UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Antimicrobial activity and cytotoxicity of extracts and an isolated compound from the edible plant *Grewia flava* against four enteric pathogens.

MAKGABO STELLA LAMOLA

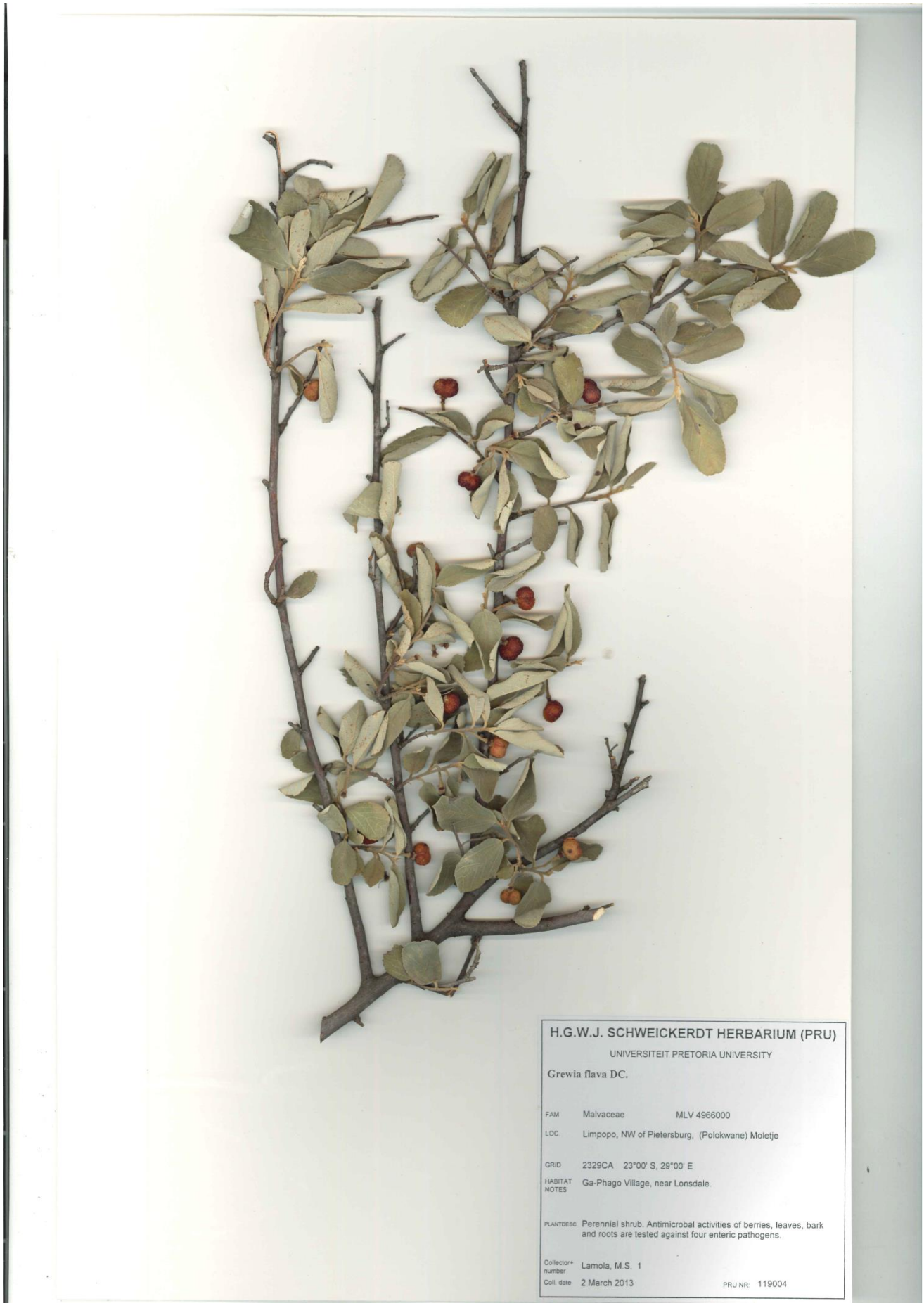
Dissertation submitted in fulfilment of the Degree Magister Scientiae (Master of Science) at the
Department of Paraclinical Sciences,
Faculty of Veterinary Sciences University of Pretoria,

SUPERVISOR: Dr FS Botha
CO-SUPERVISOR: Dr C van Wyk

March 2015

© University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA



© University of Pretoria

TABLE OF CONTENTS

© University of Pretoria

© University of Pretoria

© University of Pretoria

© University of Pretoria

ACKNOWLEDGEMENTS

I would like to thank the Phytomedicine programme management for giving me the opportunity to learn and work in their Labs, Prof Eloff, Prof McGaw, Dr Botha and Ms De Winnaar.

My Supervisor Dr F. Botha it was an honour to have been under your guidance.

My co-supervisor Dr Van Wyk, thank you for your assistance it made a big difference in my study.

My colleagues in the Phytomedicine programme, your help and support are always highly appreciated. Thanyani “Mfundisi” I was able to fractionate and draw my project structure because of your guidance. Bellona “Ausi B” and Daniel “Comrade” thank you for your support whenever I felt like I was failing, you always made sure that I regain my confidence and return to the lab. I am able to pack the column, combine fractions, run MIC and bioautography because you were there to guide me step-by-step, thank you.
Indeed we are family in the Phytomedicine Lab, may the warmth of the family continue to grow.

Dr Dzoyen you were there to assist with the cytotoxicity tests and guided me on how to calculate my $IC_{50}$and LC_{50}, continue with the good work.

Dr Leo thank you for encouraging me every day and made sure that I don’t lose faith and the spiritual food that you always offered, May the Almighty bless you.

Tharien and Emmerencia “Emmy”, you are phenomenal you always made sure that I had materials for my experiments, may the Lord bless you.

Financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

To my family thank you for always believing in me, you always told me that “you can do it”. My kids they always give the courage to wake up every morning and go to the lab, you always knew how to make me smile and be happy.

The glory and all thanks to Almighty God.

© University of Pretoria

DECLARATION

I, Makgabo Stella Lamola, declare that this Dissertation entitled, **Antimicrobial activity and cytotoxicity of extracts and an isolated compound from the edible plant *Grewia flava* against four enteric pathogens,** which I herewith submit to the University of Pretoria in partial fulfilment of the requirements for the degree Magister Scientiae (Master of Science) is my own original work, and has never been submitted for any academic award to any other institution of higher learning.

_____ _____

DATE

© University of Pretoria

LIST OF ABBREVIATIONS

µg	-	micro gram
ABTS		2-azinobis-ethylbenthiazoline-6-sulphonate
BEA	-	Benzen: Ethanol: Acetic acid
CEF	-	Chloroform: Ethyl acetate: Formic Acid
DEPT		Distortionless enhancement by polarization transfer
dH_2O	-	Distilled water
DPPH	-	2,2 diphenyl- 2- picrylhydrazine
EMW	-	Ethylacetate: Methanol: Water
FAWE	-	Formic Acid: Acetic acid: Water: Ethyl acetate
FRAP		Ferric reducing antioxidant power
g	-	gram
GC	-	Gas Chromatography
h	-	hour/s
HPLC		High performance Liquid Chromatography
IC_{50}		Inhibition concentration at 50% DPPH
INT	-	þ-iodonitrotetrazolium
L	-	Litre
LC_{50}		Lethal concentration 50.
mg	-	milligram
MIC	-	Minimal Inhibitory Concentration
min	-	minutes
ml	-	millilitre
mm	-	millimetre
mM	-	millimolar
MS	-	Mass Spectrometer
MTT		3-(4.5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide assay.
n	-	number
nm	-	nanometre
NMR	-	Nuclear Magnetic Resonance
Rf	-	Retardation factor
ROS		Biological reactive oxygen species
RNS		Reactive nitrogen species ()
SI	-	Selectivity index
TLC	-	Thin Layer Chromatography

© University of Pretoria

LIST OF TABLES

© University of Pretoria

LIST OF FIGURES

© University of Pretoria

© University of Pretoria

© University of Pretoria

ABSTRACT

Antimicrobial activity and cytotoxicity of extracts and an isolated compound from the edible plant *Grewia flava* against four enteric pathogens.

Bacterial infections of the gastrointestinal tract (GIT) causes vomiting, diarrhoea and even systemic infection. There is resistance to the commonly used antibiotics used to combat bacterial infection; there is a need for development of natural products into alternative and safer medicines. Studies have been conducted on the other species of the genus *Grewia* and there are reports of pharmacologically active compounds. There are no published studies up to date on *Grewia flava.*

This study evaluated the antimicrobial activity of extracts prepared from berries, leaves, bark and roots of the edible plant *Grewia flava* against four enteric pathogens, *Escherichia coli, Bacillus cereus, Staphylococcus aureus* and *Salmonella* Typhimurium. The genus *Grewia* was named after Nehemiah Grew, an English plant anatomist and physiologist. It is a member of the family Malvaceae, formerly Tiliaceae.

Plant material was extracted with acetone and water. The extracts were tested against the enteric pathogens using the minimal inhibitory concentration (MIC) method with gentamicin as positive control and β-iodonitrotetrazolium violet (INT) as growth indicator. Antioxidant activity of the extracts was analysed qualitatively by developing the chromatogram in three different mobile systems and spraying the plates with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and vanillin in sulphuric acid.

Quantitative determination of antioxidant activity was carried out spectrophotometrically with DPPH, a stable free radical, and Trolox as a positive control. The antimicrobial activity was further determined qualitatively by the bioautography method. The toxicity of the extracts was determined using the MTT reduction assay against Vero monkey kidney cells, with doxorubicin as a positive control.

The crude extracts of the leaves, bark and roots of *Grewia flava* were able to inhibit the growth of the enteric pathogens with MIC values ranging from 0.03 mg/ml to >2.5 mg/ml. Different compounds were visible on the plates after spraying with vanillin-H_2SO_4 reagent, and some of the compounds showed antioxidant activity when the plates were sprayed with DPPH and comparing the R_f values. Bioautography proved that the extracts were able to inhibit growth of the bacteria, *S.* Typhimurium and *B. cereus.* The acetone extracts were fractionated using

© University of Pretoria

solvent-solvent fractionation and five fractions were obtained; hexane, chloroform, butanol, 35% water in methanol and water. The chloroform fraction was effective against *B. cereus, E. coli* and *S*. Typhimurium with MIC values ranging from 0.15 mg/ml – 0.3 mg/ml. This fraction was also not toxic with a LC_{50} of 509.23 ± 24.88 µg/ml. Bioassay-guided isolation was carried out with the chloroform fraction, using open column chromatography, with silica gel as the stationary phase. The compounds were eluted with hexane: ethyl acetate solvent system at different polarities. Nine fractions were collected from the first column, and these fractions were tested for antimicrobial activity. Fraction 2 was the least effective with MIC above 1 mg/ml compared to fraction 3 which was effective against *B. cereus* and *E. coli*. On the bioautographs there was a visible clear zone with corresponding R_f on the thin layer chromatogram (TLC).

Compound 1 was isolated from fraction 2 of the first column and compound 2 from fraction 3 of the first column, and the compounds were tested for antimicrobial activity and cytotoxicity. Probably due to synergistic effects, the single or isolated compound was less effective than the mixture of compounds (i.e. the chloroform fraction and acetone crude extract). The compounds were less effective against the tested microorganisms than the fraction and extract. Compound 1 was the least toxic with LC_{50} of 39.38±3.21 µg/ml compared to LC_{50} 3.02 ± 0.03 µg/ml of the positive control compound, doxorubicin. Compound 1 was identified as Lupeol.

The indigenous edible plant, *Grewia flava* contains antimicrobial compounds, as indicated by the inherent free radical scavenging activity and could therefore be used as a natural product with little toxicity to host cells. Further *in vivo* studies have to be conducted to confirm the toxicity.

© University of Pretoria

CHAPTER 1

INTRODUCTION

For centuries the indigenous people of South Africa have relied on herbal medicine for all their primary health care, and it is estimated that millions of South Africans still use traditional remedies from as many as 700 indigenous plant species (Meyer and Afolayan, 1995). Although free health care has become well-established in the South African constitution, many rural people still rely on the cheaper traditional healing methods rather than the expensive Western treatments (Grierson and Afolayan, 1999). The use of medicinal plants in the world contributes significantly to primary health care and traditional medicines are part of the cultural and religious life of the African people, they are therefore easily available and affordable to rural people (Gurib-Fakim, 2006. Van Wyk *et al*., 2009).

Medicinal plants can be defined as plants used to prevent or cure the disease, illness or change a pathological process (Arias, 1999). Drugs and medicines derived from plants contribute largely to human health and wellbeing (Iwu *et al*, 1999). These natural products represent more than 50 % of all medicines used clinically in the world (Ciocan and Băra, 2007). Interest in natural and traditional medicine as a source of new commercial products is on the increase due to their effectiveness (CTA, 2007). Examples of products derived from plants are quinine, morphine, codeine, aspirin, atropine, reserpine and cocaine. South Africa's contribution to world medicine includes the Cape aloe (*Aloe ferox*), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum procumbens*). Interest in natural and traditional medicine as a source of new commercial products is on the increase because of its effectiveness (CTA, 2007).

The exposure to most microbial pathogens is very high, with an increased susceptibility to bacterial infection. Therefore the level of sanitation, hygiene and living conditions in most rural areas is not comparable to those of urban areas (Taylor *et al.,* 2001). The gastro- intestinal tract (GIT) is a very delicate passage that transports nutrients through the body, and bacterial infection of the GIT causes vomiting, diarrhoea and systemic disease. There are numerous bacteria species that can infect the digestive system, such as *Salmonella* spp*., Shigella* spp*. Escherichia coli, Staphylococcus aureus, Helicobacter pylori* and *Vibrio spp.* (Sekirov *et al.* 2010).

© University of Pretoria

Investigations for new classes of antibiotics have become increasingly important in recent years. There is a huge problem of antibiotic resistant bacteria such as the nosocomial Methicillin-resistant infective bacterium. The search for and development of new antibiotics that will target resistant bacterial strains are needed. Therefore the screening of plant extracts for antibacterial compounds is very important and relevant (Rabe and Van Staden, 1997). Antimicrobial substances from natural sources like plants have been investigated to achieve higher levels of food safety (Tiwara et al., 2009).

An estimated 25 million South Africans depend on traditional medicines for their primary health care (Mander, 1998), increasing the acceptance of herbal medicine as an alternative health care. South Africa has a rich diversity of medicinal plants that is reflected in the formal and informal medicinal systems practiced in the country today. The informal, oral/traditional medicinal systems of the Khoisan, Nguni- and Sotho-speaking peoples have not yet been systemised. This unfortunate total lack of detailed documentation regarding the use of traditional medicinal plants in South Africa might be due to the results of the urbanisation and cultural changes that are taking place (Van Wyk *et al*., 1997).

© University of Pretoria

CHAPTER 2

AIM AND OBJECTIVES

2.1 Aim

The aim of this *in vitro* study was to:

Identify the active antimicrobial compound(s) from the berries, leaves, bark and roots, of the indigenous edible plant *Grewia flava.*

2.2 Objectives

The objectives were to:

1. Extract the compounds from the plant materials with acetone, hot water and cold water; and analyse the antimicrobial activity of crude extracts against the gastrointestinal pathogens: *E. coli, S. aureus, B. cereus* and *S*. Typhimurium by minimal inhibition concentration (MIC)

2. Test the presence of antioxidant compounds of crude extracts using well described method

3. Determine the antioxidant and scavenging activity of the crude extracts using stable free radical DPPH (2,2 diphenyl- 2- picrylhydrazine) qualitatively and a quantitative spectrophotometric method

4. Test the toxicity of the crude extracts against the Vero monkey kidney cells using the MTT assay (3-(4.5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide)

5. Fractionate the active extract using solvent-solvent fractionation and analyse the antimicrobial activity of the fractions against the gastrointestinal pathogens using MIC and bioautography assays

© University of Pretoria

6. Isolate the active antimicrobial compounds using column chromatography

7. Characterise and elucidate the structure of the isolated compounds using NMR-structure elucidation

2.3 Null hypothesis

The traditional medicinal plant *Grewia flava* does not contain active compounds with antimicrobial properties that can be of medicinal value.

© University of Pretoria

CHAPTER 3

LITERATURE REVIEW

3.1 Introduction

Traditional medicinal plants are known to possess remedial compounds for different diseases. Compounds found in the plants are responsible for their pharmacological potential. The medicinal plants mostly possess antioxidants (Molan *et al.,* 2012*)*. Various antioxidant compounds function differently in order to fight diseases. Studies have been done on the related species of the genus *Grewia* and there were findings of pharmacologically active compounds. There are no published studies up to date on the concerned specie, *Grewia flava.*

3.1.1 The genus *Grewia*

The genus *Grewia,* family Malvacea*,* was named after Nehemiah Grew (1641-1712) an English plant anatomist and physiologist, famously known as the "Father of plant physiology" (Venn & Venn, 1978). The genus *Grewia* is a major angiosperm group (flowering plants) with approximately 400 species of flowering plants, shrubs, and trees that are widely distributed in subtropical and tropical regions depending on the species, including tropical Africa, Arabia, Madagascar, the Himalaya, India, Pakistan, Bangladesh, Thailand and Malaysia (Wali *et al*., 2012; Leistener, 2000). A number of species of the genus *Grewia* have been used as medicinal agents to treat several diseases such as skin disease, hypertension, ulcers and diarrhoea, and also for the production of food (Bowden, 1978; Raghunath, 1996; Aratanechemuge *et al.,* 2004; Juvekar *et al*., 2007).

Grewia species are rapid growers preferring rich, well-drained soil and full sun to part shade. Some species attract birds and butterflies and can be a good choice for a garden designed to attract animal visitors. Species producing edible fruits can be cultivated for culinary, as well as decorative uses. Some *Grewia* species may have bark, flowers, leaves, or roots with medicinal properties. Indigenous people harvest and use the various species of the genus for medicinal purposes. Products made from these plants are sometimes available commercially at health food stores and stores that sell indigenous products. A number of named cultivars of specific species have been developed. These cultivars have been bred to bring out traits of interest and are commercially available (McMahon, 2015).

© University of Pretoria

Grewia tiliifolia (dhaman) is a tree found in India, Sri Lanka and tropical Africa. The bark and roots are used to treat skin diseases, hypertension, ulcers and diarrhoea. The hot water extract of the bark is used as an antidote for opium poisoning in adults (Bamadi *et al.,* 2004). Lupeol an antioxidant compound isolated from *Grewia tiliifolia* is known to cause apoptosis in several cancer cells (Aratanechemuge *et al.*, 2004).

Lupeol is also an antioxidant compound isolated from the extract of *Grewia bilamellata* exhibits antimalarial activity against *Plasmodium falciparum* (Ma *et al.,* 2006).

Grewia asiatica (phalsa or falsa) is another species that was initially cultivated mainly for its sour fruits, until its high medicinal value was discovered. The fruits of this plant are used as an astringent, an anti-inflammatory agent, for blood disorders, and a fever reducer. The bark is used for the treatment of diarrhoea (Yadav, 1999).

Grewia bicolor (white raisin or mongwane) is used in Sudanese tradition to treat skin lesions and also as a tranquilizer (Yadav, 1999). The compound 6-hydroxyharman was isolated from the methanol extract of the plant and showed antibacterial properties (Jasper *et al*., 1986).

Grewia flava DC., commonly known as the “brandy bush” or “velvet raisin” (English), “fluweelrosyntjie/wilderosyntjie” (Afrikaans) and “moretlwa” (Tswana and Sepedi), is a shrub of up to 2 m tall and is widely distributed. The bark is grey-brown to black and the leaves are greyish green with fine hairs on the upper side and hairier and lighter green on the underside. The flowers are yellow and star-shaped and the berries are orange-brown of a medium size, and ripen from February until early April.

© University of Pretoria

Figure 3.1. *Grewia flava* plant, with top left picture showing the yellow star- shaped flower, top right picture shows the ripe berries ("dithletlwa"), bottom left picture shows the saw- edged leaves, the pin-red unripe berries and the grey bark and bottom right picture shows the root system.

The *Grewia flava* fruit are gathered from February onwards as they ripen and are eaten in large quantities, because they have more flesh than other Grewia species. They are also mashed, soaked for a while in water and eaten as porridge by the San (Bushmen). The beer prepared from the berries is called "Khadi". *Grewia* species have been reported to contain many alkaloids, traces of β-carbolines and triterpenoids. There is a diverse possibility that the plants involved have ethno-medicinal familiarity to the indigenous people. The primary intoxicant of "Khadi" is alcohol; and the potential pharmacological interactions is an area in serious need of in-depth study (Kalahari Plants and their traditional uses, (www.Kalahari-meerkats.com).

© University of Pretoria

3.2 Antioxidants

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules by means of protecting cells from the unstable molecule's damage, known as free radicals. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidised themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Sies, 1997). Most antioxidant molecules such as phenolic compounds, vitamins, nitrogen compounds and other antioxidant-rich metabolites are found in plants like fruit, vegetables and medicinal plants (Oluwaseun and Ganiyu, 2008).

Larson (1998) showed that antioxidant compounds have anti-inflammatory, antitumor, anti-mutagenic and other defence activity. A study by Kinsella *et al.,* (1993) showed that natural antioxidants have been of increasing interest because synthetic antioxidant compounds have a strong activity, but are associated with side effects.

3.2.1 Classification of antioxidants

3.2.1.1 Primary or natural antioxidants

Primary or natural antioxidants are the chain-breaking antioxidants which react with lipid radicals and convert them into more stable products. Antioxidants of this group are mainly phenolics in structure and include the following:

(1) Antioxidant minerals - are a co-factor of antioxidant enzymes. Absence of antioxidant minerals will definitely affect metabolism of many macromolecules such as carbohydrates. Examples include selenium, copper, iron, zinc and manganese.

(2) Antioxidant vitamins – are needed for most body metabolic functions and include vitamin C, vitamin E and vitamin B.

(3) Phytochemicals - are phenolic compounds that are neither vitamins nor minerals. These include:

- Flavonoids that are phenolic compounds that give vegetables, fruit, grains, seeds, leaves, flowers and bark their colours.
- Catechins are the most active antioxidants in green and black tea and sesamol.
- Carotenoids are fat soluble colour in fruit and vegetables.
- Beta carotene is mainly found in carrots and converted to vitamin A when the body lacks the vitamin.
- Lycopene is high mainly in tomatoes.

© University of Pretoria

- Zeaxantin is high in spinach and other dark green vegetables. The source in herbs and spices include diterpene, rosmariquinone, thyme, nutmeg, clove, black pepper, ginger, garlic and curcumin and derivatives (Hurrell, 2003).

3.2.1.2 Secondary or synthetic antioxidants

Secondary or synthetic antioxidants are phenolic compounds that perform the function of capturing free radicals and stopping the chain reactions. They include:

i. Butylated hydroxyl anisole (BHA),
ii. Butylatedhydroxyrotoluene (BHT),
iii. Propyl gallate (PG) and metal chelating agent (EDTA),
iv. Tertiary butyl hydroquinone (TBHQ) and
v. Nordihydro guaretic acid (NDGA) (Hurrell, 2003).

3.3 Gastro-intestinal pathogens

The gastrointestinal tract (GIT) is a long passage that takes food from the mouth through to the anus, where the wastes are eliminated. This large surface area allows for efficient uptake of nutrients. For the GIT to function optimally there needs to be a balance between the intestinal motility and intestinal fluid volume. The process is regulated by fluid absorption through the intestinal epithelial cells and the secretion across the intestine through the crypt cells (Martinez-Augustine *et al*., 2009). The GIT also exposes the body to potential external toxins and pathogens. Bacterial infection of the GIT causes vomiting, diarrhoea and even systemic disease. There are numerous bacterial species that can infect the digestive system, such as *Salmonella* spp*., Shigella* spp*. Escherichia coli, Staphylococcus aureus, Helicobacter pylori and Vibrio* spp*.* (Sekirov *et al.,* 2010).

Today emerging antibiotic resistance in bacteria, such as the nosocomial Methicillin-resistant infective bacterium, *Staphylococcus aureus* (MRSa)*,* has become a serious problem, and investigations for new classes of antibiotics have become increasingly important. Ideally antibiotic targets that microbes will find impossible to develop resistance to are needed. Antimicrobial substances from natural sources like plants have been investigated to achieve higher levels of food safety (Tiwar*a et al.,* 2009).

For the purpose of this study certain gastrointestinal pathogens will be used and are discussed in section 3.3.1

© University of Pretoria

3.3.1 Selected gastrointestinal (GI) microorganisms

3.3.1.1 Bacillus cereus

Bacillus cereus is a Gram-positive aerobic or facultative anaerobic, motile, spore-forming, rod-shaped bacterium that is widely distributed in the environment. It is associated mainly with food poisoning and it is increasingly reported to be a cause of serious and potentially fatal non-gastrointestinal-tract infections (Ramarao and Lereclus, 2006). The pathogenicity of *B. cereus*, whether intestinal or non-intestinal, is associated with the production of tissue-destructive exoenzymes. Among these secreted toxins are four hemolysins, three distinct phospholipases, an emesis-inducing toxin, and proteases (Lund *et al*., 2000). Outside its notoriety in association with food poisoning and severe eye infections, it has been incriminated in a multitude of other clinical conditions such as anthrax-like progressive pneumonia, fulminate sepsis, and devastating central nervous system infections, particularly in immunocompromised individuals, intravenous drug abusers, and neonates. *B. cereus* produces a potent β-lactamase conferring marked resistance to β-lactam antibiotics. Antimicrobials noted to be effective in the empirical management of a *B. cereus* infection, while awaiting antimicrobial susceptibility test results for the isolated bacteria include ciprofloxacin and vancomycin (Bottone, 2010).

3.3.1.2 Staphylococcus aureus

Staphylococci are Gram-positive spherical, facultative anaerobic bacteria that occur in microscopic clusters resembling grapes. The best-known of its nearby phylogenetic relatives are the members of genus *Bacillus* in the family Bacillaceae*,* which is on the same level as the family Staphylococcaceae*. S. aureus* can cause many forms of infection such as superficial skin lesions (boils, styes) or deep-seated infections, such as osteomyelitis and endocarditis and more serious skin infections such as furunculosis. *S. aureus* is a major cause of hospital-acquired infection of surgical wounds. It also causes food poisoning by releasing enterotoxins into food. *S. aureus* can grow at temperature ranging between 15˚C to 45˚C and at salt concentrations as high as 15%. *S. epidermidis* causes infections associated with indwelling medical devices (Foster, 1991).

© University of Pretoria

3.3.1.3 Salmonella enterica serovar Typhimurium

Salmonella enterica (formerly *Salmonella choleraesuis*) is a rod-shaped flagellated, facultative anaerobic, Gram-negative bacterium, and a member of the genus *Salmonella* that consists of a range of very closely related bacteria, many of which cause disease in humans and animals. *Salmonella enterica* is involved in causing diseases of the intestines (enteric means pertaining to the intestine). The three main serovars of *Salmonella enterica* are Typhimurium, Enteritidis and Typhi (Barron, 1996).

Salmonella enterica serovar Typhimurium (previously known as *Salmonella typhimurium)* or abbreviated to *S*. Typhimurium causes typhoid-like disease in mice. In humans *S.* Typhimurium does not cause as severe a disease as *S.* Typhi, and is not normally fatal. The disease is characterised by diarrhoea, abdominal cramps, vomiting and nausea, and generally lasts up to 7 days. Unfortunately, in elderly, young, or people with depressed immune systems, *Salmonella* infections are often fatal if they are not treated with antibiotics. When *Salmonella* is ingested it passes through the stomach to the intestine, where it binds to the wall of the intestine, and through some proteins that it produces in response to the intestinal condition. Once it is attached, it is taken to the liver or spleen. In the liver, the *Salmonella* can grow again, and be released back into the intestine. In regions with poor sanitation it can survive in the soil or in rivers and infect the next person, cow, chicken or mouse that comes in contact with it (Barron, 1996).

3.3.1.4 Escherichia coli

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium commonly found in the lower intestine of warm-blooded organisms (endotherms). Depending on the virulence factors they possess, virulent *E. coli* strains cause either non-inflammatory diarrhoea (watery diarrhoea) or inflammatory diarrhoea (dysentery with stools usually containing blood, mucus, and leukocytes). Transmission is by the faecal-oral route. A bacterium is colonised in the ileac mucosa with the help of pilli (fimbriae). Cytotoxic enterotoxins which are encoded on the plasmid induce watery diarrhoea. Plasmid-encoded invasion factors assist in the invasion of the mucosa and cytotoxic enterotoxins induce tissue damage and they induce a host inflammatory reaction with an entry of lymphocytes and resulting dysentery (Barron, 1996).

© University of Pretoria

CHAPTER 4

PRELIMINARY SCREENING AND ANTIMICROBIAL ACTIVITY

4.1 Introduction

Recent interest in the therapeutic potential of medicinal plants means more researchers are concerned with validating the ethnopharmacological usage of plants and the isolation and identification of the active components (Fennell *et al*, 2004). More of the studies dedicated to antimicrobial activity focus on the extracts (van Vuuren, 2008). This might be due to the fact that when traditional medicinal plants are prepared, efficacious compounds are not extracted individually from the plant (Street *et al*., 2008). Presence of more inactive compounds makes the screening and isolation of the active compounds tedious work (Sticher, 2008).

The Phytomedicine programme, Faculty of Veterinary Sciences, University of Pretoria [www.up.ac.za/phyto] is a research programme that investigates the use of medicinal plants growing in Africa for the benefits of its people and animals. More than 632 plant species have been screened against eight bacterial and fungal pathogens using the serial micro plate dilution and bioautography assays. Extracts with Minimal inhibitory concentration (MIC) values below eight mg/ml are considered active (Fabry *et al*., 1996), however in the Phytomedicine programme activity above 0.1 mg/ml is not considered to be significant.

4.2 Solid-liquid extraction

Extraction is the first pre-purification step in the isolation and characterisation of active compound(s). Selective removal of components from the solid plant materials involves the following:

- Mixing plant material and extractant
- Solubilisation of the solute with the shaker or sonicator
- Filtration to remove solutes and extractants from the plant residue
- Drying of the sample using freeze-drying, evaporation under vacuum or air-drying techniques and
- Recovery of solute extracts

© University of Pretoria

4.3 Plant collection and extraction

Berries, leaves, twigs and roots from the edible plant Grewia flava were collected from an open field at Moletjie Ga-Phago near Lonsdale, Polokwane, Limpopo (Grid 2329CA 23º00', 29º00' E). The plant was identified and authenticated by Magda Nel at the University of Pretoria and voucher specimens are maintained at the HGWJ Schweickerdt Herbarium of the Department of Plant Science, University of Pretoria, Hatfield campus, Pretoria, South Africa.

Leaves, twigs and roots were collected in open woven orange bags, and dried at room temperature in the shade. The leaves took 3 weeks to dry, 1 month 2 weeks for the roots and 2 months for the bark. The berries were dried under a fan on a tray. Dried leaves were ground to powder using a Kika-werk M20, bench top grinder. Powdered materials were stored in closed honey jars at room temperature in the shade and clearly marked until needed.

4.4 Materials and methods

For extraction exactly 1 g of powdered plant material from each plant part was extracted with 10 ml of technical graded acetone, methanol (Minema), distilled water and boiled distilled water in centrifuge tubes on a shaking machine for an hour. Centrifugation was carried out at 400x rpm for 10 minutes. The cold water extract was made by soaking the plant material in water overnight with occasional shaking. In the boiled water extract, the sample materials and the water was allowed to boil. The coactions were allowed to cool to room temperature and settled over night. The supernatant was filtered through Whatman No. 1 filter paper into pre-weight vials. Extraction was repeated 3 times on the pellet, with the same extractant, to exhaustively extract the plant materials. The solvents were dried under a stream of cold air.

4.4.1 Results and discussion

Yield determination

Acetone, methanol and water were the extractants used. Acetone was used due to its low toxicity and ability to extracts both the non-polar and the polar components (Eloff, 1998) and water was used because it is the only extractant traditionally used. The highest yield was from the water extract of the berries (31.6%), followed by the water extract of the leaves (29.75%).

© University of Pretoria

The lowest yield was obtained from the water extract of the bark (0.4%) followed by the acetone extracts of the bark and roots, 1.3% and 1.4% respectively as presented in

Table 4.1. Percentage yield of the 3 g *Grewia flava* plant parts: berries, leaves, bark and roots, extracted with 30 ml of acetone, methanol and water

Plant parts	Solvents	Yield(g)	Yield %
Berries	Acetone	0.131	4.37
	Water	0.949	31.63
	Methanol	0.064	2.167
Leaves	Acetone	0.065	2.17
	Water	0.8925	29.75
	Methanol	0.291	9.7
Bark	Acetone	0.039	1.3
	Water	0.013	0.43
	Methanol	0.162	5.4
Roots	Acetone	0.042	1.4
	Water	0.106	3.53
	Methanol	0.263	8.767

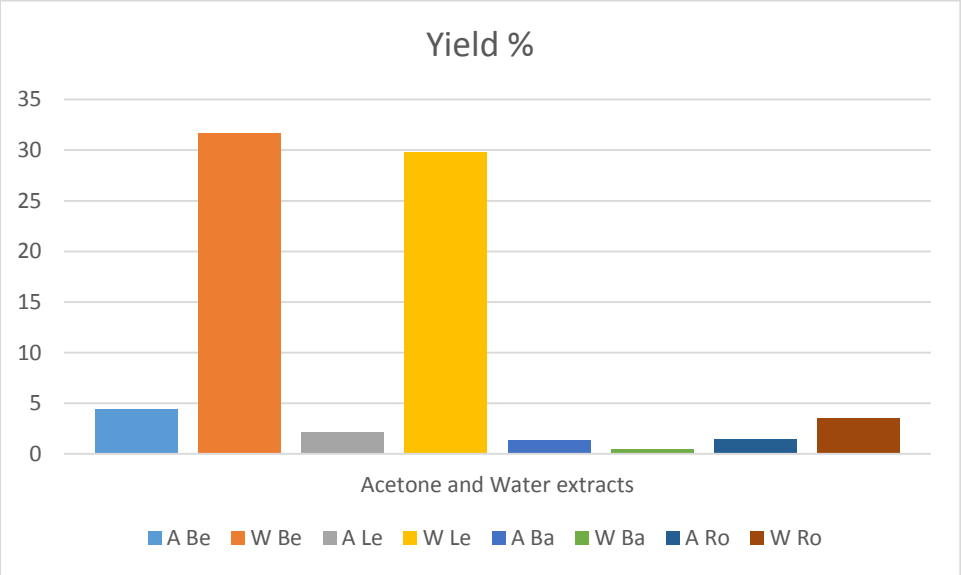


Figure 4.1. Percentage yield chart of the *Grewia flava* plant parts: berries, leaves, bark and roots, extracted with acetone and water

A Be = acetone extract of berries; W Be = water extract of berries; A Le = acetone extract of leaves; W Le = water extract of leaves; A Ba = acetone extract of bark; W Ba = water extract of bark; A Ro = acetone extract of root; W Ro = water extract of root

© University of Pretoria

4.4.2 Antimicrobial bioassays

There are different methods to determine antibacterial activity such as the disc diffusion, serial microplate-dilution and bioautography methods. Disc diffusion is a simple method to analyse a wide range of samples, but the method has limitations when analysing antimicrobial activity of oils and non-polar samples (Rasoanaivo and Ratsimamanga-Urverg 1993, Hewitt and Vincent, 2003). Minimal inhibitory concentration of an active plant extract is a sensitive method that uses a 96-well microtitre plate (Eloff 1998). The method is used to determine bacteriostatic and bacteriocidal activity at a particular concentration. Bioautography is a qualitative assay that combines thin layer chromatography with *in vitro* bioassay, and it allows the localisation of an active compound in the extract (Gibbons and Gray 1998).

4.4.2.1 Minimal inhibitory concentration

The serial micro plate dilution method by Eloff (1998), was used to determine the minimal inhibitory concentration (MIC) for the extracts using β-iodonitrotetrazolium violet (INT) as a growth indicator.

A nutrient medium of Tryptone soy agar (38 g in 1 litre) was prepared with 1% sugar and 1% dextrose. Isolates of the organisms were streaked on the agar and incubated at 37ºC for 18-24 hrs. From the prepared plates colonies were taken and sub-cultured on a fresh agar for MIC, the subcultures were incubated at 37ºC for 24 hours. Tryptone broth was prepared. Acetone extract residues were reconstituted in acetone to a concentration of 10 mg/ml. For each of the enteric bacteria used, 100 µl of the extract tested was two-fold serially diluted into 100 µl of the sterile water in a sterile 96-well microtitre plate. Similarly two-fold serial dilutions of 100 µl concentration 0.1 mg/ml gentamycin (Genta 50, Animal vebra) were used (positive control). Acetone and sterile water were used as negative controls. Each experiment and control was done in triplicate. Hundred microliters (100 µl) of each bacterial culture were added to each well. The plates were covered and incubated at 37ºC overnight. To indicate bacterial growth, 40 µl of 0.02 mg/ml INT was added and the plates were further incubated at 37ºC for 30-40 min. Bacterial growth in each well was indicated by a pink-red colour, whereas bacterial inhibition was indicated by the yellow to clear wells. Similarly the methanol, water and acytelated extracts were tested.

© University of Pretoria

4.4.2.2 Results

MIC values of the extracts against the four enteric pathogens are listed in Table 4.2. The acetone and methanol extracts of the leaves, bark and roots had better activity against the tested bacteria compared to the water and acytelated extracts. MIC values as low as 0.03 mg/ml were obtained from the methanol extracts against *S. aureus* and *S*. Typhimurium. Acetone extracts showed the lowest inhibition at a concentration of 0.07 mg/ml from the leaf, bark and root extracts against *E. coli* and *S. aureus*. Root acetylated extracts showed an effective activity against *B. cereus* and *E. coli* with a minimal concentration of 0.07 mg/ml. Water extracts were not effective against the enteric pathogens because the minimal concentration was 0.3 mg/ml.

Table 4.2. Minimal inhibitory concentrations (mg/ml) of the berries, leaves, bark and roots extracts against four enteric pathogens.

Plant parts	Solvents	MIC (mg/ml) Enteric Pathogens			
		B. cereus	*E. coli*	*S. aureus*	*S.* Typhimurium
Berries	Acetone	1.25	0.3	0.6	1.25
	Water	>2.5	1.25	>2.5	>2.5
Leaves	Acetone	0.6	0.07	0.07	0.15
	Methanol	0.07	0.07	0.03	0.03
	Acetylated	1.2	0.3	0.6	1.25
	Water	>2.5	0.3	0.3	1.25
Bark	Acetone	0.3	0.15	0.07	0.6
	Methanol	0.15	0.3	0.3	0.3
	Acetylated	1.25	0.15	0.6	1.25
	Water	>2.5	1.25	>2.5	>2.5
Roots	Acetone	0.3	0.07	0.07	0.15
	Methanol	0.3	0.6	0.15	0.3
	Acetylated	0.07	0.07	0.03	0.6
	Water	>2.5	1.25	0.3	0.6

Although the methanol extracts were also effective against the enteric pathogens, only the acetone extracts was considered for the next step, the solvent-solvent fractionation.

© University of Pretoria

4.4.3 Conclusion

Water was a good extract solvent yield wise, but the extracts were not active against the tested bacterial pathogens, with the lowest MIC of 0.3 mg/ml and the highest MIC >2.5 mg/ml. The berries were less active against any of the tested bacteria, MIC range from 0.3 mg/ml to 1.25 mg/ml from the acetone extracts and 1.25 mg/ml to >2.5 mg/ml from the water extract. Acetone was previously reported to be the best solvent because of its low toxicity to pathogens, Eloff (1998), this means that the activity of the acetone extracts is from the extracted compounds, and not the solvent used to extract them. The leaf-, bark- and root extracts showed effective activity against the tested pathogens with lowest MIC of 0.07 mg/ml. The root extracts showed the highest activity against all the tested enteric pathogens (MIC 0.3 mg/ml against *B. cereus*, 0.15 mg/ml against *S.* Typhimurium and 0.07 mg/ml against *E. coli* and *S. aureus*). The leaf extracts were effective against the tested pathogens with the lowest MIC of 0.07 mg/ml against *E. coli* and *S. aureus* and the highest MIC of 0.6 mg/ml against *B. cereus*.

4.5 Bioautography

Bioautography is a qualitative antimicrobial activity test that is efficient for activity guided separation of components (Hostettmann *et al*., 2000). Bioautography combines thin layer chromatography with bioassay in situ and allows the localisation of the active compounds in plant extracts when bacteria or fungi are sprayed on developed TLC plates, from which the eluents have been removed and incubated for a specific duration in humid chambers. Zones of inhibitions are visualised by the detection of dehydrogenase activity with a tetrazolium salt (Shai *et al*., 2008a).

4.5.1 Material and methods

Qualitative analyses of the number of antibacterial compounds were determined by the bioautography method described by Begue and Kline (1972). Exactly 100 µg of each plant extract was loaded at the baseline (1 cm wide) of a TLC plate and developed in the three mobile systems as described in section 5.3.1. TLC plates were dried at room temperature to evaporate the CEF, EMW and BEA solvents for two days. Bacterial cultures which were grown overnight in the Tryptone soy broth in an incubator at 37^{o}C were centrifuged at 3000xrpm for 10 min. The pellets were resuspended in a 10 ml of fresh broth. The dried developed plates were sprayed with the fresh bacterial cultures until completely moist using a spray gun. The moist plates were incubated at 37^{o}C in a humidified atmosphere for 18 hours. The plates were sprayed with 2 mg/ml aqueous solution of β-iodotetrazolium violet (INT from Sigma) and

© University of Pretoria

incubated for 40 min to 2 hrs depending on the organism. Bacterial growth led to an emergence of a pink-red colour from the reduction of INT into a formazan. Clear zones indicate the inhibition of the bacterial growth by the compounds present in the extract.

4.5.2 Results

Bioautography

Acetone, methanol and water extracts of the leaves, bark and roots were tested for antibacterial activity using the bioautography method. The berries were left out because they did not show effective activity during the MIC results. The TLC bioautography's of the acetone crude extracts of the *G. flava* plant parts against the enteric pathogens were determined. The antimicrobial activity was visualised in non-polar separated compounds and the intermitted polar compounds especially against the *B. cereus* and *S.* Typhimurium. Active compounds were localised by the clear zone against the pink-red plate. The compounds were able to dehydrogenase the tetrazolium salt from a pink complex to a clear zone.

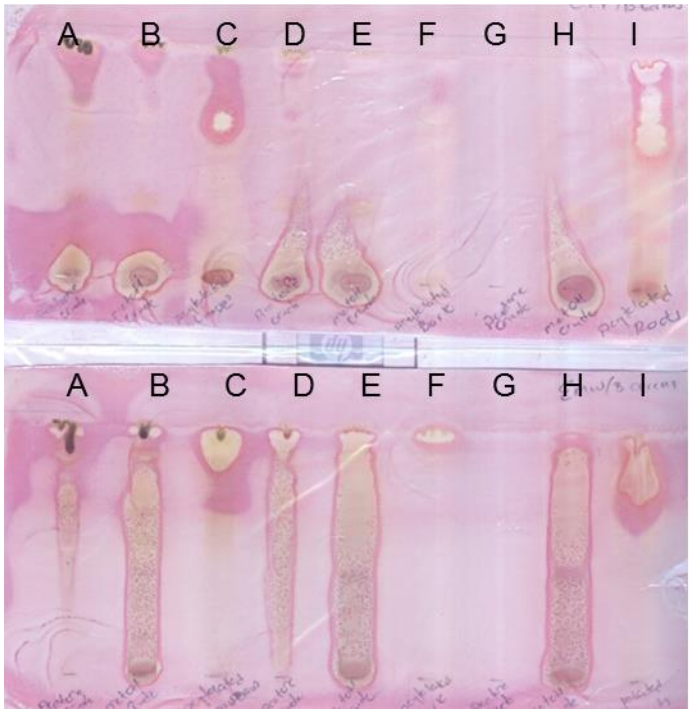


Figure 4.2. TLC bioautograms of the acetone, methanol and acetylated extracts of leaves, bark and roots of *G. flava* developed in CEF (top plate) and EMW (bottom plate) solvent systems and sprayed with *B. cereus*

© University of Pretoria

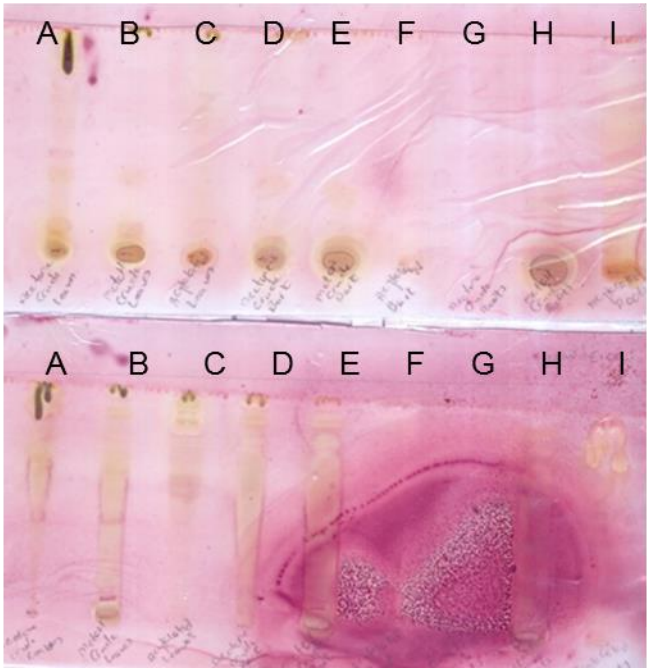


Figure 4.3. TLC bioautograms of the acetone, methanol and acetylated extracts of leaves, bark and roots of *G. flava* developed in CEF (top plate) and EMW (bottom plate) solvent systems and sprayed with *E. coli.*

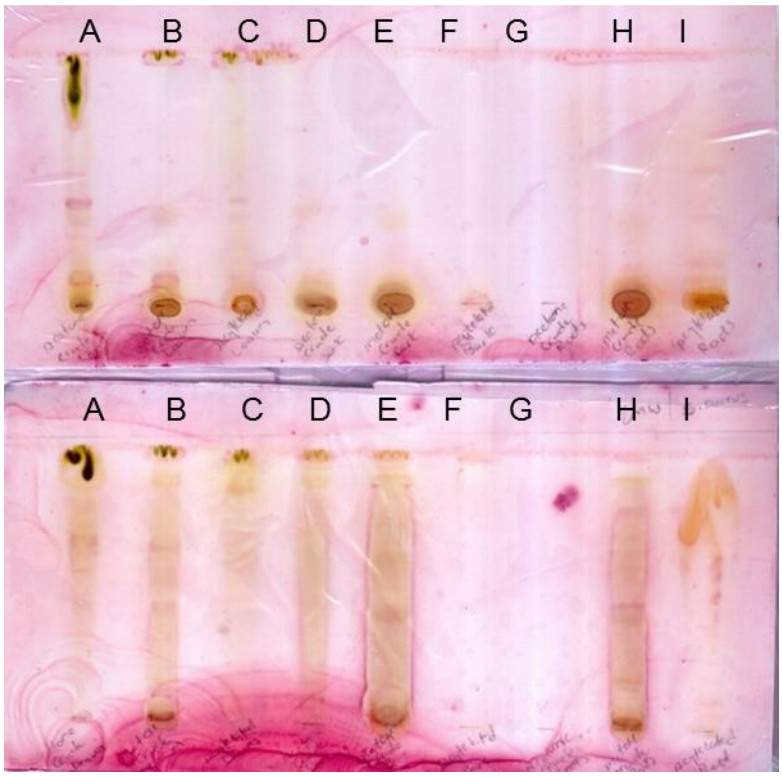


Figure 4.4. TLC bioautograms of the acetone, methanol and acetylated extracts of leaves, bark and roots of *G. flava* developed in CEF (top plate) and EMW (bottom plate) solvent systems and sprayed with *S. aureus*.

© University of Pretoria

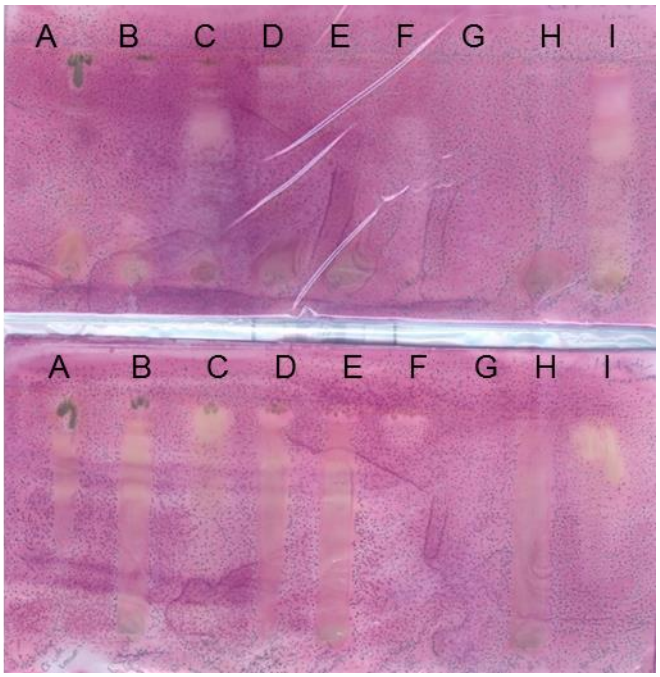


Figure 4.5. TLC bioautograms of the acetone, methanol and acetylated extracts of leaves, bark and roots of *G. flava* developed in EMW (bottom plate) and CEF (top plate) solvent systems and sprayed with *S.* Typhimurium.

4.6 Conclusion

The extracts showed the activity against the tested microorganisms as they were able to reduce the INT into a formazan, a yellow to clear zone on the TLC plates. Most of the clear zones were visible against the *B. cereus* and the *S.* Typhimurium.

© University of Pretoria

CHAPTER 5

ANTIOXIDANT ACTIVITY

5.1 Free radical scavenging activity of the extracts

There are several standardised methods for analysing the antioxidant potential of a substrate, including plant extracts and isolated compounds from the plant extracts. The method includes the measuring of the chemical process occurring in a potential application and using a biological relevant molecule or a stable free radical. The assays are mostly based on the scavenging ability against specific biological reactive oxygen species (ROS) or reactive nitrogen species (RNS), against the stable non-biological radicals and evaluation of the total reduction capacity such as: the 2.,2 diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay (Brand-Williams *et al*., 1995), 2, 2-azinobis-ethylbenthiazoline-6-sulphonate (ABTS) radical scavenging method (Re *et al*., 1999), Ferric reducing antioxidant power (FRAP) described by Benzie and Szeto (1999), Hydrogen peroxide scavenging activity (Ruch *et al.*, 1989) and other methods for evaluating antioxidants activity.

5.1.1 Qualitative analysis of free radical scavenging activity

For the qualitative detection of free radical scavengers and a number of antioxidant compounds present, DPPH or β-carotene is usually the spray reagents (Martson, 2011) onto TLC chromatograms. DPPH, a purple stable free radical complex, is reduced to a yellow complex by the antioxidant components of the extracts. The yellow spots on the TLC plates sprayed with DPPH (Sigma-Aldrich) solution, against the purple background indicates the presence of the active antioxidant compounds.

5.1.2 Quantitative analysis of free radical scavenging activity

The DPPH radical scavenging assay is a hydrogen atom transfer process mostly used to evaluate the antioxidant activity of the reductants; plant extract, phytochemicals or pharmaceutical drug (Kaviarasan *et al*., 2007). DPPH has no direct biological relevance; the process is related to inhibition of the lipid peroxide (Rekka and Kourounakis, 1991). The DPPH stable free radical, is reduced to the corresponding hydrazine when it reacts with the hydrogen donor (antioxidant) using a decolourisation mechanism (from purple to yellow), which are

© University of Pretoria

monitored by the decrease in absorbance at 515–528nm. The assay conducted with a methanol DPPH solution is considered a valid, easy and accurate assay to evaluate the radical scavenging potential of the antioxidants, since the radical compounds are stable and do not have to be generated in another assay.

5.2 Objective

Objective of this part of the study was to:

Determine the antioxidant activity of different *G. flava* plant parts against the stable free radical 2,2-1 diphenyl-2-picrylhydrazine (DPPH) qualitatively and quantitatively.

5.3 Materials and methods

5.3.1 Qualitative analysis of the antioxidant activity and extract compound profiling

The TLC-DPPH method and TLC-vanillin method of Masoko and Eloff (2007) was used. The driedacetone and water extracts were weighed and the mass of the dried residues recorded. Ten milligrams (10 mg) of the dried extract residue was weighed and a 10 mg/ml concentration extract solution was prepared. Each extract (10 µl) was loaded on the base of the thin layer chromatography silica gel 60 F_{254} aluminium backed plate (Merck). Different mobile systems were used for separation of the compounds namely: Ethyl acetate: methanol: water (EMW, polar system); chloroform: ethyl acetate: formic acid (CEF, intermediate system); and benzene: ethanol: acetic acid (BEA, non-polar system). The plates were sprayed with 0.2% DPPH in methanol to determine the free radical scavenging activity. Other plates were sprayed with vanillin in sulphuric acid (0.1 g vanillin in 28 ml methanol and 1 ml H_2SO_4) for visualising different compounds in the extracts.

5.3.2 Determination of the potential scavenging activity

The antioxidant activities of the samples were determined by the radical scavenging ability using the stable free radical DPPH, as described by Brand-Williams *et al*., (1995). Acetone and water extracts (100 µl) of the samples of different concentrations (2.5–0.01875 mg/ml), and positive controls (trolox and ascorbic acid) at different concentrations (0.5–0.375 µg/ml) were added to 100 µl of DPPH dissolved in methanol (0.2 mM) in 96-well microtitre plates.

© University of Pretoria

The change in absorbance (517 nm) was measured after 30 min incubation in the dark, with a microtitre plate reader with a 1 cm path length. The results were expressed as the percentage of the inhibition/scavenging activity.

5.4 Results

TLC fingerprinting

The TLC profiling of the crude extracts of the *Grewia flava* plant parts investigated are presented in Figures 5.2–5.5. Figure 5.1 is the TLC fingerprinting of acetone and water extracts developed in a BEA mobile system, a non-polar system that separates the non-polar compounds within the extracts.

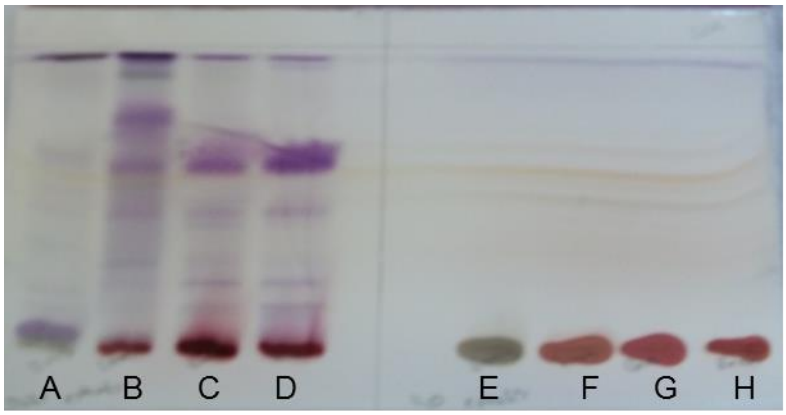


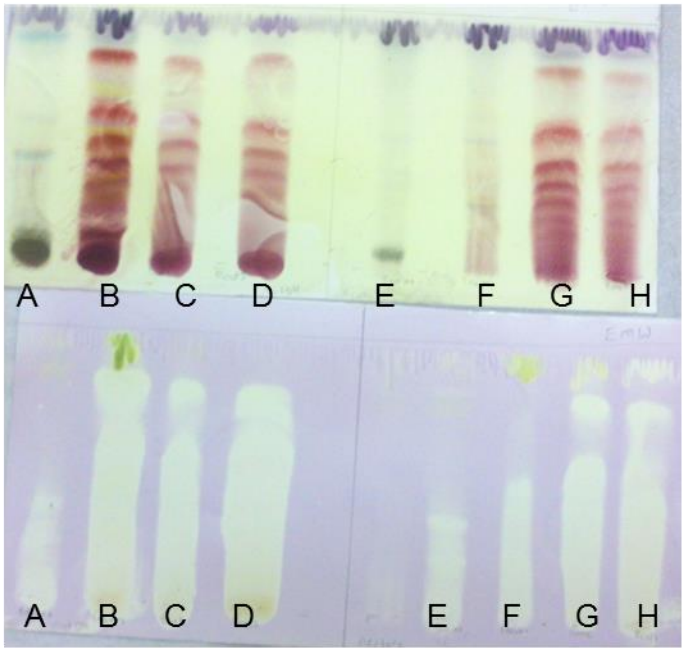
Figure 5.1. TLC chgromatogram of the acetone and water extracts of the berries, leaves, bark and roots acetone and water extracts developed in the BEA system and sprayed with vanillin in –sulphuric acid.

The chromatogram reveals a mixture of compounds where the system separated the non-polar compounds finely, which exhibited different colours when reacting to the Vanillin/H_2SO_4 spray reagent. The classes of compounds extracted include the terpenoids, which are purple or bluish purple (Taganna *et al*. 2011).

The qualitative antioxidants screening of spraying DPPH on TLC plates indicates the presence of antioxidant compounds in the crude extracts. The antioxidant compounds are visualised as yellow spots against the purple background of DPPH, as shown in Figure 5.2 and 5.3. The number of antioxidant compounds identifiable depends on the mobile system used for separation. Most of the antioxidant compounds in this species are phenolics and are polar, and are better separated with an intermediate mobile system and a polar mobile system, in this case the EMW. When reacting with vanillin in H_2SO_4, most of the compounds colour red or pink-red. Most of the compounds in the extracts of the leaves, bark and roots are highly

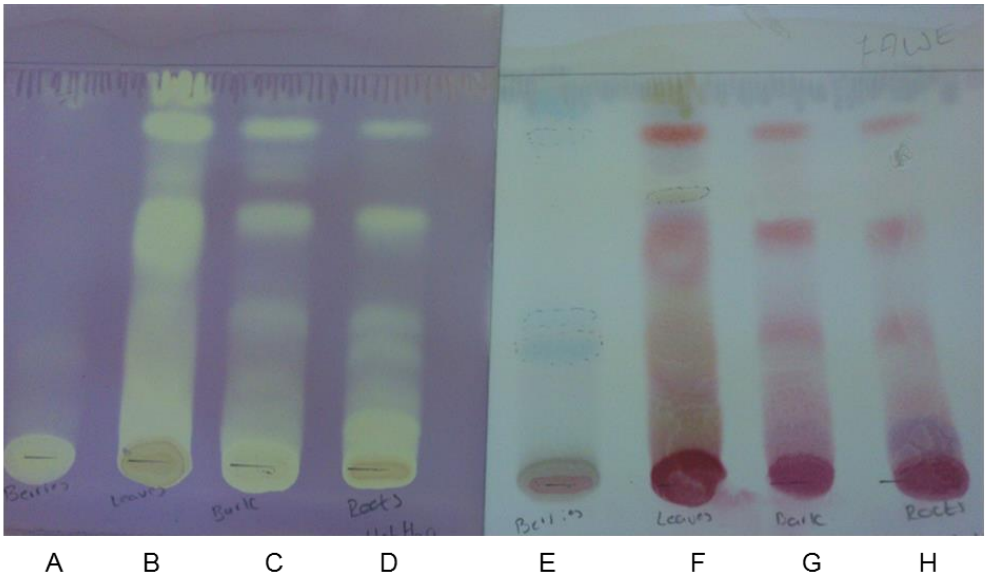
© University of Pretoria

polar compounds, probably phenolic, as they adhere to the plate because of the bond formation between the free electrons on the surface of the compounds and the silica gel on the plate. This strong bond prevents the even separation of the compounds, but causes a smear on the plate (the plate developed in the EMW mobile system Figure 5.2). The FAWE system, which is a highly polar system, was used to separate the water extracts, because water is a highly polar solvent, most of the compounds extracted are polar to highly polar compounds. The water extracts exhibited mainly antioxidant compounds when the plates were sprayed with DPPH, as the compounds were able to reduce the purple DPPH component into yellow bands, where the antioxidant potential compounds were localised (Figure 5.2 below).



A Acetone extract of berries
B Acetone extract of leaves
C Acetone extract of bark
D Acetone extract of roots
E Water extract of berries
F Water extract of leaves
G Water extract of bark
H Water extract of roots

Figure 5.2. TLC chromatogram of the berries, leaves, bark and roots extracts developed in EMW system and sprayed with vanillin-–H_2SO_4 (top plate) and DPPH (bottom plates)



A Water extract of berries
B Water extract of leaves
C Water extract of bark
D Water extract of roots

E Water extract of berries
F Water extract of leaves
G Water extract of bark
H Water extract of roots

© University of Pretoria

Figure 5.3. TLC chromatogram of the water extracts of the berries, leaves, bark and roots developed in FAWE system and sprayed with DPPH (left plate) and vanillin-H_2SO_4 (right plate).

5.4.2 Quantitative analysis of the scavenging ability

The phenolic-enriched crude extracts exhibited strong radical scavenging activity against the DPPH radical in a dose manner (Table 5.1 and Figure 5.1–5.3). The berries extract had a high anti-DPPH radical activity compared to the leaves, bark and roots, with the latter showing the effective potential of being a good scavenger for the stable free radical DPPH, 0.17 mg/ml. The leaf extracts had similar activity with the positive control Ascorbic acid, 2.2 mg/ml (leaves) and 2.8 mg/ml (ascorbic acid).

Table 5.3. IC_{50} (mg/m) of the acetone extracts against the stable free radical 0.2 mM DPPH.

Plant parts	**IC_{50} (mg/ml)**
Berries	6.2
Leaves	2.2
Bark	0.6
Roots	0.17
Vitamin C	2.8

IC_{50} = inhibition concentration at 50% DPPH
Ascorbic acid (Vitamin C) was used as a positive control

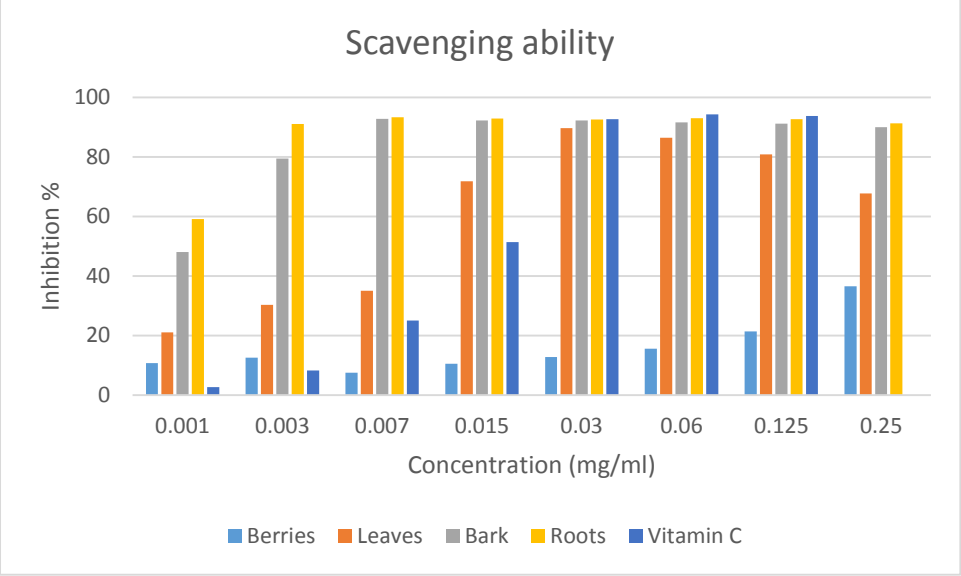
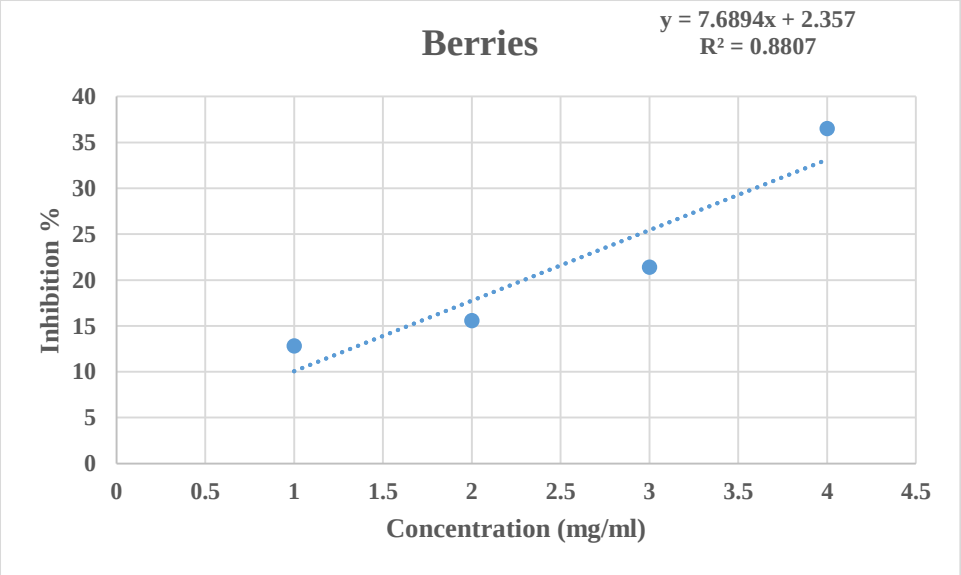


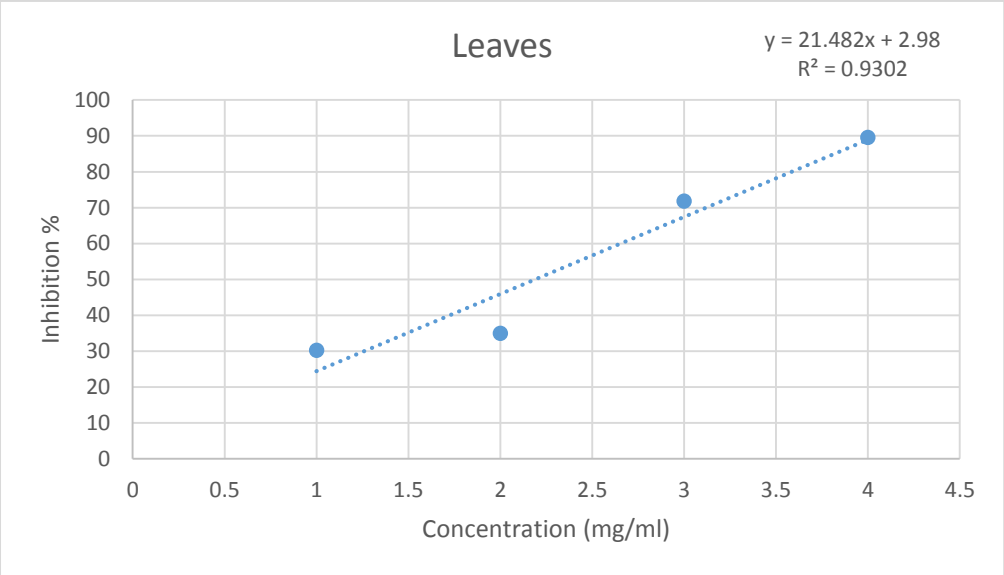
Figure 5.4. IC_{50} at different concentrations of the acetone extracts of berries, leaves, bark and roots on the stable free radical DPPH.

© University of Pretoria



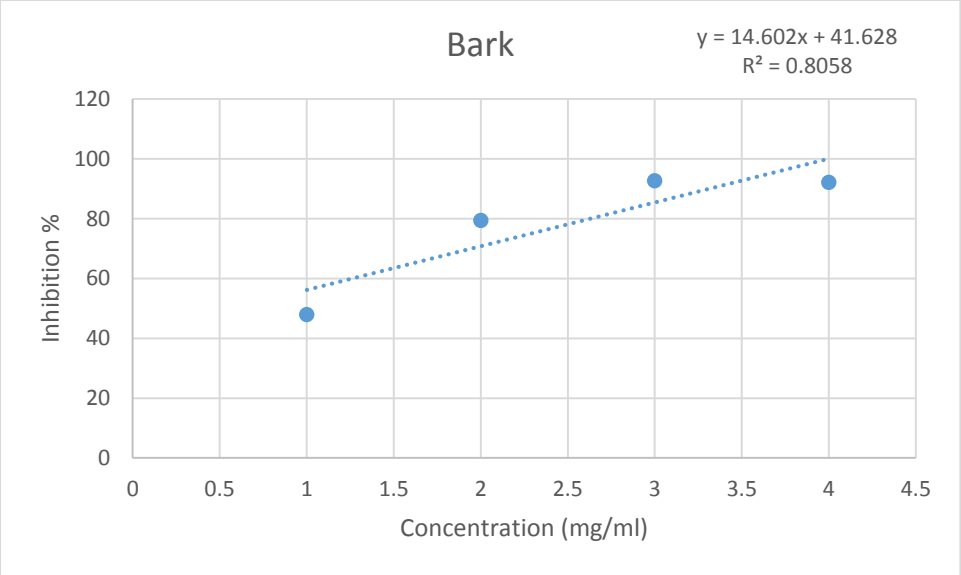
Figures 5.5. Inhibition percentage graph of the *Grewia flava* plant parts: berries extracted with acetone on the stable free radical DPPH

.



Figures 5.6. Inhibition percentage graph of the *Grewia flava* plant parts: leaves extracted with acetone on the stable free radical DPPH.

© University of Pretoria



Figures 5.7. Inhibition percentage graph of the *Grewia flava* plant parts: bark extracted with acetone on the stable free radical DPPH.

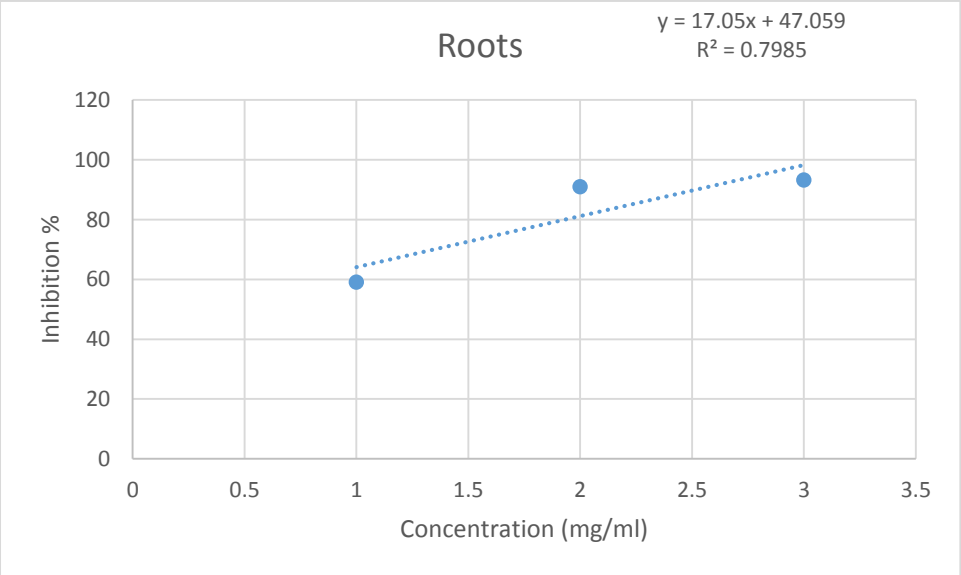


Figure 5.8. Inhibition percentage graph of the *Grewia flava* plant parts: roots extracted with acetone on the stable free radical DPPH.

5.5 Conclusion

Water extracts exhibited antioxidant active compounds, hence the water extracts might not be active against the enteric pathogens, but they are still good to isolate antioxidant compounds from them, with most of the compounds being phenolics rather than the essential oils or flavonoids. The berries are sweet when ripe and fleshier, this means the high carbohydrate content in the berries make it suitable to make the alcoholic drink “Khadi”, in which the sugars

© University of Pretoria

in the berries are fermented. In crude extracts, compounds work in a synergistic form as there are various compounds that protect the plants against different environmental factors. Some of the compounds are there to fight pathogens that infect the plant, other compounds fight the parasites that will use the plant for their survival and other compounds are there for the survival of the plant. The extracts showed a range of compounds, because acetone is able to extract both the non-polar compounds to polar compounds, and the compounds showed antioxidant activity against a stable free radical DPPH qualitatively and quantitatively. Roots exhibited high antioxidant active compounds (TLC plates) and it had the lowest LC_{50} (LC_{50} is the lethal concentration required to kill 50% of the population) against the DPPH, compared to the positive control Ascorbic acid, 0.17 mg/ml compared to 2.81 mg/ml respectively. The leaf extract also exhibited most of the antioxidant active compounds (TLC-DPPH sprayed plates) with a LC_{50} of 2.19 mg/ml.

© University of Pretoria

CHAPTER 6

CYTOTOXICITY

Medicinal plants are assumed to be non-toxic and regarded safe due to their natural origin and the long use in traditional medicine to treat various forms of diseases (Chen *et al*. 2011, Fennell *et al*. 2004). Medicinal plant preparations are administered with the hope of promoting health and treating various diseases such as infections, cold, inflammation, diarrhoea and depression. Scientific studies on efficacy and safety of some of the medicinal plants indicated that there are many phytochemicals that have cytotoxicity, genotocixity and carcinogenic effects when used chronically (Ernest 2004, Rietjens *et al*. 2005).

6.1 Objective

Objective of this part of the study was to:

Test the toxicity of the crude extracts of *G. flava* against the Vero monkey kidney cells using the MTT assay (3-(4.5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide).

6.2 Materials and Methods

The cytotoxicity of the extracts was determined against Vero monkey kidney cells using the MTT reduction assay as described by Mosman (1983), with slight modification. Cells were seeded at a density of 1×10^5 cells/ml (100 µl) in a 96-well microtitre plate and incubated at 37°C and 5% CO_2 in a humidified environment. After 24 hrs incubation, the leaf extracts (100 µl) at different concentrations were added to the wells containing the cells. Doxorubicin was used as a positive control. A suitable blank control with the equivalent concentration of acetone was also included and the plates were further incubated for 48 hrs in a CO_2 incubator. Thereafter, the medium in each well was aspirated, cells were then washed with PBS, and finally fresh medium (200 µl) was added to each well. Then 30 µl of MTT (5 mg/ml in PBS) was added to each well and the plates were incubated at 37°C for 4 hrs. The medium was aspirated from the wells and DMSO was added to solubilise the formed formazan crystals. The absorbance was measured on a Biotek Synergy microplate reader at 570 nm. Cell growth inhibition for each extract was expressed in terms of LC_{50} values, defined as the concentration

© University of Pretoria

that causes 50% inhibition of cell viability. The selectivity index (SI) values were calculated by dividing the cytotoxicity LC_{50} values by the MIC values (SI = LC_{50}/MIC). Tests were carried out in quadruplicate and each experiment was repeated three times.

6.3 Results

Cytotoxicity

The cytotoxicity was determined using the *in vitro* assay with Vero monkey kidney cells. The LC_{50} values and the selectivity index were calculated (Table 6.1). The LC_{50} values ranges between 452.69 ± 33.02 to 651.77 ± 23.44 µg/ml, while the SI was as low as 0.18 to 18.39. The acetylated leaf extract had the highest LC_{50} (lowest toxicity) of 651.77 ± 23.44. The results indicate that the extracts of *Grewia flava* are less toxic to Vero cells than the positive control doxorubicin.

Table 6.1. LC_{50} values (µg/ml) of *Grewia flava* leaf extracts against the Vero cells and Doxorubicin was used as a positive control

Extracts	**LC_{50} (µg/ml) Mean ±SD**	***Selectivity index (LC_{50}/MIC)***			
		B. cereus	***E. coli***	***S. aureus***	***S.* Typhimurium**
Acetylated	651.77 ± 23.44	0.54	2.17	1.09	0.52
Acetone	507.21 ± 2.55	0.84	7.25	7.25	3.38
Methanol	551.68 ± 2.65	7.88	7.88	18.39	18.39
Water	452.69 ± 33.02	0.18	1.51	1.51	0.36
Doxorubicin	3.02 ± 0,03	Nd	Nd	Nd	Nd

Nd = not determined

6.4 Conclusion

For the plant to survive, it needs strong and healthy roots, because minerals and water are absorbed from the soil through the roots and the healthy leaves are responsible for absorption of light. *Grewia flava* has a tap root system. In order to obtain enough roots for the experiment, many plants had to be uprooted. Hence leaves were the best plant parts to use.

Selective activity of the compounds was calculated as follows:

© University of Pretoria

Selectivity index (SI) = LC_{50} against mouse fibroblast cells / MIC in same units

The selectivity index is calculated to determine the relationship of activity of a test product to its cytotoxic concentration, the higher the number the safer the product.

The water extract of the leaves was found to be less toxic against the Vero monkey kidney cells with a LC_{50} of 452.69 ± 33.02. Acetone extracts of the leaves showed less toxicity against the Vero cells with a LC_{50} of 507.21 ± 2.55. The higher LC_{50} means lower toxicity. The acetone extracts was chosen because of its low toxicity. The leaves were chosen because they were easy to obtain and in case of this study they showed to be effective against the tested enteric pathogens and the extract was less toxic to the monkey kidney cells. It can be concluded that the indigenous edible plant *Grewia flava* is not toxic and the compounds in the plant are good to combat enteric pathogens. The next step was to fractionate the acetone extract of the leaves and isolate the bioactive compound(s).

© University of Pretoria

CHAPTER 7

SOLVENT-SOLVENT FRACTIONATION OF THE ACETONE LEAF EXTRACT OF *Grewia flava*

Plants contain different secondary metabolites and their site of synthesis is mostly restricted to a single plant part. The active chemical compounds may be stored in different parts based on the type and purpose of the compound. The compound composition may vary in leaves, bark or roots; hence the whole plant may not be used for medicinal purposes. Primary metabolites use different mechanisms against the outside factors that affect the plant whereas the secondary metabolites act as volatile attractants to promote pollination by birds and also for their nutritional role (Wink, 2004).

Antibacterial compounds are localised in different plant parts. Plant extracts are screened qualitatively by TLC or antimicrobial assay to determine the biological activity. For purification and isolation, extracts are sequentially fractionated (Verpoorte, 1989), and this strategy is bioassay-guided fractionation. Chromatographic techniques that are used to isolate compounds include column chromatography, which uses silica gel as a stationary phase and compounds are eluted with solvents at different polarity. High performance liquid chromatography and gas chromatography are the techniques used to detect compounds even at the lowest level of concentration.

7.1 Objectives

Objectives for this part of the study were to:

1. Obtain the fractions from the dried acetone extract of the *G. flava* leaves by solvent-solvent fractionation.
2. Determine the minimal inhibitory concentration of the obtained fractions against the enteric pathogens using the micro dilution plate assay.
3. Determine the cytotoxicity of the obtained fractions against the Vero cells using the MTT reduction assay.

© University of Pretoria

7.2 Materials and methods

7.2.1 Plant material collection

The plant materials were collected in the field at Moletjie Ga-Phago Village, Limpopo, South Africa, in January 2014. The plant materials were harvested and allowed to dry at room temperature and pulverized using a small desk top grinder.

7.2.2 Extraction and fractionation

Powdered leaf material (211 g) of *G. flava* was extracted with 2L of acetone shaken vigorouslyusing a shaker. Supernatant was filtered through Whatman No. 1 filter paper, the solvent was evaporated using a Büchi Rotavapor R-114 (Labotec) at 80 rpm, 50°C. Concentrated extract was dried in a pre-weighed honey jar under a stream of cool air.

Solvent-solvent fractionation

Solvent-solvent partition was carried out to fractionate the acetone extract based on the polarity of the compounds (Suffness and Dourous, 1979). The extract was reconstituted in 300 ml of chloroform and mixed with an equal volume of distilled water (dH_2O) in a separating funnel to give a chloroform fraction. The water fraction was mixed with an equal volume of n-butanol, to give the water and butanol fraction. The chloroform fraction was dried in a vacuum rotary evaporator and extracted with an equal volume of hexane and 30% water in methanol, to yield the hexane and 30% water fraction. The 30% water in methanol fraction was further diluted to 35% water in methanol and mixed with chloroform to yield the 35% water in methanol and the chloroform fraction. A total of five fractions were collected: hexane, butanol, water, 35% water in methanol, and a chloroform fraction.

TLC fingerprinting

The dried fractions were reconstituted to 10 mg/ml, and then 10 µl was loaded onto the baseline of the TLC plates. The plates were developed in a BEA solvent system. The plates were dried and vanillin-sulphuric acid was sprayed on the plates. The plates were heated to visualise the compounds.

© University of Pretoria

Antimicrobial activity

The serial micro plate dilution method by Eloff (1998), was used to determine the minimal inhibitory concentration (MIC) for the extracts using β-iodonitrotetrazolium violet (INT) as a growth indicator, as described in 4.4.2.1. In these cases the fractions were tested.

Cytotoxicity assay

The cytotoxicity of the extracts were determined against Vero monkey kidney cells using the MTT reduction assay as described by Mosman (1983), with slight modification as described in section 6.2.

7.3 Results

From the bulk extraction (211.21 g) of the leaf material, 5.05% was extracted (10.66 g). The dried extracts were then fractionated using the solvent-solvent fractionation into 5 fractions. The highest masses were obtained from the chloroform fraction and the least from the water fraction, 8.08 g and 0.175 mg respectively.

© University of Pretoria

Solvent-solvent fractionation

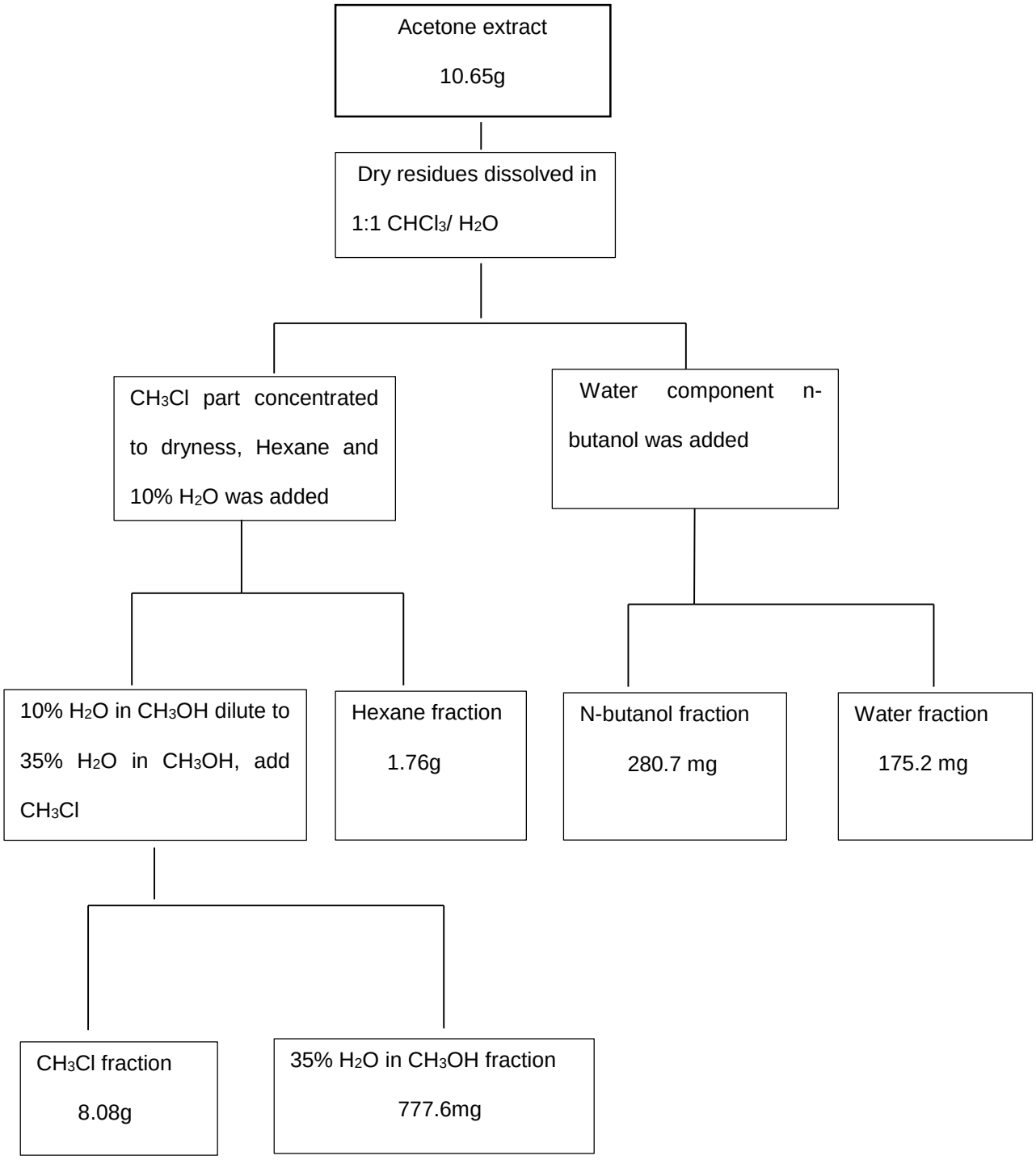


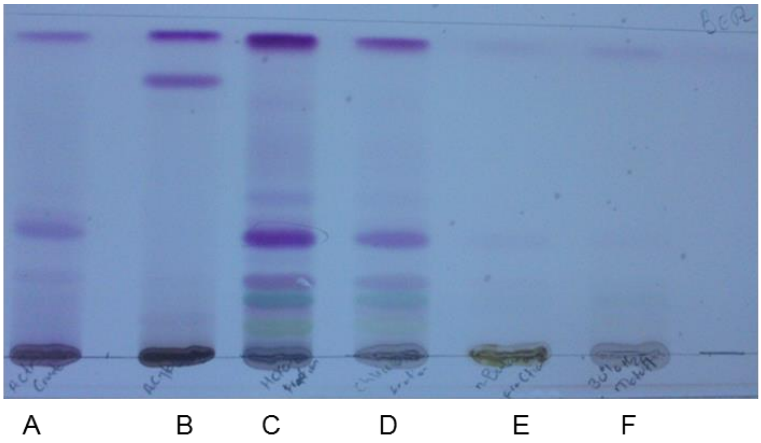
Figure 7.1. Flow chart of solvent-solvent fractionation used to categorise different compounds of *Grewia flava* leaves based on polarity.

© University of Pretoria

7.3.1 Fractions screening

TLC fingerprinting of the fractions

Compounds in the fractions were screened by TLC fingerprinting and compared with the crude acetone extracts. The TLC fingerprinting of the fractions and the crude extract were developed in BEA and CEF solvent system as shown in Figure 7.2. The hexane and chloroform fractions, non-polar fractions separated well in the BEA system whereas the butanol and the 35% water in methanol were separated well in the CEF system. The hexane and chloroform fractions appear to contain similar compounds, with a slight difference in concentration.



A Acetone crude extract of leaves
B Water fraction
C Hexane fraction
D Chloroform fraction
E n-Butanol fraction
F 35% water in methanol fraction

Figure 7.2. TLC chromatogram of the fractions developed in BEA system and sprayed with vanillin–sulphuric acid.

Minimum inhibitory concentration (MIC)

Fractions showed activity against the tested enteric pathogens with inhibitions activity lower than 1 mg/ml observed from the chloroform fractions (Table 7.1). The hexane fraction had the lowest MIC against *B. cereus* at 0.07 mg/ml. Gentamycin was used as a positive control. The MIC value of hexane and chloroform was higher than that of the crude extract beside that of *B. cereus* which was lower and the *S.* Typhimurium that was the same, in the case with chloroform fraction. The difference in the activity may be due to the synergistic activity of the compounds present together in the crude extract, but separated in the fractions.

Table 7.1. MIC (mg/ml) of the fractions against the enteric pathogens

© University of Pretoria

Fraction	MIC (mg/ml)				
	B. cereus	*E. coli*	*E. faecalis*	*S. aureus*	*S.* Typhimurium
Hexane	0.07	0.15	0.15	0.3125	1.25
Chloroform	0.15	0.15	0.3125	0.3125	0.15
Butanol	1.25	0.625	0.625	0.625	1.25
30% water in methanol	0.625	1.25	0.15	0.3125	0.3125
Gentamycin	0.01	0.07	0.07	0.03	0.03

Total activity

Determining the total activity of the fractions indicates the loss or gain of activity during fractionation may be detected. Total activity takes the MIC and the quantity present in the fractions. The total activity in fractions is used to determine the loss or gain of biological activity and to ascertain the presence of synergistic effects (Eloff 2004). The total activity of the five fractions against *B. cereus*, *E. coli*, *E. faecalis*, *S. aureus* and *S.* Typhimurium are presented in Table 7.2 below. The chloroform fraction had the highest total activity followed by hexane, 35% water in methanol and butanol with the least values. Total activity was calculated as extracted quantity/MIC.

Table 7.2. Total activity of the fractions against the tested enteric pathogens

Fractions	Total activity				
	B. cereus	*E. coli*	*E. faecalis*	*S. aureus*	*S.* Typhimurium
Hexane	25143	11733	11733	5867	1408
Chloroform	53867	53867	26933	26933	53867
Butanol	225	468	468	468	225
30% H2O:MetOH	1296	622	5184	2592	2592

Cytotoxicity

The cytotoxicity of the fractions obtained was determined against Vero monkey kidney cells using the MTT assay and Doxo was used as the positive control. The results are presented in Table 7.3. LC_{50} above 50 is considered less toxic, hence the Doxo, positive control was found to be toxic with a LC_{50} of 3.02 µg/ml. Butanol had the highest LC_{50} of 892.68 µg/ml. Selectivity index, is the ratio of the efficacy against the pathogen to the toxicity to the cells. It shows the degree at which the observed activity of a substance attributes to the toxicity of the substance. High SI value indicates good efficacy and low toxicity. A selectivity index value lower than 1 indicates that the treatment is more harmful to the cells than to the pathogen. The selectivity index was calculated by dividing the LC_{50} with the MIC. A high SI was observed from the

© University of Pretoria

hexane fraction against *B. cereus* (5.04), with the lowest SI from the 35% water in methanol fraction (0.20) against *E. coli.*

Table 7.3. LC_{50} values (µg/ml) of *Grewia flava* leaf extracts against the Vero monkey kidney cells

Extracts	LC_{50} (µg/ml)	SI (LC_{50}/MIC)				
		B. cereus	*E. coli*	*E. faecalis*	*S. aureus*	*S.* Typhimurium
Hexane	353.06±185.41	5.04	2.35	2.35	1.18	0.29
Chloroform	509.23 ± 24.88	3.39	3.39	1.7	1.7	3.39
Butanol	892.68 ± 53.15	0.74	1.49	1.49	1.49	0.74
35% H2O:MetOH	240.63 ± 53.74	0.40	0.20	1.60	0.80	0.80
Doxo	3.02 ± 0.03	Nd	Nd	Nd	Nd	Nd

7.4 Conclusion

The hexane and chloroform fractions contained similar compounds. From the TLC chromatogram, both the fractions were non-polar and most of the grouped compounds are non-polar. The MIC values for the hexane and chloroform fractions were below 1 mg/ml, which is considered an effective activity, except in the case of the hexane fraction against the *S.* Typhimurium. The chloroform fraction had the highest total activity compared to the other four fractions followed by the hexane fraction. All five fractions were shown to be less toxic or not toxic to the Vero cells, because the LC_{50} were above 100 µg/ml. The butanol fraction had the highest LC_{50}, but low selectivity indices with an SI value below 1 (0.74) against *B. cereus* and *S.* Typhimurium. Among the five fractions, the chloroform fraction had the highest yield, and was found to be effective against the tested bacteria, and it was also found to be less toxic. The next step is to isolate the active compound from the chloroform fraction using column chromatography.

© University of Pretoria

CHAPTER 8

BIOASSAY-GUIDED ISOLATION OF THE ACTIVE COMPOUND(S) FROM THE ACETONE LEAF EXTRACT OF G*rewia flava* BY COLUMN CHROMATOGRAPHY

8.1 Introduction

One of the important objectives in medicinal plant research and development is isolation, identification and characterisation of the bioactive components. Medicinal plant extracts are inherently a complex mixture of different chemical components. Separation of the active components of plant phytochemicals from inactive components are categorised into four parts namely: extraction, fractionation, chromatography and purification. Different chromatographic methods can be used for qualitative (TLC, HPLC) and for isolation open column chromatography, vacuum liquid chromatography; gas chromatography techniques can be used. The principles of separation are based on the molecular size, adsorption to the stationary phase, polarity and solubility in the mobile phase.

8.2 Column chromatography

Isolation of compounds is carried out by open column chromatography under gravitational force using silica gel, sephadex, polyamides or reverse phase mode on C8 or C18 bonded silica gel as a stationary phase. The separation of individual compounds from the complex extract mixture is based on the compound characteristics; the ability to bind to the stationary phase in the column, relative to the polarity of the mobile phase. The changing of polarity (gradient elution) of the mobile phase allows targeted compounds to be eluted in a sequential manner. The chromatographic process should be fast, not lead to compound decomposition, material loss or formation of artefacts. The method is cheap, simple and universally practised despite obvious disadvantages of being slow and often produces irreversible adsorption (Shahid, 2012).

© University of Pretoria

8.3 Genus *Grewia*

The genus *Grewia,* family Tiliaceae, is a major angiosperm group (flowering plants) with approximately 150 species of flowering plants, shrubs and trees that are widely distributed in subtropical and tropical regions depending on the species, including tropical Africa, Arabia, Madagascar, the Himalaya, India, Pakistan, Bangladesh, Thailand and Malaysia (Wali *et al*., 2012). *Grewia* species are rapid growers preferring rich, well-drained soil and full sun to part shade. Some species attract birds and butterflies and can be a good choice for a garden designed to attract animal visitors. Species producing edible fruits can be cultivated for culinary, as well as decorative uses. A number of species of the genus *Grewia* have been used as medicinal agents to treat several diseases such as skin disease, hypertension, ulcers and diarrhoea and also for the production of food (Bowden 1978, Raghunath 1996, Aratanechemuge *et al.* 2004; Juvekar *et al*. 2007).

The *Grewia flava*, commonly known as “brandy bush” or “velvet raisin” (English), “fluweelrosyntjie/ wilderosyntjie” (Afrikaans) and “moretlwa” (Tswana and Sepedi), is a shrub of up to 2m tall that is widely distributed. The bark is grey-brown to black and the leaves are greyish-green with fine hairs on the upper side and hairier and lighter green on the underside. The flowers are yellow and star-shaped and the berries are orange-brown of a medium size, and ripen from February until early April. Currently there is no published work on the medicinal value of the species.

8.4 Chemical constituents in *Grewia* species

Compounds with different activities were isolated from different *Grewia* species. More than 30 compounds have been isolated and identified. The first phytochemical research on *Grewia* was done in 1965 and Friedelin, a pentacyclic triterpenoid compound, from *G. tiliafolia,* was reported and it was also confirmed in *G. boloba (*Anjaneyulu *et al.* 1965, Ahmed *et al.,* 2009). A total of nine triterpenoids have been reported from different *Grewia* species including Lupeol, an antimalarial compound from *G. tiliafolia.* Three alkaloids from *G. bicolor* were identified (Harman, 6-Methoxyharman and 6-Hydroxyharman) and found to possess antibacterial activity (Bowden, 1978). From the methanolic extract of *G. damine* two flavones were isolated and identified as vetexin and isovetexin, which are glycosides (Jayasinghe *et al*., 2004). Among the isolated and identified compounds there are sterols, anthocyanin, coumarinolignans and the neolignans (Nair *et al.*, 2005; Ma *et al.,* 2006).

© University of Pretoria

8.5 Objective:

The objective of this part of the study was to isolate the active antimicrobial compounds obtained from the chloroform fraction.

8.6 Materials and Methods

8.6.1 First column Chromatography

Column chromatography using silica gel (Merck, Silica Gel 60) as a stationary phase was chosen for separation of compounds. About 400 g of silica was mixed with 100% hexane to form slurry and stirred using a stirring rod. The slurry was poured into a column (30 cm long and 2.5 cm wide) with the vent clogged with cotton wool; the hexane was allowed to flow out to allow the gel to set. The dried 8 g of chloroform fraction was mixed with 16 g of silica and dissolved with 20 ml of hexane. The mixture was allowed to dry completely under a stream of cold air before it was layered on the bed of the set gel in the column. Firstly, 100% hexane was gradually added to the column to remove any waxes and fats. Then the polarity was increased by addition of ethyl acetate at an interval of 5% until 100% ethyl acetate was reached.

A total of 125 fractions were collected in honey jars, and were allowed to concentrate under a stream of cold air. Fractions containing similar constituents were combined and monitored by TLC fingerprinting. The fractions were reduced to nine fractions and labelled one to nine.

8.6.2 TLC fingerprinting

The dried fractions were reconstituted to 10 mg/ml, and then 10 µl was loaded onto the baseline of the TLC plate; the plates were developed in a BEA solvent system. The plates were dried and vanillin-sulphuric acid was sprayed on the plates. The plates were heated to visualise the compounds.

© University of Pretoria

8.6.3 Antimicrobial activity

Minimal inhibitory concentration

The serial micro plate dilution method by Eloff (1998), was used to determine the minimal inhibitory concentration (MIC) for the fractions using þ-iodonitrotetrazolium violet (INT) as a growth indicator, as described in 4.4.2.1 in this case the fractions from the column were tested.

Bioautography

Qualitative analyses of the number of antibacterial compounds were determined by the bioautography method described by Begue and Kline (1972). Exactly 10 µl of the 9 fractions from the first column were loaded at the baseline (1 cm wide) of a TLC plate and developed in the BEA mobile system. TLC plates were dried at room temperature for 3 days to evaporate the solvents. Overnight bacterial cultures, which were grown in the Tryptone soy broth in an incubator at 37ºC, were centrifuged at 3000xrpm for 10 min. The pellets were resuspended in 10 ml of fresh broth. The dried developed plates were sprayed with fresh bacterial cultures until completely moist using a spray gun. The moist plates were incubated at 37ºC in a humidified atmosphere for 18 hours. The plates were sprayed with 2 mg/ml aqueous solution of iodotetrazolium violet (INT from Sigma) and incubated for 40 min–2 hrs depending on the organism. Bacterial growth led to an emergence of a pink-red colour from the reduction of INT into a formazan. Clear zones indicated the inhibition of the bacterial growth by the compounds present in the fraction.

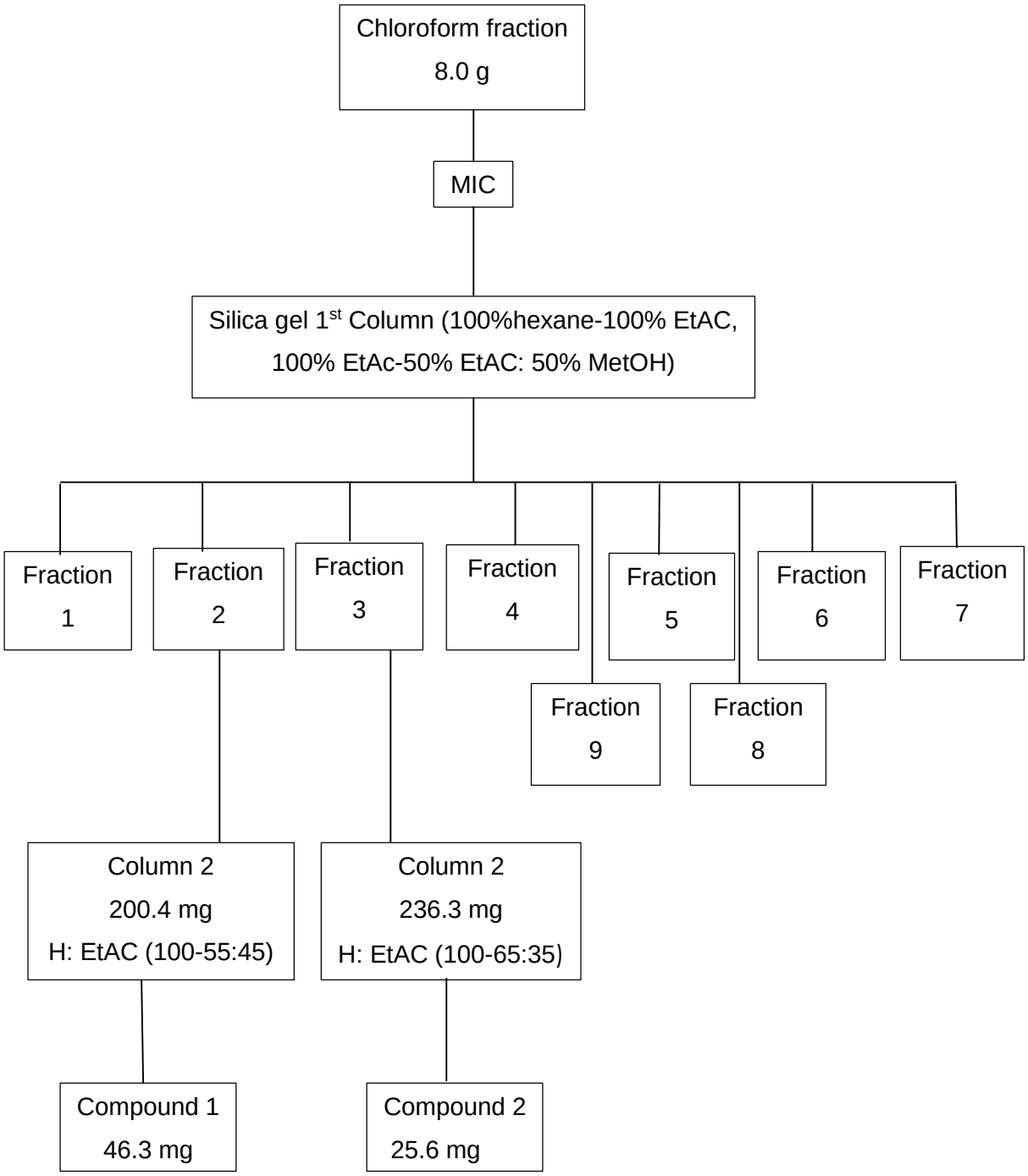
8.6.4 Cytotoxicity assay

The cytotoxicity of the fractions were determined against Vero monkey kidney cells using the MTT reduction assay as described by Mosman (1983), with slight modification as described in Chapter 6, section 6.2.

© University of Pretoria

8.7 Results

8.7.1 Column chromatography



© University of Pretoria

Figure 8.1. Schematic representation of the compound isolated from the *Grewia flava* leaves (monitored by TLC finger printing and bioautography) using column chromatography with silica gel as stationary phase.

8.7.2 Antimicrobial activity

Minimal inhibitory concentration

From the column a total of 9 fractions were obtained and they were tested for antimicrobial activity using the MIC method. Fractions 4, 5 and 9 were the most effective against all tested organisms with an MIC value ranging from 0.15 to 0.6 mg/ml. The MIC considered to be effective in the Phytomedicine Programme laboratory (MIC value <1 mg/ml). Fraction 6 and 7 were less effective against *S*. Typhimurium (MIC 1.25 mg/ml) whereas fraction 3 was less effective against *E. faecalis, S. aureus* and *S*. Typhimurium. Fraction 1, 2 and 8 were less effective against all the tested pathogens with an MIC of 1.25 and >2.5 mg/ml. These results suggest that the antimicrobial activity was due to synergistic effect and the activity is lost with each step of fractionating (Table 8.1).

Table 8.1. MIC of the 1st column fractions and the 2 isolated compounds against the tested enteric pathogens.

	MIC (mg/ml)				
Fractions	*B. cereus*	*E. coli*	*E. faecalis*	*S. aureus*	*S.* Typhimurium
1	>2.5	>2.5	>2.5	>2.5	>2.5
2	>2.5	1.25	>2.5	>2.5	>2.5
3	0.625	0.3125	1.25	1.25	1.25
4	0.3125	0.15	0.625	0.625	0.3125
5	0.3125	0.15	0.15	0.3125	0.625
6	0.3125	0.15	0.625	0.625	1.25
7	0.3125	0.3125	0.625	0.625	1.25
8	1.25	1.25	1.25	1.25	1.25
9	0.15	0.625	0.15	0.625	0.625
C1	1.25	>2.5	1.25	>2.5	>2.5
C2	1.25	>2.5	1.25	>2.5	>2.5
Gentamycin	0.01	0.03	0.03	0.03	0.03

Total activity

Total activity is calculated to determine whether the activity is being potentiated or is lost with each step to isolate the compounds. The results in Table 8.2 suggest that the activity of the

© University of Pretoria

isolated compounds were lost during first column isolation. Fraction 3 from the first column had a high total activity against *B. cereus* and a low activity against *E. faecalis, S. aureus* and *S.* Typhimurium. The results correspond with the MIC results whereby fraction 3 was less effective against the mentioned enteric pathogens. The activity was lost as compared to the total activity of the chloroform fraction against the tested pathogens.

Table 8.2. Total activity of the isolated compounds, 2 fractions from the 1st column and the chloroform fraction against the tested enteric pathogens.

	Total activity (yield/MIC)				
Compound	*B. cereus*	*E. coli*	*E. faecalis*	*S. aureus*	*S.* Typhimurium
C1	37.04	18.52	37.04	18.52	18.52
C2	20.48	10.24	20.48	10.24	10.24
Fraction 2	80.16	160.32	80.16	80.16	80.16
Fraction 3	393.83	787.67	189.04	189.04	189.04
Chloroform	53866.67	53866.67	26933.33	26933.33	53866.67

Bioautography

The antimicrobial activity of the 9 fractions was futher analysed qualitatively with a bioautography assay. The fractions were tested against the four enteric pathogens and they were found to be most effective against *E. coli*, because more zones of inhibition were visible against *E. coli*. The clear zones are formed as a result of the INT reduction by the active compound into a formazan. Guided by the bioautography results, compound 1 and 2 were isolated from fraction 2 and 3 of the first column, respectively.

Figure 8.2. Bioautography of the nine fractions of *Grewia flava* from the 1st column developed in BEA solvent systems and sprayed with *E. coli.*

© University of Pretoria

8.7.3 TLC fingerprinting

Determining the retardation value (R_f) of the isolated compounds, fractions from where the compounds were isolated and the crude extracts assist to understand whether the compound is the same or it was decomposed or created during the step towards the isolation. R_f is calculated as: R_f = solvent front/compound distance. In this case the isolated compound is the same in the crude and the fractions; R_f -1.86 and 2.42 for compound 1 and 2 respectively.

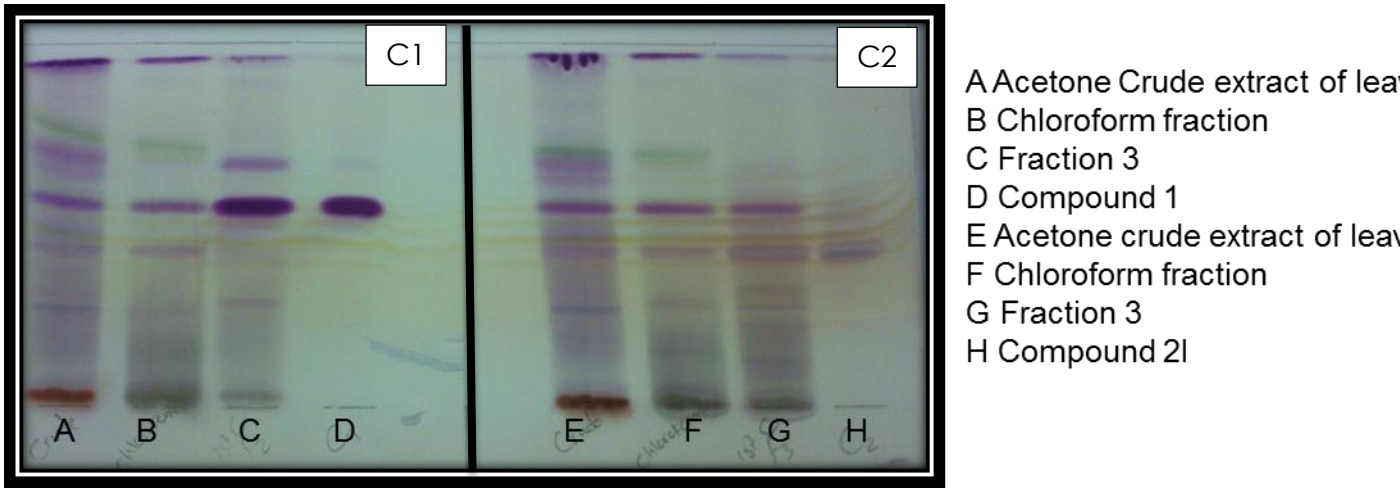


Figure 8.3. TLC chromatogram of the crude, chloroform fraction, fractions 2 and 3 from 1st column and the isolated compounds developed in BEA system and sprayed with vanillin–sulphuric acid

8.7.4 Cytotoxicity

The cytotoxicity of the isolated compound (C1) was determined against the Vero monkey kidney cells using the MTT assay. The results are presented in Figure 8.3–8.5 and Table 8.3 below. Doxorubicin was used as a positive control and it was found to be toxic with a LC_{50} of 3.02 µg/ml. The LC_{50} of the compound was calculated as the average of the three cytotoxicity experiments. Because of fewer yields of compound 2, its cytotoxicity was not determined. The cytotoxicity of compound 1 was lower than that of doxorubicin (39.38 µg/ml).

© University of Pretoria

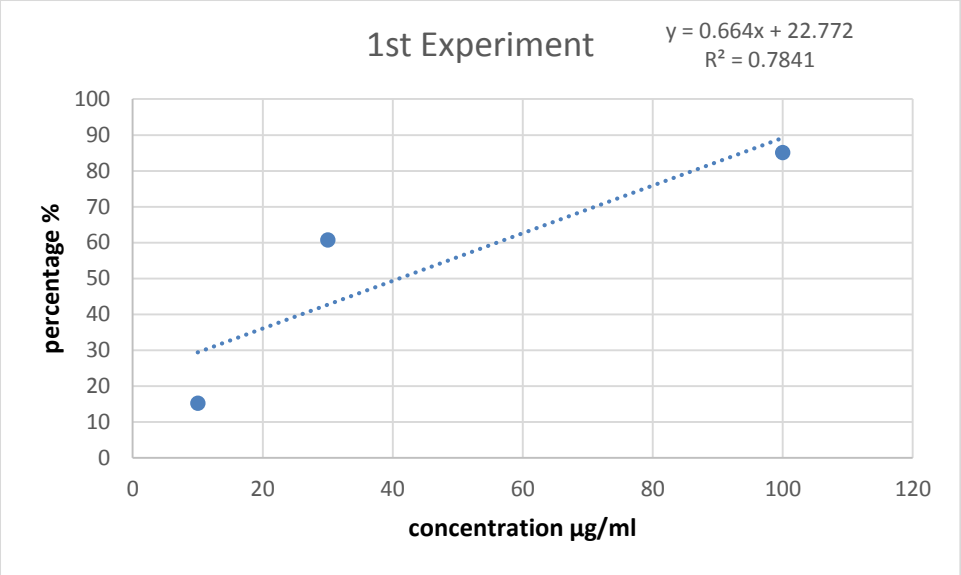


Figure 8.3. Cytotoxicity of Experiment 1 of compound 1 against Vero monkey kidney cells (LC_{50}- 41.01 µg/ml)

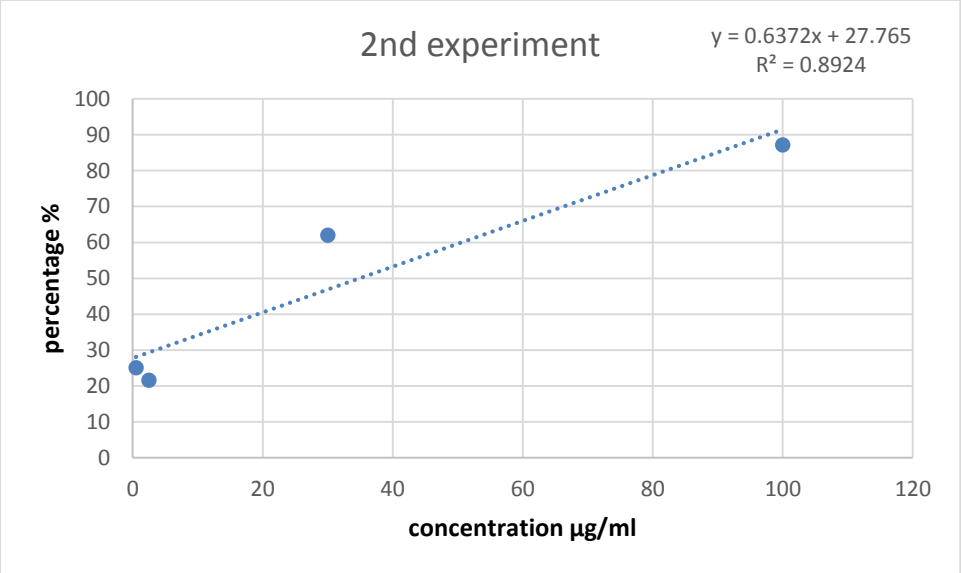


Figure 8.4. Cytotoxicity of Experiment 2 of compound 1 against Vero monkey kidney cells (LC_{50}- 35.0 µg/ml)

© University of Pretoria

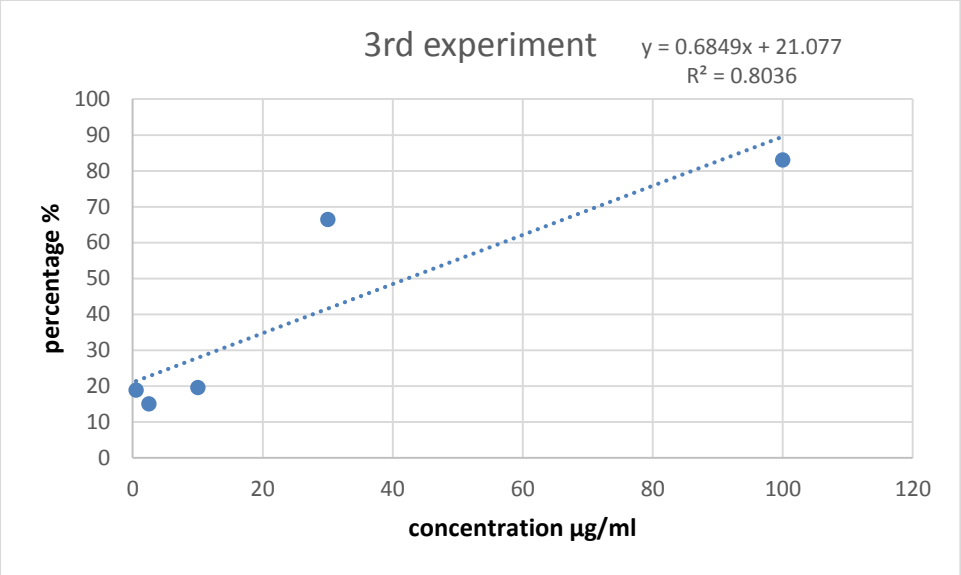


Figure 8.5. Cytotoxicity of the Experiment 3 of compound 1 against Vero monkey kidney cells (LC_{50}- 42.23 µg/ml)

The selectivity index is calculated to determine the tested product's toxicity in relation to the activity. High selectivity index means the compound is safe. In the case of isolated compound 1, the selectivity indices were very low, which suggest that although the compound is less toxic, it is not active against the tested pathogens. Results presented in

Table 8.3. LC_{50} (µg/ml) of C1 against Vero monkey kidney cells and the selectivity index against the tested pathogens.

	LC50 (µg/ml)	**SI (LC50/MIC)**				
		B. cereus	*E. coli*	*E. faecalis*	*S. aureus*	*S.*Typhimurium
C1	39.38 ± 3.21	0.03	0.02	0.03	0.02	0.02
Doxorubicin	3.02 ± 0.03	n/a	n/a	n/a	n/a	n/a

8.8 Conclusion

Two active compounds were isolated using silica gel as a stationary phase in column chromatography, following the bioassay-guided fractionation method. The isolated compounds were found to be less effective against the tested enteric pathogens and also the activity was lost. This finding suggests that the activity of the crude and fractions were due to a synergistic effect. Because of low compound yield, only compound 1 was tested for cytotoxicity and it was found that although it is not effective against the tested pathogens, it is not toxic to the Vero monkey kidney cells. The next step is to identify the isolated compound using nuclear magnetic resonance.

© University of Pretoria

CHAPTER 9

COMPOUND IDENTIFICATION AND STRUCTURE ELUCIDATION

9.1 Introduction

A structure of a biologically active compound can be determined by different spectroscopic techniques. The most commonly used techniques for elucidating structure of natural product involves Nuclear Magnetic Resonance (NMR) and mass spectrometry (MS) and to determine the molecular weight of the isolated compounds (McRae *et al*., 2007). The NMR has the advantage in determining the structure of unknown compounds, and samples are recovered after the analysis. Mass spectrometry alone is unable to characterise the complex natural products due to the occurrence of glycosides and aglycones that are present in natural products (Yang, 2006). Other techniques that can be used to determine the structure of isolated compounds includes High Performance Liquid Chromatography (HPLC), Gas Chromatography coupled with MS (GC-MS) and Ultra Violet light with other spectroscopic methods.

9.2 Objective

The objective of this part of the study was to elucidate the structure of the isolated compound from the acetone leaf extract of *G. flava*.

9.3 Materials and Methods

The structure of the bio-active compound isolated from the leaves of *G. flava* leaves was elucidated using the VJM-I-8 NMR at University of Pretoria, Chemistry department, for both the ^{1}H and ^{13}C, using Dimethyl Sulphoxide (DMSO) solvent. The spectroscopic data for the structure was interpreted by Dr Vinash Maharaj.

© University of Pretoria

9.4 Results and Discussion

The 1H NMR data showed the signals for six teriay methyl groups at 1.0, 1.0, 0.8, 1.38, 1.53 and 3.46 and one isopropenyl moiety, indicating a lupine skeleton (Mahato and Kundu, 1994). The 13C NMR and the DEPT spectra reveavealed 30 carbon signals, which were shown by the DEPT experiment to be 7 methyls, 10 methylene, 6, methane carbons, quaternary carbons and 2 olefenic carbons, Table 9.1 below.

Table **9.1.** ^{13}C NMR data of Lupeol compared with literature data (Mahato and Kundu, 1994)

Carbon position	DEPT	C-13	Literature
1	CH_2	38.31	38.7
2	CH_2	28.22	27.2
3	CH	79.24	79.0
4	C	38.95	38.6
5	CH	55.55	55.3
6	CH_2	18.23	18.5
7	CH_2	33.18	32.8
8	CH	38.31	38.80
9	CH	47.97	47.7
10	C	35.35	35.6
11	CH_2	23.91	23.6
12	CH_2	28.32	27.50
13	CH	38.96	39.0
14	C	40.23	41.80
15	CH_2	25.97	26.2
16	CH_2	27.66	27.0
17	C	32.15	32.50
18	CH	47.48	47.40
19	CH	48.21	48.10
20	C	151.17	151.1
21	CH_2	34.98	34.8
22	CH_2	37.98	37.20
23	CH_3	27.66	28.20
24	CH_3	15.80	15.5
25	CH_3	15.71	15.60
26	CH_3	16.21	16.9
27	CH_3	25.97	26.0
28	CH_3	27.69	28.40
29	CH_2	109.54	109.5
30	CH_3	19.53	19.5

© University of Pretoria

9.4.1 Structure elucidation

The compound was isolated as an amorphous colourless powder; the TLC fingerpringing indicated a single spot with the non-polar mobile systems. From the HMBC, methyl group (C-29) resonance at δH 1.0 (δC 19.53) showed correlation to a methine at δC 48.21 (C-19), methylene carbon resonance at 109.54 and unsaturated carbon resonance at δC 151.17 (C-20). The correlations are characteristic of lupeol (Fig. 9.1).

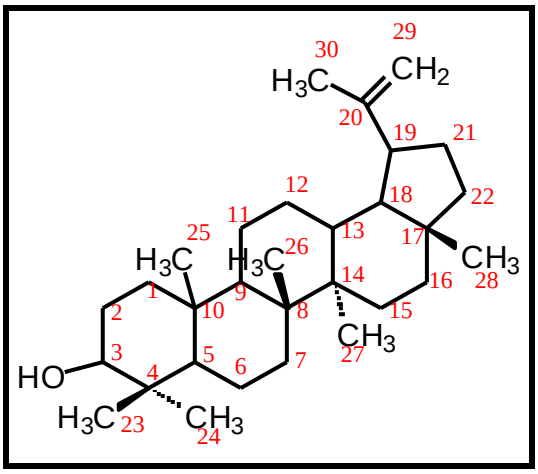


Figure 9 1. The structure of Lupeol isolated from *Grewia flava* leave**s**

Lupeol is a pharmacologically active pentacyclic triterpenoid (De las Heras *et al*., 2003) with antimicrobial, anti-inflammatory, anticancer and antimalarial properties (Shai et al., 2008b, Mathabe *et al.,* 2008). Lupeol was found to be active against *E. coli* when tested using bioautography (Shai *et al*., 2008b). It was found to be non-toxic but cytostatic, as it does not kill the Vero cells, but stop the growth and multiplication of the cells (Shai *et al.,* 2008b). *G tiliaefolia* found to contain Lupeol, and it exhibited antimalarial activity against the *Plasmodium falciparum* (Ma *et al.,* 2006). Recently there have been no reported studies on isolated compound (s) from *Grewia flava*.

9.5 Conclusion

In this study a pentacyclic triterpenoid compound Lupeol was identified. The compound was shown to be non-effective against the tested enteric pathogens and this is corroborated by the studies done by Shai *et al*., (2008b) and Mathabe *et al*., (2008). The LC_{50} in these studies is less similar to the LC_{50} in the study of Shai, *et al*., (39.38 µg/ml and 89.5 µg/ml). This is the first report of isolation and identification of a compound from *G. flava*.

© University of Pretoria

CHAPTER 10

GENERAL DISCUSSION AND CONCLUSION

10.1 Introduction

The aim of this in vitro study was to identify the active antimicrobial compound(s) from the berries, leaves, bark and roots, of the indigenous edible plant *Grewia flava***,** to determine the antimicrobial activity of the extracts of the compounds against four enteric pathogens, and to determine their cytotoxicity. A number of objectives were developed which are dicussed below:

1. To extract the compounds from the plant materials with acetone, methanol, hot water and cold water and to analyse the antimicrobial activity of crude extracts against the gastrointestinal pathogens: *E. coli, S. aureus, B. cereus* and *S.* Typhimurium by minimal inhibition concentration (MIC).

Water was a good extract solvent yield wise, but the extracts were not active against the tested bacterial pathogens, with the lowest MIC of 0.3 mg/ml and the highest MIC >2.5 mg/ml. The berries were less active against any of the tested bacteria, MIC range from 0.3 mg/ml to 1.25 mg/ml from the acetone extracts and 1.25 mg/ml to >2.5 mg/ml from the water extract. Acetone was previously reported to be the best solvent because of its low toxicity to pathogens, Eloff (1998), this means that the activity of the acetone extracts is from the extracted compounds, and not the solvent used to extract them. The leaf-, bark- and root extracts showed effective activity against the tested pathogens with lowest MIC of 0.07 mg/ml. The root extracts showed the highest activity against all the tested enteric pathogens (MIC 0.3 mg/ml against *B. cereus*, 0.15 mg/ml against *S.* Typhimurium and 0.07 mg/ml against *E. coli* and *S. aureus*). The leaf extracts were effective with the lowest MIC of 0.07 mg/ml against *E. coli* and *S. aureus* and the highest MIC of 0.6 mg/ml against *B. cereus*.

The extracts showed the activity against the tested microorganisms as they were able to reduce the INT into a formazan, a yellow to clear zone on the TLC plates. Most of the clear zones were visible against the *B. cereus* and the *S.* Typhimurium.with most of the clear zones (inhibition zones) from the acetone extracts.

© University of Pretoria

2. To test the presence of antioxidant compounds of crude extracts using the TLC-DPPH method.

Water extracts exhibited antioxidant active compounds, hence the water extracts might not be active against the enteric pathogens, but they are still good source of antioxidant compounds. Most of the compounds were phenolics rather than the essential oils or flavonoids. The berries are sweet when ripe and fleshier, this means the high carbohydrate content in the berries make it suitable for the preparation of “Khadi” a fermented drink. Compounds works in a synergistic form in crude extracts, as there are various compounds protecting the plants against different environmental factors. Some of the compounds are there to protect the plant against pathogens whereas other compounds are there for the survival of the plant. The extracts showed a range of compounds, because acetone is able to extract both the non-polar compounds to polar compounds, and the compounds showed antioxidant activity against a stable free radical DPPH qualitatively and quantitatively. Roots exhibited high antioxidant active compounds (TLC plates) and it had the lowest LC_{50} against the DPPH, compared to the positive control Ascorbic acid. The leaf extract also exhibited most of the antioxidant active compounds with a LC_{50} of 2.19 mg/ml.

3. To test the toxicity of the crude extracts against the Vero monkey kidney cells using the MTT assay

For the plant to survive, it needs strong and healthy roots, because minerals and water are absorbed from the soil through the roots and the healthy leaves are responsible for absorption of light. *Grewia flava* has a tap root system. In order to obtain enough roots for the experiment, many plants had to be uprooted. Hence leaves were the best plant parts to use. The water extract of the leaves was found to be less toxic against the Vero monkey kidney cells with a LC_{50} of 452.69 ± 33.02. Acetone extracts of the leaves showed less toxicity against the Vero cells with a LC_{50} of 507.21 ± 2.55. The higher LC_{50} means lower toxicity. The acetone extracts was chosen because of its low toxicity. The leaves were chosen because they were easy to obtain and in case of this study they showed to be effective against the tested enteric pathogens and the extract was less toxic to the monkey kidney cells. It can be concluded that the indigenous edible plant *Grewia flava* is not toxic and the compounds in the plant are good to combat enteric pathogens. The next step was to fractionate the acetone extract of the leaves and isolate the bioactive compound(s).

© University of Pretoria

4. To fractionate the active extract using solvent-solvent fractionation;

The hexane and chloroform fractions contained similar compounds. From the TLC chromatogram, both the fractions were non-polar and most of the grouped compounds are non-polar. The MIC values for the hexane and chloroform fractions were below 1 mg/ml, which is considered an effective activity, except in the case of the hexane fraction against the *S.* Typhimurium. The chloroform fraction had the highest total activity compared to the other four fractions followed by the hexane fraction. All five fractions were shown to be less toxic or not toxic to the Vero cells, because the LC_{50} were approximately 100µg/ml. The butanol fraction had the highest LC_{50}, but low selectivity indices with an SI value below 1 (0.74) against *B. cereus* and *S.* Typhimurium. Among the five fractions, the chloroform fraction had the highest yield, and was found to be effective against the tested bacteria, and it was also found to be less toxic. The next step was to isolate the active compound from the chloroform fraction using column chromatography.

5. To isolate the active antimicrobial compounds obtained from the chloroform fraction.

Two active compounds were isolated using silica gel as a stationary phase in column chromatography, following the bioassay-guided fractionation method. The isolated compounds were found to be less effective against the tested enteric pathogens and also the activity was lost. This finding suggests that the activity of the crude extracts and fractions were due to a synergistic effect. Because of low compound yield, only compound 1 was tested for cytotoxicity and it was found that although it was not effective against the tested pathogens, it is not toxic to the Vero monkey kidney cells. The next step was to identify the isolated compound using nuclear magnetic resonance.

6. To characterise and elucidate the structure of the isolated compounds using NMR-structure elucidation.

In this study a pentacyclic triterpenoid compound Lupeol was identified. The compound was shown to be non-effective against the tested enteric pathogens and this is corroborated by the studies done by Shai *et al*., (2008) and Mathabe *et al*., (2008). The LC_{50} in these studies is less similar to the LC_{50} in the study of Shai, *et al*., (39.38 µg/ml and 89.5 µg/ml). This is the first report of isolation and identification of a compound from *G. flava*.

© University of Pretoria

10.2 Recommendations for future work

As discussed above the indigenous edible plant *Grewia flava* was found to be not toxic and the compounds in the plant are good to combat enteric pathogens.

It may be worthwhile to use other techniques, like High-performance liquid chromatography (HPLC), to examine the nutritional value of the ripe berries as they may contain other properties that contribute to the antimicrobial effect of the plant on enteric pathogens.

© University of Pretoria

CHAPTER 11

REFERENCES

Ahamed B.K.M., Krishna V., and Malleshappa K.H., 2009. In vivo wound healing activity of the methanolic extract and its Isolated constituent, Gulonic Acid γ-Lactone, Obtained from *Grewia tiliaefolia*. *Planta medica*, **75(5)**, 478–482.

Anjaneyulu B., Rao V.B., Ganguly A.K., Govindachari T.R., Joshi B.S. and Kamat V.N., 1965. Chemical investigation of some plants. *Indian Journal of Chemistry*, **3**: 237-242

Aratanechemuge Y., Hibasami H., Sanpin K., Katsuzaki H., Imai K., Komiya T. (2004). Induction of apoptosis by lupeol isolated from mokumen (*Gossampinus malabarica* L. Merr) in human promyelotic leukaemia HL-60 cells. *Oncology Report*. **11**: 289−292.

Arias T.D., 1999. Glosario de Medicamentos: evaluaciony USO. Washington: *Organizacion panamricana de la salud.* p. 171.

Bamadi S., Gupta M.K., Ramaswamy S., Rai S.R., Bendell D.J., Subban R., and Bhojaraj S., 2004. Determination of betulin in *Grewia tiliaefolia* by HPTLC. *Journal of Septic Sciences,* **27**, 129–131.

Barron S. (1996). Medical Microbiology. 4th edition. University of Texas Medical Branch at Galveston, Galveston, Texas. ISBN-10: 0-9631172-1-1

Begue W.J. and Kline R.M., 1972. The use of tetrazolium salts in bioautographic procedure. *Journal of Chromatography,* **64**, 182–184.

Benzie I.F.F., and Szeto Y.T., 1999. Total Antioxidant Capacity of Teas by the Ferric Reducing/ Antioxidant Power Assay. *Journal of Agriculture and Food Chemistry*, **47**: 633−636

Bottone E.J. (2010). *Bacillus cereus*, a Volatile Human Pathogen. *Clinical Microbiology Reviews*, **23(2)**, 382–398.

Bowden B.N., 1978. Tiliaceae. Dod, B. (Ed), *Flowering plants of the world*. Oxford University Press, London, 90–91.

Brand-Williams W., Cuvelier M.E. and Berset C. (1995). Use of a free radical method to evaluate antioxidant activity. Academic Press Limited. Lebensm-Wiss. Uni. *Technol*. **28**: 25–30.

Chen X-W., Serag E.S., Sneed K.B., Zhou S-F., 2011. Herbal bioactivation, molecular targets and the toxicity relevance. *Chemico-Biological Interactions* **192**, 161–176.

© University of Pretoria

Ciocan D.I. and Băra I, (2007). Plant products as antimicrobial agents. Analeleştiinţificeale universităţii Alexandra Ioan Cuza. *Secţiunea genetic şi biologie molecular*. **3**: 151–156.

CTA Technical Centre for Agricultural and Rural Co-operation. Rural Radio Resource Pack. Medicinal plants. (`2007/03). Postal Address: Postbus 380, 6700 AJ Wageningen, The Netherlands. Telephone (31) (0) 317 467100 Fax (31) (0) 317 460067 http://www.cta.int

De las Heras B., Rodríguez B., Boscá L., and Villar A.M., 2003. Terpenoids: Sources, Structure Elucidation and Therapeutic Potential in Inflammation. *Current Topics in Medicinal Chemistry*, **3**:171-185

Eloff, J.N., 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacol.* **60**, 1–8.

Eloff J.N., 2004. Quantification the bioactivity of plant extracts during screening and bioassay-guided fractionation. *Phytomedicine* **11**, 372–381.

Ernest E., 2004. Risks of herbal medicinal products. *Pharmacoepedemiology and drug safety* **13**, 767–771.

Fabry W., Okemo P., Ansorg R., 1996. Activity of East African medicinal plants against *Heliobacter pylori*. *Chemotherapy* **42**, 315–317.

Fennell, C.W., Lindsey K.L. , McGaw L.J., Sparg S.G., Stafford G.I., Elgorashi E.E., Grace O.M., van Staden J., 2004. Assessing African medicinal plants for efficacy and safety : pharmacological screening and toxicology. *Journal of Ethnophamacology*, **94**: 205–217.

Foster T.J., (1991). Medical microbiology 4th edition. Potential for vaccination against infections caused by *Staphylococcus aureus*. Chapter 12. Microbiology Department, Moyne Institute, Trinity College, Dublin, Ireland. *Vaccine*. **9(4)**: 2217.

Gibbons S., Gray A.I., 1998. Isolation by planar chromatography. In: Natural Products Isolation. R.J.P. Cannel, ed., Humana Press, New Jersey. 209-245.

Grierson D.S and Afolayan A.J., (1999). Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. *Journal of Ethnopharmacology* **66**: 103–106.

Gurib-Fakim A., 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine* **27**, 1-93.

Hamid A.A, Aiyelaagbe O.O, Usman L.A, Ameen O.M., and Lawal A., 2010. Antioxidants: Its medicinal and pharmacological applications. *African Journal of Pure and Applied Chemistry*. **4(8)**: 142-151.

Hewitt W., Vincent S., 2003. Theory and Application of Microbiological Assay. Academic Press (Inc), London, UK, ISBN 0849318246.

© University of Pretoria

Heywood V.H., Brummitt R.K.., Culham A., and Seberg O., 2007. Flowering Plants Families of the World. Firefly Books, Richmond Hill, Ontario, Canada. ISBN 1-55407-206-9.

Hostettmann K., Martson A., Ndjoko K., and Wolfender J-L., 2000. The Potential of African Plants as a Source of Drugs. *Current Organic Chemistry*, **4**: 973-101

Hurrell R. (2003). Influence of vegetable protein sources on trace element and mineral bioavailability. *Journal of Nutrition.* **133 (9):** 2973S–2977S

Iwu M.M., Duncan A.R., and Okunji C.O., 1999. New antimicrobial of plant origin. Janick, J. (Eds), ASHS press, Alexandria, VA. 9,**8**: 51-56

Jasper M.W.J.M, Bashir A.K., Zwaving J.H., and Malingre T.M., 1986. Investigation of *Grewia bicolor* Juss. *Journal of Ethnopharmacology.* **17**, 205–211.

Jayasinghe U.L.B., Balasooriya B.A.I.S., Bandara A.G.D., and Fujimoto Y., 2004. Glycosides from *Grewia damine* and *Filicium decipiens*, *Natural Product Research*: Formerly Natural Product Letters, 18:6, 499-502

Juvekar S.S., Sakat S.S., Shah A.S.M and Wakade A.S., 2007. Anti-inflammatory activity of an aqueous extract of *Grewia tiliaefolia* leaves*. Planta Medica.* **73***, 825.*

Kaviarasan L.C., Naik G.H., Gangabhagirathi R., Anuradha C.V., and Priyadarsini K.I., 2007. *In vitro* studies on antiradical and antioxidant activities of fenugreek (*Trigonella foenum graecum*) seeds. *Food chemistry* **103**, 31–37.

Kinsella J., Frankel E., German B., and Kanner J., 1993. Possible mechanism for the protective role of antioxidants in wine and plant foods. *Food technology* **4**, 85–89.

Larson R.A., 1998. Antioxidants of higher plants. *Phytochemistry,* 27*: 969*-975.

Leistner O.A., 2000. Seed plants of Southern Africa: famielies and genera. *Strelizia* 10.

Lund T., DeBuyser M.L., Granum P.E., 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Molecular Microbiology* **38**, 254–261.

Ma C.Y., Zhang H.J., Tan G.T., Van Hung N., Cuong N.M., Soejarto D.D., Fong H.H.S. (2006). Antimalarial compounds from *Grewia bilamellata*. *Journal of Natural Products*. **69**: *346−350.*

Mahato S.B., and Kundu A.P., 1994. ^{13}C NMR spectra of pentacyclic triterpenoids-a compilation and some salient features. *Phymchemistry*, **37**, 6: 1517-1575.

Mander M., 1998. Inhibitors of cyclooxygenase and 5-lipoxygenase. *Current Medical Chemistry* **1:** 123-129.

Marston A., 2011. Thin-layer chromatography with biological detection in phytochemistry. *Journal of Chromatography* **A, 1218**: 2676-2683

© University of Pretoria

Martinez-Agustine O., Romero-Calvo I., Suarez M.D., Zarzuelo A., and Sanohez de Medina F., 2009. Molecular bases of impared water and ion movement in inflammatory bowel diseases. *Inflammatory Bowel Diseases* **15**, 114–127.

Masoko P. and Eloff J.N. (2007). Screening of twenty-four South African *Combretum* and six *Terminalia* species (Combretaceae) for antioxidant activities. *African Journal Traditional CAM.* **4 (2):** 231–239.

Mathabe M.C., Hussein A.A., Nikolova R.V., Basson A.E., Meyer J.J.M., and Lall N., 2008. Antibacterial activities and cytotoxicity of terpenoids isolated from *Spirostachys Africana. Journal of Ethnopharmacology* **116**: 194-197.

McMahon M. What Is Grewia? Osborne K (Ed), Conjecture Corporation 2003-2015. *www.wisegeeks.net* last modified 11/03/15. Visited 29/03/15.

McRae, J., Yang Q., Crawford R., and Palombo E., 2007. Review of the methods used for isolating pharmaceutical lead compounds from traditional medicinal plants. *The Environmentalist.* **27**: 165-174.

Meyer J.J.M. and Afolayan A.J. (1995). Antibacterial activity of *Helichrysumaureonitens* (Asteraceae). *Journal of Ethnopharmacol*. **47**: 109–111.

Molan A.L, Mohamad A., Abdulkhaliq F. and Mahdy S. (2012). Antioxidant activity and phenolic content of some medicinal plants traditionally used in Northern Iraq. *Journal Phytopharmacol.* ***2*****(2):** 224–233.

Mosman T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**, 55–63.

Nair M.A., Dewitt D.L., Wang H., Krempin D., and Mody W., 2005. Dietary food supplement containing natural cyclooxygenase inhibitors and methods for inhibiting pain and inflammation. US patent specification. www.patentstorm.us/patents/6818234. 11/03/15.

Oluwaseun A.A. and Ganiyu O, (2008). Antioxidant properties of methanolic extracts of mistletoes (*Viscum album*) from cocoa and cashew trees in Nigeria. *African journal of Biotechnology*, **7:** 3138–3142.

Papagiannopoulos M, Wollseifen H.R, Mellenthin A, Haber B and Galensa R. (2004). Identification and Quantification of Polyphenols in Carob Fruits (*Ceratonia siliqua*.) and Derived Products by HPLC-UV-ESI/MS^{n}. *Journal of Agriculture and Food Chemistry.* **52:** 3784–3791.

Price M.L., Butler L.G. (1977). Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *Journal of Agriculture and Food Chemistry.* **25:** 1268–1273.

Rabe T., and Van Staden J., 1997. Antibacterial activity of South African plants used for medicinal purposes. *Journal of Ethnopharmacol.* **56**, 81–87.

© University of Pretoria

Raghunath I.S., 1996. Indian medicinal plants (orient Longman, Hyderabad), Vol 3, 104–105.

Ramaroa N., Lereclus D., 2006. Adhesion and cytotoxicity of *Bacillus thuringiensis* to epithelial cells are FlhA and PIcR dependent, respectively. *Microbes and infection*, **8**: 1483-1491.

Rasoanaivo P. and Ratsimamanga-Urverg S., 1993. Biological Evaluation of Plants with Reference to the Malagasy Flora, *Napreca workshop on bioassays, Antananarivo Madagascar*.pg 9-43**:** 72-83.

Re R., Pellegrini A., Pannala A., Yang M., Rice-Evance C., 1999. Antioxidant activity applying improved ABTS radical cation decolourization assay. *Free Radical Biology and Medicine* **26**, 1231–1237.

Rekka E., and Kourounakis P.N., 1991. Effects of hydroxyethylrutenosides and free radical scavenging activity - some structural aspects. *Journal of Pharmacy and Pharmacology* **43**, 486–491.

Rietjens I.M.C.M., Boersma M.G., Van der Woude H., Jeurissen S.M.F., Schutte M.E. and Alink G.M., 2005. Flavonoids and alkenylbenzenes: mechanisms of mutagenic action and carcinogenic risk. *Mutation Research* **574**, 124–138.

Roberts M, (1990). Indigenous Healing Herbs. Southern book publishers, South Africa.*1–285.*

Ruch R.J., Cheng S.J., Klainig J.E., 1989. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catachin isolated from Chinese green tea. *Carcinogenesis*, **10**, **6**: 1003-1008

Sekirov I., Russels S.L., Antunes C.A., and Finlay B.B., 2010. Gut Microbiota in Health and Disease. *Physiolology Reviews,* **90**: 859-904.

Shahid A.A. 2012. Biological activities of extracts and isolated compounds from Bauhinia galpinii (Fabaceae) and *Combretum vendae* (*Combretaceae*) as potential antidiarrheal agents. PhD Thesis, University of Pretoria.

Shai L.J., McGaw L.J., Masoko P., Ellof J.N., 2008a Antifungual and antibacterial activity of seven traditionally used South African plant species active against *Candida albicans*. *South African Journal of Botany*, **74**: 667-684.

Shai L.J., M.A. McGaw L.J., Aderogba M.A., Mdee L.K., Eloff J.N., 2008b. Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentata* (Burm.f) C.A. Sm. leaves. *Journal of Ethnopharmacology*, **119**: 238–244.

Sies H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental Physiology*.**82**: 291–295.
Sticher, O. 2008. Natural product isolation. *Natural products report*. 517–554.

© University of Pretoria

Street, R.A., Stirk, W.A. and Van Staden, J., 2008. South African traditional medicinal plant trade-Challenges in regulating quality, safety and efficacy. *Jounal of ethnophamacology*. **119**, 705–710.

Suffness, M. and Douros, J., 1979. Drugs of plant origin. *Methods in Cancer Research*, **26**, 73–126.

Taganna J.C., Quanico J.P., Perono R.M.G., Amor E.C., and Rivera W.L. 2011. Tannin-rich fraction from *Terminalia catappa* inhibits quorum sensing (QS) in *Chromobacterium violaceum* and the QS-controlled biofilm maturation and LasA staphylolytic activity in *Pseudomonas aeruginosa. Journal of ethnophamacology.* **134**:3, 865-871.

Taylor, P., Ullah, W., Uddin, G. & Siddiqui, B. S., 2001. Ethnic uses, pharmacological and phytochemical profile of genus Grewia. *Journal of Asian Natural Products*. 37–41.

Tiwara B.K., Valdramidis V.P., O' Donnell C.P., Muthukumarappan K., Bourke P. and Cullen P.J., (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agriculture and Food Chemistry.* **57 (14)**, 5987–6000.

Van Vuuren, S.F., 2008. Antimicrobial activity of South African medicinal plants. *Journal of. Ethnopharmacology.* **119**, 462–472.

Van Wyk B.E., Van Oudtshoorn B., and Gericke N. (1997). Medicinal Plants of South Africa, 1st edition. Briza publications. Pretoria, South Africa.

Van Wyk B.-E., Van Oudtshoorn B. and Gericke N. (2009). Medicinal plants of South Africa, 2nd edition. Briza publications. Pretoria. p. 366.

Venn, J, Venn, J. A, eds. (1978). (1922–1958) "Nehemiah Grew". Alumni Cantabrigienses (10 vols) (online Ed.). Cambridge University Press.

Verpoorte, R., 1989. Some phytochemistry aspects of medicinal plant research. *Journal of Ethnophamacology* **25**, 43–59.

Wali Ullah W., Uddin G., and Siddiqui B.S., (2012): Ethnic uses, Pharmacological and phytochemical profile of genus *Grewia*, *Journal of Asian Natural Products Research*, **14:2,** 186–195.

Wink M., 2004. Phytochemical diversity of secondary metabolites. Encyclopedia of plants and crop science. Marcel Dekkar Inc. 915-919.

www.kalahari.com/ ©Kalahari Meerkat Project. Kalahari Plants and their traditional uses, pg 1-4. Visited 27/07/12.

Yadav, A.K. (1999). Phalsa: A Potential New Small Fruit for Georgia. Prospective on new crops and new uses. Jankins J (Eds). ASHS press: Alexandria, VA. 348–352.

© University of Pretoria

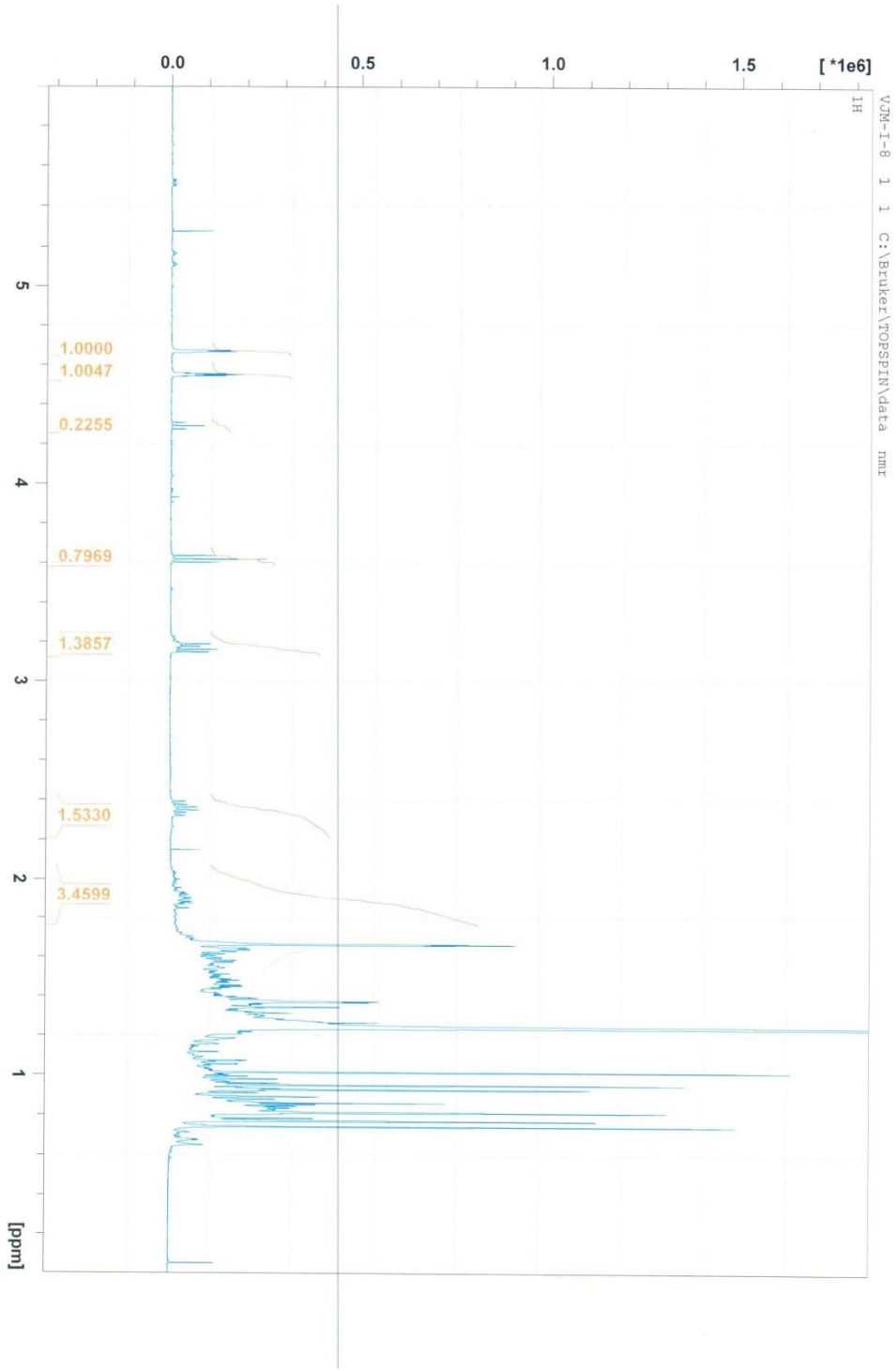
Yang Z., 2006. Online hyphenated liquid chromatography-nuclear magnetic resonance spectroscopy-mass spectrometry for drug metabolite and nature product analysis. *Journal of Pharmaceutical and Biomedical Analysis*, **40(3),** 516-27.

© University of Pretoria

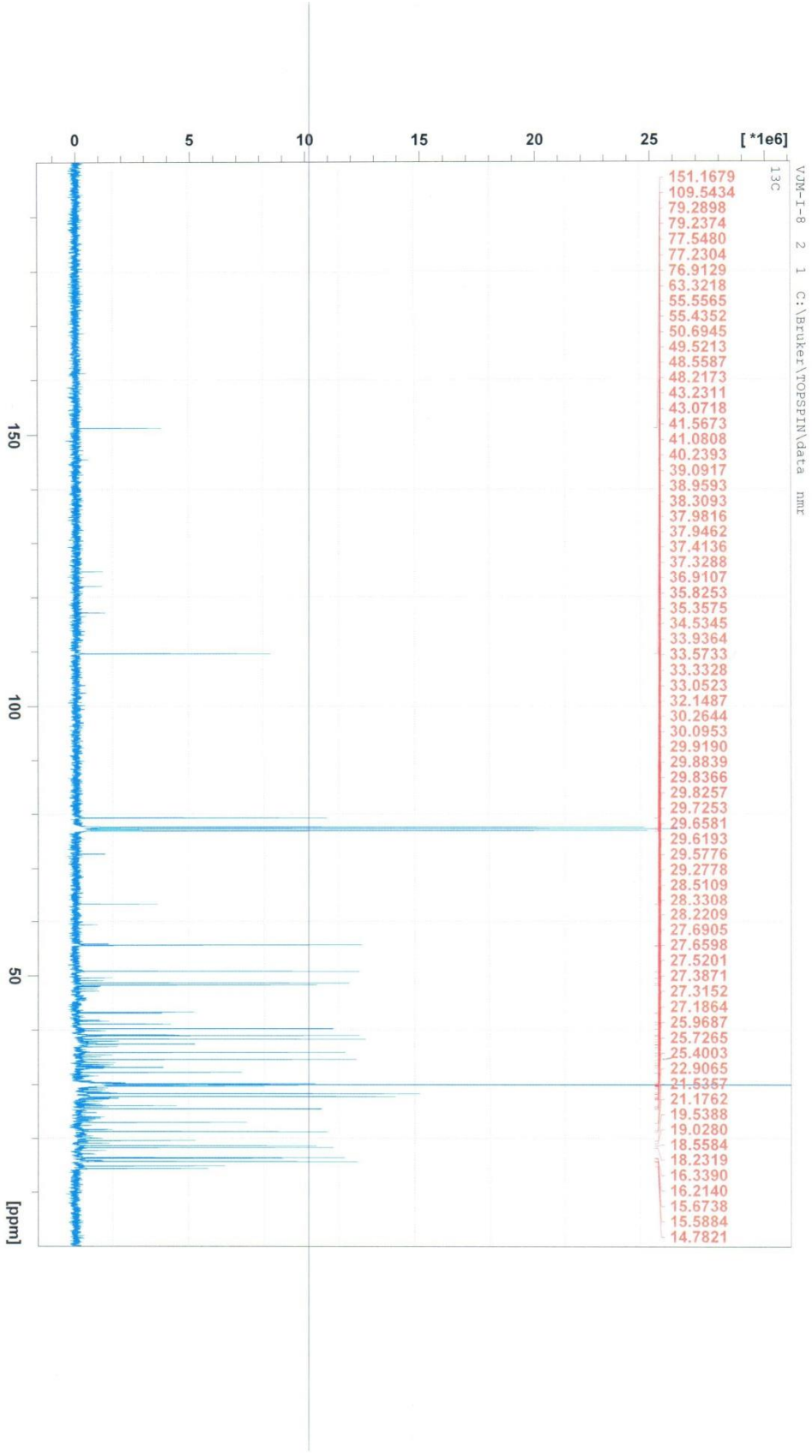
ADDENDUM

1D and 2D and Nuclear Magnetic Resonance (NMR) and mass spectrometry (MS) images

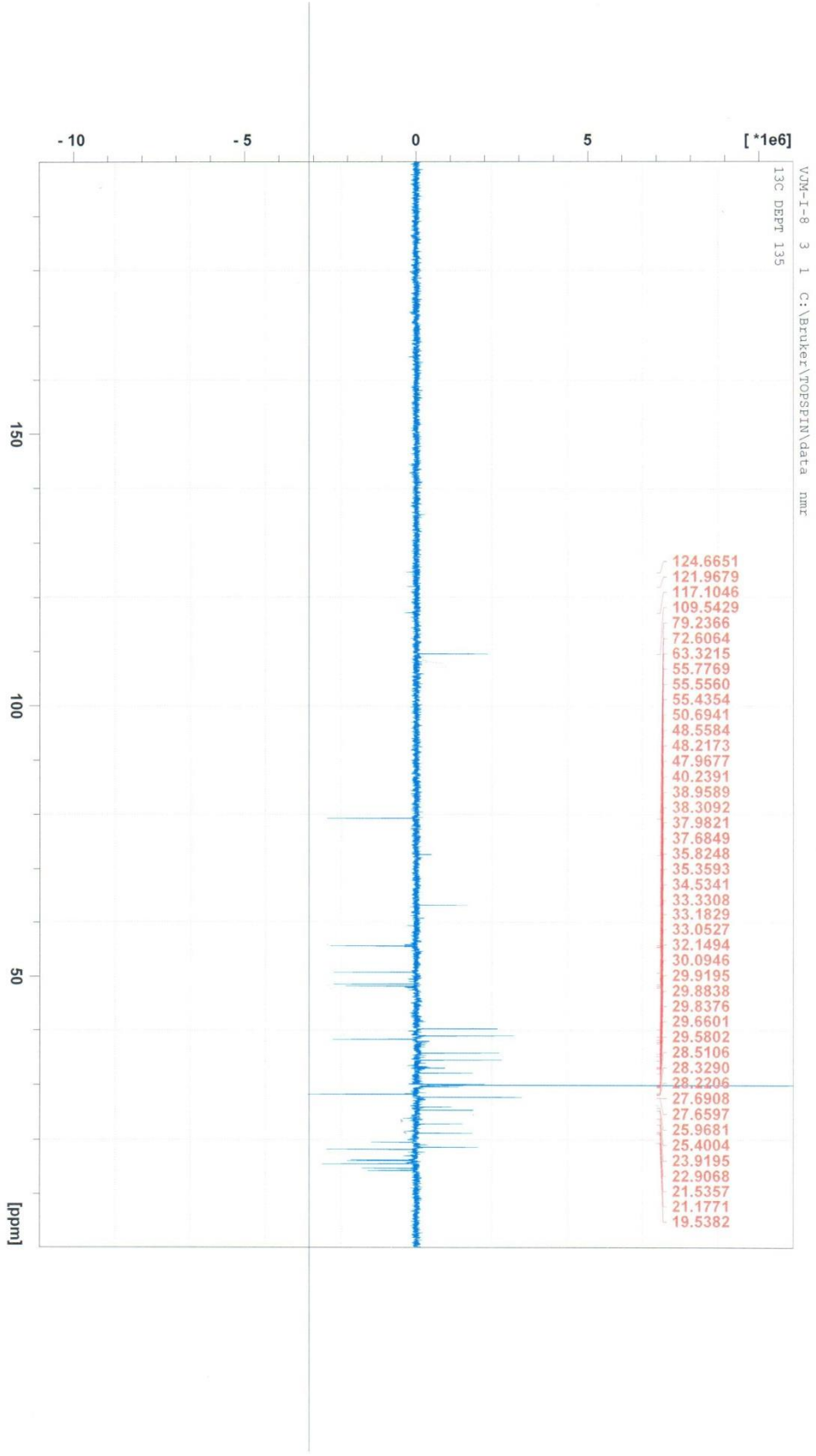
© University of Pretoria



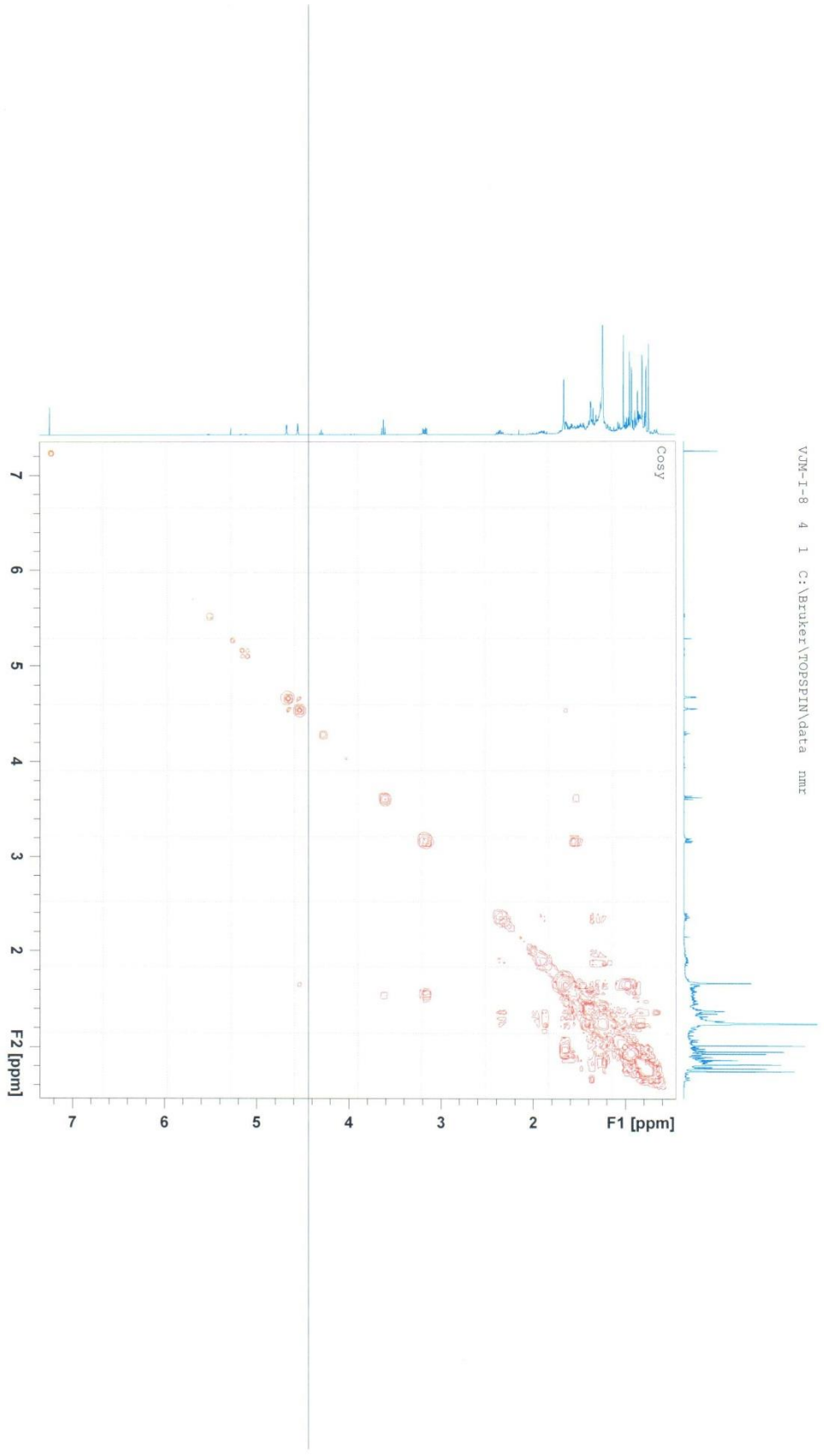
© University of Pretoria



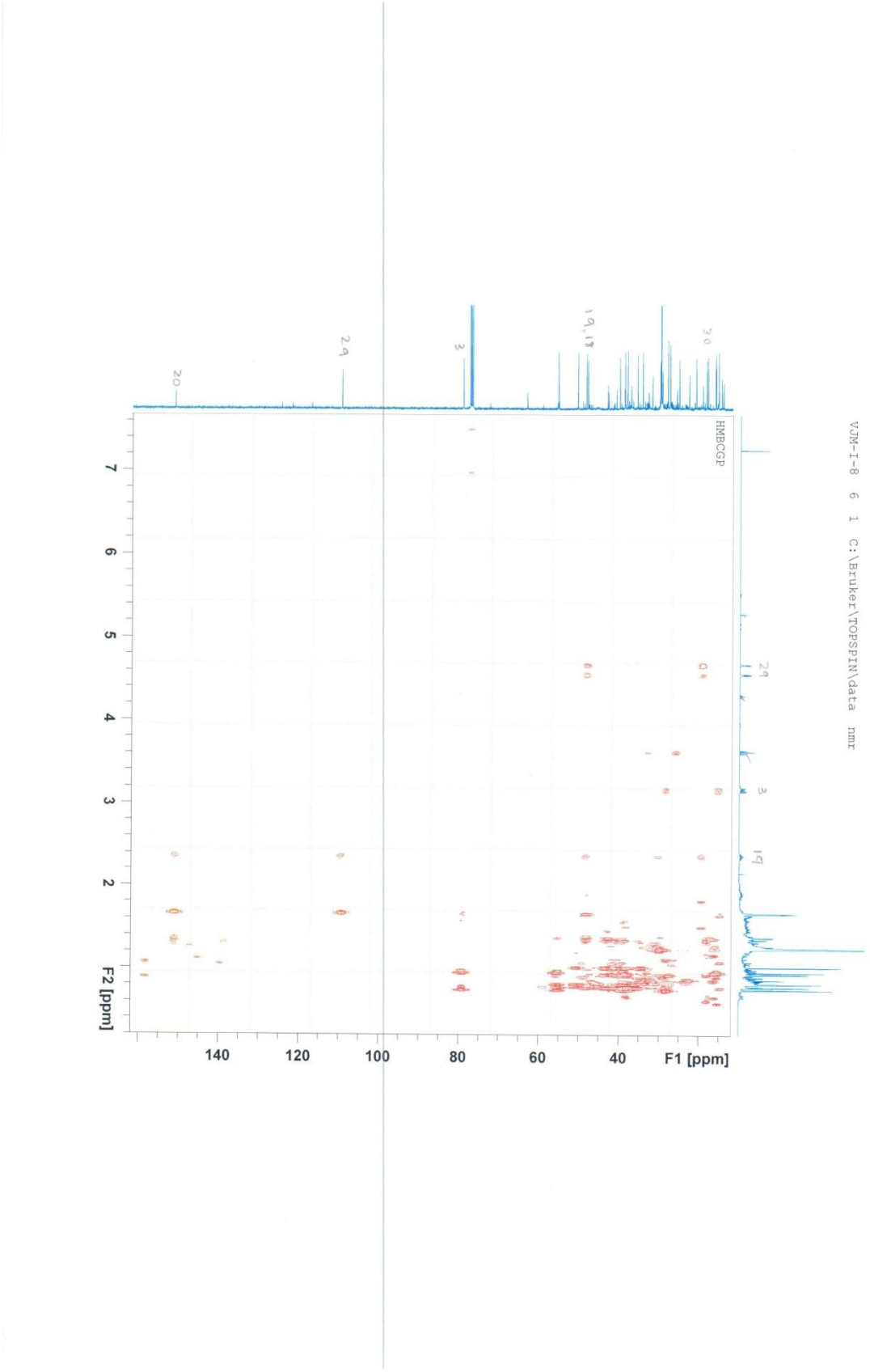
© University of Pretoria



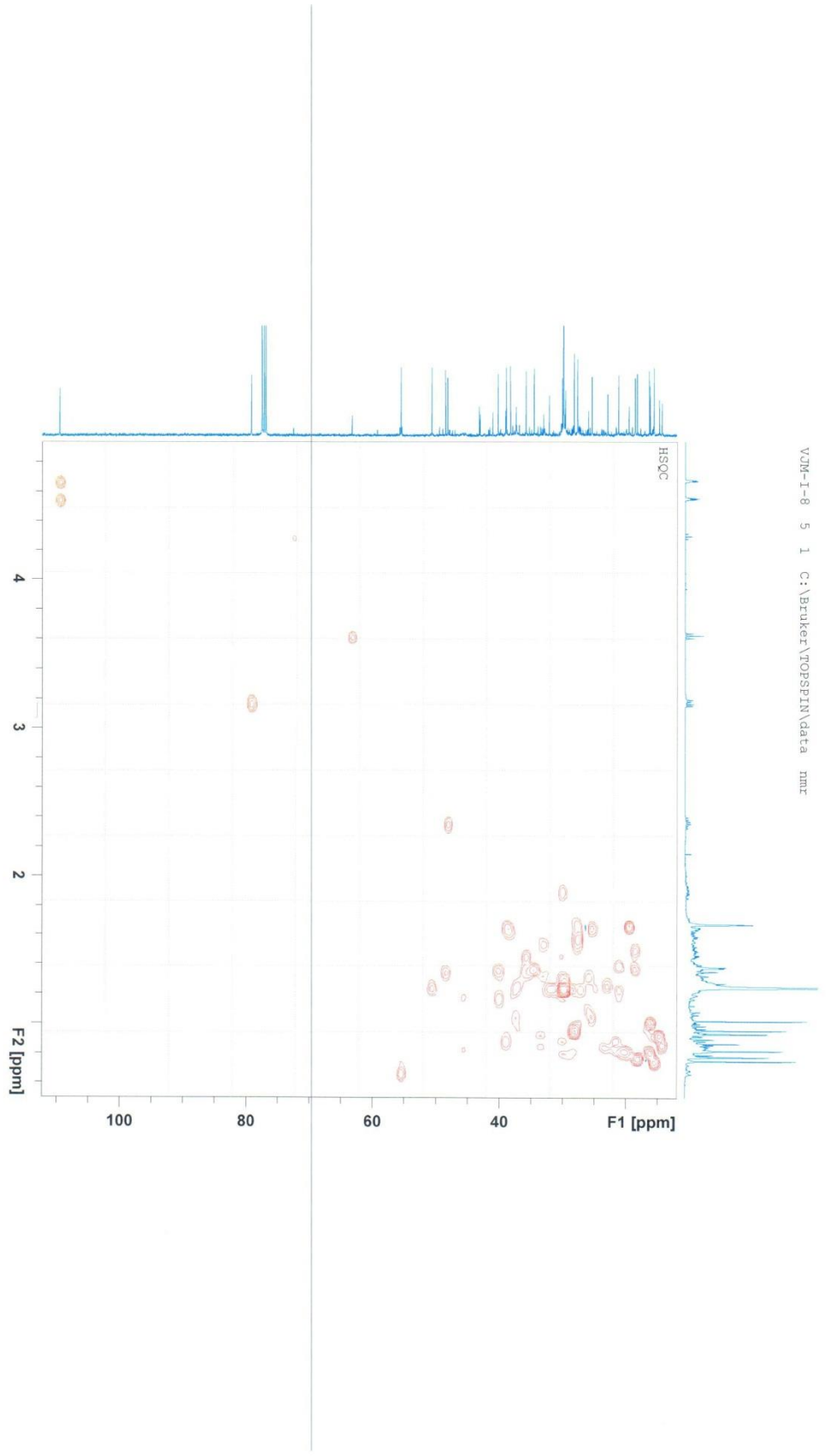
© University of Pretoria



© University of Pretoria



© University of Pretoria



© University of Pretoria