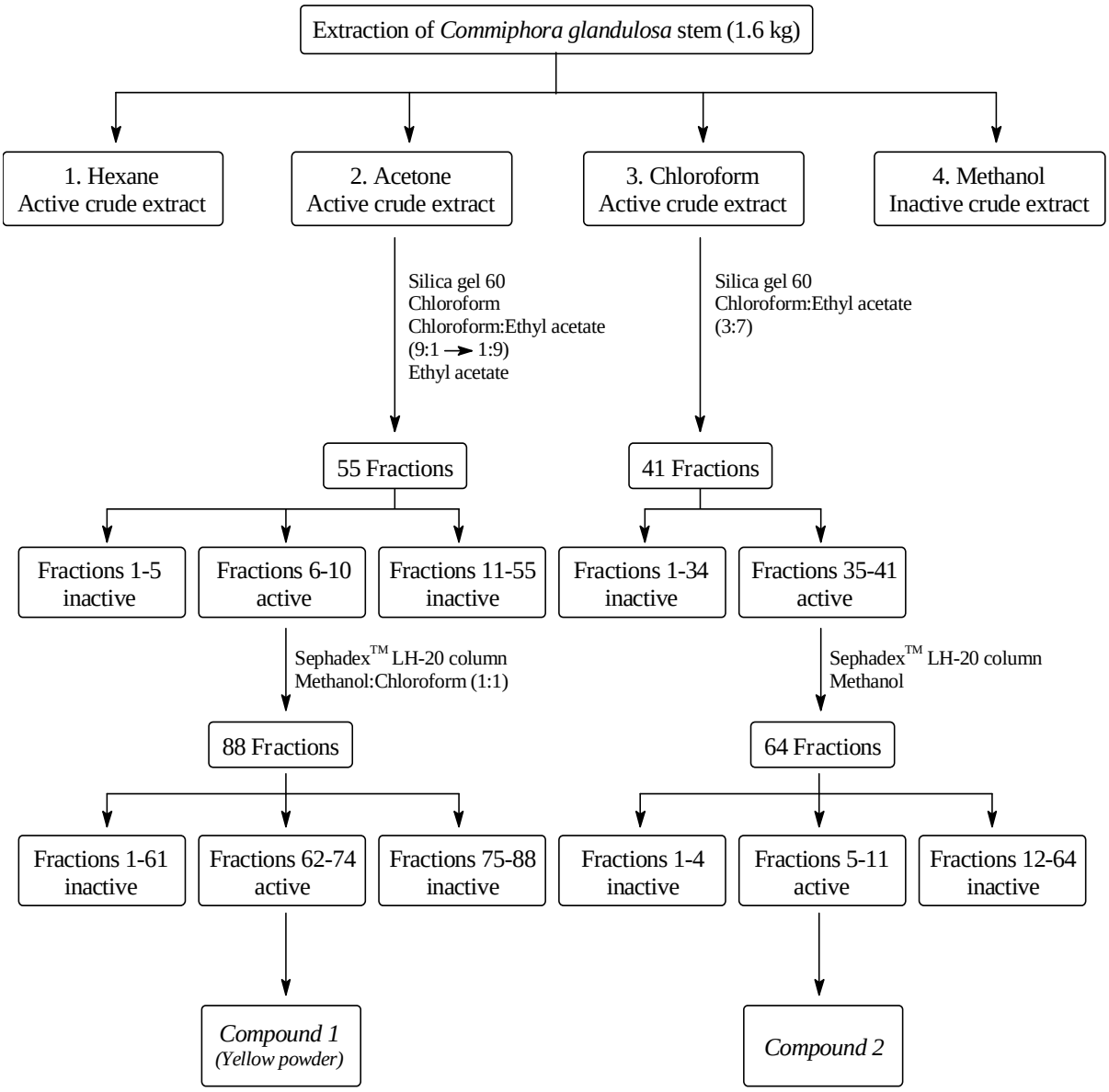


Figure 3.6: Schematic representation of the isolation and purification of compound 1 (kaempferol) and compound 2 (dihydrokaempferol) isolated from *Commiphora glandulosa* (stem).

3.2.7 Anti-oxidant activity of isolated compounds

It was evident from TLC analysis that a large number of compounds may contribute to anti-oxidant activity. In an attempt to isolate these potentially bio-active molecules, kaempferol and dihydrokaempferol were isolated. These compounds were prepared in the same way as the extracts, and were subsequently subjected to the DPPH assay, as described previously (section 3.2.2), in order to determine the anti-oxidant potential of each of the compounds, separately as well as to determine whether the combination of the two compounds will bring about a synergistic, antagonistic or additive effect (Figure 3.7).

Different concentrations of kaempferol were prepared using serial dilutions. These serial dilutions were combined with serial dilutions of dihydrokaempferol. The IC_{50} value for each combination was calculated and the isobolograms were constructed to determine the fractional IC_{50} values for kaempferol against the fractional IC_{50} value of dihydrokaempferol. The interaction between the compounds was determined through isobolographic analysis. Data points above the diagonal line serve as an indication of an antagonistic interaction between the compounds concerned, while those data points below the diagonal line provide an indication of a synergistic interaction. Data points lying on the line are indicative of a combination with an additive effect, as described by Berenbaum (1978) and Williamson (2001).

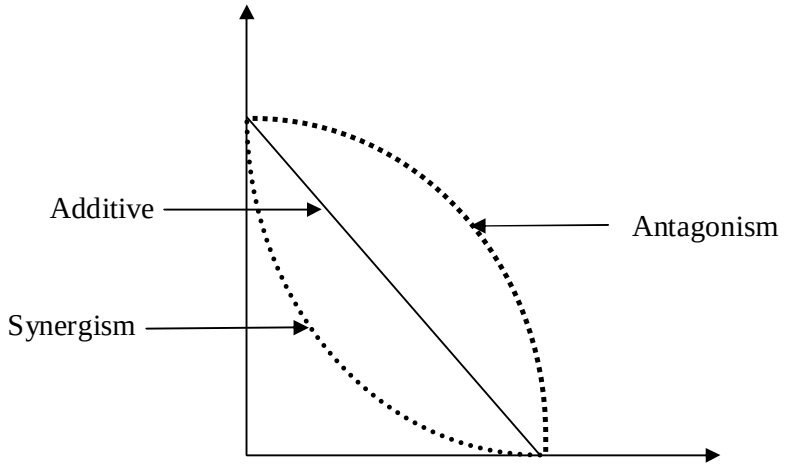
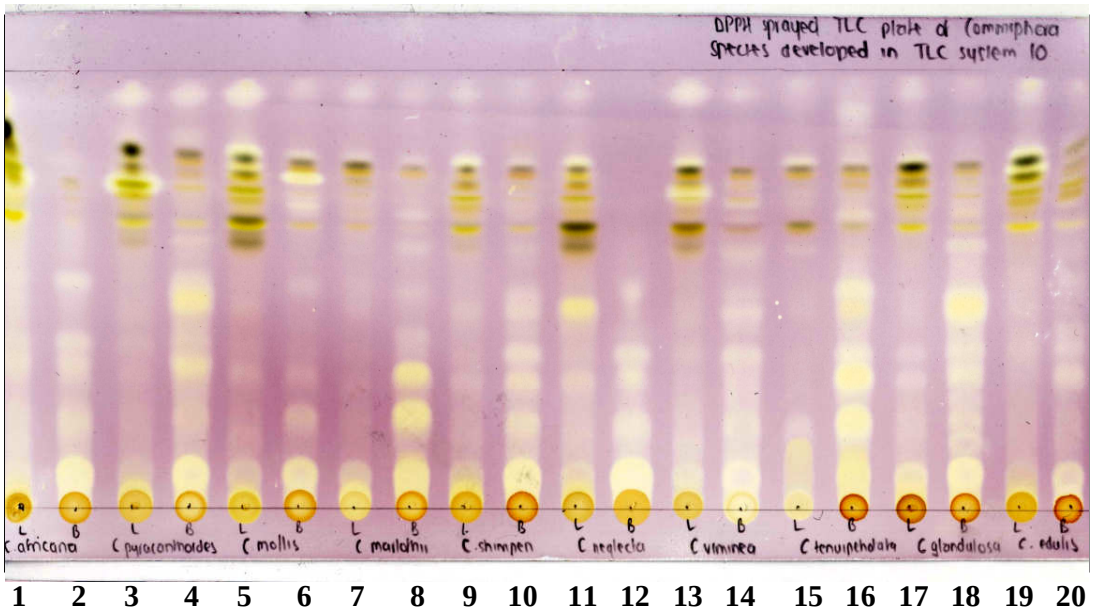


Figure 3.7: Isobologram depicting possible synergistic, antagonistic or additive effects as a result of either an interaction or a lack of interaction that exists between the compounds concerned (modified from Berenbaum, 1978).

3.3 Results

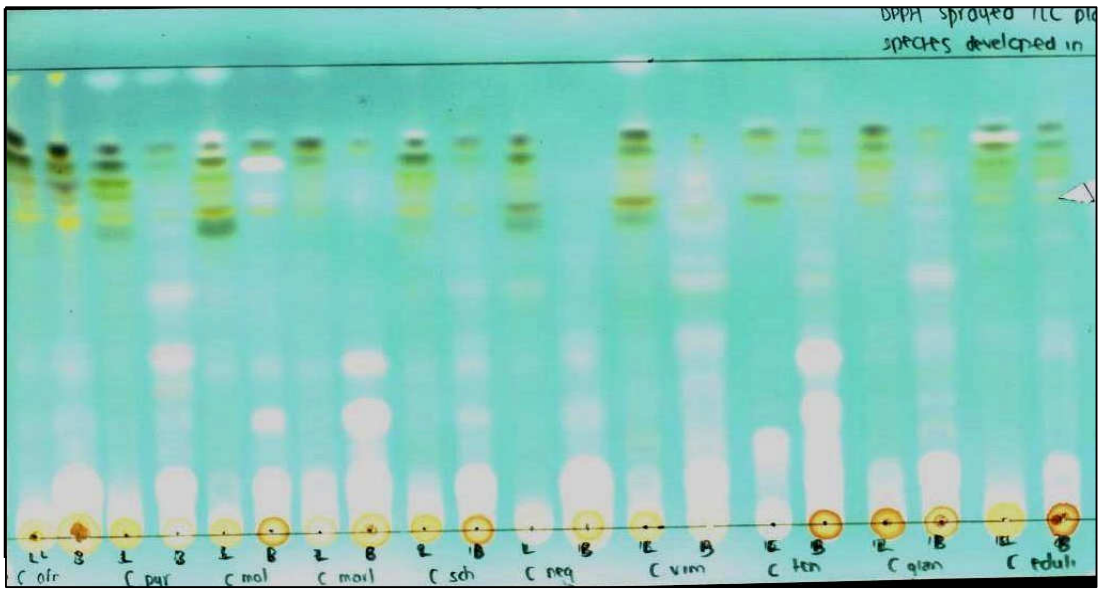
3.3.1 Screening for anti-oxidant activity using thin layer chromatography

Thin layer chromatography was performed as a preliminary screening process to determine whether *Commiphora* possessed anti-oxidant activity, as shown in Figure 3.8 (using DPPH as the spray reagent) and Figure 3.9 (using ABTS as the spray reagent). The TLC plate displays the compounds present in each of the species responsible for the anti-oxidant activity, in both the stem and the leaf extracts. This is visualised as yellow-white spots against a purple background, once sprayed with DPPH (Figure 3.8). On the TLC plate sprayed with ABTS, the anti-oxidant activity is visualized as white spots on a green background (Figure 3.9).



1. *Commiphora africana* (leaf); **2**. *C. africana* (stem); **3**. *C. pyracanthoides* (leaf); **4**. *C. pyracanthoides* (stem); **5**. *C. mollis* (leaf); **6**. *C. mollis* (stem); **7**. *C. marlothii* (leaf); **8**. *C. marlothii* (stem); **9**. *C. schimperi* (leaf); **10**. *C. schimperi* (stem); **11**. *C. neglecta* (leaf); **12**. *C. neglecta* (stem); **13**. *C. viminea* (leaf); **14**. *C. viminea* (stem); **15**. *C. tenuipetiolata* (leaf); **16**. *C. tenuipetiolata* (stem); **17**. *C. glandulosa* (leaf); **18**. *C. glandulosa* (stem); **19**. *C. edulis* (leaf); **20**. *C. edulis* (stem).

Figure 3.8: Thin layer chromatography plate, developed in a mobile phase consisting of toluene: dioxin: acetic acid (90:25:10), was used to determine anti-oxidant compounds present in the extracts of the *Commiphora* species studied, using the DPPH spray reagent. The anti-oxidant compounds are observed as yellow-white spots on a purple background.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

1. *Commiphora africana* (leaf); **2**. *C. africana* (stem); **3**. *C. pyracanthoides* (leaf); **4**. *C. pyracanthoides* (stem); **5**. *C. mollis* (leaf); **6**. *C. mollis* (stem); **7**. *C. marlothii* (leaf); **8**. *C. marlothii* (stem); **9**. *C. schimperi* (leaf); **10**. *C. schimperi* (stem); **11**. *C. neglecta* (leaf); **12**. *C. neglecta* (stem); **13**. *C. viminea* (leaf); **14**. *C. viminea* (stem); **15**. *C. tenuipetiolata* (leaf); **16**. *C. tenuipetiolata* (stem); **17**. *C. glandulosa* (leaf); **18**. *C. glandulosa* (stem); **19**. *C. edulis* (leaf); **20**. *C. edulis* (stem).

Figure 3.9: Thin layer chromatography plate, developed in a mobile phase consisting of toluene: dioxin: acetic acid (90:25:10), indicating anti-oxidant compounds present in the extracts of the *Commiphora* species studied, using ABTS spray reagent.

These TLC results provided a clear indication of the presence of free radical scavenging compounds, which prompted the colorimetric quantitative spectrophotometric analysis.

3.3.2 Colorimetric spectrophotometric assays

The anti-oxidant potential of each of the extracts of *Commiphora* species under investigation are summarised in Table 3.1 and demonstrated in Figure 3.10, in both the DPPH and ABTS anti-oxidant assays.

Table 3.1: *In vitro* anti-oxidant activity (µg/ml) of extracts from indigenous *Commiphora* species, as shown by the DPPH and ABTS assays. Results are given as mean ± s.d, n=3.

Species	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)
***C. africana* (leaf)**	43.00 ± 1.37	29.64 ± 3.81
***C. africana* (stem)**	39.44 ± 1.70	12.97 ± 1.23
***C. edulis* (leaf)**	59.70 ± 1.97	n.d.
***C. edulis* (stem)**	10.59 ± 0.50	23.75 ± 3.51
***C. glandulosa* (leaf)**	41.39 ± 1.73	12.19 ± 0.16
***C. glandulosa* (stem)**	27.27 ± 0.15	10.69 ± 1.47
***C. marlothii* (leaf)**	66.81 ± 0.43	17.66 ± 0.75
***C. marlothii* (stem)**	32.16 ± 1.72	15.67 ± 1.79
***C. mollis* (leaf)**	89.95 ± 0.04	60.11 ± 8.73
***C. mollis* (stem)**	22.17 ± 0.33	8.82 ± 0.72
***C. neglecta* (leaf)**	98.61 ± 1.97	n.d.[a]
***C. neglecta* (stem)**	10.36± 1.89	7.28 ± 0.29
***C. pyracanthoides* (leaf)**	29.32 ± 5.22	51.44 ± 0.27
***C. pyracanthoides* (stem)**	19.02 ± 0.12	18.68 ± 8.84
***C. schimperi* (leaf)**	55.30 ± 3.73	25.25 ± 1.74
***C. schimperi* (stem)**	7.31 ± 0.14	11.22 ± 3.61
***C. tenuipetiolata* (leaf)**	10.81 ± 0.56	17.47 ± 1.30
***C. tenuipetiolata* (stem)**	10.75 ± 0.36	5.10 ± 0.66
***C. viminea* (leaf)**	78.49 ± 3.46	45.89 ± 0.79
***C. viminea* (stem)**	84.01 ± 7.07	26.30 ± 0.23
Kaempferol	3.32 ± 1.27	4.27 ± 0.13
Dihydrokaempferol	301.60 ± 2.79	n.d.[a]
Vitamin C	4.18 ± 0.56	n.d.[b]
Trolox	7.03 ± 1.16	5.41 ± 0.51

n.d.[a] = not detemined as a result of insufficient plant material or compound.
n.d.[b] = not detemined, not required as a control

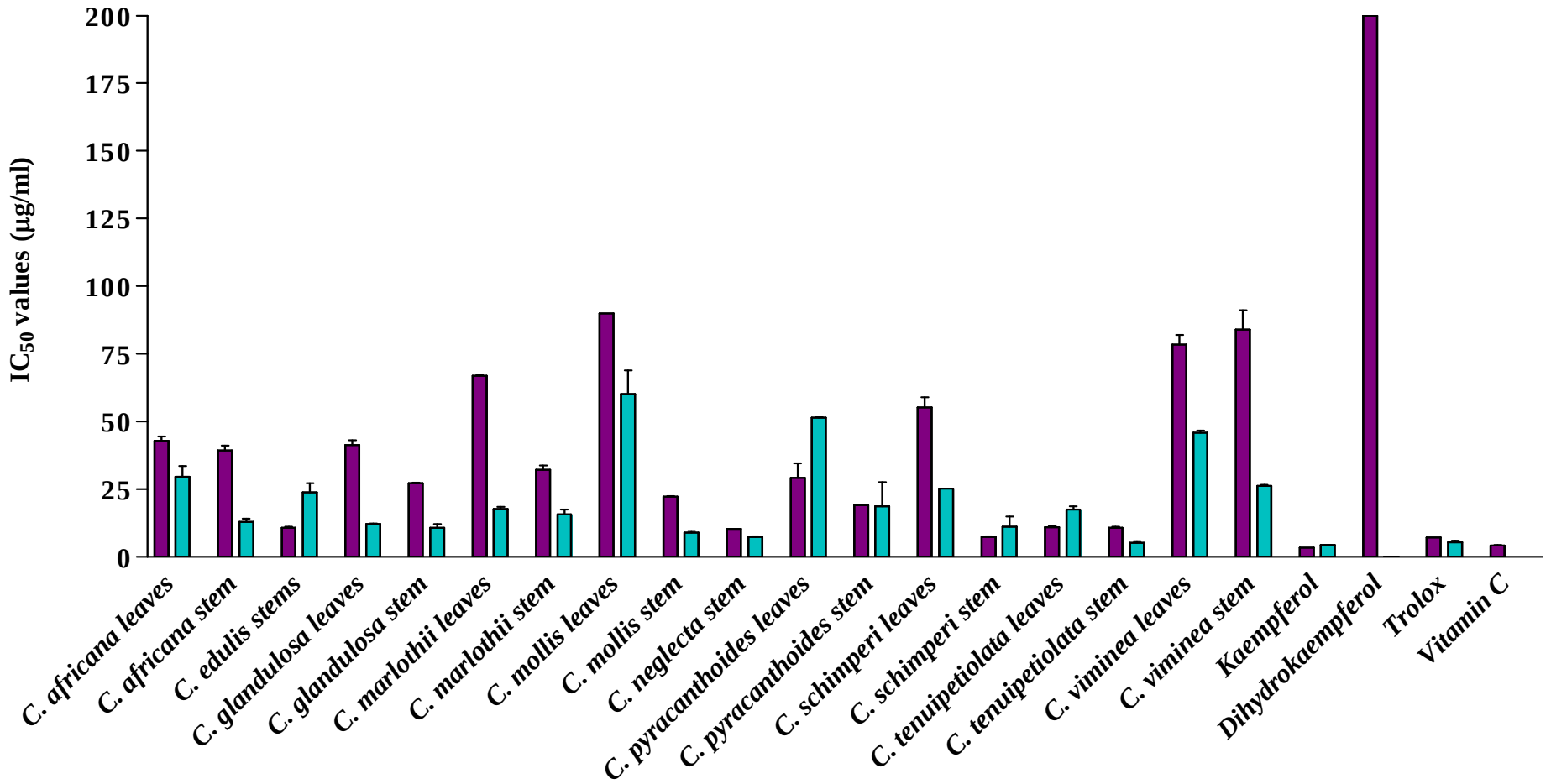


Figure 3.10: Comparative DPPH ( ) and ABTS ( ) radical scavenging capacity of each of the *Commiphora* species extracts and the isolated kaempferol and Trolox control, demonstrated by IC_{50} values with the exception of *C. edulis* leaves and *C. neglecta* leaves; the standard error of the mean of three replicates are denoted by error bars (n = 3 experiments).

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In general, the extracts of *Commiphora*, displayed poor anti-oxidant activity in the DPPH assay with IC_{50} values ranging between 7.31 ± 0.14 μg/ml and 98.61 ± 1.97 μg/ml, with the exception of the stem extracts of *C. schimperi* (IC_{50} = 7.31 ± 0.14 μg/ml), *C. neglecta* (IC_{50} = 10.36 ± 1.89 μg/ml), *C. edulis* (IC_{50} = 10.59 ± 0.50 μg/ml) and *C. tenuipetiolata* (IC_{50} = 10.75 ± 0.36 μg/ml) and the leaf extract of *C. tenuipetiolata* (IC_{50} = 10.81 ± 0.56 μg/ml), as compared to the vitamin C control which had an IC_{50} value of 4.18 ± 0.56 μg/ml. The flavonol, kaempferol (IC_{50} = 3.32 ± 1.27 μg/ml) displayed exceptional radical scavenging activity, in contrast to the activity displayed by dihydrokaempferol (IC_{50} = 301.57 ± 2.79 μg/ml) (Figure 3.10).

The results obtained in the ABTS assay differed from the results obtained in the DPPH assay, with a greater anti-oxidant activity observed for most of the species in the ABTS assay (Figure 3.10 and Table 3.1). The highest activity (in the ABTS assay) was observed in the stem extracts of *C. tenuipetiolata* (IC_{50} = 5.10 ± 0.66 μg/ml), *C. neglecta* (IC_{50} = 7.28 ± 0.29 μg/ml) and *C. mollis* (IC_{50} = 8.82 ± 0.72 μg/ml). Of the leaf extracts, *C. glandulosa* displayed the most favourable radical scavenging activity (IC_{50} = 12.19 ± 0.16 μg/ml) followed by *C. tenuipetiolata* and *C. marlothii*, which displayed similar activity (IC_{50} = 17.47 ± 1.30 μg/ml and 17.66 ± 0.75 μg/ml, respectively). Kaempferol displayed radical scavenging activity in the ABTS assay (IC_{50} = 4.27 ± 0.13 μg/ml) consistent with the activity displayed in the DPPH assay (IC_{50} = 3.32 ± 1.27 μg/ml).

3.3.3 Isolation of compounds

Isolated compounds were identified as flavonoids, viz. flavonol and dihydroflavonol. Compound **1** was isolated as a yellow powder. On the basis of the spectral data obtained for ^{1}H NMR [Table 3.2 and Figure B1 (Appendix B)] and ^{13}C NMR [Table 3.3 and Figure B2 (Appendix B)], and compared to that of relative references viz. Soliman *et al*. (2002) and Bin and Yongmin (2003), compound 1 was identified as kaempferol (Figure 3.11). It has an emprical formula of $C_{15}H_{10}O_6$.

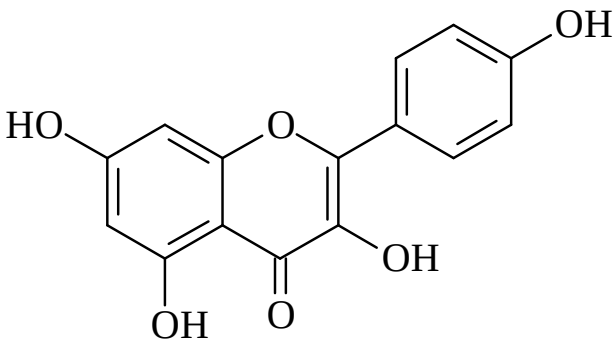


Figure 3.11: The chemical structure of kaempferol (compound 1).

Table 3.2: Comparing the experimental data of ^{1}H NMR of the aglycone kaempferol with that obtained by Bin and Yongmin (2003), Soliman *et al.* (2002) and Xu *et al*. (2005).

^{1}H- position	**K ref nucleus δ_H (ppm) (Chemical shift)**	***J* (Hz) ref**	**K exp nucleus δ_H (ppm) (Chemical shift)**	***J* (Hz) exp**
6	6.20	2.1	6.15	2.1
8	6.40	2.1	6.30	2.1
2′	7.77	8.4	7.97	8.9
3′	6.93	8.4	6.82	8.8
5′	6.93	8.4	6.82	8.8
6′	7.77	8.4	7.97	8.9

Where *J* = coupling constant

Table 3.3: Comparing the experimental data of ^{13}C NMR of the aglycone kaempferol with that obtained by Bin and Yongmin (2003), Soliman *et al.* (2002) and Xu *et al*. (2005).

^{13}C-position	**K ref1. ($DMSO-d_6$) (Chemical shift)**	**K ref2. (CD_3OD) (Chemical shift)**	**K exp. ($CDCL_3/CD_3OD$) (Chemical shift)**
2	146.7	147.0	146.4
3	135.7	136.4	135.2
4	175.8	176.4	175.3
5	156.2	157.6	156.7
6	98.2	99.0	98.4
7	163.8	165.1	160.4
8	93.4	94.3	93.8
9	160.7	161.8	163.8
10	103.0	103.9	103.1
1′	121.7	123.0	122.1
2′	129.5	130.3	129.4
3′	115.4	116.2	115.2
4′	159.2	160.2	158.7
5′	115.4	116.2	115.2
6′	129.5	130.3	129.4

The NMR spectra were in accordance with the structure of a flavonol.

Compound **2** was isolated as yellow needle-like crystals. The compound was identified as dihydrokaempferol (Figure 3.12), on the basis of the spectral data obtained for ^1H NMR [Table 3.4 and Figure B3 (Appendix B)] and ^{13}C NMR [Table 3.5 and Figure B4 (Appendix B)], and compared to the data obtained by Güvenalp and Demirezer (2005) and Bin and Yongmin (2003). Dihydrokaempferol has an empirical formula of $C_{15}H_{12}O_6$.

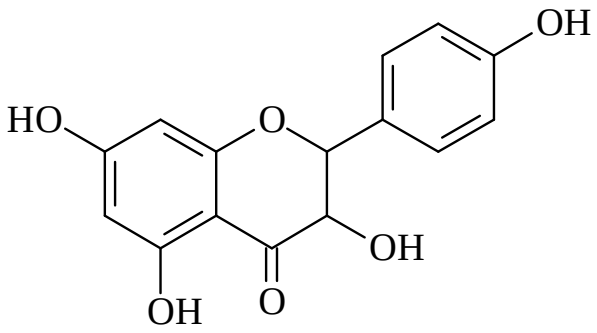


Figure 3.12: The chemical structure of dihydrokaempferol (compound 2).

Table 3.4: Comparing the experimental data of ^{1}H NMR of dihydrokaempferol with that obtained by Güvenalp and Demirezer (2005) and Xu *et al*. (2005).

^{1}H- position	D ref nucleus δ_H (ppm) (Chemical shift)	*J* (Hz) ref	D exp nucleus δ_H (ppm) (Chemical shift)	*J* (Hz) exp
2	5.04	11.0	4.99	-
3	4.60	11.0	4.58	-
6	5.85	2.0	5.85	-
8	5.91	2.0	5.95	-
2′	7.31	8.5	7.32	7.5
3′	6.78	8.5	6.83	7.5
5′	6.78	8.5	6.83	7.5
6′	7.31	8.5	7.32	7.5

Where *J* = coupling constant

Table 3.5: Comparing the experimental data of ^{13}C NMR of dihydrokaempferol with that obtained by Güvenalp and Demirezer (2005) and Xu *et al*. (2005).

^{13}C- position	D ref. (DMSO-d_6) (Chemical shift)	D exp. ($CDCL_3$/CD_3OD) (Chemical shift)
2	83.0	81.1
3	71.6	71.6
4	198.0	197.2
5	163.5	163.1
6	96.2	96.0
7	166.9	166.6
8	95.2	94.9
9	162.7	162.6
10	100.6	100.3
1′	127.7	124.1
2′	129.4	129.0
3′	115.5	117.6
4′	157.9	158.6
5′	115.1	117.6
6′	129.6	129.0

3.3.4 Isobologram construction of the interaction between the isolated compounds with anti-oxidant activity

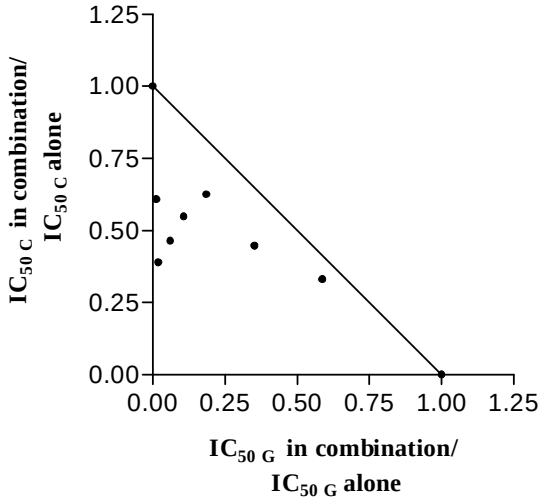
Isobolograms were constructed, as described previously to determine the synergistic, antagonistic or additive anti-oxidant effect between, firstly, *Commiphora glandulosa* (stem) and vitamin C (Table 3.6; Figure 3.13), and secondly between kaempferol and dihydrokaempferol (Table 3.7; Figure 3.14) over a range of concentrations.

The isobologram constructed to determine the interaction between *C. glandulosa* (stem) and vitamin C was performed as a means to develop the method for the interaction that exists between the two isolated compounds, kaempferol, and dihydrokaempferol. The interaction between *C. glandulosa* and vitamin C yielded a synergistic relationship (Table 3.6; Figure 3.13). The interaction that resulted between the combination of the two compounds, however, was antagonistic (Table 3.7; Figure 3.14).

Table 3.6: Data generated for the construction of the isobologram to indicate the interaction between *Commiphora glandulosa* stem extract and vitamin C, in the DPPH assay.

	Concentration		**IC_{50} values (µg/ml)**		**Ratio values**	
Plate	**G**	**C**	**G**	**C**	**G**	**C**
1 (10:0)	34.85	0.00	34.85	0.00	1.00	0.00
2 (9:1)	31.37	0.69	20.50	2.28	0.59	0.33
3 (8:2)	27.88	1.38	12.30	3.08	0.35	0.45
4 (6:4)	20.91	2.76	6.47	4.31	0.19	0.63
5 (5:5)	17.43	3.45	3.78	3.78	0.11	0.55
6 (4:6)	13.94	4.14	2.13	3.20	0.06	0.46
7 (2:8)	6.97	5.52	0.67	2.69	0.02	0.39
8 (1:9)	3.49	6.20	0.47	4.20	0.01	0.61
9 (0:10)	0.00	6.90	0.00	6.89	0.00	1.00

G = *Commiphora glandulosa* (stem), C = vitamin C.



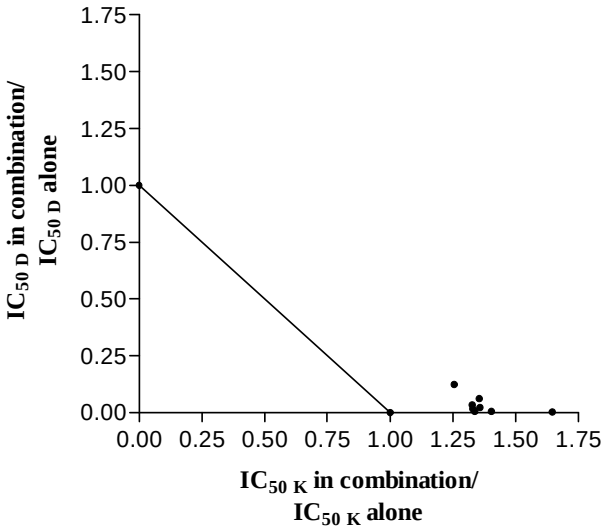
G = *Commiphora glandulosa*, C = vitamin C.

Figure 3.13: Isobologram of the interaction between *Commiphora glandulosa* (stem) and vitamin C, showing a synergistic relationship.

Table 3.7: Data generated for the construction of the isobologram to indicate the interaction between kaempferol and dihydrokaempferol, in the DPPH assay.

	Concentration		**IC_{50} values (µg/ml)**		**Ratio values**	
Plate	**K**	**D**	**K**	**D**	**K**	**D**
1 (10:0)	3.32	0.00	3.32	0.00	1.00	0.00
2 (9:1)	2.99	30.16	5.47	0.61	1.65	0.002
3 (8:2)	2.65	60.31	4.66	1.16	1.40	0.004
4 (7:3)	2.32	90.47	4.44	1.90	1.34	0.01
5 (5:5)	1.66	150.78	4.41	4.41	1.33	0.02
6 (4:6)	1.33	180.94	4.51	6.77	1.36	0.02
7 (3:7)	0.99	211.10	4.41	10.29	1.33	0.03
8 (2:8)	0.66	241.25	4.50	18.00	1.36	0.06
9 (1:9)	0.33	271.41	4.17	37.51	1.26	0.12
10 (0:10)	0.00	301.57	0.00	301.57	0.00	1.00

K = Kaempferol, D = Dihydrokaempferol.



K = Kaempferol, D = Dihydrokaempferol.

Figure 3.14: Isobologram of the interaction between isolated compounds kaempferol and dihydrokaempferol, showing an antagonistic relationship.

3.4 Discussion

3.4.1 Screening for anti-oxidant activity using thin layer chromatography

A rapid TLC evaluation of the presence of anti-oxidant activity in the *Commiphora* species under investigation was conducted to identify free radical scavenging activity and reveal the presence of compounds, if any, responsible for this activity. Most free radicals react rapidly with oxidizable substrates. Both the DPPH and ABTS spray reagents were used, and are regarded accurate with respect to measuring the anti-oxidant activity of extracts. A colour change was observed within a few seconds after plate spraying. Each extract displayed a number of active anti-oxidant bands, which further displayed a slight variation amongst species and spray reagent (Figure 3.8 and Figure 3.9). The stem extracts of each of the species displayed more active bands and at a greater intensity than those displayed by leaf extracts.

Most extracts displayed the presence of compounds scavenging both the DPPH organic radical and ABTS (Figure 3.8 and Figure 3.9). Few compounds, however only scavenged one or the other radical. This may be attributed to the nature of the compound and its consequence to scavenge only certain free radicals. Concentrated yellow spots were

observed at the origin (baseline) of the plate, indicating that the majority of the anti-oxidant compounds are of a relatively polar nature (rather than non-polar), and that the degree of intensity results from the actual concentration of the particular radical scavenging compound.

It is important to note that TLC only serves as a preliminary screening process, and that the anti-oxidant potential of the extracts may not directly correspond to the results obtained in the independent assays, as there is a definite concentration difference between the extracts analysed by TLC and those employed in the assays.

3.4.2 Colorimetric spectrophotometric method

The DPPH radical has a deep purple colour and absorbs strongly at a wavelength of 550 nm, whereas the yellowish reduction product, $DPPH_2$, does not. The radical scavenging potential of *Commiphora* species under investigation is summarised in Table 3.1 and depicted in Figure 3.10. In the DPPH assay, significant anti-oxidant efficiency was observed for only four *Commiphora* species, viz. *Commiphora schimperi* (stem), *C neglecta* (stem), *C. edulis* (stem) and *C. tenuipetiolata* (leaf and stem) with IC_{50} values of 7.31 ± 0.14 μg/ml, 10.36 ± μg/ml, 10.59 ± 0.50 μg/ml, 10.81± 0.56 μg/ml and 10.75 ± 0.36 μg/ml, respectively. When viewing the chromatographic profiles of the stem extracts (Chapter 8) of these four species, a distinct similarity is noticed between the HPLC profiles of *C. schimperi*, *C. neglecta* and *C. tenuipetiolata*. *Commiphora edulis* (stem), however, has a very different chemical profile upon comparison with the other three active species.

Upon comparison of the anti-oxidant activity, as determined by the DPPH assay, it was observed that stem extracts of *Commiphora* produced greater anti-oxidant activity than leaf extracts, with the exception of *C. africana*, *C. tenuipetiolata* and *C. viminea*. The stem and leaf extracts of these species demonstrated analogous anti-oxidant activity.

Pronounced radical scavenging activity has been reported in plants with phenolic moieties, the presence of which is common in natural anti-oxidants. These phenolic moieties include substances such as tannins, flavonoids, tocopherols, and catecheses. Tannins are, at least in part, responsible for the strong free radical scavenging activities working synergistically with other anti-oxidant substances. Organic acids and protein hydrolysates may

additionally act as anti-oxidants (Dapkevicius *et al*., 1998). The phenomenon that the stem extracts demonstrated substantial radical scavenging capacity is not completely surprising, in light of the expectancy of the high polyphenolic content.

It is, however, surprising that the leaves did not demonstrate a higher radical scavenging ability, especially with the representative flavonoid patterns identified in their chromatographic profiles (Chapter 8). The radical scavenging potential against the DPPH organic radical directly depends on the number of hydroxyl groups present in ring B of flavonoids, with an increase in the number of hydroxyl groups resulting in an increase in radical scavenging activity (Rusak *et al*., 2005). Phenols, amino and thiophenol groups are commonly known to be the active groups for DPPH scavenging. The mechanism by which DPPH is scavenged, aids in elucidating the structure-activity relationship (SAR) of the anti-oxidant and, in so doing, may be beneficial in the rational design of novel flavonoid-derived anti-oxidants with improved pharmacological profiles (Wang and Zhang, 2003).

The presence of the 6-hydroxyl group was shown to be an unfavourable structural feature of flavonoids with regard to the DPPH scavenging and anti-oxidative effects (Rusak *et al*., 2005). The radical scavenging effect is thus inhibited by the presence of 6-hydroxyl groups. In the absence of a polyhydroxylated structure on the B-ring, the C_2-C_3 double bond does not contribute to the hydrogen-donating ability of flavonoids, while the presence of both these structural entities are important structural requirements for the cytostatic effect of flavonoids (Rusak *et al*., 2005). While the flavan backbone affects and influences the anti-oxidant potential, the spatial arrangement of its substituents has a greater effect on the anti-oxidant outcome.

While the chromatographic profile of the leaf extracts of *C. africana* resembled that of *C. pyracanthoides* and *C. glandulosa* (Figure 8.3), the moderate activity displayed by these species varied with the determined IC_{50} values, the latter being 43.00 ± 1.37 μg/ml, 29.32 ± 5.22 μg/ml and 41.39 ± 1.73 μg/ml, respectively. *Commiphora africana* (leaf) and *C. glandulosa* (leaf) yielded similar chromatographic profiles and anti-oxidant activity, while *C. pyracanthoides* exhibited a higher anti-oxidant effect. Factors contributing to this activity variation may include the quantity of molecules available to react or the presence of molecules acting antagonistically to those molecules that are available with a greater scavenging potential. The flavonoid pattern displayed by all three species varied

considerably with only few flavonoids common to the leaf extracts of these three species. Limited free radical scavenging activity was displayed by *C. viminea* (leaf), *C. mollis* (leaf) and *C. neglecta* (leaf), with IC_{50} values of 78.49 ± 3.46 μg/ml, 89.95 ± 0.04 μg/ml and 98.61 ± 1.97 μg/ml, respectively. The chemical fingerprint of the leaf extract of all three species was very characteristic and differed significantly from each other as well as from the leaf extract of *C. tenuipetiolata* (Chapter 8), the latter species demonstrating greater anti-oxidant activity.

The radical scavenging activity of the *Commiphora* stem extracts under investigation was observed with IC_{50} values ranging from 7.31 ± 0.14 μg/ml (*C. schimperi*) to 84.01 ± 7.07 μg/ml (*C. viminea*). Although the HPLC profile of *C. marlothii* was significantly different from that of *C. glandulosa*, their anti-oxidant activity was similar (Figure 3.10).

Kaempferol demonstrated radical scavenging efficiency (IC_{50} = 3.32 ± 1.27 μg/ml) greater than that of both vitamin C (IC_{50} = 4.18 ± 0.56 μg/ml) and Trolox (IC_{50} = 7.03 ± 1.16 μg/ml). Rusak and colleagues (2005) investigated the anti-oxidant activity of flavonoids and determined the IC_{50} value of kaempferol to be 15 μg/ml (53.40 μmol/L), indicating a radical scavenging activity five-fold less than that determined in this study. In the study by Rusak *et al*. (2005) the scavenging activity of kaempferol and other investigated flavonoids did not correlate with their anti-oxidant activity in menadione-stressed HL-60 cells (Rusak *et al*., 2005). Kaempferol, which was shown to be the weaker of the flavonoids in scavenging DPPH, was the most effective in reducing oxidative stress in this cell line. This suggests that the anti-oxidant effect *in vivo* is not only a consequence of their electron-donating ability. The radical scavenging activity of anti-oxidants, and their affinity for lipid substrates, are important factors in their anti-oxidant activity. It is thus evident that compounds other than the polyphenolic tannins are also responsible for the radical scavenging activity of *Commiphora* species.

Systematic changes occur in the structure of flavonoids, where quercetin serves as the flavonoid from which the other flavonoids are derived or modified, as it contains all the functional elements on the C-ring, these being the 2,3-double bond, 3-hydroxy and 4-keto substitution. Kaempferol, a derivative of quercetin, has a B-ring catecholic structure and a fully substituted C-ring. Figure 3.15 demonstrates the possible mechanism, as proposed by

Tsimogiannis and Oreopoulou (2006), by which kaempferol scavenges the relatively stable organic radical.

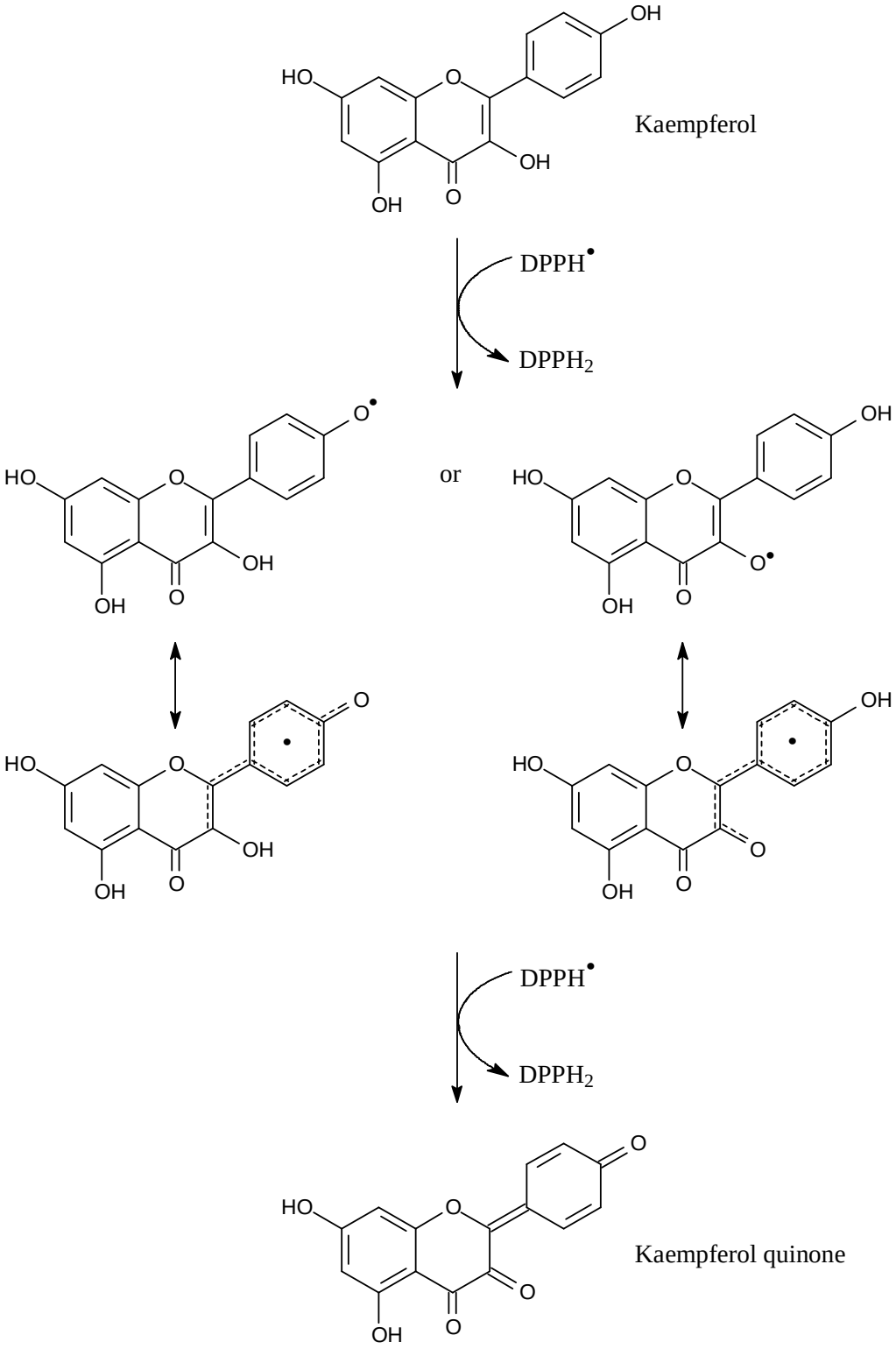


Figure 3.15: The mechanism of DPPH radical scavenging by kaempferol as proposed by Tsimogiannis and Oreopoulou (2006).

Dihydrokaempferol has poor radical scavenging activity, as demonstrated in the DPPH assay, with an IC_{50} value of 301.57 ± 2.79 μg/ml. This is an indication that not all flavonoids possess exceptional, or even moderate (as in this instance) radical scavenging efficiency. Plumb *et al.* (1999) stated that the removal of the 2,3 double bond will result in a drastic reduction in the anti-oxidant activity. In this study, this statement was found to be true upon testing of the compound in the DPPH assay, although Arts *et al.* (2003) found the contrary upon comparison to kaempferol in the ABTS assay.

The absence of significant DPPH scavenging activity in certain extracts, suggests that they possibly act as chain breaking anti-oxidants. Further studies are required for evaluation of the usefulness of the studied extracts for the treatment of disorders involving oxidative stress, and for characterisation of these active compounds. This was evident in the case of kaempferol, which exhibited the most effective ROS scavenger in menadione-stressed cells, but the lowest activity in the DPPH assay.

The results obtained in the ABTS assay (Table 3.1, Figure 3.10) differed from those obtained from the DPPH assay. Most extracts displayed greater activity in the ABTS assay, with the exception of *C. edulis* (stem), *C. pyracanthoides* (leaves), *C. schimperi* (stem) and *C. tenuipetiolata* (leaves), as summarised in Table 3.1 and demonstrated in Figure 3.10. The stem extracts were still observed to have the greater radical scavenging activity upon comparison with the leaves, with the greatest activity measured for the stem extracts of *C. tenuipetiolata* (IC_{50} = 5.10 ± 0.66 μg/ml), *C. neglecta* (IC_{50} = 7.28 ± 0.29 μg/ml), *C. mollis* (IC_{50} = 8.82 ± 0.72 μg/ml), *C. glandulosa* (IC_{50} = 10.69 ± 1.47 μg/ml), *C. schimperi* (IC_{50} = 11.22 ± 3.61 μg/ml). *Commiphora tenuipetiolata* (stem) displayed similar anti-oxidant activity to that of Trolox, the latter having an IC_{50} value of 5.41 ± 0.51 μg/ml. *Commiphora mollis* (leaves), *C. viminea* (leaves) and *C. pyracanthoides* (leaves) were still observed to have poor anti-oxidant activity, with IC_{50} values of 60.11 ± 8.73 μg/ml, 45.89 ± 0.79 μg/ml and 51.44 ± 0.27 μg/ml, respectively. Due to insufficient volumes of *C. edulis* (leaves) and *C. neglecta* (leaves) extracts, these were not tested in the ABTS assay.

In a review on flavonoids as anti-oxidants by Pietta (2000), it was stated that the major determinants for radical scavenging capability are (i) the presence of a catechol group in ring B (OH group at R_5 and R_6), which has electron donating properties and is a radical target, and (ii) a C_2-C_3 double bond conjugated with the 4-oxo group (Figure 3.16), responsible for electron delocalisation, according to three different approaches, namely the

inhibition of superoxide anion production, chelation of trace metals and scavenging ABTS. The presence of a 3-hydroxyl group in the heterocyclic ring of flavonols makes them more potent radical-scavengers than the corresponding flavones, especially in the presence of a catechol group in ring B. It was determined that the addition of an hydroxyl group in ring B further enhances the anti-oxidant capacity, while the presence of a single hydroxyl group in ring B reduces the activity. Flavanones lack a C_2-C_3 double bond with the 4-oxo group and as a result are weak anti-oxidants.

Kaempferol, demonstrated greater radical scavenging activity (IC_{50} = 4.27 ± 0.13 μg/ml) than Trolox (IC_{50} = 5.41 ± 0.51 μg/ml) (as was the case in the DPPH assay). Pannala *et al*. (2001) demonstrated that compounds containing a 4'-monohydroxyl group on the B-ring are less potent anti-oxidants; the mechanism of action occurring via the formation of a phenoxyl radical. Kaempferol, however, has a relatively high activity upon comparison with other monohydroxyl compounds. This is probably as a result of the potential for conjugation between the 4'-hydroxyl group and the 3-hydroxyl group through the conjugated C-ring. The phenoxyl radical formed possibly abstracts an electron from the radical cation to generate the di-cation and the phenolate. The structure of the B-ring is the primary determinant of the anti-oxidant activity and the electron donating ability of flavonoids.

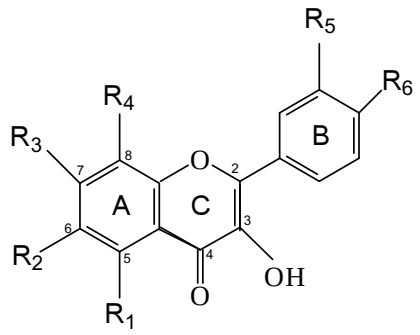


Figure 3.16: The basic chemical structure of flavonols.

The predominant mechanism of action noted by Pannala and colleagues (2001) occurred via the donation of a single electron to the radical cation, resulting in the formation of a semi-quinone. The electron donors undergo a rapid reaction with the $ABTS^+$, while the

functional hydroxyl groups are slower reacting and are taken into account only over longer time-scales.

The DPPH and ABTS assays have the same mechanism of action, but, in most cases, the results obtained from ABTS assay are higher than those from the DPPH assay, as was seen in the current study with respect to most of the *Commiphora* species. It has been reported that results from the ABTS assay do not only take into account the activity of the parent compound, but also the contribution that reaction products and other individual compounds have on the activity, which is not the case in the DPPH assay (Arts *et al*., 2003). In the ABTS assay, the total amount of radical scavenged is measured over a period of time, while most anti-oxidant assays measure the rate at which a radical is scavenged by an anti-oxidant (Arts *et al*., 2003). The total amount of $ABTS^{+}$ scavenged by a compound correlates with the biological activity in a selected group of flavonoids (Arts *et al*., 2003).

It was also determined by Lee *et al*. (2003) that the total anti-oxidant capacity of the samples being tested in the ABTS and DPPH assays correlated with the phenolic and flavonoid content. However, the determined values indicated that the DPPH assay underestimates the anti-oxidant capacity by approximately 30%, as compared to the ABTS assay. This methodological difference has previously been reported by Kim *et al*. (2002) and Arnao (2000), and may be attributed to the absorbance interruption at the specified wavelength by other compounds in the DPPH assay. The ABTS assay is also a very sensitive assay requiring only a short reaction time of approximately 4 min (versus the 30 min reaction time required for DPPH), and can be used in both organic and aqueous solvent systems (Lee *et al*., 2003) i.e. the application of the ABTS assay is for both hydrophilic and lipophilic compounds (Mathew and Abraham, 2006).

Campos and Lissi (1997) suggested that the differences observed between the DPPH and ABTS assays may also be partly as a result of the reactions, in the DPPH assay, being carried out in the absence of the reduced form of DPPH. The reduced form of ABTS is constantly present within that particular assay system. The DPPH radical is also one of a few stable and commercially available organic nitrogen radicals, which has no similarity to the highly reactive and transient peroxyl radicals.

The interaction of polyphenolic constituents with free radical species in different phases provides different perspectives on their anti-oxidant/pro-oxidant properties. Adverse biological activities could be obtained *in vitro* over a wide range of concentration levels of the flavonoids, depending on the specific assay or model system (Joubert *et al.*, 2005).

Flavonoid-enriched plant extracts may be used for their anti-oxidant properties and associated beneficial health properties. However, their use as dietary supplements should be considered with caution, in view not only of potential mutagenic properties, but any pro-oxidant effects, as well. Beneficial properties of these extracts and/or compounds may alter, due to conditions such as the availability of iron or the level of ascorbate *in vivo* (Joubert *et al.*, 2005).

3.4.3 Isolation of compounds

Approximately 9000 different flavonoids have been reported from plant sources, and with almost certainty many more are still to be discovered, as they continue to capture the interests of scientists from numerous disciplines. Based on the 10-carbon skeleton of flavonoids, they can be substituted by a range of different groups, viz. hydroxyl, methoxyl, methyl, isoprenyl and bezyl substituents (Williams and Grayer, 2004).

During the course of this study, two compounds were isolated from *Commiphora glandulosa* (stem). Both these compounds are flavonoids, viz. kaempferol (flavonol) and dihydrokaempferol (dihydroflavonol). These secondary metabolites are not novel; however, the isolation of these compounds has not been reported in *Commiphora* species previously.

Flavonoids derive their carbon skeleton from 4-coumaroyl CoA and malonyl CoA, both of which are derived from carbohydrates. The first flavonoid, naringenin, is formed from the stereospecific action by chalcone isomerase on naringenin chalcone. The dihydroflavonols, viz. dihydrokaempferol is formed by the direct hydroxylation of of flavonones (naringenin) in the 3 position. This reaction is catalysed by flavanone-3-hydroxylase (Figure 3.17). Dihydroflavonols are an important intermediate in the formation of catechins, pro-anthocyanidins and anthocyanidins. Flavonols, such as, kaempferol are formed by the introduction of a C_2-C_3 double bond, a reaction catalysed by flavonol synthase.

The kaempferol nucleus is expected to have two doublets at δ_H 6.20 and 6.40 ppm (J = 2.1 Hz), assigned to the H-6 and H-8 protons, respectively, and a pair of A_2B_2 aromatic system protons at δ_H 6.93 and 7.77 ppm (J = 8.4 Hz), assigned to H-3′, 5′ and H-2′, 6′ respectively (Harborne *et al*., 1975). The isolated compound displayed UV absorption data typical of the flavonol kaempferol, and the ^{1}H-NMR (Table 3.1) and ^{13}C-NMR (Table 3.2) spectrum displayed the characteristic signals.

In a study by Yun–Long *et al*. (1999), a comparison was made between the flavonoid contigoside A and kaempferol. It was noted that kaempferol showed an extreme downfield characteristic signal for both 5-OH and 3-OH (12.5 – 10.6 ppm).

Kaempferol has been isolated from *Delphinium consolida* L., Ranunculaceae (Perkins and Wilkinson, 1902), from *Citrus paradisi* Macf. (grapefruit), Rutaceae (Dunlap and Wender, 1962), from aerial parts of *Prunus prostrata*, Rosacea (Bilia *et al*., 1993), from the roots of *Smilax bockii* warb. Liliaceae (Xu *et al*., 2005), *Camellia oleifera* Abel., Theaceae (Bin and Yonming, 2003) and are present in the Burseraceae family.

Dihydrokaempferol was isolated as a yellow amorphous powder. The structural identity of this compound was determined through NMR spectral analysis (^{1}H-NMR - Table 3.3 and ^{13}C-NMR - Table 3.4).

Dihydrokaempferol was isolated from *Citrus paradisi* Macf. (grapefruit), Rutaceae. The presence of this compound in only trace amounts in metabolically active grapefruit leaves, fruit and stems led to the conclusion by the authors that dihydrokaempferol is a metabolic intermediate, and not an end-product (Syrchina *et al*., 1976). This supports the postulated biosynthetic pathway shown by Figure 3.17. Dihydrokaempferol had by 1966 been found in eleven plant families (Mater and Metzler, 1966).

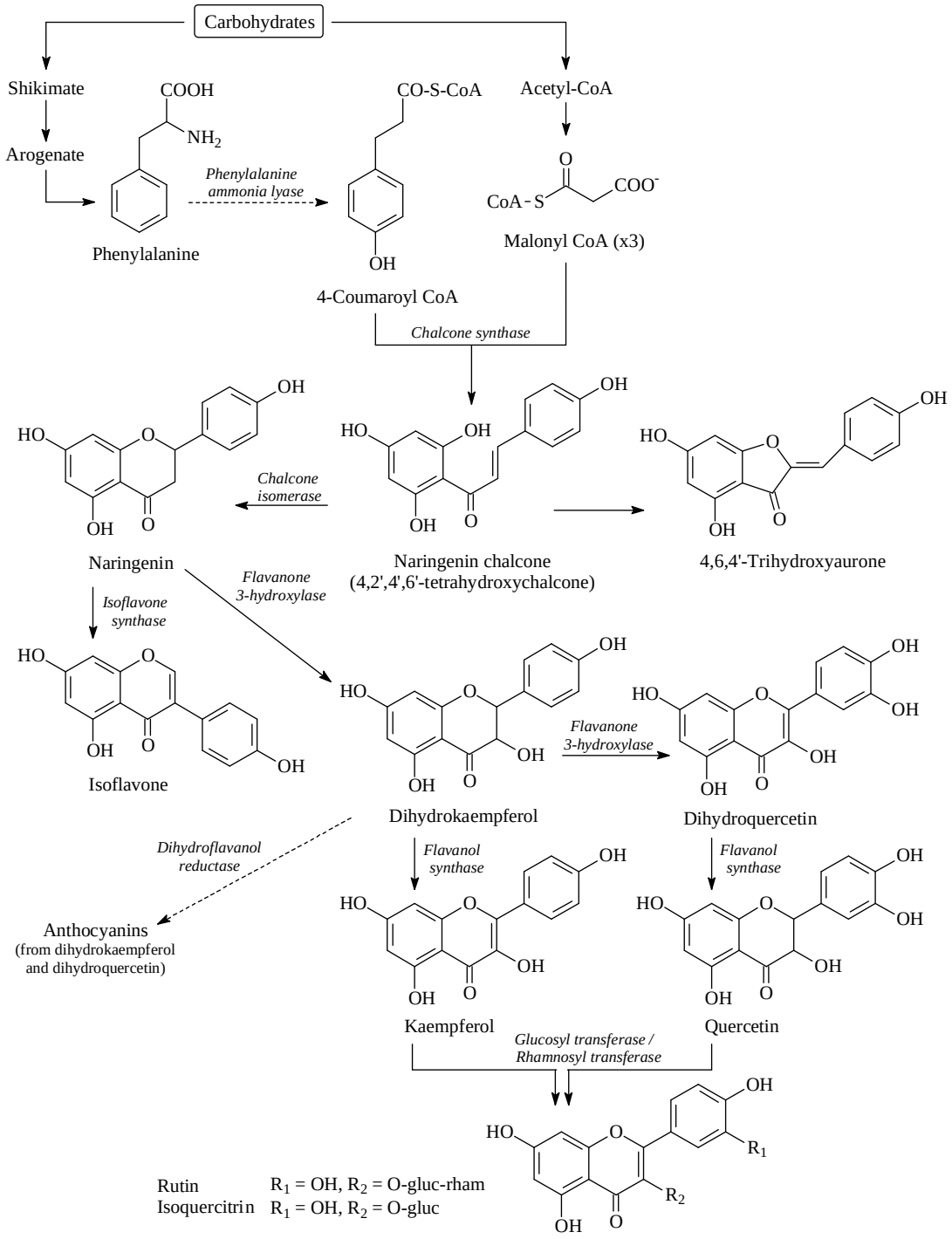


Figure 3.17: Isolated steps from the metabolic pathway of flavonoids (modified from Verhoeyen *et al*., 2002).

3.4.4 Isobologram construction of the interaction between the isolated compounds with anti-oxidant activity

The complex composition of extracts could be responsible for certain interactions (synergistic, additive or antagonistic effects) between their components or the medium (Parejo *et al*., 2002).

The investigation of the interaction between vitamin C and *Commiphora glandulosa* (stem) resulted in the formation of an isobologram displaying a pattern of synergistic effect, the data of which is presented in Table 3.6. This isobologram construction was used as a tool to determine the activity that would result from the interaction between the two isolated compounds kaempferol and dihydrokaempferol.

The investigation of the interaction between kaempferol and dihydrokaempferol resulted in the formation of an isobologram displaying an antagonistic effect. The data generated for the construction of the isobologram is presented in Table 3.7.

When kaempferol is present at a low concentration and dihydrokaempferol at a much higher concentration, the resultant IC_{50} value tends towards that of *C. glandulosa* (stem), the species extract from which the compounds were isolated. This is an indication that kaempferol acts additively towards dihydrokaempferol. The radical scavenging activity of the stem extract of *C. glandulosa* does not correlate perfectly with the radical scavenging activity depicted by the isolated compound kaempferol as a result of the presence of dihydrokaempferol, which acts antagonistically towards kaempferol.

3.5 Conclusion

Two compounds were isolated from the stem extract of *C. glandulosa*. The compounds were identified as flavonoids viz. kaempferol (flavonol) and dihydrokaempferol (dihydroflavonol). Both compounds have been previously isolated from other plant species, however, not from the genus *Commiphora*. Kaempferol has shown to be biologically active, having been assayed for various activities and as reported in the proceeding Chapters (Chapter 4 - 7).

Thin layer chromatography employed as a preliminary screening method to determine the potential anti-oxidant activity of indigenous *Commiphora* species provided a clear indication of the presence of free radical scavenging compounds, which prompted the colorimetric quantitative spectrophotometric analysis.

Most of the extracts portrayed poor anti-oxidant activity in the DPPH assay with the exception of *Commiphora schimperi* (stem), *C neglecta* (stem), *C. edulis* (stem) and *C. tenuipetiolata* (leaf and stem) with IC_{50} values of 7.31 ± 0.14 μg/ml, 10.36 μg/ml, 10.59 ± 0.50 μg/ml, 10.81 ± 0.56 μg/ml and 10.75 ± 0.36 μg/ml, respectively, when compared to vitamin C (IC_{50} = 4.18 ± 0.56 μg/ml), a known anti-oxidant. The results from the ABTS assay differed from those of the DPPH assay. Most extracts displayed higher activity in the ABTS assay with the greatest activity measured for the stem extracts of *C. tenuipetiolata* (IC_{50} = 5.10 ± 0.66 μg/ml), *C. neglecta* (IC_{50} = 7.28 ± 0.29 μg/ml), *C. mollis* (IC_{50} = 8.82 ± 0.72 μg/ml), *C. glandulosa* (IC_{50} = 10.69 ± 1.47 μg/ml), *C. schimperi* (IC_{50} = 11.22 ± 3.61 μg/ml).

The flavonol, kaempferol (IC_{50} = 3.32 ± 1.27 μg/ml) showed exceptional radical scavenging activity, in contrast to the activity displayed by dihydrokaempferol (IC_{50} = 301.57 ± 2.79 μg/ml). The investigation of the interaction between kaempferol and dihydrokaempferol resulted in an isobologram displaying an antagonistic effect, with the resultant IC_{50} value tending towards that of *C. glandulosa* (stem).

CHAPTER 4: ANTIMICROBIAL ACTIVITY

4.1 Introduction

Micro-organisms have proved to be beneficial to man in a number of ways and form an indispensable component of our ecosystem. In both terrestrial and aquatic systems, the micro-organisms enable the carbon, oxygen, nitrogen and sulphur cycles. They have, however, also proved to be harmful to mankind, specifically in their capacity to cause disease by growing on and/or within other organisms. This results in microbial colonisation, which in turn may lead to disability and death. It is, therefore, critical to minimise the growth of these micro-organisms through effective prevention and treatment of disease. Therefore, the identification of the intrinsic characteristics of a particular pathogen, its source, mode of transmission, the susceptibility of the host and the exit mechanism of the pathogen will limit the spread of the pathogen (Prescott *et al*., 1996).

4.1.1 Chemotherapeutic agents: factors affecting their effectiveness

The ideal chemotherapeutic agent has a high therapeutic index with selective toxicity, thereby resulting in lethal damage to pathogens through the inhibition of cell wall synthesis, protein synthesis or nucleic acid synthesis, as well as through the disruption of the cell membrane and the inhibition of certain essential enzymes. This results in selective disruption of the specific structure and/or function essential to bacterial growth and survival, without causing similar harmful effects to its eukaryotic host (Prescott *et al.*, 1996).

The efficacy of antimicrobial agents is influenced by a number of factors. Firstly, it is of obvious importance that the antimicrobial agent reaches the site of the infection. This greatly depends on the stability of the drug, its lipophilic or hydrophilic nature, its absorption from a specific site and the presence of blood clots or necrotic tissue, the latter of which may protect the pathogen against the antibiotic. Secondly, the susceptibility of the pathogen to the particular chemotherapeutic agent is of utmost importance, as well as the specific growth phase in which the pathogen is in at that particular stage. It is important that the pathogen be targeted while actively growing and dividing, as most antimicrobials

are only effective against bacteria in the late lag phase and exponential growth phase (Prescott *et al.*, 1996).

The efficacy of an antimicrobial agent can be estimated through the determination of the minimum inhibitory concentration (MIC), being the minimum concentration at which no microbial growth occurs after a specified exposure time to the antimicrobial agent (Prescott *et al*., 1996).

4.1.2 Drug resistance

One of the most serious threats to the successful treatment of microbial disease is the development and emergence of drug resistant pathogens, resulting from the excessive and inappropriate use of antimicrobial agents, which in turn lead to potentially serious public health problems. Resistance is defined as the ability of a microorganism to remain viable and actively multiplying under conditions that would normally have proved to be inhibitory (Prescott *et al.*, 1996). Antimicrobial agents target different microbial cellular loci, from the cytoplasmic reticulum to respiratory functions, enzymes and its genetic material. Increased drug-resistance may be brought about by the limited drug diffusion into the biofilm matrix, enzyme-mediated resistance, genetic adaptation, efflux pumps, as well as through the adaptation of the outer microbial membrane, the latter occurring either through the lack of or through the overexpression of certain membrane proteins (Cloete, 2003).

This phenomenon of increased drug resistance, combined with the multiplicity of side effects caused by existing agents and the emergence of diseases for which no treatment yet exists, makes the search for new antimicrobial agents a highly relevant and important subject for research. For centuries, plants have been used in the traditional treatment of microbial infections. This assembly of knowledge by indigenous peoples about plants and their products continue to play an essential role in health care of a great proportion of the population (Iwu *et al*., 1999).

4.1.3 Natural products and their role in drug discovery

Natural products have played a pivotal role in the discovery of antimicrobial drugs, with the drug either being completely derived from the natural product, or serving as a lead for novel drug discovery. Most antimicrobials discovered during the past 6 – 7 decades have been discovered through screening of soil samples, of which the antimicrobial efficacies were determined first *in vitro* and later *in vivo*. Examples of such naturally occurring antimicrobials are plentiful, and include drug classes such as the penicillins and cephalosporins (β-lactam being the empirically active component), all of which were discovered in or derived from fungi. Numerous other antimicrobials were derived from different filamentous strains of the bacterial genus *Streptomyces*, including streptomycin, erythromycin, tetracycline and vancomycin. Semi-synthetic modifications to these naturally-occurring drugs have brought about the production of second- and third-generation β-lactams of both the penicillin and cephalosporin classes, while complete synthesis produced yet more active compounds, specifically the second-generation erythromycins, viz. clarithromycin and azithromycin. As of the end of 1999, only the fluoroquinolones represent a totally synthetic, significant class of antibiotics (Walsch, 2000).

Research in the fields of antibiotics and natural products has declined significantly, due to a number of diverse factors. However, a substantial resurgence of interest in the topic has re-emerged during the past decade, leading to an active pursuit in the research and development of natural products. With the majority of the world's plant species not yet explored in this regard, the exploitation of the medicinal potential of these species will prove to be both interesting and challenging to scientists from diverse fields of expertise.

Plants synthesise a diverse array of secondary metabolites, which play a key role in the natural defence mechanisms employed by the plant against predation by microorganisms and insects. It is thus no surprise that these aromatic compounds have, in numerous instances, been found to be useful antimicrobial phytochemicals and, as a result, these compounds are now divided into different chemical categories: phenolics, terpenoids and essential oils, alkaloids, lectins and polypeptides, as well as polyacetylenes (Cowan, 1999). An increase in the isolation and identification of such compounds may thus contribute greatly to the success in antibiotic discovery.

4.1.4 *Commiphora* species and their known antimicrobial activity

Studies conducted on *Commiphora* species have suggested that these species may well be active against micro-organisms. The antiseptic properties of myrrh has been known since biblical times and thus has been used in the treatment of infections of the oral cavity – both alone and in combination with other herbal remedies. The treatment of gingivitis, the reduction of 4-day plaque regrowth, and the treatment of leprosy, syphilis, and most recently, of schistosomiasis and fasciolasis, all provide an indication of the success of the use of myrrh as an antimicrobial agent (Tipton *et al*., 2003).

Commiphora plants are used by the Dhofaris in the southern parts of Oman to disinfect wounds and as an antihelmintic agent (Fatope *et al*., 2003). The treatment of skin conditions such as impetigo, eczema and shingles through the application of lotions prepared from the bark of *Commiphora* prompted the investigation of *C. wightii* for antifungal activity against *Candida albicans*. The isolated flavonone, muscanone, inhibited the growth of *C. albicans*, although naringenin, also isolated from *C. wightii*, showed no inhibitory properties.

Two sesquiterpenic compounds isolated from the hexane extract of myrrh resin exhibited antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* (Tipton *et al.*, 2003). The sesquiterpene T-cadinol, isolated from *C. guidotti* Chiov. demonstrated bactericidal activity against *S. aureus* with a minimum inhibitory concentration of 24 μg/ml, as well as a fungicidal effect of 2.3 μg/ml against *Trichophyton mentagrophytes*. These activities suggest that traditional use of *Commiphora* in the treatment of wounds is congruent to the activity determined *in vitro* (Claeson *et al.*, 2003).

4.2 Materials and methods

The antimicrobial properties of the 10 species of *Commiphora* were evaluated using the MIC microtiter plate assay. Both the leaf and stem extracts were prepared (see Chapter 2, section 2.3 for extraction process). The reference stock cultures were obtained from the National Health Laboratory Services, with the exception of *Candida albicans*, which was obtained from the South African Bureau for Standards (SABS), and were maintained in the

Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg.

4.2.1 Minimum inhibitory concentration assay

4.2.1.1 Principle of the method

Minimum inhibitory concentration (MIC) assays investigate the *in vitro* susceptibility of organisms to antimicrobial agents. This method employs different dilutions of the antimicrobial agent and quantitatively investigates the lowest concentration at which visible microbial growth inhibition is achieved. Microbial growth is visualised through the addition of the tetrazolium salts, specifically *p*-iodonitrotetrazolium (INT, Sigma-Aldrich). The assay is based on the detection of dehydrogenase activity in living cells by being converted from a colourless solution to an intensely coloured formazan (red) product (Eloff, 1998). This method yields reproducible results within one doubling dilution of the end point of the activity.

4.2.1.2 Protocol

The MIC of the plant extracts was determined by serial dilution, as described by Eloff (1998), with the lowest concentration being beyond the concentration where no growth inhibition of test organisms was observed. These dilution experiments were performed in sterile 96-well microtiter plates.

Stock solutions of the respective plant extracts were prepared in 1.5 ml microcentrifuge tubes (Eppendorff) by dissolving dry plant extract in dimethylsulphoxide (DMSO, Saarchem) to a final concentration of 64 mg/ml. Dimethylsulphoxide was the solvent of choice as a result of the insolubility of certain extracts in acetone. Aliquots of 100 µl of the stock solution were transferred aseptically into the top row of microtiter plate (row A), which already contained 100 µl aliquots of sterile water, thereby resulting in a 50% dilution of the stock solution to 32 mg/ml. After adequate mixing of the contents of each well, 100 µl aliquots of row A were transferred from row A to the corresponding wells in row B (also containing 100 µl aliquots of sterile water), followed by mixing and resulting in yet another 50% dilution of the plant extract (to 16 mg/ml). This process was repeated

for every row, resulting in 100 µl aliquots ranging in concentration from 32 mg/ml (row A) to 0.25 mg/ml (row H). This was followed by addition of 100 µl of liquid microbial culture grown in Tryptone Soya Broth (TSB, Oxoid) to each well. This yielded a final volume of 200 µl in each well and final extract concentrations ranging from 16 mg/ml in row A to 0.125 mg/ml in row H (Figure 4.1). The microtiter plates were incubated at 37°C, overnight for bacteria and 48 hours for yeasts (as yeasts require a greater time period for growth).

In order to determine the range of antimicrobial activity of *Commiphora* species, six different microbial (reference) cultures were used and are listed below. Inoculums were prepared by mixing 500 µl of a 24 hour broth suspension with 50 ml of TSB.

- *Staphylococcus aureus* (ATCC 6358) (Gram-positive bacteria)
- *Bacillus cereus* (ATCC 11778) (Gram-positive bacteria)
- *Klebsiella pneumoniae* (NCTC 9633) (Gram-negative bacteria)
- *Pseudomonas aeruginosa* (ATCC 9027) (Gram-negative bacteria)
- *Candida albicans* (ATCC 10231) (yeast)
- *Cryptococcus neoformans* (ATCC 90112) (yeast)

After incubation at 37°C, 50 µl of a 40% (w/v) solution of INT was added to each well as an indicator of microbial growth. The plates were incubated at ambient temperature (25°C) and the MIC values visually determined after 6 hours. The lowest concentration of each extract displaying no visible growth was recorded as the minimum inhibitory concentration.

In order to determine the sensitivity of the microorganisms, two positive control experiments were conducted: (1) for bacterial strains, Ciprofloxacin (Sigma-Aldrich) at a starting concentration of 0.01 mg/ml in sterile water, and (2) for yeast strains, Amphotericin B (Sigma-Aldrich), at a starting concentration of 0.01 mg/ml in DMSO and water (where 1 mg/ml was prepared in DMSO, and diluted to 0.01 mg/ml in sterile water thereafter). The final concentrations for these experiments ranged from 2.50×10^{-3} mg/ml (row A) to 1.95×10^{-5} mg/ml (row H). A negative control experiment was conducted using only DMSO.

4.2.2 Death kinetic assay

4.2.2.1 Principle of the assay

Death kinetic studies are often referred to as time-kill studies, and are used to determine the rate at which the antimicrobial agent kills pathogens over time, as well as the extent at which the activity occurs.

Upon introduction of a micro-organism into a new environment, its growth displays a lag phase during which no cell growth occurs, after which its growth enters the exponential phase, where microbial cell multiplication occurs at an exponential rate (doubling in number at regular intervals). This exponential phase is followed by the stationary phase, the latter of which usually results from the depletion of nutrients (carbon, nitrogen and/or oxygen sources) (Prescott *et al*., 1996).

However, upon exposure to a constant concentration of an antimicrobial agent, the organism will remain within the lag phase for a certain amount of time. This is followed by a log-linear killing phase, during which the number of microbial colonies are decreased until it enters into a second lag phase. Re-growth may occur after this second lag phase, but documentation of this phenomenon rarely occurs as time-kill studies are usually performed over a 24 hour period only (Li, 2000; Tam *et al*., 2005).

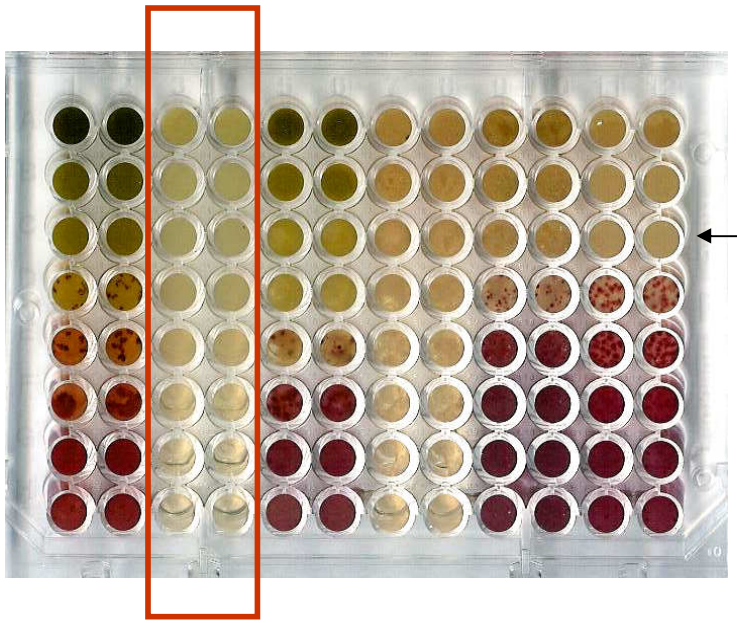
4.2.2.2 Protocol

Based on the preliminary promising results obtained from the MIC determination, *C. marlothii* (stem) was identified as a suitable candidate for the inactivation broth death kinetic assay, as described by Lattaoui and Tantaoui-Elaraki (1994), as well as the slightly modified method by Christoph *et al*. (2001).

Serial dilutions of the plant extract starting at a concentration of 16 mg/ml

MIC at a concentration of 4 mg/ml

A	**16**	**16**	**16**	**16**	**16**	**16**	**16**	**16**	**16**	**16**	**16**	**16**
B	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
C	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**
D	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**
E	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
F	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**
G	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**
H	**0.12**	**0.12**	**0.12**	**0.12**	**0.12**	**0.12**	**0.12**	**0.12**	**0.12**	**0.12**	**0.12**	**0.12**
	1 CAS	***2*** CAS	***3*** CGS	***4*** CGS	***5*** CMS	***6*** CMS	***7*** CPS	***8*** CPS	***9*** CVS	***10*** CVS	***11*** CES	***12*** CES



Complete inhibition of micro-organisms by CGS, at a concentration of 0.125 mg/ml.

CAS – *Commiphora africana* stem, CGS – *C. glandulosa* stem, CMS – *C. marlothii* stem, CPS – *C. pyracanthoides* stem, CVS – *C. viminea* stem, CES – *C. edulis* stem.

Figure 4.1: Representative 96-well microtiter plate, indicating final concentrations of plant extracts (left)**;** A 96-well microtiter plate prepared for use in the MIC assay. Red wells indicate the absence of inhibitory activity (or the presence of *p*-iodonitrotetrazolium) (right).

Preparation of the bacterial inoculum

Staphylococcus aureus (ATCC 6538) was cultured overnight on Tryptone Soya Agar (TSA, Oxoid) at 37°C, after which the resulting colonies were removed from the agar and used to inoculate a sterile 0.9% sodium chloride (NaCl, Labchem) solution. Of this resulting cell suspension, serial dilutions were prepared in 0.9% NaCl in order to obtain a suspension with an appropriate colony count, of 1 x 10^6 CFU/ml. The latter was obtained in the lowest dilution, which provided a bacterial cell suspension with a final colony count of approximately 100 colonies per plate.

Preparation of the test solutions

A series of test solutions were prepared containing specified amounts (% w/v) of the dried extract of *C. marlothii* (for preparation, see Chapter 2, section 2.3) added to TSB, to a final volume of 45 ml. Solutions were prepared in final plant extract concentrations of 0.125%, 0.25%, 0.5%, 0.75% and 1.0% w/v (in acetone), and were stabilised at 37°C in a shaking water bath, after which 5 ml of the bacterial inoculum (as prepared above) was added for evaluation of growth inhibition.

Preparation of the inactivation broth

Inactivation broth, in which the growth of *S. aureus* is known to be completely inhibited, was prepared containing 0.1% w/v peptone (Oxoid), 5% w/v lecithin (Merck) and 5% w/v yeast extract (Oxoid) (Christoph *et al*., 2001), which was autoclaved to ensure sterility. The inactivation broth was initially vortexed to ensure complete mixing of the ingredients before use.

Death kinetic assay

A 1 ml sample of each incubated test solution (containing plant extract, TSB and bacterial cells, as described above), was removed at standard time intervals of 0, 5, 15, 30, 60, 120 and 240 min as well as at 8, 24 hours and 48 hours, and added to 9 ml of inactivation broth for instantaneous inactivation of any further microbial growth. All samples were analysed in duplicate, except for those taken at 48 hours, which were taken for the sole purposes of qualitative evaluation of re-growth. Four serial dilutions, from the resulting solutions, were prepared in 0.9% NaCl solution, of which 100 µl aliquots were spread out onto TSA plates. The plates were subsequently incubated at 37°C for 24 hours. After the incubation period, the colonies on each of the plates were counted and the results expressed in a $\log_{10}$

reduction time kill plot of colony forming units (CFU)/ml versus time. A control experiment was conducted using the same broth formulation, in the absence of any extract, but with acetone.

4.3 Results

4.3.1 Minimum inhibitory concentration

All extracts displayed activity against Gram-positive and Gram-negative bacteria, as well as the yeasts (Table 4.1).

The activity against *Staphylococcus aureus* (ATCC 6538) ranged from 1.00 mg/ml to 8.00 mg/ml. The extracts exhibiting the strongest activity against *S. aureus* were from *C. glandulosa* (stem), *C. marlothii* (both leaf and stem), *C. pyracanthoides* (stem) and *C. viminea* (stem).

Greater activity was observed against *Bacillus cereus* (ATCC 11778), when compared to the above-mentioned activity against *S. aureus*, with activities ranging from 0.005 mg/ml to 8.00 mg/ml. In general, the *Commiphora* stem extracts displayed higher activity than the leaf extracts, with the activity of *C. marlothii* (stem), *C. viminea* (stem), *C. glandulosa* (both the leaf and stem) and *C. pyracanthoides* (stem) being the greatest. Kaempferol was isolated from *C. glandulosa* (stem) as described in Chapter 3 (section 3.2.4), and its antimicrobial activity against the 6 micro-organisms was tested. The MIC value of kaempferol, prepared and treated in a similar way to the extracts, was also determined against *S. aureus* and *B. cereus*, and found to be 0.25 mg/ml.

Antimicrobial activity against Gram-negative bacteria was shown to be less effective than the activity against Gram-positive bacteria. The sensitivity with respect to *Pseudomonas aeruginosa* was similar amongst all the species (8.00 mg/ml), indicating poor antimicrobial activity. Activity with respect to *Klebsiella pneumoniae*, however, varied between the different species, with the highest sensitivity obtained by *C. pyracanthoides* (leaf) with a MIC value of 1.00 mg/ml. The MIC value of kaempferol against *P. aeruginosa* and *K. pneumoniae* was also determined to be 0.25 mg/ml.

The activity observed against the yeasts, *Candida albicans* and *Cryptococcus neoformans* were significant in almost all 10 species of *Commiphora*.

4.3.2 Death kinetic assay

The results obtained for the death kinetics of *C. marlothii* (stem) are shown in Figure 4.2. The antibacterial activity was observed to begin after approximately 30 min of the exposure of *S. aureus* to the different concentrations of plant extract. This observation was made through the reduction in colony forming units over time. All concentrations exhibited antibacterial activity, with a complete bactericidal effect being achieved by all test concentrations by the 24th hour. It was noted that by the 48th hour, re-growth had begun in the two lowest test concentrations of *C. marlothii* (stem), viz. 0.125% and 0.25%.

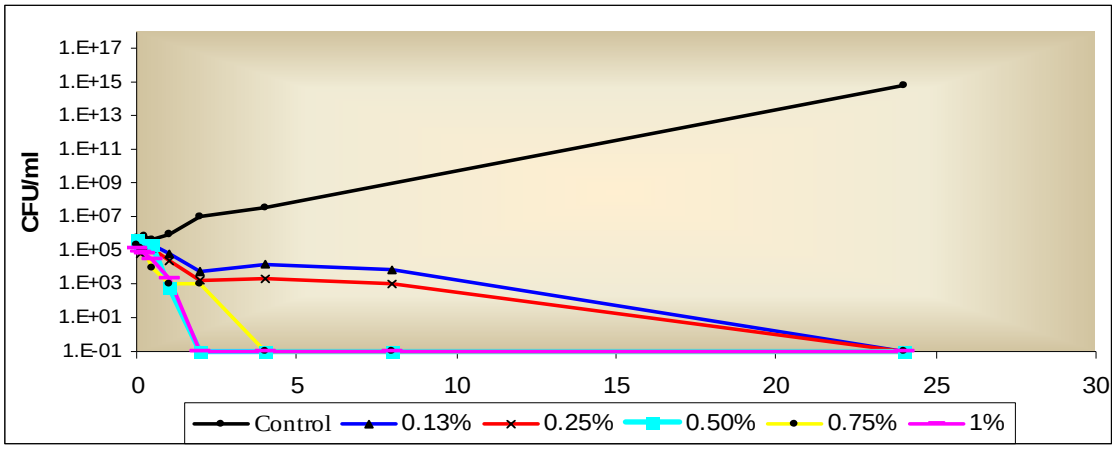


Figure 4.2: Log_{10} reduction time kill plot of *Commiphora marlothii*.

4.4 Discussion

This study was conducted specifically to determine the *in vitro* antimicrobial activity of indigenous *Commiphora* species, and to determine whether the therapeutic properties of some of the species used in traditional medicine correlates with laboratory-generated findings.

Table 4.1 MIC values (mg/ml) obtained for extracts of indigenous *Commiphora* species against *Staphylococcus aureus, Bacillus cereus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans* and *Cryptococcus neoformans.* n = 3.

Species	*S. aureus* ATCC 6538	*B. cereus* ATCC 11778	*K. pneumoniae* NTCC 9633	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*C. neoformans* ATCC 90112
***C. africana* (stem)**	4.00	4.00	4.00	8.00	2.00	4.00
***C. africana* (leaf)**	8.00	4.00	4.00	8.00	2.00	0.25
***C. edulis* (stem)**	4.00	4.00	8.00	8.00	4.00	2.00
***C. edulis* (leaf)**	8.00	4.00	2.00	8.00	4.00	2.00
***C. glandulosa* (stem)**	1.00	0.005	4.00	8.00	1.00	1.00
***C. glandulosa* (leaf)**	4.00	0.01	2.00	8.00	4.00	0.50
***C. marlothii* (stem)**	1.00	0.31	4.00	8.00	1.00	1.00
***C. marlothii* (leaf)**	1.00	2.00	2.00	8.00	2.00	1.00
***C. mollis* (stem)**	4.00	2.00	4.00	8.00	2.00	1.00
***C. mollis* (leaf)**	8.00	8.00	2.00	8.00	1.00	8.00
***C. neglecta* (stem)**	8.00	4.00	4.00	8.00	2.00	0.50
***C. neglecta* (leaf)**	8.00	4.00	4.00	8.00	2.00	2.00
***C. pyracanthoides* (stem)**	1.00	0.04	8.00	8.00	0.50	1.00
***C. pyracanthoides* (leaf)**	2.00	2.00	1.00	8.00	1.00	0.25
***C. schimperi* (stem)**	4.00	2.00	4.00	8.00	1.00	1.00
***C. schimperi* (leaf)**	8.00	4.00	4.00	8.00	1.00	0.50
***C. tenuipetiolata* (stem)**	4.00	2.00	2.00	8.00	0.50	1.00
***C. tenuipetiolata* (leaf)**	2.00	2.00	2.00	8.00	0.50	1.00
***C. viminea* (stem)**	1.00	0.23	4.00	8.00	1.00	0.25
***C. viminea* (leaf)**	8.00	2.00	2.00	8.00	1.00	0.25
Kaempferol	0.25	0.25	0.25	0.25	0.25	0.25
Amphotericin B (positive control)	-	-	-	-	1.25×10^{-3}	1.25×10^{-3}
Ciprofloxacin (positive control)	2.5×10^{-3}	3.13×10^{-4}	2.5×10^{-3}	2.5×10^{-3}	-	-
DMSO (negative control)	16	16	8	16	16	16

4.4.1 Minimum inhibitory concentration (MIC) assay

All plant species with MIC values of up to 8 mg/ml are considered to possess at least some degree of inhibitory effect, and any concentration exceeding this should not be considered effective, according to Fabry *et al*. (1998).

The majority of the crude stem and leaf extracts of the 10 *Commiphora* species exhibited moderate to relatively good activity against Gram-positive pathogens, with few extracts exhibiting weak antimicrobial activity. The MIC values ranged from 0.005 mg/ml to 8.00 mg/ml. All active extracts displayed concentration-dependent antimicrobial activity. Aligiannis *et al.* (2001) proposed a classification system based on MIC results obtained for plant materials, which was consequently described and implemented by Duarte *et al*. (2005).

Strong microbial inhibitors possessed MIC values as low as 0.50 mg/ml; a clear indication that the MIC value of 0.005 mg/ml obtained for *C. glandulosa* (stem) against *Bacillus cereus* indicates exceptional antimicrobial activity. Four other extracts demonstrated a strong inhibitory effect against the Gram-positive pathogen, *B. cereus***,** these being *C. glandulosa* (leaf, MIC = 0.01 mg/ml), *C. marlothii* (stem, MIC = 0.31 mg/ml), *C. pyracanthoides* (stem, MIC = 0.04 mg/ml) and *C. viminea* (stem, MIC = 0.23 mg/ml).

Bacillus cereus is a causative agent of both gastrointestinal infections (diarrheal and emetic type of food poisoning) and non-gastrointestinal infections (post traumatic wound and burn infections. ophthalmic infections, endocarditis, postoperative meningitis and urinary tract infections) (Kotiranta *et al*., 2000). This will make the discovery of an antimicrobial agent as effective as *Commiphora* spp. against this pathogen highly significant.

Moderate microbial inhibitors are described by Aligiannis *et al.* (2001) as those plant species with MIC values ranging between 0.60 mg/ml and 1.50 mg/ml. Amongst the species investigated in the present study, the species displaying moderate inhibition against the Gram-positive bacterium *Staphylococcus aureus* were *C. glandulosa* (stem), *C. marlothii* (stem and leaf), *C. pyracanthoides* (stem) and *C. viminea* (stem), all of which yielded a MIC value of 1.00 mg/ml.

Weak microbial inhibitors are classified as those agents with MIC values of between 1.60 mg/ml and 8.00 mg/ml (Aligiannis *et al*., 2001). None of the extracts in the present study yielded MIC values in excess of 8.00 mg/ml against Gram-positive bacteria, and all species thus posses at least some antimicrobial effect, as shown in Table 4.1. The results obtained against the Gram-positive bacteria thus support the traditional use of *Commiphora*, and hold potential in the treatment of colds, wound healing and as an antiseptic.

In general, the Gram-negative bacteria displayed the least sensitivity towards the extracts, and all of the plant extracts exhibited poor and unvaried activity against *Pseudomonas aeruginosa*, indicating the resistance of this bacterium to the plant extracts. The activity against *Klebsiella pneumoniae* was, however, far more promising with variability amongst the plant extracts, with the leaf extract of *C. pyracanthoides* yielding the most promising activity (moderate inhibition: MIC = 1.00 mg/ml). This was to be expected, as Gram-negative bacteria offer a much more complex barrier system against permeation of foreign substances (in this case, the antimicrobial agent). This is attributed to the specialised cell wall structure and especially the presence of the outer envelope, as shown in Figure 4.3, resulting in the impermeability of these micro-organisms to biocides and antibiotics, and at times, resulting in regulation and prevention of their passage to the target region (Denyer and Maillard, 2002). Resistance to the plant extracts is, thus, exhibited to a far greater extent by the Gram-negative bacteria than by Gram-positive bacteria (Lin *et al.*, 1999).

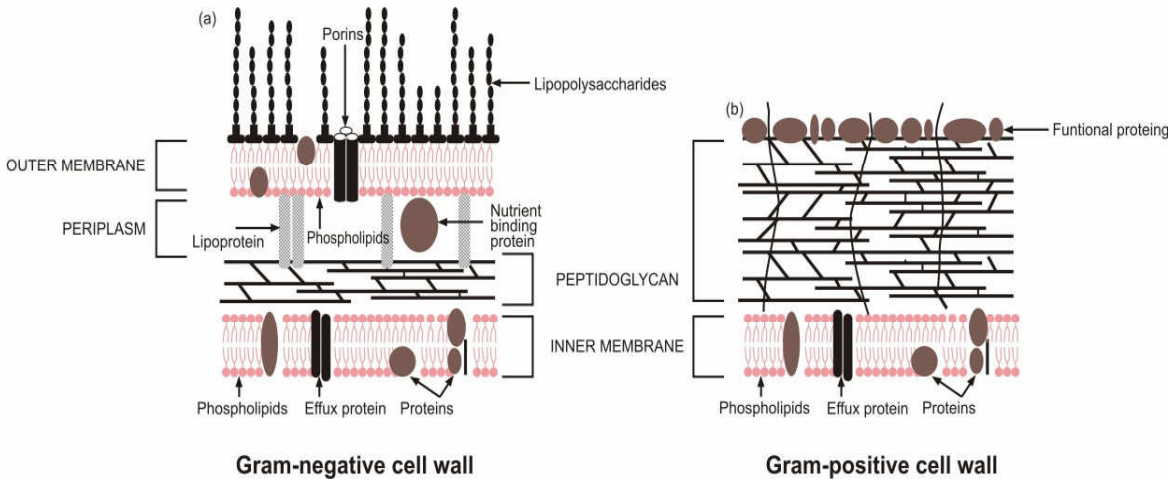


Figure 4.3: The comparative structural complexity of the outer membranes and cell walls of Gram- negative and Gram- positive bacteria (Denyer and Maillard, 2002).

The lipophilic or hydrophilic nature of compounds also plays a role in the activity, or lack thereof, against the micro-organisms. Compounds considered to be more effective against Gram negative bacteria are considerably less lipophilic. This is as a result of the structure of the Gram-negative cell wall (Figure 4.3), which also has a higher lipid content (Linfield *et al.*, 1982). Interactions of lipophilic compounds with hydrophilic parts of the membrane will bring about a more toxic effect against the micro-organism (Sikkema *et al.*, 1995).

The resistance may also be aided by additional resistance mechanisms used by the micro-organism, such as decreased accumulation of the antimicrobial agent within the cell (Denyer and Maillard, 2002), as well as the fact that many harmful agents, including antibiotics are either hydrophobic or relatively large hydrophilic compounds, and are thus hardly able to penetrate the outer membrane. It has also been suggested that the polysaccharide constituents of the outer membrane aid the bacterial cell in evasion of phagocytosis and protect the deeper parts of the outer membrane from complement and antibody binding (Vaara, 1992). In comparison, Gram-positive bacteria possess a much thicker peptidoglycan layer, which does not act as an effective barrier to permeation, and inhibitors are thus able to pass through more easily (Scherrer and Gerhardt, 1971). The current results are consistent with the pattern of *in vitro* activity emerging from other studies.

Apart from the antimicrobial activities observed towards the four bacterial strains, the results obtained in this study demonstrates the importance of investigating natural products for antimicrobial activity against yeasts such as *Candida albicans* and *Cryptococcus neoformans*. The screening of *Commiphora* spp., both in the present study and in those conducted on non-indigenous species (Fatope *et al*., 2003; Claeson *et al*., 2003; Tipton *et al.*, 2003) has yielded fairly good antimicrobial activity against both *C. albicans* and *C. neoformans.*

Greater activity was observed against *C. neoformans* than against *C. albicans*, with MIC values ranging from 0.125 mg/ml (in the leaf extracts of *C. africana, C. pyracanthoides* and *C. viminea* and the stem extract of *C. viminea*) to 8.00 mg/ml in the leaf extract of *C. mollis*. The relevance of these results is clear in the light of the fact that *C. neoformans* is a known pathogen of the respiratory tract, causing pulmonary infections which may spread

to the skin, bones and central nervous system, and occasionally resulting in meningitis (Prescott *et al.*, 1996).

On the other hand, *C. albicans* is an opportunistic pathogen able to cause both systemic and local fungal infections, especially in patients with compromised immune systems and those undergoing antibiotic therapy over extended periods of time (Duarte *et al.*, 2005). There thus exists an ever-increasing need for the development of novel and improved drugs for the management of fungal infections. The inhibitory activity of *Commiphora* spp. against *C. albicans* ranged from 0.50 mg/ml to 8.00 mg/ml, and is mainly ascribed to the widespread ability of flavonoids to inhibit the spore germination of fungal plant pathogens, thereby also suggesting possible use against fungal infections in man (Harborne and Williams, 2000). A prenylated flavanone (Wachter *et al.*, 1999) and flavan (Valsaraj *et al.*, 1997) have also shown to be active against *C. albicans*.

Kaempferol, a flavonol, was isolated from the bark of *C. glandulosa* and displayed significant antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as against the yeasts, with an MIC value of 0.25 mg/ml for all 6 micro-organisms tested. This supports existing evidence that hydroxylation on the B-ring is important for antimicrobial activity, and that the antimicrobial activity is probably due to inactivation of the bacterial membrane (Cowan, 1999) and to interferences with the permeability of fungal membranes (Havsteen, 1983).

The activity of an isolated and purified compound is normally expected to be higher than that of the crude extract. Kaempferol, the active compound isolated during the course of this study, and having shown promising activity against all the relevant micro-organisms, showed a four-fold increase in activity against *S. aureus*, than the *C. glandulosa* stem extract from which it was isolated (Table 4.1). The same results were, however, not achieved against *B. cereus*, against which the crude extract yielded a greater inhibitory effect than an equivalent dose of the isolated compound. This could be as a result of a number of compounds working synergistically or additively within the extract, thereby resulting in a significantly greater antimicrobial effect. However, this explanation remains highly hypothetical, as the exact mechanisms of action of many phytomedicines are as yet unknown. Only once a full explanation of this mechanism is available, will conclusive

evidence regarding the identity of the compounds responsible for biological activity be possible.

Kaempferol has in previous studies demonstrated clear antimicrobial activity against *S. aureus* (DSM 20231), moderate activity against *Staphylococcus epidermidis* (FOMK), *Bacillus subtillis* (ATCC 6633) and *P. aeruginosa* (ATCC 9027), although no activity was observed against *C. albicans* (ATCC 10231) (Rauha *et al.*, 2000). The results for kaempferol, as obtained in the present study, indicated strong antimicrobial activity against both of the Gram-negative bacteria (*P. aeruginosa* and *K. pneumoniae*). While Rauha and co-workers (2000) observed no activity against the yeast *C. albicans* (ATCC 10231), the antimicrobial activity against the said yeast, as observed in the present study, was strong, with a MIC value of 0.25 mg/ml. Such variations in antimicrobial data are, however, frequent and is accounted for by species-specific growth requirements and the associated difficulty to cultivate under identical conditions (e.g. incubation temperature, growth medium and atmosphere), resulting in notable differences between the resulting batches of biomass. Further causes of these differences in antibacterial activity may well be found in the inherent differences in strain sensitivity, and the mode of and choice of solvent for extraction. Discrepancies have also been reported in the antibacterial activity of flavonoids as a result of the different assays employed in their investigation, especially in those assays that depend on the diffusion rate (certain antibacterial flavonoids have a low rate of diffusion) (Cushnie and Lamb, 2005).

The presence of flavonoids and flavonoid derivatives was confirmed by HPLC-UV analysis (Chapter 8) in the crude extracts of the investigated *Commiphora* species. Plants synthesise flavonoids, and are thus not just constitutive agents, but accumulate as phytoalexins in response to microbial infection (Dixon *et al.*, 1983; Grayer and Harborne, 1994) and are richest in antibacterial agents after the flowering stage of their growth is complete. Those plants taken from a stressful environment have shown to be particularly active against micro-organisms. It is thus not surprising that they have been found to exert *in vitro* antimicrobial activity against many micro-organisms (Cowan, 1999). The observed antimicrobial activity may be attributed to the presence of these flavonoids. In a review by Cushnie and Lamb (2005) examples of flavonoids with antimicrobial activity were referenced. Examples of such flavonoids are apigenin, galangin, pinocembrin, ponciretin, genkwanin, sophoraflavanone G and its derivatives, naringin and naringenin,

epigallocatechin gallate and its derivatives, luteolin and luteolin 7-glucoside, quercetin, various quercetin glycosides, kaempferol and its derivatives. Other flavones (Iniesta-Sanmartin *et al*., 1990; Encarnacion *et al*., 1994; Sato *et al.*, 1996) flavone glycosides (Ng *et al.,* 1996), isoflavones (Dastidar *et al*., 2004; Chacha *et al*., 2005) flavanones (Kuroyanagi *et al*., 1999; Chacha *et al*., 2005; Yenesew *et al*., 2005), isoflavanones (Osawa *et al.*, 1992; Yenesew *et al*., 2005), isoflavans (Li *et al*., 1998), flavonols (Nishino *et al*., 1987; Kuroyanagi *et al.*, 1999) flavonol glycosides (Kuroyanagi *et al*., 1999 and chalcones (Gafner *et al*., 1996; Chacha *et al*., 2005) with antibacterial activity have been identified. Hydroxylated phenolic compounds generally have a broad range of activity, which is attributed to their ability to complex with the bacterial cell wall (Cowan, 1999). Flavonoids are hydroxylated phenolic compounds occurring as a C_6–C_3 unit attached to an aromatic ring. Flavonoids have the ability to complex with bacterial cell walls, with probable targets in the microbial cell being surface-exposed adhesions, cell wall polypeptides and membrane-bound enzymes. A possible mechanism of action of flavonoids (as with quinines) is the irreversible complexation with nucleophilic amino acids in extracellular and soluble proteins. This leads to inactivation and loss of function of the proteins (Tsuchiya *et al*., 1996). A study by Mori *et al*. (1987) demonstrated that flavonoids (myricetin, robinetin, (-)-epigallocatechin) inhibit DNA synthesis in *Proteus vulgaris* fairly strongly, while in *S. aureus*, RNA synthesis was affected (protein and lipid synthesis were affected to a lesser degree). The mechanism proposed was the possible intercalation or hydrogen bonding of the B-ring of the flavonoids with the stacking of nucleic acid bases. It has been reported that the more the hydroxylation, the greater the antimicrobial activity noted (Sato *et al.*, 1996). Ohemeng *et al*. (1993) determined that DNA gyrase was inhibited to different extents by certain flavonoid compounds. This activity was limited to those compounds with B-ring hydroxylation (such as quercetin, apingenin and 3,6,7,3′,4′-pentahydroxyflavone), with the exception of 7,8-dihydroxyflavone. In contrast, however, it was determined that flavonoids with no hydroxyl groups on their B rings are more active against micro-organisms than those with hydroxyl groups (Chabot *et al.*, 1992). This supports the hypothesis that the membrane of the micro-organism is the microbial target of flavonoids, as greater lipophilicity may possibly cause a greater disruption to the membranes of the micro-organisms.

In a study by Tsuchiya and Iinuma (2000), it was suggested that intensive antibacterial activity by flavonoids such as sophoraflavanone G may be attributed to the membrane

interference through the reduction of membrane fluidity of both the outer and inner layers of the bacterial cell membranes. The flavonoid quercetin was shown to cause an increase in the permeability of the inner bacterial membrane, and a dissipation of the membrane potential, necessary for ATP-synthesis, membrane transport and motility (Mirzoeva *et al*., 1997).

In general, methanolic extracts are high in their alkaloid, coumarin and tannin, content (Fabry *et al.*, 1998). As the methanolic extracts of the bark of most of the *Commiphora* spp. under investigation were highly active, this may with a high degree of certainty be ascribed to the presence of polyphenol compounds such as tannins, the latter of which are known to have a wide range of non-specific anti-infective actions. The specific mode of antimicrobial action may be affected by enzyme inactivation, through adhesion and through cell envelope transport proteins. However, it is important to note that, if tannins were solely responsible for the activity presented by these results, this activity would be observed against all organisms and would not be limited to Gram-positive bacteria or yeasts. The current hypothesis is thus that tannins are at least partially responsible for the antibiotic activity. Coumarins have also demonstrated antimicrobial activity, especially against *C. albicans* (Cowan, 1999).

4.4.2 Death kinetic assay

The efficacy of *Commiphora marlothii* (stem) extract against *Staphylococcus aureus* was displayed by a killing rate at ca. 30 min of exposure of all the extract concentrations tested. A complete bactericidal effect was achieved after 120 min at extract concentrations of 0.5% (w/v) and 1%, with no re-growth after 48 hours. Surprisingly, this was not the case at an extract concentration of 0.75% (w/v), where a complete bactericidal effect was only achieved at 240 min. Furthermore, bactericidal activity was observed at all extract concentrations after 24 hours, with no re-growth after 48 hours, except at an extract concentration of 0.13% (w/v). Apart from the surprising result achieved at 0.75% (w/v) extract (as described above), an overall concentration dependent effect was exhibited.

4.5 Conclusion

All crude stem and leaf extracts of the 10 indigenous *Commiphora* spp. under investigation exhibited concentration-dependent activity against both Gram-positive and Gram-negative bacteria, as well as against yeasts. The most promising activity was displayed against the yeasts, with the greater inhibitory activity being observed against *C. neoformans* than against *C. albicans*, by the leaf extracts of *C. africana*, *C. pyracanthoides* and *C. viminea* and the stem extract of *C. viminea*. This serves as a clear indication of the potential of these extracts for further chemical and pharmacological studies as antifungal agents.

The antimicrobial activity exhibited by *C. glandulosa* (stem) against *B. cereus* indicates exceptional antimicrobial activity. Four other extracts demonstrated a strong inhibitory effect against *B. cereus*, these being *C. glandulosa* (leaf), *C. marlothii* (stem), *C. pyracanthoides* (stem) and *C. viminea* (stem). Amongst the species investigated in the present study, the species displaying the promising activity against the Gram-positive bacterium *S. aureus* were *C. glandulosa* (stem), *C. marlothii* (stem and leaf), *C. pyracanthoides* (stem) and *C. viminea* (stem).

In general, the Gram-negative bacteria displayed the least sensitivity towards the extracts, and all of the plant extracts exhibited poor and unvaried activity against *P. aeruginosa*, indicating the resistance of this bacterium to the plant extracts. The activity against *K. pneumoniae* was, however, far more promising with variability amongst the plant extracts. *Commiphora pyracanthoides* (leaf) was the most active.

Kaempferol was active against the Gram-positive bacteria *S. aureus* and *B. cereus*. However, it was less active against *B. cereus* than the stem extract as a whole, a result which may well serve as an indication that the overall activity of the extract may be attributed to the presence of other synergistically-acting compounds. Significant activity was displayed by this compound against Gram-negative bacteria, and against the yeasts, as was displayed against the Gram-positive bacteria.

It is at this stage important to note that the failure of a plant extract to demonstrate *in vitro* activity during the general screening process does not necessarily imply a total absence of inherent medicinal value. The possible presence of synergistic interactions between the

different plant constituents in crude preparations may result in activities that are not exhibited by isolated compounds, and should not be excluded.

The death kinetic assay conducted on the crude extract of *C. marlothii* (stem) indicated that this activity is bacteriostatic with bactericidal activity being achieved against *S. aureus* by all extract concentrations after 24 hours, with no re-growth after 48 hours, except at an extract concentration of 0.13% (w/v).

CHAPTER 5: ANTI-INFLAMMATORY ACTIVITY

5.1 Introduction

5.1.1 Inflammatory response process

Inflammation is a physiological response process that is generated by the body in the event of injury, infection or irritation. In the acute stages of the inflammatory process, inflammation serves a vital role in the healing process by the body. Chronic inflammation, however, involves the release of a number of mediators, resulting in the proliferation of fibroblasts, vascular endothelium, as well as lymphocytes, plasma cells and macrophages (Brooks *et al*., 1998). The release of all these mediators can contribute to chronic degenerative diseases such as arthritis, cancer, heart disease, Alzheimer's disease, diabetes and asthma, which may increase disease-associated morbidity.

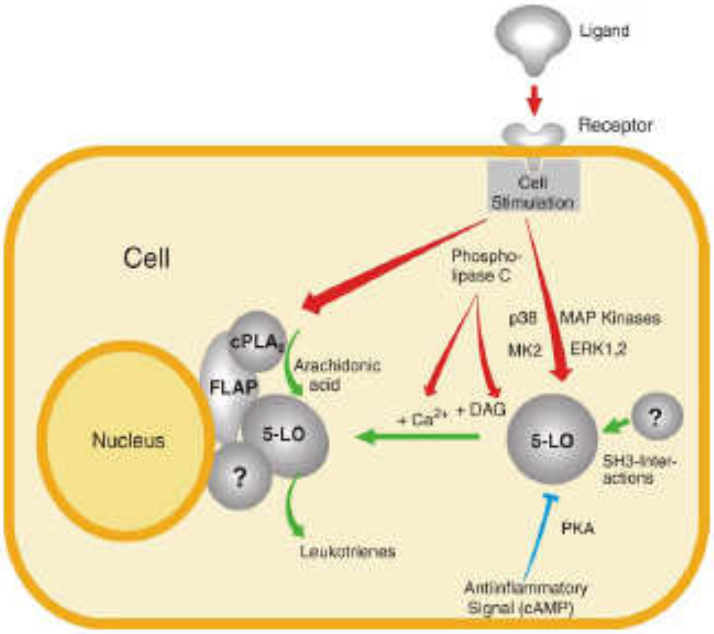
The treatment of inflammatory diseases is largely based on interrupting the synthesis or action of these mediators that drive the host's response to the injury. Examples of such mediators are kinins, substance P, cytokines, chemokines, intermediaries of apoptosis, lipoxins and leukotrienes (Gilroy *et al*., 2004).

Inflammation in injured cells is both initiated and maintained by the overproduction of prostaglandins and leukotrienes, which are produced by separate enzymatic pathways, viz. the cyclo-oxygenase (COX) and lipoxygenase (LOX) pathways, respectively. Both the prostaglandins as well as the leukotrienes are biosynthesised on demand from arachidonic acid, which is a 20-carbon fatty acid, derived from the breakdown of cell membrane phospholipids by any number of phospholipase A_2 (PLA_2) isoforms.

5.1.2 The lipoxygenase system

5-Lipoxygenase presents either in the cytosol or the nucleus of a resting cell (depending on the cell), as a soluble enzyme. Upon cellular stimulation, 5-LOX and cytosolic phospholipase A_2 ($cPLA_2$) co-migrate to the nucleus. It is here that $cPLA_2$ liberates arachidonic acid from the membrane phospholipids. Arachadonic acid is the main substrate

for the lipoxygenase enzyme, and is presented to 5-LOX for metabolism by the 5-lipoxygenase activating protein (FLAP) (Figure 5.1). The metabolism of arachidonic acid produces leukotrienes and lipoxins via the LOX pathway (Werz and Steinhilber, 2006).



5-LO = 5-lipoxygenase, $cPLA_2$ = cytosolic phospholipase A_2, FLAP = 5-Lipoxygenase activating protein, PKA = protein kinase A, cAMP = cyclic adenosine monophosphate, MK2 = kinases, ERK1,2 = extracellular signal-regulated kinase 1/2.

Figure 5.1: The translocation of 5-lipoxygenase and cytosolic phospholipase A_2, upon cellular stimulation, to the nuclear membrane, followed by the substantial generation of leukotrienes (Werz and Steinhilber, 2006).

5-Lipoxygenase, catalysing the oxidation of arachidonic acid, produces 5(*S*)-hydroxyperoxyeicosatetraenoic acid (5-HPETE), a hydroperoxide intermediate, which undergoes dehydration, resulting in the formation of leukotriene A_4 (LTA_4) (Figure 5.2). Enzymatic hydrolysis of LTA_4, as well as conjugation with other substances, leads to the formation of inflammatory mediators (Ford-Hutchinson *et al.*, 1994). These inflammatory mediators are responsible for the powerful chemo-attractive effects on the eosinophils, neutrophils and macrophages, as well as the increased release of pro-inflammatory cytokines by macrophages and lymphocytes. Other inflammatory mediators (such as

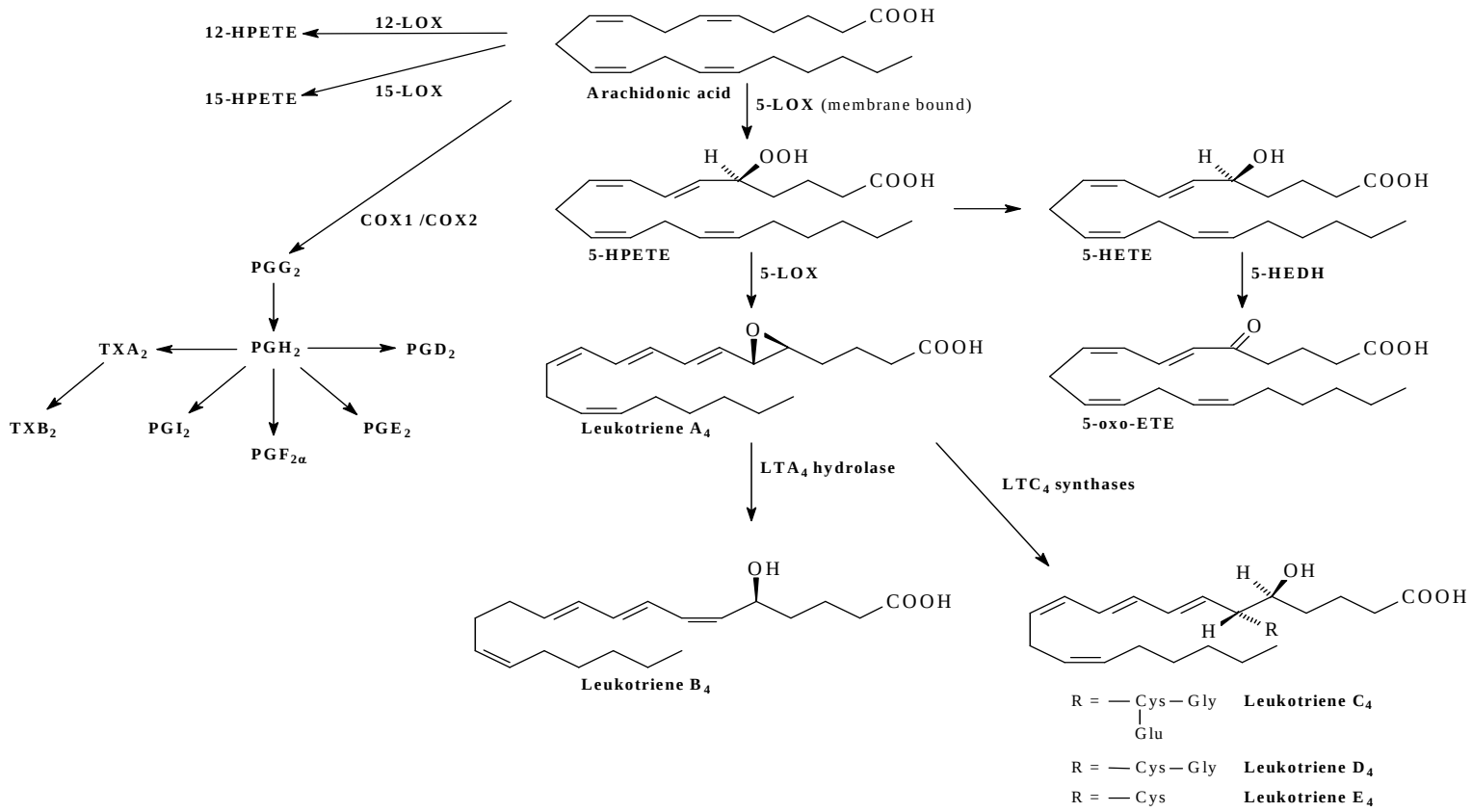
histamine and immunoglobulin E, cause spasms in the smooth muscle of bronchi and blood vessels, playing an eminent role in asthmatic attacks.

Two other pathways in the LOX system, 15-LOX and 12-LOX, are responsible for the production of lipoxins, the latter having the potential to counteract the pro-inflammatory effects of the leukotrienes. 5-Lipoxygenase is inhibited by quinones, hydroxyquinones, and a variety of phenolic compounds, including certain flavonoids such as, quercetin, kaempferol, morin, myricetin and cirsiliol (Kim *et al.*, 2004).

A range of therapies exists for the treatment of inflammation. In most cases, these therapies, however, also have undesirable side effects. An urgent need therefore exists for the research and development of additional or alternative therapies. Man has always looked to nature for the treatment of wounds and topical infections, and it is important to consider that, while traditional principles exist, these need to be evaluated pharmacologically. These traditional remedies are important, not only for their active principles, but also for the synergistic effects of a number of active constituents, especially when such a large proportion of traditional medicines are used as remedies for the treatment of skin conditions and wound healing (Bodeker *et al.*, 1999).

When wounding occurs in the skin, it is accompanied almost immediately by pain, reddening and oedema of the surrounding tissue. At the onset of the inflammatory process, arachidonic acid is converted to eicosanoids and leukotriene B_4 (LTB_4) by LOX (Spector *et al.*, 1988), which is coupled with the production of prostaglandins and thromboxanes by cyclo-oxygenase (COX). The leukotrienes present at the onset of inflammation are also responsible for the maintenance thereof. As leukotrienes thus play a major role in the pathophysiology of chronic inflammatory diseases, it has been suggested that 5-LOX inhibitors may thus be useful in the treatment of various conditions (Zschocke *et al.*, 1999).

Leukotrienes have been identified as mediators of a number of inflammatory and allergic reactions. These include rheumatoid arthritis, inflammatory bowel disease, atopic dermatitis, psoriasis, chronic urticaria, asthma (Claesson and Dahlen, 1999) and allergic rhinitis (Samuelsson *et al.*, 1987; Lewis *et al.*, 1990).



12-LOX = 12-lipoxygenase, 12-HPETE = 12-hydroperoxyeicosatetraenoic acid, 15-LOX = 15- lipoxygenase, 15-HPETE = 15-hydroperoxyeicosatetraenoic acid, COX-1 = cyclo-oxygenase-1, COX-2 = cyclo-oxygenase-2, PGG_2 = prostaglandin G_2, PGH_2 = prostaglandin H_2, PGD_2 = prostaglandin D_2, PGE_2 = prostaglandin E_2, $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$, PGI_2 = prostaglandin I_2 (prostacyclin), TXA_2 = thromboxane A_2, TXB_2 = thromboxane A_2, 5-LOX = 5-lipoxygenase, 5-HPETE = 5-hydroperoxyeicosatetraenoic acid, 5-HETE = 5-hydroxyeicosatetraenoic acid, 5-HEDH = 5-hydroxyeicosanoid dehydrogenase, LTA_4 hydrolase = leukotriene A_4 hydrolase, LTC_4 synthases = leukotriene C_4 synthas

Figure 5.2: Schematic representation of the 5-lipoxygenase pathway and simplified scheme of the generation of other eicosanoids from arachidonic acid, indicating the cyclo-oxygenase pathway (modified from Werz and Steinhilber, 2006).

The 5-LOX pathway has also recently been linked to the development of atherosclerosis, osteoporosis and certain types of cancers (Werz and Steinhilber, 2006).

As a result of the pathophysiological implications of 5-LOX products and the potential benefits of anti-leukotriene therapy, different strategies have been employed (targeting PLA_2, 5-LOX, FLAP, LTA_4 hydrolase and leukotriene C_4 (LTC_4) synthase) with 5-LOX being the ideal and most promising target (Werz and Steinhilber, 2006).

5.1.3 The cyclo-oxygenase-1 and cyclo-oxygenase-2 enzyme system

The COX enzyme systems are responsible for the production of both prostaglandins and prostacyclins through the metabolism of arachidonic acid. Prostaglandins, for which the isoform COX-2 is the key synthetic enzyme, induce vasodilation, pain and increased capillary permeability. Prostaglandins also prevent the formation of ulcers by a mechanism independent of their antisecretory properties and by protecting the gastric mucosa. Flavonoids and sesquiterpene lactones, isolated from plants, have been shown to possess both anti-inflammatory and anti-ulcerogenic activity (Abad *et al.*, 1994).

5.1.4 *Commiphora* species and their anti-inflammatory effects

Myrrhanol A and myrrhanone A are triterpenes isolated from *Commiphora myrrha*, which possess potent anti-inflammatory activity. These compounds were isolated from the gum resins of guggul and myrrh, in a 50% aqueous methanolic extract. Their anti-inflammatory activity was demonstrated against four inflammatory processes, this being a clear indication that these compounds are plausible anti-inflammatory agents (Kimura *et al.*, 2001).

In a study by Meselhy (2003) guggulsterone (4,17(20)-pregnadiene-3,16-dione), a plant sterol isolated from the gum resin of *Commiphora mukul*, suppressed inflammation by inhibiting nitric-oxide synthetase expression induced by lipopolysaccharides in macrophages. The activation of nuclear factor-kappa B (NF-κB) is found in most inflammatory diseases, but modulation by guggulsterone results in the interference in the inflammatory process.

Myrrh oil was tested for its *in vitro* ability to lessen interleuken-1β (IL-1β)-stimulated production of cytokines viz. IL-6 and IL-8 by human gingival fibroblasts and epithelial cells, in a study by Tipton *et al.* (2003). The fibroblasts in the inflamed gingival produce IL-6 and IL-8. Interleukin-8 is a cytokine significant in acute inflammation as well as neutrophil chemotaxis. It was found that fibroblasts present in the inflamed gingival were able to participate in local inflammation process through the production of these cytokines. The addition of myrrh oil, however, resulted in decreased production of these fibroblasts, thereby decreasing the production of the pro-inflammatory cytokines, and ultimately resulting in a decreased involvement of these cytokines in gingivitis and periodonitis.

Sesquiterpene compounds exhibit considerable anti-inflammatory activity by inhibiting the transcription factors responsible for the transcription of genes encoding numerous cytokines, one of which being IL-6.

Extracts from *Commiphora mukul* (Duwiejua *et al.*, 1993; Sosa *et al.*, 1993) and *Commiphora incisa* (Duwiejua *et al.*, 1993) resins were studied for their anti-inflammatory activity. Significant inhibition of both the maximal oedema response and the total oedema response was observed. Two octanodammarane triterpenes, mansubinone and mansumbinoic acid, were isolated and tested. Both compounds exhibited significant anti-inflammatory activity, with mansumbinoic acid reducing joint swelling, warranting further investigation of this compound as an anti-inflammatory agent (Duwiejua *et al.*, 1993). Sosa *et al.* (1993) highlighted the role of guggulsterones in the anti-inflammatory activity of *C. mukul*, however, it was deduced that since this steroidal fraction represented only 24% of the raw extract, its activity was considered to be too low to entirely account for the anti-inflammatory effect.

Despite the traditional use of *Commiphora* in the treatment of wounds, other inflammatory skin disorders and its intensive usage in rheumatoid arthritis, there has not been, to our knowledge, any previous study concerning the actual therapeutic anti-inflammatory properties of indigenous species. It is thus considered an apt subject for research, and even more so when combined with a pharmacological evaluation of the mechanism by which these ailments are treated.

5.1.5 Flavonoids - their anti-inflammatory potential

Flavonoids have long been utilised for their anti-inflammatory capacity in Chinese medicine, as well as in the cosmetic industry in the form of crude plant extracts. Numerous studies have demonstrated their potential *in vitro* and *in vivo*, with some flavonoids inhibiting chronic inflammation in several experimental animal models (Kim *et al.*, 2004).

Kaempferol decreased the production of prostaglandin E_2 by lipopolysaccharide (LPS)-stimulated human cells by the inhibition of COX 2, in a study by Miles *et al*. (2005). This decrease in the inflammatory mediator production by human whole blood cultures may contribute to the anti-atherogenic properties; however this would require further investigation.

Yoshimoto *et al.* (1983) reported that certain flavonoids were relatively selective inhibitors of the 5-LOX enzyme, and certain structural characteristics are required to produce this anti-inflammatory activity. A cathechol (3',4'-dihydroxyl) structure in ring B (R_5 and R_6), of flavonoid derivatives appeared necessary to inhibit 5-LOX, as indicated by the most active compounds cirsiliol and pedalitin. A 6-OH (R_2) group on pedalithin results in an inhibitory activity equal to that of cirsiliol. The modification of the 5-OH (R_1) group of cirsiliol, and the demethylation at position 7, both reduce the inhibitory effect of the flavones (Figure 5.3). Flavone derivatives with no substituents produced a decreased inhibitory activity.

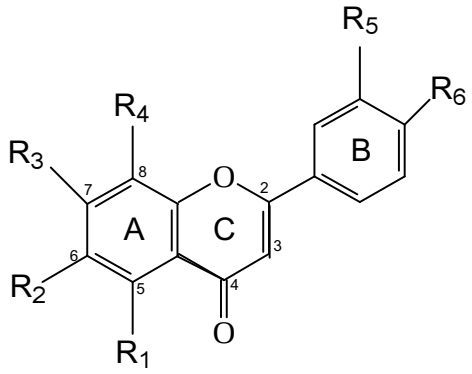


Figure 5.3: The basic chemical structure of flavones (Harborne *et al.*, 1975).

The structural components of 5,8-dihydroxy-6,7,4'-trimethoxyflavone and cirsimaritin indicates most structural requirements are present thus, causing some activity. Flavones proved to be more potent COX inhibitors, while flavonols showed preferential LOX inhibitory activity, strongly inhibiting 5-LOX, and being far less active against 12-LOX. The important moieties are the C_2-C_3 double bond, 5,7-hydroxyl groups on the A-ring and 4'- or 3',4'-hydroxyl groups on the B-ring. The C-3 hydroxyl group found in flavonols is favourable for inhibitory activity against the LOX enzyme. The C6 and C8 substituted flavones and flavonols also exhibit good anti-inflammatory activity. Exceptional activity is demonstrated by prenylated flavonoids, an example of which is shown in Figure 5.4 (Kim *et al*., 2004)

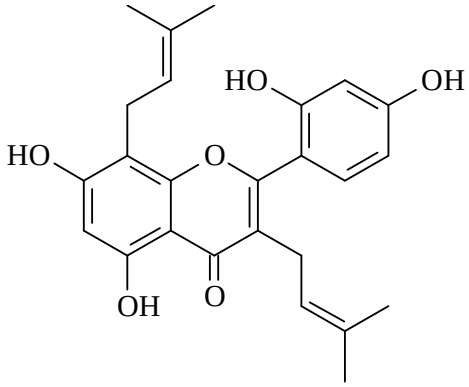


Figure 5.4: The chemical structure of a prenylated flavonoid, kuwanon C (Kim *et al*., 2004).

It is thus not only valuable to evaluate flavonoids, and other isolated plant compounds, for their potential anti-inflammatory activity and establishing the mechanisms by which they are effective, but also as a possible new class of anti-inflammatory agents.

The objective of this study is to determine the anti-inflammatory activity of the 10 indigenous *Commiphora* species studied, and to determine the anti-inflammatory effect of the isolated compound kaempferol.

5.2 Materials and methods

The anti-inflammatory activity of the plant extracts was determined using the *in vitro* 5-LOX assay. This assay measures the inhibitory activity against the 5-LOX enzyme, which is a key enzyme in the metabolism of arachidonic acid that is responsible for the formation of leukotrienes (which play a pivotal role in the pathophysiology of chronic inflammatory and allergic diseases) as first determined by Sircar *et al.* (1983) and later modified by Evans (1987).

5.2.1 Principle of the assay

Lipoxygenases (LOX) are dioxygenases that catalyse the addition of molecular oxygen to polyunsaturated fatty acids containing a 1,4-pentadiene group. The 5-LOX enzyme thus converts its substrate, arachidonic acid, to the conjugated diene product 5-hydroxy-6,8,11,1-eicosatetraenoic acid (5-*S*-HETE), which, in turn, is converted to LTA_4, and then to LTB_4, by LTA_4 hydrolase. For the purposes of this experiment, linoleic acid was used as the substrate, as it shares a high degree of structural similarity to arachidonic acid (containing the 1,4-pentadiene group in question), it is far easier to handle as well as having a stronger affinity for the 5-LOX enzyme resulting in greater UV absorbance readings (Baylac and Racine, 2003). The experiment specifically determines increases in absorbance at 234 nm as a result of the formation of conjugate double bonds in linoleic acid hydroperoxide (from a 1,4-diene to a 1,3-diene), as used in the biochemical evaluation of the LOX pathway of soybean plants submitted to wounding (Vieira *et al.*, 2001).

5.2.2 Protocol

5.2.2.1 Preparation of plant samples

Extracts of both the leaf and bark were prepared, as described in Chapter 2, section 2.3. The samples were prepared by dissolving these plant extracts in DMSO (Saarchem) and Tween® 20 (Merck).

The isolated compound, kaempferol, from stem extract of *C. glandulosa* (Chapter 3) was prepared in the same way as the extracts, to determine any potential anti-inflammatory activity present.

5.2.2.2 5-Lipoxygenase assay

The standard assay mixture contained 10 µl of the plant extract dissolved in a solution of DMSO and Tween® 20 with the starting concentration of 100 µg/ml, in a 3 ml cuvette. The addition of 0.1 M potassium phosphate buffer (pH 6.3, 2.95 ml), prepared with analytical grade reagents, which was maintained at 25°C in a thermostated waterbath, and 45 µl of 100 µM linoleic acid (purity ≥ 99%, Fluka), followed. The enzymatic initiation of the reaction occurred upon addition of 100 units of the isolated 5-LOX enzyme (Cayman), the latter diluted with 12 µl of 0.1 M potassium phosphate buffer (pH 6.3) and maintained at 4°C until required (Figure 5.5).

Increases in absorbance were recorded at 234 nm for 10 min, using a UV-VIS Analytikjena Specord 40 spectrophotometer, connected to a computer equipped with Winaspect® software. Serial dilutions of the extracts were prepared for those species that exhibited anti-inflammatory activity, and these were assayed in a similar way. The results were plotted and initial reaction rates were determined from the slope of the straight-line portion of the curve.

The percentage of enzyme inhibition attributable to each of the extracts was then determined by comparison with the negative control, the latter comprising DMSO and Tween® 20 in the absence of plant extract. The percentage enzyme inhibition (calculated using equation 5.1), which denotes the anti-inflammatory activity, was plotted against the concentration of plant extract (µg/ml). The IC_{50} values were determined (being the concentration at which 50% inhibition is achieved) from the dose-response curves using Enzfitter® (version 1.05) software.

Equation 5.1

$$\% \text{ 5-lipoxygenase inhibition} = \frac{100 - (\text{5-HETE with inhibitor})}{(\text{5-HETE without inhibitor})} \times 100$$

Nordihydroguaiaretic acid (NDGA, Cayman), an inhibitor of the 5-LOX enzyme, was used as a positive control in this assay.

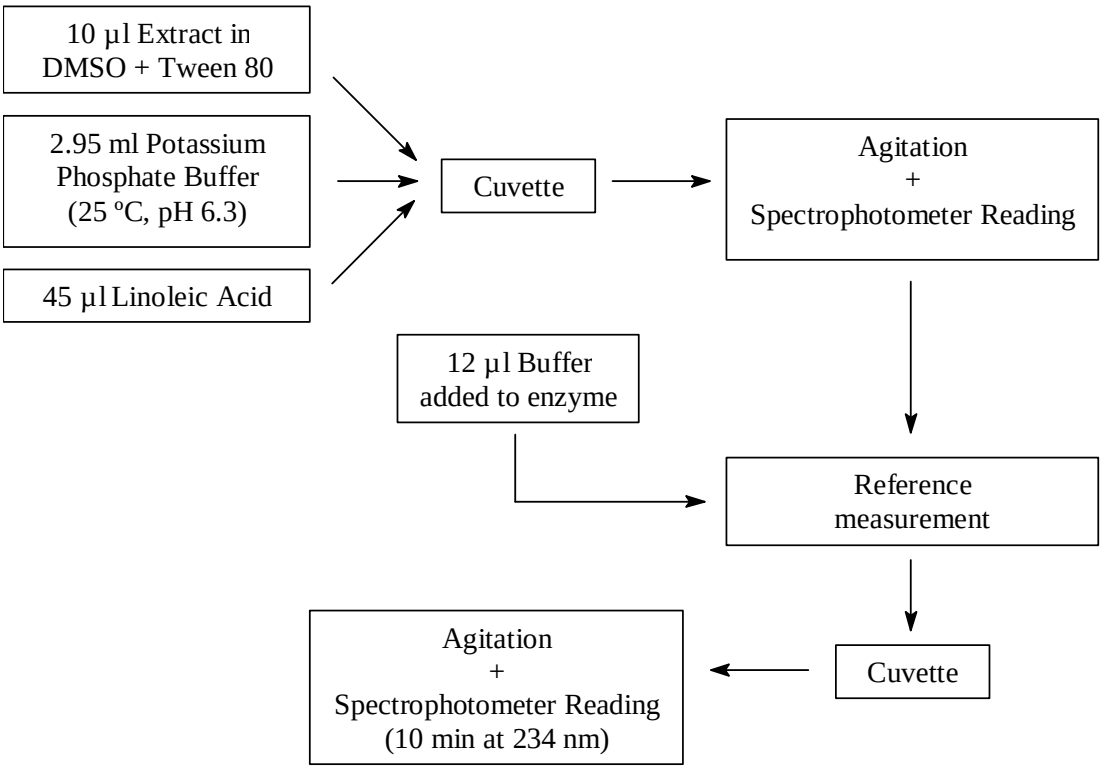


Figure 5.5: Schematic representation of the 5-lipoxygenase assay.

5.3 Results

The *in vitro* anti-inflammatory activity for each of the species of *Commiphora*, for both the leaf and stem extracts, indicated as the percentage 5-LOX enzyme inhibition, together with their corresponding IC_{50} values, are shown in Table 5.1. No results were obtained for the leaf extracts of *C. neglecta* and *C. edulis*, as there was no sufficient plant material available.

Table 5.1: The percentage 5-lipoxygenase enzyme inhibitory activity of *Commiphora* species stem and leaf extracts *in vitro* at 100 μg/ml and their corresponding IC_{50} values.

Species	5-lipoxygenase inhibition at 100 μg/ml (%)	IC_{50} (μg/ml)
***C. africana* (stem)**	8.8	n.d.[b]
***C. africana* (leaf)**	16.7	n.d.[b]
***C. edulis* (stem)**	100	55.61 ± 1.25
***C. edulis* (leaf)**	n.d.[a]	n.d.[a]
***C. glandulosa* (stem)**	100	66.16 ± 3.61
***C. glandulosa* (leaf)**	70	50.54 ± 14.70
***C. marlothii* (stem)**	13.8	n.d.[b]
***C. marlothii* (leaf)**	34.7	n.d.[b]
***C. mollis* (stem)**	87.5	53.98 ± 1.59
***C. mollis* (leaf)**	26.4	n.d.[b]
***C. neglecta* (stem)**	100	61.65 ± 8.98
***C. neglecta* (leaf)**	n.d.[a]	n.d.[a]
***C. pyracanthoides* (stem)**	100	27.86 ± 4.45
***C. pyracanthoides* (leaf)**	48.6	n.d.[b]
***C. schimperi* (stem)**	100	58.38 ± 13.88
***C. schimperi* (leaf)**	80	76.22 ± 4.84
***C. tenuipetiolata* (stem)**	100	53.58 ± 10.44
***C. tenuipetiolata* (leaf)**	25	n.d.[b]
***C. viminea* (stem)**	66.2	62.97 ± 11.64
***C. viminea* (leaf)**	51.4	n.d.[b]
Kaempferol	100	19.09 ± 6.41
Control NDGA	100	4.95 ± 0.07

n.d.[a] = not determined, as there was no sufficient plant material available.

n.d.[b] = not determined, as serial dilutions were prepared for the extracts exhibiting anti-inflammatory activity at 100 μg/ml of over 55%.

Commiphora myrrha, used in aromatherapy for its anti-inflammatory properties, has demonstrated *in vitro* inhibitory activity against the 5-LOX enzyme in a study by Baylac and Racine (2003). The measure of the activity was defined according to an arbitrary scale of IC_{50} values, where an IC_{50} value above 100 μg/ml was inactive, between 51 μg/ml and100 μg/ml was poorly active (+), between 31 μg/ml and 50 μg/ml was moderately active (++), between 10 μg/ml and 30 μg/ml showed good activity (+++) and an IC_{50} value of less than 10 μg/ml showed excellent activity (++++). *Commiphora myrrha* essential oil (volatile components), as well as the resinoid obtained from the gum (non-volatile component) showed good anti-inflammatory activity (+++).

All the stem extracts displayed good to moderate activity at the starting concentration of 100 μg/ml, and serial dilutions were thus prepared and assayed, with the exception of *C. africana* and *C. marlothii*. IC_{50} values ranging from 27.86 ± 4.45 μg/ml to 66.16 ± 3.61 μg/ml were obtained. *Commiphora pyracanthoides* (stem) displayed good activity against the 5-LOX enzyme (IC_{50} = 27.86 ± 4.45 μg/ml).

The leaf extracts of eight species displayed minimal inhibition of the 5-LOX enzyme, and serial dilution assays were therefore not performed on these extracts, with the exception of *C. schimperi* and *C. glandulosa*, which yielded IC_{50} values of 76.22 ± 4.84 μg/ml and 50.54 ± 14.70 μg/ml, respectively. The isolated compound kaempferol, displayed good activity against the 5-LOX enzyme, with an IC_{50} value of 19.09 ± 6.41 μg/ml.

Nordihydroguaiaretic acid (NDGA), an inhibitor of the 5-LOX enzyme (positive control), was determined to exert excellent (++++) inhibitory effects on the 5-LOX enzyme. Figure 5.6 depicts the percentage 5-LOX enzyme inhibition for each of the extracts at a concentration of 100 μg/ml, revealing the extensive activity of the stem extracts as compared to the inhibitory effects of the leaf extracts.

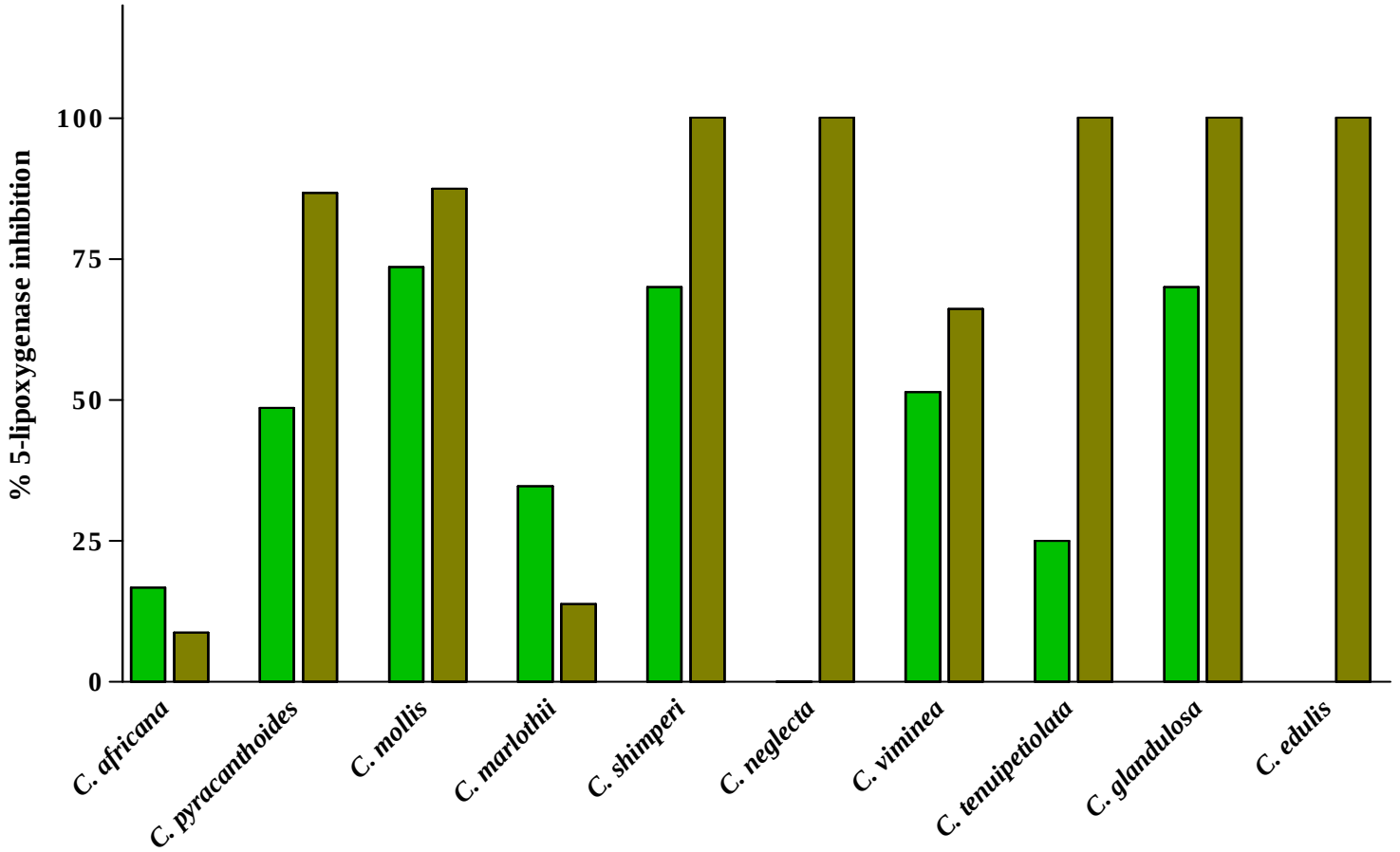


Figure 5.6: The percentage 5-lipoxygenase enzyme inhibition by *Commiphora* leaf ( ) and stem ( ) extracts at a concentration of 100 µg/ml.

5.4 Discussion

The results obtained for the stem and leaf extracts of each of the species of *Commiphora* indicate that anti-inflammatory activity varies widely amongst the species. It is clear that, through inhibition of the enzyme, most of the stem extracts completely inhibit the formation of the 5-LOX products at 100 μg/ml. Complete inhibition (100%) at 100 μg/ml was observed for the stem extracts of *C. edulis* (IC_{50} = 55.61 ± 1.25 μg/ml), *C. glandulosa* (IC_{50} = 66.16 ± 3.61 μg/ml), *C. neglecta* (IC_{50} = 61.65 ± 8.98 μg/ml), *C. schimperi* (IC_{50} = 58.38 ± 13.88 μg/ml), and *C. tenuipetiolata* (IC_{50} = 53.58 ± 10.44 μg/ml), all of which, when compared to *C. myrrha*, display only poor activity (+).

The activity exhibited by these extracts was one of a concentration dependant effect such that increasing concentrations of extract produced a greater inhibition of the 5-LOX enzyme. Of the extracts the most promising activity was observed for the stem extract of *C. pyracanthoides* (yielding complete inhibition at 100 μg/ml and an IC_{50} value of 27.86 ± 4.45 μg/ml), which is comparable to that of *C. myrrha,* with an IC_{50} value ranging between 10 μg/ml and 30 μg/ml, indicating good anti-inflammatory activity.

The leaf extracts for all species displayed inhibitory effects inferior to that of the stem extracts (Table 5.1). The greatest activity amongst the leaf extracts was displayed by *C. glandulosa,* which yields an IC_{50} value of 50.54 ± 14.70 μg/ml. The leaf extracts indicated higher flavonoid content than the stems (Chapter 8). However, it is due to the specific structural characteristics required by flavonoids to produce a 5-LOX inhibitory effect, that the lack of promising activity by the leaf extracts may be explained.

The absence of an inhibitory effect on the 5-LOX enzyme by *C. marlothii* (leaf and stem) *C. africana* (leaf and stem), as well as that of the leaf extract of *C. tenuipetiolata*, is not necessarily indicative of a total lack of anti-inflammatory activity by these species. The 5-LOX pathway is by far not the only pathway involved in the inflammatory process (Figure 5.1), and certain compounds present within each of the species may act at other sites or may follow other modes of action such as 5-LOX activating protein (FLAP), 8-, 12- or 15-LOX, COX-1 or COX-2.

Two flavonoid derivatives were isolated from the stem extract of *C. glandulosa* (Chapter 3, section 3.2.4 and section 3.2.5). Flavonoids have a wide range of biological activities, which include anti-inflammatory, antibacterial and anticarcinogenic effects, all of which are mediated by different mechanisms. The activities of the different flavonoids are highly dependent on their chemical structures and selected phenolic compounds have been shown to inhibit the pathways of both COX and 5-LOX (Ferrandiz and Alcaraz, 1991), although the exact mechanism of pathway and/or enzyme inhibition is not known. Flavonoids also inhibit the biosynthesis of eicosanoids, a product of the LOX (and COX) pathway (Moroney *et al.*, 1988).

Kaempferol, the isolated flavonol, exhibits anti-inflammatory activity (Bezáková *et al.*, 2004). In a study by Della *et al*. (1988), kaempferol, when compared to the synthetic anti-inflammatory agent indomethacin, showed a potent, prolonged anti-inflammatory effect. The *in vitro* anti-inflammatory activity of kaempferol against 5-LOX was determined to be 19.09 ± 6.41 μg/ml. The presence of kaempferol in other species was verified by HPLC data analysis (Chapter 8), and was found to be present in the stem extracts of *C. pyracanthoides* and *C. tenuipetiolata*, and in the leaf extracts of *C. mollis* and *C. neglecta*. Kaempferol may contribute partly to the overall anti-inflammatory activity of the crude stem extracts of *C. glandulosa* and *C. tenuipetiolata*; however, the total anti-inflammatory activity of the crude extracts is far less than the isolated compound. This could be attributed to the presence of kaempferol in only low concentrations (Chapter 8). The decreased activity may also be attributed to the presence of compounds, which may act antagonistically.

Commiphora pyracanthoides was observed to have amongst numerous compounds, kaempferol. The anti-inflammatory activity observed in this case, however, was comparable to that of the isolated compound, also only present in small amounts. It is therefore evident that kaempferol may not be the major contributor of the anti-inflammatory activity observed, but rather suggests that there are a number of compounds, occurring within the plant that may be acting synergistically to produce the anti-inflammatory activity observed.

In a review study by Kim *et al.* (2004), flavonols such as quercetin, morin, myricetin including kaempferol, were found to be 5-LOX inhibitors that were less active against 12-

LOX, but were stronger inhibitors than flavones. Exceptions to this finding were the flavone derivatives including cirsiliol and its analogues, being strong inhibitors of 5-LOX. Based on cirsiliol molecule, C_6 and C_8 alkyloxyflavones have a B-ring 3',4'-dihydroxyl group, and some were found to be potent 5-LOX inhibitors (IC_{50} value in the 10 μM range).

In a study by Yoshimoto *et al.* (1983), it was reported that certain flavonoids were relatively selective inhibitors of the 5-LOX enzyme, and that certain structural characteristics are required to produce this anti-inflammatory activity. The minimal requirement for inhibition of 5-LOX is the presence of the keto group at C_4 with the absence of substitution at $C_{2'}$ (Abad *et al*., 1994). A cathechol structure in ring B (a vicinal diol at R_5 and R_6), as mentioned previously appeared necessary to inhibit 5-LOX. The 4'-hydroxyl in the B-ring, C_2-C_3 double bond in the C-ring and the 5,7-hydroxyl groups on the A-ring are all characteristic of kaempferol, which explains the favourable anti-inflammatory activity (+++).

Quercetin, a flavonoid sharing structural similarities with kaempferol, is a potent inhibitor of 5-LOX isolated from rat basophilic leukemia cells. Quercetin also bears some structural resemblance with NDGA (Hope *et al*., 1983). Nordihydroguaiaretic acid putatively blocks the formation of 5-LOX products, exerting a significant effect with a determined IC_{50} value of 4.95 ± 0.07 μg/ml. This suggests that the structural resemblance may also apply to kaempferol, and that there is a probable similarity in the mechanism of action.

The 5-LOX pathway has also been implicated in cardiovascular disease, including atherosclerosis, stroke, myocardial infarction and the weakening of large artery walls and the formation of aneurysms (Osher *et al.*, 2006; Werz and Steinhilber, 2006). The presence of the 5-LOX pathway, the production of leukotrienes and presence of the enzymes concerned, as well as leukotriene receptors is expressed in diseased tissue. Genetic studies have been carried out on mice linking the 5-LOX pathway to atherosclerosis, and population genetic studies involving humans, correlates genotypes of 5-LOX, FLAP and LTA_4 hydrolase to cardiovascular disease, as shown in studies by Dwyer *et al*. (2004), Helgadottir *et al*. (2004), Helgadottir *et al.* (2005), and Helgadottir *et al*. (2006). *Commiphora myrrha* has shown to possess 5-LOX inhibitory activity (Baylac and Racine, 2003) and *C. mukul* and guggulsterone have been used for the treatment of atherosclerosis

(albeit the mode of action being to inhibit the oxidative modification of low-density lipoproteins, which would otherwise lead to the accumulation of cholesterol in foam cells and atherogenesis).

5.5 Conclusion

Significant inhibitory activity against the 5-LOX enzyme was observed for the majority of *Commiphora* stem extracts. The leaf extracts investigated did not possess promising activity against the 5-LOX enzyme with the exception of *C. schimperi* and *C. glandulosa*. It is important that recognition be given to the other inflammatory pathways within the cascade, as a lack of inhibitory activity against the 5-LOX enzyme cannot exclude the possibility of alternative inhibitory effects.

As kaempferol, isolated from *C. glandulosa* (stem), is known to exhibit anti-inflammatory activity, the activity of *C. glandulosa* (stem), *C. pyracanthoides* (stem) and *C. tenuipetiolata* (stem) extracts may be attributed partly to the presence of this compound.

5-Lipoxygenase is the key enzyme responsible for the biosynthesis of leukotrienes, which play a pivotal role in the inflammatory response to rheumatoid arthritis and injury and may additionally act as mediators in asthmatic responses, myocardial ischaemia, cancer and psoriasis. The traditional use (by the Himba tribe) of *Commiphora* stem extracts for rheumatoid arthritis and other inflammatory conditions is scientifically validated by the *in vitro* determination of the anti-inflammatory activity.

CHAPTER 6: ANTICANCER ACTIVITY

"Let food be thy medicine and medicine be thy food" (Hippocrates, as quoted by Treasure, 2005).

6.1 Introduction

Despite the therapeutic advances made in understanding the processes involved in carcinogenesis, cancer has become one of the most serious medical problems today. The worldwide mortality rate increases annually, with more than seven million deaths occuring per year. For this reason, cancer chemotherapy has become a major focus area of research. Different lifestyles, risk factors (such as age, gender, race, genetic disposition) and the exposure to different environmental carcinogens, lead to the varying patterns of cancer incidence (Chang and Kinghorn, 2001). At least 35% of all cancers worldwide result from an incorrect diet, and in the case of colon cancer, diet may account for 80% of these cases (Reddy *et al*., 2003).

Cancer, a cellular malignancy that results in the loss of normal cell-cycle control, such as unregulated growth and the lack of differentiation, can develop in any tissue of any organ, and at any time. The tissues most affected by cancer and which have a relative survival rate of five years are the oesophagus, lung, pancreas, stomach, liver, ovaries and breasts (Chang and Kinghorn, 2001).

Studies have revealed that certain cancers are more common in people of certain cultures than others. Cancers of the lung, colon, prostate and breast are very common in Western countries, and not as prevalent in the Eastern countries. Cancers of the head, neck and of the cervix are most common in India, while stomach cancer is most prevalent in Japan. In South Africa, cancer diagnosis statistics for four major groupings are available from 1986; these groups include 75.2% South African blacks, 13.6% caucasians (mainly of European descent), 8.6% coloured (mixed race) and 2.6% Asian (Indian/East Asian descent) (Albrecht, 2003). There were 60 172 new cancer cases reported to the cancer registry in 1998 and 60 343 new cases in 1999. Overall statistically, the ethnic groups at highest risk are the South African caucasians, comprising 45.2% and 46.4% in 1998 and 1999

respectively, and South African blacks, with 39.4% in 1998 and 36.8% in 1999 (Mqoqi *et al.* 2004). Table 6.1 demonstrates the incidence rates of major cancers in these groups.

Table 6.1: Incidence rates of the major cancers in the caucasian and black population of South Africa (Albrecht, 2003).

Cancer type	South African Caucasians	South African Blacks
Oesophageal	5%	25%
Breast	64%	13%
Prostate	43%	13%
Cervix	9%	40%
Colon	23%	3%

The search for potential anticancer agents from natural products dates back to 1550 BC. Scientific research reports only started emerging in the 1960's, with investigations by Hartwell and colleagues (Pettit, 1995), on the application of podophyllotoxin and its derivatives as anticancer agents. This growing international trend towards chemoprevention was initiated in an attempt to reduce the incidence of cancer. "Chemoprevention" is defined as a process to delay or prevent carcinogenesis in humans through the ingestion of dietary or pharmaceutical agents. This also implies the identification of chemical entities (specifically cytotoxic entities) that are effective against a range of cancer cell lines, although less active or non-toxic against the normal (healthy) cell population. The search for such anticancer agents from plant sources started in the 1950's, and plant products have proven to be an important source of anticancer drugs (Cragg and Newman, 2005). This directly results from the biological and chemical diversity of nature, which allows for the discovery of completely new chemical classes of compounds.

The discovery and development of plant-derived compounds led to the first cures of human cancer, specifically upon administration of these compounds in combination with synthetic agents. Of the 121 medications being prescribed for use in cancer treatment, 90 are sourced from plants. It was also determined that approximately 74% of these discoveries were as a result of an investigation into the claims made by folkloric tradition (Shishodia and Aggarwal, 2004). Examples of these compounds used as cytotoxic drugs are shown in Table 6.2.

Table 6.2: Cytotoxic drugs developed from plant sources.

Therapeutic agent	Chemical compound	Mechanism of action	Treatment of cancer type	Plant source	Reference
Vinblastine, vincristine	Alkaloids	Inhibition of tubulin polymerization	Hodgkin's disease	*Catharanthus roseus*	Mans *et al*. (2000)
Etoposide, teniposide	Epipodophyllotoxin	Inhibition of topoisomerase II	Testicular cancer, and small cell lung carcinoma, leukaemias, lymphomas	*Podophyllum peltatum*	Lee (1999), Mans *et al*. (2000)
Paclitaxel, docetaxel	Taxanes	Promotion of tubulin stabilization	Ovarian and breast carcinoma	*Taxus brevifolia*	Mans *et al*. (2000)
Irinotecan, topotecan, 9-aminocampothecin, 9-nitrocamptothecin	Alkaloid	Inhibition of topoisomerase I	Advanced colorectal cancer, also active in lung, cervix and ovarian cancer	*Camptotheca acuminata*	Srivastava *et al*. (2005)
Homoharingtonine	Alkaloid	Inhibition of DNA polymerase α	Various leukaemias	*Harringtonia cephalotaxus*	Mans *et al*. (2000)
4-Ipomeanol	Pneumotoxic furan derivative	Cytochrome P-450-mediated conversion into DNA-binding metabolites	Lung cancer	*Ipomoea batatas*	Mans *et al*. (2000)
Elliptinium	Semi-synthetic derivative from ellipticine	Inhibition of topoisomerase II	Advanced breast cancer	*Bleekeria vitensis*	Mans *et al*. (2000)
Flavopiridol	Synthetic flavone derived from plant alkaloid rohitukine	Inhibition of cyclin-dependent kinases	Encouraging results noted in a variety of solid and haematological malignancies, in patients with colorectal, prostate, lung, renal carcinoma, non-Hodgkin's lymphoma and chronic lymphocytic leukaemia	*Amoora rohituka, Dysoxylum binectariferum*	Mans *et al*. (2000)

Carcinogenesis is the transformation of a normal cell to a cancerous cell through many stages, which occur over a number of years or even decades. The first stage of carcinogenesis is the initiation stage, which involves the reaction between the carcinogens and the DNA of the cells. Inhibiting this early stage of cancer is an important strategy in cancer prevention or treatment. Promotion is the second stage and may occur slowly over an extended period of time, ranging from several months to years. Beneficial effects may arise from a change in lifestyle and diet, which may result in the individual not developing cancer during his or her lifetime. The third stage is the progressive stage, involving the spread of the cancer. It is evident that, upon entering into this stage, preventative factors such as diet have less of an impact (Reddy *et al.*, 2003).

A number of mechanisms exist by which phytochemicals aid in the prevention of cancer. This preventative action most probably results from the additive or synergistic effects of a number of phytochemicals, since cancer is a multi-step process. Proposed mechanisms by which phytochemicals may prevent cancer include: (i) anti-oxidant and free radical scavenging activity; (ii) antiproliferative activity; (iii) cell-cycle arresting activity; (iv) induction of apoptosis; (v) activity as enzyme cofactors; (vi) enzyme inhibition; (vii) gene regulation; (viii) activity as hepatic phase I enzyme inducers, and (ix) activity as hepatic phase II enzyme inducers. Oxidative damage to DNA, proteins and lipids, resulting from an increase in oxidative stress, is considered to be one of the most important mechanisms contributing to the development of cancer. As the oxidative damage is linked to the multi-step process of carcinogenesis, this may be prevented, or at least limited, by the consumption of anti-oxidants (mechanism (i), as described above) (Liu, 2004).

6.1.1 Natural products and carcinogenesis defence

A number of natural products are used as chemoprotective agents against commonly occurring cancers. The phytochemicals that most often appear to be protective against cancer are curcumin, genistein, resveratrol, diallyl sulfide, (S)-allyl cystein, allicin, lycopene, ellagic acid, ursolic acid, catechins, eugenol, isoeugenol, isoflavones, protease inhibitors, saponins, phytosterols, vitamin C, lutein, folic acid, beta carotene, vitamin E and flavonoids, to name but a few (Reddy *et al.*, 2003).

These phytochemicals suppress the inflammatory processes that lead to transformation, hyperproliferation, and initiation of carcinogenesis. The inhibitory influences of these phytochemicals may ultimately suppress the final steps of carcinogenesis viz. angiogenesis and metastasis.

Anti-oxidants, such as flavonoids, are found in a wide variety of plant extracts, fruits and vegetables, beverages and herbs. As mentioned, one of the most important contributions to the development of cancer is the oxidative damage to DNA (Fan et al., 2000). Permanent genetic alterations may occur in those cells where DNA is damaged and where division of this DNA occurs before it can be repaired. These cells may begin to divide more rapidly and result in carcinogenesis (Reddy *et al*., 2003).

6.1.2 Flavonoids - a source of anticancer agents

It is well established that natural products are an excellent source of compounds with a wide variety of biological activities. This has extended the field of research for potential anticancer compounds, some of which are already extensively used (as mentioned previously - Table 6.2).

Flavonoids are a group of polyphenolic secondary metabolites present in a wide variety of plants, and display a large number of biochemical and pharmacological properties, including cancer preventative effects (Williams and Grayer, 2004). It is the elicited biochemical interferences by flavonoids that are associated with their capacity to control cell growth. A number of mechanisms by which flavonoids are able to prevent carcinogenesis have been reported. These mechanisms include their free radical scavenging ability, the modification of enzymes to activate or detoxify carcinogens, and the inhibition of the induction of the transcription factor activator protein activity by tumour promoters (Canivence-Lavier *et al*., 1996; Shih *et al*., 2000; Moon *et al*, 2006).

Tumour promotion of cells results in the formation of benign tumour cells, which may progress to malignant tumours. Flavonoids have the ability to interfere with the different steps of this process, as illustrated in Figure 6.1.

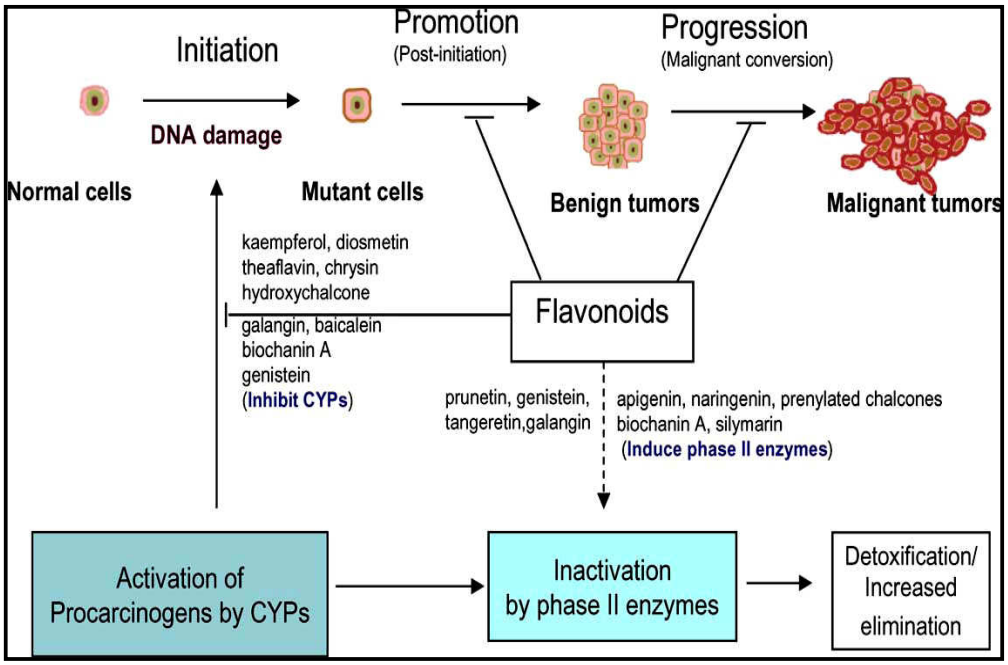


Figure 6.1: Flavonoids that block or suppress multi-stage carcinogenesis (Moon *et al.*, 2006).

Flavonoids such as kaempferol, diosmetin, theaflavin and biochanin A are able to inhibit the activation of procarcinogens to their electrophilic species (Figure 6.1) by hepatic phase I enzymes, or their subsequent interaction with DNA. These flavonoids may be considered as inhibiting agents, as they inhibit tumour initiation. Flavonoids may also suppress the promotional and progressional steps in multi-stage carcinogenesis by affecting cell-cycle progression, angiogenesis, invasion and apoptosis (Moon *et al*., 2006).

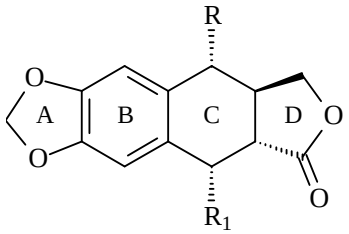
The high consumption of soy products by women in Japan and the Far East has led to a much lower incidence of breast cancer in these women than those in the Western world. The isoflavones in these products bind to estrogen receptors in the body, thereby blocking the cancer-promoting effects of estrogen (Reddy *et al*., 2003).

6.1.3 The investigation of *Commiphora* as an anticancer agent

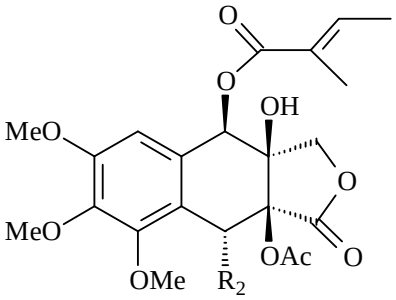
Recent phytochemical studies on *Commiphora erlangeria* revealed the presence of four lignans, erlangerin A - D, of which C and D closely resemble the structure of podophyllotoxin. Podophyllotoxin and 4-deoxypodophyllotoxin have been identified as antitumour lignans (Figure 6.2) (Habtemariam, 2003). The biological activity of these compounds was due to an inhibitory effect on the cell growth of two human (HeLa and Eahy926) and two murine (L929 and RAW 264.7) cell lines.

Sesquiterpenoids are being identified more often as having cytotoxic properties against cancer cell lines. The activity of extracts of *Commiphora myrrha* was tested against the MCF-7 breast cancer cell line, which is known to be resistant to anticancer drugs. A novel furanosesquiterpenoid identified as rel-1S,2S-epoxy-4R-furanogermacr-10(15)-en-6-one, exhibited weak cytotoxic activity against the MCF-7 cell line. The mechanism by which the cytotoxicity occurs is through the inactivation of a specific protein, Bcl-2 (Zhu *et al*., 2001).

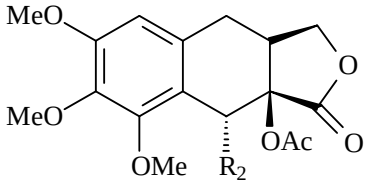
Singh *et al*. (2005) studied the molecular mechanism by which guggulsterone (Figure 6.3), isolated from *Commiphora mukul*, induces apoptosis using PC-3 human prostate cancer cells. The viability of these cells was significantly reduced upon treatment with guggulsterone, in a concentration-dependent manner. Guggulsterone-mediated suppression results from induction of apoptosis, and not the perturbation of cell-cycle progression. This apoptotic induction is a result of the activation of caspase-9, caspase-8 and caspase-3, which is, in part, mediated by Bax and Bak, which are pro-apoptotic B-cell lymphoma-2 (Bcl-2) protein family members.



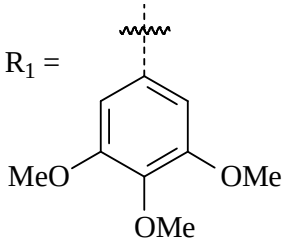
Podophyllotoxin: R = OH; 4-Deoxypodophyllotoxin: R = H

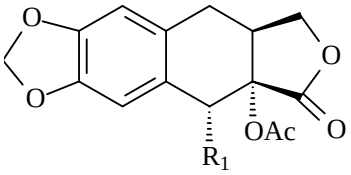


Erlangerin A

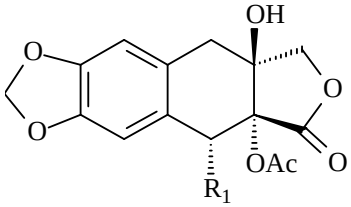


Erlangerin B





Erlangerin C



Erlangerin D

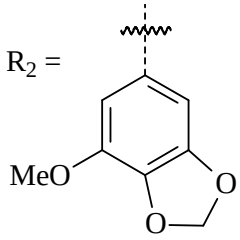


Figure 6.2: The chemical structures of podophyllotoxin, 4-deoxypodophyllotoxin and Erlangerin A - D (Habtemariam, 2003).

The expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL was initially increased, but a marked decline was noticed after the 16 to 24 hour incubation.

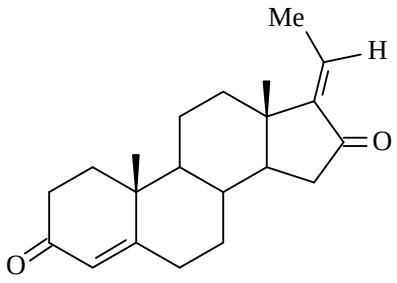


Figure 6.3: The chemical structure of guggulsterone (Aggarwal and Shishodia, 2006).

Figure 6.4 demonstrates the molecular targets of dietary agents and natural products, which may influence the prevention and therapy of cancers (Aggarwal and Shishodia, 2006). The molecular targets highlighted in orange are indicative of the influences of *C. mukul* in its mechanisms of action in cancer cell suppression. In a study by Zhu *et al*. (2001), *C. mukul* exhibited a decreased cellular viability in MCF-7 and PC-3 cancer cells, with the IC_{50} value in both cells being 14.3 μg/ml.

As indicated in Table 6.2, phytochemicals present in medicinal plants possess substantial anticancer activity, and investigations conducted on *Commiphora* species indicate a true potential for the treatment and/or prevention of cancer. Despite this, to the best of our knowledge, no investigations have been carried out on the potential effects of South African indigenous species. It is for this reason that the *in vitro* anticancer activity of 10 indigenous *Commiphora* species was evaluated.

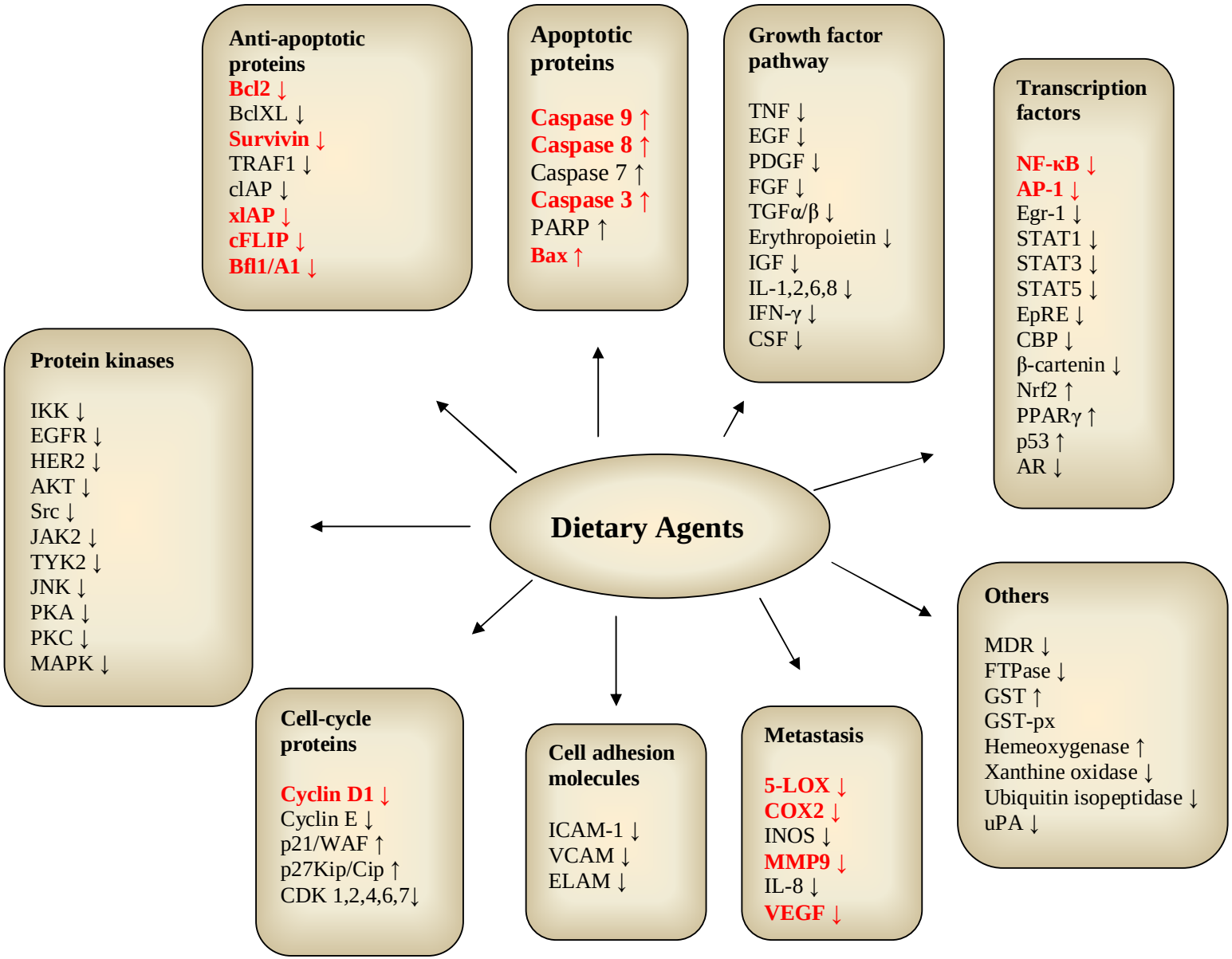


Figure 6.4: Molecular targets of dietary agents for the prevention and therapy of cancers. Highlighted in orange are the targets of guggulsterone isolated from *Commiphora mukul* (Aggarwal and Shishodia, 2006). (Refer to abbreviation list).

6.2 Materials and methods

6.2.1 Principle of the method

The sulforhodamine B (SRB) assay is an antiproliferative assay used to assess the growth inhibition of cells. This colorimetric assay indirectly estimates the viable cell number by staining total cellular proteins, and was performed according to Monks *et al.* (1991). The ability of extracts of indigenous *Commiphora* species to inhibit the *in vitro* growth of three human cancer cell lines, namely the colon adenocarcinoma (HT-29), breast adenocarcinoma (MCF-7), and the neuronal glioblastoma (SF-268) cancer cell lines, was evaluated.

6.2.2 Protocol

6.2.2.1 Cell lines and cell culture

The HT-29, MCF-7 and SF-268 cell lines were obtained from the National Cancer Institute (USA). The MCF-7 and SF-268 cells were routinely maintained in 75 cm^3 flasks (Nunclon™) in Roswell Park Memorial Institute Media-1640 (RPMI-1640) (Cambrex Bioproducts), supplemented with 5% foetal bovine serum (FBS, Highveld Biological). HT-29 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Highveld Biological) supplemented with 5% FBS, 1 ml per 500 ml media of 10 mg/ml penicillin-G, 10 mg/ml sodium streptomycin sulphate (Highveld Biological) and 5 ml per 500 ml media of 2 mM L-glutamine (Cambrex Bioproducts). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Once the cells were at 75% confluency, they were subcultured by aspirating the media and replacing it with 0.1 mM (500 µl) phosphate buffered saline (PBS, Highveld Biological) (pH 7.4) and 1 ml 0.05% (2 mg/ml) trypsin-EDTA (Highveld Biological). The flasks were incubated at 37°C for 10 - 15 min, until the majority of cells had lifted. The trypsin was then inactivated by the addition of experimental media (antibiotic-free, serum-supplemented RPMI-1640 or DMEM medium).

A single cell suspension was formed by the gentle pipetting action. The cell suspension was centrifuged at 1000 rpm (Sorvall T6000D) for 3 min, and the supernatant discarded. The pellet was resuspended in its respective medium, an aliquot of which was stained with 0.2% (w/v) trypan blue (Sigma-Aldrich) and the cells were counted using a haemocytometer. This single cell suspension, with a cell viability of greater than 95%, was then diluted in culture medium to obtain a standard cell suspension of 150 000 cells/ml.

6.2.2.2 Preparation of plant samples

The plant samples were prepared from a stock solution of extracts in methanol at a concentration of 50 mg/ml. The following final well concentrations were prepared using experimental medium: 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml and 6.25 μg/ml**,** as shown in Figure 6.5. The following controls were prepared: (i) methanol in experimental media as a negative control, (ii) plant extract with experimental media and no cells, (iii) positive control: 5′-fluorouracil (Fluka). DNA synthesis is necessary for cancer cell proliferation. 5′-Fluorouracil inhibits the activity of thymidylate synthase, thereby blocking the synthesis of deoxythymidine monophosphate (dTMP) and in so doing, blocks DNA synthesis (Noordhuis *et al.*, 2004).

6.2.2.3 The sulforhodamine B assay

Aliquots of 100 μl of 150 000 cells/ml cell suspension was seeded into a 96-well microtiter plate. The plates were incubated at 37°C for 24 hours to facilitate the attachment of the cells to the bottom of the wells. No cells were seeded into the blank wells, instead, 200 μl of media was added (Figure 6.5). Plant extract and serial two-fold dilutions with cell culture medium (100 μl) was added to the wells already containing 100 μl of cell suspension, in triplicate. The control wells contained no cells, only experimental media, in the presence of the test sample. The control wells were used to aid in the determination of any background absorbance produced by the extract. The plates were incubated for a further 48 hours at 37°C.

On completion of the 48 hour incubation period, the cells were fixed to the bottom of the well by layering the medium with 50 μl of ice cold 50% w/v trichloroacetic acid (TCA, Saarchem). Trichloroacetic acid was prepared at 50 g/100 ml in distilled water. The plates

were incubated at 4°C for 1 hour, after which the supernatant was washed from the wells (washed out 5 times) with water to remove any excess amounts of TCA, experimental media or any other low molecular weight metabolites. The plates were inverted and left overnight to air dry.

Once dry, 100 µl of SRB (Sigma-Aldrich), which was prepared at a concentration of 0.4% w/v in1% acetic acid, was added to all wells to stain the fixed cells and aid in assessing the cell growth. Sulforhodamine is a water-soluble dye that binds electrostatically to the basic amino acids of cellular proteins, synthesised by viable cells (Voigt, 2005). The microtiter plates were left to stain for 10 - 20 min, after which the excess dye was discarded and the microtiter plates washed five times with 1% acetic acid to remove any unbound dye. Trisma base (Merck) (pH 10.5, 200 µl, 10 mM) was added to all wells (under mild basic conditions the dye can be extracted from the cells and solubilised for measurement) and the plate was shaken at 960 rpm for 3 min on a microtiter plate reader (Labsystems iEMS Reader MF) equipped with the Ascent® version 2.4 software program, dissolving bound dye present in the wells. The absorbance was then read at 492 nm. The colour intensity of each well corresponds to the number of viable cells, an indication of the inhibitory effect of the extracts or test compounds added (Figure 6.5, right). The percentage inhibition of cells was calculated (using equation 6.1 below). Using the Enzfitter® (version 1.05) software, the concentration that inhibits 50% of cellular growth (IC_{50} value) was determined from the log sigmoid dose-response profile for each sample.

Equation 6.1

$$\% \text{ cell inhibition} = \frac{(1 - \text{abs test sample} - \text{Mean abs control sample} - \text{abs blank})}{\text{Mean (abs control} - \text{abs blank)}} \times 100$$

abs = absorbance at 492 nm

The plant extracts depicting activity of more than 80% inhibition of cell growth at 100 µg/ml, were subsequently re-evaluated with dilutions prepared in experimental media.

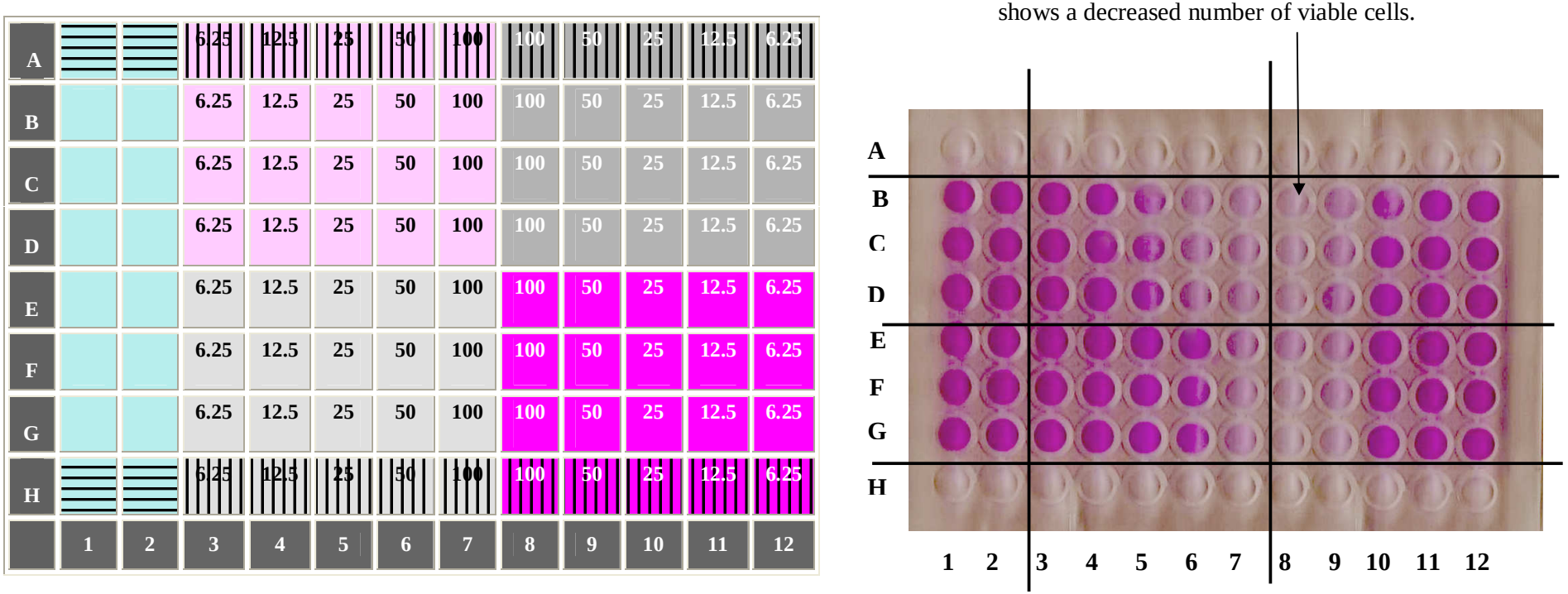


Figure 6.5: Representative 96-well microtiter plate, indicating concentrations of plant extracts (left) where,

A 96-well microtiter plate prepared for use in the SRB assay. Pink wells are an indication of stained cells (right).

Kaempferol, isolated from the stem extract of *C. glandulosa* (Chapter 3) was prepared in the same way as the extracts. The *in vitro* ability of this compound to inhibit the growth of the three cell lines was also determined.

6.3 Results

The inhibition of cancer cell proliferation and cellular viability by solvent extracts of indigenous *Commiphora* species and the flavonol, kaempferol, against three human cancer cell lines was evaluated and is detailed in Table 6.3. The percentage cell growth inhibition at 100 μg/ml and IC_{50} values for those plant extracts that showed more than 80% inhibition of cell viability (at 100 μg/ml) was evaluated for all cell lines tested. This would require that the inhibitory concentration at which 50% of the cells are inhibited (IC_{50} value) be 30 μg/ml (Suffiness and Pezzuto, 1990). For those *Commiphora* species exhibiting anticancer activity, the inhibitory effect appeared to be concentration-dependent, increasing as the concentration of extract increased (Figure 6.6).

The most active extracts from the investigated *Commiphora* species against the HT-29 cells were *C. glandulosa* (leaf and stem) and *C. marlothii* (leaf) (Figure 6.7 and Table 6.3). For all other species, the percentage growth inhibitions at 100 μg/ml was observed to be less than 80%, failing the prescreen-select criterion and were thus not subjected to further testing. The IC_{50} values for these species could thus not be determined. No significant activity was observed for the extracts from *C. africana* (leaf) and *C. schimperi* (stem) against HT-29 cells, at 100 μg/ml (Figure 6.7).

Extracts from *Commiphora glandulosa* (stem) and *C. pyracanthoides* (stem) exhibited the most significant inhibitory activity against the MCF-7 cells, with IC_{50} values of 24.28 ± 0.18 μg/ml and 20.60 ± 0.73 μg/ml, respectively (Table 6.3). The MCF-7 cells exhibited the highest sensitivity to indigenous *Commiphora* species, with extracts from *C. edulis* (leaf and stem), *C. glandulosa* (leaf and stem), *C. marlothii* (leaf), *C. pyracanthoides* (leaf and stem), *C. schimperi* (stem) and *C. viminea* (stem) all possessing a percentage cell growth inhibition greater than 80% at 100 μg/ml.

Table 6.3: The percentage cell growth inhibition (CGI) of colon adenocarcinoma cell line (HT-29), breast adenocarcinoma cell line (MCF-7) and neuronal cell line (SF-268) on exposure to stem and leaf extracts of indigenous *Commiphora* species, kaempferol and reference compound 5′-fluorouracil, and the IC_{50} values of the respective species. Results are given as mean ± s.d, n=3.

Species	%CGI of HT-29 at 100 μg/ml	IC_{50} value HT-29 (μg/ml)	%CGI of MCF-7 at 100 μg/ml	IC_{50} value MCF-7 (μg/ml)	%CGI of SF-268 at 100 μg/ml	IC_{50} value SF-268 (μg/ml)
***C. africana* (stem)**	29.01 ± 3.42	n.d.	53.00 ± 4.82	n.d.	0.04 ± 3.43	n.d.
***C. africana* (leaf)**	0.01 ± 0.92	n.d.	31.10 ± 3.75	n.d.	0.02 ± 4.71	n.d.
***C. edulis* (stem)**	36.26 ± 2.54	n.d.	80.50 ± 4.68	67.85 ± 5.46	24.32 ± 4.69	n.d.
***C. edulis* (leaf)**	33.18 ± 1.69	n.d.	82.10 ± 1.99	50.34 ± 4.25	47.53 ± 3.67	n.d.
***C. glandulosa* (stem)**	86.41 ± 1.93	57.89 ± 2.04	95.80 ± 3.09	24.28 ± 0.18	86.20 ± 3.35	70.32 ± 2.45
***C. glandulosa* (leaf)**	90.25 ± 0.42	52.34 ± 1.95	89.20 ± 4.37	39.58 ± 2.11	83.21 ± 3.60	71.45 ± 1.24
***C. marlothii* (stem)**	59.13 ± 1.83	n.d.	77.40 ± 2.09	n.d.	0.05 ± 2.58	n.d.
***C. marlothii* (leaf)**	82.48 ± 3.45	72.12 ± 3.14	84.20 ± 2.72	63.27 ± 1.51	35.43 ± 4.83	n.d.
***C. mollis* (stem)**	29.38 ± 1.90	n.d.	74.30 ± 1.64	n.d.	57.40 ± 6.65	n.d.
***C. mollis* (leaf)**	42.97 ± 4.59	n.d.	60.80 ± 3.75	n.d.	0.01 ± 3.97	n.d.
***C. neglecta* (stem)**	14.38 ± 2.77	n.d.	76.80 ± 3.41	n.d.	28.91 ± 3.75	n.d.
***C. neglecta* (leaf)**	18.88 ± 2.66	n.d.	61.45 ± 6.98	n.d.	0.01 ± 4.03	n.d.
***C. pyracanthoides* (stem)**	77.46 ± 2.62	n.d.	80.65 ± 2.23	20.60 ± 0.73	80.00 ± 3.77	69.36 ± 1.64
***C. pyracanthoides* (leaf)**	36.57 ± 2.23	n.d.	86.50 ± 1.37	35.51 ± 3.57	94.85 ± 1.92	68.55 ± 2.01
***C. schimperi* (stem)**	0.02 ± 3.47	n.d.	88.30 ± 1.88	83.14 ± 5.10	0.02 ± 3.59	n.d.
***C. schimperi* (leaf)**	7.43 ± 1.37	n.d.	24.90 ± 5.43	n.d.	0.05 ± 3.72	n.d.
***C. tenuipetiolata* (stem)**	10.63 ± 2.45	n.d.	71.00 ± 0.70	n.d.	36.72 ± 6.02	n.d.
***C. tenuipetiolata* (leaf)**	15.42 ± 1.39	n.d.	28.26 ± 3.93	n.d.	4.73 ± 2.83	n.d.
***C. viminea* (stem)**	48.00 ± 2.03	n.d.	88.90 ± 4.92	96.02 ± 0.42	21.76 ± 4.58	n.d.
***C. viminea* (leaf)**	53.79 ± 4.82	n.d.	59.20 ± 5.44	n.d.	5.63 ± 4.37	n.d.
Kaempferol	71.46 ± 0.89	9.78 ± 0.01	82.39 ± 1.35	20.21 ± 3.29	89.25 ± 0.72	43.83 ± 2.04
5′-Fluorouracil	96.76 ± 1.19	7.00 ± 2.20	98.81 ± 0.93	1.11 ± 0.31	20.70 ± 0.86	n.d.

n.d. – not determined: IC_{50} values not determined as the percentage cell growth inhibition at 100 μg/ml was less than 80%.

Commiphora glandulosa (leaf and stem) and *C. pyracanthoides* (leaf and stem) (100 µg/ml) were the only two species exhibiting percentage cell growth inhibition of greater than 80% in SF-268 cells. The IC_{50} values calculated (Table 6.3) indicates that the inhibitory effect of the two species did not differ significantly ($p > 0.05$) for this cell line. No inhibitory activity on SF-268 cell growth was noted for extracts tested from *C. africana* (leaf and stem), *C. marlothii* (stem), *C. mollis* (leaf), *C. neglecta* (leaf) and *C. schimperi* (leaf and stem) (Figure 6.7).

The inhibition of the cancer cell proliferation by kaempferol in all three-cancer cell lines was determined, with IC_{50} values of 9.78 ± 0.01 µg/ml in HT-29 cells, 20.21 ± 3.29 µg/ml in MCF-7 cells and 43.83 ± 2.04 µg/ml in SF-268 cells.

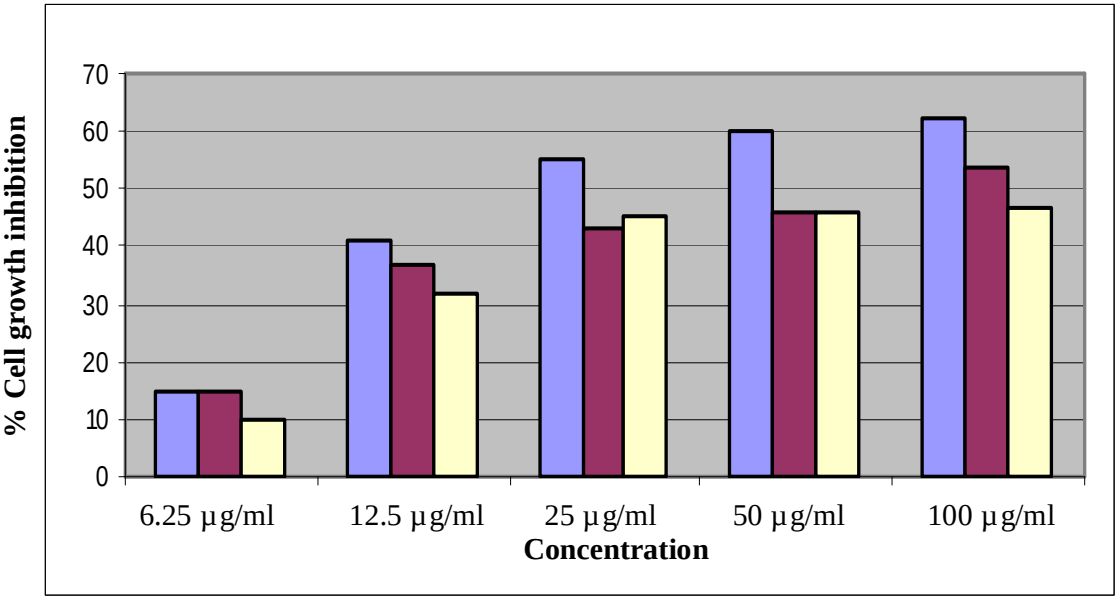


Figure 6.6: Percentage cell growth inhibition of extracts from *Commiphora neglecta* (leaf) ( ) against the MCF-7 cell line, *C. viminea* (leaf) ( ) against the HT-29 cell line and *C. edulis* (leaf) ( ) against the SF-268 cell line, indicating the concentration-dependent inhibitory effect.

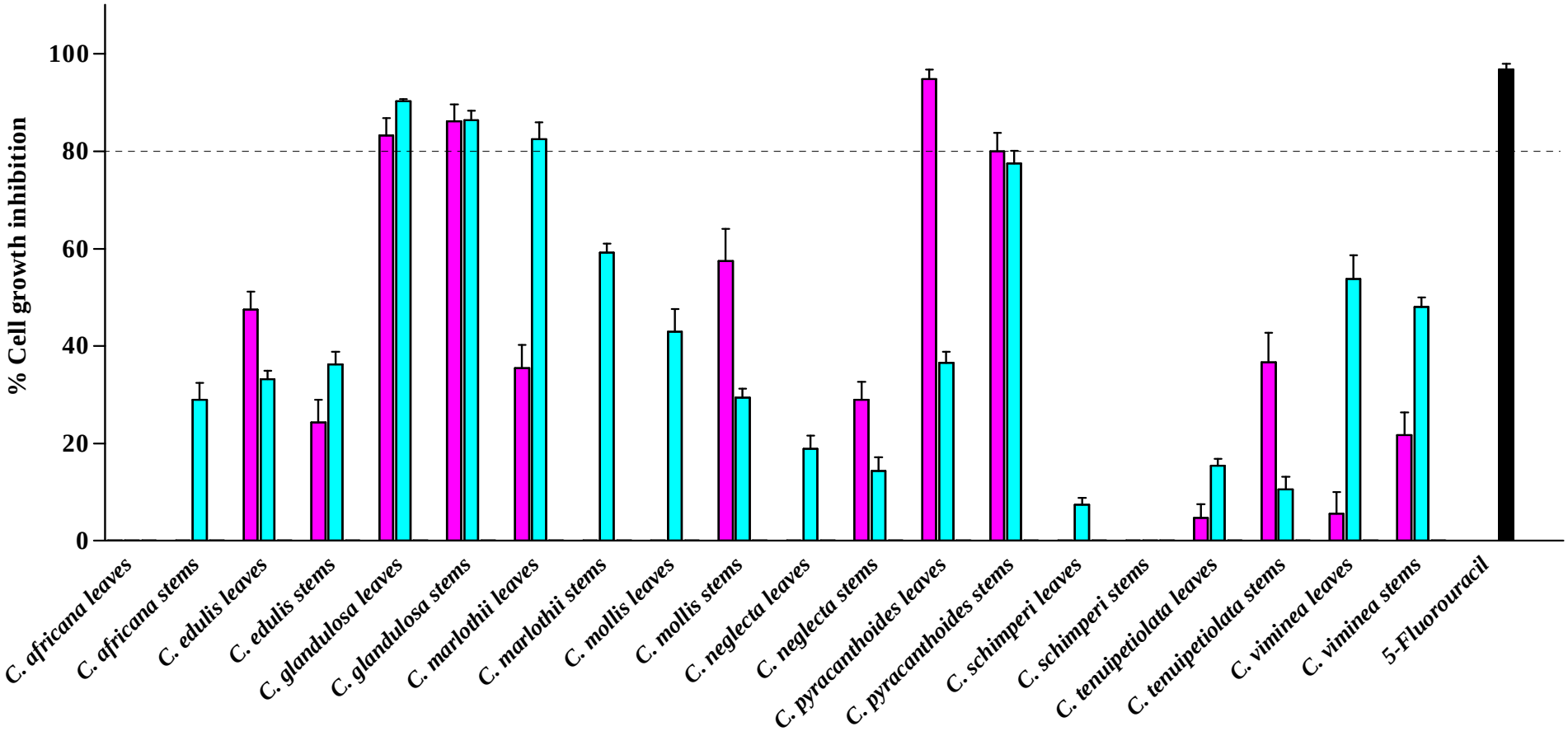


Figure 6.7: Representative antiproliferative activity at 100 μg/ml of indigenous *Commiphora* species under investigation and 5′-fluorouracil in the SRB assay against two cancer cell lines - the neuronal SF-268 ■ and colon adenocarcinoma HT-29 ■ cell lines; the standard error of the mean of three replicates are denoted by error bars (n = 3 experiments).

6.4 Discussion

The evaluation of the cytotoxic potential of indigenous *Commiphora* species was investigated using the SRB assay. The results are generally expressed as percentage cell growth inhibition at 100 μg/ml, while those indicating a percentage inhibition of greater than 80% at 100 μg/ml were expressed as IC_{50} values, the latter defined as the concentration causing 50% cell growth inhibition. After a continuous 48 hour exposure of the cells to the extracts, in accordance with the "NCI three-cell line screen", the activity observed was most certainly reflective of a cancer type-specific sensitivity. The extracts of only a few indigenous *Commiphora* species exhibited an inhibitory effect greater than 80% at 100 μg/ml on the cancer cells. The inhibitory activity of the extracts showed some concentration-dependance (Figure 6.6), which increased with an increase in extract concentration. The most promising activity against the HT-29 cells was presented by extracts of *C. glandulosa* (leaf and stem), with IC_{50} values of 52.34 ± 1.95 μg/ml and 57.89 ± 2.04 μg/ml, respectively, and extracts of *C. marlothii* (leaf), with an IC_{50} value of 72.12 ± 3.14 μg/ml. At 100 μg/ml, the leaf extracts exhibited a slightly higher inhibitory effect. However, comparing the results to that of 5′-fluorouracil (IC_{50} = 7.00 ± 2.20 μg/ml), the inhibitory activity of the most active extract against this cell line was seven-fold less active, an indication that no extract was as effective as 5′-fluorouracil.

No trend was found upon comparison of the leaf and stem extracts and their activity against the three cell lines. In general, the leaf extracts were found to be more active than the stem extracts against the HT-29 cell line, with the exception of *C. africana* and *C. pyracanthoides*, while the stem extracts were observed to be more active against the MCF-7 and SF-268 cell lines, with the exception of *C. edulis*, *C. marlothii* and *C. pyracanthoides* (Table 6.3).

The extracts of each of the species were analysed using HPLC (Chapter 8), and it was determined that the leaf extracts contain varying amounts of flavonoids. Flavonoids are known to have anticancer activity, as discussed in section 6.1.2 above, and may to some degree contribute to the observed activity. The percentage cell growth inhibition observed at the highest concentration for extracts from *C. mollis* (leaf) was determined to be 42.97 ± 4.59% against the HT-29 cells.

This leaf extract showed similar flavonoid patterns to *C. africana* and *C. schimperi* (Chapter 8, Table 8.1), and similarities in their HPLC chromatograms.

The leaf extracts of *Commiphora africana* (% CGI = 0.01 ± 0.92%) and *C. schimperi* (% CGI = 7.43 ± 1.37%) demonstrated a decreased inhibitory effect against the HT-29 cell line; which differed from that of *C. mollis*. The presence of other compounds may be attributing to the difference in inhibitory effect. This may be as a result of antagonistic relationships that may exist between the compounds present and further investigations would thus need to be conducted.

Although flavonoids are emerging as prospective anticancer drug candidates, the understanding of the structure-activity relationship surrounding this activity is not well understood. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations and degree of polymerisation (Calabrò *et al*., 2004). Flavonoids are effective scavengers of reactive oxygen species (ROS), and the anticancer activities observed suggest a dependence on their anti-oxidant and chelating properties. It is known that reactive oxygen and nitrogen metabolites are involved in cancer processes. Pro-inflammatory cytokines, apoptosis signaling and redox-response transcription factors are dependent on these free radicals (Rusak *et al*., 2005).

Nagao *et al*. (2002) reported the anticancer activity of flavonoids with respect to their structure-activity relationship, especially that of flavones (Figure 6.8). These flavones possess hydroxyl groups at the C_5 (R_1) and C_7 (R_3) positions in ring A, and at C'_3 (R_5) and C'_4 (R_6) in ring B, important for enhanced activity. The influence of other substituents at other positions is, however, not clear. Compounds bearing a free hydroxyl group in the flavone series exhibit anticancer activity by a mechanism that involves topoisomerase I inhibition. The presence of flavones in certain species of *Commiphora* such as *C. africana* (leaf) and *C. edulis* (leaf) (Chapter 8) may contribute to the inhibitory activity. However, the identification of these flavones and their chemical structures is imperative for the attributable activity.

Flavonoids are potent antiproliferative agents, in which the C_2–C_3 double bond and the lack of the 6-hydroxyl group are important structural requirements for their cytostatic effects (Rusak *et al*., 2005). Kaempferol, for example, possesses the C_2-C_3 double bond and

additionally lacks the 6-hydroxyl group (Chapter 3, Figure 3.11), and has been shown to possess cancer chemopreventative properties against the lung cancer cell line A549.

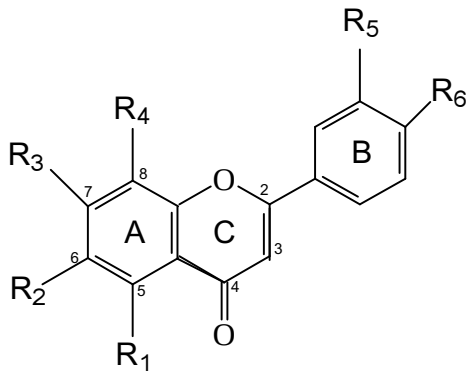


Figure 6.8: The basic chemical structure of flavones (Harborne *et al.*, 1975).

The treatment of estrogen receptor-positive breast cancer cells with kaempferol also resulted in the time- and dose-dependent decrease in cell number, especially in MCF-7 cells (Hung, 2004).

Kaempferol has been used with another flavonoid, quercetin, in order to determine their effects in reducing the proliferation of cancer cells. These flavonoids were more effective together than independently in their action against the human cancer cell lines, showing synergistic activity *in vitro* (Ackland *et al.*, 2005). It was also found that kaempferol aids in reducing the resistance of cancer cells to anticancer drugs, and in so doing aids in the fight against cancer.

Breast cancer is the most common malignancy in which growth is linked to hormonal factors. Most primary breast cancers contain estrogen receptor alpha (ER-α) that requires estrogens or estrogenic activity for tumour growth. Estrogens interact with both the ER-α and the estrogen receptor beta (ER-β) to modulate the expression of genes involved in regulating growth, differentiation and survival of the cancer cells. ER-α is imperative in breast cancer progression, and for this reason, endocrine therapies for ER-positive breast cancer are aimed at the use of anti-estrogens. Recently, researchers have shown that

kaempferol is a growth inhibitor for ER-positive breast cancer cells. Kaempferol blocks ER activity through the inhibition of ER-α expression, resulting in ER-α aggregation (in the nuclei), as well as the induction of ER-α degradation by a different pathway. These findings therefore suggest that the kaempferol-mediated modulation of ER-α expression and function may, in part, be responsible for the *in vitr*o anti-proliferative effects observed. The efficacy of this hormonal regime and the potential use of kaempferol to treat breast cancer are yet to receive clinical confirmation (Hung, 2004).

The success of cancer chemotherapy greatly depends on the absence of multiple-drug resistance (MDR). One of the most studied mechanisms of drug resistance is characterised by the accumulation, resulting from over-expression, of P-glycoprotein (Pgp). P-glycoprotein is a pump that catalyses the efflux of drugs from the cells, resulting in the reduction of drug accumulation and therefore the access of drugs to their target sites. If Pgp confers MDR on cancer cells, it would be of great importance to develop agents that would inhibit the Pgp-mediated efflux of drugs, and thus reverse MDR. Such a compound is kaempferol. Through the *in vitro* inhibition of Pgp-mediated drug efflux, there is an increase in the intracellular accumulation and cytotoxicity of chemotherapeutic drugs within the cancer cells. This was demonstrated when kaempferol was combined with vinblastine, resulting in a significant increase in the anticancer activity of vinblastine on the cervical carcinoma cell line KB-V1, (Khantamat *et al*., 2004).

The inhibition of cancer cell proliferation by kaempferol in all three-cancer cell lines was determined (Table 6.3), with IC_{50} values of 43.83 ± 2.04 μg/ml in SF-268 cells, 20.21 ± 3.29 μg/ml in MCF-7 cells, and 9.78 ± 0.01 μg/ml in HT-29 cells. The IC_{50} values of kaempferol when compared to 5′-fluorouracil indicated no significant difference in the HT-29 cells, however the activity observed for kaempferol against the MCF-7 cells was significantly less active than that of 5′-fluorouracil. The activity of the isolated compound was determined to be far greater in the SF-268 and HT-29 cell lines than that observed in *C. glandulosa* (stem), the species from which it was isolated. This indicates possible antagonistic effects by other compounds present within the crude extract. However, no difference was observed in the IC_{50} values of *C. glandulosa* (stem) and the MCF-7 cells.

Existing research has indicated that a tumour is a dynamic system, consisting of cancer cells, often of multiple classes, supporting stroma normal cells, and frequently

lymphocytes. Tumours are highly individualistic, and researchers have concluded that each tumour has its own individual drug response spectrum (Hoffman, 1991). This phenomenon was shown to be true by the current comparison of inhibitory activity of the three different cell lines, and their sensitivity to the stem and leaf extracts of investigated *Commiphora* species. The MCF-7 cells exhibited the most sensitivity to indigenous *Commiphora* species. Extracts from *Commiphora africana* (leaf and stem) displayed cancer cell specificity against the MCF-7 cell line, where the percentage cell growth inhibition at 100 μg/ml was found to be 53.00 ± 4.82% and 31.00 ± 3.75% for the stem and leaf extracts, respectively. No cell inhibition was observed in SF-268 cells, as well as no cell inhibition by the leaf extract against HT-29 cell line, with only slight inhibitory activity by the stem extract (29.00 ± 3.42%) against this cell line. The least potent extracts against all three cell lines were *C. schimperi* (leaf) and *C. africana* (leaf) (Table 6.3).

The activity presented by the extracts against the MCF-7 cell line was promising. In a study by Campbell *et al*. (2002), 14 crude plant extracts (not including any purified fractions) were tested against MCF-7 cells, one of which was *C. myrrha*. The results were presented as IC_{50} values, with *C. myrrha* having a reported IC_{50} value of > 0.7 mg/ml. In comparison to this study, the stem extracts of *C. glandulosa* and *C. pyracanthoides* are 30-fold and 33-fold more potent. Of the extracts presenting an inhibitory concentration of greater than 80% at 100 μg/ml (Table 6.3) against the MCF-7 cells, *C. viminea* (stem) was found to be the least potent. The IC_{50} value determined for this species was 96.02 ± 0.42 μg/ml, exhibiting a 7-fold greater inhibitory effect than that exhibited by *C. myrrha*.

As mentioned previously, existing research suggests that lipoxygenase-catalysed products have a substantial influence on the development and progression of human cancers. Upon comparison with normal tissues, lipoxygenase metabolites viz. leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) (LTB_4, 5-HETE and 12-HETE), are found at elevated levels in lung, prostate, breast, colon, as well as in skin cancer cells (Steele *et al*., 1999). It has been proposed that LTs may exert a greater role than prostaglandins (PGs) in stimulating tumour growth, depending on the type of cancer (Wallace, 2002). Agents blocking this lipoxygenase-catalysed activity, such as lipoxygenase inhibitors (5-lipoxygenase and its associated enzymes and 12-lipoxygenase) or leukotriene receptor antagonism may be effective in interfering with signaling events needed for tumour

growth. In a study by McCormick and Spicer (1987), nordihydroguaiaretic acid (NDGA), a 5-lipoxygenase (5-LOX) inhibitor, inhibited induced rat mammary tumour development *in vivo*, indicating the role of lipoxygenase products in this tumour development. In a study by Hussey and Tisdale (1996), certain colon adenocarcinoma cell lines treated with 5-LOX inhibitors resulted in the inhibition of growth stimulation. Boswellia (*Boswellia serrata* Roxb.) derived from the same family as *Commiphora*, Burseraceae, is a potent 5-LOX inhibitor as a result if its boswellic acids (pentacyclic triterpenes). In low micromolar concentrations apoptosis is induced in glioma cells with promising effects in patients (two uncontrolled trials) with intracranial tumors (Wallace, 2002). Pentacyclic triterpenes are also present in *Commiphora* resins, and have been investigated for their anti-inflammatory activity, with promising results (Duwiejua *et al.*, 1993).

Extracts that showed 5-lipoxygenase inhibitory activity such as *C. edulis* (stem), *C. glandulosa* (stem), *C. mollis* (stem), *C. neglecta* (stem), *C. schimperi* (stem), and *C. tenuipetiolata* (stem) (Chapter 5) displayed definite cell growth inhibition in the MCF-7 cells (Table 6.3), with the exception of *C. schimperi* (leaf), which displayed low inhibitory activity. The isolation of anti-inflammatory agents from these extracts may lead to promising anticancer compounds.

6.5 Conclusion

The ability of extracts of indigenous *Commiphora* species and the flavonol, kaempferol, to inhibit the *in vitro* growth of three human cancer cell lines, was evaluated using the sulforhodamine (SRB) antiproliferative assay. The results were expressed as the concentrations causing 50% cell growth inhibition and have been summarised in Table 6.3.

The inhibition of cell proliferation and viability appeared to be highly dose-dependent. While certain species were cancer type-specific, other cancer cell lines were less sensitive to the extracts resulting in a much lower degree of selectivity.

The most active *Commiphora* species against the HT-29 cells were *C. marlothii* (leaf) and *C. glandulosa* (leaf and stem). The MCF-7 cells exhibited the highest sensitivity to extracts indigenous *Commiphora* species, with *C. edulis* (leaf and stem), *C. glandulosa* (leaf and stem), *C. marlothii* (leaf), *C. pyracanthoides* (leaf and stem), *C. schimperi* (stem), and *C.*

viminea (stem) all possessing an inhibition greater than 80% at 100 μg/ml. Extracts from *Commiphora glandulosa* (leaf and stem) and *C. pyracanthoides* (leaf and stem) were the two most active against the SF-268 cells, with IC_{50} values of 71.45 ± 1.24 μg/ml, 70.32 ± 2.45 μg/ml, 68.55 ± 2.01 μg/ml and 69.36 ± 1.64 μg/ml, respectively.

The inhibition of the cancer cell proliferation by kaempferol in all three-cancer cell lines was determined, with IC_{50} values of 9.78 ± 0.01 μg/ml in HT-29 cells, 20.21 ± 3.29 μg/ml in MCF-7 cells and 43.83 ± 2.04 μg/ml in SF-268 cells.

This study has, without a doubt, proven the existence of compounds with potential *in vitro* anticancer activity in different extracts of *Commiphora* species. Isolation and identification of these compounds is imperative and may lead to the development of novel treatments in the global struggle against cancer and cancer-related ailments.

CHAPTER 7: CYTOTOXICITY OF INDIGENOUS *COMMIPHORA* SPECIES

"In all things there is a poison, and there is nothing without a poison. It depends only upon the ***dose*** *whether a poison is a poison or not"* (*Paracelsus, 1490 – 1541* as quoted by Stumpf, 2006).

7.1 Introduction

The age old importance of natural products derived from plants cannot be underestimated, and even more so in lieu of the fact that these products serve as a rich source of lead compounds for the development and production of modern chemotherapeutic agents. The importance of prerequisite extensive toxicological studies on plant products is thus magnified, in order to ensure safe utilisation and minimisation of potentially harmful or poisonous effects.

Plants produce toxins and present toxicity in a multitude of complex ways, and although vertebrates have developed a number of mechanical and biochemical defence mechanisms against these, few systems of the vertebrate body are completely immune to damage by all toxins from plant origins. The plant itself influences toxicity, as well as the interrelationship between the dose, absorption, detoxification and excretion (Douglas, 2006).

With regards to plants, primary metabolites are defined as those compounds required for basic metabolic processes, while secondary metabolites are unique to a set of species within a phylogenetic group, and are thus of prime importance in taxonomic research. The importance of these compounds is usually of an ecological nature as they are used as defences against predators, parasites and diseases, as well as to facilitate certain reproductive processes such as colouring agents and attractive smells. The study of secondary compounds, their identity, complexity and quantity, as well as other available data, leads to definite characteristics relating to their toxicity. In terms of basic terminology, the word "toxic" can be considered synonymous to "poisonous", and these poisonous principles result from specific essential plant functions. All plants differ in their

cytotoxic profiles, as the levels of toxic components are dependent on the stage of plant growth, the environment, the season and plant part used. Examples of plant compounds with potential toxic effects include alkaloids, glycosides, oxalates, phytotoxins (toxic proteins similar to bacterial toxins), polypeptides, amines and resins (van Wyk *et al*., 2002).

Plants developed self-protective defence mechanisms against potentially toxic secondary compounds. This entails the physical movement of potential toxins to metabolically inactive sites such as the bark, or chemical conversion to nontoxic compounds via specific chemical reactions. These compounds also provide an efficient defence mechanism against herbivores and insects (Mello and Silva-Filho, 2002).

7.1.1 *Commiphora* and its cytotoxic properties

Generally *Commiphora* species are commonly considered to be toxicologically harmless plants, with the exception of five species, namely *C. erlangeriana*, *C. staphyleifolia*, *C. unilobata*, *C. guidotti* and *C. boiviniana*. The resins of these species are considered by the local communities to be extremely poisonous to humans and animals alike (Neuwinger, 1996).

The toxic property of the resin of *C. erlangeriana* has lead to its traditional use as arrow poison for hunting purposes and in tribal wars (Habtemariam, 2003). The toxic effect has since been hypothesized and attributed to a direct effect, similar to that of podophyllotoxin, which inhibits protein, RNA and DNA synthesis on mammalian cells (Habtemariam, 2003). Toxicity studies were consequently also conducted on *C. myrrha* and *C. guidotti*, although, in contrast to *C. erlangeriana*, these resins were found to be non-toxic at the concentrations tested (Mekonnen *et al.*, 2003).

The effect of myrrh oil on cell viability was determined by Tipton *et al.* (2003), using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which measures the metabolic reduction of the tetrazolium salt to a formazan dye product. Normal human gingival fibroblast cells, gingival fibroblasts from patients with aggressive periodontitis and human gingival epithelial cells were exposed to myrrh oil for 24 and 48 hours. Cell viabilities were unaffected when exposed to 0.0001 - 0.001% myrrh oil, with a

noted decrease in cell viability after 24 hours at 0.0025% myrrh oil, and a reduction in cell viability by 60 - 85% after exposure of cells to an excess of 0.005% myrrh oil. This percentage was then lowered to 0.0005% for a 48 hour exposure period, which showed an approximate 30 - 50% decrease in viability of the epithelial cells, but no change had occurred in the viability of the fibroblasts. The relative sequence of sensitivity to myrrh oil was observed to be human gingival epithelial cells > normal human gingival fibroblast cells > gingival fibroblasts from patients with aggressive periodontitis (Tipton *et al.*, 2003).

In an effort to verify the efficacy of traditional medicine, Krishnaraju *et al.* (2005) collected several medicinal plants from various geographical locations, based on available ethnopharmacological information. Amongst the plants collected were *Commiphora wightii* and *C. myrrha.* The toxicity of these medicinal plants was determined using the brine shrimp lethality bioassay, a simple and inexpensive assay based on the ability of a compound or extract to kill laboratory-cultured brine shrimp (*Artemia nauplii*). The gum resin of *C. wightii* and the oleo-resin of *C. myrrha* were tested at various concentrations (1-5000 μg/ml). The percentage brine shrimp lethality was determined by comparing the mean number of surviving larvae in the treated tubes with that of the control (untreated) tubes. No lethality was noted for either *Commiphora* species (*C. wightii* LC_{50} = 1600 μg/ml, *C. myrrha* LC_{50} >5000 μg/ml) when compared to the positive control podophylotoxin (LC_{50} = 3.1 μg/ml).

Indigenous *Commiphora* extracts have shown to be of potential therapeutic value in the assays investigating certain biological activities, viz. anti-oxidant (Chapter 3), antimicrobial (Chapter 4), anti-inflammatory (Chapter 5), and anticancer (Chapter 6). The traditional use for medicinal purposes also increases the importance of assessing the toxicity of the selected species.

7.2 Materials and methods

7.2.1 Cytotoxicity

Proliferation assays are widely used in cell biology for the determination of growth expression factors, cytokines and nutrients, as well as for the screening of cytotoxic or chemotherapeutic agents. In this study, the tetrazolium cellular viability assay (Mosmann, 1983) was used to determine the cytotoxicity of the stem and leaf extracts from the 10

chosen indigenous *Commiphora* species on transformed human kidney epithelium (Graham) cells.

7.2.2 Principle of the method

The determination of the effect on Graham cell proliferation was achieved by the MTT cellular viability assay. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide is a tetrazolium salt dye used in a colourimetric assay, which measures the mitochondrial conversion or modification of the yellow substrate to an insoluble dark blue/purple formazan product. Substrate modification is brought about by the cleavage of MTT by NADH-generating succinic dehydrogenase present in the mitochondria of living cells, with only living cells containing active mitochondria are able to yield a colour change. As an increase in mitochondrial enzyme activity leads to a linear increase in the production of formazan dye, the measured quantity of formed formazan dye is directly correlated to the number of metabolically active cells, yielding an accurate measurement of cell viability and thus toxicity (if any). As the formazan dye is insoluble in the reaction medium, it is solubilised by the addition of DMSO or isopropanol, and the colour intensity is measured spectrophotometrically.

7.2.3 Protocol

7.2.3.1. Cell culture maintenance

Transformed human kidney epithelium (Graham) cells were continuously maintained in HAM F10 culture medium [(9.38 g/L HAM F10 powder (Highveld Biological) and 1.18 g/L $NaHCO_3$ (Saarchem)], which was supplemented with 5% (v/v) foetal calf serum (FCS, Highveld Biological) and 0.5 mg/ml gentamicin sulphate. The FCS was inactivated at 56°C for 1 hour before use. The cells were maintained as a sub-confluent monolayer, fed three times weekly, and incubated at 37°C in a humidified atmosphere of 5% CO_2. The adherent cells were detached by the addition of 1 ml 0.25% trypsin/ 0.1% Versene EDTA (Highveld Biological). The trypsin was then inactivated by addition of experimental media (antibiotic free serum supplemented HAM F10 medium). Once the cells were resuspended in the experimental media and a single cell suspension obtained, the trypsin was removed by centrifugation at 1500 rpm (Sorvall T6000D) for 5 min. The pellet was resuspended

yielding a single cell suspension, then 1 ml of cells was used to re-seed a new culture and the remainder used in the MTT assay. The resulting single cell suspension was stained with 0.2% (w/v) trypan blue (Sigma) and the number of cells per ml of cell suspension was determined using a haemocytometer. Cell suspensions with cell viabilities in excess of 97% were diluted in experimental media to obtain a final cell concentration of 250 000 cells/ml.

7.2.3.2 Preparation of plant samples

Stock solutions (50 mg/ml methanol) of the respective extracts were used to prepare plant samples in experimental medium at concentrations of 2000 µg/ml, 1500 µg/ml, 1000 µg/ml and 200 µg/ml. When plated out in 96-well microtiter plates, the final concentrations of the extracts were 200 µg/ml, 150 µg/ml, 100 µg/ml, and 20 µg/ml, as depicted in Figure 7.1.

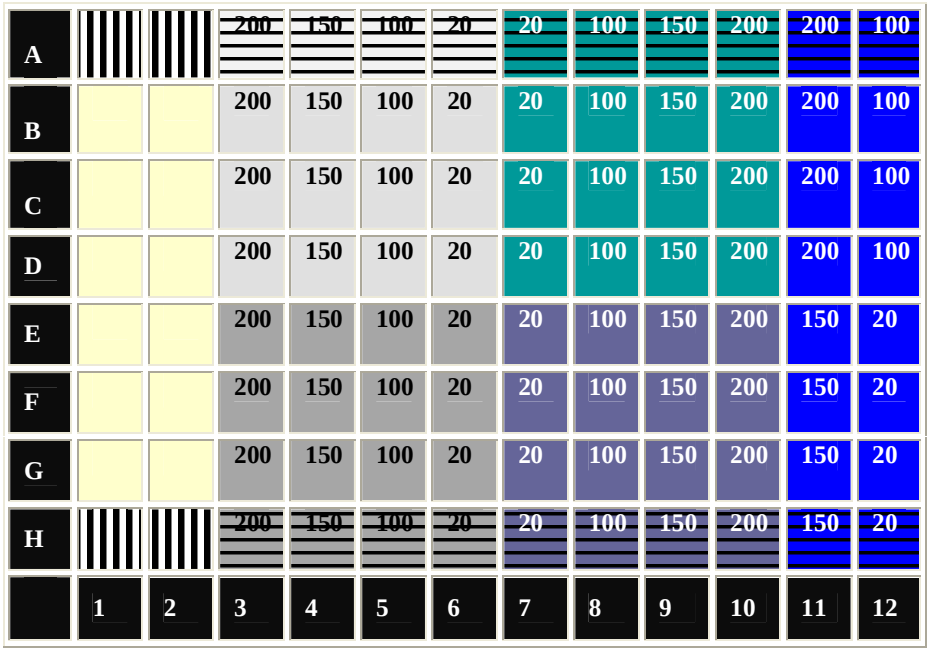


Figure 7.1: Representative 96-well microtiter plate indicating final concentrations of plant extracts and arrangement of controls prepared for use in the MTT assay where

represents plant extract 1
represents plant extract 2
represents plant extract 3
represents plant extract 4
represents plant extract 5
represents experimental media in absence of both plant extract and Graham cells
represents methanol in experimental media, no plant extracts only cells
represents experimental media with respective plant extract, in absence of Graham cells

The isolated compound, kaempferol, from the stem extract of *C. glandulosa* (Chapter 3) was prepared in the same way as the extracts. The toxicity of this compound was also determined, as it demonstrated potential therapeutic value in a number of biological assays (Chapters 3 – 6). Determining the cytotoxicity of isolated compounds may also aid in understanding the cytotoxicity of specific extracts.

7.2.3.3 The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cellular viability assay

Aliquots of 180 µl of the 0.25 million cells/ml cell suspension were seeded into the 96-well microtiter plate, as detailed in Figure 7.1. The plates were then incubated at 37°C in 5% CO_2 for 6 hours to facilitate attachment of the cells to the bottom of the wells. No cells were seeded into the blank wells (instead, 200 µl of experimental media was added). Plant extracts (20 µl) were added in triplicate to the wells already containing 180 µl of cell suspension. The controls comprised of: (i) methanol in experimental media (negative control), (ii) plant extract with experimental media in the absence of Graham cells (colour control), (iii) experimental media in absence of both plant extract and Graham cells (blank control), and (iv) quinine (Fluka) as the control. Control wells aided in the determination of any background extract absorbance, especially in the event of colour interferences or interaction of the extract with the MTT solution. Each plate contained four wells for the blank cell-free control (Figure 7.1). Once the extracts were added, the plates were incubated for 44 hours, after which 40 µl of a 12 mM (50 mg/ml) MTT (USB^{TM}) solution in phosphate buffered saline (pH 7.4) (prepared with NaCl (8 g), KCl (0.3 g), $Na_2HPO_4 \cdot 2H_2O$ (0.73 g) and KH_2PO_4 (0.2 g) in one liter of distilled Millipak® 40 water which was then autoclaved for one hour at 120°C and at 1.5 kgf/cm^2 pressure, stored at 4°C until required), was added to each well before being incubated for a further 4 hours.

After a total incubation period of 48 hours, 200 µl of supernatant was carefully removed and discarded, after which 150 µl of DMSO was added for dissolution and measurement of the formed formazan crystals. For this purpose, a Labsystems iEMS Reader MF microplate reader, equipped with Ascent® version 2.4 software, was employed, agitating the plates at 1020 rpm for 4 min, while measuring and recording absorbances at a test wavelength of 540 nm and a reference wavelength of 690 nm. The results were expressed in terms of percentage cellular viability, calculated using equation 7.1, taking the relevant controls into

account. The IC_{50} value for each sample was determined from a log sigmoid dose response curve generated by Enzfitter® (version 1.05) software. The assay was performed in triplicate.

Equation 7.1

$$\% \text{ cell viability} = \frac{(\text{Drug treated abs} - \text{Mean abs of cell free control})}{\text{Mean abs of untreated cell control} - \text{Mean abs of cell free control}} \times 100$$

7.3 Results

The toxicity results obtained for the 10 indigenous *Commiphora* species are summarised in Table 7.1 and Figure 7.2. *Commiphora glandulosa* (stem) proved to be most toxic (IC_{50} value of 30.5 μg/ml), being 4.5-fold more toxic than quinine (control). The IC_{50} values for all other extracts were in excess of 95 μg/ml, with *C. africana* (stem and leaf), *C. mollis* (leaf), *C. neglecta* (stem), *C. schimperi* (leaf) *C. tenuipetiolata* (stem and leaf), and *C. viminea* (stem) yielding IC_{50} values in excess of 200.0 μg/ml.

Figure 7.3 demonstrates the percentage cell viability of the stem extracts of *Commiphora* against the MCF-7 cell line (Chapter 6) when compared with that of the transformed human kidney epithelium cells, at 100 μg/ml. The percentage cell viability of the transformed human kidney epithelium cells in the presence of the extracts is observed to be higher for the species, than the percentage cell viability of the MCF-7 cell line, with the exception of *C. glandulosa* (100% cell death). In contrast to the result obtained for *C. glandulosa*, *C. edulis* has a cell viability of 100%. The inhibitory activity of this extract against the MCF-7 cell line was greater resulting in a percentage cell viability of only 19.00%.

Table 7.1: The cytotoxicity of extracts of indigenous *Commiphora* species, kaempferol and quinine, against the transformed human kidney epithelium cells. Results are given as mean ± s.d, n=3.

Species	IC_{50} value (μg/ml)
***C. africana* (leaf)**	>200.0
***C. africana* (stem)**	>200.0
***C. edulis* (leaf)**	99.5 ± 0.71
***C. edulis* (stem)**	194.0 ± 8.48
***C. glandulosa* (leaf)**	106.5 ± 3.53
***C. glandulosa* (stem)**	30.5 ± 3.54
***C. marlothii* (leaf)**	97.5 ± 0.71
***C. marlothii* (stem)**	123.0 ± 4.24
***C. mollis* (leaf)**	>200.0
***C. mollis* (stem)**	172.0 ± 1.41
***C. neglecta* (leaf)**	111.5 ± 4.95
***C. neglecta* (stem)**	>200.0
***C. pyracanthoides* (leaf)**	104.0 ± 7.07
***C. pyracanthoides* (stem)**	101.5 ± 0.71
***C. schimperi* (leaf)**	>200.0
***C. schimperi* (stem)**	136.5 ± 0.71
***C. tenuipetiolata* (leaf)**	>200.0
***C. tenuipetiolata* (stem)**	>200.0
***C. viminea* (leaf)**	141.5 ± 7.78
***C. viminea* (stem)**	>200.0
Kaempferol	>150.0
Quinine (control)	136.1 ± 4.06

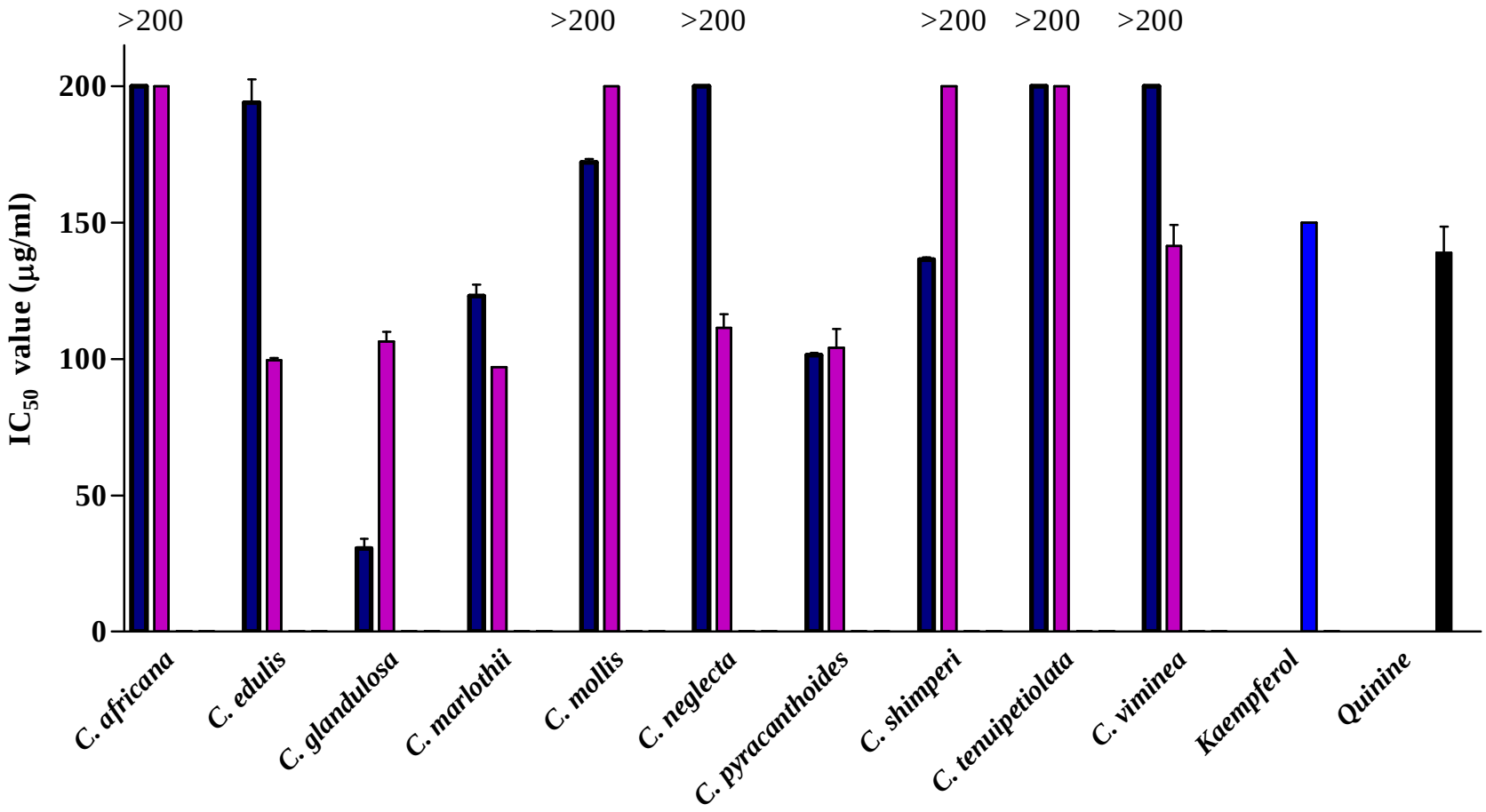


Figure 7.2: The IC_{50} values depicting the cytotoxicity of the 10 stems ( ) and leaves ( ) of indigenous *Commiphora* species; the standard error of the mean of three replicates are denoted by error bars (n = 3 experiments).

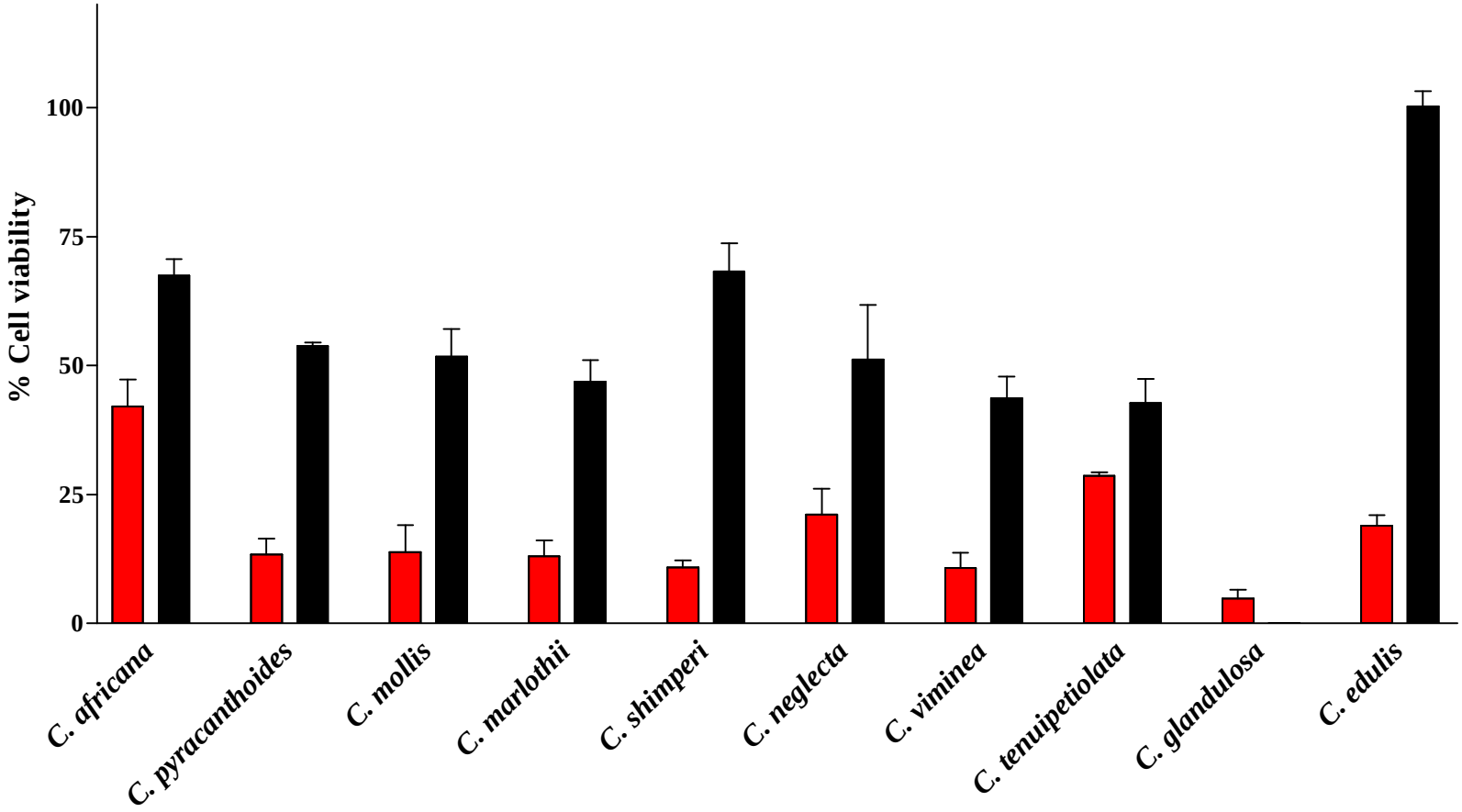


Figure 7.3: A comparison between the cytotoxicity elicited by *Commiphora* species on the normal human transformed kidney epithelium cells (■) in the MTT assay and the breast adenocarcinoma MCF-7 (■) cell line in the SRB assay.

7.4 Discussion

As described above, the toxicity of the majority of the species under investigation were generally similar, with IC_{50} values in excess of >200.0 μg/ml for *C. africana* (stem and leaf), *C. mollis* (leaf), *C. neglecta* (stem), *C. schimperi* (leaf) *C. tenuipetiolata* (stem and leaf), and *C. viminea* (stem) (Table 7.1). These results therefore strongly suggest a lack of relative *in vitro* cytotoxicity in the majority of the *Commiphora* species, both in stem and leaf extracts, and thus at least some probability of *in vivo* non-cytotoxic potential.

During this study, the IC_{50} value of the plant-derived compound quinine, generally considered to be relatively safe, was determined to be 136.06 ± 4.06 μg/ml. All extracts [with the exception of *C. glandulosa* (stem)] with IC_{50} values in excess of >100.0 μg/ml can, with relative certainty, be considered non-toxic. The HPLC chromatograms of all the stem extracts of the selected *Commiphora* species, with exception of *C. viminea,* shared a number of similarities and demonstrated IC_{50} values of >100.0 μg/ml (including *C. viminea*). *Commiphora glandulosa* (stem) was observed to have the highest degree of cytotoxicity (IC_{50} = 30.5 ± 3.54 μg/ml), and its cytotoxicity further indicated a definite concentration dependence, with increased stem extract concentrations resulting in parallel increases in cell death. The HPLC chromatogram also showed compounds unique to this species, to which some of the toxicity may be attributed (Chapter 8, Table 8.2). The leaf extract of *C. glandulosa* (IC_{50} = 106.5 ± 3.53 μg/ml) differed from that of the stem, with the stem extract being approximately three times more toxic.

Commiphora glandulosa was additionally observed to be active in a number of bio-activity assays, demonstrating antimicrobial activity (Chapter 4), anti-inflammatory activity (Chapter 5), as well as anticancer activity (Chapter 6). Therefore, these results require further clarification regarding the nature and possible basis of these promising activities, the latter being either ascribed to truly selective and potentially therapeutic activity, or simply to the overall cytotoxicity in general. The toxicity observed in the current study may therefore serve as a strong indication that all other activities observed with respect to *C. glandulosa* may not be specific.

The leaf extracts of each of the species were analysed using HPLC. It was concluded that they contain flavonoids in varying amounts (Chapter 8). Flavonoids are known to be

cytoprotective or antiproliferative (Woerdenbag *et al*., 1994; Pessoa *et al*., 2000), refer to Chapter 6, as well as cytotoxic (Wang and Mazza, 2002; Matsuo *et al*., 2005), however, only few flavonoids are cytotoxic to mammalian cells (Neuwinger, 1996). The leaf extracts of *C. africana*, *C. mollis* and *C. schimperi* showed similar flavonoid patterns (Chapter 8, Table 8.1) and similarities in their HPLC chromatograms (Chapter 8), an indication of the chemotaxonomic similarity. It is thus not surprising that these three species had similar cytotoxicity (IC_{50} >200.0 μg/ml). *Commiphora tenuipetiolata* (leaf) was also observed to have an IC_{50} value of >200.0 μg/ml, although, its flavonoid pattern varied considerably from that of the aforementioned species.

The extracts of the stem and leaf of *C. africana* showed reasonably similar cytotoxicity, with IC_{50} values of >200.0 μg/ml. This relationship was observed to be true for *C. pyracanthoides*, *C. tenuipetiolata* and *C. marlothii*.

It is important to note, however, that the apparent overall cytotoxicity of the plant does not necessarily exclude all possibilities regarding the presence of potential, even specific therapeutically active compounds. The IC_{50} value of the flavonol kaempferol, isolated from the stem extract of *C. glandulosa*, was determined to be in excess of 100.0 μg/ml, an indication of the relative safety of this compound. Antimicrobial, anti-inflammatory, anti-oxidant and anticancer investigations were conducted on the compound – yielding positive results in all cases (Chapters 3 - 6). It is clear that, in spite of the presence of toxic compounds within a crude plant extract, further investigations regarding non-toxic principles of potential therapeutic value cannot, and should not, be excluded.

In Angiosperms, flavonoids function to protect plants from predators and infectious agents, shield plants from UV-B radiation, act as signaling molecules in plant-bacterium symbioses, and are the primary pigments that attract pollinators and seed dispersers. Kaempferol, at a concentration of >100 μg/ml was observed to be non-toxic against the human kidney epithelial cells.

It is evident, however, that toxicity towards certain cell lines may be specific. In a study by Wang and Mazza (2002), the cellular viability of kaempferol (prepared in DMSO), and other flavonoids, was determined by the MTT assay against the RAW 264.7 mouse monocyte/macrophage cell line, where it 'exhibited marked cytotoxicity' at 18 μg/ml.

While kaempferol may be relatively inert to the human kidney epithelial cells, potential toxicity against other cell lines cannot be excluded. Kaempferol is not a dead-end product, but serves as a precursor to dihydroquercetin, which in turn leads to the production of (±)-catechin, while quercetin (similar in structure to kaempferol) does not. In a study determining their phytotoxicity, kaempferol was shown to be phytotoxic, while quercetin was not (Bais *et al*., 2003). It is therefore imperative that all factors be taken into consideration upon investigation of the activity of compounds and extracts, with special emphasis on the possibility of bioconversion to distinct compounds. *In vitro* studies can therefore not be considered sufficient and additional investigations are required both in *in vitro* and *in vivo*.

Kaempferol was determined, through HPLC analysis (Chapter 8), to be present in the stem extracts of *C. pyracanthoides* and *C. tenuipetiolata*, as well as in the leaf extracts of *C. mollis* and *C. neglecta* (Table 8.1), all of which exhibited very different cytotoxicity (IC_{50} >100.0 μg/ml) to that of *C. glandulosa* (stem) (IC_{50} = 30.5 ± 3.54 μg/ml), a further indication that this compound is not contributing to any toxicity within the selected species.

The toxicity of a compound or extract against the transformed human kidney epithelium cells (Graham cells, as in the MTT assay) versus its cytotoxicity against cancer cell lines (as in the SRB assay) may be compared. Data from several hundred agents screened in a MTT assay, in parallel with the SRB assay, indicated that under similar experimental conditions and within the applied limits of data analyses, the MTT and SRB assays generally yielded similar results (Rubinstein *et al*., 1990; Monks *et al*., 1991). Cytotoxicity against the Graham cells was minimal, with the percentage cell viability being far greater than the percentage cell viability of the cancer cell lines, with the exception of *C. glandulosa* (Figure 7.3). This indicates that the activity is selective, and that the isolation of the particular compounds responsible for this activity may prove to be invaluable.

Different variables may affect the overall toxicity of a plant, extract or isolated compound. It is thus important that, when determining the toxicity profile (or any biological activity) of an extract or plant, further studies be conducted to verify whether *in vitro* activity is, in fact, a true reflection of the *in vivo* capacity of the plant.

7.5 Conclusion

In the present study, *Commiphora* species were found to be generally non-toxic, with the exception of *C. glandulosa* (stem), which was toxic to transformed human kidney epithelium (Graham) cells. Toxicity to this cell line was shown to be a concentration-dependent occurrence, and proved to be the case with respect to *C. glandulosa*. While the toxicity against the cancer cell lines was more prevalent, cytotoxicity against the Graham cells was minimal. This indicates that the activity is selective.

It is important to correct the misguided belief that herbal medicines do not cause adverse effects, and even though there may be no evidence of cytotoxicity *in vitro*, the possibility of *in vivo* cytotoxicity cannot be excluded.

CHAPTER 8: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

8.1 Introduction

Chromatography is a specialised separation technique that is often employed as a highly accurate tool in chemical analysis. It may, to a limited extent, also be used for preparative purposes, particularly in the isolation of relatively small quantities of compounds or materials of comparatively high intrinsic value. Chromatography is widely considered to be the most powerful science available to the modern analyst (Scott, 1995). In a single step process, it can separate a mixture into its individual components and simultaneously provide a quantitative estimate of each constituent. Samples may be in the form of a gas, liquid or solid and can range in complexity from a single compound for identification and/or quantification, to a multiple component mixture containing widely differing chemical species. The analysis can be carried out on a complex instrument or simply, through the use of relatively inexpensive thin layer plates (Scott, 1995).

The advantages of high performance liquid chromatography (HPLC) over other forms of liquid chromatography include (Scott, 1995):

- an HPLC column can be used a number of times without regeneration
- the resolution achieved is credible
- the technique is less dependent on the skill of the operator thereby greatly improving the reproducibility
- the instrumentation lends itself to automation and quantification
- analytical time is decreased
- it provides an analytical alternative for the large number of organic compounds that are too unstable or insufficiently volatile for analysis by gas chromatography (GC).

The separation process of chromatography is achieved by distributing the components of a mixture between two phases, that being a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes

are eluted from the system in the order of their increasing distribution coefficients with respect to the stationary phase; *ipso facto* a separation is achieved (Scott, 1995).

The advances made in natural product research directly result from the use of isolation techniques, nuclear magnetic resonance (NMR), and from the specific separation and analytical methods adopted, with HPLC being the most widely used analytical method. This is as a result of the high degree of adaptability and sensitivity of HPLC, as well as the accurate quantitative determination of substituents in complex mixtures. The presence of a photo diode array (PDA) detector in HPLC analysis allows for the detection of compounds by comparing the HPLC retention time and UV spectra, with a greater degree of certainty (Molnár-Perl and Füzfai, 2005). HPLC may serve as a chemotaxonomic tool in identifying the presence of certain compounds within a range of samples and as an example, to confirm that these compounds exist within different plant species.

8.1.1 Flavonoids

Polyphenolics are used extensively (among other compounds) as chemical markers in botanical chemosystematic studies. Of widespread taxonomic occurrence, flavonoids have been classed as the most useful chemosystematic markers. On the basis of their presence, plant families have been included in or excluded from specific orders (Lai Fang *et al.*, 2001). These markers are helpful in assessing intra-specific variation or relationships among closely related species.

Flavonoids are responsible for the colour astringency (Fukui *et al.*, 2003) and often the bitter taste of certain natural products, and also demonstrate a variety of bioactivities. Of all the plant polyphenols, flavonoids are of particular interest due to their high prevalence. They are characterised into five subclasses: anthocyanins, flavonols, flavones, catechins and flavonones (Molnár-Perl and Füzfai, 2005).

Flavonols and flavones are of particular importance, as they possess anti-oxidant and free radical scavenging activity. The specific role of flavonoids is directly related to their chemical structures, and their chemical analysis is therefore of utmost importance. A number of methods have been employed for the quantitative and qualitative determination of these flavonoids. This can been accomplished through the use of thin layer

chromatography (TLC) with UV-Vis absorption, (Blouin and Zarins, 1988; Hertog *et al.*, 1992; Gil *et al*., 1995), gas chromatography (Molnár-Perl and Füzfai, 2005), HPLC coupled with diode-array ultraviolet (DAD-UV) detector (Justesen *et al*., 1998; Wang and Huang, 2004), mass spectroscopy (Molnár-Perl and Füzfai, 2005) as well as through the use of capillary electrophoresis (Wang and Huang, 2004). The chromatographic analysis of flavonoids is a complex process, specifically as a result of their highly specialised chemical, physical and structural properties. These compounds readily undergo transformation, oxidation and reduction processes, as well as intra- and inter-molecular rearrangements. The high degree of variation in the basic flavonoid structure (Figure 8.1) is ascribed to hydroxylation, methoxylation, the degree of polymerisation, as well as the type of conjugation, such as glycosylation, malonylation or sulphonation (Molnár-Perl and Füzfai, 2005). The typical UV spectra of the flavonoids will be discussed later in the Chapter.

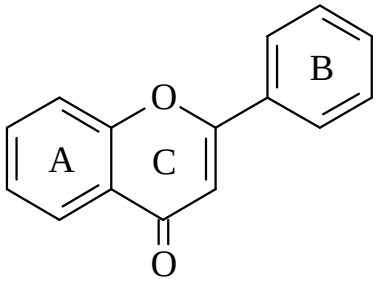


Figure 8.1: The basic chemical structure of flavonoids (Harborne *et al*., 1975).

8.1.2 Non-volatile chemical constituents found in *Commiphora* species

Flavonoids have captured the interest of scientists from numerous disciplines due to their structural diversity, the biological and ecological significance, and for their health-promoting properties (Williams and Grayer, 2004). The major flavonoid components of the flowers of *Commiphora mukul* were identified as quercetin, quercetin-3-*O*-α-L-arabinoside, quercetin-3-*O*-β-D-galactoside, quercetin-3-*O*-α-L-rhamnoside, quercetin-3-*O*-β-D-glucuronide (Kakrani, 1981). The flavonoid pelargonidin-3,5-di-*O*-glucoside is an anthocyanidin also isolated from *C. mukul* flowers.

Bioassay guided fractionation of a crude CH_2Cl_2-MeOH extract from *Commiphora africana* led to the isolation of the dihydroflavonol glucoside phellamurin (Ma *et al.*, 2005). This flavonoid was identified as the active compound responsible for the cleavage activity of the DNA strand, which resulted in the relaxation of supercoiled plasmid DNA.

An ethanolic extract of the air-dried trunk of *Commiphora wightii* yielded a new antifungal flavanone, namely muscanone, as well as an already known flavanone, naringenin (Fatope *et al*., 2003). Muscanone was identified as 3-*O*-(1",8",14"trimethylhexadecanyl)naringenin, which is active against *Candida albicans in vitro*.

Commiphora mukul resin was extracted with alcohol by Hanuš *et al*. (2005). After removal of the solvent, the extract was partitioned between water and ether. Two crystalline compounds were isolated from the unsaponiable portion of the ether-soluble residue and identified as myricyl alcohol and β-sitosterol (Hanuš *et al*., 2005).

High performance liquid chromatography was the method employed for fingerprinting and quantitatively determining *E*- and *Z*-guggulsterones, two closely related steroidal ketones, in the resin of *C. mukul* (Mesrob *et al*., 1998).

Very little is known regarding the non-volatile chemical constituents of indigenous *Commiphora* species. The objective of this study is therefore to produce chromatographic profiles for the 10 indigenous *Commiphora* species studied.

8.2 Materials and methods

Extracts for HPLC analysis were prepared by extraction with chloroform: methanol (1:1) with subsequent dilution with methanol to a final concentration of 50 mg/ml. Analysis of 10 µl extract aliquots was conducted on a Phenomenex Aqua C_{18} column (250 mm x 2.1 mm), using a Waters 2690 HPLC system equipped with a 996 PDA detector, at a constant mobile phase flow rate of 0.2 ml/min. The mobile phase consisted acetonitrile: aqueous formic acid (10 mM) at a starting concentration of 10% acetonitrile in 90% 10 mM aqueous formic acid. This ratio was altered through a linear gradient to 90% acetonitrile and 10% 10 mM aqueous formic acid at 40 min. The latter ratio was maintained for 10 min before returning to initial conditions. Nebuliser gas flow was maintained at 30 l/h at 80ºC,

with the expansion region at 90ºC and the source temperature at 225ºC. Isolated compounds were injected separately and compared to the HPLC fingerprint for each extract, the latter obtained by injection of the extracts, as described above. Data integration, analysis and compound identification were achieved by using the Empower® software. The flavonoids were tentatively identified.

8.3 Results

The stacked HPLC chromatograms of the selected *Commiphora* stem and leaf extracts are presented in Figure 8.2 and Figure 8.3, respectively. The retention times, UV absorbance maxima and the percentage integration areas for detected peaks are detailed in Figures A1 - A20 and Tables A1 - A20 (Appendix A).

The presence of flavonoids was detected in the leaf extracts, with flavonols and flavones being most the prominently detected flavonoid derivatives. Table 8.1 presents the HPLC-UV results of the tentatively identified flavonoid derivatives detected in the leaf extracts, their retention times (minutes), the flavonoid type, and an indication of the concentration present in each extract. The flavonoids are identified by their characteristic bands, absorbed at respective wavelengths (Figure 8.4).

Table 8.2 demonstrates compounds present in the stem extracts of the investigated *Commiphora* species. Few compounds are common to the different species, an indication that compounds can be utilised as chemotaxonomic markers.

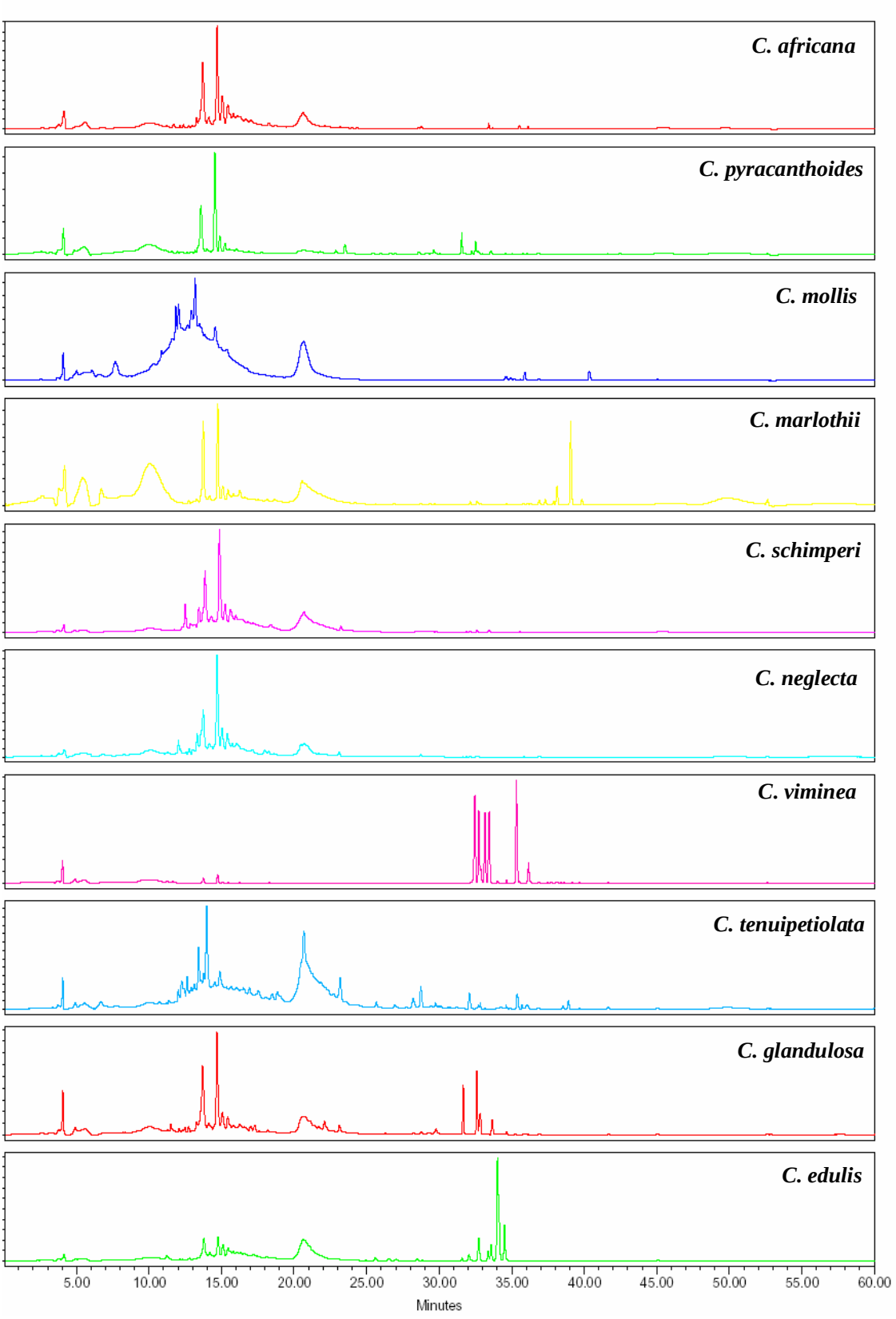


Figure 8.2: HPLC chromatograms of 10 indigenous *Commiphora* stem extracts.

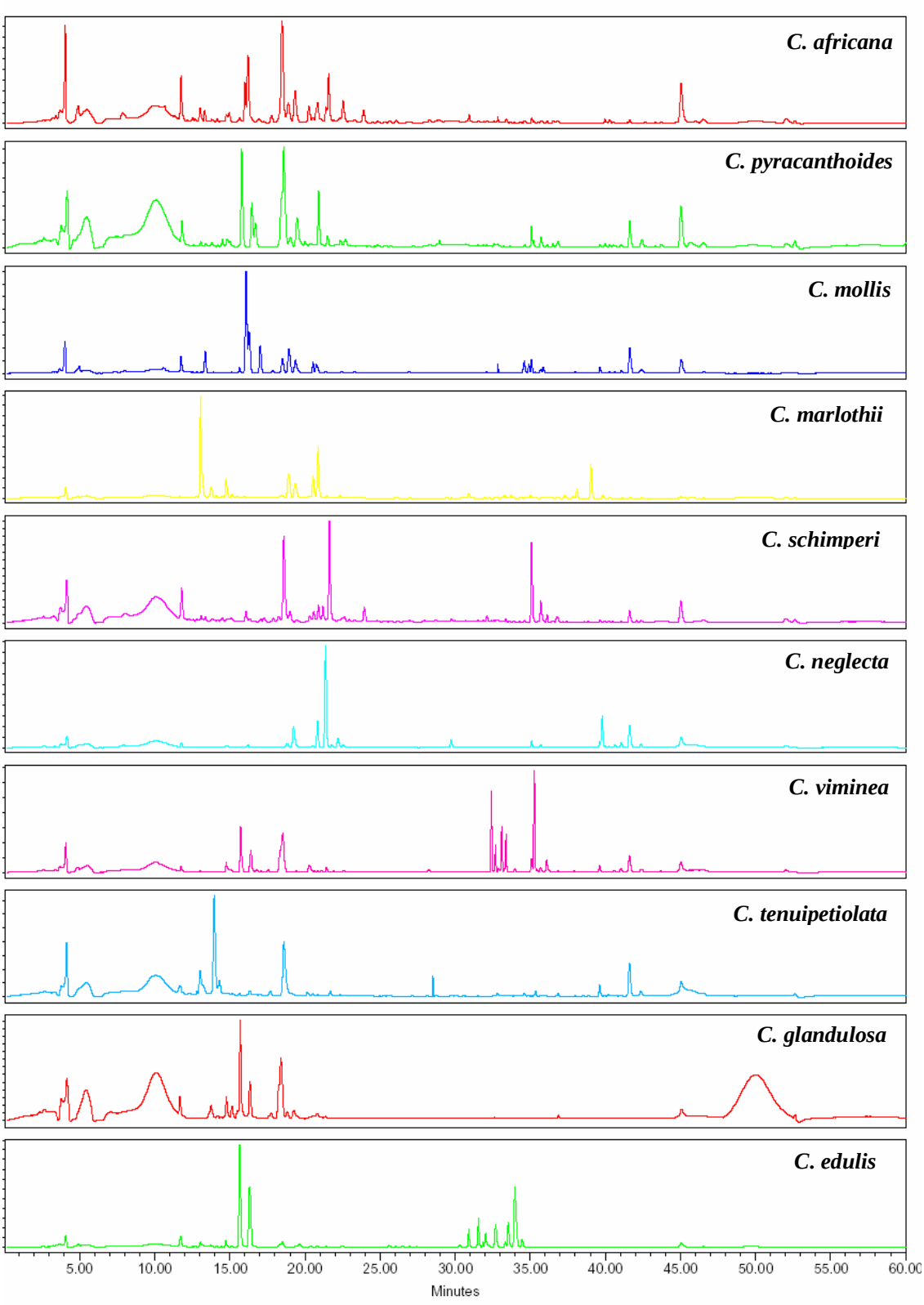


Figure 8.3: HPLC chromatograms of 10 indigenous *Commiphora* leaf extracts.

Table 8.1: HPLC-UV maxima of the tentatively identified flavonoid derivatives present in the *Commiphora* leaf extracts.

Rt (min)	UV λ_{max} Band I (nm)	UV λ_{max} Band II (nm)	Flavonoid type	CAL	CEL	CGL	CMaL	CML	CNL	CPL	CSL	CTL	CVL
15.74	348.0	269.7	Flavone	●	●●●	●				●●			●●
15.74	337.3	240.3	Flavone						●				
16.01	357.5	260.3	Flavonol	●				●●●			●		
16.22	354.0	260.0	Flavonol	●●				●●	●				
16.39	348.0	255.6	Flavone		●●●	●				●			●
17.03	354.0	261.5	Flavonol					●●					
17.78	356.3	255.6	Flavonol	●		●		●				●	
18.25	354.0	253.2	Flavonol				●				●		
18.51	336.1	269.1	Flavone	●●●	●	●●	●	●		●●●	●●●		●●●
18.51	364.4	253.2	Flavonol						●			●●●	
18.95	354.0	255.6	Flavonol	●		●	●●	●●	●		●		
19.41	354.0	255.6	Flavonol	●●	●	●	●●	●	●●	●	●		●
20.54	354.0	255.0	Flavonol	●				●			●		
20.85	349.2	265.0	Flavone	●				●	●●				
20.85	354.0	255.6	Flavonol				●●				●		
21.21	354.0	255.6	Flavonol								●		
21.44	345.6	265.0	Flavone	●	●		●	●	●●●	●	●●		●
21.58	349.2	255.6	Flavone	●●									
21.70	349.2	252.0	Flavone									●	
22.20	348.0	265.0	Flavone				●		●				
22.54	348.0	265.0	Flavone						●				
23.90	342.0	263.8	Flavone	●							●		

Table 8.21 *continued*: HPLC-UV results of the tentatively identified flavonoid derivatives present in the *Commiphora* leaf extracts.

Rt (min)	UV λ_{max} Band I (nm)	UV λ_{max} Band II (nm)	Flavonoid type	CAL	CEL	CGL	CMaL	CML	CNL	CPL	CSL	CTL	CVL
29.79	363.4	266.2	Flavonol (Kaempferol)					●	●				
31.49	345.6	247.3,	Flavone		●●		●						
33.28	348.0	267.4,	Flavone				●						
33.72	342.0	263.8	Flavone				●						
35.01	351.6	253.2,	Flavonol				●						

where: Rt – represents the retention time in minutes, CAL = *C. africana* (leaf), CEL = *C. edulis* (leaf), CGL = *C. glandulosa* (leaf), CMaL = *C. marlothii* (leaf), CML = *C. mollis* (leaf), CNL = *C. neglecta* (leaf), CPL = *C. pyracanthoides* (leaf) CSL = *C. schimperi* (leaf) CTL = *C. tenuipetiolata* (leaf), CVL = *C. viminea* (leaf). ● = low concentration (0.2 – 5.0%), ●● = moderate concentration (5.0 – 15.0%), ●●● = high concentration (15.0 – 40.0%), an arbitrary classification of the concentration, where the concentration is a measure of the percentage integration area.

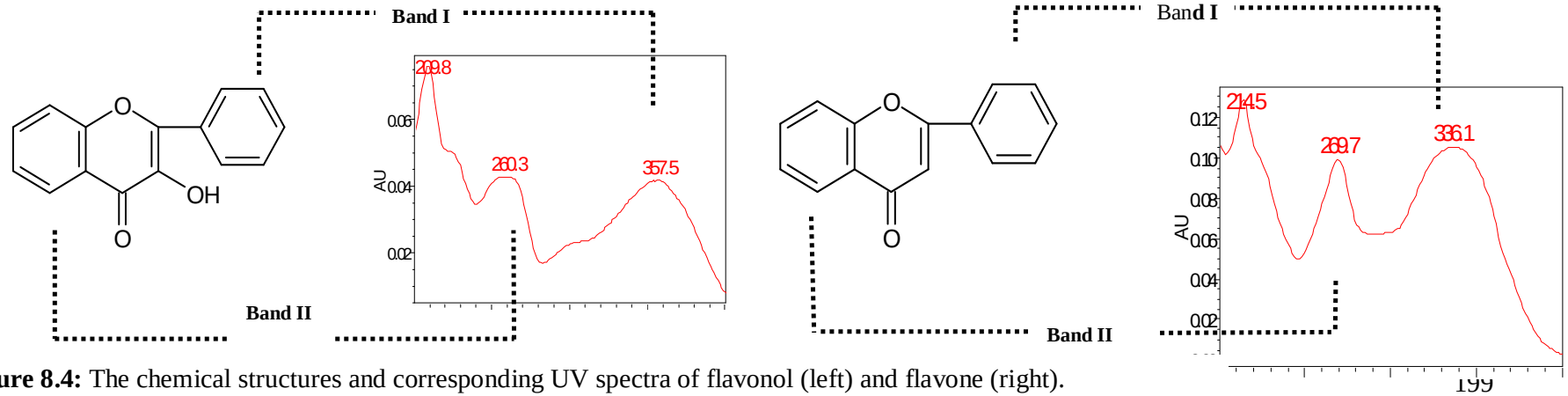


Figure 8.4: The chemical structures and corresponding UV spectra of flavonol (left) and flavone (right).

Table 8.2: HPLC results of the compounds, expressed in percentage area, detected in *Commiphora* stem extracts.

Peak	UV λ_{max} (nm)	Rt (min)	CAS	CES	CGS	CMAS	CMS	CNS	CPS	CSS	CTS	CVS
1	**205.1**	**4.00 - 4.17**	5.02	1.55	6.85	7.66	2.91	2.39	6.94	1.96	3.83	4.36
2	**207.4**	**5.40 - 5.50**	5.15	2.08	0	17.55	1.6	3.14	9.67	2.53	0.29	3.13
3	**207.4**	**9.90 - 10.20**	11.96	0	0	11.68	0	10.17	6.74	0	0	3.37
4	**218.0; 278.0**	**11.622**	0.67	0	0.76	0	8.07	0	0	0.1	0	0.28
5	**205.1; 278.0**	**11.853**	0	0	0	0	6	0.17	0.39	0.25	0	0
6	**203.9; 278.0**	**12.04 - 12.15**	0.39	0	0.55	0	12.08	2.44	0	0	1.91	0
7	**206.3; 275.6**	**12.50 - 12.67**	0	0	0	0	5.76	0.14	0	5.79	9.67	0
8	**202.8; 278.0**	**12.80 - 12.92**	0.46	0	0	0	7.1	0	0	1.58	2.56	0
9	**202.8; 278.0**	**13.179**	1.75	0.24	1.77	0	10.59	0	0	1.14	2.93	0
10	**202.8; 278.0**	**13.30 - 13.48**	0	0.5	0	0	4.15	4.37	0	4.89	10.99	0
11	**202.8; 278.0**	**13.50 - 13.80**	18.24	7.41	19.03	10.4	0	14.48	16.24	19.51	3.94	0.85
12	**201.6; 279.2**	**13.81 - 14.20**	2.15	2.2	2.24	0	0.85	2.21	0	3.79	16.58	0
13	**225.0; 280.3**	**13.81 - 14.20**	0	0	0	0.56	0	0	1.06	0	0	0
14	**202.8; 278.0**	**14.50 - 14.87**	21.26	5.39	20.82	9.75	2.09	21.61	23.46	25.33	7.81	1.5
15	**202.8; 279.2**	**15.00 - 15.28**	6.48	3.13	3.74	1.77	0	5.9	2.09	5.16	0	0.14
16	**202.8; 279.2**	**15.30 - 15.60**	5.14	3.41	2.56	1.72	0.62	5.2	0	4.25	0	0.15
17	**201.6; 278.0**	**20.50 - 20.70**	11.18	22.95	10.8	13.35	19.98	7.29	4.36	16.29	14.37	0
18	**232.0; 281.5**	**31.6**	0	0.42	5.46	0	0	0	3.17	0	0	0
19	**206.3; 234.4; 270.9**	**32.40 - 32.50**	0	0	0	0	0	0	0	0	0	16.83
20	**239.1; 295.7**	**32.40 - 32.50**	0	0	0	0	0	0	2.58	0	0	0
21	**206.3; 234.4; 269.7**	**32.60 - 32.70**	0	0	0	0	0	0	0	0	0	15.27
22	**229.7; 294.5**	**32.60 - 32.70**	0	4.75	8.29	0.65	0	0	0.81	0	0	0
23	**232.0; 274.4**	**32.8**	0	0	4.13	0	0	0	0	0	0	0

Peak	UV λ_{max} (nm)	Rt (min)	CAS	CES	CGS	CMAS	CMS	CNS	CPS	CSS	CTS	CVS
24		**33.00 – 33.38**	0	0	0	0	0	0	0	0	0	11.99
25	**262.6; 308.8**	**33.40 - 33.58**	0	2.81	0	0	0	0	0	0.24	0	0
26	**208.6; 237.9; 273.3**	**33.40 - 33.58**	0	0	0	0	0	0	0	0	0	13.65
27	**262.6; 304.0; 363.4**	**34**	0	24.66	0	0	0	0	0	0	0	0
28	**262.6; 363.4; 484.8**	**34.5**	0	6.29	0	0	0	0	0	0	0	0
29	**207.4; 234.4; 269.7**	**35.3**	0	0	0	0	0	0	0	0	0	18.82
30	**246.1**	**39**	0	0	0	8.92	0	0	0	0	0	0

Where: Rt – represents the retention time in minutes, %A = percentage area, CAS = *C. africana* (stem), CES = *C. edulis* (stem), CGS = *C. glandulosa* (stem), CMaS = *C. marlothii* (stem), CMS = *C. mollis* (stem), CNS = *C. neglecta* (stem), CPS = *C. pyracanthoides* (stem) CSS = *C. schimperi* (stem) CTS = *C. tenuipetiolata* (stem), CVS = *C. viminea* (stem). Blue highlights the dominant compounds common to most species.

Kaempferol was isolated from *C. glandulosa* (stem), with a retention time of 30.53 min. The absorbance maximum of kaempferol was determined to be 266.3 nm and 363.9 nm (Figure 8.5). This flavonol was shown to be present in the leaf extracts of *C. marlothii* and *C. neglecta* and the stem extracts of *C. pyracanthoides*, and *C. tenuipetiolata*, however, only in small quantities.

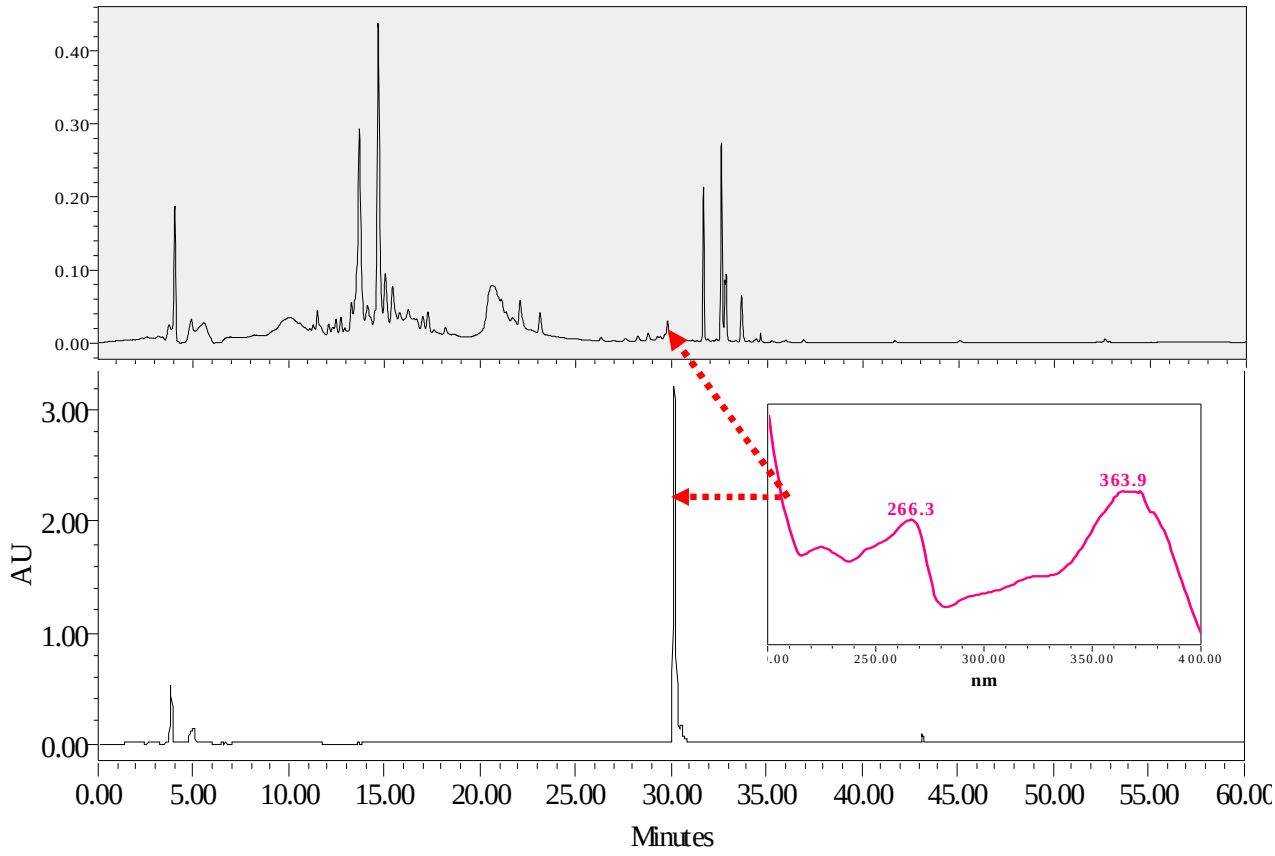


Figure 8.5: The chemical structure, corresponding UV spectrum (insert), and HPLC chromatogram of kaempferol.

8.4 Discussion

HPLC can be a valuable tool in plant chemosystematics assisting in the characterisation of species on the basis of their secondary metabolite contents (Lai Fang *et al*., 2001). The HPLC chromatograms of the crude stem extracts (Figure 8.2) differed significantly from the leaf extracts (Figure 8.3). The leaf extracts revealed the presence of a number of flavonoid compounds (Table 8.1). Establishing the class of flavonoid being represented may in some cases be sufficient to draw conclusions (Lattanzio *et al.*, 1996). These flavonoids were not observed to be present within the stem extracts, with the exception of kaempferol detected in small amounts in *Commiphora glandulosa*, *C. pyracanthoides* and *C. tenuipetiolata*. It is, however, expected that the leaf flavonoid content be higher in the leaves as a result of the increased synthesis of UV-absorbing flavonoids and polyphenols. This may serve as protection against the harmful effects of UV-B radiation, functioning as anti-oxidant and UV filters in plants.

Typical flavone and flavonol UV spectra exhibit two major absorption peaks in the region of 240 – 400 nm (Mabry *et al*., 1970). These absorption peaks occur at wavelengths 300 – 380 nm and 240 –280 nm, and are classified as Band I and Band II, respectively. Band I is associated with UV absorption due to ring B (cinnamoyl) of the typical flavonoid structure (Figure 8.1), and provides information on both the type of flavonoid and the oxidation pattern, while Band II is associated with the absorption of ring A (benzoyl). In identifying and distinguishing flavones from other flavonoids, the absorbance maxima occur between 304 and 350 nm for Band I (Figure 8.4), while the absorbance maxima for flavonols occur between 352 and 385 nm (Harborne, 1973). These groups are easily identified and were observed to be the predominant groups amongst the flavonoids present.

Isoflavones and flavanones demonstrate similar UV spectra. This is due to the relative or total absence of conjugation between the A- and B-rings. For both these flavonoids, Band II exhibits an intense absorption peak, which, for isoflavones, occurs at a wavelength region of between 245 nm and 270 nm, and for flavanones between 270 nm and 295 nm, with Band I in all instances represented as only a shoulder or low intensity peak (Harborne, 1973). The presence of these compounds, however, was not detected.

Common compounds elute at approximately the same retention times. *C. africana* (leaf), *C. edulis* (leaf), *C. glandulosa* (leaf), *C. pyracanthoides* (leaf) and *C. viminea* (leaf), contained a flavone with an approximate retention time of 15.74 min and absorbance maxima of 269.7 nm and 348.0 nm (Table 8.1). This flavone was absent in the stem extracts of the species. The percentage detected within these species varied significantly, with the highest levels present in *C. edulis* (Table A4, Appendix A). *Commiphora neglecta* (leaf) contained a flavone at approximately the same retention time of 15.74 min, but the observed absorption maxima differed slightly (240.3 nm and 337.3 nm). A flavonol at this same retention time was present only in *C. mollis* (leaf), with absorbance maxima 265.0 nm and 354.0 nm (Table 8.1).

The presence of four other flavonoids in the leaf extracts of *C. edulis* was observed at retention times of 16.39 min (flavone), 18.51 min (flavone), 19.41 min (flavonol) and 21.44 min (flavone). This flavonoid pattern is similar to that presented by the leaves of *C. glandulosa, C. pyracanthoides* and *C. viminea* (Figure 8.4 and Table 8.1).

The HPLC-UV results also suggest that *C. africana* (leaf), *C. mollis* (leaf), and *C. schimperi* (leaf) share similar chemical profiles when ascertaining the presence of flavonoids. *Commiphora marlothii* (leaf) and *C. neglecta* (leaf) also have similar flavonoid profiles.

A flavone with absorption maxima of approximately 269.1 nm and 336.1 nm and a retention time of 18.519 min is present in the leaf extracts of eight of the species, namely *C. edulis, C. glandulosa, C. mollis, C. marlothii, C. pyracanthoides, C. schimperi* and *C. viminea*, with the percentage integration area being greatest for *C. africana*, *C. pyracanthoides, C. schimperi* and *C. viminea* (Table 8.1). A slight variation exists in the remaining two species, *C. neglecta* and *C. tenuipetiolata*, as the flavonoid present at this retention time is a flavonol with absorption maxima at 253.0 nm and 364.0 nm. Extracts from *C. africana*, *C. edulis, C. glandulosa, C. mollis, C. marlothii, C. neglecta, C. pyracanthoides, C. schimperi* and *C. viminea* were observed to possess a peak of varying percentage integration area at a retention time of approximately 19.41 min, which displayed a flavonol pattern in the UV spectra, with absorption maxima of 255.6 nm and 354.0 nm (Table 8.1). All species, with the exception of *C. glandulosa* and *C. tenuipetiolata*, have a flavone present in the extracts of the leaves with a retention time of 21.44 min and the absorption maxima 265.0 nm and 345.6 nm.

A very prominent compound in the HPLC chromatogram of *C. africana* (leaf) is present at a retention time of 4.05 min with absorption maxima of 207.4 nm and 278.0 nm. This compound has a percentage integration area of 11.14% (Table A2, Appendix A) and is present in all the leaf extracts, but at a lesser percentage integration area. This compound was also observed in the stem extracts of all 10 species.

All 10 *Commiphora* leaf extracts possess a compound at a retention time of 45.068 min (Figure 8.3) with an absorbance maximum of 267.4 nm, a compound that may serve as a chemotaxonomic marker. This compound has a percentage integration area of 7.50% in *C. africana* (Table A2, Appendix A) and the percentage integration was observed to be small in other *Commiphora* leaf extracts.

The HPLC profiles (Figure 8.2) and UV spectra of the stem extracts of these indigenous *Commiphora* species indicate that a similarity exists in their chemical fingerprints. At the retention time of approximately 13.72 min the absorption maxima was determined to be 202.8 nm and 278.0 nm. This particular compound occurs, in varying concentrations, in all the HPLC chromatograms, with the exception of *C. mollis* (stem) (Table 8.2, Peak 11). It is the dominant compound in *C. africana*, *C. glandulosa*, *C. neglecta*, *C. pyracanthoides* and *C. schimperi*, with percentage integration areas of over 14.00% (Table 8.2). Another major compound present in the aforementioned species occurs at a retention time of around 14.72 min, with absorption maxima of 202.8 nm and 278.0 nm (Figure 8.6). This compound is present in all species, and has a higher percentage integration area than that eluting at 13.73 min for all species (Table 8.2).

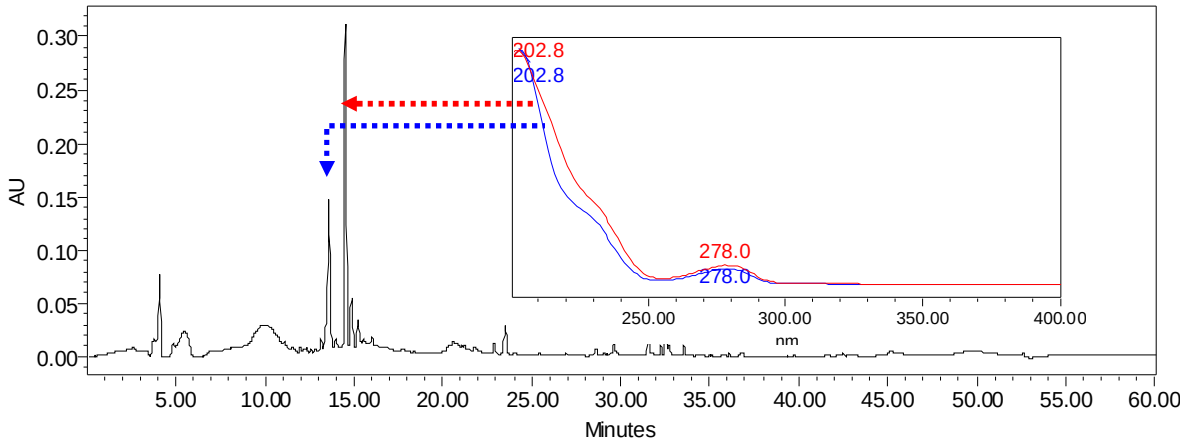


Figure 8.6: Chromatogram of *Commiphora pyracanthoides* (stem) with UV absorption maxima (insert) of compounds eluting at retention times 13.73 min and 14.72 min.

Common to all stem extracts, with the exception of *C. viminea*, is the compound eluting at the retention time of approximately 20.60 min. This compound has an absorption maximum of 281.6 nm, and is observed to be most prominent in *C. edulis* and *C. mollis* (percentage integration area is >20.00%). This compound is present in high percentages in all stem extracts with the exception of *C. pyracanthoides* (percentage integration = 4.36%).

Certain similarities in peak areas, retention time and absorbance maxima have been noted in the stem and leaf chromatograms of *C. viminea* (Figure A19 and Figure A20, Appendix A), as well as that of *C. edulis* species (Figure 8.7).

Commiphora viminea has a group of four prominent peaks at a retention time of between 32.40 min and 33.50 min in the HPLC chromatogram of the stem extract (Figure A19, Table A19, Appendix A) with distinctly high percentage integration areas. These compounds are absent in all other stem profiles (Figure 8.2). The presence of a number of peaks with similar retention times and absorbance maxima was observed in *C. viminea* leaf extract (Figure A20, Appendix A).

Two particular compounds with UV absorbance maxima of 262.6 nm, 304.0 nm and 363.4 nm occur in the HPLC chromatogram of the stem and leaf extracts of *C. edulis* only. These two compounds elute at the retention times of approximately 34.02 min and 34.50 min (Figure 8.7). The percentage integration area of these compounds is: 24.66% and 6.29% (Table A3, Appendix A) respectively in the stem extract, while in the leaf extract, the percentage integration areas are slightly less, being 16.53% and 1.60% respectively (Table A4, Appendix A).

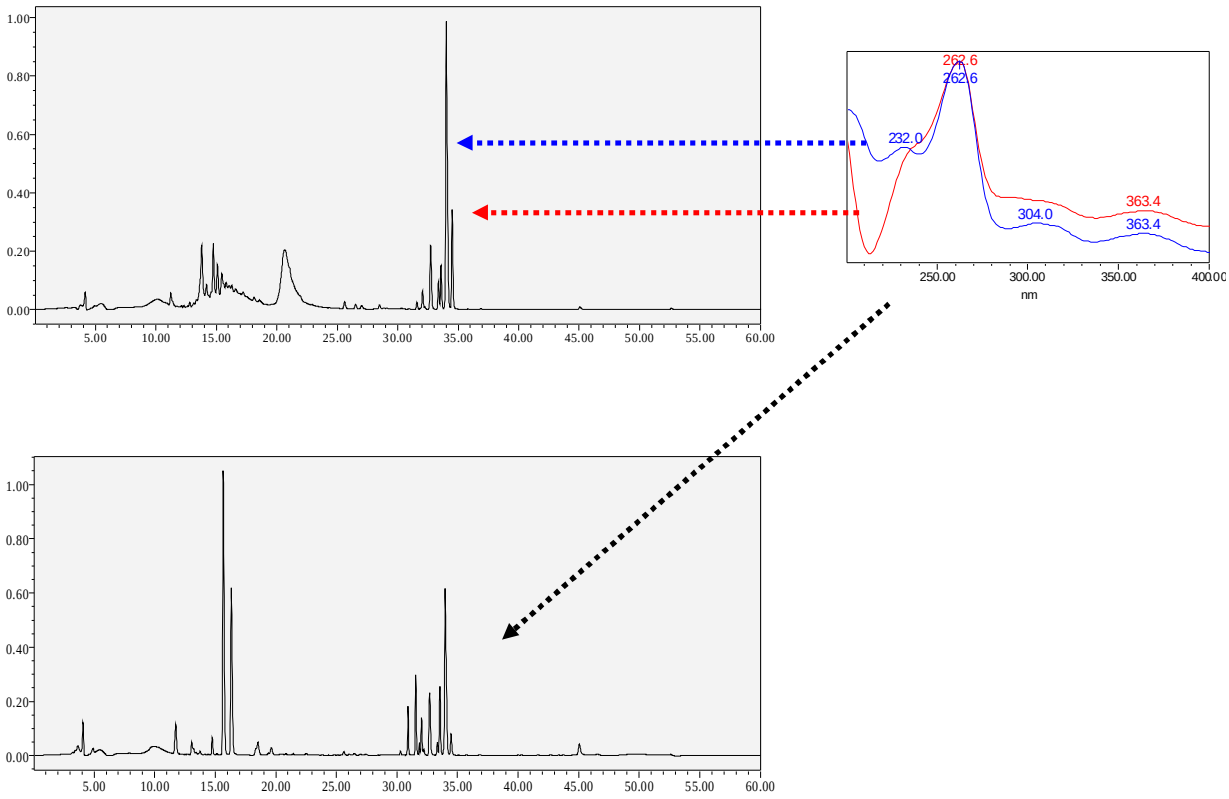


Figure 8.7: Chromatogram of *Commiphora edulis* (stem, top) and *Commiphora edulis* (leaves, bottom), with UV absorption maxima of compounds eluting at the retention times of approximately 34.02 min and 34.50 min.

The lack of detection of certain compounds in the extracts is not as a result of the complete absence of those compounds, but may be as a result of the presence of only trace amounts, which were not detected by HPLC/UV.

The effect of oxidation patterns on the UV spectra of flavones and flavonols is an important distinguishing characteristic. Band I provides information about the type of flavonoid, as well as its oxidation pattern. On increasing the oxidation of the B-ring in flavones and flavonols, a bathochromic shift in Band I occurs with each additional oxygen function. Changes in the B-ring oxygenation pattern do not result in any Band II shifts. Band II may, however, appear as either one or two peaks (which are designated as IIa and IIb, with IIa being the peak at the longer wavelength). Kaempferol is a flavonol isolated from *C. glandulosa* (stem) (as described in Chapter 3), with an oxidation pattern on the A- and C-ring at the 3, 5 and 7 position and on the B-ring at the 4' position, resulting in the absorbance maxima of Band I at 367 nm. If kaempferol is compared to a flavonol with a different oxidation pattern such as myricetin, which has the same oxidation pattern on the A- and C- ring but the B-ring differs in that it's pattern does not only include the 4' position but the 3' and 5', then the end result is an absorbance maximum of 374 nm (Harborne, 1973).

Kaempferol was observed to have a UV absorbance maximum of 266.3 nm and 363.9 nm, at a retention time of around 30.537 min (Figure 8.5). This compound was observed in the extracts of the following *Commiphora* species: *C. pyracanthoides* (stem), *C. tenuipetiolata* (stem), *C. mollis* (leaf), and *C. neglecta* (leaf), albeit in small quantities.

Fluorescent detectors, refractive index detectors and HPLC-DAD, coupled with mass spectroscopy (MS), are other methods that may be utilised to detect other compounds for which the specific column and detectors utilised, was unable to detect. Compounds that lack a chromophore do not allow for UV detection and it is for this reason that a limited number of compounds were detected.

8.5 Conclusion

As a result of the limited adequacy of traditional morphological taxonomy, where morphological and anatomical classifications prove complicated in certain instances, other sources of information are sought after. Chemosystematic data will aid in the precise classification within the genera, complementing the other methods for reliable taxonomic classification. It is apparent that HPLC can be of valuable use in plant chemosystematics assisting to characterise species, assessing taxonomic relationships amongst species on the basis of their secondary metabolite contents (Lai Fang *et al*., 2001).

The HPLC-UV profile chromatograms of the stem and leaf extracts of the 10 *Commiphora* species under investigation were obtained. The presence of flavonoids, predominantly flavone and flavonol structures, was noted in the leaf extracts of the species, while the stem extracts contained few flavonoids and at much lower percentage integration areas. A number of peaks were observed to be present at the same retention time with identical absorption maxima, an indication of the similarities that exist between the species. Other compounds present in the stem extracts of *Commiphora* indicated a definite relationship between species.

It is thus evident that HPLC-UV may be used as a tool in identifying species that belong to the same chemotaxonomic group. Similarities and differences were noted in the chromatograms of the leaves and stems of a certain species. This aids in the explanation of the differences in biological activities that exists for the leaves and stems, and may justify the use of the bark, rather than the leaf, traditionally, or vice-versa. In general, structures, distributions and percentage occurrence of secondary metabolites are valuable tools in providing taxonomic markers as well as defining evolutionary pathways. A number of these metabolites are common in many species (such as kaempferol, Chapter 3) but some of them are characteristic to a particular family, genus or only to a single species. In fact, the specific constituents of certain species have been used for systematic determination. Groups of secondary metabolites are used as markers for chemotaxonomical classification, which is based on the assumption that systematically related plants will show similar chemical characteristics. For these purposes, widespread distribution of simple compounds are less valuable than more complex

compounds, formed in long reaction chains by the mediation of many enzymes and specified by many different genes (Lattanzio *et al*., 1996).

HPLC analysis and compound identification with respect to the flavonoids is tentative. These flavonoids will need to be confirmed, as was seen in the case of kaempferol. Once confirmed, the isolation of these flavonoids and other secondary metabolites can be carried out. The HPLC analysis results could form the basis of a more detailed study of leaf phenolics, and any marked similarity in their flavonoid pattern may draw reasonable conclusions of the interspecies relationships within *Commiphora*. Flavonoids, too, exert multiple biological activities, and identification may provide a major source of information and a better understanding of the *in vitro* biological activities that certain species possess.

It is also important to note that not all compounds can be identified through the use of HPLC-UV, as those compounds that are poor chromophores will not be detected. It is for this reason that further analysis be conducted.

CHAPTER 9: GENERAL CONCLUSIONS

The objectives of the study were to:

(i) determine the *in vitro* anti-oxidant activity of the crude extracts of both the leaf and stem of 10 species of *Commiphora*.

(ii) isolate and identify of the chemical compounds responsible for the anti-oxidant activity in the most active species, through bioassay-guided fractionation using column chromatography, TLC and nuclear magnetic resonance (NMR).

(iii) determine the inhibitory effects of each of the extracts on the growth of selected bacteria and yeast and to investigate the death kinetics of a selected species against a specific micro-organisms.

(iv) determine the *in vitro* anti-inflammatory activity of the crude extracts.

(v) determine the anticancer activity of both the leaf and stem extracts against three human tumour cell lines.

(vi) determine the cytotoxicity of each of the species.

(vii) produce a phytochemical profile of the plant extracts using high performance liquid chromatography (HPLC).

The following appropriate conclusions were drawn on the basis of experimental data.

i. Anti-oxidant Activity

Most of the extracts portrayed poor anti-oxidant activity in the DPPH assay with the exception of *Commiphora schimperi* (stem), *C neglecta* (stem), *C. edulis* (stem) and *C. tenuipetiolata* (leaf and stem). The results from the ABTS assay differed from that of the DPPH assay, with most extracts displaying higher ABTS-scavenging activity with the most active species being *C. tenuipetiolata* (stem), *C. neglecta* (stem), *C. mollis* (stem), *C. glandulosa* (stem) and *C. schimperi* (stem).

The flavonol, kaempferol, displayed exceptional free radical scavenging activity, in contrast to the activity displayed by dihydrokaempferol (dihydroflavonol).

The investigation of the interaction between kaempferol and dihydrokaempferol resulted in the construction of an isobologram displaying an antagonistic effect, with the resultant IC_{50} value tending towards that of *C. glandulosa* (stem).

It is important to note that the failure of a plant extract to demonstrate *in vitro* activity during the general screening process does not necessarily imply a total absence of inherent medicinal value. The possible presence of synergistic interactions between the different plant constituents in crude preparations may result in activities that are not exhibited by isolated compounds, and should not be excluded. Furthermore, the plant or extract may react differently *in vivo*.

ii. Isolation of biologically active compounds

Two compounds were isolated from the stem extract of *C. glandulosa*. The compounds were identified as flavonoids viz. kaempferol (flavonol) and dihydrokaempferol (dihydroflavonol). Both compounds have been previously isolated from other plant species, however, not from the genus *Commiphora*. Kaempferol was detected in small amounts in the stem extracts of *C. glandulosa*, *C. pyracanthoides* and *C. tenuipetiolata*, and in the leaf extracts of *C. neglecta* and *C. mollis*.

iii. Antimicrobial Activity

All crude stem and leaf extracts of the 10 indigenous *Commiphora* spp. under investigation exhibited concentration-dependent activity against both Gram-positive and Gram-negative bacteria, as well as against yeasts. Highly pronounced anti-microbial activity was displayed against the yeasts, which serves as a clear indication of the potential of these extracts for further chemical and pharmacological studies.

Kaempferol, which has been documented to possess antimicrobial activity, was isolated from *C. glandulosa*. In this study, kaempferol was shown to have an MIC value of 0.25 mg/ml with respect to activity against the Gram-positive bacteria *S. aureus* and *B. cereus*. However, the isolated kaempferol was found to be less active against *S. aureus* than the stem extract as a whole, the latter yielding an impressive MIC-value of 0.005 mg/ml, a result which may well serve as an indication that the

overall activity of the extract may be attributed to synergism amongst other compounds.

vi. Anti-inflammatory Activity

The *in vitro* anti-inflammatory activity of the crude extracts was investigated, by their ability to inhibit the 5-LOX enzyme, which is involved in the inflammatory process. The stem extracts were observed to possess some inhibitory activity, the most active being *C. pyracanthoides*, while the leaf extracts displayed only limited 5-LOX inhibition, with the exception of *C. schimperi* and *C. glandulosa*. Kaempferol (flavonol), isolated from *C. glandulosa* (stem), exhibited good anti-inflammatory activity. The 5-LOX inhibitory activity of *C. glandulosa* (stem), *C. pyracanthoides* (stem) and C. *tenuipetiolata* (stem) extracts may be attributed partly to the presence of kaempferol within these extracts (as verified by HPLC analysis).

The traditional use (by the Himba tribe) of *Commiphora* stem extracts for rheumatoid arthritis and other inflammatory conditions is thus supported by the *in vitro* determination of the anti-inflammatory activity.

v. Anticancer Activity

The *in vitro* inhibitory activity of extracts of both the stem and leaf of indigenous *Commiphora* species on cell growth in MCF-7, SF-268 and HT-29 cells was determined. The inhibition of cell proliferation and viability was determined to be highly dose-dependent. Certain *Commiphora* extracts were highly cancer type specific.

No trend was found upon comparison of the leaf and stem extracts and their activity against the three cell lines. In general, the leaf extracts were found to be more active than the stem extracts against the HT-29 cell line, with the exception of *C. africana*, *C. edulis* and *C. pyracanthoides*, while the stem extracts were observed to be more active against the MCF-7 and SF-268 cell lines, with the exception of *C. edulis*, *C. marlothii* and *C. pyracanthoides*. The most promising activity against the HT-29 cells was presented by *C. glandulosa* (leaf and stem) and *C. marlothii* (leaf). The MCF-7 cells exhibited the most sensitivity to indigenous *Commiphora* species. *Commiphora*

africana (leaf and stem) displayed cancer cell specificity against the MCF-7 cell line, and the other cell lines were less sensitive to the inhibitory effects of this extract. Considerable cytotoxicity against the SF-268 cells by *Commiphora glandulosa* (leaf and stem) and *C. pyracanthoides* (leaf and stem) was observed.

The inhibition of cancer cell proliferation of kaempferol in all three-cancer cell lines was determined. The activity of the isolated compound was determined to be far greater in the SF-268 and HT-29 cell lines than that observed in *C. glandulosa* (stem), the species from which it was isolated. This indicates possible antagonistic effects by other compounds present within the crude extract.

vi. Cytotoxicity

Commiphora glandulosa stem extract also produced significant cytotoxicity against the Graham (transformed human kidney epithelial) cells, which may explain its activity in the different biological assays, however, the isolated compound was observed to be relatively safe. In spite of the presence of toxic compounds within a plant in its crude form, further investigations regarding non-toxic compounds of potential therapeutic value cannot and should not be excluded.

Cytotoxicity of all other extracts against the Graham cells was minimal, with the percentage cell viability being far greater than the percentage cell viability of the cancer cell lines. This indicates that the activity is selective against the cancer cell lines and that the isolation of the particular compounds responsible for this activity may prove to be invaluable.

vii. Non-volatile chemical constituents found in *Commiphora* species

The HPLC chromatograms of the stem and leaf extracts of the 10 *Commiphora* species under investigation were obtained. Similarities and differences were noted in the chromatograms of the leaf and stem extracts of a certain species. This aids in the explanation of the differences in biological activities that exists for the leaves and stems, and may justify the use of the stem, rather than the leaf, traditionally, or vice-

versa. The presence of flavonoids was noted in the leaf extracts of the species, while the stem extracts contained few flavonoids and at much lower concentrations.

The HPLC-UV results also suggest that *Commiphora africana* (leaf), *C. mollis* (leaf) and *C. schimperi* (leaf) share similar chemical profiles when ascertaining the presence of flavonoids, while *C. marlothii* (leaf) and *C. neglecta* (leaf), are similar.

As modern cultures and scientific advances spread around the world, the breadth of the knowledge store of traditional healers still remains crucial. The full significance of the indigenous knowledge forfeited may not be realised. It is thus important that the knowledge be documented and the traditional use given some credence through modern scientific studies. *Commiphora* is one such example.

CHAPTER 10: RECOMMENDATIONS FOR FURTHER RESEARCH

This study intends to contribute towards the knowledge base of plant species with therapeutic potential. It should be noted that it acutely encompasses only 10 indigenous species, and the biological activities and phytochemistry of other indigenous *Commiphora* species should also be investigated. Furthermore, material for this study was only collected from a single site. To account for possible geographical and chemotypic variation material should be studied from several populations.

i. Anti-oxidant activity

- No single testing method is capable of providing a comprehensive profile of the anti-oxidant capacity of a plant species, due to the complexity of oxidation-anti-oxidation processes, and therefore different methods are to be used to determine the anti-oxidant potential.

ii. Antimicrobial activity

- Isolation and structural elucidation of the compound/s responsible for the antimicrobial activity of *C. marlothii* and the determination of the presence of these compounds in any of the other species through the use of HPLC-UV should be investigated.

- Determination of the concentration of compounds within each of the species, and whether synergistic, antagonistic or additive effects are evident.

- The antimicrobial activity of investigated *Commiphora* species was determined for collection strains, but further antimicrobial investigations, which may be of interest, may be carried out on clinical isolates, especially in the case of active extracts.

iii. Anti-inflammatory Activity

- The active extracts of *Commiphora* species require further investigation, specifically in terms of the isolation, identification and characterisation of the compounds responsible for the anti-inflammatory activity. Once isolated, these compounds can be combined to

determine their anti-inflammatory relationship. Investigations involving different combinations of selected *Commiphora* extracts, or combinations of *Commiphora* extracts with other potentially active plant extracts, should be carried out to assess whether synergistic or antagonistic effects are produced.

- Additionally, the effects of these active compounds on COX-1 and COX-2, including their selectivity and effect on the suppression of the COX-2 gene expression, requires investigation and clarification. The effectiveness of interaction of these extracts and isolated compounds, including the flavonoid derivatives, with other pro-inflammatory biochemical pathways may be assessed, and the possible structure-activity relationships determined. This will provide a better understanding of the possible mode of action.

v. Anticancer

- This study has, without a doubt, proven the existence of a compound or compounds with potential *in vitro* anticancer activity in different species and extracts of *Commiphora*. Activity-guided fractionation, isolation and identification of these compounds is imperative and may lead to the development of novel treatments in the global struggle against cancer and cancer-related ailments.

vi. Toxicity

- While indigenous *Commiphora* species were observed to be non-toxic against the transformed kidney epithelial cells, it is important to note that in order to establish a toxicity profile, other cell lines should be investigated such as the liver HepG2 cells, as well as *in vivo* studies.

vii. Phytochemical investigation

- It is recommended that a thorough geographical variation study is performed on each species to explain the diversity between individuals within a population and between populations.
- Research on the non-volatile compounds warrant further study, as the HPLC assignments are tentative only. Further analysis must be conducted using different detectors and chromatographic techniques such as liquid chromatography – mass

spectrometry, to provide a greater insight of the phytochemical composition of this species.

- Plants produce a wide range of secondary metabolites, which may prove to be invaluable in development of drugs, flavours, fragrances, dyes, anti-oxidants and insecticides. It is thus important to locate and determine the role of these secondary metabolites in plants and unravel their biosynthesis. While HPLC has proven to be a valuable tool in detecting certain secondary metabolites, low or very high molecular weight molecules go unnoticed. Finding new leads for drug development, and determining the biosynthesis of such products, thus requires a different approach which researchers are developing. Metabolomics investigates the end products of cellular functions. The levels of these metabolites are viewed as a response of the biological systems to environmental or genetic manipulation (Maloney, 2004). The use of metabolomics in plant studies will enable the characterisation and differentiation of genotypes and phenotypes based on the levels of metabolites, and also aid in the rapid screening of multiple extracts giving an exceptionally broad overview of the chemistry. This may also provide a means of improving the production of certain metabolites in plants through genetic engineering.

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APPENDIX A - HPLC DATA

***Commiphora africana* (stem)**

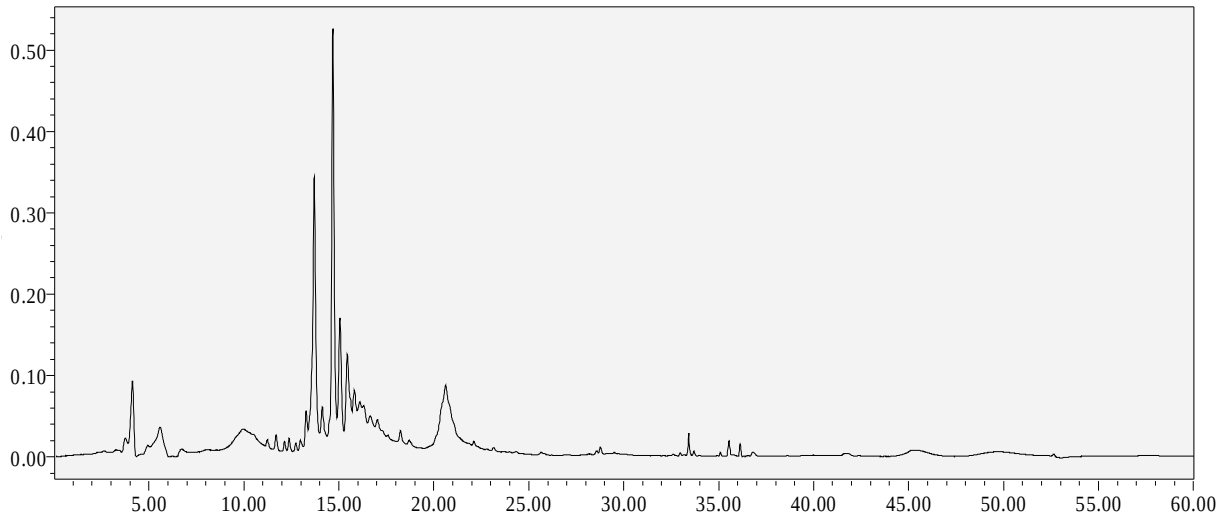

Figure A1: HPLC chromatogram of *Commiphora africana* stem extract.

Table A1: Retention time, percentage integration area and UV maxima for peaks from *Commiphora africana* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.763	263.8 ; 307.6 ; 363.4	1.22
4.133	205.1 ; 278.0	5.02
4.941	262.6	0.72
5.589	207.4 ; 345.6	5.15
6.740	275.6 ; 332.5	1.42
9.968	206.3 ; 289.8	11.96
11.696	218.0 ; 278.0	0.67
12.137	203.9 ; 278.0	0.39
12.376	205.1 ; 278.0 ; 334.9	0.52
12.736	203.9 ; 278.0	0.27
12.981	202.8 ; 278.0	0.46
13.701	202.8 ; 278.0	18.24
14.125	201.6 ; 279.2	2.15
14.686	202.8 ; 278.0	21.26
15.056	202.8 ; 279.2	6.48
15.442	202.8 ; 279.2	5.14
15.815	202.8 ; 279.2	1.50
20.626	281.5	11.18
33.422	241.4 ; 283.9 ; 331.3	0.60

***Commiphora africana* (leaf)**

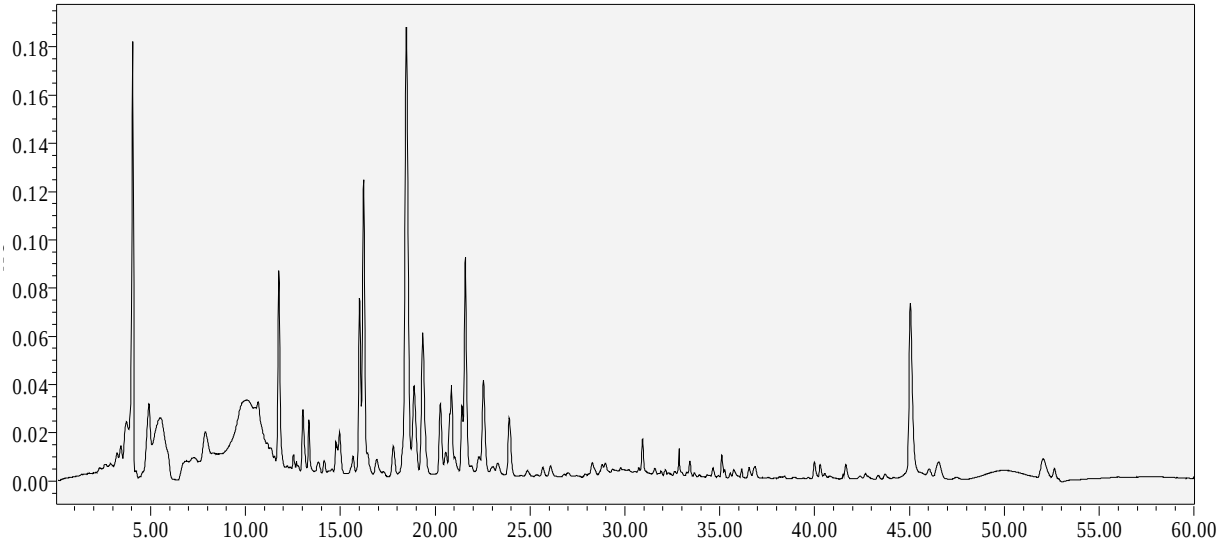

Figure A2: HPLC chromatogram of *Commiphora africana* leaf extract.

Table A2: Retention time, percentage integration area and UV maxima for peaks from *Commiphora africana* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.726	263.8 ; 358.5	1.96
4.051	207.4 ; 278.0	11.14
4.909	202.8 ; 260.3 ; 325.4	2.13
7.880	255.6 ; 321.8	1.99
11.758	219.1 ; 278.0	5.20
14.954	239.1	1.24
16.013	209.8 ; 260.3 ; 356.3	4.43
16.222	208.6 ; 260.3 ; 354.0	8.31
17.788	230.9 ; 255.6 ; 356.3	1.13
18.482	209.8 ; 265.0 ; 343.2	19.52
18.887	205.1 ; 254.4 ; 354.0	4.02
19.344	203.9 ; 255.6 ; 354.0	5.78
20.270	222.7 ; 278.0	2.20
20.853	228.5 ; 265.0 ; 349.2	2.72
21.414	265.0 ; 345.6	1.56
21.587	255.6 ; 348.0	6.31
22.548	245.0 ; 345.6	2.90
23.908	263.8 ; 342.0	2.11
30.934	246.1 ; 269.7 ; 318.3	0.71
45.050	267.4	7.50

***Commiphora edulis* (stem)**

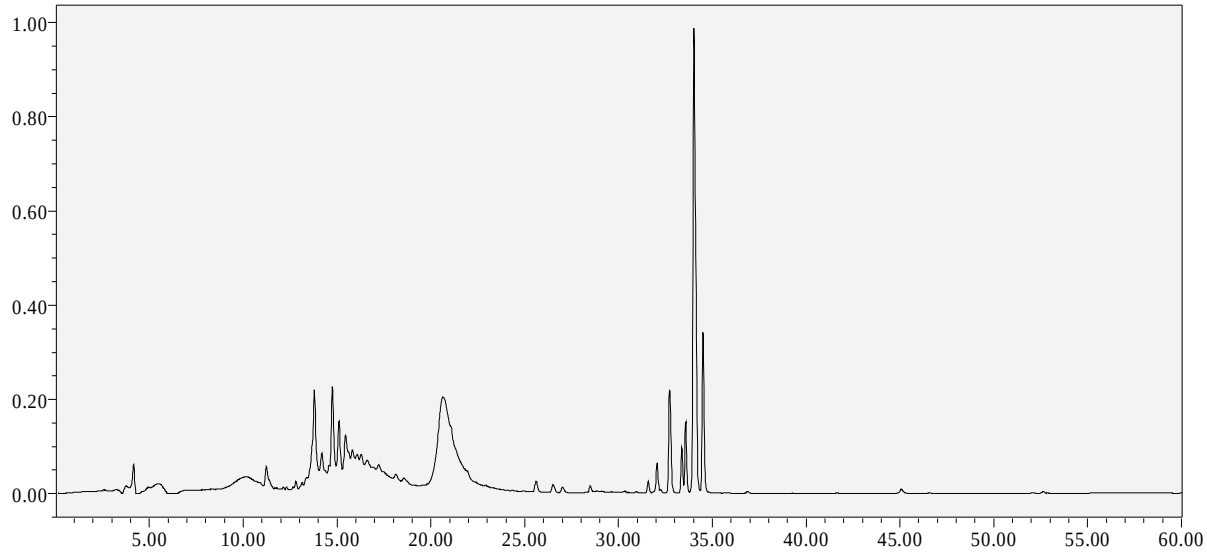

Figure A3: HPLC chromatogram of *Commiphora edulis* stem extract.

Table A3: Retention time, percentage integration area and UV maxima for peaks from *Commiphora edulis* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.754	201.6 ; 265.0 ; 307.6	0.67
4.152	206.3 ; 278.0	1.55
5.494	206.3 ; 278.0	2.08
11.233	202.8 ; 278.0	1.31
12.134	201.6 ; 278.0	0.07
12.308	201.6 ; 278.0 ; 319.4	0.08
12.807	201.6 ; 221.5 ; 278.0	0.34
13.767	201.6 ; 278.0	7.41
14.188	201.6 ; 278.0	2.20
14.754	201.6 ; 278.0	5.39
15.099	201.6 ; 278.0	3.13
15.456	201.6 ; 278.0	3.41
15.812	201.6 ; 278.0	1.44
20.645	201.6 ; 278.0	22.95
32.059	259.1 ; 313.5	1.14
32.726	259.1 ; 308.8	4.75
33.371	262.6 ; 308.8 ; 354.0	1.64
33.582	260.3 ; 304.0	2.81
34.021	232.0 ; 262.6 ; 304.0	24.66
34.508	262.6 ; 363.4	6.29

***Commiphora edulis* (leaf)**

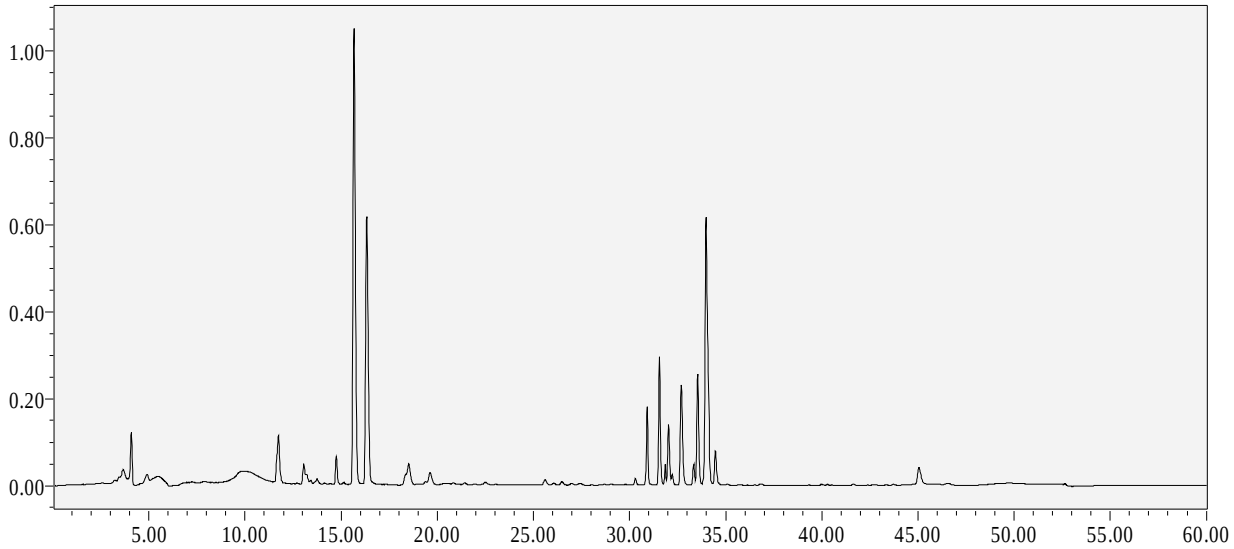

Figure A4: HPLC chromatogram of *Commiphora edulis* leaf extract.

Table A4: Retention time, percentage integration area and UV maxima for peaks from *Commiphora edulis* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.664	263.8 ; 320.6	1.37
4.083	207.4 ; 278.0	2.40
10.133	207.4 ; 278.0	2.95
11.745	219.1 ; 288.6 ; 326.6	2.92
13.057	226.2 ; 312.3	1.57
14.743	202.8 ; 278.0	1.16
15.668	210.9 ; 269.7 ; 348.0	24.65
16.329	210.9 ; 255.6 ; 348.0	15.72
18.517	214.5 ; 269.7 ; 336.1	2.08
19.391	255.6 ; 351.6	0.15
19.630	207.4 ; 226.2 ; 254.4 ; 348.0	0.89
30.305	262.6 ; 302.8 ; 363.4	0.28
30.920	232.0 ; 286.3 ; 318.3	2.72
31.557	247.3 ; 345.6	4.92
31.862	240.3 ; 285.1	0.58
32.032	257.9 ; 314.7	2.64
32.220	250.9 ; 343.2	0.43
32.696	259.1 ; 312.3	5.30
33.549	259.1 ; 304.0 ; 360.4	4.95
33.985	262.6 ; 304.0 ; 363.4	16.53
45.057	267.4	1.23

***Commiphora glandulosa* (stem)**

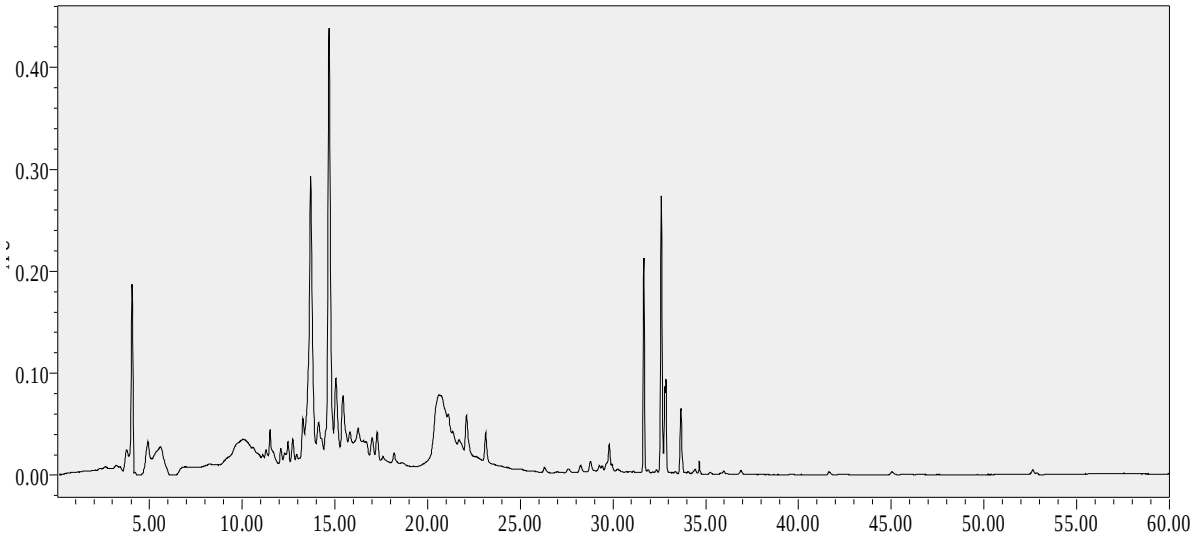

Figure A5: HPLC chromatogram of *Commiphora glandulosa* stem extract.

Table A5: Retention time, percentage integration area and UV maxima for peaks from *Commiphora glandulosa* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.760	296.9 ; 366.8	1.39
4.057	206.3 ; 278.0	7.00
4.919	205.1 ; 260.3	1.46
12.726	278.0	0.80
13.275	201.6 ; 278.0	1.81
13.691	201.6 ; 278.0	19.39
14.122	278.0	2.28
14.682	202.8 ; 278.0	21.22
15.055	201.6 ; 278.0	3.81
15.438	278.0	2.95
20.560	278.0	5.49
22.091	232.0 ; 278.0	1.92
23.130	229.7 ; 287.4	1.29
29.791	243.8 ; 266.2 ; 296.9 ; 363.4	0.96
31.659	229.7 ; 282.7	5.57
32.605	229.7 ; 295.7	8.45
32.842	235.6 ; 268.5	4.21
33.660	272.1 ; 356.3	2.42
34.648	265.0 ; 329.0 ; 365.6	0.22

***Commiphora glandulosa* (leaf)**

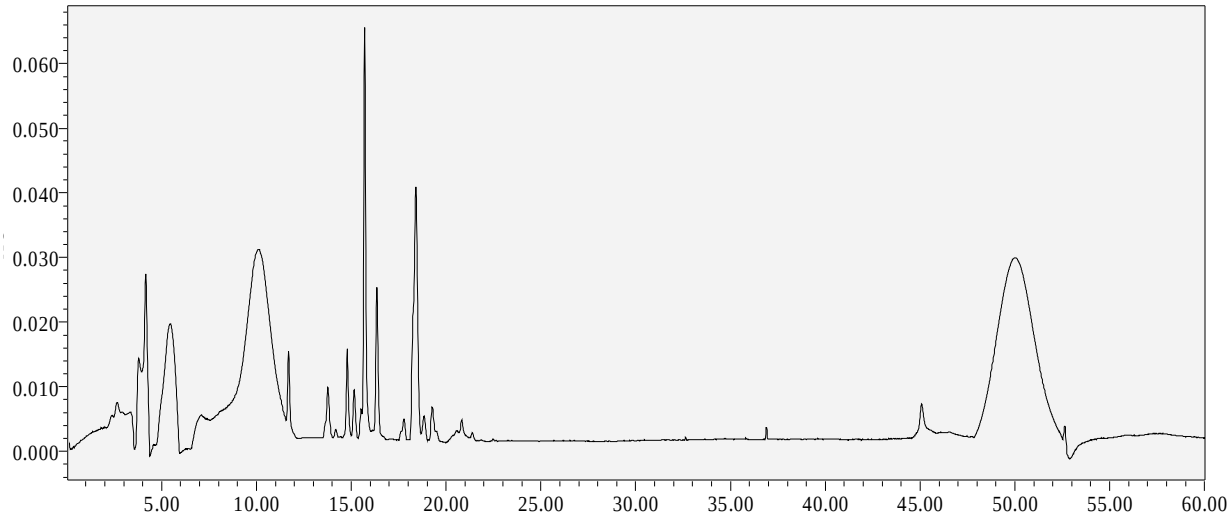

Figure A6: HPLC chromatogram of *Commiphora glandulosa* leaf extract.

Table A6: Retention time, percentage integration area, and UV maxima for peaks from *Commiphora glandulosa* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.798	206.3 ; 261.5	1.75
4.158	207.4 ; 314.7	3.32
4.579	206.3 ; 267.4 ; 314.7	0.16
5.448	207.4 ; 279.2	7.38
7.085	207.4 ; 293.4	2.32
10.094	207.4	27.49
11.697	219.1 ; 278.0	1.07
14.787	205.1 ; 228.5 ; 279.2 ; 313.5	0.87
15.147	230.9 ; 279.2 ; 313.5 ; 366.8	0.54
15.522	233.2 ; 279.2 ; 313.5 ; 366.8	0.29
15.709	213.3 ; 269.7 ; 348.0	4.10
16.346	215.6 ; 255.6 ; 348.0	1.71
18.410	215.6 ; 269.7 ; 336.1	5.08
18.834	254.4 ; 291.0 ; 350.4	0.37
19.272	255.6 ; 350.4	0.59
21.381	241.4 ; 263.8 ; 313.5	0.06
36.893	279.2 ; 312.3 ; 337.7	0.07
45.085	263.8 ; 312.3 ; 337.7	0.44
50.028	272.1 ; 324.2	35.04
54.423	272.1 ; 291.0 ; 333.7 ; 312.3	5.25

***Commiphora marlothii* (stem)**

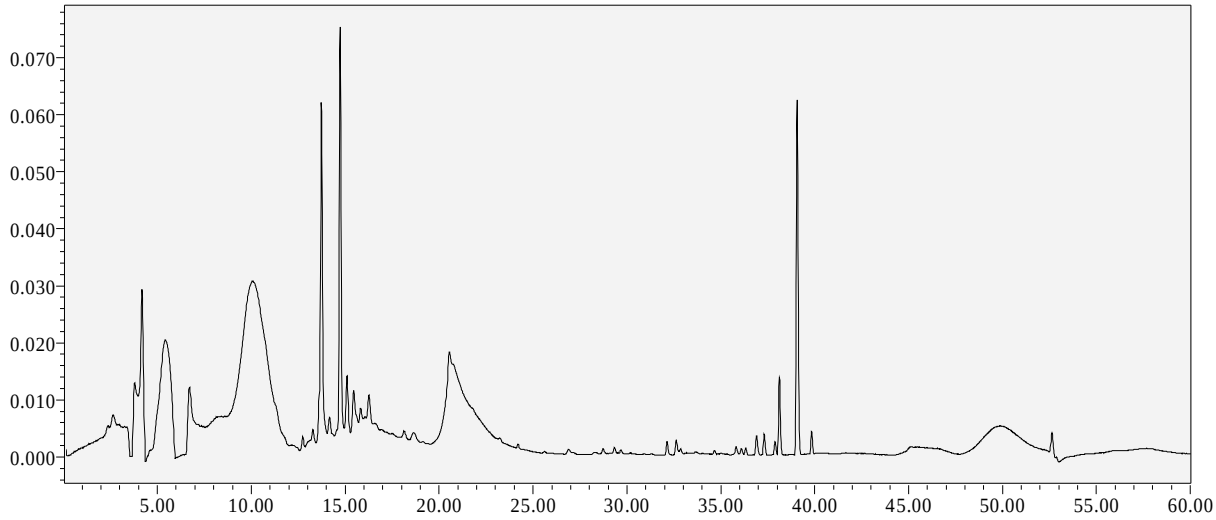

Figure A7: HPLC chromatogram of *Commiphora marlothii* stem extract.

Table A7: Retention time, percentage integration area and UV maxima for peaks from *Commiphora marlothii* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.364	207.4 ; 276.8	2.86
3.798	207.4 ; 288.6	3.04
4.177	207.4 ; 283.9	6.08
5.426	207.4 ; 281.5	13.52
6.707	215.6 ; 270.9	1.96
10.067	207.4 ; 278.0	23.38
13.735	201.6 ; 278.0	8.02
14.730	202.8 ; 278.0	7.52
15.092	228.5 ; 279.2	1.37
15.453	229.7 ; 279.2	1.32
15.823	232.0 ; 279.2	0.65
16.262	230.9 ; 281.5 ; 336.1	1.10
20.554	228.5 ; 281.5	16.14
32.142	288.6	0.24
32.626	275.6 ; 326.6	1.62
36.902	275.6	0.40
37.312	252.0	0.41
38.119	248.5	1.47
39.065	246.1	6.88

***Commiphora marlothii* (leaf)**

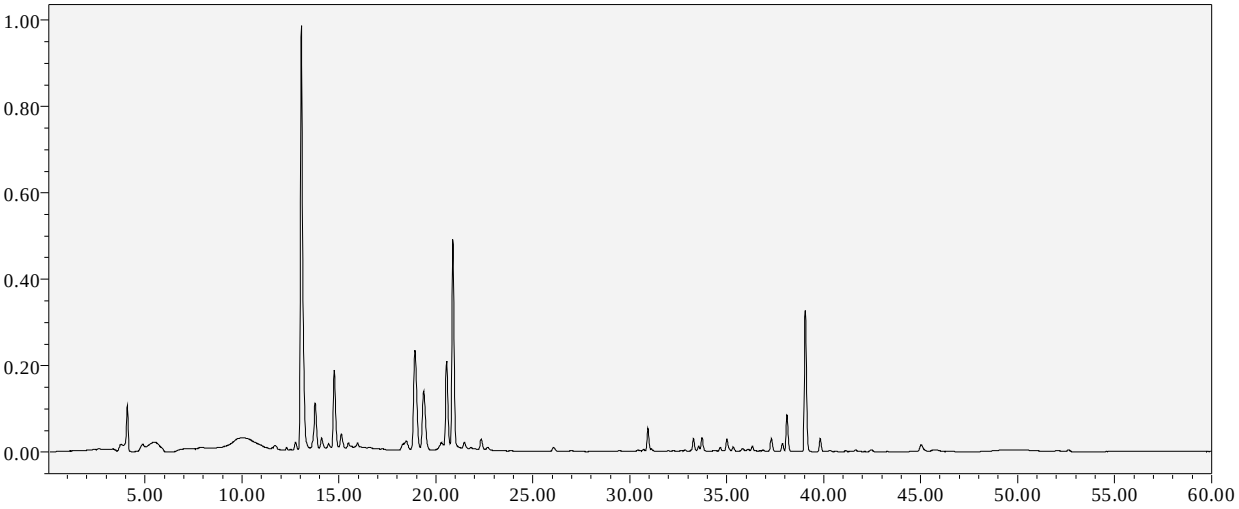

Figure A8: HPLC chromatogram of *Commiphora marlothii* leaf extract.

Table A8: Retention time, percentage integration area and UV maxima for peaks from *Commiphora marlothii* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.766	267.4	0.69
4.083	207.4 ; 278.0	2.66
13.065	226.2 ; 311.1	28.47
13.773	201.6 ; 278.0	3.64
14.763	201.6 ; 278.0	5.53
15.133	222.7 ; 312.3	1.31
15.499	201.6 ; 226.2 ; 278.0	0.84
15.956	225.0 ; 278.0	1.98
18.468	226.2 ; 269.7 ; 336.1	1.24
18.917	203.9 ; 255.6 ; 354.0	9.12
19.372	203.9 ; 255.6 ; 354.0	5.35
20.282	223.8 ; 278.0	0.42
20.552	203.9 ; 255.6 ; 354.0	5.45
20.874	202.8 ; 255.6 ; 354.0	12.79
21.470	234.4 ; 265.0 ; 348.0	0.34
22.334	265.0 ; 348.0	0.65
30.934	240.3 ; 269.7 ; 318.3	1.13
38.107	245.0 ; 308.8	2.15
39.051	245.0	8.22
45.030	268.5	0.62

***Commiphora mollis* (stem)**

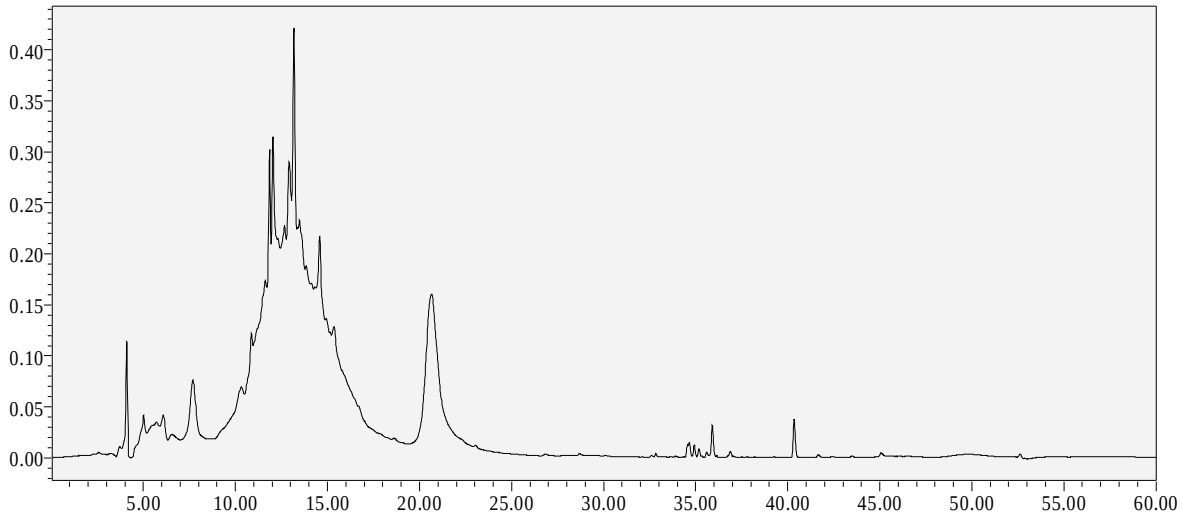

Figure A9: HPLC chromatogram of *Commiphora mollis* stem extract.

Table A9: Retention time, percentage integration area and UV maxima for peaks from *Commiphora mollis* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
4.091	206.3 ; 278.0	2.91
4.717	206.3	0.45
5.011	205.1 ; 278.0	2.30
5.515	206.3 ; 278.0	1.60
5.711	206.3 ; 278.0	1.67
6.081	206.3 ; 269.7	1.92
6.548	205.1 ; 272.1	1.00
7.687	206.3 ; 269.7	4.33
10.283	206.3 ; 278.0	0.85
10.869	205.1 ; 278.0	2.00
11.622	206.3 ; 275.6	8.07
11.853	205.1 ; 278.0	6.00
12.038	203.9 ; 278.0	12.08
12.665	205.1 ; 276.8	5.76
12.919	205.1 ; 278.0	7.10
13.179	203.9 ; 278.0	10.59
13.480	205.1 ; 278.0	4.15
13.842	205.1 ; 276.8	0.85
14.573	203.9 ; 279.2	2.09
20.644	202.8 ; 278.0	19.98
34.653	265.0	0.55

***Commiphora mollis* (leaf)**

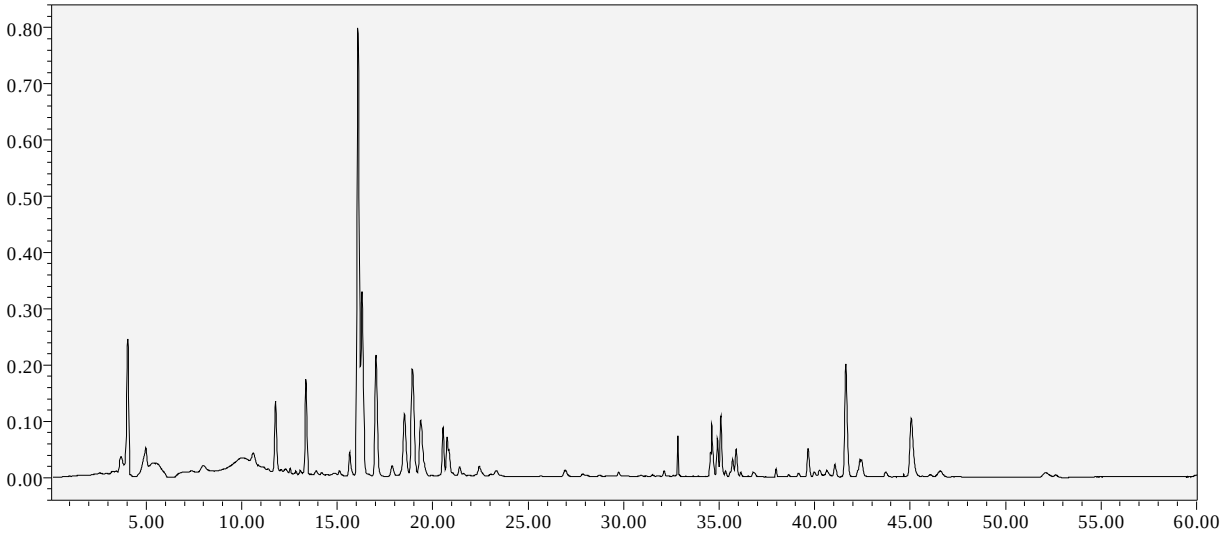

Figure A10: HPLC chromatogram of *Commiphora mollis* leaf extract.

Table A10: Retention time, percentage integration area and UV maxima for peaks from *Commiphora mollis* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.669	261.5	1.16
4.031	207.4 ; 278.0	6.12
4.979	261.5	1.48
11.769	219.1 ; 278.0	2.98
13.367	202.8 ; 278.0	4.04
15.651	209.8 ; 261.5 ; 354.0	1.05
16.088	208.6 ; 259.1 ; 357.5	22.45
16.304	208.6 ; 259.1 ; 356.3	9.75
17.034	208.6 ; 261.5 ; 354.0	6.33
18.517	214.5 ; 269.7 ; 336.1	3.70
18.940	203.9 ; 255.6 ; 354.0	6.56
19.380	203.9 ; 255.6 ; 354.0	3.80
20.545	203.9 ; 255.6 ; 354.0	1.83
20.756	216.8 ; 268.5 ; 339.7	2.15
21.406	265.0 ; 354.6	0.35
32.122	308.8	1.52
34.620	265.0	2.52
34.917	265.0	1.56
35.892	263.8	1.21
41.638	246.1 ; 278.0 ; 325.4	5.98
42.382	260.3	1.45
45.065	268.5	4.11

***Commiphora neglecta* (stem)**

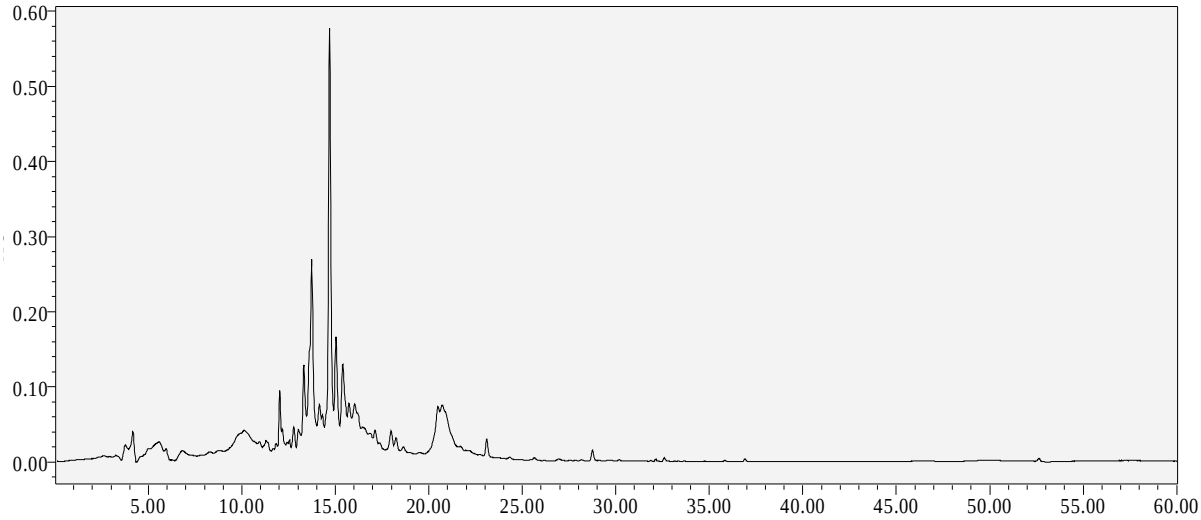

Figure A11: HPLC chromatogram of *Commiphora neglecta* stem extract.

Table A11: Retention time, percentage integration area and UV maxima for peaks from *Commiphora neglecta* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.768	202.8 ; 263.8	1.36
4.166	206.3 ; 304.0	2.39
4.629	202.8 ; 260.3	0.25
5.532	202.8 ; 278.0	3.14
6.832	275.6 ; 337.3	1.49
10.131	206.3 ; 289.8	10.17
11.298	207.4 ; 263.8	1.04
11.830	278.0	0.17
12.028	221.5 ; 275.6	2.44
13.020	201.6 ; 278.0	1.05
13.316	202.8 ; 278.0	4.37
13.730	201.6 ; 278.0	14.84
14.146	278.0	2.21
14.299	278.0	1.15
14.689	202.8 ; 278.0	21.61
15.035	201.6 ; 278.0	5.90
15.395	201.6 ; 278.0	5.20
15.735	201.6 ; 278.0	1.81
16.033	201.6 ; 278.0	2.66
20.488	281.5	3.67
20.712	278.0	7.29

***Commiphora neglecta* (leaf)**

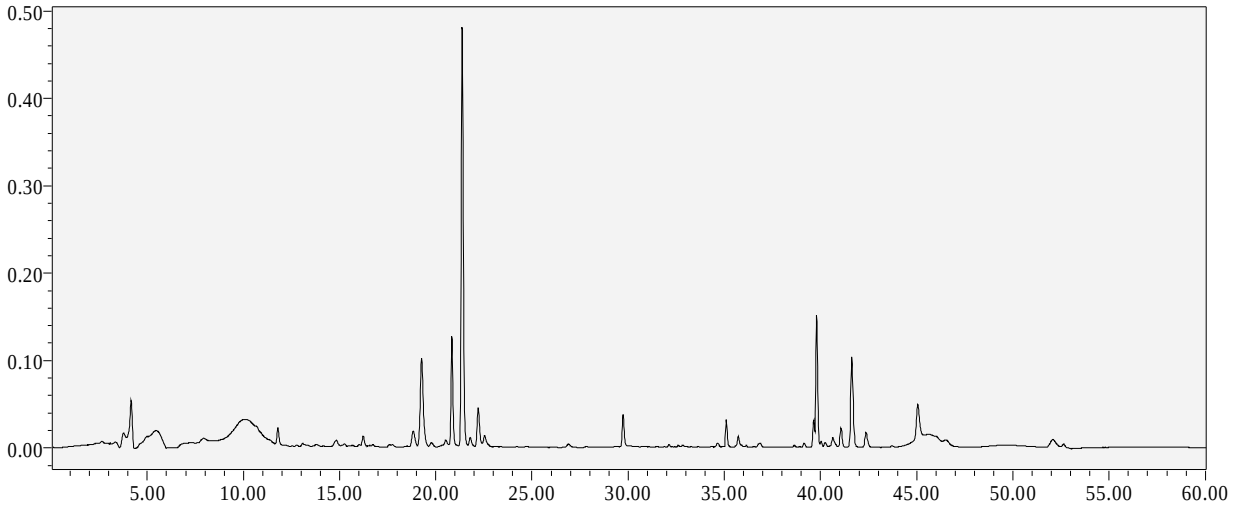

Figure A12: HPLC chromatogram of *Commiphora neglecta* leaf extract.

Table A12: Retention time, percentage integration area and UV maxima for peaks from *Commiphora neglecta* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.771	202.8 ; 261.5 ; 368	1.75
4.164	205.1 ; 307.6	4.29
5.457	207.4 ; 278.0	7.54
10.115	207.4 ; 278.0	5.10
11.791	219.1 ; 278.0	1.00
16.226	229.7 ; 260.3 ; 354.0	0.67
18.823	255.6 ; 354.0	1.68
19.259	205.1 ; 255.6 ; 354.0	8.41
20.837	265.0 ; 348.0	6.13
21.373	265.0 ; 348.0	26.87
21.793	254.4 ; 352.8	0.63
22.209	265.0 ; 348.0	2.88
29.737	265.0 ; 363.0	1.91
35.102	240.0	1.36
39.801	243.8 ; 331.3 ; 365.6	7.67
40.637	278.0	1.01
41.060	278.0	1.37
41.624	248.5 ; 273.3 ; 325.4	6.84
42.366	265.0	1.11
45.054	268.5 ; 337.3	3.29
52.066	268.5 ; 365.6	0.97

***Commiphora pyracanthoides* (stem)**

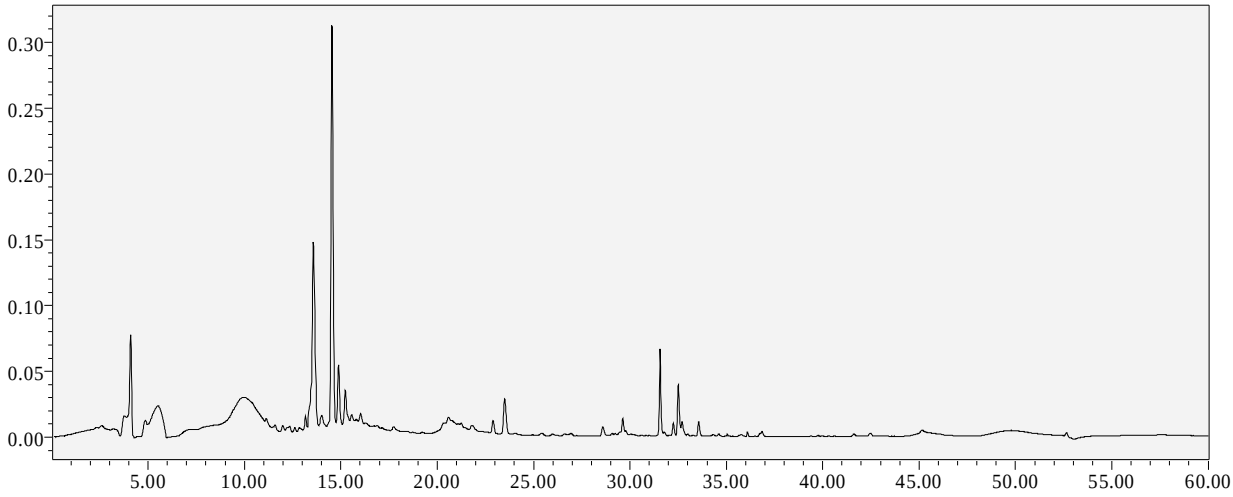

Figure A13: HPLC chromatogram of *Commiphora pyracanthoides* stem extract.

Table A13: Retention time, percentage integration area and UV maxima for peaks from *Commiphora pyracanthoides* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.776	206.3 ; 267.4 ; 307.6	2.05
4.101	206.3 ; 278.0	6.70
4.857	206.3 ; 265.0	1.57
5.511	206.3 ; 278.0	9.33
10.013	206.3 ; 278.0	12.64
12.348	221.5 ; 278.0 ; 331.3	0.58
13.170	205.1 ; 227.4 ; 278.0	0.61
13.584	202.8 ; 278.0	15.67
14.004	202.8 ; 225.0 ; 280.3	1.06
14.537	202.8 ; 279.2	23.46
14.879	202.8 ; 279.2 ; 369.2	3.39
15.233	202.8 ; 279.2	2.09
20.340	229.7 ; 267.4 ; 348.0	0.63
20.588	229.7 ; 281.5	4.36
21.797	236.7 ; 280.3	1.17
22.893	233.2 ; 288.6	0.78
23.498	263.8 ; 344.4	2.50
31.561	234.4 ; 293.4	3.17
32.508	237.9 ; 295.7	2.58
32.705	243.8 ; 274.4	0.81
33.568	272.1 ; 350.4	0.70

***Commiphora pyracanthoides* (leaf)**

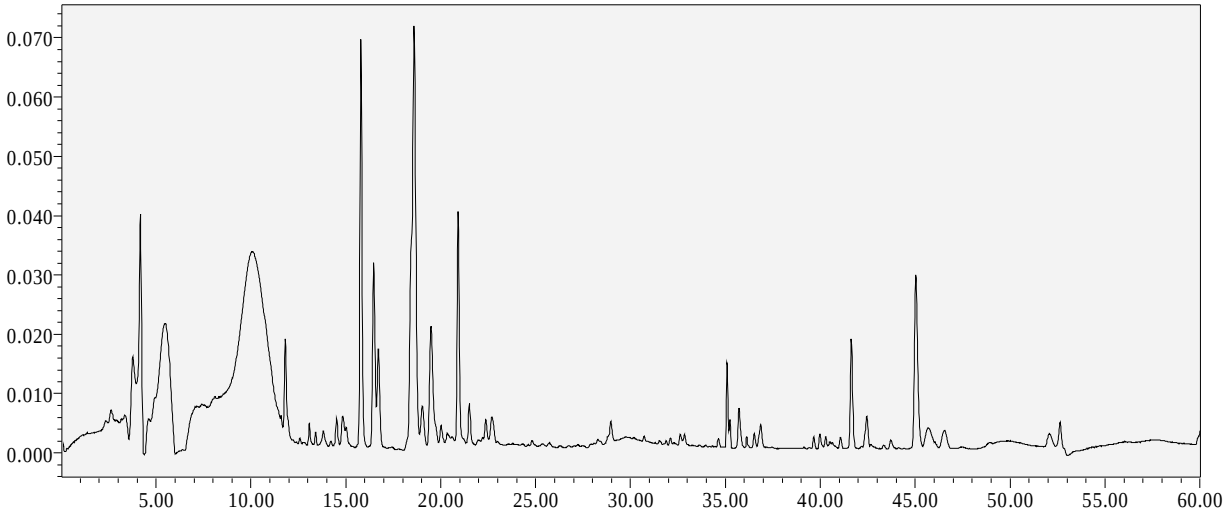

Figure A14: HPLC chromatogram of *Commiphora pyracanthoides* leaf extract.

Table A14: Retention time, percentage integration area and UV maxima for peaks from *Commiphora pyracanthoides* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.776	201.6 ; 265.0 ; 370.4	2.92
4.170	206.3 ; 281.5	6.29
4.625	265.0 ; 327.8	1.02
4.952	262.6	1.87
5.486	205.1 ; 278.0	12.57
10.061	205.1 ; 278.0	14.01
11.817	219.1 ; 278.0	1.66
15.795	213.3 ; 269.7 ; 348.0	7.40
16.470	214.5 ; 255.6 ; 348.0	3.84
16.713	255.6 ; 354.0	2.20
18.598	214.5 ; 269.7 ; 336.1	15.16
19.037	255.6 ; 354.0	1.07
19.490	206.3 ; 255.6 ; 354.0	3.71
20.917	255.6 ; 354.0	4.06
21.509	265.0 ; 343.2	0.72
22.695	246.1 ; 308.8	0.69
28.970	278.0 ; 363.4	5.89
35.093	243.8 ; 278.0	1.89
41.636	278.0	2.27
45.040	267.4	4.76

***Commiphora schimperi* (stem)**

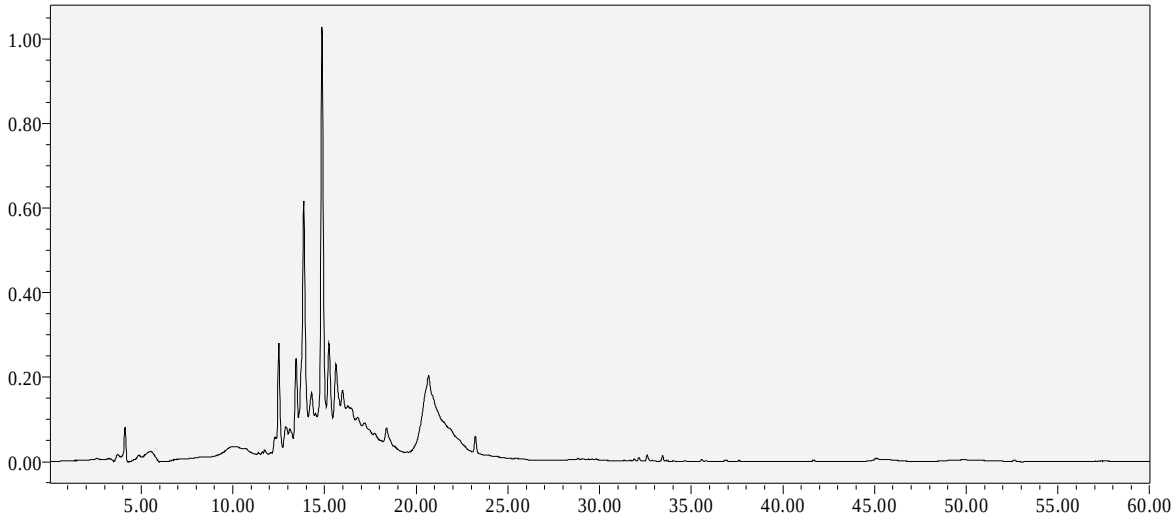

Figure A15: HPLC chromatogram of *Commiphora schimperi* stem extract.

Table A15: Retention time, percentage integration area and UV maxima for peaks from *Commiphora schimperi* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.717	263.8 ; 307.6	0.60
4.116	206.3 ; 278.0	1.73
4.623	263.8	0.11
4.877	206.3 ; 263.8	0.67
5.512	206.3 ; 278.0	2.36
10.112	207.4 ; 278.0	2.13
12.506	205.1 ; 278.0	5.11
12.880	202.8 ; 278.0	1.39
13.108	202.8 ; 278.0	1.01
13.446	202.8 ; 278.0	4.31
13.860	202.8 ; 278.0	17.22
14.295	201.6 ; 278.0	3.35
14.862	202.8 ; 278.0	22.36
15.243	201.6 ; 278.0	4.56
15.625	201.6 ; 278.0	3.75
15.982	201.6 ; 280.3	1.33
18.384	201.6 ; 278.0	1.59
20.673	281.5	18.60
32.596	241.4 ; 294.5	0.25
33.438	245.0 ; 288.6 ; 332.5	0.21
45.080	269.7 ; 330.1	1.40
49.866	278.0 ; 324.2	1.65

***Commiphora schimperi* (leaf)**

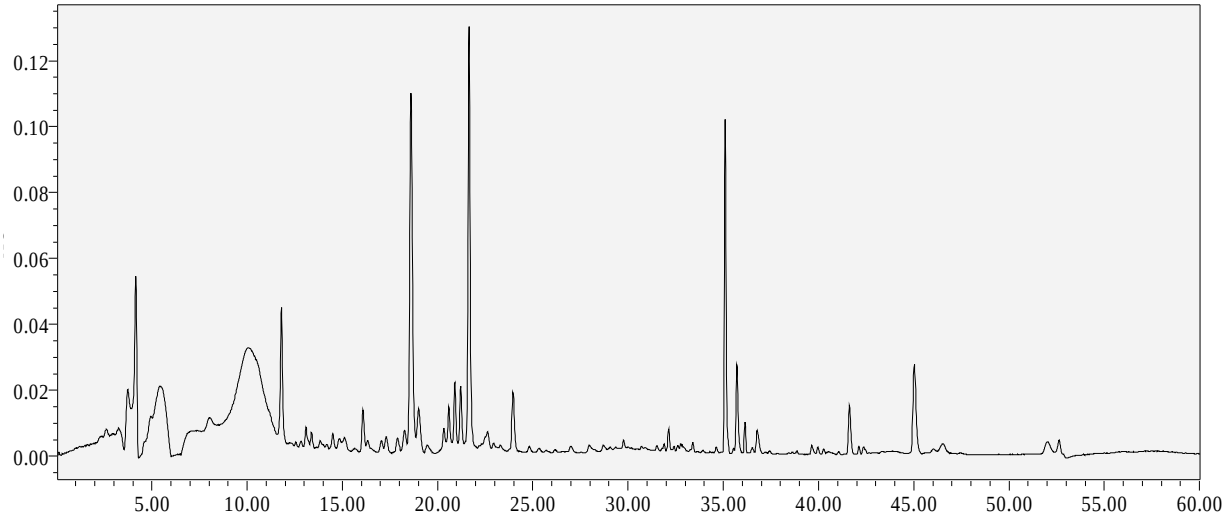

Figure A16: HPLC chromatogram of *Commiphora schimperi* leaf extract.

Table A16: Retention time, percentage integration area and UV maxima for peaks from *Commiphora schimperi* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.739	201.6 ; 265.0	3.83
4.152	207.4 ; 278.0	8.08
4.658	201.6 ; 265.0	0.64
4.951	201.6 ; 260.3	2.88
5.436	201.6 ; 278.0	12.49
11.806	219.1 ; 278.0 ; 323.0	4.38
16.087	229.7 ; 260.3 ; 356.3	1.29
18.270	253.2 ; 354.0	1.05
18.604	208.6 ; 261.5 ; 349.2	15.80
19.004	254.4 ; 354.0	2.33
20.593	255.6 ; 354.0	1.52
20.905	255.6 ; 354.0	2.17
21.210	255.6 ; 354.0	2.05
21.645	255.6 ; 348.0	13.96
23.961	263.8 ; 339.7	2.37
29.761	265.0 ; 308.8 ; 365.6	0.34
35.101	237.9	9.07
35.714	278.0	2.72
39.647	278.0 ; 308.8 ; 337.3 ; 365.6	1.03
41.626	278.0 ; 325.4	1.80
45.028	268.5	4.36

***Commiphora tenuipetiolata* (stem)**

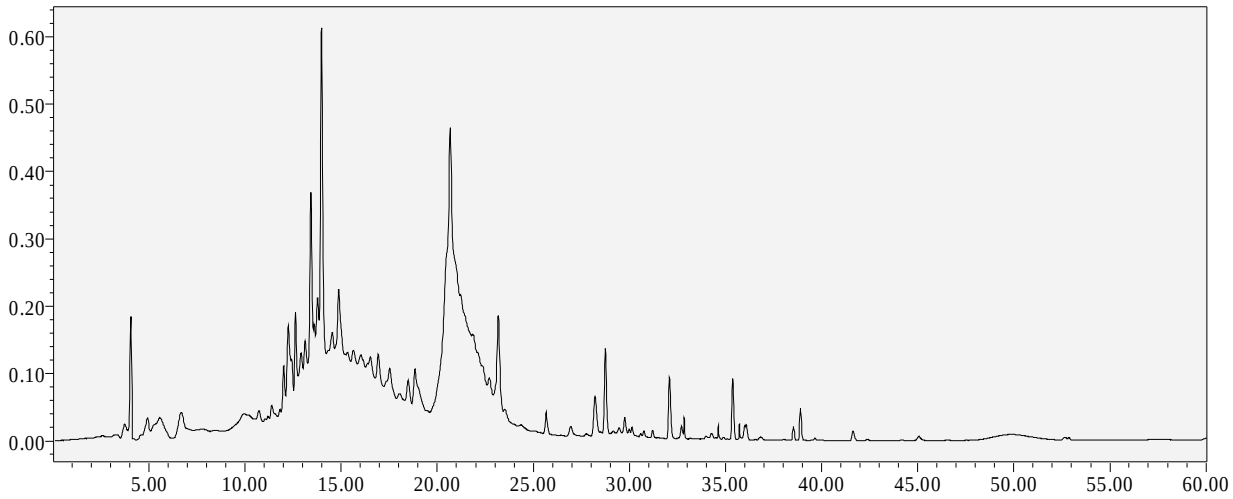

Figure A17: HPLC chromatogram of *Commiphora tenuipetiolata* stem extract.

Table A17: Retention time, percentage integration area and UV maxima for peaks from *Commiphora tenuipetiolata* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
4.055	207.4 ; 278.0	3.34
6.682	215.6 ; 270.9	1.02
12.010	205.1 ; 270.9	1.68
12.249	201.6 ; 278.0	5.42
12.623	201.6 ; 278.0	3.06
12.913	202.8 ; 278.0	2.26
13.124	202.8 ; 273.3	2.58
13.426	202.8 ; 278.0	9.19
13.774	202.8 ; 278.8	3.47
13.977	203.9 ; 278.0	14.53
14.537	202.8 ; 359.4	2.78
14.874	202.8 ; 276.8 ; 352.8	4.22
20.672	283.9	22.53
23.176	225.0 ; 288.6	4.07
28.210	239.1	2.09
28.747	228.5 ; 288.6	3.07
29.755	243.8 ; 266.2 ; 320.6 ; 363.4	0.52
32.085	229.7 ; 287.4	2.27
32.851	261.5	0.71
34.630	273.3	0.93
35.379	241.4 ; 318.3	2.04
38.898	247.3 ; 306.4 ; 334.9	1.00

***Commiphora tenuipetiolata* (leaf)**

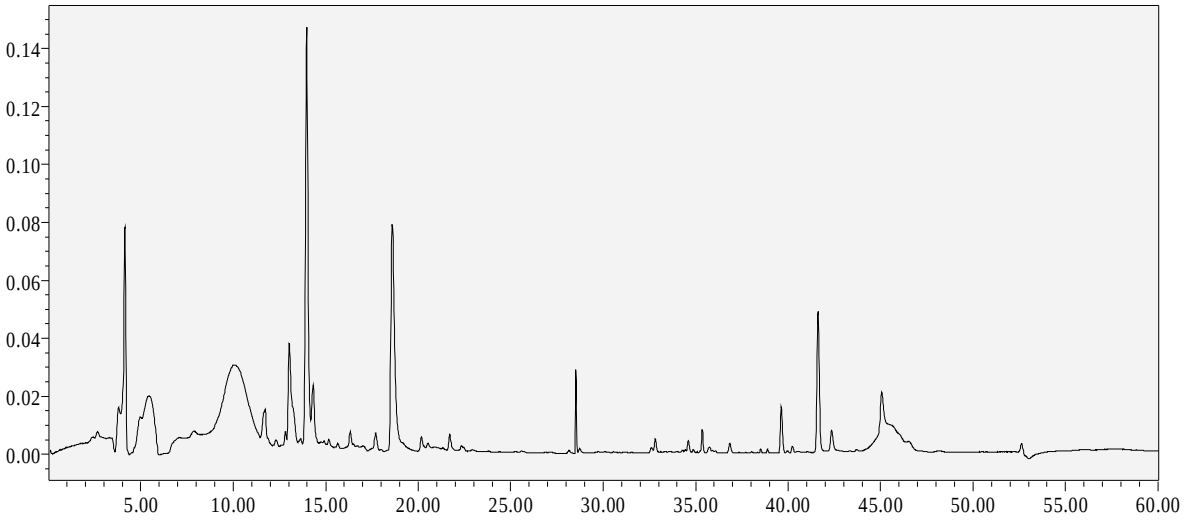

Figure A18: HPLC chromatogram of *Commiphora tenuipetiolata* leaf extract.

Table A18: Retention time, percentage integration area and UV maxima for peaks from *Commiphora tenuipetiolata* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
2.655	205.1 ; 279.2 ; 291.0	7.39
4.144	207.4 ; 278.0	6.71
4.979	201.6 ; 278.0	2.07
5.437	205.1 ; 278.0	7.43
6.767	210.9 ; 269.7	0.27
9.233	205.1 ; 278.0	7.99
10.096	207.4 ; 278.0	23.01
11.714	219.1 ; 288.6 ; 326.6 ; 399.2	1.76
13.026	223.5 ; 278.0 ; 399.2	4.45
13.969	221.5 ; 278.0	13.10
14.323	228.5 ; 253.2 ; 349.2	1.91
15.167	233.2 ; 268.2	0.13
16.329	254.4 ; 349.2	0.34
17.703	255.6 : 356.3	0.50
18.604	253.2 ; 264.4	9.83
21.704	252.0 ; 349.2	0.40
28.531	254.4 ; 272.1	1.17
35.360	246.1 ; 315.9	0.46
39.635	291.0 ; 327.8 ; 368.0	1.11
41.615	272.1 ; 324.2	3.99
42.358	272.1 ; 324.2	0.60
45.068	266.2 ; 331.3	1.45

***Commiphora viminea* (stem)**

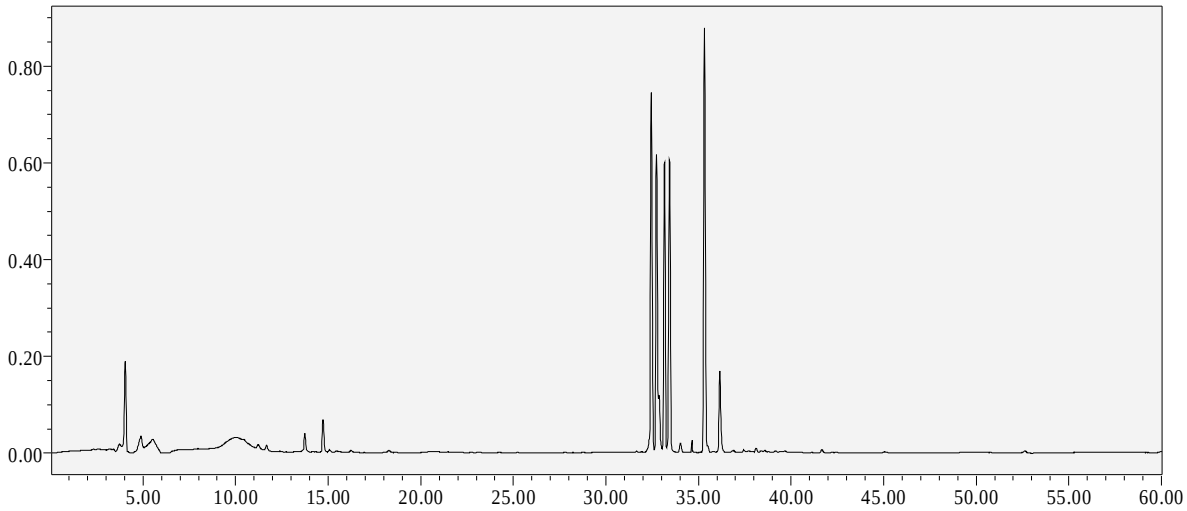

Figure A19: HPLC chromatogram of *Commiphora viminea* stem extract.

Table A19: Retention time, percentage integration area and UV maxima for peaks from *Commiphora viminea* stem extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.725	202.8 ; 263.8 ; 343.2	0.64
4.040	207.4	4.36
4.890	205.1 ; 259.1	1.70
5.521	206.3 ; 278.0	3.13
6.872	203.9	1.07
9.964	205.1 ; 278.0	3.37
13.743	202.8 ; 278.0	0.85
14.725	202.8 ; 278.0	1.50
32.454	206.3 ; 269.7	16.83
32.734	207.4 ; 234.4 ; 269.7	15.27
33.160	208.6 ; 236.7 ; 274.4	11.99
33.440	208.6 ; 237.9 ; 273.3	13.65
34.022	246.1 ; 278.0	0.44
34.650	265.0 ; 308.8	0.25
35.324	207.4 ; 234.4 ; 269.7	18.82
36.157	226.2 ; 269.7 ; 329.0	3.82

***Commiphora viminea* (leaf)**

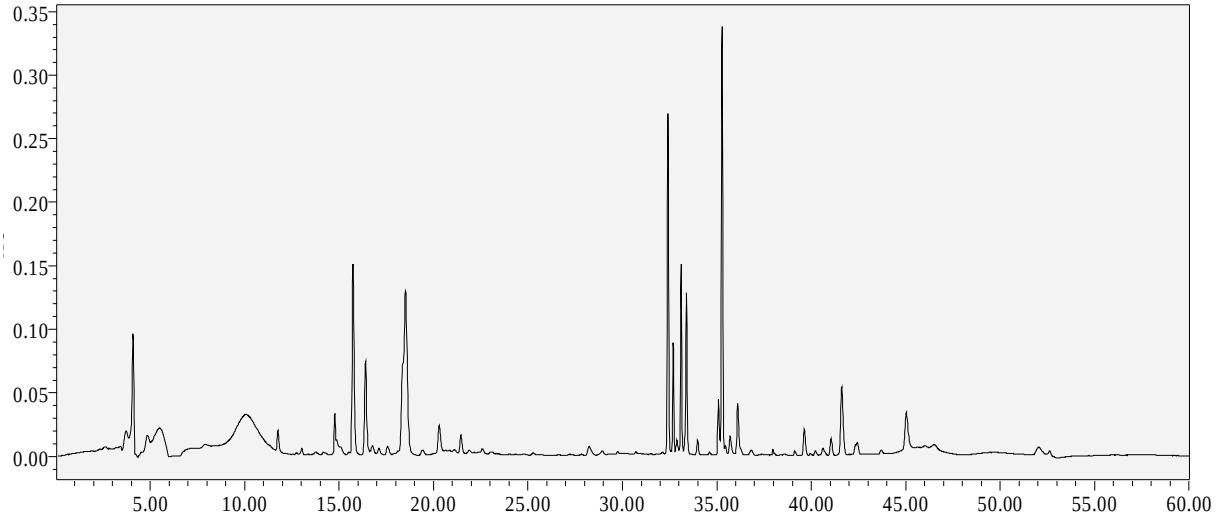

Figure A20: HPLC chromatogram of *Commiphora viminea* leaf extract.

Table A20: Retention time, percentage integration area and UV maxima for peaks from *Commiphora viminea* leaf extract.

Retention time (min)	UV maxima (nm)	% Integration area
3.726	203.9 ; 262.6 ; 339.7	1.69
4.085	207.4 ; 278.0	5.57
4.857	203.9 ; 262.6 ; 311.1	0.76
14.778	203.9 ; 229.7 ; 278.0	1.56
15.740	212.1 ; 269.7 ; 348.0	8.85
16.397	213.3 ; 255.6 ; 348.0	4.68
18.519	214.5 ; 269.7 ; 336.1	15.31
19.416	255.6 : 356.3	0.32
20.301	222.7 ; 278.0 ; 323.0 ; 336.1	1.48
21.445	265.0 ; 348.0	0.85
28.242	246.1	2.22
32.413	206.3 ; 235.6 ; 269.7	10.70
32.689	237.9 ; 269.7 ; 366.8	3.00
32.894	281.5 ; 366.8	0.64
33.117	208.6 ; 239.1 ; 274.4 ; 366.8	5.02
33.394	208.6 ; 240.3 ; 273.3 ; 366.8	5.04
33.981	247.3 ; 274.4	0.58
35.088	239.1	2.10
35.281	207.4 ; 237.9 ; 269.7 ; 339.7	13.70
36.112	269.7	2.49
41.615	272.1 ; 324.2	3.82
45.033	266.2 ; 340.9	2.61

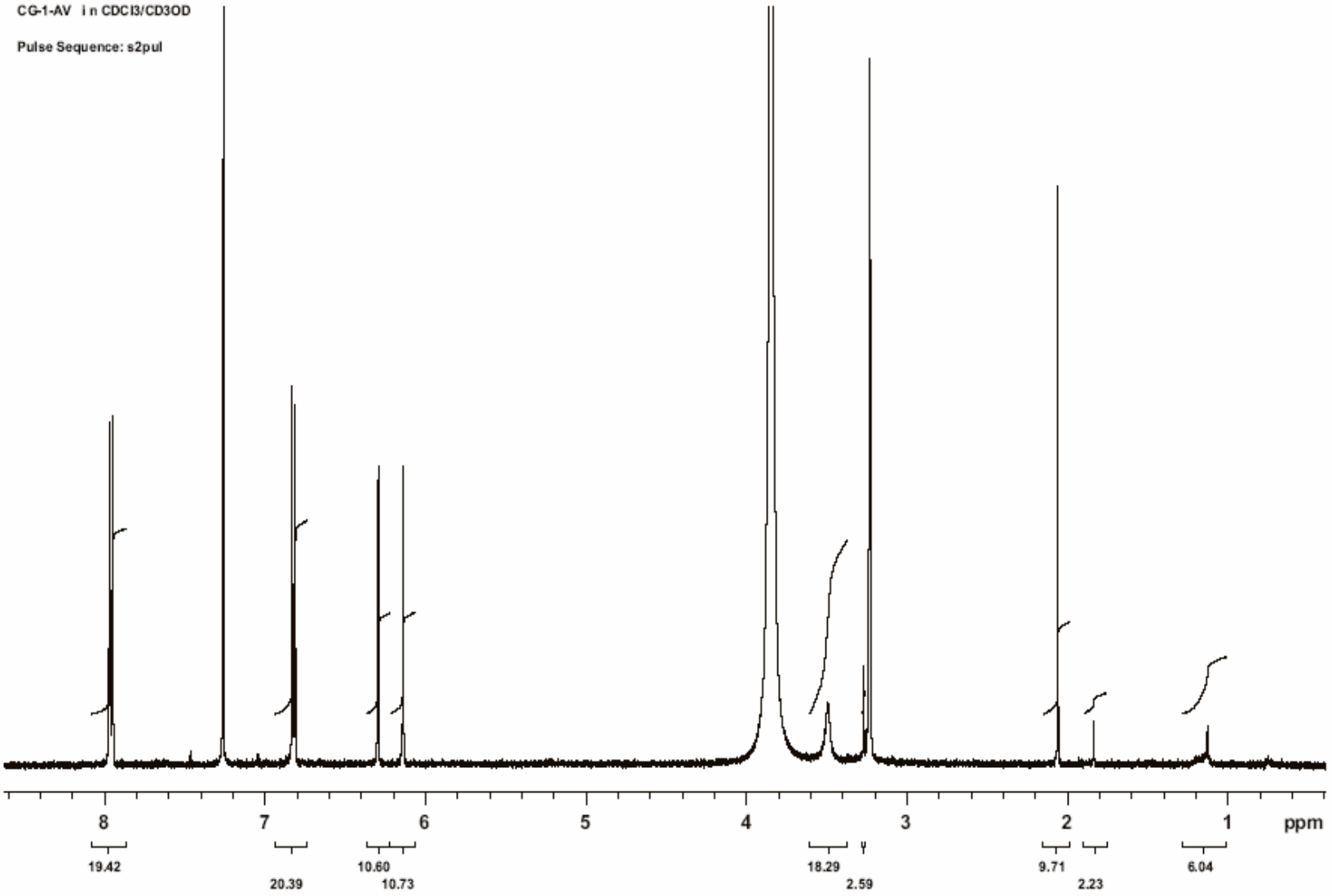

Figure B1: ^{1}H NMR spectrum of Compound **1**

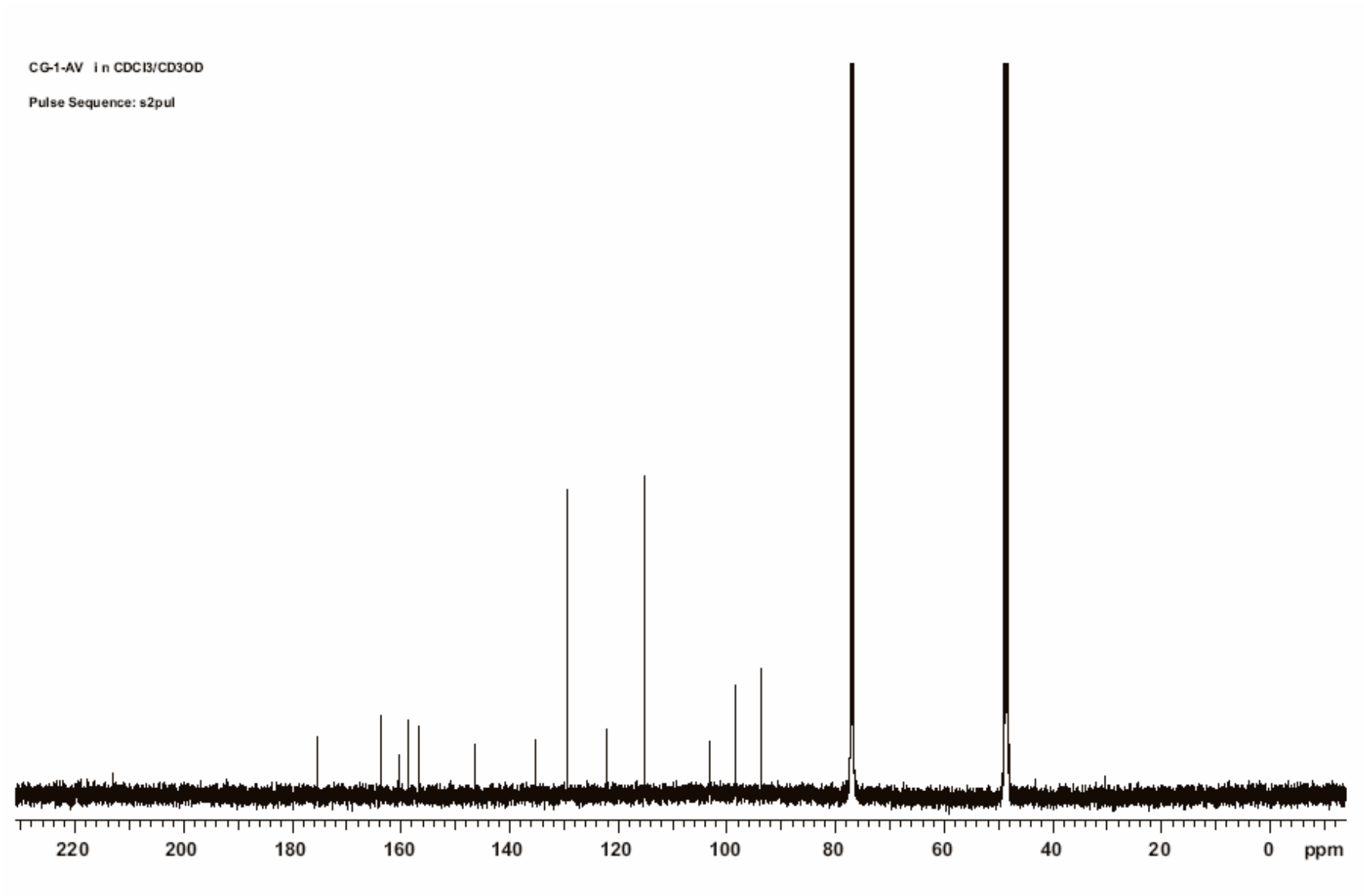

Figure B2: ^{13}C NMR spectrum of Compound **1**

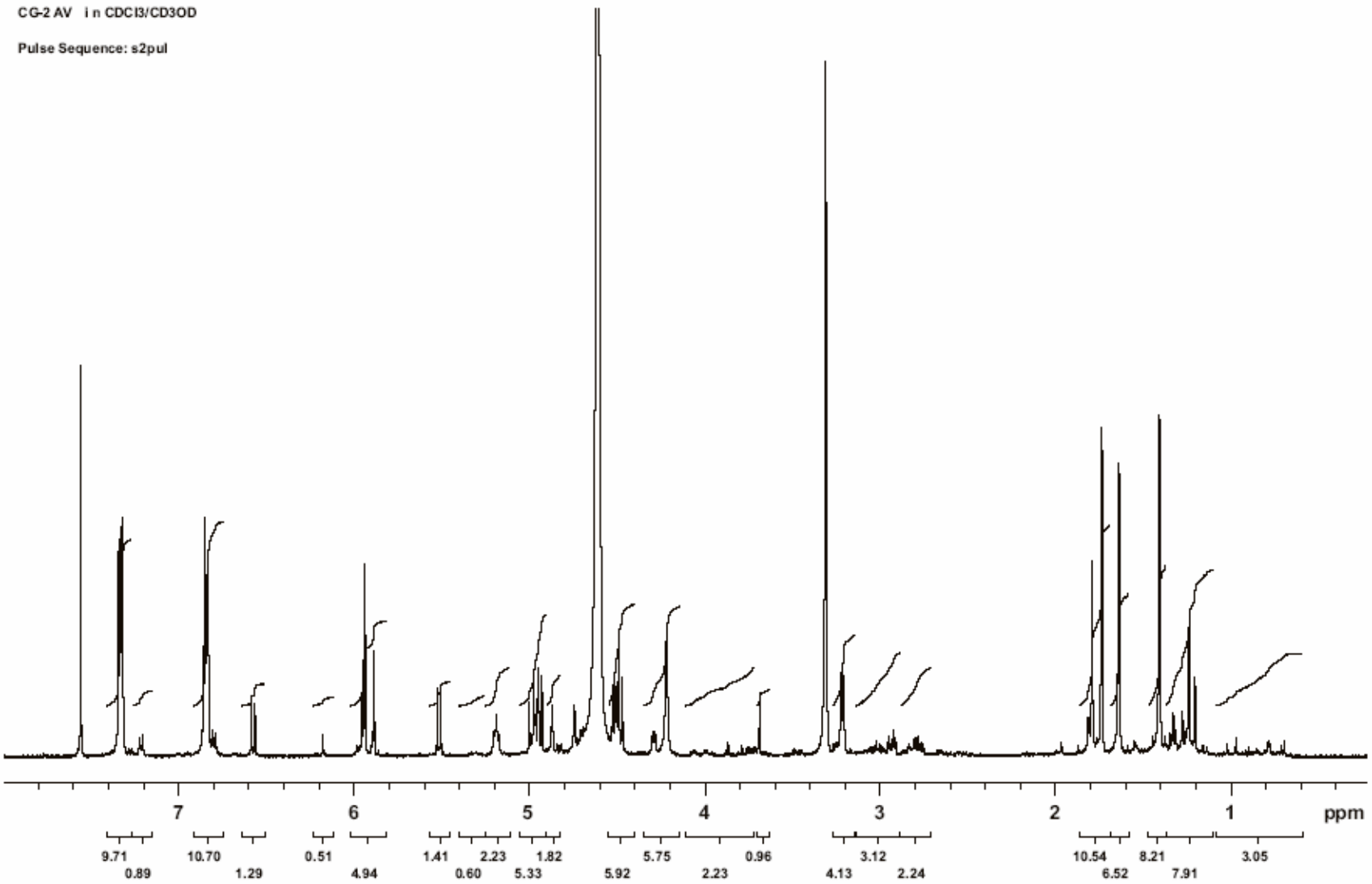

Figure B3: ^{1}H NMR spectrum of Compound **2**

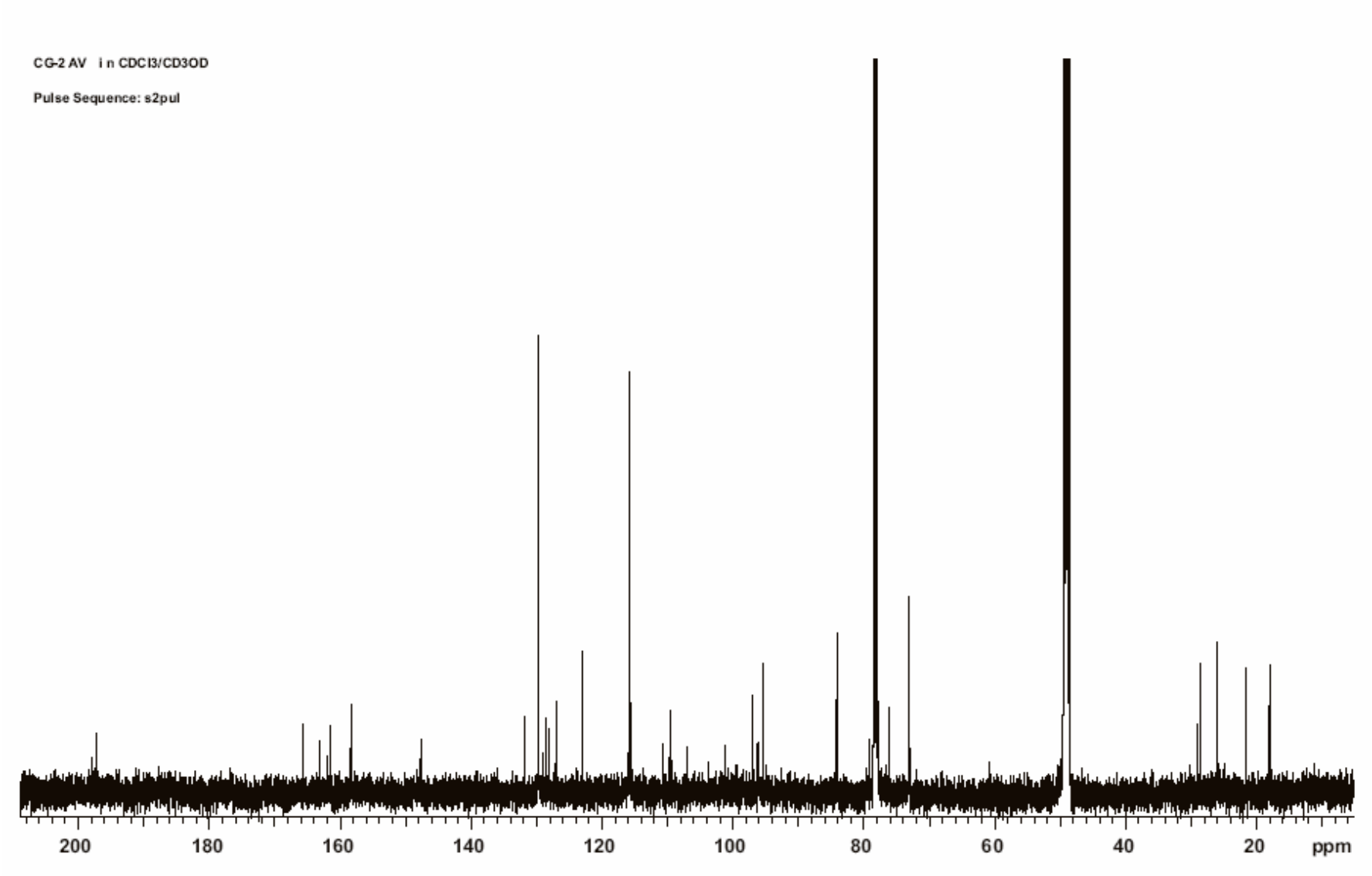

Figure B4: ^{13}C NMR spectrum of Compound **2**

APPENDIX C – ABSTRACTS PRESENTED AT CONFERENCES

The pharmacological activity of ten species of *Commiphora* indigenous to South Africa.

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Commiphora (Burseraceae) is used traditionally in southern for the treatment of stomach ailments, ulcers, fevers, and as a remedy for snake and scorpion bites. In various parts of western Africa, the macerated stem is used in the treatment of rheumatic conditions. The resin of a number of *Commiphora* species is applied topically for wound healing. It has been documented that certain *Commiphora* species possess antibacterial and antifungal properties. Cytotoxic and cytostatic activities have also been reported.

Extracts of both the bark and leaf of ten *Commiphora* species were prepared to test the *in vitro* antimicrobial, antioxidant, cytotoxic as well as anticancer activities.

The antimicrobial efficacy [minimum inhibitory concentration (MIC) microtiter plate assay] ranged between 1 mg/ml and 8 mg/ml with respect to the Gram-negative bacteria. Greater sensitivities were observed for Gram-positive organisms when tested on *C. marlothii, C. pyracanthoides* and *C. glandulosa,* with the MIC value of ≤ 0.125 mg/ml against *Bacillus cereus.*

Extracts generally exhibited poor anti-oxidant activity in the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, with the exception of *C. schimperi* (stem), *C. neglecta* (stem), *C. tenuipetiolata* (stem and leaf), and *C. edulis* (stem), which possessed IC_{50} values ranging between 7.31 μg/ml and 10.81 μg/ml. The activity was also assessed qualitatively using a TLC plate.

The effect of *Commiphora* extracts on the growth of human tumour cell lines (SF-268 and MCF-7) was observed using the sulforhodamine B (SRB) assay. *C. pyracanthoides* (leaf and bark) and *C. glandulosa* (leaf and bark) were active in both cell lines. Some species exhibited

anticancer activity with some degree of cytotoxicity against transformed human kidney epithelium cells as assessed by the MTT (3-[4,5-dimethylthiazol-2yl]-2,5 diphenyltetrazolium bromide) cellular viability assay, indicating a degree of selectivity against the different tumour cell lines.

The biological activity of ten species of *Commiphora* indigenous to South Africa.

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Department of Pharmacy and Pharmacology, Faculty of Health Sciences, University of the Witwatersrand, York Road, Parktown 2193, South Africa

Commiphora (Burseraceae) is used **traditionally** in southern and western Africa for the treatment of stomach ailments, ulcers, fevers, rheumatic conditions and as a remedy for snake and scorpion bites.The resin of a number of *Commiphora* species is applied topically for wound healing**. Documented** uses of *Commiphora* species (not indigenous to Southern Africa) are antibacterial and antifungal properties, cytotoxic and cytostatic activities, as well as antioxidant activity.

Solvent extracts of both the bark and leaf of ten indigenous *Commiphora* species were prepared to test the *in vitro* antimicrobial, antioxidant, anti-inflammatory, anticancer, as well as cytotoxicity activity.

The antimicrobial efficacy against Gram-positive, Gram-negative bacteria and yeasts was assessed using the MIC microtiter plate assay. The MIC with respect to Gram-negative bacteria ranged between 1 mg/ml and 8 mg/ml. Greater sensitivities were observed for Gram-positive organisms when tested on *C. marlothii, C. pyracanthoides* and *C. glandulosa,* with the MIC value of $\leq$ 0.125 mg/ml against *Bacillus cereus.*

Extracts generally exhibited poor anti-oxidant activity in the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, with the exception of *C. schimperi* (stem), *C. neglecta* (stem), *C. tenuipetiolata* (stem and leaf), and *C. edulis* (stem), which possessed IC_{50} values ranging between 7.31 μg/ml and 10.81 μg/ml. The activity was also assessed qualitatively using a TLC plate.

The anti-inflammatory activity of the extracts was carried out through the use of the *in vitro* 5-LOX assay. The anti-inflammatory activity displayed by each of the extracts at 100ppm

varied. The species that displayed the greatest 5-LOX anti-inflammatory activity were *C. pyracanthoides* and *C. glandulosa.*

The effect of *Commiphora* extracts on the growth of human tumour cell lines (SF-268 and MCF-7) was ascertained using the sulforhodamine B (SRB) assay. *C. pyracanthoides* (leaf and bark) and *C. glandulosa* (leaf and bark) were active in both cell lines. Some species exhibited anticancer activity with some degree of cytotoxicity against the representative normal cell population assessed by the MTT (3-[4,5-dimethylthiazol-2yl]-2,5 diphenyltetrazolium bromide) cellular viability assay.

Bio-autographic guided isolation was used to try isolate the anti-oxidant compound(s) from the most active species.

The antibacterial and anti-oxidant activity of South African indigenous *Commiphora* species and the isolated compounds from *C. glandulosa*.

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[2] School of Chemistry, University of KwaZulu-Natal, Corner, Scottsville, Pietermaritzburg 3209, South Africa
[3] School of Pharmacy, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa. *Correspondence: ViljoenAM@tut.ac.za

Commiphora species (from which Myrrh is obtained) has been a source of several novel and bioactive natural compounds. The botanical diversity of this genus in South Africa warrants a study of this plant group, to provide scientific evidence for the traditional use of *Commiphora* species in African healing rites. Traditionally members of this genus are used in southern Africa for the treatment of ulcers, fevers, and as a remedy for snake and scorpion bites. The resin of some *Commiphora* species is applied topically for wound healing. Documented uses of *Commiphora* include antibacterial and antifungal properties, as well as anti-oxidant activity.

In vitro antimicrobial efficacy was determined against Gram-positive, Gram-negative bacteria and yeasts using the MIC microtitre plate assay. Using death kinetics studies (time-kill studies), the rate at which the antimicrobial agent kills pathogens over a 24 hour period was determined. *Commiphora marlothii* (stem) was identified as a suitable candidate for the death kinetics assay (MIC = 1 mg/ml against *S. aureus*). The antibacterial activity was observed to begin at ca. 30 min of the exposure of *S. aureus* to the different concentrations of plant extract, as observed through the reduction in colony forming units (CFU) over time. All concentrations exhibited antibacterial activity, with complete bactericidal effect achieved by all test concentrations by the 24th hour.

The *in vitro* anti-oxidant activity of the leaf and stem extracts of ten *Commiphora* species was investigated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) assays. Isolated compounds were subjected to the DPPH assay to determine the anti-oxidant potential of each of the compounds, separately and in combination to determine possible synergistic, antagonistic or additive interactions. Extracts generally exhibited poor anti-oxidant activity in the DPPH

assay, with the exception of *C. schimperi* (stem), *C. neglecta* (stem), *C. tenuipetiolata* (stem and leaf), and *C. edulis* (stem), which possessed IC_{50} values ranging between 7.31 μg/ml and 10.81 μg/ml. The flavonol, kaempferol (IC_{50} = 3.32 μg/ml) showed exceptional radical scavenging activity, in contrast to the activity displayed by dihydrokaempferol (IC_{50} = 301.57 μg/ml), their combination being antagonistic. The results obtained in the ABTS assay differed significantly from the results obtained in the DPPH assay, with a greater anti-oxidant activity observed for most of the species. The best activity was observed for the stem extracts of *C. neglecta* (IC_{50} = 7.28 μg/ml) and *C. mollis* (IC_{50} = 8.82 μg/ml).