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Antioxidant and antibacterial activities of ethanol extract and flavonoids isolated from *Athrixia phylicoides*

by

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submitted in partial fulfilment of the requirements for the degree of

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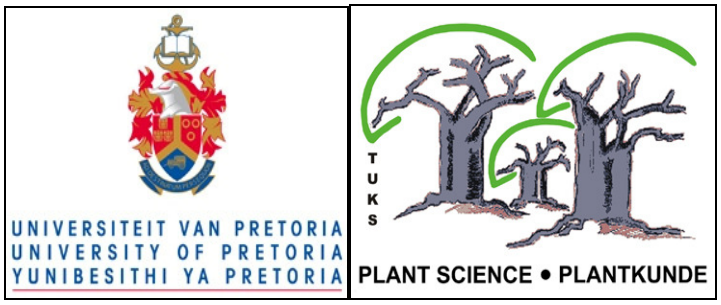

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DECLARATION

I the undersigned, declare that these studies except were acknowledge in the text, is my own work and has not previously submitted to this or any other institution. I empower the University of Pretoria to reproduce for the purpose of research the whole or any part of the contents in any manner whatsoever.

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Date.....

DEDICATION

This thesis is dedicated to my late grandmother, Maria Mamayila N'wapitirosi Mavundza, who passed away in March 2007. She played a vital role in my upbringing and my studies.

Etlela hikurhula ntombi ya ka Nkuna!

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AIDS	Acquired immune deficiency syndrome
ATCC	American type culture collection
^{13}C-NMR	Carbon-nuclear magnetic resonance
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1, 2 –diphynyl-2-picrylhydrazyl
EC_{50}	Half maximal inhibitory concentration
^{1}H-NMR	Proton-nuclear magnetic resonance
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl
MIC	Minimum inhibitory concentration
NMR	Nuclear magnetic resonance
TLC	Thin layer chromatography
TPC	Total phenol content
UV	Ultra violet light
WHO	World health organisation
XTT	2, 3-bis- (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

ABSTRACT

Antioxidant and antibacterial activities of ethanol extract and flavonoids isolated from *Athrixia phylicoides*

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Degree: MSc (Plant Science)

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The ethanol extract of *A. phylicoides* was investigated for its antioxidant activity using the DPPH scavenging method. The extract showed good antioxidant results with a EC_{50} value of 10.64 ± 0.0842 µg/ml. The extract was also tested for antibacterial activity against microorganisms (*Staphylococcus aureus, Enterococus faecalis, Bacillus cereus, Bacillus subtilis, Bacillus pumilus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia*) commonly known to pose a threat in the wellbeing of man. All tested microorganisms were significantly inhibited by the extract with the MIC values ranging from 3.13 µg/ml to 6.25 µg/ml. Folin-Ciocalteu's reagent method was used to determine total phenolic content of dried and freshly prepared crude extract of *A. phylicoides*. Higher total phenolic content (28.28 ± 0.019 mg GAC/100g) and antioxidant activity (EC_{50}, 10.64 ± 0.084 µg/ml) was

observed in the dried extract compared to the fresh extract with a TPC value of 23.04 ± 0.003 mg GAC/100g and EC_{50} of 13.97 ± 0.066 µg/ml.

Bioassay-guided fractionation of ethanolic extract from aerial parts of *Athrixia phylicoides* using silica and sephadex column chromatography led to the isolation of four known flavanoids, 5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavon-3-ol (**1**), 3-0-demethyldigicitrin (**2**), 5,6,7,8,3',4'-hexamethoxyflavone (**3**) and Quecertin (**4**). Due to the low yield**,** no further tests were done on compound **3**. A DPPH-scavenging assay was performed to evaluate the antioxidant activity of the isolated compounds. All the tested compounds showed potent antioxidant activity with EC_{50} values ranging from 1.27 to 3.41 µg/ml. Compound **4** showed a higher antioxidant activity (EC_{50}, 1.27 µg/ml) than vitamin C ($EC_{50,}$ 2.66 µg/ml) used as a control. The MIC values of the isolated compounds against tested microorganisms varied from 20 to more than 40 µg/ml. All the tested compounds showed no activity against *S. aureus, B. pumilus, K. pneumonia* and *P. aeruginosa* at the highest concentration tested (40 µg/ml). These compounds together with the extract were further analyzed by XTT assay on Vero cells. The extract showed a low toxicity effect on the cells at lower concentrations exhibiting EC_{50} value of 107.8 ± 0.129 µg/ml. Compound **4** showed minimal toxicity effect on the cells with a EC_{50} value of 81.38±0.331 µg/ml, compared to Compound **1** and **2** which exhibited EC_{50} values of 27.91 ± 0.181 µg/ml and 28.92 ± 0.118 µg/ml respectively. The results obtained from this study provide a clear rationale for the medicinal uses of *Athrixia phylicoides.*

Keywords: *Athrixia phylicoides;* Compounds; Antioxidant activity; DPPH; $EC_{50;}$ Cytotoxicity; IC_{50}.

Chapter 1

Introduction

1.1 Background

Throughout the ages, humans have relied on nature for their basic needs for the production of foods, shelters, clothing, means of transportation, fertilizers, flavours, fragrances, and not least medicines (Newman *et al*., 2000; Gurib–Fakim, 2006). Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies (Gurib–Fakim, 2006). The use of plants as medicine dated back to early man. Certainly, the great civilisations of the ancient Chinese, Indians, and North Africans provided written evidence of man's ingenuity in utilising plants for the treatment of a wide variety of diseases (Phillipson, 2001; Samuelsson, 2004). These medicines took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations. The first records, written on clay tablets in cuneiform, are from Mesopotamia and date from about 2600 BC; among the substances that were used were oils of *Cedrus* species (Cedar) and *Cupressus sempervirens* (Cypress), *Glycyrrhiza glabra* (Licorice), *Commiphor*a species (Myrrh) and *Papaver somniferum* (poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation (Gurib–Fakim, 2006).

Through periods of trial, error and success, the herbalists and their apprentices have accumulated a large body of knowledge about medicinal plants (Iwu *et al*., 1999). In recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early

19th century (Samuelsson, 2004). Most of the clinical drugs that are currently in use were derived from plants and were discovered because of their use in traditional medicine. Asprin (antipyretic), atropine, cocaine, codeine, digitoxin, respine (hypertension) and quinine in addition to morphine (pain killer) are a few examples of drugs, which were discovered through the study of ethnobotany (Newman *et al*., 2000; Gilani and Attaur-Rahman, 2005). Table 1.1 lists just a few of the many examples of drugs derived from plants. Significantly, 74% of these were obtained because of chemical studies directed at the isolation of the active substance from the plants used in traditional medicine (Newman *et al*., 2000). Today, despite advances in pharmacology and synthetic organic chemistry, the reliance on plants, remains largely unchanged (Phillipson, 2001). The vast majority of people on this planet still rely on their traditional medicinal plants for their everyday health care needs. According to World Health Organisation (WHO), about 80% of the world's population, primarily those of developing countries rely on plant-derived medicines for their healthcare (Gurib-Fakim, 2006). This means that about 3.5 to 4 billion people in the world rely on plants as a source of drugs. Of the 250 000 species of higher plants known to exist on earth, only a relative handful have been thoroughly studied for all aspects of their potential therapeutic value in medicine. As a result, the pharmacology industry has invested vast resources into screening the active constituents of medicinal plants from all over the world (Finimh, 2001).

Table 1.1 Plants used in traditional medicine which have given useful modern drugs (Gurib–Fakim, 2006).

Botanical names	English names	Indigenous use	Origin	Use in biomedicine	Biological active compounds
Ahdatoda vasica	-	Antispasmodic, antiseptic, insecticides, fish poison	India, Sri Lanka	Antispasmodic, oxytocic, cough suppressant	Vasicin (lead molecule for Bromhexin and Ambroxol)
Catharanthus roseus	Periwinkle	Diabetes, fever	Madagascar	Cancer chemotherapy	Vincristine, Vinblastine
Condrodendron tomentosum	-	Arrow poison	Brazil, Peru	Muscular relaxation	D-Tubocurarine
Gingko biloba	Gingko	Asthma, anthelmintic (fruit)	Eastern China	Dementia, cerebral deficiencies	Ginkgolides
Harpogophytum procumbens	Devil's claw	Fever, inflammatory conditions	Southern Africa	Pain, rheumatism	Harpagoside, Caffeic acid
Piper methysticum	Kava	Ritual stimulant, tonic	Polynesia	Anxiolytic, mild stimulant	Kava pyrones
Podophyllum peltatum	May apple	Laxative, skin infections	North America	Cancer chemotherapy, warts	Podopphyllotoxin and lignans
Prunus africana	African plum	Laxative, Old man's diseases'	Tropical Africa	Prostate hyperplasia	Sitosterol

1.2 Herbal medicines in South Africa

Southern Africa boast an amazing floral diversity, with an estimated nearly 30 000 species of higher plants many of which are endemic to the region (Fennell *et al*., 2004; Light *et al*., 2005; Thring and Weitz, 2006). Around 147 plant families are used traditionally for medicinal purposes. The most prominent of these, with over 50 species each are the Fabaceae, Asteraceae, Euphorbiaceae, Rubiaceae and Orchidaceae families (Louw *et al*., 2002). It has been estimated that approximately 3000 plant species are used as medicines (Light *et al*., 2005).

In South Africa, as in most developing countries, traditional herbal medicine still forms the back bone of rural health care. The uses of traditional medicines are prevalent in regions where western medicines are inaccessible due to their unavailability and high cost. It is however, largely due to cultural importance of traditional medicines that the demand for these herbal remedies remains so high (Light *et al*., 2005). It is estimated that 27 million South Africans (about 60%) depend on traditional herbal medicines. There are now about 200 000 registered traditional healers in South Africa (Thring and Weitz, 2006). Traditional healers (Figure 1.1) are commonly called “tin’anga” (Xitsonga), “inyanga” and “isangoma” (isiZulu), “ixwele” and “amaqhira” (Xhosa), “nqaka (Sotho) and “bossiedokter” and “kruiedokter” (Afrikaans). They use many diffrent traditional medicines derived from plants for various ailments and the practical knowledge regarding the healing powers of plants is passed on to the next generation by word of mouth and experience (Louw *et al*., 2002).

Figure 1.1 South African traditional healers (www.aids.org.za).

The exponential growth of the South African population in the latter half of the twentieth century has led to an almost exponential increase in the demand for medicinal plants. Demand for plant-derived medicine has created a trade in indigenous plants in South Africa. More than 700 plant species are known to be actively traded throughout the country in informal medicinal plant markets (Figure 1.2), which contributes to a multi-million rand hidden economy. The trading business is currently estimated to be worth approximately R270 million a year (Dold and Cocks, 2002; Light *et al*., 2005,). Among medicinal plants being traded are *Warburgia salutaris*, *Siphonochilus aethiopicus*, *Aloe ferox*, *Agathosma* spp., *Harpagophytum procumbens*, *Pelargonium sidoides*, *Elaeodendron transvaalense*, *Alepiadea amatymbica*, *Erythrophleum lasianthum* and *Xysmalobium undulatum*

(Fennell *et al*., 2004). Currently there are no regulations to monitor and control the trading of these plants. Many South African medicinal plants are harvested from the wild and their biodiversity is highly threatened. The excessive harvesting has resulted in many species becoming extremely rare with some facing extinction, especially those outside protected areas (Fennell *et al*., 2004; Street *et al*., 2008). The prescription and uses of traditional medicines is also not regulated, as a result there is always the danger of misadministration, especially of toxic plants (Dold and Cocks, 2002; Fennell *et al*., 2004). Poisoning by traditional medicines is very common in South Africa, especially in children. Misidentification of a plant is a common reason for poisoning, for example, 11% of 442 children were admitted in Ga-Rankuwa hospital after mistakly ingested the seeds of *Jatropha curcas* L. (Euphorbiceae) (Street *et al*., 2008).

Figure 1.2 Informal markets of medicinal plants in South Africa (www.gardenafica.org.za).

Table 1.2 Selected indigenous medicinal plants (Van Wyk *et al*., 1997).

Species	**Common name**	**Medicinal uses**
Acacia karroo	Sweet thorn	Diarrhoea, dysentery
Adansonia digitata	Baobab	Fever, diarrhoea, haemoptysis
Agathosma betulina	Khoi	Stomach, kidney complaints
Aloe ferox	Bitter aloe	Laxative, arthritis, eczema
Aspalathus linearis	Rooibos tea	Milk substitute for infants
Elaeodendron transvalensis[*]	Saffronwood	Stomach cleaner, fever
Catharanthus roselis	Periwinkle	Diabetes, rheumatism
Cyclopia intermedia	Honeybush tea	Herbal tea, urinary system
Helichrysum odoratissimu	Everlastings	Wounds, fever, colds, coughs
Psidium guajava	Guava	Malaria, diabetes, ulcer
Punica granatum	Pomegranate	Tapeworms, stomach ache
Rauvolfia caffra	Quinine tree	Fever, malaria, insomnia
Scadoxus punicelis	Red paintbrush	Coughs, colds
Schotia brachypetala	Weeping boer-bean	Heart burn, diarrhoea
Sclerocarya birrea	Marula	Diarrhoea, stomach pains
Securidaca longepedunculata	Violet tree	Coughs, chest pains, headaches
Trichilia emetica	Natal mahogany	Stomach and internal complaints
Warburgia salutaris	Pepper-bark tree	Colds, headaches, malaria
Zantedeschia aethiopia	Arum lily	Asthma, heartburn, sore throats
Zanthoxylum capense	Small knobwood	Stomach ache, fever
Ziziphus mucronata	Buffalo-thorn	Wounds, boils, sores, burns

*Previously called *Cassine transvalensis.*

1.3 Teas

1.3.1 Botany of tea

The tea plant, *Camellia sinensis,* is a perennial evergreen shrub that belongs to the *Theaceae* family. There are two varieties of tea, *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* (Weisburger, 1997; Dufresne and Farnworth, 2000;; Wang *et al*., 2000; Wu and Wei, 2002; Schmidt, *et al*., 2005). *C. sinensis* var. *sinensis,* known as China tea, is grown extensively in China, Japan, and Taiwan, while *C. sinensis* var. *assamica* (known as Assam tea) predominates in south and south-eastern Asia, including Malaysia and, more recently Australia (Chan *et al*., 2007). Tea is a shrub grown in about 30 countries worldwide and it can attain a height of 20-30 m. It is grown in a wide range of latitudes ranging from 45°N to 30°S and longitude from 150°E to 60°W (Mudau, 2006). It grows better in tropical and subtropical regions with high humidity, adequate rainfall, and slightly acidic soil, from sea level to high mountains (Chan *et al*., 2007). Tea is often planted in the highlands at a density of 5000 to 10 000 plants per hectare and maintained as a low evergreen shrubs of 1 to 1.5 m height by pruning during harvesting. Tea leaves are harvested the whole year round in tropical countries whereas in temperate countries harvesting is done seasonally. To make fine quality tea, the two youngest leaves and the terminal bud are plucked (Mudau, 2006; Chan *et al*., 2007).

1.3.2 Types of teas

Depending on the treatment of the harvested leaf, teas are classified into three major types: green tea (unfermented), oolong (semi-fermented), and black (Fully fermented) (Weisburger, 1997; Wang *et al*., 2000; Luczaj and Skrzydlewska, 2005; Schmidt *et al*., 2005). The leaves of *C. sinensis* contain specific polyphenols and an

enzyme, polyphenol oxidase. As soon as the leaves are chopped the enzyme is activated and the polyphenols oxidised. Green tea, consumed mainly in Japan, China and Korea is produced when freshly harvested leaves of *C. sinensis* are subjected to withering, and then pan-fried/steamed prior to rolling/shaping and drying (Figure 1.3) (Santana-Rios *et al*., 2001).

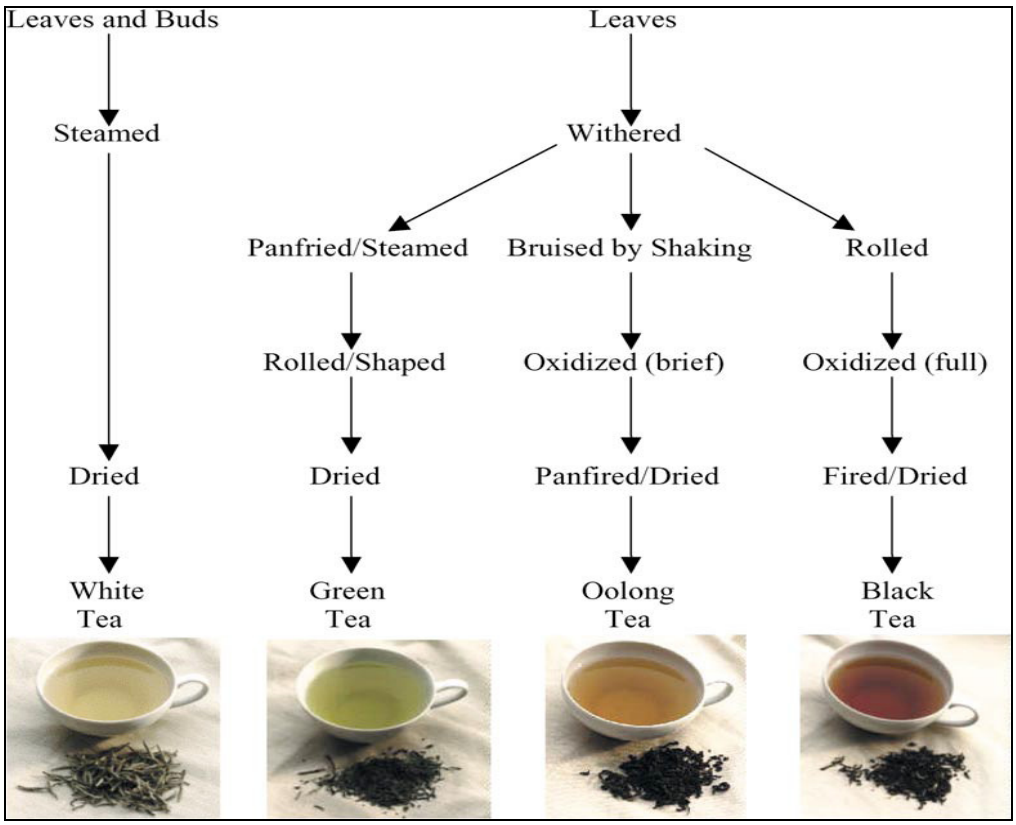


Figure 1.3 Tea manufacturing processes (Santana-Rios *et al*., 2001).

Black tea, which represents >90% of the total consumption worldwide, follows some of the processing steps used for green tea but with the critical difference that the leaves are bruised, crushed, or broken, thus allowing the polyphenol oxidases in the leaf to generate theaflavins, thearubigins and other complex polyhenols from endogenous catechins and this is termed fermentation (Wang *et al*., 2000; Santana-Rios *et al*., 2001). This fermentation process gives black tea its typical colour and the

strong, astringent favour. Oolong tea, popular in China and Japan, goes through an intermediate process involving withering, bruising, brief oxidation, and frying/drying (Santana-Rios *et al*., 2001). The frying process ends the oxidation; hence oolong tea is called semi-fermented tea. The characteristics of oolong tea are between that of black and green tea (Wang *et al*., 2000). White tea, which has received little attention, it is the least processed tea when compared to the other three. The leaves are steamed and dried only.

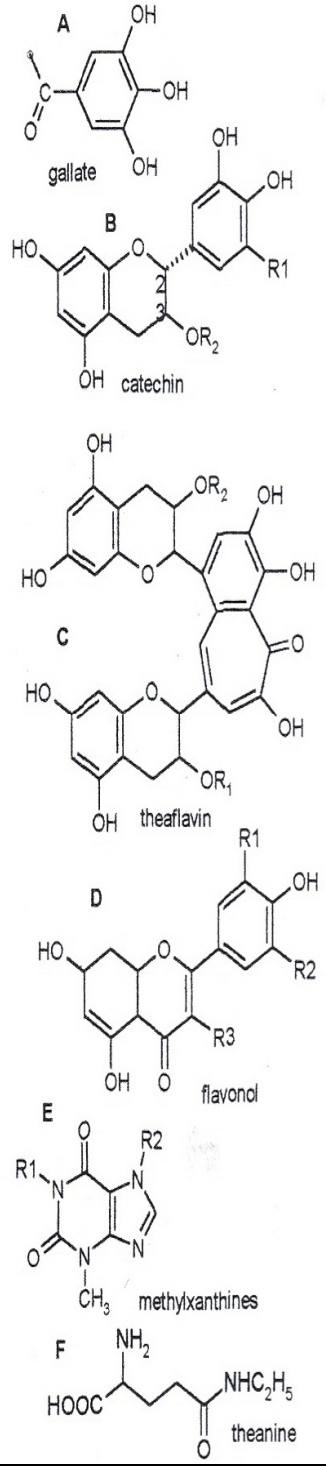
1.3.3 Chemical composition of tea

The chemical composition of tea has been thoroughly studied. It is a complex that includes: polyphenols, catechins, caffeine, amino acids, carbohydrates, protein, chlorophyll, volatile compounds, fluoride, minerals, and other undefined compounds (Wu and Wei, 2002). The major constituents of tea are listed in Table 1.3, among these, polyphenols and catechins constitute the most interesting group of tea leaf components (Dufresne and Farnworth, 2001). The main constituents of green tea leaves belong to the polyphenol group accounting for 25-35% on a dry weight basis (Balentine *et al*., 1997). Important and characteristic tea polyphenols are the flavanols of which catechins are predominant and the major ones are: (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (+)-catechin (C), and (+)-gallocatechin (GC). (Dufresne and Farnworth, 2000). The flavonols are mainly kaemferol, myricertin, and their glycosides. Tea also contains many amino acids; theanine is the most abundant, accounting for 50% of the total amino acids, and is found nowhere else than in tea leaves. It is also a good source of phenolic acids mainly caffeic, quinic and gallic acids. Tea contains caffeine,

which is about one third of coffee, the most well known source of caffeine. Green tea also contains vitamin C (Dufresne and Farnworth, 2000; 2001).

Table 1.3 Major components of tea (Dufresne and Farnworth, 2001).

	occurence (% dry weight)		
	green tea	black tea	structure
Catechins	**30-42**	**10-12**	
epigallocatechin gallate	11		B (-)2,3-cis R1=OH R2=A
epicatechin gallate	2		B (-)2,3-cis R1=H R2=A
gallocatechin gallate	2		B (+)2,3-trans R1=OH R2=A
epicatechin	10		B (-)2,3-cis R1=R2=H
epigallocatechin			B (-)2,3-cis R1=OH R2=H
gallocatechin			B (+)2,3-trans R1=OH R2=H
catechin			B (+)2,3-trans R1=R2=H
Teaflavin		**3-6**	
theaflavin-3-gallate			C R1=OH R2=OH
theaflavin-3'-gallate			C R1=A R2=OH
theaflavin-3,3'-digallate			C R1=OH R2=A
Thearubigens		**12-18**	C R1=A R2=A
Theogallin	**2-3**		
Proanthoocyanidin			
Flavonols	**5-10**	**6-8**	
quercetin			D R1=OH R2=H R3=OH
kaempferol			D R1=R2=H R3=OH
rutin			D R1=OH R2=H R3=O-rutinose
Methylxanthines	**7-9**	**8-11**	
caffeine	3-5		E R1=R2=CH_3
theobromine	0.1		E R1=H R_2=CH_3
theophylline	0.02		E R= CH_3 R2=H
Amino acids			
theanine	4-6		F
Organic acids			
caffeic acid			
quinic acid	2		
gallic acid			
Volatiles			
linalool			
delta-cardinene			
geraniol			
nerolidol			
alpha-terpineol			
cis-jasmone			
indole			
beta-ionone			
1-octanal			
indole-3-carbinol			
beta-caryophyllene			



1.3.4 Health benefits of tea consumption

Tea is one of the most widely consumed beverage in the world, next only to water and well ahead of coffee, beer, wine and carbonated soft drinks (Du Toit *et al*., 2001; Schmidt, *et al*., 2005; Cheng, 2006). It was progressively introduced all around the world by traders and travellers (Balentine *et al*., 1997). The scientific community has recently turned its attention to tea as it is alleged that tea is good for health. Several epidemiological studies, experimentation with animals, and *in vitro* studies lead to the conclusion that tea has potentially protective for wide variety of health conditions (Dufresne and Farnworth, 2000). Numerous studies have demonstrated that aqueous extracts or the major polyphenols that green tea possess are antimutagenic, antidiabetic, antioxidant, antibacterial, anti-inflamatory, antitumor, hypocholesterolemic, and above all, cancer-preventive in variety of experimental animal models systems (Dufresne and Farnworth, 2001).

1.3.4.1 Antioxidant activity

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions. They prevent damage that can be caused by free radicals to cellular components (Javanmardi *et al*., 2003). They are therefore critical for maintaining optimal cellular and systemic health and well-being. The over production of free radicals can trigger chain reactions which may cause oxidative damage to sensitive biological structures, such as DNA lipids, proteins (Wang *et al*., 2000; Dimitrios, 2006; Naithani *et al*., 2006).This damages has been associated with an increased risk of cardiovascular diseases, cancer and other chronic diseases. The health benefits associated with tea consumption have been attributed, in part, to the antioxidant

activity of abundant phenolic compounds. They are natural antioxidants and are considered to be responsible for the carcinogenic and antimutagenic properties of tea, as well as the protective action against cardiovascular diseases (Dimitrios, 2006).

1.3.4.2 Anticancer activity

Many studies have indicated that tea and its constituents, mainly EGCG, are antimutagenic and anti-inflammatory by intercepting carcinogenic agents and by reducing oxidant species before they damage the DNA (Dufresne and Farnworth, 2000). Green tea polyhenols, particularly EGCG, not only inhibits an enzyme required for cancer cell growth, but also kills cancer cells with no ill effect on healthy cells. Catechins also protects cell membranes against oxidation, keeps the reactive oxygen species in confined zones and probably blocks the cell membrane receptors required for cancer cell growth (Bushman, 1998). Promotion and progression of cancer pathology are retarded even at late stages by tea in a variety of cancers in target organs as indicated in many studies conducted on rodents (Blot *et al*., 1997). Quercetin, kaempferol, and myricetin were found to be able to inhibit carcinogen-induced tumours in rats and mice (Wang *et al*., 2000).

1.3.4.3 Antibacterial activity

Green tea catechins have demonstrated antibacterial activity against both gram-positive and gram-negative bacteria which can be harmful to humans. Tea extracts inhibit enteric pathogens such as *Staphylococcus aureus*, *S. epidermis*, and *Plasiomonas shigelloides* (Dufresne and Farnworth, 2000; 2001). Black and green tea extracts can also kill *Helicobacter pylori* associated with gastric, peptic and duodenal ulcer diseases. Tea polyphenols can selectively inhibit the growth of clostridia and

promote the growth of bifid bacteria in human large intestine. The bacterial balance in intestinal micro-flora may be important for the prevention of colon cancer (Diker and Hascelik, 1994).

Antimicrobial activity against cariogenic and periodontal bacteria has been reported (Dufresne and Farnworth, 2000). The polyphenols in green tea can prevent teeth from decaying by inhibiting the biological activities of the cariogenic streptococci, *Streptococcus mutans* (Sakanaka *et al*., 1989) and *S. sobrinus* (Sakanaka *et al.,* 1990). Tea extracts not only prevent the growth of *S. mutans* but also hinder the synthesis of insoluble glucans by glucosyltransferase, and the sucrose-dependant bacterial cell adherence to teeth and epithelium, by reducing collagenase activity (Mitscher *et al*., 1997).

1.3.4.4 Antiviral activity

Tea can have a beneficial effect against viral infection. Tea extracts have been used as therapy in cholera patients in epidemic areas and for the prevention of influenza virus infections (Wang *et al*., 2000). Tea polyphenols strongly inhibits rotavirus propagation in monkey cell culture and influenza A and B virus in animal cells (Dufresne and Farnworth, 2001). EGCG agglutinates and inhibits influenza A and B viruses in animal cell cultures (Mitscher *et al*., 1997). It is reported that several flavonoids including EGCG and ECG inhibit retrovirus human immunodeficiency virus (HIV) propagation by inhibiting reverse transcriptase, an enzyme allowing the establishment of the virus in host cells (Dufresne and Farnworth, 2001). An antiviral activity has been found against HIV virus enzymes and against rotaviruses and

anteroviruses in monkey cell cultures when treated with EGCG (Mitscher *et al*., 1997).

1.3.4.5 Antidiabetic activity

Diabetes is associated with high blood glucose content (Zeyuan *et al*., 1998). High blood glucose levels in aged rats, an indicator of diabetes frequently observed in the aging population can be reduced with green tea. Tea suppresses the activity of glucose transporters in the intestinal epithelium and is believed to reduce dietary glucose intake. A reduction in oxidation damage to lymphocyte DNA has been observed in diabetic patients receiving quercetin and tea (Dufresne and Farnworth, 2001).

1.4 Herbal tea and its medicinal values

Herbal teas are not true teas at all; they differ from the leaves of traditional black teas in that they are made from plant parts such as flowers, roots, barks and seeds. They can also be prepared from blends of different plants, and because of this they have distinctively different flavours, colours and aromas (Phelan and Rees, 2003; Araya, 2005). Since herbal teas are caffeine free they have been used as an alternative to beverages such as coffee, cocoa and tea by many individuals. Many herbal teas have a long history of use in Europe and the Far East countries. The infusion of herbal teas was widespread in Europe long before the arrival of black tea and some of the current favourites such as chamomile, peppermint and rosehips have long been well known standards. The flowers of chamomile, for example, started to be used as a medicine by the ancient Romans, and they are still used as folk medicine in Europe today (Araya, 2005).

Throughout history herbal teas played a great role in everyday living in many societies, not only for their flavour but for their medicinal value as well. They were quite often taken medicinally to cure coughs, sore throats, fever, aches and headaches (Pietta, 2000). The consumption of tea is a very ancient habit and legends from China indicate that it was initiated about five thousand years ago. An archaeological report by Jelinek in 1978 suggests that the infusion of leaves from different wild plants and also from the tea tree was probably already practised more than 500 000 years ago (Dufresne and Farnworth, 2001). Herbal tea, which is generally a polyherbal formulation made up of different medicinal plants, is also considered as a source of antioxidants. These antioxidants found in herbal tea play an important role as a part of a healthy diet (Naithani *et al*., 2006). Herbal teas are reported to contain natural antioxidants such as vitamin A, B_6, C, E, polyphenols (flavonoids, flavanols, flavonols, isoflavones, quercetin, catechin, epicatechin and others), co-enzyme Q_{10}, carotenoids, selenium, zinc and phytochemicals. The protective role of herbal tea against many diseases have been attributed to the antioxidant activity they possess (Atoui *et al*., 2005).

1.5 The selected model plant: *Athrixia phylicoides*

1.5.1 Plant description

Athrixia phylicoides (Figure 1.4) is a herbaceous belonging to the Asteraceae family. It is an indigenous South African plant; commonly known as Bushmen tea, or bush tea (Araya, 2005; Mudau, 2006). It is a small, sprawling, attractive aromatic shrub growing from about 50 cm to 1 m in height. It has thin (<1 cm in diameter), white and woolly stems (Roberts, 1990). The leaves are simple, alternate, linear to broadly lanceolate, tapering to a sharp point, very shortly stalked, auriculate at the

base, light grey-green, smooth on upper surface, white-woolly below, the margins are entire or slightly revolute. The inflorescence head is sessile or sub-sessile, terminal and axillary, in large subcorymbose panicles. Involucral bracts are 10 mm long, campanulate, straw coloured, and the many ray flowers mauve, magenta or pink, and the disk flowers are yellow. Based on the soil and the area where the plant grows the colour of the flowers may vary from the palest pink to all shades of pink and mauve to deep purple. It flowers from May to July in the coastal areas and from mid to late summer inland (Roberts, 1990; Shackleton, 2006).

Figure 1.4 Description of *A. phylicoides.* (**A**) Shrub, (**B**) Stem, and (**C**) Flowers (www.plantzafrica.com).

1.5.2 Distribution

A. phylicoides grows naturally in the mountainous parts of South Africa; from the Eastern Cape in the south, to the Soutpansberg in the Limpopo Province in the north (Van Wyk and Gericke, 2000). It is usually found in high altitude grasslands and prefers moist, well-drained yellow brown soils with loose shale or gravel. The plant prefers the cool, moist, southern and south-eastern slopes of the mountains where fog or mist may supply supplementary moisture (Van Wyk and Gericke, 2000).

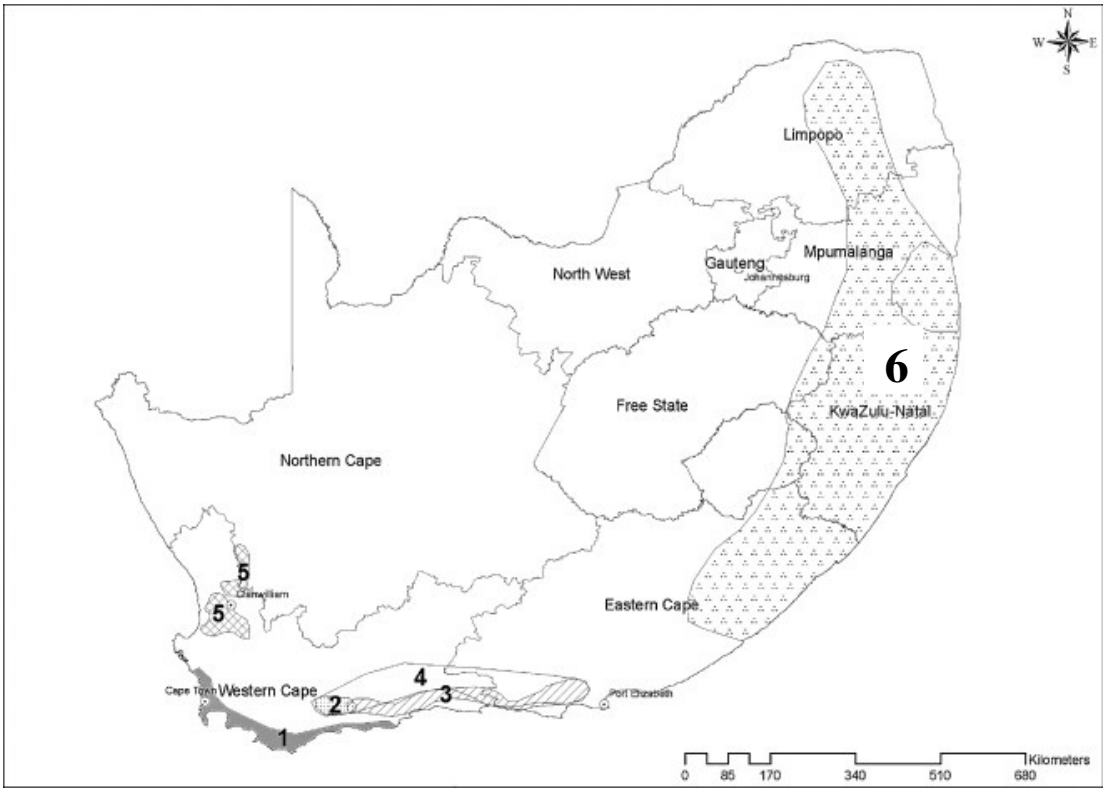


Figure 1.5 Geographical distribution of *A. phylicoides* (**6**) (Joubert *et al*., 2008).

1.5.3 Medicinal uses

Bush tea is a multipurpose plant, as it is used as an herbal tea, for making of brooms and it may have medicinal and aphrodisiac properties (Van Wyk and Gericke,

2000). The people of South Africa have used bush tea for many years as a medicinal or herbal tea and throughout history people have gathered this plant from the mountainous regions of their homeland to prepare the tea. As a medicinal herbal tea, it is used for cleansing or purifying the blood, treating boils, bad acne, infected wounds and cuts, for washing (Joubert *et al*., 2008). The tea is also wonderful for coughs and colds, and for loss of voice and is used for infected throats as a gargle (Roberts, 1990). It can also be used as a treatment for diabetes, high blood pressure, heart conditions, stomach-ache, headaches and as a stimulant. The Sotho people in addition use a strong brew preparation for sore feet, which are washed and the bandage with castor oil leaves (*Ricinis communis*), both having a deep acting effect on hard skin and the muscles of the feet (Roberts, 1990). It is also believed to have aphrodisiac properties by Vhavenda people. They are reported to use extracts from soaked roots and leaves as anthelmintics. In some areas of South Africa people grow this plant nearby their homes, because it is pleasant to drink and for its medicinal properties (Roberts, 1990). The stems of bush tea are tied up in bundles for brooms and traded on a small scale in the Limpopo Province (Van Wyk and Gericke, 2000).

1.6 Aim and objectives of the study

A series of investigations has been initiated to validate the uses of *Athrixia phylicoides* as a medicinal herbal tea. An experiment to evaluate *A. phylicoides* for cytotoxicity, antioxidant activity, caffeine content and the presence of pyrrolizidine alkaloids was intiated by McGaw *et al*. (2007). The study concluded that *A. phylicoides* possessed higher antioxidant activity and phenol content compared to Rooibos tea. The study also reported the absence of caffeine and pyrrolizidine alkaloids from the extract of *A. phylicoides*. Another study to investigate the variation

in the polyphenolic content of the tea leaves with season and with nitrogen application was conducted by Mudau *et al*. (2006). The total phenolic content showed definite seasonal variation; a content of 11.8 mg/g was detected in March, 10.8 mg/g in April and September, while the highest concentration was obtained in June and July, 35.5 and 35.9 mg/g respectively. Addition of nitrogenous fertilizer supplements resulted in significant ($P \leq 0.001$) increased concentrations of total polyphenols in *A. phylicoides* in all seasons. Mogotlane *et al*. (2007) also reported the increase in total antioxidant content after the application of nitrogen, potassium and phosphorus in different seasons. In another study a novel compound was discovered; new flavonoid (5-hydroxy-6,7,8,3',4'.5'-hexamethoxy flavon-3-ol was isolated from the leaves of *A. phylicoides* (Mashimbye *et al*., 2006).

This study was initiated to investigate the antioxidant activity of *A. phylicoides*, using DPPH scavenging activity. The antioxidant activity of the extract was compared against the activity of vitamin C as a standard control. Vitamin C is considered as the most important water-soluble antioxidant. It can directly scavenge several types of radicals and is currently the most widely used vitamin supplement worldwide (Klimczak *et al*., 2007). The second objective was to compare the phenol content of dried and fresh extracts and their correlation to antioxidant activities. The antibacterial activities of *A. phylicoides* have never been conducted before, so this study will determine the extracts' ability in the inhibition of common, known microorganism. The final objective is to isolate the active compounds and test their antioxidant activity and cytotoxicity.

1.7 Scope of the thesis

The antioxidant activity of a crude extract against DPPH oxidant is described in chapter 2. Chapter 3 describes the antibacterial activity of a crude *A. phylicoides* extract. The effect of drying on the phenolic content and antioxidant activity of the crude extract is explained in chapter 4. Chapter 5 deals with the isolation, identification and evaluation of the antioxidant activity of the isolated compounds of *A. phylicoides*. Chapter 6 describes the cytotoxicity effect of the isolated compounds. Chapter 7 deals with the general discussion and the conclusions of this study. Chapter 8 is the appendix of the ^{1}H-NMR and ^{13}C-NMR spectrum of the isolated compounds.

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Chapter 2

Antioxidant activity of *Athrixia phylicoides*

2.1 Introduction

Oxidation is the transfer of electrons from one atom to another and represents an essential part of aerobic life and our metabolism, since oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP. When the transfer of unpaired electrons happens free radicals will be formed (Pietta, 2000). Free radicals play important roles in the human being, such as energy production, phagocytosis, regulation of cell growth and intercellular signalling, and the synthesis of biologically important compounds. However, when they are overproduced they have a negative impact. They can cause damage to the biological systems in the body, promoting the development of various diseases. They attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates, and DNA, and induce oxidations, which causes membrane damage, protein modification and DNA damage (Aruoma, 1998; Atoui *et al*., 2005; Pietta, 2000). This oxidative damage is considered to play a causative role in aging and several degenerative diseases associated with it, such as cardiovascular diseases, cancer, cataracts, immune system decline, and brain dysfunction (Percival, 1998; Young and Woodside, 2001). Free radical formation is controlled by various beneficial compounds known as antioxidants. Antioxidants are capable of stabilizing, or deactivating free radicals before the latter attack cells and biological targets. They are therefore critical for maintaining optimal cellular and systemic health and well being (Percival, 1998).

Throughout the world, tea is the most popular beverage after water, and has attracted much interest because of its reported health benefits. Herbal teas' role in the treatment and cure of diseases has been attributed to the antioxidant properties of their constituents, mainly phenolic compounds (Inova *et al*., 2005). The antioxidant activity of phenols against free radicals and other reactive species is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators (Rice-Evans *et al*., 1997).

There are several different methods that have been developed to measure antioxidant activity. These methods differ in terms of their assay principles and experimental conditions. They are typically based on the inhibition of the accumulation of oxidised products. The generation of free radical species is inhibited by the addition of antioxidants and this give rise to a reduction of the endpoints by scavenging the free radicals. A reliable method to determine the antioxidant activity of a sample involves the measurement of the disappearance of free radicals such as DPPH (1, 1-diphenyl-2-picrylhydrazyl). The objective of this study was to determine the ability of the plant extract in scavenging DPPH.

2.2 Materials and methods

2.2.1 Plant material

The aerial parts (stem, leaves, and bark) of *A. phylicoides,* which are used traditionally as herbal teas, were collected from Muhuyo village, Venda in the Limpopo Province in South Africa. A voucher specimen was prepared and identified at the H.G.W.J. Schweikerdt Herbarium, University of Pretoria.

2.2.2 Preparation of the extract

Plant material was air dried in the laboratory (under shade) for five weeks. Small cuts weighing 1500 g were soaked for 48 hours in 5l of ethanol and then homogenised. The filtrates were evaporated to dryness under reduced pressure and the residue dissolved in 1 % DMSO to achieve a concentration of 100 mg/ml. The extract was then stored in a cold room for further use.

2.2.3 DPPH free-radical scavenging assay

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assay was carried out using the method as previously described by Rangkadilok *et al.* (2007); with slightly modifications. A stock solution (1 mg/ml) of crude extract was prepared in an ethanol solvent. Briefly, 200 µl of distilled water was added to the first top wells of a 96-well ELISA plate and the remaining wells were filled with 110 µl as a medium. The plant extract (20 µl) was added to the first top wells and then double diluted. Later, 90 µl of 90 µM DPPH methanolic solution was added to each well to give final concentrations of 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250 and 500 µg/ml of extract per ml of DPPH solution. Ascorbic acid (Vitamin C) was used as the positive control, ethanol was used as the negative control and distilled water as a blank. The plate was covered with aluminium foil and left to stand for an hour at room temperature.

2.2.4 Spectrophotometric assay

The radical scavenging capacities of the extract was determined by using a BIO–TEK Power Wave Multiwell plate reader (A.D.P., Weltevreden Park, South Africa) to measure the disappearance of DPPH at 550 nm. The free radical-

scavenging activity was calculated as a percentage inhibition of the DPPH radical by the plant sample or by ascorbic acid according to the formula:

$$\%\ inhibition = \frac{(absorbance\ of\ the\ control - absorbance\ of\ the\ plant\ sample)}{absorbance\ of\ the\ control} \times 100$$

The percentage of DPPH radical-scavenging was plotted against the plant extract concentration (µg/ml) to determine the concentration of the extract required to scavenge DPPH by 50% (called EC_{50}).

2.2.5 Thin Layer Chromatography (TLC) assay

For the qualitative assay, the ethanol extract (10 µl) was applied onto a TLC plate (Merck Silica gel 60 F_{254}). The plate was then developed using the solvent system hexane/ethyl acetate (7:3). After drying in a stream of cold air, the plate was sprayed with 0.2 % methanolic DPPH and was incubated for 30 minutes at room temperature to detect the number of antioxidant compounds present in the extract. The presence of antioxidants compounds was revealed within 5 minutes as white spots against the purple background on the plate.

2.2.6 Statistical analysis

Each of the measurements and experiments described above were carried out in triplicate, and the results are reported as the mean and standard deviation. EC_{50} was estimated by the sigmoid non-linear regression using SigmaPlot 2000 Demo (SPSS Inc., Chicago, IL, USA).

2.3 Results and discussion

The antioxidant activity of the *A. phylicoides* extract was evaluated using the DPPH free radical-scavenging assay. It is a reliable method to determine the antioxidant activity of a sample, and it measures the disappearance of free radicals after reaction with antioxidant (Bandoniene and Murkovic, 2002). The measurement of the consumption of the DPPH radical allows one to determine exclusively the intrinsic ability of a substance to donate hydrogen atoms or electrons to the oxidant. The method is based on the reduction of the DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of a non-radical stable DPPH-H molecule. The formation of the stable DPPH-H changes the colour of the DPPH from purple to yellow, and as a result the absorbance also decreases (Paixao *et al*., 2007; Bandoniene and Murkovic, 2002).

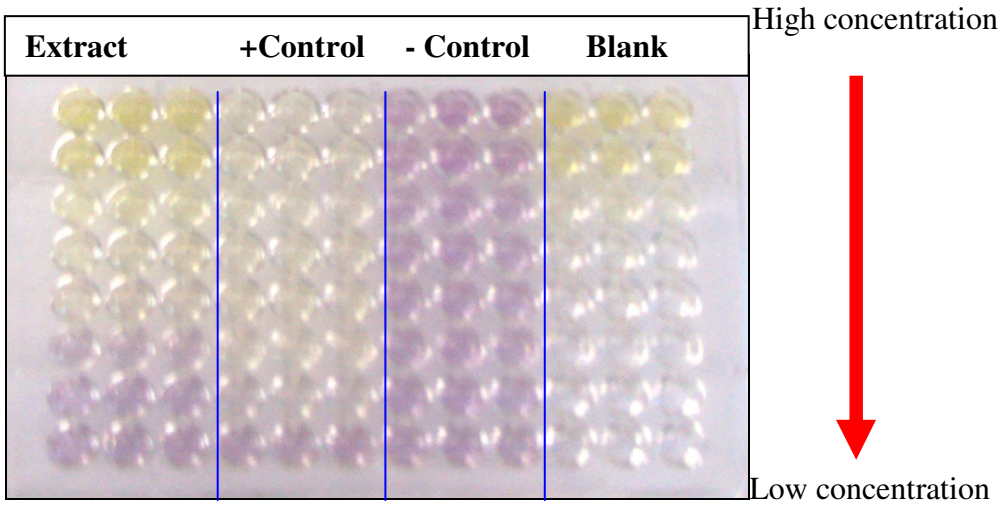

Figure 2.1 Microtitre plate showing the reaction between the DPPH and the extract.

Figure 2.1 shows the reduction of DPPH after reacting with the extract. During the reaction a colour change was observed, the purple colour gradually changing to yellow. At higher concentrations, the yellow colour was easily observed and at lower

concentrations the purple colour was in excess. According to Paixao *et al*. (2007), the degree of colour change is correlated to the concentration of the antioxidant while the discoloration of DPPH indicates the scavenging efficacy of the extract (Narasimhan *et al*., 2006). In our study, the yellow colour represents the high and purple colour the low scavenging activity of the extract. These results are in agreement with those reported by Paixao *et al*. (2007), while studying the relationship between antioxidant capacity and total phenolic content of red, rose and white wines.

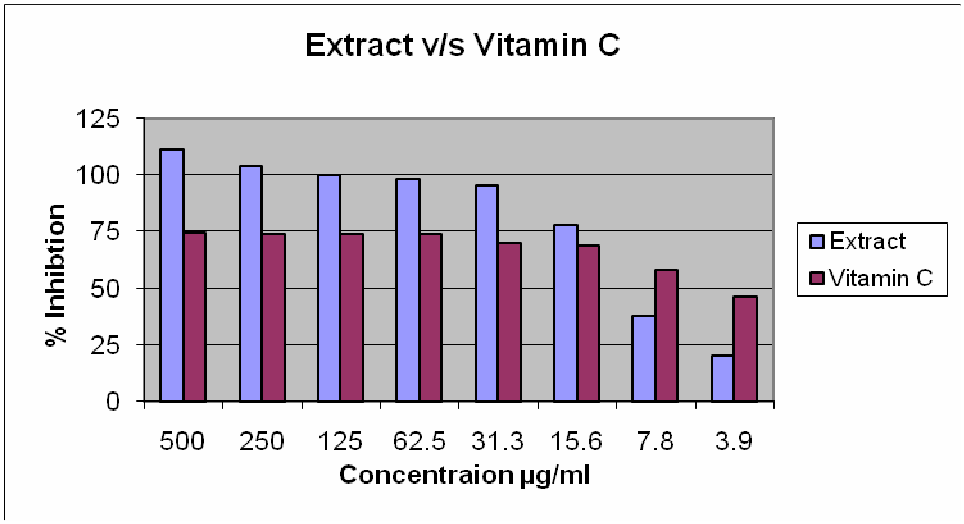

Figure 2.2 The DPPH inhibition activity of the extract and vitamin C.

The percentage inhibition of the DPPH radical by the crude extract and by vitamin C at different concentrations is shown in Figure 2.2. The extract showed a concentration-dependent radical scavenging activity. The percentage of DPPH inhibited was 111.34 ± 0.01 at higher (500 µg/ml) and 20.151 ± 0.021 at lower (3.9 µg/ml) concentrations tested. Vitamin C showed a DPPH inhibition percentage of 74.43 ± 0.001 at higher and 46.61 ± 0.009 at lower concentrations tested.

The concentration of an antioxidant needed to decrease the initial DDPH concentration by 50% (EC_{50}) was used to measure the antioxidant activity of the crude extract (Sanchez *et al*., 1998; Du Toit *et al*., 2001). The EC_{50} value of the extract was found to be 10.64 ± 0.0842 µg/ml compared to 4.11 µg/ml of vitamin C. The antioxidant activity of the extract expressed as vitamin C equivalence was found to be 38.62 mg/g dry weight. The results obtained from this study showed the potential of *A. phylicoides* of being a good antioxidant agent. A ethanol extract of *A. phylicoides* has previously been reported to be a potent free radical scavenger compared to Rooibos and *Athrixia elata* (McGaw *et al*., 2007).

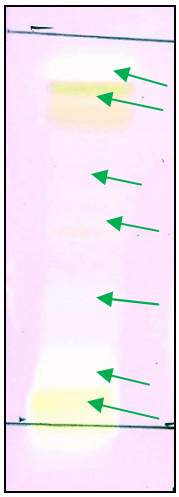

Figure 2.3 TLC plate showing the presence of antioxidant compounds.

A TLC based qualitative antioxidant assay was used in order to observe the presence of antioxidants compounds that may be found in the sample. Figure 2.3 shows the TLC plate after spraying with DPPH. Antioxidant compounds appeared as white spots (green arrows) on the purple background of the plate. It has been reported that the roles of tea in disease preventing and cure have been attributed to the

antioxidant properties of phenolic compounds presents in the plants (Inova *et al*. 2005). In this study these active compounds were isolated and identified.

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Chapter 3

Antibacterial activity of *Athrixia phylicoides*

3.1 Introduction

Bacterial and fungal pathogens are capable of causing serious diseases in humans and animals. Infectious diseases account for approximately one-half of all deaths in tropical countries (Iwu *et al*., 1999). People who are deficient in the production of circulating antibodies are highly susceptible to respiratory infections by gram-positive bacteria while the people who are deficient in T cell functions are highly susceptible to infection by fungi and viruses, as well as by bacteria that grow predominately intracellularly (Stanier *et al*., 1985).

Bacteria are unicellular organisms, that can rapidly produce many toxins; powerful chemicals that damage specific cells in the tissues they've invaded. Toxins produced by pathogenic bacteria, could result in serious complications (Lall, 2001). Recently, the pathogenicity of some gram-positive bacteria has initiated acute awareness among people (Dellat, 1997). A gram-positive bacterium, *Staphylococcus aureus* has the ability to produce a number of different toxins, of which, the enterotoxins are responsible for a common type of food poisoning, and exotoxins causes necrosis of the skin and lyses of red blood cells during the development of boils or other local abscesses (Dellat, 1997). These organisms are frequently spread by means of the lymphatic system and the blood. Hence, S*taphylococcus* infections often develop into more serious diseases such as pneumonia, meningitis, endocarditis, osteomylitis and many other dangerous diseases. Most of the *Bacillus* organisms are usually straight rods with parallel sides that may be arranged in varying

configurations. *Bacillus cereus* and *B. subtilis* are associated with some outbreaks of food poisoning which causes human eye infections (Dellat, 1997). Gram-negative bacteria causes impaired function in the human body which may be acute, and manifest at short notice and can be relatively long in duration. *Escherichia coli* is the most frequent cause of urinary tract infections, which may take the form of cystitis, pyelitis, pyelonephritisas well as appendicitis, peritonitists, postoperative wound infection, infantile diarrhoea and others. These bacteria can also causes secondary infections of the lungs (Dellat, 1997).

Long before mankind discovered the existence of microbes, the idea that certain plants had healing potential, indeed, that they contained what we would currently characterize as antimicrobial principles, was well accepted (Rios and Recio, 2005). Historically, plants have provided a good source of anti-infection agents; ementine, quinine, and berberine remain highly effective instruments in the fight against microbial infections (Iwu *et al*., 1999). Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by micro-organisms has increased (Nascimento *et al*., 2000). Antibiotic resistance has become a global concern (Parekh and Chanda, 2007). In general, bacteria have the genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents (Nascimento *et al*., 2000). The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has lead to the screening of several medicinal plants for their potential antimicrobial activity (Parekh and Chanda, 2007). Currently, there is an increasing interest in plant-derived medicine to fight microbial diseases (Palombo and Semple, 2001). Plants contain numerous biological active compounds, many of which

have been shown to have antimicrobial activity (Lopez *et al*., 2001; Karaman *et al*., 2001). The antibacterial activity of *A. phylicoides* has not been evaluated before. This study was aimed at investigating the antibacterial activity of the *A. phylicoides* by preliminary *in vitro* bioassay screening, using a ethanol extract.

3.2 Materials and methods

3.2.1 Preparation of the extract

The plant materials were collected and prepared as mentioned in chapter 2. The extract was dissolved in 10% DMSO to a concentration of 100mg/ml for the antibacterial assay.

3.2.2 Antibacterial activity

3.2.2.1 Microorganisms

The microorganisms used in this study were the following: Gram-positive; *Staphylococcus aureus* (ATCC 12600), *Bacillus cereus* (ATCC 11778), *Enterococus faecalis* (ATCC 29212), *B. subtilis, B. pumilus* (ATCC 21356) and the Gram-negative; *Pseudomonas aeruginosa* (ATCC 25922), *Escherichia coli* (ATCC 11775), *Klebsiella pneumonia* (ATCC 27736). All the microorganisms were obtained from the Department of Microbiology, University of Pretoria. Each organism was maintained on a nutrient broth for 24 hours before testing.

3.2.2.2 Minimum inhibitory concentration (MIC) assay

The Minimum Inhibition Concentration (MIC) of the crude extract was determined using the micro-dilution method on 96 well micro-plates, as previously described by Eloff (1998). The ethanol crude extract was dissolved in 10% DMSO to obtain a stock solution of 100 mg/ml. This experiment was carried out in triplicate. Briefly, 100 µl of the nutrient broth was added to each well on a micro-plate. One hundred microlitres (100 µl) of 100 mg/ml crude extract was added into the first wells on the row. Serial dilution was then carried out to yield volumes of 100 µl per well, with the final concentrations ranging from 25.00 to 0.19 mg/ml. One hundred microlitres (100 µl) of the cultured bacteria was added to each well to give a final volume of 200 µl/well. The same procedure was done for all the bacteria. Streptomycin and distilled water were used as positive and negative controls respectively. The plates were sealed and incubated overnight at 37 ºC. After the overnight incubation an indicator of bacterial growth, 40 µl of 0.2 mg/ml ρ-iodonitrotetrazolium violet (INT) was added to all the micro-plate wells and incubated for a further 30 minutes. Bacterial growth was indicated by the red/pink colour while colourless results indicated the inhibition of bacteria growth in each well. The lowest concentration of the extract that inhibited bacterial growth was defined as the MIC value of the extract.

3.2.2.3 Direct bioautography assay

The direct bioautography method described by Lall and Meyer (2000) was used to detect the antibacterial compounds. Briefly, 20 µl of the ethanol extract (20 mg/ml) was applied to a TLC plate (Merck Silica gel 60 F_{254}). The plate was developed in (hexane: ethyl acetate (7:3) and carefully dried over cold air for the

complete removal of the solvents. A 48 hour old *Staphylococcus aureus* culture in nutrient broth was centrifuged at 3000 × g for 20 minutes, the supernatant was discarded and the pellet re-suspended in fresh nutrient broth. A fine spray was used to spray the bacterial suspension onto the TLC plate. The plate was then dried until it appeared translucent and then incubated at 37 °C for 48 hour under humid conditions. After incubation, the plate was sprayed with an aqueous solution of ρ-iodonitrotetrazolium violet (0.2 mg/ml). The plate was then re-incubated at 37 °C for 30 min. Antibacterial compounds can be observed as clear spots against the reddish background of the plate.

3.3 Results and discussion

The determination of the MIC involves a semi-quantitative test procedure which gives an approximation of the least concentration of an antimicrobial that is needed to prevent microbial growth. The MIC assay method is widely used and is an accepted criterion for measuring the susceptibility of organisms to inhibitors (Lambert and Pearson, 2000). The MIC values of extract on different microorganisms are reported in Table 3.1. The crude extract showed positive inhibitory activity against all the tested microorganisms with MIC values ranging from 3.13 to 6.25 mg/ml. All plant extracts with MIC values below 8 mg/ml are considered to possess some antimicrobial activity (Fabry *et al*., 1998). The lowest MIC value of 3.13 mg/ml was obtained for the entire Gram-positive microorganism except for *B. cereus,* which exhibited a MIC value of 6.25 mg/ml. All the Gram-negative microorganisms gave a MIC value of 6.25 mg/ml. These MIC values demonstrate that the Gram-positive bacteria appeared to be more susceptible than gram-negative ones to the inhibitory effects of the extract. Similar observations were made by Lall and Meyer (2000),

Meyer and Afolayan (1995) and Tshikalange *et al*. (2005), while studying the antibacterial activity of *Hyptis veriticillata*, *Helichrysum aureonitens* and selected medicinal plants used in treatment of sexually transmitted diseases.

Table 3.1 MIC values (mg/ml) of the crude extract from *Athrixia phylicoides*.

Bacterial species	**Gram** +/-	**MIC** (mg/ml)
Staphylococcus aureus	+	3.13
Bacillus cereus	+	6.25
Bacillus subtilis	+	3.13
Bacillus pumilus	+	3.13
Enterecoccus faecalis	+	3.13
Pseudomonas aeruginosa	-	6.25
Escherichia coli	-	6.25
Klebsiella pneumonia	-	6.25

MIC, Minimum Inhibitory Concentration

The weak activity obtained against Gram-negative bacteria is not surprising as, in general, these bacteria are more resistant than gram-positive ones (Paz *et al*., 1995; Rabe and Van Staden, 1997). The reason for the differences in sensitivity between Gram-positive and gram-negative bacteria could be attributed to the morphological difference between them (Nikaido and Vaara, 1985; Palombo and Semple, 2001; Tadeg *et al*., 2005). Gram-negative bacteria have an outer phospholipidic membrane that carries structural lipopolysaccharide components making the cell walls impermeable to lipophilic solutes. Gram-positive bacteria, on

the other hand, are more susceptible having only an outer petidoglycan layer which is not an effective permeable barrier (Nostro *et al*., 2000; Tadeg *et al*., 2005). Therefore, the cell walls of Gram-negative organisms which are more complex than the gram-positive ones acts as a diffusional barrier making them less susceptible than Gram-positive to the antimicrobial agents (Nostro *et al*., 2000; Palombo and Semple 2001).

Bacterial growth inhibition was also seen as clear spots on the TLC plate sprayed with *S. aureus*. Generally, the extent of the inhibitory effects of extract could be attributed to their phenolic composition (Baydar *et al*., 2004). Phenols are the predominant active compounds in medicinal plants, with gram-positive bacteria being the most susceptible microorganisms (Rios and Recio, 2005). It has been reported that the preventing activity of diseases by herbal teas is attributed to phenolic compounds, and bush tea leaves are rich in phenols, which have an antibacterial and antimicrobial activities. However, the isolation of the active compounds will provide a better explanation of the antibacterial activity of *A. phylicoides*.

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Chapter 4

Effect of drying on the phenolic content and antioxidant activity of *Athrixia phylicoides*

4.1 Introduction

Phenolic compounds are secondary plant metabolites found in both edible and inedible plants. They have been reported to have multiple functions, and are very important for the normal growth, development and defence mechanism of a plant (Rusak *et al*., 2008; Caillet, 2006). These compounds are capable of modulating the activity of many enzymes, not only in plants, but in animals and humans, suggesting their involvement in biochemical and physiological processes (Rusak *et al*., 2008). There is a growing body of evidence indicating that certain plant phenols also play a vital role in human health and diseases prevention. They have been reported to have biological effects, including antioxidant activity, which are helpful against human cancers, arteriosclerosis, ischaemis and inflammatory diseases that are partially caused by exposure to oxidative stress (Asami *et al*., 2003; Kähkönen *et al*., 1999; Rusak *et al*., 2008). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which plays an important role in absorbing and neutralising free radicals (Javanmardi *et al*., 2003).

Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health (Gurib-Fakim, 2006). These chemical constituents are responsible for the plant's efficacy in the treating of illness and diseases, as well as its cytotoxicity (Fennel *et al*., 2004). The

biological activities are mostly affected by the chemical changes happening within the plant material. Post harvest treatments may affect the chemical composition and biological activity of plant material; this includes the common practise of drying and re-dissolving plant extracts, filtering, heating and the use of liquid nitrogen to grind plant material (Fennel *et al*., 2004). Medicinal plants are either used as fresh or dried materials (Capecka *et al*., 2005). Traditionally, *A. phylicoides* is harvested in the wild and sun dried. The fine twigs and leaves are then removed and used as a herbal tea. The purpose of the present study was to compare the antioxidant activity and its correlation with thr total phenolic content between the fresh and the dried plant material of *A. phylicoides*.

4.2 Materials and methods

4.2.1 Preparation of the extract

Plant material that has not been dried after harvesting was used to prepare the fresh extract. The dry extract was prepared by air drying it in the laboratory at room temperature (under shade) for five weeks.

4.2.2 Determination of the total phenolic content

The amount of total phenolic content in the extracts was determined spectrophotometrically using Folin-Ciocalteu's reagent according to a modified method by Naithani *et al*. (2006). Briefly, 1ml of plant extract (sample), distilled water and 50% Folin-Ciocalteu reagent were mixed thoroughly and incubated in a water-bath at 25°C. After an interval of 3 min, 2 ml of 2% saturated aqueous sodium carbonate solution was added and the mixture was further incubated in the water bath

for another 60 min. The absorbance of the resulting blue colour was measured at 750 nm against a blank sample. Gallic acid (2, 5, 7, 10 and 15 µg/ml) was used as a standard to obtain a standard curve. All the determinations were performed in triplicate (n = 3).

4.2.3 DPPH free-radical scavenging assay

The DPPH free-radical scavenging assay was conducted as previously described in chapter 2.

4.3 Results and discussion

The total phenolic content (TPC) and antioxidant activities of dried and fresh ethanolic extracts of *A. phylicoides* are shown in Table 4.1.

Table 4.1 Total phenolic content and antioxidant activities of dry and fresh extracts.

Extracts	**TPC** (mg GAC/100g)	**EC_{50}** (µg/ml)
Dry	28.28 ± 0.019	10.64 ± 0.084
Fresh	23.04 ± 0.003	13.97 ± 0.066

Results are means ± SD (n = 3)

In the two extracts tested, the highest level of TPC was found in the dry extract compared to the fresh extract. The dried extract gave a TPC value of 28.28±0.019 mg GAC/100g and fresh extract gave a total of 23.04 ± 0.003 mg GAC/100g. The same findings were reported by Capecka *et al*. (2005), where the

drying of oregano and peppermint resulted in a considerable increase of total phenolics when compared to the freshly harvested plant materials.

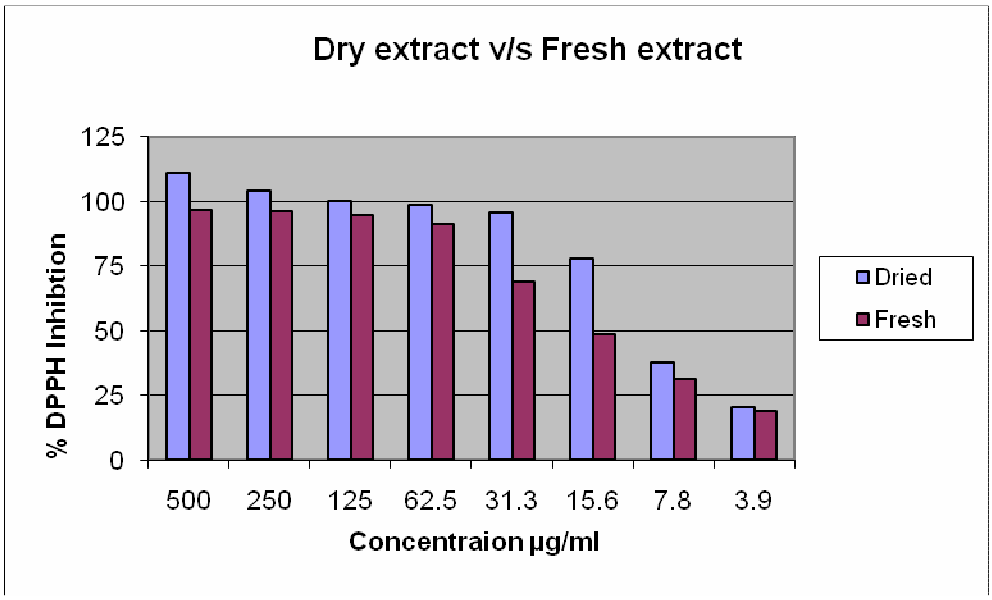

Figure 4.1 The DPPH inhibition activities of dried and fresh extracts.

The percentage inhibition of DPPH by the fresh and a dry extracts are shown in Figure 4.1. The dried extract had a higher antioxidant activity with a EC_{50} value of 10.64 ± 0.084 µg/ml when compared to the fresh extract with a EC_{50} value of 13.97 ± 0.066 µg/ml. The lower the EC_{50}, the higher the antioxidant activity (Chan *et al.*, 2007).

This study reports a positive correlation between phenolic content and antioxidant activity. Our results suggest that the antioxidant capacity of the dried extract results from the contribution of the phenolic compounds, since the highest levels of phenolic content was detected in the dry extract. Additionally, it has been well-documented that the antioxidant effect of plant products are mainly due to

phenolic compounds, such as flavonoids, phenolic acids, tannins and phenolic diterpenes (Rao *et al*., 2007).

In this study, the drying of plant material after harvesting had a significant impact on the chemical composition and the biological activity of an extract. The reason may be due to the rupturing and degradation of the cell membranes during drying, which would result in a greater release of compounds during extraction (Stafford *et al*., 2005). Proper drying conditions are largely overlooked by the plant collectors and traders. Inadequate drying may also result in chemical changes of the plant products (Street *et al*., 2008). These chemical changes cannot be detected by the human senses, thus, consumers are not able to determine the quality of the plant materials easily (Stafford *et al*., 2005). The results obtained from this study conclude that to be of good medicinal value, adequate drying of *A. phylicoides* is necessary.

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Chapter 5

Isolation and purification of the antioxidant compounds from *Athrixia phylicoides*

5.1 Introduction

A. phylicoides DC. (Bush tea) is an aromatic shrub belonging to the family Asteracea. A decoction of leaves and twigs are widely used by many South Africans as a herbal tea. Plant infusions are used as medicinally, as a blood purifier or cleanser, for treating boils, headaches, infested wounds and cuts while a solution is used as a foam bath. The Vhavenda people drink the extracts made from the leaves and roots as a aphrodisiac, while the Zulu people use a decoction of the roots as a cough remedy and purgative (Mashimbye *et al*., 2006; McGaw *et al*., 2007). Ethanol extract of *A. phylicoides* showed excellent inhibition activity of DPPH when used as antioxidant agent and thus lead us to isolate active the active compounds of this extract. In this chapter, the isolation and purification of the bioactive compounds, their chemical structures and inhibitory activities as oxidant are described.

5.2 Materials and methods

5.2.1 Preparation of the extract

About 4 kg of plant material was used to prepare the crude extract in the manner as mentioned in chapter 2.

5.2.2 Isolation and identification of antioxidant compounds

The extract (110 g) was dissolved in a minimal amount of methanol solvent and mixed with 160 g of silica gel. The mixture was then left overnight in an open space to dry into a fine powder. A 10×70 cm glass column, filled with 1.5 kg silica gel, was used for the isolation. The column was eluted with a solvent gradient of hexane: ethyl acetate in 100:0 to 0:100 ratios. The column was then washed with ethyl acetate (100 %), methanol: ethyl acetate (2:8), and 100 % methanol. A total of 34 fractions of 1000 ml each were collected and concentrated to dryness under reduced pressure. Fractions containing the same compounds as determined by the TLC plates were combined and concentrated to dryness under reduced pressure, which resulted in twelve fractions. These twelve fractions were assayed qualitatively for antioxidant activity. Fraction F (46.45 g) showed more antioxidant compounds as compared to the other fractions. Fraction F was then chromatographed on a silica gel column eluted with *n*-hexane-ethyl acetate mixtures of increasing polarity followed by 100% methanol. A total of 20 sub-fractions were obtained. The chromatography of these sub-fractions on a sephadex column eluted with 100% methanol resulted in four pure compounds.

5.2.3 Antioxidant activity of the isolated compounds

The antioxidant activities of the isolated compounds were determined as described in chapter 2. They were tested at the final concentrations of between 100 and 0.8 µg/ml.

5.2.4 Antibacterial activity of isolated compounds

The antibacterial activities of isolated compounds were determined as described in chapter 3. They were tested at the final concentrations of between 40 and 0.31 µg/ml.

5.3 Results and discussion

5.3.1 Isolation and identification of compounds

The column chromatography (Figure 5.1) yielded 34 fractions which were pooled together according to their TLC profiles and this resulted in 12 pooled fractions (Figure 5.2).

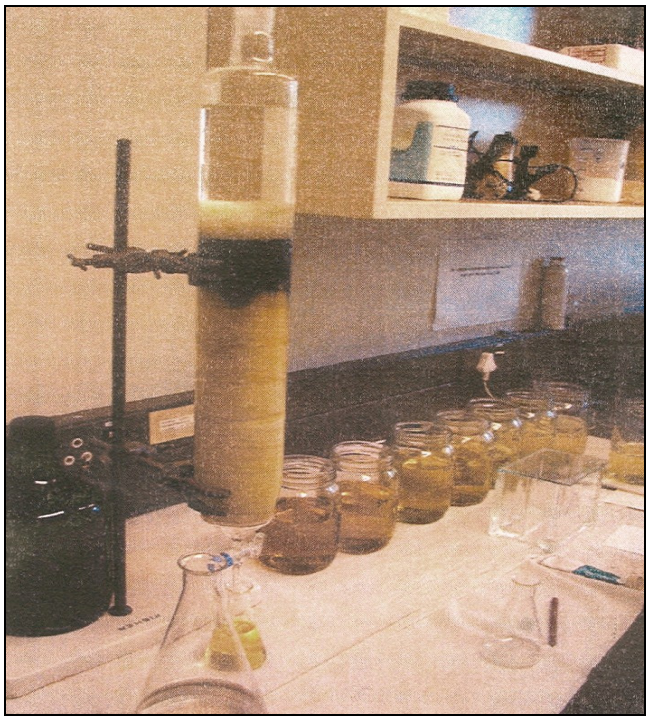

Figure 5.1 Column chromatography

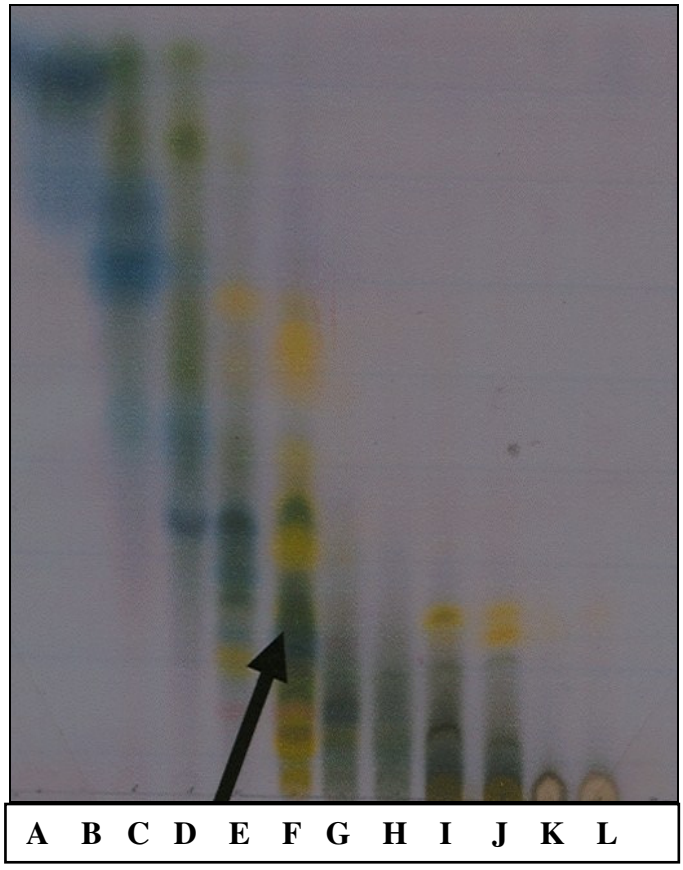

Figure 5.2 TLC plate of 12 pooled fractions sprayed with vanillin reagent**.**

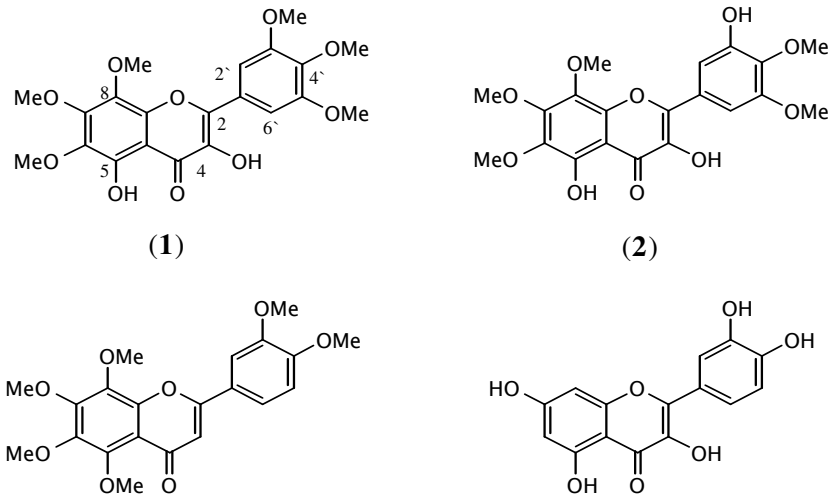

Figure 5.3 Chemical structures of isolated compounds. **(1)** 5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavon-3-ol, **(2)** 3-0-demethyldigicitrin, **(3)** 5,6,7,8,3',4'-hexamethoxy flavone and **(4)** Quecertin.

Fraction F yielded four flavonoids: **1** (166 mg), **2** (94 mg), **3** (8 mg) and **4** (41 mg) respectively (Figure 5.3). The TLC plates which showed these compounds were examined under UV light (245 and 366 nm) after development and also dipped in vanillin (15 g vanillin, 500 ml ethanol and 10 ml concentrated 98% sulphuric acid) and then heated to detect the compounds not absorbing UV light. The ^{1}H NMR and ^{13}C-NMR data of the isolated compounds are attached in chapter 8.

Compound **1** was isolated as a yellowish powder on chromatography using Sephadex columns. The ^{1}H NMR spectrum (Table 5.1) showed five singlets at δ_H 3.95, 3.94, 3.89, 3.88 (x 2), 3.83 (δ_C 56.0 (2 Me groups), 60.1, 60.8, 60.9, 61.5, and 61.5 ppm), due to six methoxy groups. Two aromatic protons singlets at δ_H 7.43 ppm, one-proton singlet at δ_H 12.40 of a strongly hydrogen-bonded hydroxyl group indicated the presence of a phenolic hydroxyl group, in addition to a carbonyl group at δ_C 179.3 ppm. Previous data with COSY, HMQC, HMBC and NOESY showed the structure of compound **1** as 5-hydroxy-6,7,8,3', 4',5'-hexamethoxyflavon-3-ol (Figure 5.3), which had been isolated from the same source before (Mashimbye *et al*., 2006). Compound **2** showed the same pattern of ^{1}H and ^{13}C NMR as those of compound **1** except for the ring B which showed a splitting of the two proton into singlets, indicating a change of the substitution pattern of ring B and removing one methoxyl from the C-3`. This compound has previously been isolated from plant extracts of *Zieridium pseudobtusifolium* and its structure is also supported by the ^{13}C NMR data published by Johannes *et al.* (1994). Compound **3** was isolated as yellow powder and identified based on the NMR data (^{1}H and ^{13}C) which showed six methoxyl groups, and 1,3,4-trisubstituted ring B pattern (doublet signal at 6.82 (*J*=8.4 Hz); a proton signal at 7.51 (d, *J*=2.2 Hz): and a signal at 7.58 (dd, *J*=2.2, 8.4 Hz), in addition a singlet signal at 7.24 of H-3. The structure of the compound was identified as

5,6,7,8,3',4'-hexamethoxyflavone. Compound **4** showed a typical signal of quercetin, a widely distributed flavonol and its NMR spectra was identical to those published in literature. It has previously been isolated from the aerial parts of *Hypericum hyssopifolium* (Cakir *et al*., 2003), leaves of *Castanea crenata* (Lee *et al*., 1999), aerial parts of *Epimedium brevicornum*, flowers of *Campsis radicans*, roots of *Aster tataricus*, seeds of *Cuscuta chinensis,* and fruits of *Cornus officinalis* (Cai *et al*., 2004).

Table 5.1 ^{1}H-NMR and ^{13}C-NMR data of the isolated compounds.

	Compound 1		**Compound 2**		**Compound 3**		**Compound 4**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2		155.14		145.4		155.3		147.4
3		130.49		136.3		137.7		136.4
4		179.30		179.3		178.5		176.5
5		147.95		147.9		148.2		161.4
6		127.08		135.9		135.4	6.38 d, 0.8	98.9
7		148.82		153.4		152.6		164.6
8		122.23		133.2		132.4	6.16, d, 0.8	94.0
9		144.83		145.1		144.3		156.8
10		106.01		105.3		105.3		103.6
1`		125.53		126.4		121.9		122.6
2`	7.43	139.06	7.37 s	108.1	7.51, d, 2.2	110.6	7.65 d, 0.6	115.7
3`		153.20		152.2		148.2		145.7
4`		140.70		137.5		151.0		148.4
5`		153.20		149.4	6.82 8.4	115.8	6.86,d, 8.4	116.3
6`	7.43	139.06	7.42 s	104.3	7.58, dd, 2.2, 8.4	122.4	7.52, dd, 0.6, 8.4	121.6
3-OMe	3.89	60.84	4.01	61.3				
5-OMe						55.27		
6-OMe						59.5		
7-OMe	3.94	60.85	3.98	62.1		60.0		
8-OMe	3.95	61.5	3.98	61.8		60.8		
3'-OMe	3.88	56.0				55.44		
4`-OMe	3.83	60.1	3.92	61.2		55.21		
5'-OMe	3.88	56.0	3.86	55.9				
3-OH	6.49							
5-OH	12.46							

5.3.2 Antioxidant activity of the isolated compounds

Antioxidant activities of the isolated compounds from aerial parts of *A. phylicoides* were evaluated *in vitro* using the DPPH scavenging assay. Due to a low yield of compound **3,** no further tests were done on this compound. Figure 5.4 show the DPPH scavenging activities of the tested compounds and vitamin C. All the tested

compounds showed a potent DPPH radical scavenging activity. Among the tested compounds, the most potent radical scavenger was compound **4** ($EC_{50,}$ 1.27 ± 0.25 µg/ml), followed by compound **1** ($EC_{50,}$ 2.73 ± 0.10 µg/ml), with compound **2** ($EC_{50,}$ 3.41 ± 0.09 µg/ml) as the least active compound (Table 5.2). It is reported that the lower the EC_{50} value, the higher the antioxidant activity of the sample (Chan, *et al*., 2007; Banerjee *et al*., 2005; Loo *et al*., 2008). Compound **4** (quercetin) has been reported to be the most potent scavenger of flavonoid compounds (Boots *et al*., 2008). The antioxidant activity shown by quercetin is attributed to the presence of the catechol group in the B ring and the OH group at position 3 of the AC ring within the molecule (Papiez *et al*., 2008; Kumarasamy *et al*., 2002; Heijnen *et al*., 2002).

Table 5.2 The EC_{50} values of the isolated compounds.

Compounds	**EC_{50}** (µg/ml)
1	2.73±0.10
2	3.41±0.09
3	N^a
4	1.27±0.25
Vitamin C	2.66±0.05

N^a, not assayed

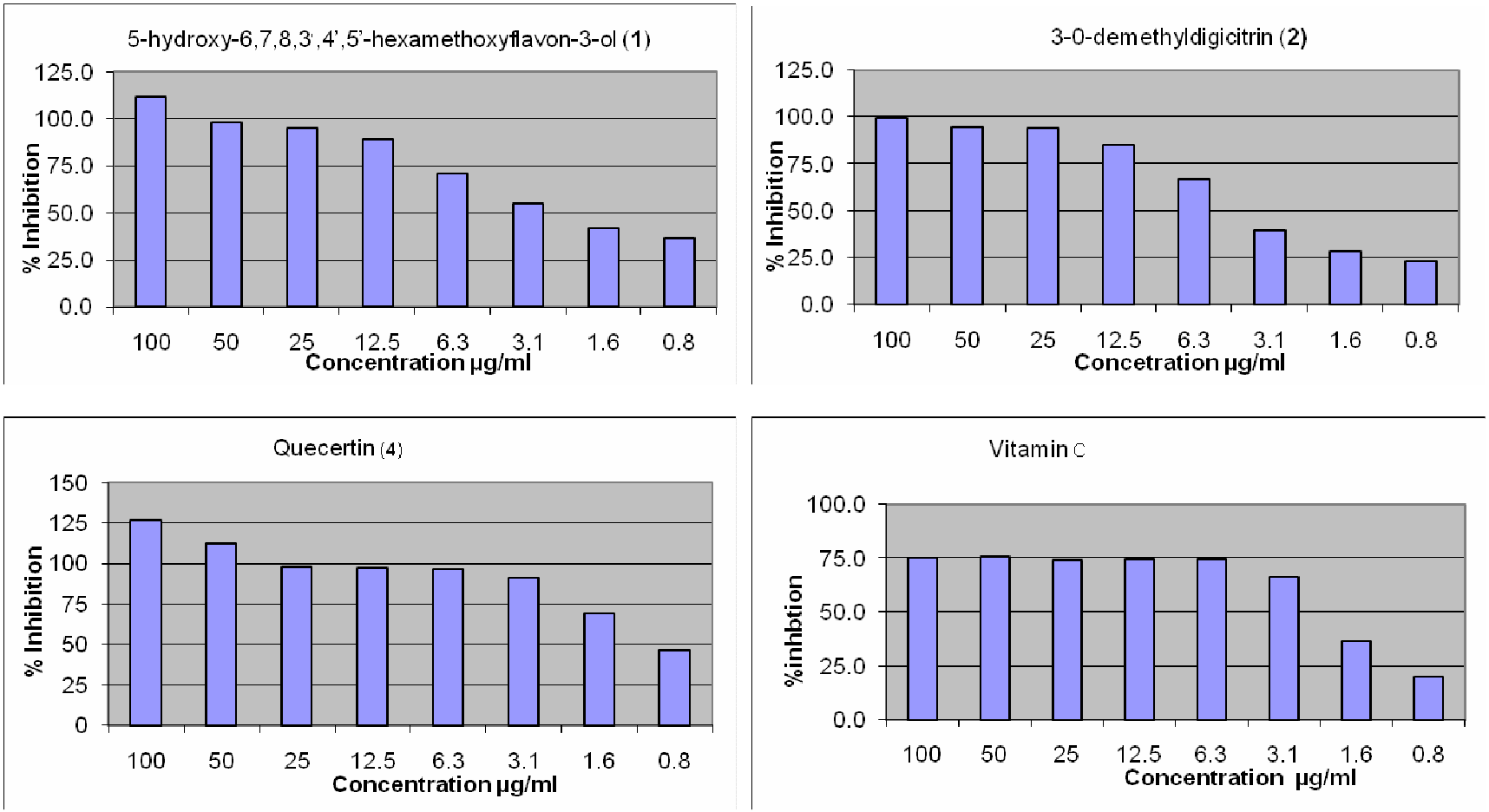

Figure 5.4 The DPPH inhibition activities of the isolated compounds and vitamin C.

In our results, it is important to note that compound **4** (quercetin) showed a higher antioxidant activity compared to the standard control (vitamin C) with a EC_{50} of 2.66 ± 0.05 µg/ml. Loo *et al*. (2008) reported that three compounds isolated from *Rhizophora apiculata* showed a higher scavenging activity than vitamin C. It is well-documented that flavonoids such as quercetin, catechin and kaempferol are more potent antioxidants agents than vitamins C and E (Chow *et al*., 2005).

5.3.4 Antibacterial activity of the isolated compounds

Compound **1** showed activity against four microorganisms with MIC values between 20 and 40 µg/ml (Table 5.3). Compounds **2** had MIC value of 40 µg/ml against *E. faecalis* and *E. coli* while compound 4 showed activity (20 µg/ml) against *B. subtilis* and *E. faecalis.*

Table 5.3 The MIC values of isolated compounds.

Microorganisms	MIC of tested compounds (µg/ml) **1**	**2**	**4**	**PC**
Bacillus cereus	40	>40	>40	2.5
Bacillus subtilis	40	>40	20	2.5
Staphylococcus aureus	>40	>40	>40	10
Bacillus pumilus	>40	>40	>40	20
Enterecoccus faecalis	20	40	20	2.5
Escherichia coli	20	40	>40	2.5
Klebsiella pneumonia	>40	>40	>40	10
Pseudomonas aeruginosa	>40	>40	>40	10

PC, positive control (Streptomycin)

All the isolated compounds showed no activity against *S. aureus, B. pumilus, K. pneumonia* and *P. aeruginosa* at the highest concentration tested (40 µg/ml). The inhibitory activities of isolated compounds were the same against Gram-positive and Gram-negative microorganisms. Tested compounds exhibited similar MIC values against individual microorganisms, possibly due to similarities between structures and hence structure-activity relationship (Martini *et al*., 2004).

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Chapter 6

Cytotoxicity of *Athrixia phylicoides* extract and the isolated compounds

6.1 Introduction

It has been estimated that 80% of people living in developing countries are almost completely dependent on traditional medical practices for their primary health care needs (Gurib–Fakim, 2006). The potential toxicity of the traditional medicines is an important consideration when studying their biological activities (McGaw *et al*., 2007). Plant extracts might be very toxic as they contain many different compounds; therefore it is very important to investigate cytotoxicity of both crude extracts and isolated compounds. Many plant extracts and isolated compounds can be evaluated for cytotoxicity by using human cell lines (prostate, stomach, liver colon and etc.) and animal cells such as monkey kidney cells (Don *et al*., 2006). In this study, the toxicity test of the *A. phylicoides* ethanol extract and the isolated compounds was performed on Vero cells.

6.2 Materials and methods

6.2.1 Preparation of the extract and isolation of the compounds

The ethanol extract was prepared as described in chapter 2 and the compounds were isolated as described in chapter 5.

6.2.2 Cell culture

The cytotoxicity screening of the *A. phylicoides* extract and the isolated compounds were tested against Vero cell lines. Cells were cultured in Eagle's minimal essential media (MEM) supplemented with 1.5 g/l sodium bicarbonate, 2mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/ml penicillium, 10 µg/ml streptomycin, 0.25 µg/ml fungizone, and 10 % fetal bovine serum at 37 ºC in a humidified atmosphere with 5% CO_2. The cells were sub- cultured in a 1:3 ratio every second to third day after trypsinization of the confluent cultures (American Tissue Culture Collection).

6.2.3 Toxicity screening (XTT viability assay)

The cytotoxicity of the crude extract and the pure compounds isolated from *A. phylicoides* was investigated by the XTT colorimetric assay using the Cell Proliferation Kit II (Roche Diagnostics GmbH) as previously described by Tshikalange (2007). On the first day of the experiment, the outer wells of the 96-well micro-titre plate (Figure 6.1) were filed with 200 µl of incomplete medium while the inner wells were filed with cell suspension. The plate was then incubated overnight at 37 °C in a humidified atmosphere with 5 % CO_2. Hundred micro-litres (100 µl) of the extract/compound dilutions were dispensed into the cell-containing wells of the sample plate in duplicate. The final concentrations of the crude extract in the wells were 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00, and 400.00 µg/ml. The final concentrations of the pure compounds in the wells were 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, and 200 µg/ml. Control wells received a final concentration of 1 % (for the crude extract) or 0.5 % (for the pure compounds) DMSO in complete medium. Zelaralenone was used as positive control. The plate was then incubated for 3 days.

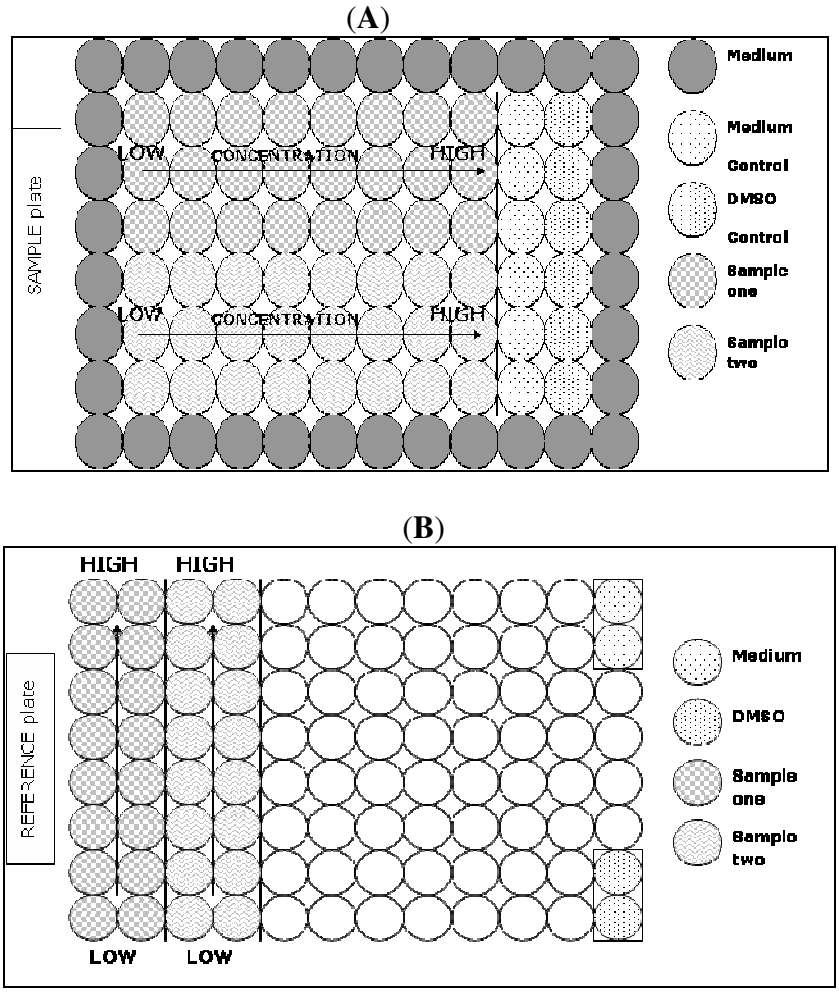

Figure 6.1 Plate design for the cytotoxicity assay (**A**, Sample and **B**, Reference plate).

Reference plates (without cells), containing 100 µl of medium and the diluted extract/compound were also prepared in duplicate. These plates were also incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 3 days. On day 4, 50 µl of sodium 3-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate (XTT) reagent was added to the wells and re-incubated for 1 to 4 hours. The optical densities were then measured at 450 nm (690 nm as reference wavelength) with an Eliza plate reader (KC Junior program). The 690 nm reference wavelength values were subtracted from their corresponding 450 nm wavelength values. The reference plate values were then subtracted from their corresponding

sample values. Cell viabilities were assessed by comparing sample values to the control values. The concentration of the extract/compounds at which 50% (IC_{50}) of the Vero cells were alive until the 4^{th} day was considered as to be the highest concentration which is non-toxic to the cells. The IC 50 values were then calculated by the Graph Pad Prism 4 programme.

6.3 Results and discussion

The cytotoxicity effects of the crude extract of *A. phylicoides* and the isolated compounds on the growth of Vero cells are shown in Figure 6.2 and Table 6.1. The crude extract showed no toxicity on Vero cells with cell viability of more than 140% at the lowest concentration tested (3.13 µg/ml). Toxicity effects were seen at the higher concentration tested (400 µg/ml), with cell viability of less than 40%. Ethanol extract from *A. phylicoides* have been reported to be highly toxic on Vero cells using the MTT cytotoxicity assay (McGaw *et al*., 2007). All the isolated compounds were toxic against the Vero cells at the highest concentration tested (200 µg/ml). Compound **4** showed minimal toxicity ($IC_{50,}$ 81.38 ± 0.331 µg/ml) as compared to compound **2** ($IC_{50,}$ 28.92 ± 0.118 µg/ml) and **1** ($IC_{50,}$ 27.91 ± 0.181 µg/ml). Compound **4** is reported to be a potent antitumor agent (Chow *et al*., 2005). Johannes *et al*. (1994) reported the high toxicity of compound 2 against carcinoma cells.

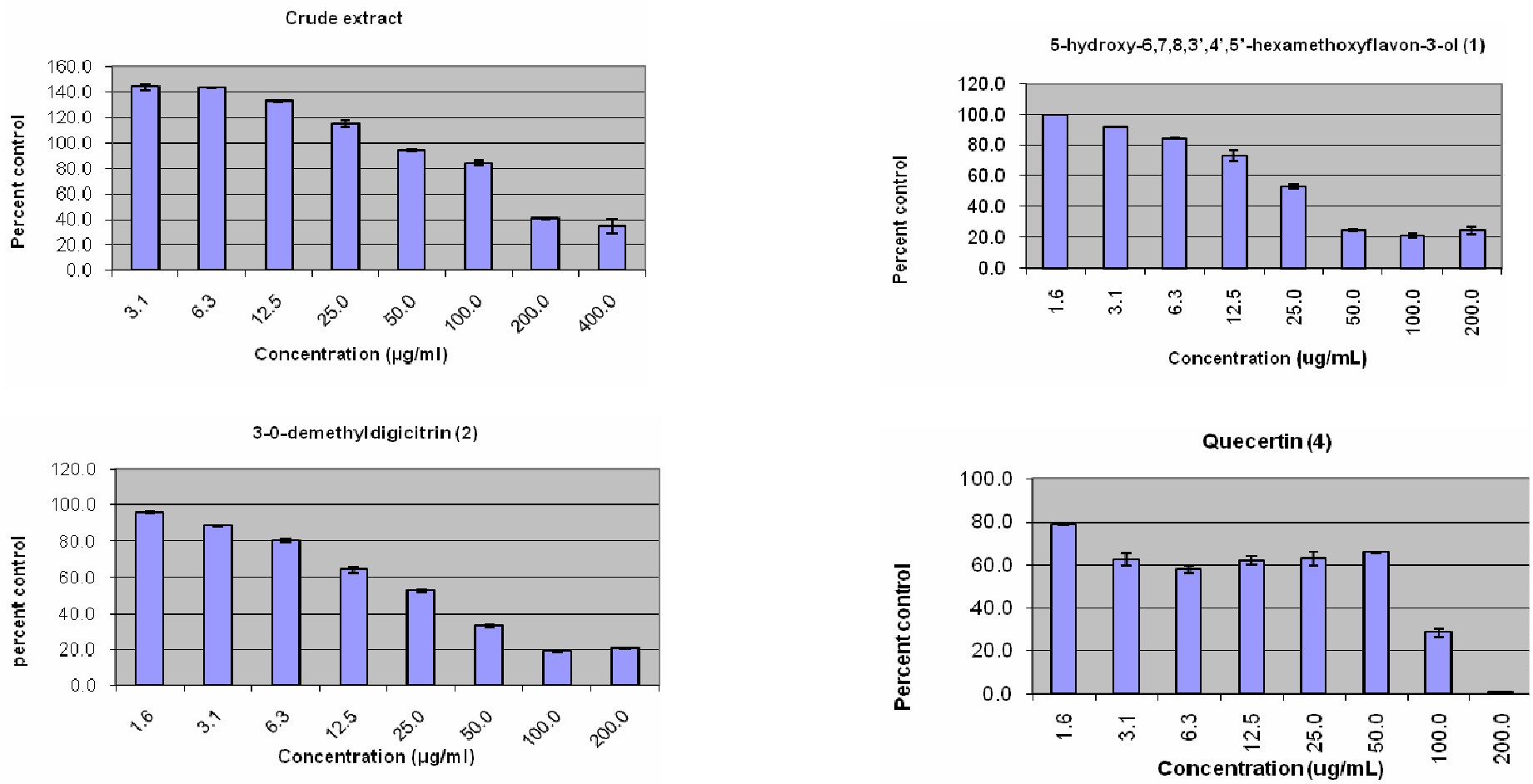

Figure 6.2 The cytotoxicty effect of *A. phylicoides* extract and the isolated compounds on the growth of the Vero cell line.

Table 6.1 The IC_{50} values of the crude extract and the isolated compounds.

Plant extract/compound	**IC_{50}** (µg/ml)
Crude extract	107.8±0.129
1	27.91±0.181
2	28.92±0.118
3	N[a]
4	81.38±0.331
Zelaralenone	2.6±0.31

N[a], Not assayed

Since many people in developing countries depend on traditional medicinal plants for their primary health care; it is very important to study the cytotoxic effects of the plant in use. *In vitro* cytotoxicity is necessary to define basal cytotoxicity such as the intrinsic ability of a compound to cause cell death as a result of damage to several cellular functions (Bouaziz *et al*., 2006). According to our IC_{50} values, the crude extract showed little toxicity on Vero cells compared that of the isolated compounds. To our best knowledge, there are no toxic reports of traditionally prepared (aqueous) *Athrixia phylicoides* since it has been discovered as a beverage many years ago. Aqueous extracts prepared from the same species have been reported to be not toxic on Vero cells (McGaw *et al*., 2007), and Wistar rat model following sub-chronic ingestion (Chellan *et al*., 2008). The cytotoxicity of all the compounds isolated from *A. phylicoides* against Vero cells are reported for first time in this study.

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Chapter 7

General discussion and conclusion

7.1 Introduction

Medicinal plants continue to play a central role in the healthcare systems of a large proportion of the world's population. About 80% of people living in the developing world are almost completely dependent on plant derived medicines for their healthcare (Prozesky *et al*., 2001). Today, more pharmacognostic investigations of plants are carried out to find novel drugs or templates for the development of new therapeutic agents. Many useful drugs that are currently in use for different diseases were derived from medicinal plants and then developed because of their use in traditional medicine (Gurib–Fakim, 2006). With the emergence of new diseases and resistant to already available drugs, many medicinal plants will continue to be the best source of new and active drugs. There is still a large number of higher plant species that have never been investigated for their chemical or biologically active constituents.

Previous studies have reported antioxidant activity and cytotoxicity of crude extract from *A. phylicoides* (McGaw *et al*., 2007). Mashimbye *et al*. (2006), isolated a new flavonoid (5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavon-3-ol) from the leaves of this plant. The aim of this study was to investigate the antioxidant, antibacterial activities and cytotoxicity of ethanol extract and isolated compounds from *A. phylicoides.* The phenolic content in dried and fresh crude extract was also investigated.

7.2 Antioxidant activity of *A. phylicoides*

Antioxidant activity of the ethanol extract was evaluated using the DPPH scavenging method. Our results indicated that the ethanol crude extract of *A. phylicoides* is a potent DPPH radical scavenger. The ethanol extract from *A. phylicoides* has previously been reported to be a more potent free radical scavenger as compared to Rooibos tea (McGaw *et al*., 2007). Herbal teas are reported to be rich in phenolic compounds (Atoui *et al*., 2005). The antioxidant activity shown by *A. phylicoides* could be attributed to these compounds.

7.3 Antibacterial activity of *A. phylicoides*

The antibacterial activity of the crude extract was determined by using the micro-dilution method on 96-well micro-plates. The results obtained in this study demonstrated that the extract has *in vitro* antibacterial activity against all the tested microorganisms, exhibiting MIC values ranging from 3.13 to 6.25 mg/ml. This was the first study of antibacterial activity done on *A. phylicoides*. However, our results are in line with the findings by other studies which reported the inhibition of the growth of microorganisms by herbal teas (Joubert *et al*., 2008).

7.4 Effect of drying on the phenolic content and antioxidant activity of *A. phylicoides*

Folin-Ciocalteu's reagent method was used to determine the total phenolic content of dried and fresh material crude extract of *A. phylicoides*. In our study, the total phenolic content and antioxidant activity was higher in the dried extract than in fresh the fresh material extract. During drying of the fresh plant materials, there are

enzymatic processes taking place which may lead to significant changes in the phytochemicals (Capecka *et al*., 2005).

7.5 Antioxidant and antibacterial activity of the isolated compounds

The silica column chromatography of the ethanol extract of *A. phylicoides* lead to the isolation of four flavonoids: (5-hydroxy-6,7,8,3',4',5'-hexamethoxyflavon-3-ol **1**), (3-0-demethyldigicitrin **2**), (5,6,7,8,3',4'-hexamethoxyflavon-3-ol **3**), and (quecertin **4**). Compound **4** was found to be a potent radical scavenger followed by compound **1** with **2** as the least active compound. The MIC values of the isolated compounds against tested microorganisms varied from 20 to more than 40 µg/ml. All the compounds showed no activity against *S. aureus, B. pumilus, K. pneumonia* and *P. aeruginosa* at the highest concentration tested (40 µg/ml).

7.6 Cytotoxicity of *A. phylicoides* extract and the isolated compounds

The cytotoxicity of the crude extract and the pure compounds isolated from *Athrixia phylicoides* was investigated by the XTT colorimetric assay. The crude extract showed little or no toxicity on the growth of the Vero cell lines, exhibiting IC_{50} value of 107.8 ± 0.129 µg/ml. Compound **4** was found to be less toxic with a IC_{50} value of 81.38 ± 0.331 µg/ml compared to compound **1** and **2** exhibiting a IC_{50} values of 27.91 ± 0.181 and 28.92 ± 0.118 µg/ml respectively.

7.7 Conclusion

The crude extract showed good antioxidant and antibacterial activities. The isolated compounds exhibited good antioxidant activity, but a toxicity effect was seen against Vero cell lines. The toxicity effect shown by the isolated compounds cannot be used to overlook the uses of the crude extract; there have been no toxic reports for the traditionally prepared (aqueous) *A. phylicoides* extract since it was discovered as a beverage many years ago. The results of this study provide a clear rationale for the medicinal uses of *A. phylicoides*. It is also recommended that the isolated compounds should be analysed for antidiabetes, anticancer and antiviral activities.

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Chapter 8

Appendix: ^{1}H-NMR and ^{13}C-NMR spectrum of isolated Compounds