THE PHYTOCHEMISTRY AND BIOLOGICAL ACTIVITIES OF *ATHRIXIA PHYLICOIDES*

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DECLARATION

I, Kumeshnie Padayachee declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature……..

……day of……2011

DEDICATION

I dedicate this research report to
my husband, Prinevin
my son, Praveen
and
my parents, Kassey and Savie

For always loving and supporting me
and
for all the sacrifices you have made for me.

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ABSTRACT

Herbal medicines are an important part of the African culture and tradition and about 80% of Africa's population relies on traditional remedies for their primary health care needs (WHO, 2008). *Athrixia phylicoides* is widely used as a traditional remedy, but despite its substantial use, literature on its chemical composition and biological activities is limited. In this study, the chemical composition of the essential oil was determined using gas chromatography combined with mass spectroscopy (GC/MS) and analysis resulted in the identification of 182 compounds. The major compounds identified in selected samples were α-pinene, β-pinene, caryophyllene oxide, β-caryophyllene, myrcene and spathulenol. Based on the traditional uses of *A. phylicoides*, various *in vitro* biological activities were investigated. The extensive use of *A. phylicoides* for the treatment of boils, sores, bad acne, infected wounds and cuts, prompted the antimicrobial (bacterial and fungal) study using the minimum inhibitory concentration (MIC) assay. The methanol extract and essential oil were more selective for the Gram-positive bacteria than the Gram-negative bacteria. The extract exhibited stronger activity against all micro-organisms tested compared to the essential oil, with the highest activity against *S. aureus*, *B. cereus* and *B. subtilis* (MIC = 1 mg/ml). The bioactive compound, (4-hydroxyphenyl) propyl coumaroate was isolated from the extract and proved to be most active against *S. aureus* (MIC = 19.5 µg/ml). Using the DPPH$^{\bullet}$ assay, a comparative anti-oxidant study was performed. The anti-oxidant activity of the aqueous extract of *A. phylicoides* (IC_{50} = 14.01 ± 2.68 µg/ml) was greater than rooibos and Ceylon (black) tea (IC_{50} > 25.00 µg/ml); comparable to green rooibos and honeybush tea (IC_{50} = 18.01 ± 4.06 µg/ml; 18.02 ± 4.27 µg/ml, respectively), but less active than green tea (IC_{50} = 9.64 ± 0.96 µg/ml). The antimalarial activity was determined using the [^{3}H] hypoxanthine incorporation method. The essential oil exhibited greater antimalarial activity against the chloroquine-resistant *Plasmodium falciparum* (FCR-3) strain (IC_{50} = 1.006 ± 0.06 µg/ml) than the methanol extract. The 5-lipoxygenase assay was used to assess the anti-inflammatory activity of the methanol extract and essential oil. Only the essential oil displayed anti-inflammatory activity (IC_{50} = 25.68 µg/ml). Low safety indices were reported for both the methanol extract (SI = 2.28) and essential oil (SI = 1.77). The *in vitro* biological activities may validate the use of *A. phylicoides* in traditional medicine.

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LIST OF ABBREVIATIONS AND SYMBOLS

% Percent

˚**C**: Degrees celsius

CAT: Catalase

CFU: Colony forming unit

CO_2: Carbon dioxide

COSY: Correlation spectroscopy

COX: Cyclooxygenase

DEPT: Distortionless enhanced polarization transfert

DPPH: 2,2-diphenyl-1-picrylhydrazl

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EC_{50}: Extract concentration 50%

EDTA: Ethylenediaminetetraacetic acid

EIMS: Electron ionization mass spectrometry

eV: Electron volt

FID: Flame ionization detection

FCS: Foetal calf serum

GC: Gas chromatography

GC/MS: Gas chromatography coupled to mass spectrometry

GPX: Gluthathione peroxidase

Gram -ve: Gram-negative

Gram +ve: Gram-positive

H_2O_2: Hydrogen peroxide

HEPES: [N-(2-hydroxethyl)piperazine-N'-(2-ethanesulfonic acid)]

HP: Hewlett Packard

HPETE: Hydroperoxyeicosatetraenoic

HPLC: High pressure liquid chromatography

IC_{50}: Inhibitory concentration (Concentration that reduced the effect by 50%)

INT: ρ-Iodonitrotetrazolium

LC_{50}: Lethal concentration 50%

LT: Leukotrienes

m: Meter

mg: Milligram

MH_Z: Megahertz

ml: Millilitre

mm: Millimeter

mM: Millimolar

MIC: Minimum inhibitory concentration

min: Minutes

MS: Mass spectroscopy

MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

n: Number of repetitions

N_2: Nitrogen

$NaHCO_3$: Sodium hydrogen carbonate

ND: Not determined

NIST: National Institute of Standards and Technology

NDGA: Nordihydroguaiaretic

NMR: Nuclear magnetic resonance

No.: Number

NSAIDs: Non-steroidal anti-inflammatory drugs

$O_2^{\bullet}$: Superoxide radical

$OH^{\bullet}$: Hydroxyl radicals

PG: Prostaglandin

pH: Potential hydrogen

ppm: Parts per million

rpm: Revolutions per minute

RPMI 1640: Roswell Park Memorial Institute 1640

RRI: Relative retention index

SANBI: South African National Botanical Institute

SI: Safety index

s.d.: Standard deviation

SOD: Superoxide dismutase

t: Trace

TEAC: Trolox™ equivalent anti-oxidant capacity

TLC: Thin layer chromatography

TXA_2: Thromboxane

µl: Microlitre

µm: Micrometer

µM: Micromolar

UV: Ultraviolet

UV-VIS: Ultraviolet-visible

WHO: World Health Organisation

v/v: Volume per volume

w/v: Weight per volume

w/w: Weight per weight

XTT: Sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate

Chapter 1 – General introduction

1.1 Medicinal plants and their place in our society

"Medicinal plants are something of the future, not the past" (van Wyk *et al.,* 2002).

Herbal medicines are an important part of the culture and tradition of African people (Fennell *et al*., 2004). The World Health Organisation (WHO) estimates that in some African countries, up to 80% of the population relies on traditional *materia medica* (medicinal plants) for their primary health care needs (WHO, 2008).

Africa is a continent endowed with an enormous wealth of plant resources. Southern Africa has well over 30 000 species of higher plants. In South Africa, approximately 3 000 species have been used for several centuries as medicine for the prevention and treatment of diseases (van Wyk *et al.,* 2002). Some renowned African medicinal plants include *Acacia senegal* (gum Arabic), *Agathosma betulina* (buchu), *Aloe ferox* (Cape aloes), *Artemisia afra* (African wormwood), *Aspalathus linearis* (rooibos tea), *Boswellia sacra* (frankincense), *Catha edulis* (khat), *Commiphora myrrha* (myrrh), *Harpagophytum procumbens* (devil's claw), *Hibiscus sabdariffa* (hibiscus or roselle), *Hypoxis hemerocallidea* (African potato) and *Prunus africana* (African cherry or red stinkwood) (van Wyk and Wink, 2004).

It is believed that the profound knowledge of medicinal plants was developed through trial and error over several centuries and the most important remedies were passed on verbally from one generation to the next. The founders and users of the medicinal plants may not have understood the scientific rationale behind the efficacy of these plants, but knew from personal experience that they were highly effective (van Wyk and Wink, 2004).

History reveals that for centuries plants have been the main source of medicine for most cultures. The long history of herbal medicine and the number of allopathic drugs derived from plant material (Table 1.1) is an indicator that medicinal plants are effective and can stimulate the discovery of new compounds for the treatment of new and old diseases.

Table 1.1: Examples of active compounds isolated from plants.

Plant	Active compound	Use/Action	Literature source
Aloe ferox	Aloe emodin Chrysophanol Aloin	Purgative	Kambizi *et al*., 2004
Artemisia annua	Artemisinin	Antimalarial	Clark, 1996
Atropa belladonna	Atropine	Anticholinergic	De Smet, 1997; Rates, 2001
Camellia sinesis	Theophylline Caffeine	Bronchodilator CNS stimulant	De Smet, 1997
Cassia spp.	Sennosides A&B	Laxative	De Smet, 1997
Catharanthus roseus	Vinblastine & Vincristine	Antitumor	De Smet, 1997; Rates, 2001
Chondodendron tomentosum	Tubocurarine	Skeletal muscle relaxant	De Smet, 1997
Cinchona ledgeriana	Quinine Quinidine	Antimalarial Antiarrhythmic	De Smet, 1997; Rates, 2001
Colchicum autumnale	Colchicine	Anti-gout	De Smet, 1997
Digitalis purpurea	Digoxin	Cardiotonic	De Smet, 1997; Rates, 2001
Ephedra sinica	Ephedrine	Sympathomimetic	De Smet, 1997
Gaultheria procumbens	Methyl salicylate	Rubefacient	De Smet, 1997
Helichrysum cymosum	Helihumulone	Antibacterial and antifungal (yeasts)	van Vuuren *et al*., 2006
Hyoscyamus niger	Hyocyamine	Anticholinergic	De Smet, 1997
Larrea divaricata	Nordihydroguaiaretic acid	Anti-oxidant	De Smet, 1997
Mucuna deeringiana	Levodopa	Anti-parkinsonism	De Smet, 1997
Papaver somniferum	Codeine Morphine Noscapine	Analgesic/antitussive Analgesic Antitussive	De Smet, 1997; Rates, 2001
Physostigma venenosum	Physostigmine	Cholinesterase inhibitor	De Smet, 1997
Pilocarpus jaborandi	Pilocarpine	Parasympathomimetic	De Smet, 1997
Podophyllum peltatum	Podophyllotoxin	Topical treatment	De Smet, 1997
Rauwolfia serpentine	Reserpine	Antihypertensive	De Smet, 1997
Salix capensis	Catechol 2-Hydroxybenzyl alcohol	Antibacterial	Masika *et al*., 2005
Salvia chamelaeagnea	Carnosol	Antibacterial	Kamatou *et al*., 2007
Terminalia sericea	Anolignan	Antibacterial	Eldeen *et al*., 2006

The integration of medicinal plants into the mainstream healthcare system in South Africa presents its own challenges with the following to overcome (WHO, 2008 *adapted*):

- National policy and regulation: The prescription and use of medicinal plants is currently not regulated. The result is that there is always the danger of misadministration, especially of toxic plants.
- Patient safety and use: It is believed that because herbal medicines are natural, they are safe. Herbal medicines can cause harmful adverse reactions, if the medicine is of a poor quality, taken incorrectly or in conjunction with modern medicines.
- Scientific evidence: Evidence from *in vivo* studies to evaluate the safety and efficacy of traditional medicines is limited.
- Sustainability: Herbal medicines are collected from wild plant populations and cultivated from medicinal plants. Poorly managed collections and cultivations could lead to the extinction of endangered plant species and destruction of natural resources.
- Knowledge: The use of African traditional medicine is not well documented and this is becoming increasing urgent due to the rapid loss of the natural habitat of some of the plants.

Since only one-third of all diseases can be treated efficiently with currently available innovative drugs (Muller *et al.,* 2000), the need for research and development of medicinal plants is on the increase. Phytochemical and pharmacological studies of medicinal plants are essential to provide us with sound scientific data on their safety and efficacy and possibly provide mankind with a sound solution to the current problem of ineffective drugs.

1.2 *Athrixia phylicoides*

Athrixia phylicoides DC. is a well-known, multi-purpose, versatile plant and according to the literature, it is used as a tea and a medicine (van Wyk and Gericke, 2000). A recent survey revealed that *A. phylicoides* is still frequently utilised as a tea and a medicine by both rural and urban dwellers in South Africa (Figure 1.1A) and will be purchased if commercially available (Rampedi and Olivier, 2005). But despite the substantial demand for *A. phylicoides*, little scientific data on its chemical composition, toxicity and pharmacological activity has been published. In contrast to the lack of scientific data available for *A. phylicoides*, extensive

A

B

C

D

E

Figure 1.1: **A:** Speaking to a local woman collecting *A. phylicoides* in Haenertsberg.
B & C: *Athrixia phylicoides* during the non-flowering seasons.
D & E: *Athrixia phylicoides* during the flowering season.
[Source: Myself (A&C), A.M. Viljoen (B&E) and G. Mbambezeli (D)]

research on the chemical composition, pharmacological activity and toxicity has been performed on another well-known and frequently consumed tea, Rooibos tea (Rabe *et al*, 1994; von Gadow *et al*., 1997; Manzocco *et al*., 1998; McGaw *et al*., 2007; Almajano *et al*., 2008; Marnewick *et al*., 2009). Guided by the medicinal uses (Table 1.2) and the substantial demand for *A. phylicoides*, this study encompasses a phytochemical and pharmacological investigation of the indigenous plant. The biological activities investigated were based on the traditional uses of *A. phylicoides*. Its use as a substitute for tea prompted the investigation as an anti-oxidant agent and the comparative anti-oxidant activity with other commercially available teas. *Athrixia phylicoides* is also used to treat boils, sores, bad acne, infected wounds and cuts, and therefore its antimicrobial activity was studied. The anti-inflammatory activity of the plant was investigated because of its use to treat sore feet and sore throats. According to the WHO, malaria has been responsible for nearly one million deaths in 2008 as a result of drug-resistant strains of *Plasmodium falciparum* (WHO, 2010). These statistics prompted the investigation of *A. phylicoides* as an antimalarial agent. A toxicity study was conducted due to the substantial use and consumption of *A. phylicoides*, and the lack of safety data for the volatile oils.

1.2.1 Name derivation and common names

The genus name *Athrixia* is derived from the Greek word *thrix* meaning hair describing the appearance of the leaves. *Athrixia phylicoides* is commonly known as Bushman's tea, Bush tea, Zulu tea (English), Boesmanstee, Bostee (Afrikaans) and Icholocholo, Itshelo, Umthsanelo (Zulu) (Mbambezeli, 2005).

1.2.2 Description

Athrixia belongs to the Asteraceae family which is the largest family of flowering plants comprising of about 1 100 genera and 20 000 species. Nine species of the genus *Athrixia* occur in southern Africa, *A. phylicoides* and *A. elata* (wildtee) being the most common.

Athrixia phylicoides is an erect shrub with small aromatic leaves (Figure 1.1B/C). The upper surface of the leaf is dark green. The lower surface of the leaf is covered by a mass of fine woolly hair giving the shrub a silvery appearance. *Athrixia phylicoides* bears small purple flowers in late autumn (van Wyk and Gericke, 2000) (Figure 1.1D/E).

Table 1.2: Traditional uses of *A. phylicoides* as a medicine by various ethnic groups.

Medicinal use/ailments	Ethnic group	Part of plant used	Literature source
Tea	All communities	Not specified	Plowes and Drummond, 1976
	Vhavenda	Branches and leaves	Mabogo, 1990
	Zulu, Xhosa and Vhavenda	Leaves	Hutchings *et al*., 1996
Sore throats	Sotho and Xhosa	Leaves chewed	Roberts, 1990; Hutchings *et al*., 1996
Sore feet	Sotho	Decoction of leaves for bathing sore feet.	Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996
		A strong tea used as a soothing wash	Roberts, 1990
Cough remedy	All communities	Not specified	Plowes and Drummond, 1976
	Zulu	Root decoctions	Watt and Breyer-Brandwijk, 1962; Roberts, 1990; Hutchings *et al*., 1996
	Sotho and Xhosa	Leaves chewed	Roberts, 1990; Hutchings *et al*., 1996
Anti-helmintics	Vhavenda	Extracts from soaked leaves and roots	Mabogo, 1990; Hutchings *et al*., 1996
Aphrodisiac	Vhavenda	Root infusions	Hutchings *et al*., 1996; van Wyk and Gericke, 2000
Boils, bad acne, infected wounds and cuts	All communities	Leaves	Plowes and Drummond, 1976; Roberts, 1990
'Blood purifier' for sores and boils	Zulu	Plant infusion (no part specified)	Hutchings *et al*., 1996
Purgative	Zulu	Root decoctions	Watt and Breyer-Brandwijk, 1962; Roberts, 1990; Hutchings *et al*., 1996

1.2.3 Distribution

Athrixia phylicoides grows in the eastern hilly and mountainous parts of South Africa, from the Eastern Cape in the south to the Soutpanberg in the Limpopo Province in the north (Figure 1.2).

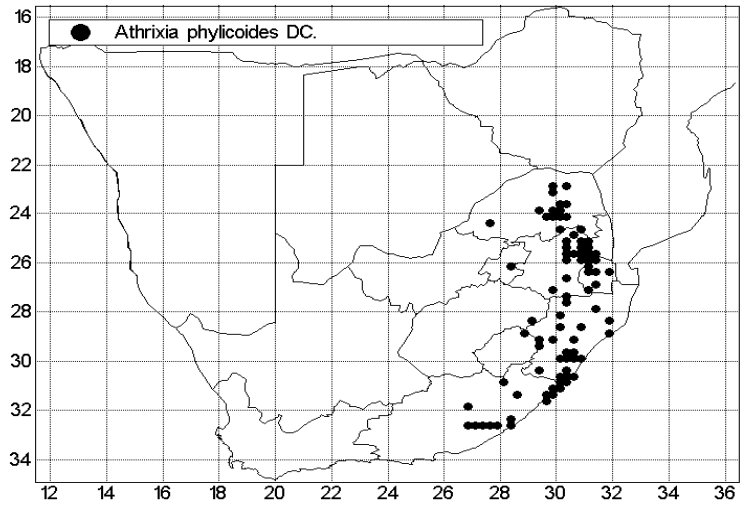


Figure 1.2: Distribution of *A. phylicoides* in South Africa (SANBI).

1.2.4 Traditional uses

Athrixia phylicoides is a multi-purpose plant with a variety of uses such as a tea, a medicine and for the making of brooms (van Wyk and Gericke, 2000). The attractive plant with its small purple flowers and silvery leaves is very useful for decorative purposes, alone in a vase or as foliage in flower arrangements (Olivier and De Jager, 2005). *Athrixia phylicoides* is also used to make baskets and is burnt as incense at ceremonies such as weddings and funerals (Olivier and De Jager, 2005). The medicinal use of *A. phylicoides* by various ethnic groups in South Africa is summarised in Table 1.2.

1.2.5 A review of previous studies on *A. phylicoides*

Most of the literature refers to the extensive traditional use of *A. phylicoides* (van Wyk and Gericke, 2000; Olivier and De Jager, 2005; Rampedi and Olivier, 2005) and thus, it is remarkable that only limited research has been conducted on this indigenous plant. This

implies that very little information on the chemical composition, toxicity and pharmacological activity of *A. phylicoides* is available.

Phytochemical studies conducted on *A. phylicoides* revealed the presence of chemical compounds known to possess pharmacological activity, such as flavonoids (5-hydroxy-6,7,8,3′,4′,5′-hexamethoxyflavon-3-ol; 3-O-demethyldigicitrin; 5,6,7,8,3′,4′-hexamethoxy-flavone and quercertin) and polyphenols (Mudau *et al*., 2006; Mashimbye *et al*., 2006; Mavundza *et al*., 2010). Further analysis revealed the absence of caffeine (CNS stimulant) stimulant) and pyrrolizidine alkaloids (plant toxins associated with disease in humans and animals) in *A. phylicoides* (McGaw *et al.*, 2007). A study on the nutrient content of *A. phylicoides* revealed the presence of approximately 13% non-structural carbohydrates, 8% proteins, 2.5% fatty acids, minerals (calcium, magnesium, phosphorus, potassium, sodium, iron, manganese, zinc, copper, aluminium, sulphur and fluoride) and traces of tannins and vitamins B_1, B_2, C and E (Olivier and Rampedi, 2008).

The effect of fermentation temperature and time on the chemical composition of *A. phylicoides* was investigated by Hlahla *et al*. (2010). The study revealed that neither fermentation temperature nor time had any significant influence on the anti-oxidant content, however, increasing fermentation temperature increased polyphenols and decreased tannin content. In addition increasing fermentation time increased both polyphenols and tannin content.

Few reports have been undertaken on the anti-oxidant and antimicrobial activity of *A. phylicoides*. McGaw *et al.* (2007) reported that the ethanol extracts of *A. phylicoides* exhibited anti-oxidant activity comparable to that found in rooibos when using Trolox™equivalent anti-oxidant capacity (TEAC) assay. It has also been reported that the ethanol extract of *A. phylicoides* displayed anti-oxidant activity in the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) free radical assay (Mavundza *et al*., 2007, 2010). Mavundza *et al*. (2007) reported that the ethanol extract displayed antimicrobial activity against five micro-organisms tested.

Some toxicity studies have been conducted on *A. phylicoides* (McGaw *et al.*, 2007; Chellan *et al*., 2008; Mavundza *et al*., 2010). *Athrixia phylicoides* traditional preparations (decoctions and infusions), aqueous and ethanol extracts were tested for toxic effects to brine shrimp larvae and the Vero kidney cell line. The traditional preparations and aqueous extract had little effect in both assays, but the ethanol extract was toxic. In another study, the ethanol extract showed low toxicity on Vero kidney cells (Mavundza *et al*., 2010). Chellan *et al*. (2008) investigated the cytotoxic effect of the aqueous extract of *A. phylicoides* on Wistar rats for three months, but the extract did not cause any morbidity or mortality.

Some reviews have been undertaken on *A. phylicoides*. In a review conducted by Joubert *et al*. (2008), it was noted that due to its substantial use, *A. phylicoides* is at high risk of over-exploitation. The review also confirmed that very few studies have been conducted on *A. phylicoides*, and to date it is the only indigenous South African tea that has been subjected to a sub-chronic toxicity study. In another review by Gulumian and Savolainen (2008), the results of the toxicity study conducted by Chellan *et al*. (2008) on *A. phylicoides* was discussed.

1.3 Objectives of the study

The objectives of the study were to:

- Investigate the chemical composition of the essential oil using gas chromatography (GC) and gas chromatography combined with mass spectrometry (GC/MS).
- Evaluate the antimicrobial activity of the essential oil and methanol extract on selected micro-organisms using the minimum inhibitory concentration method.
- Isolate and identify the compound(s) responsible for the antimicrobial activity.
- Investigate the anti-oxidant activity of the aqueous extract of *A. phylicoides* and to compare its activity to other commercially available teas.
- Investigate the effect of the methanol extract and essential oil on *Plasmodium falciparum*, the protozoan responsible for malaria.
- Investigate the anti-inflammatory activity of the methanol extract and essential oil of *A. phylicoides*.
- Determine the toxic properties of the methanol extract and essential oil.

Chapter 2 – Plant collection and preparation of samples

2.1 Collection of plant material

A total of seven samples of *A. phylicoides* were collected from two natural populations over a period of four months from February 2004 to May 2004. The sample populations were Haenertsberg in Limpopo and Dundee in KwaZulu-Natal. Pooled samples (a number of many individual plants) and two individual plants were collected (Table 2.1). Professors J. Olivier and A.M. Viljoen assisted with the collection of plants. The taxonomic identification of all samples was confirmed by botanists at the South African National Botanical Institute (SANBI) in Pretoria. Voucher specimens have been maintained at the Department of Pharmacy and Pharmacology at the University of Witwatersrand. Pooled samples for both the essential oils and extracts have been used in the antimicrobial, anti-oxidant, antimalarial, anti-inflammatory and toxicity studies.

Table 2.1: Plant collection, voucher and sample reference numbers, and percentage oil yield.

Plant Population	Month of collection	Type of sample	Voucher no.	Sample reference no.	Yield (%)
Haenertsberg	February	Pooled sample	AV914	1A	0.22
Haenertsberg	February	Individual	AV916	1B	0.17
Haenertsberg	February	Individual	AV917	1C	0.18
Haenertsberg	March	Pooled sample	AV936	2A	0.10
Dundee	March	Pooled sample	AV958	2B	0.17
Haenertsberg	April	Pooled sample	AV977	3A	0.47
Haenertsberg	May	Pooled sample	AV999	4A	0.31

2.2 Preparation of samples

2.2.1 Isolation of essential oils

The fresh aerial parts of the plant were allowed to dry for 24 hours at room temperature. Conventional hydrodistillation was then carried out using a Clevenger-type apparatus for three hours, to selectively isolate the essential oils from the aerial parts of the plant (Figure 2.1). The pure essential oils were collected and the essential oil yield (Table 2.1) was recorded prior to storing the essential oils in amber vials at 4 °C.

Figure 2.1: Hydrodistillation of plant material using the Clevenger-type apparatus.

2.2.2 Preparation of the solvent extracts

Fresh aerial parts were dried at room temperature for seven days and pulverized into a fine powder, using a mill (Diaf R/S, sieve no. 2). The pulverized aerial parts (5 g) were macerated for 24 hours with methanol (50 ml) at room temperature, than filtered through Whatman® No.1 filter paper (pore size: 20-25 μm). The extraction solvent was evaporated at 35 °C using a rotavapor (Büchi Rotavapor R-114). The extracts were then concentrated to dryness in the fume-hood. The concentrated extract was stored at 4 °C prior to analysis. A yield of 0.04% was recorded for extract of sample 1A. It is important to note that various solvent systems (acetone, methanol, dichloromethane, chloroform and ethanol) were assayed before methanol

was finally selected as the extracting solvent because this solvent extracted compounds over a range of polarities.

2.2.3 Preparation of aqueous tea infusions

Aqueous tea extracts were prepared by pouring boiling distilled water (250 ml) onto dry leaves (1.7 g), followed by stirring with a magnetic stirrer. The leaves were brewed for 15 min. The aqueous extracts were strained and filtered through Pasteur pipettes plugged with cotton wool. Aliquots of the filtered extracts were freeze-dried and the yield of the extracts was recorded (Table 2.2).

Table 2.2: Botanical name, common name and percentage aqueous extract yield.

Botanical name	Common name	Yield (%)
Aspalathus linearis	Green rooibos tea	1.06
Aspalathus linearis (fermented)	Rooibos tea	1.48
Athrixia phylicoides	Bush tea (Zulu tea)	0.59
Camellia sinesis	Green tea	0.94
Camellia sinesis (fermented)	Black tea (Ceylon)	0.88
Cyclopia intermedia	Honeybush tea	1.93

Chapter 3 – Composition of essential oils of *Athrixia phylicoides*

3.1 Introduction

Essential oils are volatile, natural, complex compounds formed by aromatic plants as secondary metabolites. Initially essential oils were thought to be waste products or storage products; however, in recent times we have begun to uncover the biological and ecological roles of essential oils. Research has shown that essential oils can act as pollination agents, dispersal agents, defense compounds against insects and animals and as antimicrobial agents (Hili, 2001).

Essential oils have been used for centuries in medicine. It is believed that Arnald de Villanova was the first doctor to use essential oils therapeutically in the 13th century. By the 17th century essential oils were widely used by pharmacists as topical preparations. During the 19th and 20th century much research was conducted on the nature of essential oils and aromatic compounds. Physical and chemical procedures were established to analyze aromatics (Hili, 2001). Today essential oils are used in perfumes, make-up, sanitary products, dentistry and agriculture, as food preservers and additives and as natural remedies (Bakkali *et al*., 2008). The use of essential oils as natural remedies has triggered a renewed interest in essential oil research. In recent years many researchers have dedicated time to establish the relationship between essential oils and their biological activities (Hammer *et al*., 1999; Hernandez *et al*., 2007; van Vuuren *et al*., 2007).

Even though the literature describes *A. phylicoides* as a well-known, multi-purpose, versatile plant, utilised as a tea and a medicine (van Wyk and Gericke, 2000), to date no data on the essential oil composition of this plant has been published. This study is the first report on the essential oil composition of *A. phylicoides*.

The aim of this aspect of the study was to determine the chemical composition of the essential oil of *A. phylicoides*.

3.2 Materials and methods

Essential oils were distilled from the aerial parts of the plant using hydrodistillation as explained in Section 2.2.1 and were analysed using gas chromatography (GC) and gas chromatography combined with mass spectrometry (GC/MS) in collaboration with Professor Başer at the Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey.

3.2.1 Principle of the method

Gas chromatography is a separating technique comparing retention times of the chromatogram peaks with those of reference compounds run under identical conditions to identify the peaks. Mass spectrometry records a fragmentation pattern of a substance and provides information about the molecular structures. Since the mass spectrum of a substance is unique to that substance, the spectrum can be compared to previously recorded spectra to identify the substance (Gillespie *et al*., 1994).

3.2.2 Gas chromatography

Analytical gas chromatography was performed using the Hewlett Packard 6890 system. An HP-Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with nitrogen gas as the carrier gas (1 ml/min). The oven temperature was kept at 60 °C for 10 min and programmed to increase to 220 °C at a rate of 4 °C/min, then kept constant at 220 °C for 10 min and then programmed to increase to 240 °C at a rate of 1 °C/min. The split ratio was adjusted to 50:1. The injector temperature was set at 250 °C. The percentage amounts were obtained from electronic integration measurements using flame ionization detection (FID) set at 250 °C. Alkanes were used as reference points in the calculation of relative retention indices (RRI).

3.2.3 Gas chromatography combined with mass spectrometry

Analysis of the essential oils by GC/MS was performed using a Hewlett Packard (HP) 1800A GCD system operating under the following conditions: column: HP-Innowax (60 m x 0.25 mm, 0.25 μm film thickness); temperatures: injection port 250 °C, column 60 °C for 10 min, 4 °C/min to 220 °C, 220 °C for 10 min, 1 °C/min to 240 °C (total = 80 minutes). Percentage

compounds were calculated from total ion chromatograms, by the computer. To identify the essential oil components, the mass spectra and retention indices of the components were compared to the Başer, Adams and Wiley Libraries of essential oil constituents.

3.3 Results and discussion

3.3.1 Essential oil yield

The essential oil yield among the samples studied ranged from 0.1 to 0.47% (w/w), with the highest essential oil yield recorded in April (0.47% w/w) and May (0.31% w/w) (Figure 3.1). The flowering season of *A. phylicoides* is late autumn (van Wyk and Gericke, 2000). A higher oil yield had been recorded during the flowering months of April and May as compared to the non-flowering months of February and March. Since the amount of essential oil was highest during the flowering months, it is possible that the plant increases the amount of oil to favour pollination (Palá-Pául *et al*., 2001). A similar trend was observed in the leaf oil yield of *Agathosma ovata*, with values ranging from 0.23% in early spring to 0.85% in late autumn (flowering season) (Viljoen *et al*., 2006).

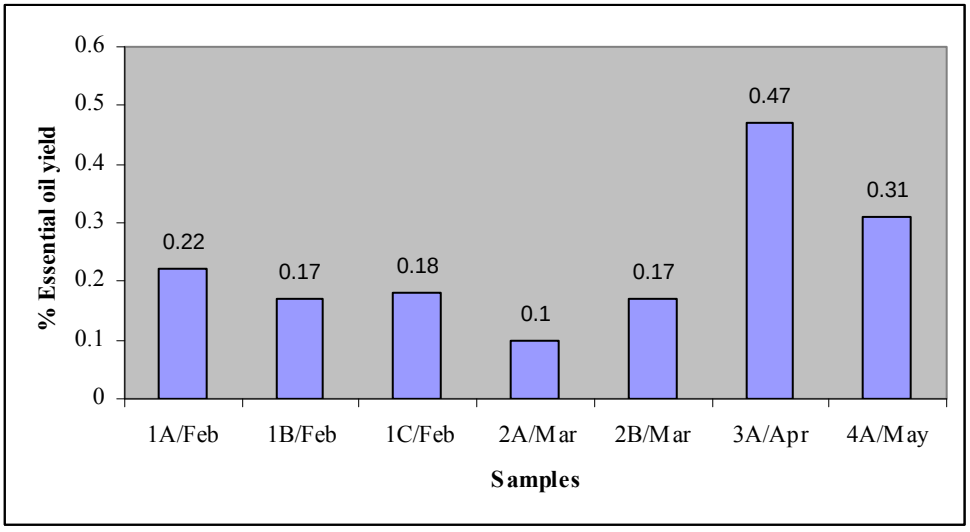


Figure 3.1: Percentage essential oil yield for samples of *A. phylicoides*.

In a study performed by Möller *et al*. (2006), it was reported that the glandular trichomes present on the leaf surfaces of *A. phylicoides*, which are believed to be responsible for

essential oil synthesis, bursts open during late autumn. The time at which the trichomes bursts open coincided with the highest essential oil yield reported in this study.

3.3.2 Essential oil composition

The chemical compounds identified in seven samples using GC/MS analysis are reported in Table 3.1. The essential oil compositions determined by GC and GC/MS revealed 182 components representing 75.4% to 99.8% of the total oil composition.

Table 3.1: Essential oil composition of seven samples of *A. phylicoides*.

RRI	Compound	1A	1B	1C	2A	2B	3A	4A
1014	Tricyclene	t	t	t	-	t	t	t
1032	**α-Pinene**	**13.7**	4.6	1.9	1.5	0.8	**13.9**	**14.8**
1035	α-Thujene	0.1	t	0.1	t	t	0.3	0.2
1048	2-Methyl-3-buten-2-ol	t	t	t	t	t	-	-
1050	Decene	t	t	t	-	-	t	t
1072	α-Fenchene	-	-	-	-	-	t	t
1076	Camphene	0.1	-	t	-	t	0.1	0.1
1093	Hexanal	-	-	-	-	-	t	t
1100	Undecane	-	-	t	t	t	5.0	2.5
1118	**β-Pinene**	**10.2**	7.0	2.5	1.1	1.0	**12.6**	**12.9**
1132	Sabinene	0.8	0.9	0.3	t	0.1	1.0	0.8
1134	Thuja-2(4),10-diene	t	-	0.2	t	0.1	t	t
1174	**Myrcene**	4.1	1.5	0.2	-	t	**11.6**	9.9
1176	α -Phellandrene	-	-	-	t	-	0.5	t
1183	*ρ*-Mentha-1,7(8)-diene (=*Pseudolimonene*)	-	-	-	t	t	t	t
1195	Dehydro-1,8-cineole	t	t	t	-	t	t	t
1203	Limonene	1.0	0.9	1.0	1.8	0.7	4.0	3.6
1213	1,8-Cineole	0.3	0.1	1.8	2.0	2.5	2.3	2.6
1218	β-Phellandrene	t	-	-	t	-	0.5	0.2
1225	(Z)-3-Hexenal	-	-	-	-	-	t	t

RRI	Compound	1A	1B	1C	2A	2B	3A	4A
1244	2-Pentyl furan	-	-	-	-	t	t	t
1246	(Z)- β -Ocimene	t	t	-	-	-	1.7	0.7
1247	6-Methyl-2-heptanone	-	t	t	t	t	-	-
1255	γ-Terpinene	-	-	-	-	t	0.2	t
1257	1-Dodecene	t	-	t	-	t	-	-
1266	(*E*)-β-Ocimene	t	-	-	-	-	1.0	0.2
1280	*ρ*-Cymene	2.8	4.0	1.4	0.9	1.0	2.1	2.5
1290	Terpinolene	-	-	-	-	-	0.1	t
1327	(Z)-3-Hexenyl acetate	0.2	0.2	0.1	-	-	-	-
1348	6-Methyl-5-hepten-2-one	t	-	0.1	0.1	t	-	t
1356	1-Tridecene	t	-	-	0.1	0.1	t	t
1382	*cis*-Allo-ocimene	-	-	-	-	-	t	t
1384	α-Pinene oxide	0.2	0.2	0.3	t	0.1	-	t
1391	(Z)-3-Hexenol	t	t	0.1	-	-	t	t
1393	3-Octanol	0.1	t	0.1	t	0.1	t	t
1400	Nonanal	-	0.1	-	t	t	-	-
1402	Tetradecane	-	-	-	t	-	-	-
1405	4,8-Dimethyl-1,3,7-nonatriene	0.3	0.3	0.4	0.1	0.1	t	0.2
1429	Perillen	0.3	0.8	0.7	t	0.1	t	0.1
1432	7-α-(H)-Silphiperfol-5-ene	t	t	t	0.1	0.1	t	t
1437	α-Thujone	t	t	t	t	t	t	-
1439	γ-Campholene aldehyde	-	-	t	-	-	-	-
1443	Dimethyltetradecane	t	t	0.1	0.7	0.1	t	t
1450	*trans*-Linalool oxide (*Furanoid*)	t	t	0.1	0.1	-	-	-
1451	β-Thujone	t	0.3	0.3	t	t	t	t
1452	α,*ρ*-Dimethylstryene	-	-	-	-	-	t	t
1452	1-Octen-3-ol	0.1	t	t	t	0.1	0.1	0.1

RRI	Compound	1A	1B	1C	2A	2B	3A	4A
1458	*cis*-1,2-Limonene epoxide	-	-	t	-	t	-	-
1460	7β-(H)-Silphiperfol-5-ene	t	t	-	-	t	t	t
1466	α-Cubebene	t	t	0.1	0.1	0.1	0.1	0.1
1475	Menthone	-	t	-	-	-	-	-
1477	4,8-Epoxyterpinolene	-	t	-	-	-	-	-
1478	*cis*-Linalool oxide (*Furanoid*)	t	t	0.1	0.1	0.1	t	t
1481	Longipinene	t	-	-	-	-	-	-
1493	α-Ylangene	0.1	0.1	0.1	t	t	0.1	0.1
1495	Bicycloelemene	-	-	-	-	-	t	t
1496	3-Nonanol	t	-	-	-	-	-	-
1497	α-Copaene	0.4	0.2	0.6	t	0.3	0.5	0.4
1499	α-Campholene aldehyde	t	-	-	0.2	-	-	-
1503	Isomenthone	-	t	-	-	-	-	-
1507	(*E,E*)-2,4-Heptadienal	-	-	-	-	-	0.1	t
1528	α-Bourbonene	t	t	t	t	t	t	t
1532	Camphor	-	-	t	t	-	-	-
1535	β-Boubonene	0.1	0.1	0.2	0.2	t	0.1	0.2
1544	α-Gurjunene	t	t	-	-	t	t	t
1549	β-Cubebene	-	t	-	t	-	0.1	-
1553	Linalool	1.3	0.5	1.7	0.1	2.4	1.1	1.0
1556	*cis*-Sabinene hydrate	-	-	-	-	0.1	-	-
1557	1-Nonen-3-ol	t	0.1	0.1	-	-	-	t
1559	8,9-Limonene epoxide I	-	-	-	-	t	-	-
1562	Octanol	-	-	-	t	-	-	-
1571	*trans*-*ρ*-Menth-2-en-1-ol	t	0.1	0.1	t	t	t	t
1574	8,9- Limonene epoxide II	-	-	t	-	t	-	-
1586	Pinocarvone	0.3	0.2	0.7	0.2	0.3	0.1	0.2
1589	β-Ylangene	0.1	t	1.3	0.1	0.3	0.3	0.2

RRI	Compound	1A	1B	1C	2A	2B	3A	4A
1592	Nopinone	-	t	0.6	0.1	0.1	-	-
1594	*trans*-β-Bergamotene	-	0.2	-	t	t	0.5	0.1
1600	β-Elemene	0.6	2.4	0.3	1.1	0.1	0.3	t
1602	β-Copaene	-	t	0.1	0.3	0.1	0.1	t
1604	6-Methyl-3,5-heptadien-2-one	-	t	-	-	-	-	-
1611	Terpinen-4-ol	0.2	2.7	0.2	0.2	0.1	0.4	0.5
1612	**β-Caryophyllene**	8.3	5.4	0.3	0.2	2.2	**11.9**	**10.0**
1628	Aromadendrene	0.1	0.1	t	0.4	0.1	-	t
1638	*cis*-*ρ*-Menth-2-en-1-ol	-	0.1	-	-	-	t	t
1639	*trans*-*ρ*-Mentha-2,8-dien-1-ol	-	-	t	t	0.1	-	-
1648	Myrtenal	0.6	0.7	1.7	0.3	0.8	0.1	0.3
1661	Alloaromadendrene	0.3	0.2	0.2	0.2	0.1	0.2	0.2
1668	(Z)-β-Farnesene	-	-	-	-	-	0.1	0.1
1670	*trans*-Pinocarveol	0.7	0.9	2.4	0.4	1.4	0.1	0.4
1677	*epi*-Zonarene	-	-	-	-	-	t	0.1
1678	*cis*-*ρ*-Mentha-2,8-dien-1-ol	-	-	-	t	0.1	-	-
1683	*trans*-Verbenol	-	0.1	1.4	0.3	t	t	0.3
1687	α-Humulene	0.9	0.1	-	-	1.2	0.4	0.5
1688	Selina-4,11-diene *(=4,11- Eudesmadiene)*	-	-	-	0.1	0.1	0.1	t
1695	(*E*)- β-Farnesene	-	0.1	-	t	t	0.1	0.1
1700	*ρ*-Mentha-1,8-dien-4-ol *(=Limonen-4-ol)*	-	-	-	0.1	t	-	-
1704	γ-Muurolene	0.5	0.8	0.6	1.0	0.5	0.2	1.1
1706	α-Terpineol	-	t	t	t	t	-	-
1708	Ledene	-	t	-	-	t	t	0.1
1719	Borneol	t	t	t	0.1	0.1	t	t
1720	*trans*-Sabinol	t	-	-	-	-	0.1	t
1725	Verbenone	0.3	0.1	0.8	0.2	0.4	-	t

RRI	Compound	1A	1B	1C	2A	2B	3A	4A
1726	**Germacrene D**	2.7	0.1	-	-	t	**13.5**	5.5
1740	α-Muurolene	0.7	0.4	0.7	0.7	0.8	0.4	0.7
1741	Valencene	0.1	t	t	t	t	-	-
1742	β-Selinene	0.1	-	t	0.7	t	0.1	0.2
1751	Carvone	-	-	0.1	0.2	0.2	-	-
1755	Bicyclogermacrene	0.2	0.1	t	-	-	2.4	0.4
1758	(*E,E*)-α-Franesene	t	-	-	-	-	0.2	0.1
1760	1-Heptadecene	t	-	-	-	-	-	-
1764	*cis*-Chrysanthenol	-	0.1	0.1	-	0.3	-	-
1773	δ-Cadinene	0.4	t	t	t	t	0.9	1.0
1776	γ-Cadinene	0.6	0.6	0.3	0.6	0.1	0.3	0.6
1783	β-Sesquiphellandrene	-	-	-	-	-	t	t
1784	(*E*)-α-Bisabolene	t	-	-	-	-	t	t
1797	*ρ*-Methyl acetophenone	-	-	0.1	t	0.1	-	-
1804	Myrtenol	0.4	0.6	1.6	0.4	0.9	0.1	0.3
1807	α-Cadinene	0.1	t	-	-	0.1	0.1	0.1
1845	*trans*-Carveol	0.1	t	0.4	0.2	0.3	t	0.1
1853	*cis*-Calamenene	0.2	0.3	0.1	0.1	0.1	0.1	0.3
1857	Geraniol	-	0.1	t	-	-	0.1	t
1864	*ρ* -Cymen-8-ol	0.1	0.4	0.2	0.1	t	t	0.1
1868	(*E*)-Geranyl acetone	t	t	t	0.2	0.2	t	0.1
1882	*cis*-Carveol	-	-	0.1	0.1	0.2	t	t
1900	*epi*-Cubebol	0.3	t	0.2	0.2	0.2	0.2	0.2
1937	α-Calacorene I	-	0.3	0.1	0.1	0.1	0.1	t
1945	1,5-Epoxy-salvial(4)14-ene	0.2	0.5	1.3	1.5	1.1	t	0.3
1949	(Z)-3-Hexenyl nonanoate	t	t	0.2	t	t	-	t
1953	Palustrol	-	0.2	0.2	t	t	t	t
1957	Cubebol	t	0.2	-	0.2	0.3	0.2	0.2
1984	γ-Calacorene	0.1	0.1	0.2	t	0.2	t	-
2001	Isocaryophyllene oxide	1.4	1.3	4.0	0.7	4.0	0.1	0.7

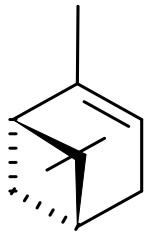
RRI	Compound	1A	1B	1C	2A	2B	3A	4A
2008	**Caryophyllene oxide**	6.2	**21.2**	**21.4**	**34.2**	**18.5**	1.0	4.1
2033	Epiglobulol	-	-	-	0.1	0.1	-	-
2037	Salvial-4(14)-en-1-one	0.2	0.4	0.1	0.2	0.2	t	0.2
2041	Pentadecanal	0.1	-	-	0.2	-	t	t
2050	(*E*)-Nerolidol	-	-	t	-	-	t	t
2057	Ledol	0.2	0.2	0.2	0.3	0.1	0.1	0.1
2071	Humulene epoxide-II	0.2	0.2	0.9	0.9	0.7	t	0.1
2080	1,10-di-epi-Cubenol	0.2	0.3	0.1	0.1	t	0.1	0.2
2088	1-*epi*-Cubenol	0.7	0.2	0.4	0.3	0.6	0.3	0.5
2098	Globulol	0.2	0.1	-	1.2	0.6	0.2	0.3
2104	Viridiflorol	0.2	0.1	0.1	0.4	0.3	0.1	0.1
2113	Cumin alcohol	-	-	t	0.1	t	-	-
2131	Hexahydrofarnesyl acetone	-	t	0.3	-	t	t	0.1
2144	Rosifoliol	0.1	0.1	t	t	0.1	t	0.1
2151	Salviadienol	0.4	0.3	0.3	0.1	0.1	t	0.2
2164	**Spathulenol**	4.1	8.5	2.7	9.9	**12.6**	1.5	3.4
2169	6-*epi*-Cubenol	0.1	0.4	0.1	0.2	-	0.1	0.2
2179	Nor-Copaonone	-	t	0.1	-	-	-	-
2187	T-Cadinol	0.7	1.0	0.5	0.3	0.3	0.3	0.6
2209	T-Muurolol	0.6	0.9	0.5	0.4	0.3	0.3	0.6
2214	Torreyol	0.4	0.3	0.3	0.3	0.2	0.3	0.3
2219	Dimyrcene II-a	-	0.1	0.1	-	-	0.3	0.1
2228	Isospathulenol	0.1	t	-	0.2	0.2	-	-
2247	*trans*-α-Bergamotol	0.4	0.1	0.1	0.1	0.4	0.2	0.3
2249	Torilenol	1.0	2.2	0.8	0.2	0.3	t	0.3
2255	α-Cadinol	1.1	2.0	1.3	1.1	0.9	0.5	1.0
2268	4,7-Dimethyl-1-tetralone	-	0.1	0.3	0.1	-	-	-
2269	Dimyrcene II-b	-	0.3	-	-	-	0.1	0.1
2273	Selin-11-en-4α-ol	0.1	0.1	0.1	0.7	0.1	-	-
2287	4-Oxo-α-ylangene (= *Mustakone*)	-	t	0.3	0.5	0.3	-	-

RRI	Compound	1A	1B	1C	2A	2B	3A	4A
2315	Eudesma-4(5),7-dienol isomer[¥]	1.1	0.8	0.4	0.1	0.2	0.3	0.6
2316	9-Geranyl-*ρ*-cymene	t	t	t	-	t	t	t
2320	Caryophylla-2(12),6(13)-dien-β-ol (=*Caryophylladienol I*)	0.2	0.2	0.5	0.3	0.5	0.1	0.2
2323	14-nor-Cadin-5-en-4-one*	0.3	0.2	0.6	0.5	1.0	-	-
2389	Caryophylla-2(12),6-dien-5α-ol (=*Caryophyllenol I*)	0.1	0.1	0.1	0.4	0.3	0.1	0.1
2395	*cis*-Calamenenol[¥]	0.2	0.1	0.1	t	t	t	t
2415	Eudesma-4(5),7-dien-1β-ol	0.9	0.9	1.0	t	0.6	0.2	0.4
2425	*trans*-Calamenenol[¥]	0.4	0.3	0.5	0.8	1.3	t	0.1
2427	Farnesyl acetone	-	t	-	-	0.1	t	t
2433	Caryophylla-2(12),6-dien-5β-ol (=*Caryophyllenol II*)	0.4	0.5	1.4	2.0	0.2	0.1	0.2
2489	Demethoxyencecaline	-	-	t	-	-	t	t
2500	Pentacosane	-	-	t	-	0.2	-	-
2552	Oplopanone	-	0.5	0.6	-	-	-	-
2554	Geranyl linalool	0.2	0.1	0.6	-	0.2	0.2	0.6
2570	14-Hydroxy-α-muurolene	0.1	0.2	0.1	t	-	t	t
2575	*ent*-4β,10α-Dihydroxy-aromadendrane[¥]	0.1	t	0.3	0.4	1.5	t	0.1
2651	*ent*-4β,10α-Dihydroxy-aromadendrane isomer[¥]	-	-	t	0.4	0.6	t	-
2622	Phytol	-	-	-	-	0.2	0.1	0.1
2700	Heptacosane	-	-	-	-	0.3	-	-
2816	Unknown[†]	0.9	1.5	2.0	1.1	3.3	0.3	1.1

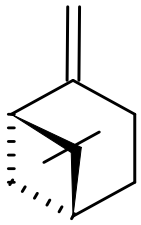
RRI	Compound	1A	1B	1C	2A	2B	3A	4A
2908	Unknown[††]	2.0	1.1	1.0	3.6	2.1	0.1	0.6
2931	Hexadecanoic acid	0.2	t	t	t	t	t	t
	Total	**81.2**	**87.0**	**75.4**	**82.1**	**76.8**	**99.8**	**95.3**

RRI Relative retention indices calculated against *n*-alkanes;
t Trace (< 0.1%); % calculated from FID data;
***** Correct isomer not characterized;
[¥] Tentatively identified from Wiley, Adams, NIST libraries;
[†] Mass spectrum of unknown constituent: EIMS, 70 eV, m/z (rel. int.): 107(100), 121(82), 81(76), 93(71), 43(64), 159(63), 55(58), 177(54), 220(44), 69(41), 189(24), 202(22).
[††] Mass spectrum of unknown constituent: EIMS, 70 eV, m/z (rel. int.): 193(100), 91(92), 123(86), 107(82), 133(75), 81(69), 41(61), 55(60), 175(54), 147(52), 236(48), 69(38), 153(26), 218(17).
Bold text represents major compounds.

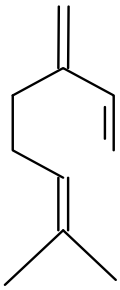
Major compounds: Major compounds (>10%) identified in the volatile oil of *A. phylicoides* studied were α-pinene, β-pinene, myrcene, germacrene D, β-caryophyllene, spathulenol and caryophyllene oxide (Figure 3.2).



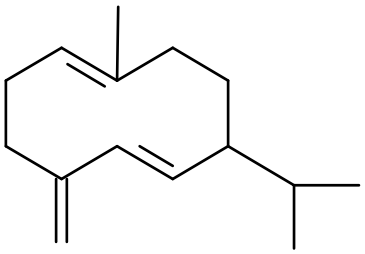
α-pinene



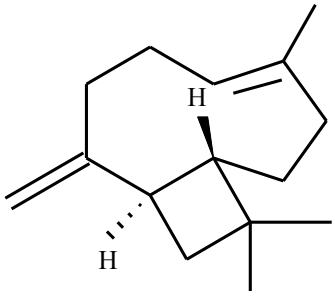
β-pinene



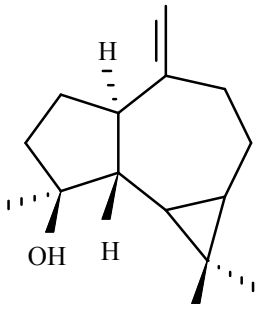
myrcene



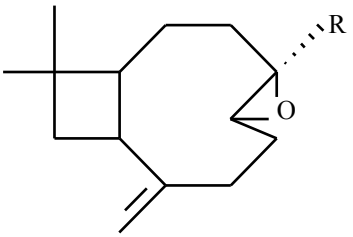
germacrene D



β-caryophyllene



spathulenol



caryophyllene oxide

Figure 3.2: Chemical structures for major compounds identified in the essential oil of *A. phylicoides.*

Essential oils are known to exhibit various pharmacological activities, however the constituents responsible for these activities remain largely unidentified (van Zyl *et al*., 2006). Some of the major essential oil constituents present in *A. phylicoides* are known to possess pharmacological activities. Essential oil constituents, β-caryophyllene ($10 \leq IC_{50} \leq 30$ ppm); germacrene D ($10 \leq IC_{50} \leq 30$ ppm) and α-pinene ($31 \leq IC_{50} \leq 50$ ppm) exhibited anti-inflammatory activity against the 5-lipoxygenase enzyme (Baylac and Racine, 2003). In a study conducted by van Zyl *et al*. (2006), it was reported that α-pinene displayed potent antimalarial activity against the chloroquine- resistant strain of *P. falciparum* (FRC-3) (IC_{50} = 1.2 ± 0.2 μM). The toxicity of some of the major essential oil constituents of *A. phylicoides* have also been investigated. Caryophyllene oxide has been reported to have cytotoxic activity (IC_{50} values range from 147 to 351 μM) against human tumour cells (Sibanda *et al*., 2004). In another study, it has been reported that α-pinene (IC_{50} = 172.2 ± 13.4 μM) and β-pinene (IC_{50} = 166.7 ± 19.6 μM) displayed some degree of toxicity against human kidney epithelial cells (van Zyl *et al*., 2006).

Quantitative variation in major compounds of the essential oil in the seven samples studied has been observed (Figure 3.3). No specific trend was noticed for the major compounds, however remarkable fluctuations in quantities included caryophyllene oxide (1.0% to 34.2%), β-pinene (1.0% to 12.9%), α-pinene (0.8% to 14.8%), spathulenol (1.5% to 12.6%), germacrene D (0% to 13.5%), β-caryophyllene (0.2% to 11.9%) and mycrene (0% to 11.6%). The most

conspicuous variation in major compounds was noted for caryophyllene oxide, ranging from 1.0% to 34.2%. α-Pinene was found to be the main constituent of samples 1A/Feb (13.7%), 3A/Apr (13.9%) and 4A/May (14.8%) and caryophyllene oxide was the main constituent of samples 1B/Feb (21.2%), 1C/Feb (21.4%), 2A/Mar (34.2%) and 2B/Mar (18.5%). Spathulenol (12.6%) was identified as a major compound in sample 2B/Mar only. It was also noted that the essential oil compositions of samples 3A/Apr and 4A/May were similar (Figure 3.3). The major compounds represented between 21.2% and 63.5% of the total oil composition (Table 3.1).

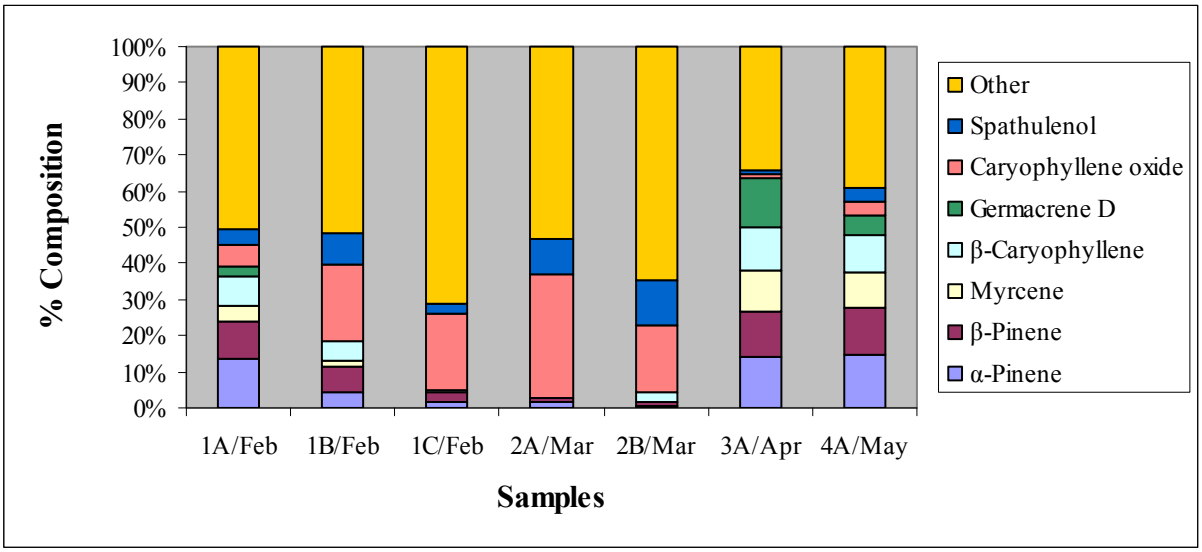


Figure 3.3: Major compounds identified in *A. phylicoides*.

The results from this study revealed that variation in essential oil composition exists in the same species (*A. phylicoides*) collected from the same location. The composition of the essential oils can be affected by many factors such as development or reproductive stages, seasons, location, temperature, extraction method and the conditions of analysis (Putievsky *et al*., 1986; Sangwan *et al*., 2001; Kim and Lee, 2004). In a study by Simmons *et al*. (1987), it was reported that the variation in essential oil composition of *Eucalyptus ovata* and *Eucalyptus camphora* could be attributed to the leaf ageing effect. Kamatou *et al*. (2008) reported seasonal variation in essential oil composition for three South African *Salvia* species. The composition of the essential oil of sage has been found to change under the influence of temperature (Avato *et al*., 2005). The variation in essential oil composition noted in this study

may be as a result of various factors, such as temperature, age of the leaves, development stages and genetic factors.

In a study conducted on *Athrixia elata*, the following constituents were found in the aerial parts: squalene, germacrene D, caryophyllene, α-humulene and cinnamates (Bohlmann *et al*., 1982). Some of the constituents found in *A. elata* are also present in *A. phylicoides* in varying quantities: germacrene D – trace to 13.5%; caryophyllene – 0.2 to 11.9% and α-humulene – 0.1 to 1.2%.

3.4 Conclusions

- A higher percentage essential oil yield was recorded for *A. phylicoides* collected during the flowering months of April and May (0.47% and 0.31%) compared to the non-flowering months (0.1% to 0.2%).
- Seven major compounds (>10%) were identified in the samples of *A. phylicoides* studied: caryophyllene oxide, spathulenol, germacrene D, β-caryophyllene, β-pinene, α-pinene and myrcene.
- Variation in the essential oil composition of *A. phylicoides* from within the same population and between populations has been noted.

Chapter 4 – Antimicrobial activity and isolation of bioactive compound

4.1 Introduction

The discovery of antimicrobial agents during the twentieth century substantially reduced the threat posed by infectious diseases. Antimicrobial agents combined with improvements in sanitation, housing and nutrition, and the advent of widespread immunization programmes, ensured a dramatic drop in deaths from diseases that were previously widespread, untreatable and frequently fatal. Over the years, antimicrobial agents have saved lives, eased the suffering of millions of people and contributed to increasing life expectancy (WHO, 2002).

This significant advancement in medicine is now under severe threat by another recent development, the emergence and spread of micro-organisms that are resistant to antimicrobial agents (WHO, 2002). The consequences of resistant micro-organisms are severe. Infections caused by resistant micro-organisms do not respond to treatment with antimicrobial agents, for example nosocomial infections caused by methicillin resistant *Staphylococcus aureus* will not respond to methicillin and community acquired pneumonia caused by strains resistant to penicillin will not respond to penicillin and even sometimes cephalosporins. The result is prolonged illness, increasing the risk of death. In addition, the prolonged illness leads to longer periods of infectivity, which can increase the number of infected people moving in the community and thus expose the general population to the risk of contracting a resistant strain of the infection (WHO, 2002). With the continuous escalation of antimicrobial resistance, the acceptance of traditional medicine as an alternative form of health care by South Africans has increased. For this reason, medicinal plants have become the focus of intense study to determine whether their traditional uses can be supported by pharmacological effects or are merely based on folklore (Rabe and van Staden, 1997).

A survey of the literature revealed that the leaves of *A. phylicoides* have been used for the treatment of boils, sores, bad acne, infected wounds and cuts (Table 1.2) (Plowes and

Drummond, 1976; Roberts, 1990; Hutchings *et al*., 1996). This information prompted an investigation into the *in vitro* antimicrobial activity of *A. phylicoides*.

The aims of this study were to:

- investigate the essential oil and methanol extract of the indigenous plant *A. phylicoides* for potential antimicrobial activity.
- isolate and identify the compound/s exhibiting antimicrobial activity.

4.2 Materials and methods

4.2.1 Minimum inhibitory concentration assay

The anitmicrobial activity of the essential oil and methanol extract of *A. phylicoides* was assessed using the minimum inhibitory concentration microdilution (MIC) assay (Eloff, 1998).

4.2.1.1 Principle of the method

The minimum inhibitory concentration (MIC) is the lowest concentration at which the growth of the bacteria is inhibited. The ρ-iodonitrotetrazolium (INT) microplate bioassay technique was used to determine the MIC (Eloff, 1998). Microbial growth is indicated by the presence of a reddish colour, which is produced when INT (colourless product) is reduced by metabolically active micro-organisms to the corresponding intensely coloured formazan. Therefore the MIC value is the concentration of the first well in which no colour change is observed (Figure 4.1).

4.2.1.2 Protocol

Five Gram-positive bacteria: *Staphylococcus aureus* (ATCC 12600), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Staphylococcus epidermidis* (ATCC 2223) and *Bacillus subtilis* (ATCC 6051); four Gram-negative bacteria: *Klebsiella pneumoniae* (NCTC 9633), *Escherichia coli* (ATCC 8739), *Yersinia entercolitica* (ATCC 23715) and *Salmonella typhimurium* (ATCC 14028) and two yeasts: *Candida albicans* (ATCC 10231) and *Candida tropicalis* (ATCC 750) were used to evaluate the antimicrobial activity of the essential oil and methanol extract of *A. phylicoides*.

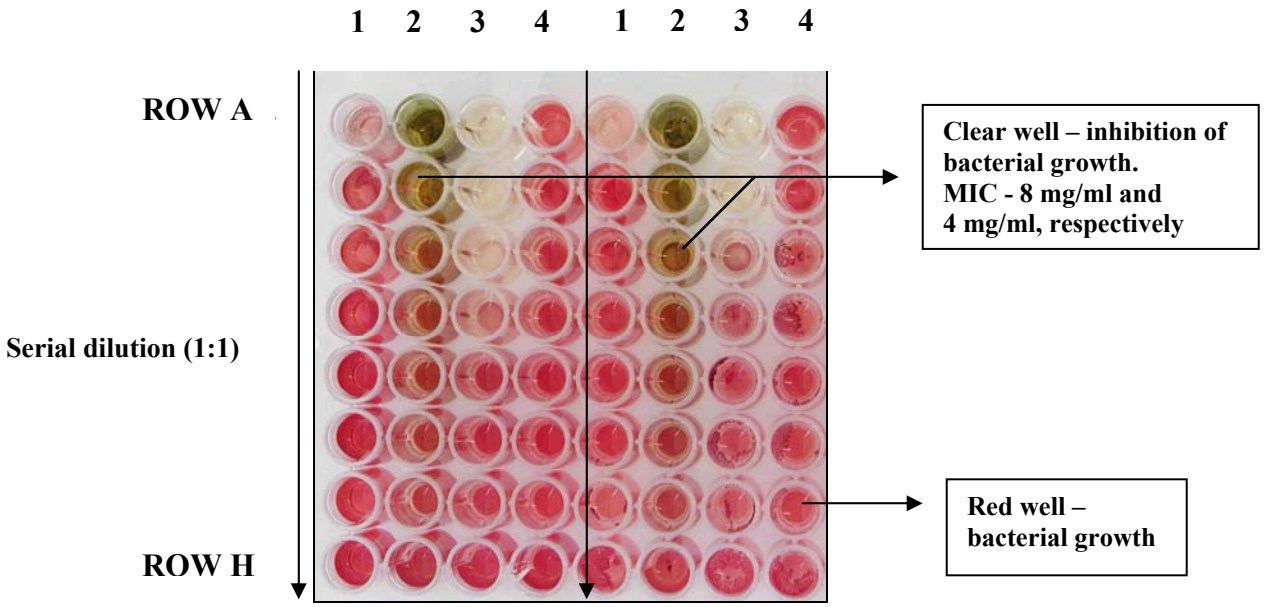


Figure 4.1: Microtitre plate for *Salmonella typhimurium* where column 1 is the essential oil (concentrations decreasing from 32 mg/ml in row A to 0.25 mg/ml in row H), column 2 is the extract (concentrations decreasing from 16 mg/ml in row A to 0.125 mg/ml in row H), column 3 is a positive control and column 4 is a negative control.

The test solutions were prepared by diluting the essential oil and methanol extract in acetone to achieve a final concentration of 128 mg/ml and 64 mg/ml, respectively. The inoculum was prepared by mixing 1 ml of the respective bacteria and yeast grown overnight with 99 ml of fresh Tryptone Soya broth. INT was prepared by dissolving 40 mg in 100 ml sterile water.

For each micro-organism, columns in the microtitre plate were reserved for positive and negative controls. Commercial antimicrobials (ciprofloxacin and amphothericin B) were used as positive controls for the bacteria and yeast, respectively. Ciprofloxacin (1 mg/ml) and amphothericin B (1 mg/ml) was diluted (1:10) with sterile water and DMSO, respectively to achieve a concentration of 0.01 mg/ml. For negative controls, wells containing acetone (100 µl) and no plant extract were prepared for the observation of normal bacterial and fungal growth. In the microtitre plate, 100 µl sterile water was added to each well. The test solution (100 µl) was added to each well in row A of the plate. Serial dilutions (1:1) were made in the

microtitre plates. Inoculum (100 µl), yielding an inoculum size of 1 x 10^6 CFU/ml was then added to each well to achieve final concentrations of 32 mg/ml in row A to 0.25 mg/ml in row H for the essential oil and 16 mg/ml in row A to 0.125 mg/ml in row H for the extract, respectively. The plates were incubated at 37 °C for 24 hours for bacteria and 48 hours for yeast. After incubation, 50 µl INT (0.04% w/v) was added to each well and the plates were left at room temperature for six hours for bacteria and 24 hours for yeast before MIC's were determined visually. All organisms were tested in duplicate (two columns each) for the essential oil and the methanol extract (Figure 4.1). The minimum inhibitory assays were repeated and the mean was calculated.

4.2.2 Isolation of the bioactive compound

The methanol extract of *A. phylicoides* was selected to isolate the bioactive compound/s, since it displayed favourable antimicrobial activity.

4.2.2.1 Column chromatography

Column chromatography was conducted to isolate the compound/s responsible for antimicrobial activity (Figure 4.2). Silica mixed with hexane and ethyl acetate was poured into the column and allowed to settle for a few minutes. The plant extract dissolved in ethyl acetate was carefully pipetted along the rim of the column. The column was eluted successively with hexane:ethyl acetate (6:4), then hexane:ethyl acetate (7:3) followed by 100% ethyl acetate and finally stripped with 100% methanol. The collection of these crude fractions was based on colour change. The fractions were collected in 250 ml conical flasks and spotted on TLC plates. TLC plates were developed in hexane:ethyl acetate (6:4). Fractions displaying similar chromatographic profiles on the TLC plates were combined to yield four main fractions (A-D).

4.2.2.2 Thin layer chromatography and bio-autographic assay

A direct antimicrobial bioassay on a thin layer chromatography plate was employed using the agar-overlay method with *Staphylococcus aureus* (ATCC 12600) as the test organism to isolate the bioactive compound. *S. aureus* was chosen as the test organism because it is one

Figure 4.2: Preparing a silica column for column chromatography used to separate fractions.

of the most difficult bacterial strains to treat with conventional antibiotics (Esposito *et al.*, 2009). Each of the four fractions were spotted on a TLC plate (Alugram® Sil G/UV_{254}, 0.200 mm) and developed in a hexane:ethyl acetate (6:4) solvent system. The TLC plate was then dried and sterilised under UV light (254 mm) for one hour. Tryptone Soya agar (15 ml) was poured into a petri dish and allowed to solidify. The sterilised TLC plate was placed onto the agar surface with the silica side facing upwards. An overlayed layer of Tryptone Soya agar (15 ml) containing 150 µl of *S. aureus* (approximate inoculum size, 1 x 10^6 CFU/ml)) was placed on top of the TLC plate. The petri-dish was placed in the refrigerator (4 °C) for one hour to allow the compounds on the TLC plate to diffuse into the agar. The plate was then removed and incubated at 37 °C for 24 hours. At the end of the incubation period, the TLC plate was sprayed with INT (0.04% w/v) to visualise the inhibition zone. The inhibition zone was indicated by the clear zone on the red background (Figure 4.3). On evaluation of the bio-autographic assay of the four fractions, the best zone of inhibition was observed around Fraction D and was thus selected for further purification (Figure 4.3).

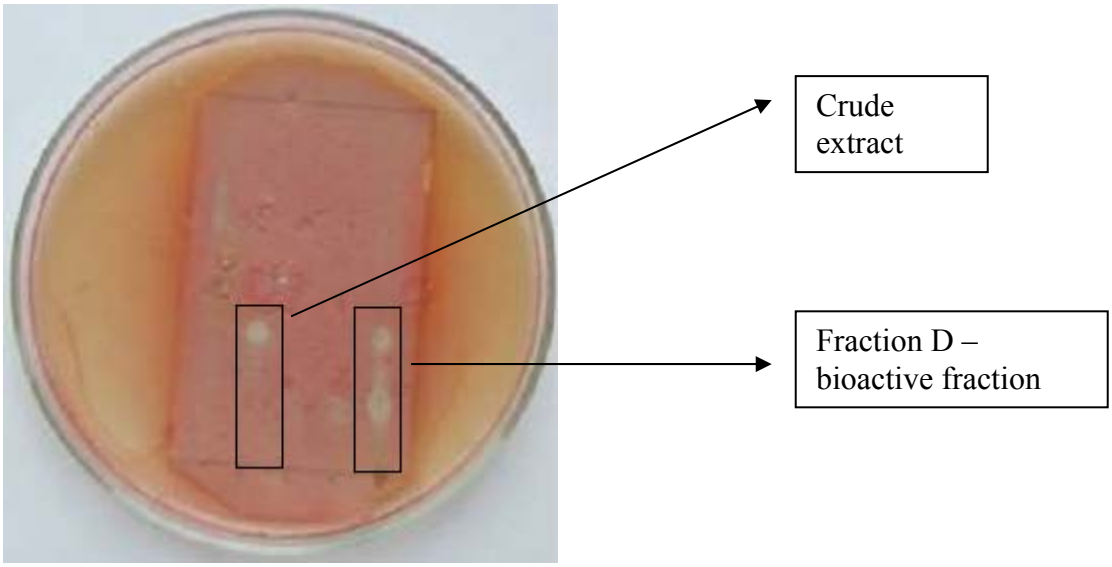


Figure 4.3: Bio-autographic assay of *A. phylicoides* against the test pathogen *S. aureus*. (Inhibition zones are shown by clear spots on the red background.)

4.2.2.3 Bioactivity-guided fractionation and isolation

Column chromatography yielded four fractions of which Fraction D was the most bioactive (Figure 4.3). A second column was prepared in order to fractionate Fraction D. A total of 57 sub-fractions were collected and spotted on TLC plates. The bioactivity-guided fractionation procedure is summarized in Figure 4.4.

4.2.2.4 Identification, elucidation and minimum inhibitory concentration of the isolated compound

4.2.2.4.1 Nuclear magnetic resonance

The isolated compound was structurally characterized using nuclear magnetic resonance (NMR) and MS and data compared to those found in the literature in collaboration with Professor F.R. van Heerden at the University of KwaZulu-Natal. Spectra were recorded on a Varian Inova 2000 300 MHz spectrometer. All spectra were recorded at 25 ˚C in denatured chloroform and the chemical shifts were recorded in ppm referenced to tetramethylsilane. Electron ionization MS of the isolated compounds was performed by direct inlet at 70 eV on the GC-MS QP 2010 GC coupled to MS.

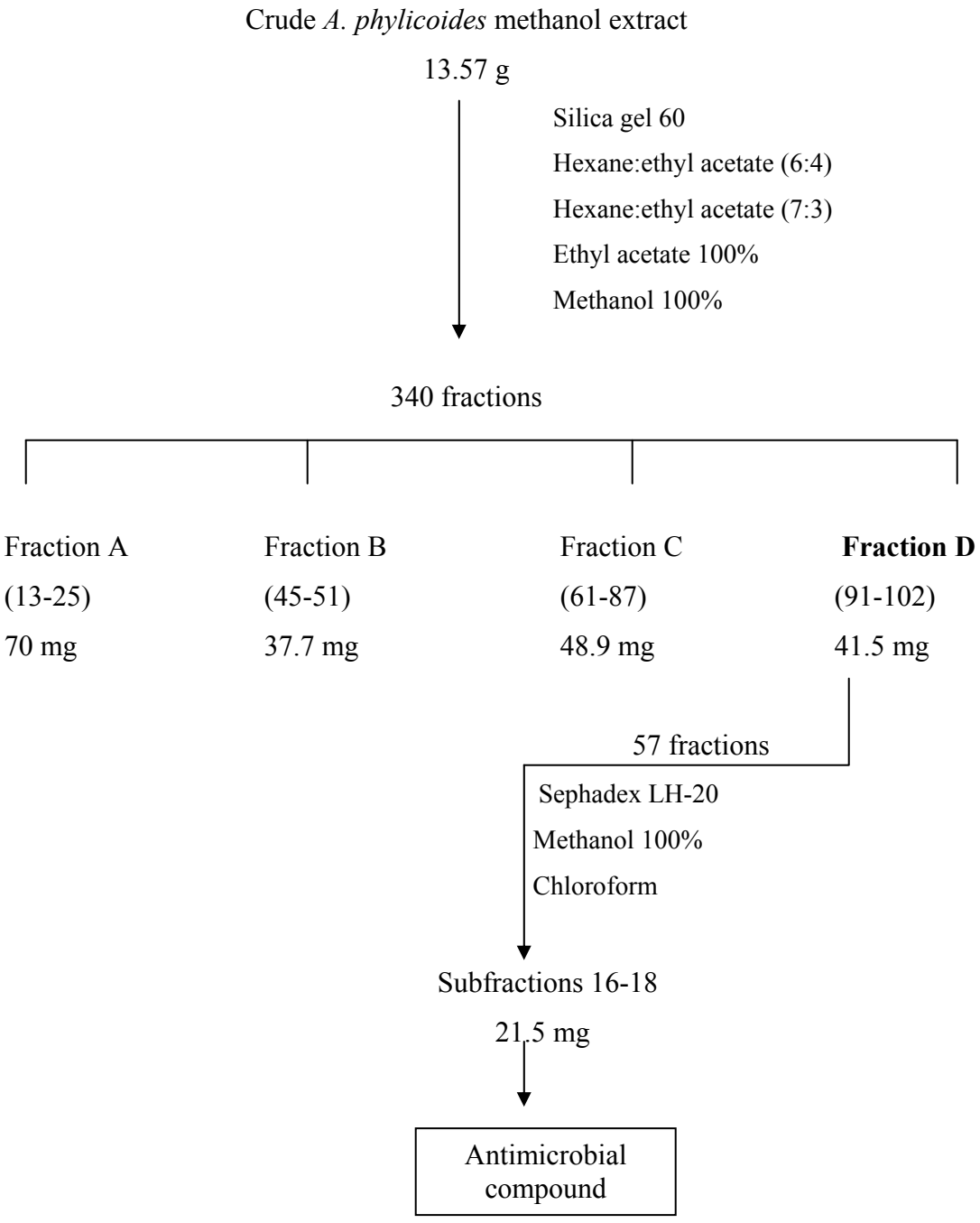


Figure 4.4: Bioactivity-guided fractionation procedure (All fractions and sub-fractions not listed had low activity and were not considered for further purification).

4.2.2.4.2 Antimicrobial activity of the isolated compound

The antimicrobial activity of the isolated compound of *A. phylicoides* was assessed using the MIC assay as described in Section 4.2.1. Three micro-organisms were chosen for the MIC assay, i.e. one Gram-positive bacterium: *Staphylococcus aureus* (ATCC 12600); one Gram-negative bacterium: *Escherichia coli* (ATCC 8739) and one yeast *Candida albicans* (ATCC 10231). Only three micro-organisms could be used in the MIC assay because of a limited quantity of isolated compound. Commercial antimicrobials (ciprofloxacin and amphothericin B) were used as positive controls for the bacteria and yeast, respectively.

4.3 Results and discussion

4.3.1 Antimicrobial activity: Extract, essential oil and bioactive compound

The MIC assay revealed that both the extract and essential oil were active against some of the test organisms (Table 4.1). The methanol extract displayed stronger antimicrobial activity towards all test organisms than the essential oil. The average MIC values obtained for the extract against each organism ranged from 1 to 6 mg/ml; while the average MIC values obtained for the essential oil against each organism ranged from 3 to >32 mg/ml. It has been reported that an essential oil with a MIC ≤2 mg/ml, an extract with a MIC <8 mg/ml and a bioactive compound with a MIC value ranging between 64 – 100 µg/ml are considered to possess noteworthy antimicrobial activity (Fabry *et al.*, 1998; Gibbons, 2004; van Vuuren, 2008). The highest sensitivities for the methanol extract were against *S. aureus*, *B. cereus* and *B. subtilis* (MIC value: 1 mg/ml) and the essential oil was moderately active against *B. cereus* (MIC value: 3 mg/ml).

The MIC assay revealed that *A. phylicoides* bioactive compound displayed antimicrobial activity and was most active against the Gram-positive bacterium, *S. aureus* (MIC value: 19.5 µg/ml). The bioactive compound also displayed some antimicrobial activity against the Gram-negative bacterium, *E. coli* and the yeast, *C. albicans*.

The bioactive compound was 51 times more active compared to the methanol extract against *S. aureus,* 26 times more active compared to the methanol extract against *C. albicans* and 24 times more active compared to the methanol extract against *E. coli*.

Table 4.1: Antimicrobial activity of *A. phylicoides* methanol extract, essential oil and bioactive compound (n = 2).

Test Organism	Essential Oil (mg/ml)	Extract (mg/ml)	Bioactive compound (µg/ml)	Control (µg/ml)
Bacillus cereus (ATCC 11778)	3	1	ND	0.6 (a)
Bacillus subtilis (ATCC 6051)	6	1	ND	0.3 (a)
Enterococcus faecalis (ATCC 292192)	> 32	4	ND	0.3 (a)
Staphylococcus aureus (ATCC 12600)	24	1	19.5	0.6 (a)
Staphylococcus epidermidis (ATCC 2223)	24	3	ND	0.6 (a)
Escherichia coli (ATCC 8739)	12	3	125	0.15 (a)
Klebsiella pneumoniae (NCTC 9633)	12	6	ND	0.3 (a)
Salmonella typhimurium (ATCC 14028)	> 32	6	ND	0.6 (a)
Yersenia entercolitica (ATCC 23715)	12	3	ND	0.3 (a)
Candida albicans (ATCC 10231)	6	2	78	0.15 (b)
Candida tropicalis (ATCC 750)	4	1.5	ND	0.15 (b)

(a) Ciprofloxacin
(b) Amphothercin B
ND – Not determined

The plant extract, the bioactive compound and the essential oil displayed better activity against the Gram-positive bacteria than the Gram-negative bacteria. These results are consistent with results from previous *in vitro* studies with plant extracts and essential oils (Hernandez *et al.*, 2007; Nyiligira *et al*., 2008; Mahboubi and Haghi, 2008; Paraskeva *et al*., 2008; Lalli *et al*., 2008). It is believed that the difference in structure, that is, the hydrophobic lipopolysaccharide in the outer membrane of Gram-negative bacteria provides an additional barrier to agents making them more resistant to the inhibitory effects of agents compared to Gram-positive bacteria (Delaquis *et al*., 2002; Mahboubi and Haghi, 2008).

In a study conducted by Mavundza *et al*. (2007), the antimicrobial activity of the ethanol extract of *A. phylicoides* (collected from Venda in the Limpopo Province, South Africa in mid-July) was investigated. The micro-organisms tested were *S. aureus* (ATCC 12600), *B. cereus* (ATCC 11778), *E. faecalis* (ATCC 292192), *E. coli* (ATCC 11775) and *Mycobacterium smegmatis* (ATCC X). The extract displayed inhibitory activity against all micro-organisms tested. These results are congruent with the results obtained in this study, in which the methanol extract also displayed inhibitory activity against *S. aureus*, *B. cereus*, *E. faecalis* and *E. coli* (Table 4.1).

Athrixia phylicoides plant extract had MIC values below 8 mg/ml for all organisms tested (Table 4.1) and the bioactive compound had MIC values of 19.5 µg/ml and 78 µg/ml for *S. aureus* (Gram-positive bacteria) and *C. albicans* (yeast) respectively. As such it can be concluded that *A. phylicoides* plant extract possesses some antimicrobial activity (Fabry *et al.*, 1998; Gibbons, 2004). Furthermore the antimicrobial activities exhibited by the crude plant extract and the bioactive compound of *A. phylicoides* against *S. aureus* (MIC values: 1 mg/ml and 19.5 µg/ml) must be emphasised. Infections caused by *S. aureus* are difficult to treat because often it does not respond to conventional antibiotic therapy, which can result in high morbidity and substantial costs (Esposito *et al.*, 2009). In addition, *S. aureus* has been recognised as a significant cause of nosocomial infections (Esposito *et al*., 2009). This noteworthy antimicrobial activity of *A. phylicoides* extract and bioactive compound against *S. aureus* (Table 4.1) can therefore pave the way for further *in vitro* and possibly *in vivo* tests to yield compounds that can improve the treatment of infections caused by *S. aureus*. The *in*

vitro antimicrobial activity of *A. phylicoides* obtained from this study, justifies its use as a traditional remedy to treat boils, sores, bad acne, infected wounds and cuts (Table1.2).

4.3.2 Bioactivity-guided fractionation and isolation

Two active substances were visible on the TLC plate (Figure 4.3) for Fraction D, whereas only one active substance was visible for the crude extract. However following the TLC of fractions of the second column which separated the constituents of Fraction D, only active substance was visible for both the fractions and the crude extract. It can therefore be concluded that the second compound for Fraction D was probably due to degradation during isolation or due to concentration effects. Only one active compound was present in the *A. phylicoides* methanol extract.

4.3.3 Elucidation and identification of the isolated compound

Based on the ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY spectra and comparison with data obtained in the literature, the compound was identified as (4-hydroxyphenyl)propyl coumaroate (Figure 4.5). The chemical shift data (Table A.1) as well as the ^{13}C and ^{1}H spectra (Figures A.1 and A.2) for (4-hydroxyphenyl)propyl coumaroate has been included in Appendix A.

(4-Hydroxyphenyl)propyl coumaroate has been previously identified in *A. elata*, however, no data has been published on any of the biological activities of the compound (Bohlmann and Zdero, 1977; Bohlmann *et al*., 1982). (4-Hydroxyphenyl)propyl coumaroate is an ester of coumaric acid.

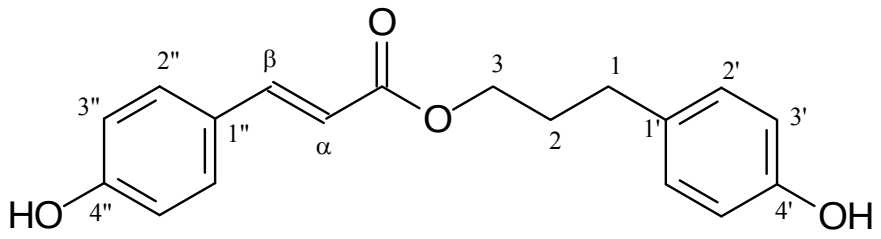


Figure 4.5: Structure of (4-hydroxyphenyl)propyl coumaroate isolated from *A. phylicoides.*

Coumarins are found in many families of plants such as Apiaceae, Asteraceae, Leguminoseae, Rosaceae, Rubiaceae, Rutaceae and Solanaceae (Weinmann, 1997). Their structural diversity can greatly influence their biological activities (Kostova, 2005). Several papers have been published on the antimicrobial activity of coumarins. The antimicrobial properties of coumarins were first recognized in 1945 when an investigation with dicoumarol was conducted and it was found to inhibit the growth of several strains of bacteria (Goth, 1945). Smyth *et al*. (2009) evaluated the antimicrobial activity of 43 naturally occurring and synthetic coumarins against six strains of bacteria, using the MIC microtitre assay. Approximately half of the coumarins tested were found to have varying antimicrobial activity with MICs ranging from 1.56 to >1000 µg/ml. The antimicrobial activity of three coumarins (aesculetin, prenyletin and haplopinol) isolated from *Haplopappus multifolius* were investigated and it was found to inhibit the growth of three bacterial strains (*Sarcina lutea*, *E. coli* and *S. aureus*) with MICs ranging from 250 to 1000 µg/ml (Chiang *et al*., 1982).

4.4 Conclusions

- The plant extract of *A. phylicoides* (MIC range: 1 to 6 mg/ml) exhibited greater antimicrobial activity than the essential oil (MIC range: 3 to >32 mg/ml).
- Greater antimicrobial activity was displayed against Gram-positive bacteria than Gram-negative bacteria.
- Bioactivity guided fractionation led to the isolation of (4-hydroxyphenyl)propyl coumaroate.
- (4-Hydroxyphenyl)propyl coumaroate was most active against the Gram-positive bacteria, *S. aureus* with a MIC of 19.5 µg/ml compared to a MIC of 1 mg/ml for the methanol extract and a MIC of 0.6 µg/ml for ciprofloxacin (positive control).
- The MIC assay results of *A. phylicoides* provided preliminary evidence to support its use for the treatment of boils, sores, bad acne, infected wounds and cuts.

Chapter 5 – Anti-oxidant activity

5.1 Introduction

Free radicals such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet}$) and hydroxyl radicals ($OH^{\bullet}$) are unstable, highly reactive molecules. They are produced as a normal consequence of biochemical processes in the body and as a result of increased exposure to environmental and/or dietary xenobiotics. Free radicals are major contributors to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction (Lee *et al*., 2003; Atoui *et al*., 2005).

Anti-oxidants are free radical scavengers that inhibit the oxidative mechanisms which lead to degenerative diseases. Mammalian cells possess defence mechanisms (anti-oxidant enzymes and small molecule anti-oxidants) for the detoxification of free radicals. The anti-oxidant enzymes include superoxide dismutase (SOD), which dismutes superoxide to hydrogen peroxide and oxygen; catalase (CAT), which converts H_2O_2 into H_2O and oxygen and gluthathione peroxidase (GPX), which destroys toxic peroxides. In addition, small molecule anti-oxidants are available *in vivo* (gluthathione, bilirubin and melatonin) or obtained from the diet (α-tocopherol, β-carotene, Vitamin A and E, flavonoids, zinc and selenium) (Lee *et al.,* 2003).

When an imbalance in oxidant and anti-oxidant processes exists (oxidative stress), the excess free radicals induce peroxidation of polyunsaturated fatty acids in the cell membrane lipid bi-layer. This causes a chain reaction of lipid peroxidation, thus damaging the cellular membrane and causing further oxidation of the membranes lipids and proteins. Subsequently, cell contents, including DNA are damaged. This free radical damage precedes the degenerative diseases (Cook and Samman, 1996; Gramza and Korczak, 2005).

Traditionally, carotene, Vitamin E and C are referred to as dietary anti-oxidants. In recent years there has been particular interest in the anti-oxidant activity and health benefits of other phytochemicals.

Tea is an important source of dietary polyphenols. The types and amounts of polyphenols present in tea will differ dependant on the variety of leaf, growing environment, processing, manufacturing, particle size of ground tea-leaves and infusion preparation (Gramza and Korczak, 2005). The results of many studies showed the potential anti-oxidant properties of polyphenols (Kähkönen *et al*., 1999; Miliauskas *et al*., 2004; Aoshima *et al.*, 2007; Conforti *et al*., 2008). However, the relationship between the total phenolic content and anti-oxidant activity are in-conclusive. Some authors have found a correlation between the total phenolic content and anti-oxidant activity while others found no relationship. Kähkönen *et al*. (1999) and Conforti *et al*. (2008) found no correlation between the total phenolic content and anti-oxidant activity, and have concluded that the difference in anti-oxidant activity is attributed to the type of phenolic constituents and not the amount of phenolic constituents. However, other authors confirm that the higher the polyphenol content, the higher the anti-oxidant activity (Miliauskas *et al*., 2004; Aoshima *et al.*, 2007).

The growing interest in the anti-oxidant activity of tea and the potential health benefits led to the study of the anti-oxidant activity of commercially available teas with *A. phylicoides*.

The aims of this study were to:

- confirm that *A. phylicoides* possess anti-oxidant activity.
- compare the anti-oxidant activity of *A. phylicoides* to that of other commercially available teas.

5.2 Materials and methods

The anti-oxidant activities of rooibos tea (green and fermented), green tea, Ceylon (black) tea and honeybush tea were compared to *A. phylicoides* (Bush tea) using the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) free radical scavenging assay. Rooibos, green, Ceylon and honeybush tea were purchased at a local supermarket. Green rooibos tea was sourced from Rooibos Ltd in Clanwilliam and a pooled sample of *A. phylicoides* (Bush tea) (Figure 5.1) was used in the analysis. The botanical and common names of the samples tested are listed in Table 5.1.

Figure 5.1: Commercial packs of teas tested for anti-oxidant activity.

Table 5.1: Botanical names and common names of samples tested in DPPH$^{\bullet}$ assay.

Botanical name	**Common name**
Aspalathus linearis	Green rooibos tea
Aspalathus linearis (fermented)	Rooibos tea
Athrixia phylicoides	Bush tea (Zulu tea)
Camellia sinesis	Green tea
Camellia sinesis (fermented)	Black tea (Ceylon)
Cyclopia intermedia	Honeybush tea

5.2.1 Principle of the method

DPPH$^{\bullet}$ is a stable free radical with a dark violet colour. Anti-oxidant compounds donate electrons to DPPH$^{\bullet}$, resulting in decolourization from deep violet to light yellow. The degree of decolourization is proportionate to the number of electrons accepted by the DPPH$^{\bullet}$ and is measured with a UV/VIS light spectrophotometer (Miliauskus *et al*., 2004).

5.2.2 Protocol

A 96 µM DPPH$^{\bullet}$ solution was prepared in HPLC grade methanol and stored at 4 °C in the dark. Tea extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 10 mg/ml. For the first dilution, 50 µl of the stock solution was added to 950 µl of DMSO to

obtain a concentration of 500 µg/ml, but following the addition of the DPPH$^{\bullet}$, there was a final concentration of 100 µg/ml in the well. Six serial dilutions (1:1) were performed thereafter using DMSO.

Using a 96-well microtitre plate, 50 µl of the stock solutions and serial dilutions was plated out in triplicate from rows B to G. In row A and H, 50 µl of DMSO was added, which acted as a negative control. In columns 2, 4, 6, 8, 10 & 12 HPLC grade methanol (200 µl) was added and in columns 1, 3, 5, 7, 9 & 11 DPPH$^{\bullet}$ (200 µl) was added. L-Ascorbic acid (Vitamin C) was used as a positive reference.

The plate was shaken for 2 minutes and left to stand for 30 minutes at room temperature. Thereafter, the absorbance was read at 550 nm using an UV-spectrophotometer and the percentage decolourization (the percentage free radical scavenging activity of the test compound) was calculated. The IC_{50} values were calculated using Enzfitter® software. Each experiment was done in triplicate and the mean and standard deviation (s.d.) reported.

5.3 Results and discussion

All the aqueous extracts showed free radical scavenging activity ranging from 9.64 µg/ml to 29.26 µg/ml (Figure 5.2). The results of the *in vitro* assay showed that green tea possessed the highest anti-oxidative activity (IC_{50} = 9.64 ± 0.96 µg/ml) and *A. phylicoides* (Bush tea) (IC_{50} = 14.01 ± 2.68 µg/ml) had greater anti-oxidant properties than fermented rooibos tea, honeybush tea, green rooibos tea and Ceylon tea (IC_{50} > 18.00 µg/ml). *A. phylicoides* was at least 2.5 times less active than the reference compound, Vitamin C (L-Ascorbic acid).

The anti-oxidant activity of the aqueous tea extracts tested in this study using the DPPH$^{\bullet}$ assay decreased in the order: green > *A. phylicoides* (Bush) > green rooibos > honeybush > fermented rooibos > Ceylon (black) (Figure 5.2). These results are in agreement with a previous study in which the DPPH$^{\bullet}$ free radical scavenging method was utilised to determine the anti-oxidant activity of green, rooibos (fermented, unfermented and semi-fermented), black and oolong tea extracts (concentration of tea extracts: 50 mg/100 ml). The anti-oxidant activity and percentage inhibition was as follows: green (90.8%) > unfermented rooibos

(86.6%) > fermented rooibos (83.4%) > semi-fermented rooibos (81.9%) > black (81.7%) > oolong (71.2%) (von Gadow *et al*., 1997).

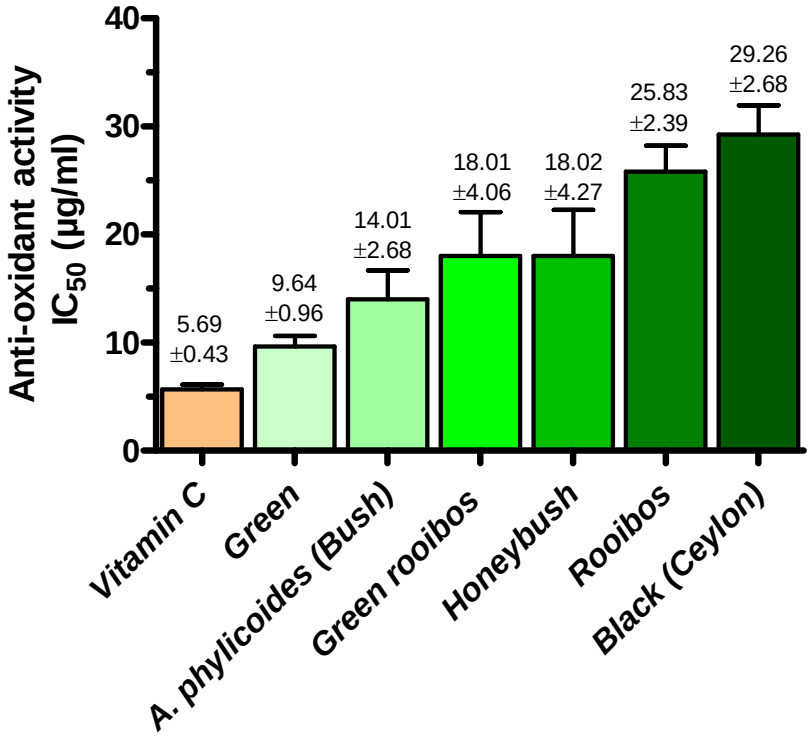


Figure 5.2: *In vitro* anti-oxidant activity of aqueous tea extracts.

In another study, the DPPH• assay was utilized to determine the anti-oxidant activity of fruits, vegetables and teas and the amount of anti-oxidants needed to decrease the initial DPPH• absorbance by 50% (EC_{50}) was determined. It was concluded from the study that in general, honeybush tea (EC_{50} ranges from > 8000 to > 1000 µg/ml) exhibited lower anti-oxidant activity than green (EC_{50} ranges from 120 to 320 µg/ml), rooibos (EC_{50} ranges from 620 to 1100 µg/ml) and black tea (EC_{50} ranges from 230 to 750 µg/ml) (du Toit *et al*., 2001). The results from this study deviated with regard to anti-oxidant activity of honeybush tea from the du Toit *et al*. (2001) study (Figure 5.2). Honeybush tea (IC_{50} 18.02 ± 4.27 µg/ml) exhibited greater anti-oxidant activity than both rooibos and black tea and lower anti-oxidant activity than green tea.

According to McGaw and co-authors (2007), the decoction (50 g of dried aerial parts boiled in 1.5 L water for 15 min) and the infusion (1.5 L of boiling water poured over 50 g of dried aerial parts and allowed to stand for 15 min) of *A. phylicoides* revealed higher anti-oxidant activity than *A. elata* in the Trolox™ equivalent anti-oxidant capacity (TEAC) assay. In the same study the anti-oxidant activity of the *A. phylicoides* decoction was higher than rooibos (TEAC content: 0.269 ± 0.015 vs. 0.257 ± 0.001) and the anti-oxidant activity of the *A. phylicoides* infusion was similar to that of rooibos (TEAC content: 0.248 ± 0.012 vs. 0.257 ± 0.001). The comparative results from the McGaw *et al.* (2007) study deviates from the results obtained in the $DPPH^{\bullet}$ assay, in which *A. phylicoides* (Bush tea) displayed greater anti-oxidant activity than fermented rooibos tea. The difference in results can be attributed to the different methods utilised to determine antioxidant activity. However, the McGaw *et al.* (2007) study supports the conclusion that *A. phylicoides* (Bush tea) possesses some anti-oxidant activity.

In another study, the anti-oxidant $DPPH^{\bullet}$ scavenging activity of the ethanol extract of *A. phylicoides*, harvested in Venda in the Limpopo Province (South Africa) in mid-July was investigated. The concentration of the extract required to scavenge 50% $DPPH^{\bullet}$ (EC_{50}) was calculated. The results revealed that the ethanol extract displayed some degree of anti-oxidant activity (EC_{50} = 10.64 ± 0.08 µg/ml) (Mavundza *et al*., 2007). These results further supports the conclusion that *A. phylicoides* (Bush tea) possesses some anti-oxidant activity. The difference in results between the aqueous extract from this study (IC_{50} = 14.01 ± 2.68 µg/ml) and the ethanol extract (EC_{50} = 10.64 ± 0.08 µg/ml) from the Mavundza *et al*. (2007) study can be attributed to the difference in extraction solvents used, locations from where samples were collected and the time of collection of samples.

Previous studies have shown the potential anti-oxidant properties of polyphenols (Kähkönen *et al*., 1999; Miliauskas *et al*., 2004; Aoshima *et al*., 2007; Conforti *et al*., 2008). Polyphenols have been found in *A. phylicoides*, however, no consistent correlation between the anti-oxidant activity and total phenolic content could be found (McGaw *et al.*, 2007).

According to Manzocco and co-authors (1998), tea exhibits a significant health protecting activity due to its high flavonoid content. Flavonoids are the most abundant compounds in

fresh tea leaves and extracts. Flavonoids have long been recognised to have strong anti-oxidant activity and are therefore considered to be responsible for the anticarcinogenic and antimutagenic properties of tea, as well as its protective action against cardiovascular disease. However, it is well known that the tea manufacturing processes can greatly affect the content of flavonoids. Tea flavonoids are very reactive species, which can easily undergo enzymatic and chemical reactions, which may be responsible for changes in the anti-oxidant properties of the product (Manzocco *et al*., 1998).

The anti-oxidant activity of the commercially available teas assessed in this study, are in agreement with the results of previous studies, in which the effects of manufacturing processes on anti-oxidant activity of tea extracts were investigated (von Gadow *et al*., 1997; Manzocco *et al*., 1998). Green tea and green rooibos tea are produced from fresh leaves, preventing oxidation of flavonoids and therefore exhibit stronger anti-oxidant activity than black tea and fermented rooibos tea, respectively (Figure 5.2). Both black tea and fermented rooibos tea are subjected to chemical modifications during processing. The greater free radical scavenging activity of *A. phylicoides* compared to black and fermented rooibos tea can be attributed to the fact that *A. phylicoides* has not been subjected to any processing. It is not known how *A. phylicoides* free radical scavenging activity will be affected if it is subjected to manufacturing processes like other commercially available teas.

The literature revealed that *A. phylicoides* is used by the local people as a medicinal product and as a beverage (van Wyk and Gericke, 2000). A recent survey conducted in a rural area in Limpopo and three black urban areas in Gauteng revealed that *A. phylicoides* is still a very popular beverage in the rural area and is known and utilised by some urban residents as a tea. It was further indicated by urban residents that *A. phylicoides* would be purchased to be enjoyed as a beverage if it was commercially available (Rampedi and Olivier, 2005). The favourable anti-oxidant property of *A. phylicoides*, in comparison to other commercially available teas (Figure 5.2) supports its traditional use as a beverage.

5.4 Conclusions

- *A. phylicoides* (bush tea) exhibited anti-oxidant activity (IC_{50} = 14.01 ± 2.68 µg/ml) against the free radical scavenging ($DPPH^{\bullet}$) assay.
- *A. phylicoides* (bush tea) anti-oxidant activity was greater than unfermented rooibos and Ceylon (black) tea (IC_{50} > 25.00 µg/ml); but comparable to green rooibos and honeybush tea ((IC_{50} = 18.01 ± 4.06 µg/ml; 18.02 ± 4.27 µg/ml, respectively), but less than green tea (IC_{50} = 9.64 ± 0.96 µg/ml).
- The *in vitro* anti-oxidant results of *A. phylicoides* against the free radical scavenging ($DPPH^{\bullet}$) assay provided preliminary evidence to support its use of as a health-promoting beverage.

Chapter 6 – Antimalarial activity

6.1 Introduction

As recent as the early 1900's, human malaria was endemic across every continent except Antarctica (Girard *et al*., 2007). Despite intense efforts to control and eradicate malaria, the disease continues to be one of the greatest health problems facing Africa. The WHO estimated that at least 247 million cases of malaria occurred in 2008 with nearly one million deaths, mostly among children living in Africa (WHO, 2010).

Malaria, transmitted by the bite of an infected female *Anopheles* mosquito is caused by four species of *Plasmodium*, which are *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (Kaur *et al*., 2009). *P. falciparum* is the most dangerous of the human malaria parasites and is responsible for the majority of infections in Africa (Pillay *et al*., 2008). The life cycle of a malaria parasite is presented in Figure 6.1. Symptoms appear seven days or more (usually 10–15 days) after exposure and include fever, headache, chills and vomiting (WHO, 2010).

Although a number of advances have been made towards the understanding of the disease, the increasing prevalence of drug-resistant strains of *P. falciparum* to standard antimalarial drugs necessitates a continuous effort to search for new antimalarial drugs with new modes of action, used alone or in combination.

History reveals that the majority of antimalarial drugs have been derived from medicinal plants. These include the quinoline-based antimalarials such as quinine as well as artemisinin and its derivatives (Clarkson *et al*., 2004; Bourdy *et al*., 2008). Since the majority of antimalarials originated from plants, an investigation of the chemical components of traditional medicinal plants could lead to the development of new antimalarial drugs. South Africa with its rich diversity of plants is an ideal place to screen plants for antiplasmodial activity (van Wyk, 2008). The mortality of malaria and therefore the pressing need to discover new antimalarials substantiates the investigation of *A. phylicoides* for antiplasmodial activity.

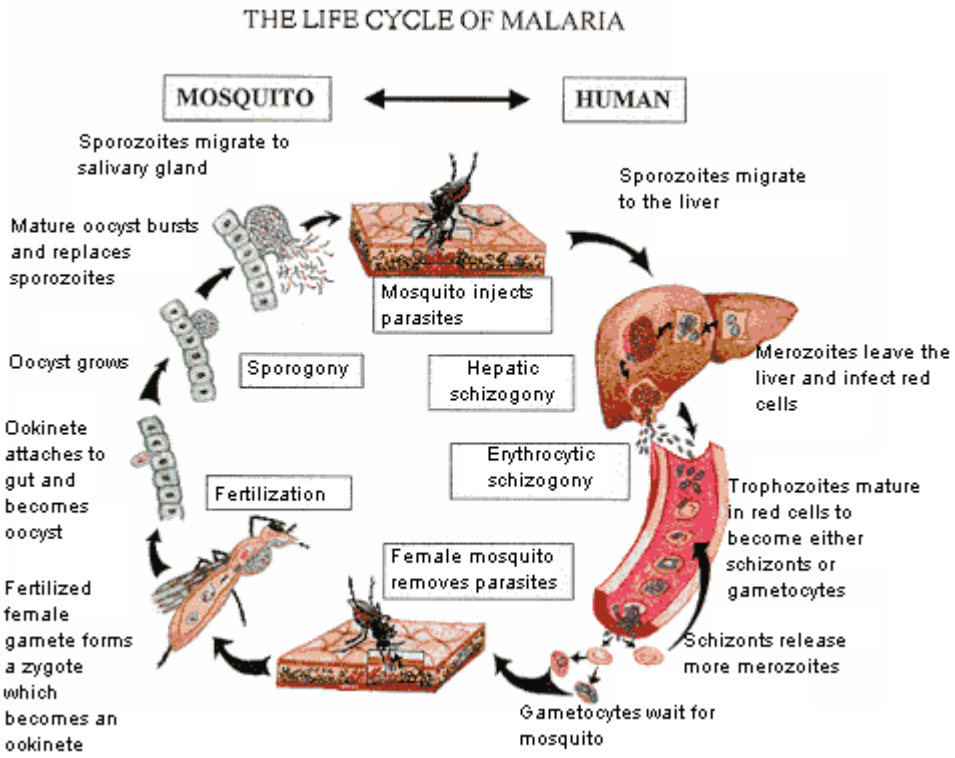


Figure 6.1: The life cycle of the malaria parasite (http://www.emro.who.int/rbm/Images/MalariaLifeCycle-1.gif).

The aim of this study was to investigate the essential oil and methanol extract of the indigenous plant *A. phylicoides* for potential antiplasmodial activity.

6.2 Materials and methods

6.2.1 Parasite suspension

The parasites were grown continuously in culture medium and maintained at a 5 – 10% haematocrit. The culture medium consists of Roswell Park Memorial Institute media (RPMI-1640), HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), D-glucose, hypoxathine, gentamicin, 5% (w/v) $NaHCO_3$ and 10% (v/v) heat-inactivated human plasma (Desjardin *et al.*, 1979). The stock culture was adjusted to a haematocrit of 1% and parasitemia of 0.5% for addition to the microtitre plates.

6.2.2 Tritrated hypoxanthine incorporation assay

The antimalarial activity of the methanol extract and essential oil of *A. phylicoides* was assessed *in vitro* on the chloroquine-resistant *Plasmodium falciparum* (FCR-3) strain using the tritrated hypoxanthine incorporation assay (Desjardin *et al*., 1979; van Zyl *et al*., 2006) in collaboration with Dr RL van Zyl, Department of Pharmacy and Pharmacology, University of Witwatersrand.

6.2.3 Principle of the method

The human malaria parasite *P. falciparum* was exposed to different concentrations of the test compounds for a single cycle of parasite growth (48 hours). The *in vitro* uptake of [^{3}H]-hypoxathine by malaria parasites was used as an indicator of parasite growth. Hypoxanthine is required by the parasites for their DNA synthesis and is only supplied by the human host. The standard antimalarial, quinine was used as a positive control.

6.2.4 Protocol

The methanol extract was dissolved in DMSO to make a stock solution of 10 mg/ml, subsequent dilutions made in experimental medium (RMPI-1640, glucose and HEPES buffer). The essential oil (10 µl) was diluted with 90 µl of DMSO to make a stock solution of 10%; whilst quinine (control) was dissolved in the experimental medium.

For each dilution, 25 µl (extract or control) or 2 µl (essential oil) of the various dilutions were plated out in triplicate in a 96-well plate. To ensure a total of 25 µl per well, 23 µl experimental medium was added to the wells containing essential oils. The parasite suspension 200 µl was then added to each well. Untreated parasites in eight wells and uninfected red blood cells in four wells were used as controls.

After preparation, the plates were placed in a humidified airtight candle jar with a gaseous atmosphere of 5% O_2, 5% CO_2 and 90% N_2. The candle jar was then incubated at 37 °C for 24 hours. Following incubation, 25 µl of [^{3}H]-hypoxanthine was added to each well. The plate was then returned to the candle jar and incubated for a further 18 hours at 37 °C. After the second incubation, parasitic ^{3}H-DNA was harvested on glass fiber filter mats with a cell

harvester. The filter mats were dried and the incorporated [^{3}H]-hypoxanthine was counted with a liquid scintillation counter.

The inhibitory effects of the test compounds were expressed as percentage parasite growth of untreated parasitised and erythrocyte controls. The percentages were plotted against their respective concentrations and the log sigmoid dose-response curve was constructed using the Enzfitter® software. The IC_{50} values (the concentration required to kill 50% parasite) determined from the log sigmoid dose-response curve was calculated as a measure of the antimalarial activity of the solvent extract and essential oil. The experiment was repeated four times for each test compound and the mean and standard deviation reported.

6.3 Results and discussion

The methanol extract of *A. phylicoides* displayed no significant *in vitro* activity against the chloroquine-resistant *P. falciparum* (FCR-3) strain (IC_{50} value = 83.489 ± 5.482 µg/ml). However, the essential oil showed promising *in vitro* activity against the chloroquine-resistant *P. falciparum* (FCR-3) strain (IC_{50} value = 1.006 ± 0.06 µg/ml) when compared to the control quinine (0.034 ± 0.002 µg/m) (Table 6.1).

Table 6.1: *In vitro* antimalarial activity of *A. phylicoides*.

Sample	**Antimalarial activity: IC_{50} ± s.d. (µg/ml)**	**n**
Methanol extract	83.489 ± 5.482	4
Essential oil	1.006 ± 0.06	4
Quinine	0.034 ± 0.002	6

Malaria is a major health problem and new treatments are urgently needed to curb and eradicate this endemic. As a result antimalarial studies have been performed on several plants indigenous to South Africa (Prozesky *et al*., 2001; Clarkson *et al*., 2004). To date no data has been published on the antimalarial activity of *A. phylicoides.*

The antimalarial activity in this study varied greatly between the essential oil and methanol extract (Table 6.1). The essential oil showed good antimalarial activity (1.006 ± 0.06 µg/ml).

According to Clarkson and co-authors (2004), solvent extracts with an IC_{50} value ≤10 μg/ml were classified as having promising antimalarial activity. Therefore it can be concluded that the methanol extract exhibited poor antimalarial activity (IC_{50} value = 83.489 ± 5.482 μg/ml).

Physical properties of essential oils, including low density (~0.94 g/ml) and the ability to readily diffuse across cell membranes (Boyom *et al*., 2003) may partly explain why the essential oil is more active than the methanol extract.

It has been reported that sesquiterpenoids and their derivatives possess numerous biological properties, including antimalarial activity (Boyom *et al*., 2003; Kamatou *et al*., 2005). In a study on antimalarial use of the volatile oil from leaves of *Virola surinamensis* (Rol.) Warb, nerolidol (an acyclic oxygenated sesquiterpene) was identified as one of the active constituents (Lopes *et al*., 1999). α-Pinene (IC_{50} = 1.2 ± 0.2 μM) and (E & Z)–(±)-nerolidol (IC_{50} = 0.9 ± 0.3 μM) have been reported to have potent antimalarial activity (van Zyl *et al*., 2006). In literature, the antimalarial activity of essential oils of other plant species have been reported frequently. Kamatou and co-authors (2005) reported that the essential oils of *Salvia* species displayed good antimalarial activity against the chloroquine-resistant *P. falciparum* (FCR-3) strain (IC_{50} values ranged from 1.23 ± 0.31 to 4.38 ± 1.07 μg/ml). *Lippia multiflora* exhibited similar antimalarial activity against the chloroquine-resistant (FcB1) and chloroquine-sensitive (F32) strains of *P. falciparum* (Valentin *et al*., 1995). Nerolidol and linalool were thought to be the constituents responsible for the antimalarial activity of the essential oil of *L. multiflora*. Boyom *et al*. (2003) investigated the antimalarial activity of *H. crispiflorus* against *P. falciparum* and found that the oils were also active (IC_{50} = 2.0 μg/ml).

Athrixia phylicoides essential oil samples studied consists of the following major sesquiterpenoids and their derivatives in varying quantities: caryophyllene oxide (1.0 – 34.2%); spathulenol (1.5 – 12.6%); germacrene D (trace amounts – 13.5%); β-caryophyllene (0.2 – 11.9%) (Figure 3.3). The acyclic oxygenated sesquiterpene, (*E*)-nerolidol is also present in trace amounts in some samples. In addition, the monoterpene α-pinene (0.8 – 14.8%), which has previously, exhibited antimalarial activity (IC_{50} = 1.2 ± 0.2 μM) (van Zyl *et al*., 2006) is also present (Table 3.1). From the literature review it can be concluded that these

constituents of the essential oil could contribute to the overall antimalarial activity of *A. phylicoides*. These preliminary studies on the antimalarial activity of the essential oils can contribute towards the discovery of agents which may assist with prophylaxis of malaria. However, further *in vitro* and *in vivo* studies are required to understand the mechanism of action of the essential oil.

6.4 Conclusions

- The essential oil of *A. phylicoides* exhibits antimalarial activities against the chloroquine-resistant *Plasmodium falciparum* (FCR-3) strain (IC_{50} = 1.006 ± 0.06 μg/ml).
- The methanol extract of *A. phylicodes* exhibits poor antimalarial activities against chloroquine-resistant *Plasmodium falciparum* (FCR-3) strain (IC_{50} = 83.489 ± 5.482 μg/ml).

Chapter 7 – Anti-inflammatory activity

7.1 Introduction

Inflammation is the body's reaction in response to injury, infections, chemicals and poisons and is characterized by redness, swelling, heat and pain. The inflammatory response has survival value but if inappropriately deployed, as occurs in many diseases, may be deleterious. For this reason it is essential to understand the process of inflammation and the mediators responsible for this process (Dale and Haylett, 2004).

Two important groups of mediators responsible for the inflammatory response are prostaglandins and leukotrienes. The pathway by which prostaglandins are formed, can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and aspirin that inhibit the cyclooxygenase enzyme (Figure 7.1). These drugs are commonly utilized for the management of diseases associated with inflammation such as rheumatoid arthritis, oesteoarthritis, acute bursitis and tendonitis (Dale and Haylett, 2004).

The use of NSAIDs for the management of these diseases provides more substrate for the alternate pathway of the arachidonic acid casade (Figure 7.1), in which the 5-lipoxygenase enzyme converts arachidonic acid into biologically active leukotrienes. Leukotrienes are the major pathophysiological mediators of the inflammatory response, since they are more potent than the prostagladins with regard to the increase in vascular permeability, adhesion of leukocytes to the vessel wall and oedema production. Leukotrienes mediate the inflammatory response, which can lead to various diseases, such as asthma, inflammatory bowel disease and allergic rhinitis. It is therefore essential to discover compounds that can inhibit the 5-lipoxygenase enzyme. A study conducted on South African medicinal plants revealed that some plants such as the aqueous extract of *Melianthus comosus* (IC_{50} = 13.84 ppm) and the essential oil of *Ballota africana* (IC_{50} = 29.99 ppm) exhibited 5-lipoxygenase inhibitory activity when compared to the control, nordihydroguaiaretic acid (NDGA) (IC_{50} = 5.0 ppm) (Frum and Viljoen, 2006).

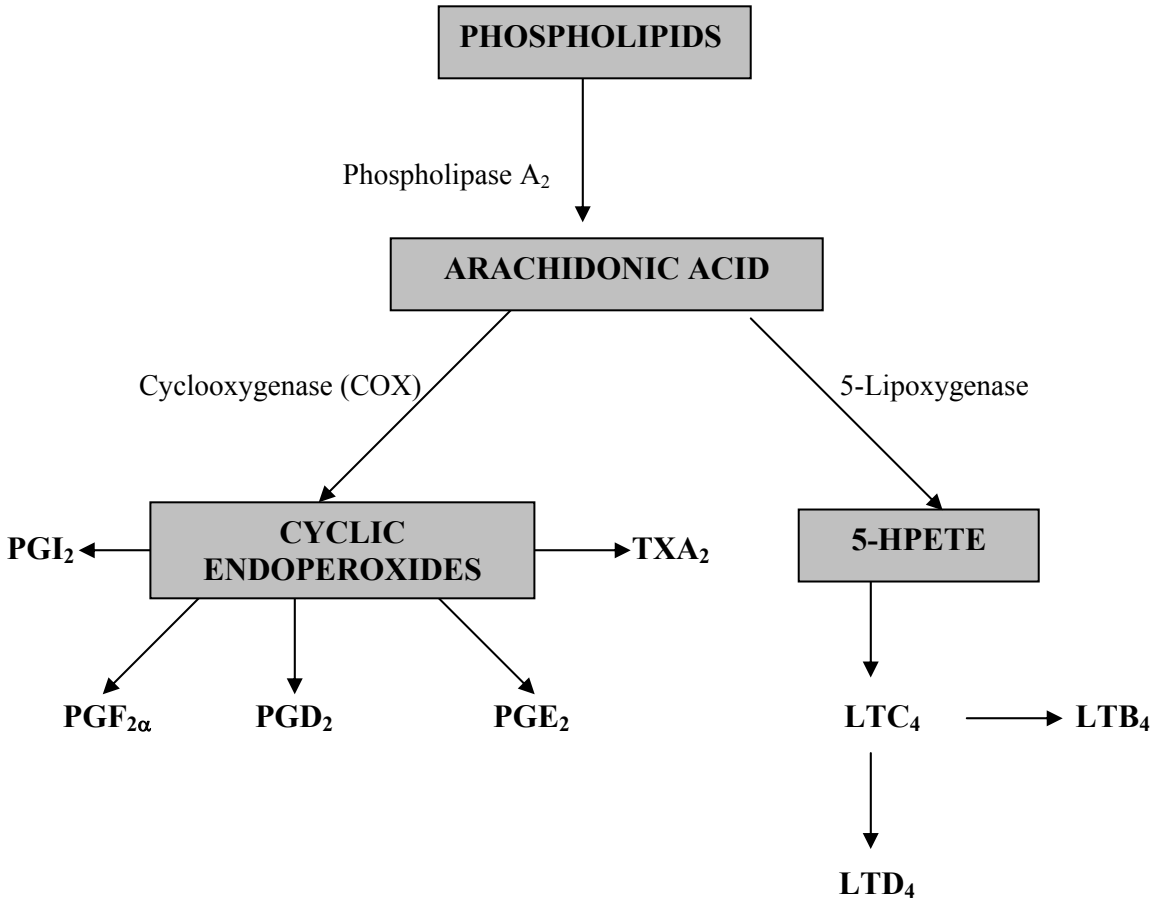


Figure 7.1: Inflammatory cascade, where PG is prostaglandins, LT is leukotrienes, TXA_2-thromboxane and HPETE is hydroperoxyeicosatetraenoic acid (Dale and Haylett, 2004).

It was reported that a decoction or strong tea of *A. phylicoides* leaves has been used in traditional medicine by Sotho speaking people to treat sore feet, and the leaves are chewed by Sotho and Xhosa speaking people to treat sore throats (Table 1.2) (Watt and Breyer-Brandwijk, 1962; Roberts, 1990; Hutchings *et a*l., 1996). These traditional uses imply that the indigenous plant may possess anti-inflammatory activity.

The aim of this study was to determine whether the non-volatile compounds and the essential oil of *A. phylicoides* possess anti-inflammatory activity by specifically inhibiting the 5-lipoxygenase enzyme.

7.2 Materials and methods

The anti-inflammatory activity of the essential oil and methanol extract of *A. phylicoides* was performed *in vitro* using the 5-lipoxygenase assay of Sicar *et al*. (1983) as modified by Evans (1987).

7.2.1 Principle of the method

5-Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1,4-pentadiene structures. In the body, arachidionic acid (biological substrate) is oxidized to hydroperoxyeicosatetraenoic acid (HPETE's) by the 5-lipoxygenase. In the *in vitro* assay, linoleic acid is used as the substrate for 5-lipoxygenase enzyme. Linoleic acid is oxidized *in vitro* to a conjugate diene by 5-lipoxygenase, which absorbs at 234 nm. 5-Lipoxygenase activity is evaluated by the spectrophotometric measurement of the conjugated diene at 234 nm. The initial reaction rate was measured with the UV-VIS spectrophotometer and the decrease of this initial reaction rate is a measure of the sample's inhibitory activity (Baylac and Racine, 2003).

7.2.2 Protocol

The test compounds were dissolved in DMSO and Tween® 20 to achieve a starting concentration of 100 µg/ml. The potassium phosphate buffer (pH 6.3) was prepared by mixing 50 ml of 0.1 M potassium dihydrogen phosphate and 9.7 ml of 0.1 M sodium hydroxide.

In a 3 ml cuvette maintained at 25 °C in a thermostated bath, 10 µl of the test compound dissolved in DMSO and Tween® 20 was mixed with 2.95 ml of pre-warmed potassium phosphate buffer (pH 6.3) and 45 µl of linoleic acid (≥ 99%) from Fluka. Thereafter 100 U of the 5-lipoxygenase (enzyme) diluted in an equal volume of ice-cold potassium phosphate buffer (pH 6.3) (stored at 4 °C), was added to the cuvette to initiate the enzymatic reaction. The same mixture was prepared as above, but only with DMSO and Tween® 20 (no test sample) to act as a negative control.

The inhibitory activity of the test compounds on the enzyme was assessed by recording the absorbance using a UV-VIS spectrophotometer at 234 nm, of the conjugated diene formed from linoleic acid. The initial reaction rate was determined from the slope of the straight line portion of the curve. The percentage inhibition of enzyme activity was calculated by comparison with the negative control. The concentration that gave 50% inhibition (IC_{50}) was calculated using Enzfitter® software. Nordihydroguaiaretic acid was used as a positive control in this assay.

7.3 Results and discussion

Results obtained for the 5-lipoxygenase assay for the methanol extract and essential oil of *A. phylicoides* were quite contrasting. The essential oil displayed *in vitro* 5-lipoxygenase inhibitory activity with an IC_{50} value of 25.68 µg/ml, whereas the methanol extract was found to be inactive at the starting concentration of 100 µg/ml (IC_{50} > 100 µg/ml). The positive control, NDGA inhibited enzyme activity at an IC_{50} value of 5.0 µg/ml. Only one test was performed for both the essential oil and methanol extract due to insufficient quantities of samples remaining after the completion of all other assays.

An essential oil with a $10 \leq IC_{50} \leq 30$ ppm (= µg/ml) is defined as a good 5-lipoxygenase inhibitor (Baylac and Racine, 2003). Therefore, it can be concluded that the essential oil of *A. phylicoides* is a good 5-lipoxygenase inhibitor (IC_{50} = 25.68 µg/ml).

Essential oil constituents, terpenic hydrocarbons (*d*-limonene, $10 \leq IC_{50} \leq 30$ ppm (= µg/ml) and α-pinene, $31 \leq IC_{50} \leq 50$ ppm (= µg/ml)) and sesquiterene hydrocarbons (β-caryophyllene and germacrene D, $10 \leq IC_{50} \leq 30$ ppm (= µg/ml)) exhibited inhibitory activity against the 5-lipoxygenase enzyme (Baylac and Racine, 2003). These compounds could have contributed to the overall anti-inflammatory activity of *A. phylicoides* since they were present in varying concentrations in most samples studied (limonene: 0.7% to 4.0%; α-pinene: 0.8% to 14.8%; β-caryophyllene: 0.2% to 11.9%; germacrene D: trace amounts to 13.5%) (Table 3.1).

Traditionally the leaves of *A. phylicoides* are chewed for the treatment of sore throats and a decoction or strong tea of *A. phylicoides* is used for bathing of sore feet (Table 1.2). The

chewing of the plant can result in the release of the essential oils from the plant. The *in vitro* result obtained from this study confirms that the essential oil of *A. phylicoides* inhibits the 5-lipoxygenase enzyme, providing scientific evidence to justify its traditional use.

It is important to note that the absence of 5-lipoxygenase inhibitory activity of the methanol extract of *A. phylicoides* does not necessarily imply that the extract is devoid of any anti-inflammatory activity. The activity may be observed at concentrations greater than 100 µg/ml as assayed in this study. The inflammatory cascade (Figure 7.1) is very complex and the extract may exert pharmacological effects on other target sites of the cascade, such as the cyclooxygenase enzyme or phospholipase A_2 enzyme. Therefore further studies (e.g. COX-1, COX-2 and phospholipase A_2 inhibition studies) are required to confirm whether or not the extract of *A. phylicoides* displays inhibitory activity at any of the other target sites of the inflammatory cascade.

7.4 Conclusions

- The essential oil exhibited inhibitory activity against the 5-lipoxygenase enzyme (IC_{50} = 25.68 µg/ml).
- The methanol extract was inactive against the 5-lipoxygenase enzyme at a concentration of 100 µg/ml.
- The *in vitro* anti-inflammatory results of the essential oil of *A. phylicoides* against the 5-lipoxygenase enzyme, provided preliminary evidence to support its use as a treatment of sore feet and sore throats.

Chapter 8 – Cytotoxicity

8.1 Introduction

Herbal remedies are rapidly gaining popularity throughout the world as a result of dissatisfaction with conventional medicines. It is a widely held belief that herbal preparations are "natural" and are therefore intrinsically harmless (Bateman *et al*., 1998). In South Africa, traditional or herbal remedies form a significant part of day-to-day living. While many traditional remedies may be of benefit, some may have little or no effect, and others may be cytotoxic. Cytotoxicity is a measure of toxicity of a substance to living cells of the human body. Cytotoxicity of herbal remedies may be attributed to several factors such as hepatotoxicity of main constituents, contamination of preparations by heavy metals or micro-organisms, and adverse reactions due to age, and genetic and concomitant disease characteristics of the user (Bateman *et al*., 1998). Some classical examples of plants that are toxic to humans include foxglove, poison hemlock and aconite (Wittstock and Gershenzon, 2002).

In South Africa, the process for registration and use of conventional medicines is clearly defined and all medicines must have proven quality, safety and efficacy. However traditional remedies are not subjected to the same process. Therefore, the potential toxicity of traditional remedies becomes an important consideration when studying the traditional use of plants. Very little information is available in the literature on the safety and toxicity of *A. phylicoides*, in contrast to other commercially available teas such as rooibos tea and honeybush tea. A number of studies have addressed aspects of safety and toxicity of rooibos and honeybush, for example, it was reported that in a ten week study, chronic consumption of rooibos and honeybush by rats did not cause any adverse effects in the kidney or liver (Marnewick *et al*., 2003). Several studies have also addressed tea-drug interactions (Jang *et al*., 2004; Mertens-Talcott *et al*., 2006; Matsuda *et al*., 2007). The lack of toxicity data for the volatile oils of *A. phylicoides* and the pharmacological activities displayed in the previous Chapters prompted the investigation of the cytotoxicity properties to determine whether *A. phylicoides* is safe for consumption.

The aim of this study was to:

- evaluate the cytotoxic properties of the essential oil and methanol extract of *A. phylicoides*.

8.2 Materials and methods

8.2.1 Microculture tetrazolium cellular viability assay

The cytoxicity properties of the methanol extract and essential oil of *A. phylicoides* was assessed on human kidney epithelial cells using the microculture tetrazolium cellular viability assay, in collaboration with Dr RL van Zyl, Department of Pharmacy and Pharmacology, University of Witwatersrand (Mosmann, 1983; van Zyl *et al*., 2006).

8.2.2 Principle of the method

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) is a tetrazolium dye which is used to measure the cellular conversion of the dye into an insoluble formazan product by active mitochondria of living cells. The conversion of MTT to the insoluble compound is monitored by a shift in absorbance. The amount of formazan produced is directly proportional to the number of active cells. The MTT assay evaluates cellular viability in the presence of a test compound.

8.2.3 Protocol

The human kidney epithelial cells were maintained in culture medium consisting of HAM F10, 5% (v/v) foetal calf serum (FCS) and 0.5 mg/ml gentamicin sulphate. HAM F10 solution consists of HAM F10 medium (9.38 g) and $NaHCO_3$ (1.18 g) in a total volume of one litre of sterile MilliQ™ water. The culture medium was replaced three times a week. Once the cells reached confluency, they were trypsinised with 4 ml of 0.25% Trypsin containing EDTA (0.1%) to obtain a single cell suspension. The cells were centrifuged at 1500 rpm for 5 minutes to remove the trypsin. Once the cells were resuspended in experimental medium, 1 ml was used to seed a new culture and the remaining was used in the MTT assay. The experimental medium was prepared the same way as the culture medium except no gentamicin sulphate was included. The trypsinised cell suspension (50 µl) was stained with an equal

volume of Trypan blue (0.2% w/v) and the number of cells were counted using a haemocytometer. The experimental medium was used to adjust the cell suspension (≥ 95% cell viability) to 0.25 million cells/ml.

The methanol extract were dissolved in DMSO to make a stock solution of 10 mg/ml. Subsequent dilutions were made in experimental medium. The essential oil (10 µl) was diluted with 90 µl of DMSO to make a stock solution of 10% and subsequent dilutions were made in DMSO. Quinine (negative control) was dissolved in the experimental medium.

Kidney epithelial cell suspension (180 µl) was plated out in a 96-well plate and incubated at 37 °C for six hours to allow the cells to adhere to the well. For each dilution, 20 µl (extract or control) or 2 µl (essential oil) of the various dilutions were plated out in triplicate in a 96-well plate. To ensure a total of 200 µl per well, 18 µl experimental medium was added to the wells containing essential oils. Each plate contained 10 wells for the untreated cell control and two wells for the blank cell-free control. The plates were incubated in 5% CO_2 at 37 °C. After 44 hours of incubation 40 µl of MTT (12mM) was added to each well and re-incubated for further four hours. The supernatant (180 µl) was removed from each well and replaced with 150 µl of DMSO to stop the reaction and to solubilise the formazan crystals. The plates were shaken for four minutes and the absorbance was read at a test wavelength of 540 nm and a reference wavelength of 690 nm. The results were expressed as percentage cellular viability of the drug and cell-free controls. The percentage cellular viability data was plotted against their respective concentrations and log sigmoid dose-response curves were generated by the Enzfitter® software. The concentration which inhibits 50% of cellular growth (IC_{50} value) was determined from the log sigmoid dose-response profile. The experiment was repeated four times and the mean and standard deviation (s.d.) calculated. Quinine, a plant -derived compound considered to be relatively safe, was used as a negative control.

The safety index relates the dose of the drug required to produce a desired effect to that which causes an undesired effect. The toxicity of the methanol extract and essential oil was compared to their antimalarial activity, using the safety index Equation 1.

$$\text{Safety Index} = \frac{\text{Toxicity}}{\text{Activity}}$$

Equation 1

8.3 Results and discussion

The toxicity of the methanol extract and essential oil of *A. phylicoides* was assessed using the MTT assay and the results obtained are presented in Table 8.1. The essential oil proved to be more toxic than the methanol extract. The safety indices of the samples and control were calculated and are presented in Table 8.1. The safety index of the control, quinine (SI = 4001.74) was appreciably higher than the methanol extract (SI = 2.28) and the essential oil (SI = 1.77). The low safety indices of the methanol extract and the essential oil implied that they have low degrees of selectivity for *P. falciparum*.

Table 8.1: *In vitro* cytotoxic properties of *A. phylicoides*.

Sample	**Toxicity: IC_{50} ± s.d. (µg/ml)**	**n**	**Antimalarial activity IC_{50} ± s.d. (µg/ml)**	**Safety index (SI)**
Methanol extract	189.893 ± 12.658	4	83.489 ± 5.482	2.28
Essential oil	1.776 ± 0.2298	4	1.006 ± 0.06	1.77
Quinine	136.059 ± 4.057	6	0.034 ± 0.002	4001.74

The cytotoxicity of the extract of *A. phylicoides* had been investigated in two *in vitro* studies and one *in vivo* study. McGaw *et al*. (2007) investigated the cytotoxicity of the *A. phylicoides* against the Vero monkey kidney cell line (MTT assay) and brine shrimp eggs (Solis *et al*., 1993 method) and the concentration required to kill 50% of cells (LC_{50}) was calculated. It was found that the ethanol extract displayed low toxicity (Vero cell: LC_{50} = 252 µg/ml and Brine shrimp: LC_{50} = 394 µg/ml) and the aqueous preparations (i.e. aqueous extract, decoction and infusion) had no effect on either assay method. In another study using the sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) colorimetric assay, the cytotoxicity of the ethanol extract against Vero cells lines was investigated. The extract displayed low toxicity (IC_{50} = 107.8 ± 0.13 µg/ml)

(Mavundza *et al*., 2010). Chellan *et al*. (2008) investigated the effect of high doses of aqueous extract of *A. phylicoides* (30 to 180 mg) on Wistar rats for a period of three months. No mortality or morbidity was reported. Histopathology showed no signs of any extract induced toxicity in the liver, kidneys or gastrointestinal tract and all laboratory results (serum alkaline phosphatase, creatinine and urea levels) were normal.

No data has been published on the cytotoxicity of the essential oils of *A. phylicoides*. The essential oil is about 77 times more toxic than the control, quinine and has a low safety index of 1.77. The essential oil composition in Table 3.1 was evaluated to identify compounds possibly contributing to the toxic effects noted. Caryophyllene oxide has been reported to have cytotoxic activity (LC_{50} values range from 147 to 351 μM) (Sibanda *et al*., 2004). The cytotoxicity of *P. serrulata* essential oil can be partly explained by the presence of α-humulene, caryophyllene oxide, γ-elemene and δ-elemene (Jie *et al*., 2007). In a study conducted by van Zyl *et al*. (2006), it was reported that α-pinene (IC_{50} = 172.2 ± 13.4 μM), β-pinene (IC_{50} = 166.7 ± 19.6 μM), (E- & Z-)-(±)-nerolidol (IC_{50} = 5.5 ± 1.2 μM) and 1,8-cineole (IC_{50} = 69.1 ± 9.4 μM) displayed some degree of toxicity. Caryophyllene oxide, α-pinene and β-pinene have been identified as major constituents of the essential oil (Figure 3.3). Nerolidol, α-humulene and 1,8-cineole are either present in trace amounts or very small percentages in some of the samples studied (Table 3.1). It can therefore be assumed that caryophyllene oxide, α-pinene and β-pinene together with the minor constituents (nerolidol, α-humulene and 1,8-cineole) synergistically contribute to the cytotoxicity of the essential oil.

The cytotoxicity of the essential oil was compared to the antimalarial results in Table 6.1. The results from the MTT assay suggest that the antimalarial activity (Table 6.1) of the essential oil may be due to general cytotoxic effects and this was further supported by the low safety index of the essential oil (SI = 1.77). This implied that the essential oil had a greater affinity for human kidney epithelial cells than for *P. falciparum*.

The extensive traditional use of *A. phylicoides* aqueous extracts as a tea and medicine (Table 1.2) and the lack of adverse effects published in the literature (van Wyk and Gericke, 2000; Olivier and De Jager, 2005; Rampedi and Olivier, 2005), may be an indication of the lack of

toxicity of this species. This hypothesis that *A. phylicoides* is unlikely to be toxic was supported by the studies conducted by McGaw *et al*. (2007) and Chellan *et al*. (2008). However, further *in vivo* testing is needed to confirm the safety profile of *A. phylicoides*.

8.4 Conclusions

- The essential oil of *A. phylicoides* (IC_{50} = 1.776 ± 0.2298 μg/ml) is more toxic than the methanol extract (IC_{50} = 189.893 ± 12.658 μg/ml).
- Both the essential oil (SI = 1.77) and methanol extract (SI = 2.28) of *A. phylicoides* have low safety indices.

Chapter 9 – General Conclusions and Recommendations

9.1 Summary

For centuries eastern countries have been using herbal remedies in the form of teas to treat infections, ailments and diseases, however, in recent years herbal teas have gained popularity in western countries for their health-related properties, including antimicrobial activity and anti-oxidant activity (Almajano *et al*., 2008; Chan *et al*., 2010). One of the most beneficial effects of tea is its anti-oxidant activity. Anti-oxidants play a role in the prevention of cardiovascular disease, cancers and neurodegenerative diseases (Kris-Etherton *et al*., 2002). As such research on teas and their effects on human health have intensified. This report focused on the health-related properties as well as the toxicity of *A. phylicoides*.

The results of the *in vitro* biological activities and the compounds identified in this study are given in Table 9.1. The methanol extract of *A. phylicoides* was found to be more active against the micro-organisms tested compared to the essential oil; whereas the essential oil displayed greater antimalarial and anti-inflammatory activity compared to the methanol extract (Table 9.1). The essential oil was also found to be more toxic when compared to the methanol extract of *A. phylicoides* (Table 9.1). The aqueous extract of *A. phylicoides* exhibited anti-oxidant activity comparable to that of commercially available teas (green, green rooibos, honeybush, rooibos and Ceylon) (Table 9.1). Seven major compounds (>10%) were identified in the essential oil and one bioactive compound was identified in the methanol extract of *A. phylicoides*. The bioactive compound was found to be 24 to 51 times more active against micro-organisms tested compared to the methanol extract of *A. phylicoides* (Table 9.1).

This report is the first consolidated report in which the essential oil composition and the biological activities of both the essential oil and methanol extract of *A. phylicoides* has been investigated. It has revealed that *Athrixia phylicoides* possesses antimicrobial, anti-oxidant anti-inflammatory and antimalarial activity. As such this report paves the way for the successful marketing of *A. phylicoides* against the commonly known rooibos and honeybush tea.

9.2 Recommendations for future work

- It is recommended that a seasonal and geographical variation study is performed on *A. phylicoides* to explain the variations in essential oil composition noted in the samples studied.
- The chemotypic variation be investigated for solvent extracts using liquid chromatography and HPLC.
- Since the anti-inflammatory activity was only performed on one sample, it is recommended that the assay be repeated to confirm the results obtained in this report.
- A study on the anti-oxidant activity of the essential oil and methanol extract should be performed to obtain a complete anti-oxidant profile of *A. phylicoides*.
- Since the aqueous extract was only tested for anti-oxidant activity, to obtain a complete profile the other assays conducted in this study should be done.
- Studies should be performed to identify the active compounds responsible for anti-inflammatory and antimalarial activity, and toxicity of the essential oil of *A. phylicoides* and for the anti-oxidant activity of the aqueous extract of *A. phylicoides*.

Table 9.1: Summary of the phytochemistry and *in vitro* biological activities of the essential oils and extracts of *A. phylicoides*.

	Antimicrobial activity MIC range (mg/ml)	Anti-oxidant activity (µg/ml)	Antimalarial activity (µg/ml)	Anti-inflammatory activity (µg/ml)	Cytotoxicity (µg/ml)	Compounds identified
***A. phylicoides* essential oil**	Gram +ve bacteria: 3 to >32. Gram –ve bacteria: 12 to >32. Yeasts: 4 to 6.	ND	1.006 ± 0.06	25.68	1.776 ± 0.2298	Major compounds (>10%): caryophyllene oxide, spathulenol, germacrene D, β-caryophyllene, β-pinene, α-pinene and myrcene
***A. phylicoides* methanol extract**	Gram +ve bacteria: 1 to 4. Gram –ve bacteria: 3 to >6. Yeasts: 1.5 to 2.	ND	83.489 ± 5.482	>100	189.893 ± 12.658	(4-Hydroxyphenyl) propyl coumaroate
(4-Hydroxyphenyl) propyl coumaroate	Gram +ve bacteria: 0.0195 Gram –ve bacteria: 0.125 Yeasts: 0.078	ND	ND	ND	ND	ND
Aqueous tea extracts	ND	Green: 9.64 ± 0.96 *A. phylicoides:* 14.01 ± 2.68 Green rooibos: 18.01 ± 4.06 Honeybush: 18.02 ± 4.27 Rooibos: 25.83 ± 2.39 Ceylon: 29.26 ± 2.68	ND	ND	ND	ND

ND: Not determined; Gram +ve: Gram-positive; Gram –ve: Gram-negative

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Appendix A: NMR data for the (4-hydroxyphenyl)propyl coumaroate.

Table A.1: NMR data (in $CDCl_3$ + CD_3OD) of the compound isolated from *A. phylicoides.*

Position	δ_C	δ_H
1	30.9*	2.62 (2H, t, *J* 7.5 Hz)
2	30.9 *	1.94 (2H, quintet, *J* 7.3 Hz)
3	64.2	4.14 (2H, t, *J* 6.5 Hz)
1'	132.6	
2'	129.7	7.00 (2H, d, *J* 8.5 Hz)
3'	115.5	6.72 (2H, d, *J* 8.6)
4'	155.3	
5'	115.5	6.72 (2H, d, *J* 8.6)
6'	129.7	7.00 (2H, d, *J* 8.5 Hz)
1"	126.3	
2"	130.4	7.40 (2H, d, *J* 8.7 Hz)
3"	116.2	6.80 (2H, *d*, *J* 8.7 Hz)
4"	159.9	
5"	116.2	6.80 (2H, *d*, *J* 8.7 Hz)
6"	130.4	7.40 (2H, d, *J* 8.7 Hz)
α	114.7	
β	145.6	7.56 (1H, d, *J* 16.0 Hz)
=O	168.6	

CD_3OD: methanol; $CDCl_3$: chloroform; d: doublet; t: triplet, δ_C: carbon shift; δ_H: proton shift; *J*: coupling constant; Hz: hertz.
* Assignment may be interchanged.

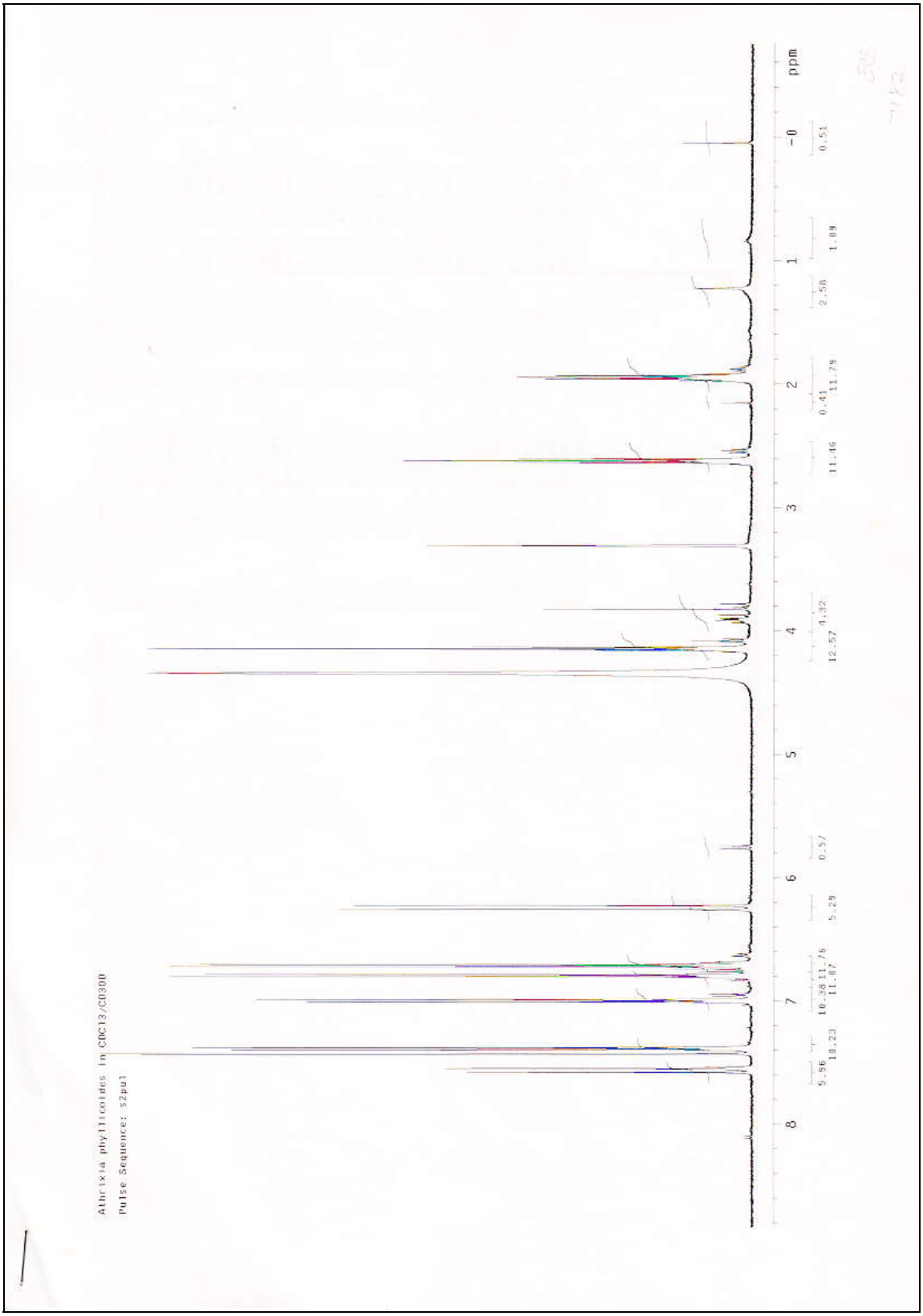

Figure A.1: The NMR carbon spectra (^{1}H) for (4-hydroxyphenyl)propyl coumaroate.

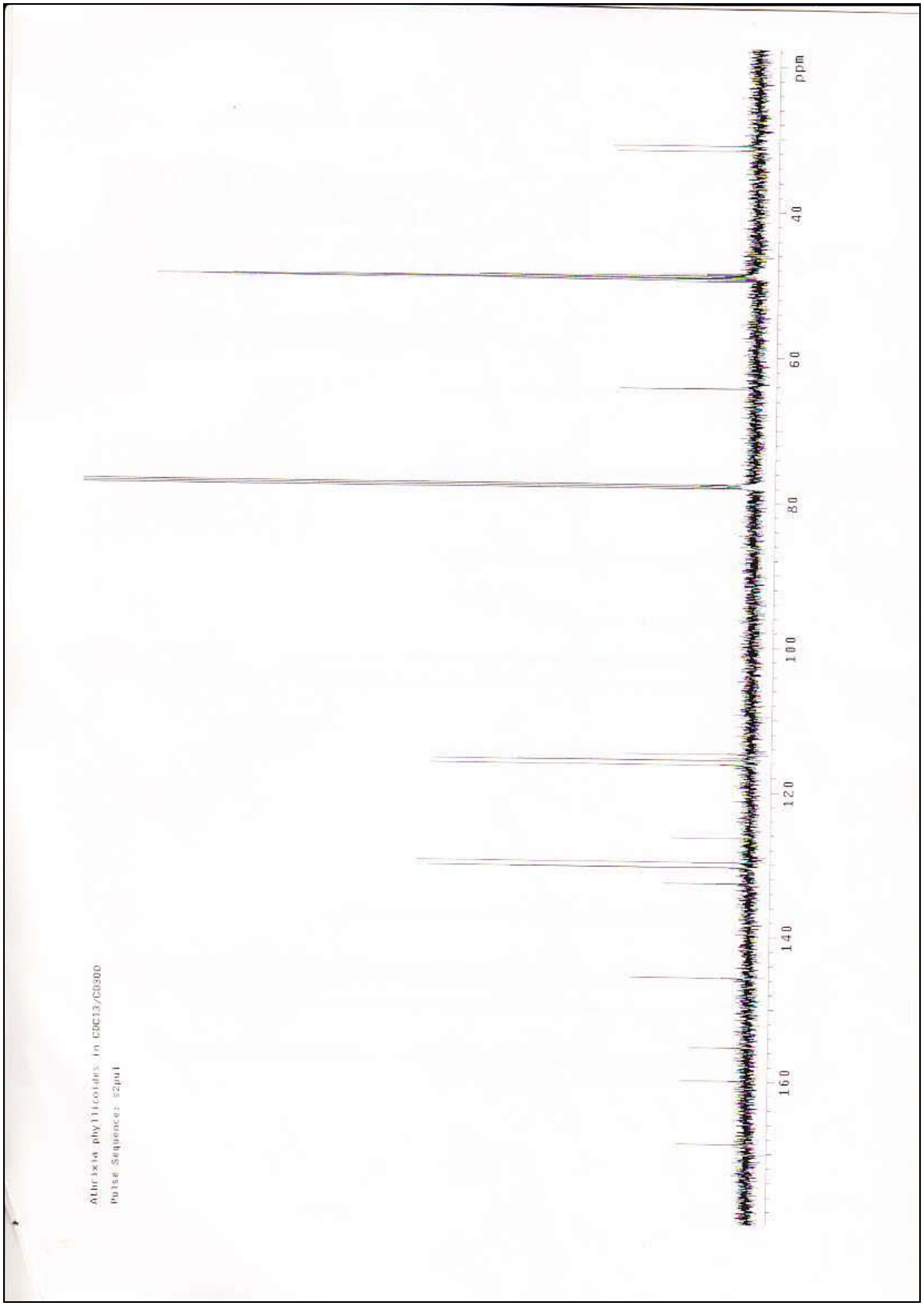

Figure A.2: The NMR carbon spectra (^{13}C) for (4-hydroxyphenyl)propyl coumaroate.