Chemo-morphological Characterisation and Some Biological Activities of *Pelargonium sidoides* DC.

By

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DEDICATION

I dedicate this thesis to my family.

DECLARATION

I, **Pretty Gqamile Mthiyane**, declare that: The research reported in this thesis, except where otherwise indicated, is my original research.

- This thesis has not been submitted for any degree or examination at any university.
- This thesis does not contain other persons' data, pictures, graphs and other information, unless specifically acknowledged
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SYMPOSIUM PRESENTATION FROM THIS WORK

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ABBREVIATIONS

µg	: microgram
µl	: microliter
ARC	: Agricultural Research Council
ATTC	: American Type Culture Collection
CA	: Cluster Analysis
CDD	: Diarrheal Diseases Control Program
cm	: centimeters
CMC	: carboxymethyl cellulose
conc.	: Concentration
DMSO	: Dimethylsulfoxide
E coli	: *Escherichia coli*
E	: East
EWM-	: ethyl water methanol
g/ml	: gram per milligram
HPLC	: High Pressure Layer Chromatography
hr	: hour
IZD	: Inhibition Zone Diameter
km	: Kilometers
KZN	: KwaZulu Natal
LBC	: Ligulate flowers with brown centre
LYC	: Ligulate flowers with Yellow centre
m	: metre
MBC	: Minimum Bactericidal Concentration
mg/ml	: milligram per millilitre
MIC	: Minimum Inhibitory Concentration
ml	: millilitre
mm	: millimetre
Nm	: nanometre
^{o}C	: degrees Celsius

P. sidoides	: *Pelargonium sidoides*
PCA	: Principal Component Analysis
S. aureus	: *Staphylococcus aureus*
S. flexineri	*: Shigella flexineri*
TB	: Tuberculosis
TBC	: tubular flower with Brown centre
TLC	: Thin Layer Chromatography
TYC	: tubular flowers with yellow centres
UV	: Ultra Violet
UZ	: University of Zululand
VOPI	: Vegetable and Ornamentals Plant Institute
WHO	: World Health Organisation

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ABSTRACT

Pelargonium sidoides DC (Geraniaceae) is one of several medicinal plants indigenous to South Africa. Various concoctions prepared from the plant are used for the treatment of tuberculosis, diarrhea, gonorrhea and fevers. The aim of this study was to evaluate the variability in qualitative and quantitative traits in *P. sidoides* accessions, correlate different morphotypes with the phytochemical content, and to the antimicrobial activity of *P. sidoides* extracts.

Morphological characterisation of accessions of *P. sidoides* was done using IPGRI/IITA/BAMBNET list for Bambara groundnut. Principal Component Analysis (PCA) and Cluster Analysis (CA) were used to evaluate the morphological variability and to reveal the groups of different morphotypes. The PCA revealed that the first three principal components exhibited Eigenvalues greater than 1 and explained 74.170% of the total variability, contributing the entire variable to the morphological variation of the accessions established at the University of Zululand. Cluster analysis was able to group the morphotypes into two major groups with each group having two sub-groups. Nine groups of the morphotypes were selected and screened for coumarins (umckalin, esculin and scopoletin) using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). Coumarins are known to exhibit moderate antibacterial and significant immunomodulatory capabilities. Variation was observed in the phytochemical content (umckalin) of morphotypes of *P. sidoides.* The content of umckalin varied between 13.90 mg/ml and 4.41 mg/ml in the roots of morphotypes of *P. sidoides* and 0.15mg/ml and 3.90 mg/ml in the leaves of morphotypes of *P. sidoides*.

Methanolic extracts from leaves and roots of selected morphotypes were screened for antimicrobial activity against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATTC 6538, *Shigella flexineri* and *Salmonella ssp*. The antimicrobial activity was determined by agar-well diffusion method. The minimum inhibitory concentration (MIC) of active extracts was determined using the micro-plate dilution assay. Significant variation was observed among all accessions for all the investigated traits. The high morphological

variation that was observed among morphotypes did not affect the phytochemical content and antimicrobial activity of *P. sidoides.* Most of the extracts showed relatively high antimicrobial activity against the tested bacterial strains with the inhibition zones ranging between 8.0 and 12.0 mm for leaves and 15.0 and 20.5 mm for the roots. The MIC values for active extracts ranged between 1.5 to 5 mg/ml.

In vivo antidiarrheal activity of *P.sidoides* methanolic root extracts in rats (*Sprague-Dawley) was* investigated*.* In the castor oil induced diarrhea experiment, the rats that did not receive the *P*. *sidoides* plant extracts showed typical diarrheal signs, stools were too wet. *P. sidoides* extracts inhibited castor oil induced diarrhea in *Sprague-Dawley* rats at doses of 100, 200 and 400 mg/kg. The extracts reduced the weight of feacal pellets with extracts treated groups showing lower diarrheal severity than control rats.

The variants utilised in this study seem to have similar compounds and could be all utilized in future research on cultivation practices. It was concluded that leaves of the plants may be harvested instead of roots to minimise the complete removal of the plants. The results of this study suggest that *P. sidoides* extracts possess anti-microbial activities against some of the tested microorganisms which are significant pathogens in humans. *P. sidoides* roots have the potential for the treatment of diarrhea.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 Background

In Africa, many plant species have been identified as having medicinal value and 80% of the population depend on traditional medicine for primary health care (Van Niekerk, 2009). These plant species are either utilised as folk medicines or exploited for commercial trade. As a result of this demand for both local and commercial uses, a number of medicinal plants have been harvested unsustainably (Kokwaro, 1991). High unemployment due to low levels of formal education has been cited as one of the reasons why people have turned to collection of medicinal plants for sale. In some African countries, the number of medicinal plant collectors has increased rapidly (Cunningham, 1993). In many developing countries where resources are poor, medicinal plants play an important role with the supply of compounds for the discovery and development of drugs for their health care needs (Kong *et al*., 2003). In South Africa, medicinal plants are collected from the wild for local use and they are threatened by large scale harvesting for export trade (Lange, 1997; Van Niekerk, 2009).

Cultivation of medicinal plants in controlled environments out of their wild populations, as an alternative to the collection in the wild has been suggested by several researchers (Losos and Glor, 2003; Keirung and Fabricius, 2005; and White, 2007). Thus, domestication is a potential way of dealing with this upsurge in the unsustainable harvesting of medicinal plants. For cultivation of these plant species to be feasible, they need to be domesticated and this domestication must be preceded by morphological characterisation to understand the diversity of the wild populations. Morphological variation among populations is a requirement to the formation and characterisation of the species (Losos and Glor, 2003). Morphological variation in plants results in divergence as a result of artificial selection which is commonly associated with cultivation in managed environments out of their wild populations (Zohary and Hopf, 1993).

P. sidoides DC is one of the medicinal plant species that is exploited unsustainably in South Africa (Cunningham, 1998; Lange 1997). The plant belongs to the family Geraniaceae and is indigenous to South Africa especially in the parts of the Eastern Cape Province and the Free State (Kolodziej, 2000). *P. sidoides* is recognised in the field as an erect, woody to herbaceous sub-shrub. Its leaves are clustered, velitinous, rosulate, ovate- cordate with long stalks. The inflorescences are formed by flowering branches more or less throughout the year (Dreyer and Marias, 2000).

It is known by vernacular names in different parts of South Africa. In the Western Cape (Afrikaans and Khoi Khoi) it is known as *rabas, rabassam, rooi rabas, rooiwortel, t'namie, heyntame*. In Zululand (IsiZulu), it is known as *ishaqa, uvendle* or *isandla* sonwabu). In the Eastern Cape (IsiXhosa) it is known as *iyeza lezikhali, ikhubalo, uvendle* or *icwayiba*. In Lesotho (SeSotho) it is known as *khoaara e nyenyane (*Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996; Van Wyk and Gericke, 2000).

P. sidoides roots are traditionally used for their medicinal properties. The most uses of the plant are associated with the treatment of infections including bacterial infections of the respiratory tract like tuberculosis and coughs (van, Wyk *et. al*., 1997). It is also used in the treatment of diarrhea, gonorrhea, stomachache, colic, dysentery (Matsiliza and Barker, 2001; Lewu *et al.,* 2007). The local people of the Eastern Cape use the plant to treat dysentery in cattle and it is a remedy for worms in calves (Lewu *et al*., 2007; Smith, 1966). Freshly harvested leaves of the plant are used for the treatment of wounds in humans and livestock (Batten and Bokelmann, 1966).

There are two strategies that would promote the conservation of wild *P. sidoides* populations for future use, to the benefit of people and the biodiversity of the natural environment. The first option would be to develop and implement sustainable harvesting practices for wild populations. The second would be to effectively cultivate *P. sidoides* plants in order to supply local and international demand, thus reducing the harvest of roots from the wild. A combination of good cultivation and wild harvest practices may provide for the sustainable harvest of *P. sidoides* in the future.

1.2 Statement of the Problem

The value of *P. sidoides* has become known to the world and has led to the development of commercial products like Umckaloabo®, which is used for the treatment of bacterial and fungal infections. Traditionally, only the roots are used for medicinal formulations. During the establishment of demonstration grow-out blocks at the University of Zululand and Agricultural Research Council Vegetable and Ornamental Plant Institute (ARC-VOPI), it was observed that there was extensive morphological variation amongst plants. The aim of the demonstration block was to determine the feasibility of production of *P. sidoides* in these areas for commercial and traditional use. The morphological variations observed led to wonder if there may be differences in the chemical composition of morphotypes. Currently, no knowledge is available on the variation in the chemical composition of the varying morpho-types. Positive pharmacological activities of *P. sidoides* have been reported, but not related to its morphological characteristics. Additionally, if the plant material (aerial and underground parts) is supplied for commercial use, it is important to verify the medicinal activity. In this study, the main aim was to determine if the morphological variation observed in plants was correlated to its chemical properties. If there were chemical differences in the morphotypes, then we would have to select the best lines for production. If there was no chemical difference, then all the plants can be harvested and there would be no need to be concerned with morphological variation.

The purpose of the research, therefore, was to investigate the interrelationship between the morphological characters and chemicals in the accessions of *P. sidoides* and further link morphotypes with their biological activities.

1.3 Aim

The aim was to investigate the interrelationship between morphological variations and phytochemicals and evaluate antimicrobial and antidiarrheal activities of cultivated *P. sidoides* in the northern part of KwaZulu Natal.

1.4 Specific Objectives

The specific objectives were:

1. To establish and characterise morphological traits in the cultivated accessions of *P. sidoides*.
2. To analyse the chemical composition (coumarins) of selected *P sidoides* morphotypes using TLC and HPLC.
3. To determine the correlation between different morphotypes and types of coumarins
4. To determine the anti-microbial activity of extracts of *P. sidoides* on selected microbes
5. To determine the antidiarrheal activity of extracts of *P. sidoides* using *Sprague-Dawley* rats

1.5 Hypotheses

The hypotheses were:

1. There is no difference in morphological characteristics of *P. sidoides* accessions at the University of Zululand the cultivated accessions of *P. sidoides*.
2. *P. sidoides* morphotypes do not contain coumarins
3. There is no relationship between morphological characteristics and chemical composition of *P. sidoides*.
4. Extracts of *P. sidoides* have no effects on microbial growth.
5. Extracts of *P. sidoides* have no antidiarrheal effects on *Sprague-Dawley* rats

1.6 Dissertation outline

The dissertation is set out in six chapters and the schematic presentation illustration of experimental procedures followed in the project documentation is shown in **Figure 1.1**

Chapter two brings together the literature review, where it explains scientific classification, botanical description, ecology, morphological variation versus phytochemical content, types of coumarins identified, and some biological activities of *P. sidoides* including antimicrobial and antidiarrheal reported by previous authors.

Chapter three presents the methodology used in the study. The materials used during the study are outlined. The methodology explaining the establishment of the *P. sidoides* plants, screening of the phytochemicals and evaluation of some biological activities (antimicrobial and antidiarrheal assay) is presented.

Chapter four summarizes the data from the experiments without discussing their implications. The data are organised in the form of tables, figures and photographs. The section concentrates on general trends and differences.

Chapter five emphasises the interpretation of the data. Obtained data are related to the existing theory and information. This section includes the suggestions for the improvement of the techniques and experimental designs based on the previous and conducted studies.

Chapter six provides the summary of the study, the conclusions and recommendations made concerning the findings of the research. The conclusions bring together the field experience, laboratory work and the literature that bring perceptions into the study.

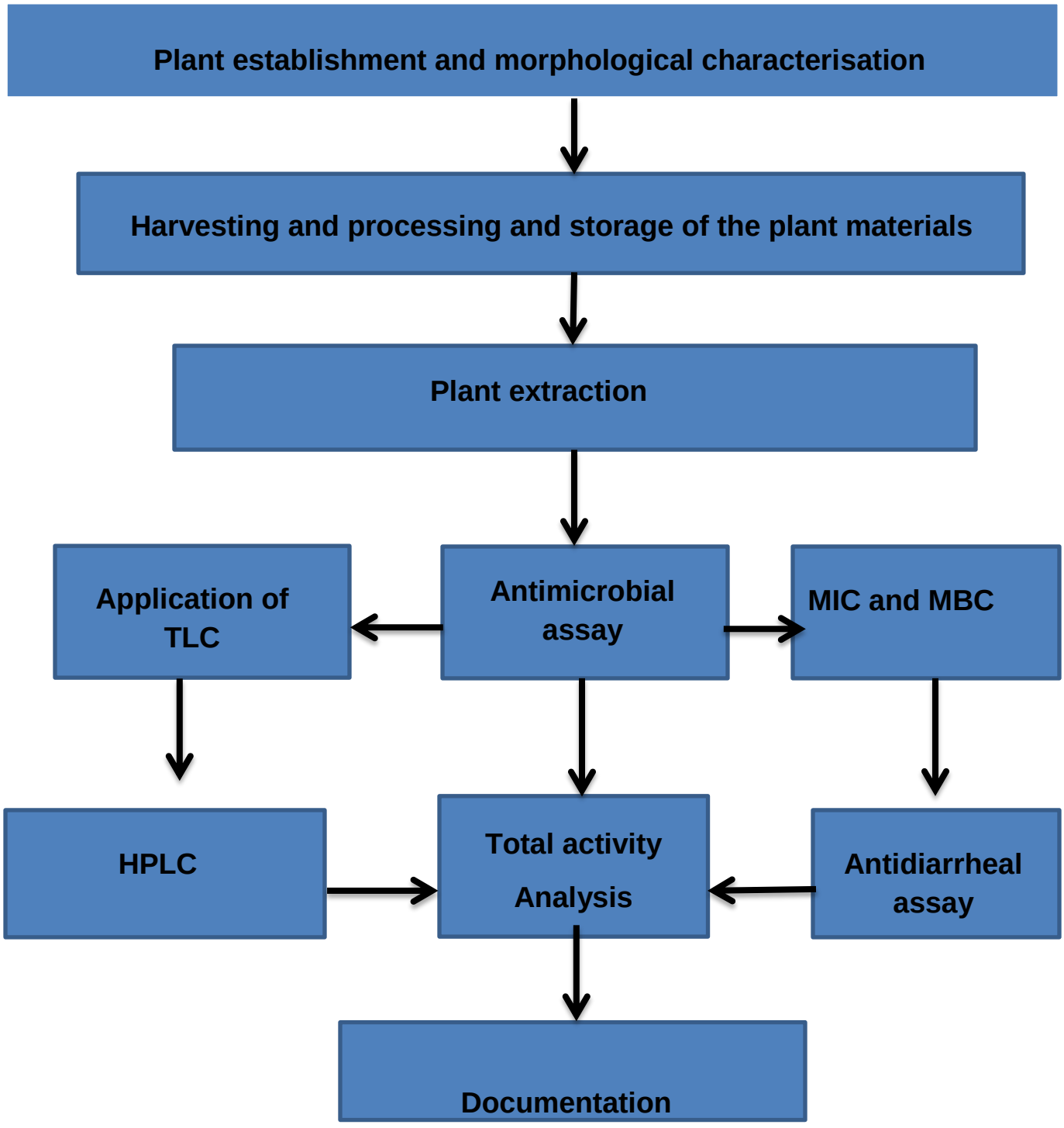


Figure 1.1 The schematic presentation illustration of experimental procedures followed in the project

CHAPTER 2

2. LITERATURE REVIEW

2.1 Exploitation of medicinal plants

Worldwide, medicinal plants have always been a primary source of medicine and still provide man with new remedies. According to van Wyk and Wink (2004), the use of traditional medicines is the oldest method in the world. In many developing countries, where the level of education is low and rates of unemployment are high, the over-exploitation of medicinal plants is increasing. This is occurring as a result of Western medicines not being easily accessible because of the high costs. Medicinal plants are an important aspect of the daily lives and play a vital part of South Africa's cultural heritage (Hutchings *et al*., 1996). It is estimated that there are about 200 000 indigenous traditional healers in South Africa. About 60 % of the Black population consults traditional healers, who are usually available in addition to modern medical services. In Africa, medicinal plants are still collected from the wild populations for traditional use and export trade.

P. sidoides is also occurring in South Africa and Lesotho, there has been no Red List assessment carried out for Lesotho. However, there have been two field surveys of *P. sidoides* populations in Lesotho; one by Newton *et al*. (2008) conducted as part of a training exercise for the Lesotho CITES Scientific Authority and a second by De Castro *et al*. (2010) as part of a resource assessment of P. *sidoides* across its entire range. The findings of both these studies indicate that there is limited localised decline due to incorrect harvesting practices and that rangeland degradation due to overgrazing is also a threat to this species.

Many medicinal plants that are in danger of extinction comprise species that are growing and reproducing slowly. Medicinal plants need to be conserved as there is rapid increase of informal trade in traditional medicines and unsustainable harvesting practices. Development and implementation of sustainable harvesting practices for wild populations and effective cultivation *P. sidoides* plants are the two interlinked options that would promote the conservation of wild *P. sidoides* populations for future use and to the benefit of people and the biodiversity of the natural environment.

2.2 Scientific classification and general botanical description of *P. sidoides*

The genus *Pelargonium* (Geraniaceae) comprises approximately 270 distinct species of perennial small shrubs of which about 80% occur in southern Africa with the centre of diversity in the Eastern Cape Province (Van der Walt and Vorster, 1988). *P sidoides* is scientifically classified as Kingdom *Plantae*, division *Magnoliophyta*, class *Magnoliopsida*, order Geraniales, family Geraniaceae, genus *Pelargonium* and species *Pelargonium sidoides* (Van der Walt and Vorster, 1988).

Pelargonium sidoides (**Figure 2.1a and b**) is recognised in the field as, an erect, woody to herbaceous sub-shrub with clustered, velutinous, rosulate, mildly aromatic velvety leaves and long-stalked (Dreyer and Marias, 2000). The leaf shape is described as ovate-cordate. The inflorescences are formed by flowering branches more or less throughout the year. It has distinctive dark, deep-purple (almost black) flowers. *P. sidoides* is similar to *Pelargonium reniforme Curt* (**Figure 2.2**). The major distinguishing trait between the two species is flower colour. *P. sidoides* has maroon deep purple flowers and linear to spatulate petals, with green sepals containing white margins, while *P. reniforme* has pink flowers, oblanceolate to ovate petals and red sepals with pink margins (Van der Walt and Voster 1988; Dreyer and Marias, 2000).

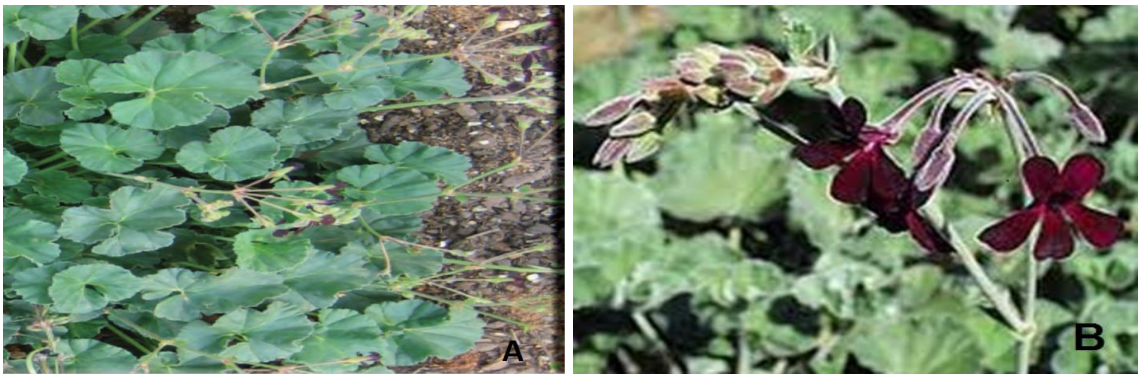


FIGURE 2.1 *P. sidoides* a) plant showing clustered leaves b) flowers and c) roots (Brendler and van Wyk 2008).

FIGURE 2.2 *Pelargonium reniforme* flowers and leaves

2.3 Ecology

P. sidoides plants are said to thrive in direct sunlight and are found predominantly on sand or loamy soils from near sea level to 2300 meters above sea level. It is able to survive in night chills below 0ºC without permanent physiological damage. Maximum

temperature ranges between 20ºC and 25ºC and result in maximum leaf growth and high essential oil content (Weiss, 1997). The species is characterised as drought tolerant, but in prolonged drought conditions, it shows poor vegetative growth (Weiss, 1997). Ideal soil for *P. sidoides* should be rich in organic matter with soil pH 5.5 to 6.5. The rainfall in the majority of the distribution range, falling mostly in summer, is between 200 and 800 mm per year (Dreyer and Marais, 2000; and Van der Walt and Vorster, 1988). The distribution of this species is very widespread across the central to eastern parts of Southern Africa occurring in the Eastern Cape, Free State, North West, Gauteng and Mpumalanga Provinces of South Africa (White, 2006) while in Lesotho it has been recorded at 2746 m (Newton *et al.* 2008).

2.4 Relationship between morphological variation and phytochemical content of medicinal plants

Based on the literature review, this aspect of research has not been reported for *P. sidoides*. Cases where morphological variation also resulted in chemical variation in other plant species have been reported in literature. For example, the antioxidant activities of the medicinal plant, basil (*Ocimum basilicum L.)* antioxidant activities were studied so as to determine the relationship between the morphological characteristics and its essential oil. The study revealed a greater variability for each observed characteristics (Marotti *et al*., 1996). Its leaves showed that they were differing in shapes, sizes, colours and weights, and the plants differed in height, weight, branching and leafing. Cultivars with similar phenotypic characters were considered as a homogenous group and four groups were distinguished. The essential oil of the basil varieties revealed marked differences in qualitative and quantitative composition. A high content of linalool was characterized in the composition of the essential oils. The results obtained in this study suggested that it is not always possible to establish a correlation between morphological characteristics and chemotype (Marotti *et al*., 1996).

Another example where differences in morphological characteristics resulted in differences in phytochemical content was in the species *Calendula officinalis* by Paim *et*

al (2010). It was reported that plants with tubular flowers and a yellow centre (TYC), and tubular flowers with brown centre (TBC) produced higher levels of flavonoids (1.41 and 1.44% respectively) when compared to ligulate flowers with a yellow centre (LYC) and ligulate flowers with a brown centre (LBC) (0.89 and 0.95% respectively).

The phytochemical variations and morphological characteristics in *Satureja hortensis* L (Hadian *et al*., 2010) are summarized in Table 2.1. It is apparent that the results showed variation in essential oil content of 30 accessions of *Satureja hortensis*. In the reported study, thymol (29-43%) was the major component of wild accessions of *S. hortensis*. This study also showed instances where the differences in morphological characteristics resulted in differences in phytochemical content in accessions of *S. hortensis*.

Table 2.1 Morphology versus phytochemicals in the genus *Satureja hortensis* L. (Hadian *et al*., 2010)

Morphological characteristics			**Phytochemical content**				
Growth type	**Stem color**	**Leaf color**	**α – pinene**	**Myrcene**	**p-cymene**	**У-terpinene**	**Alpha thujene**
Semi-upright	Slightly brownish green	Green	2.5	2.5	11.7	24.2	2.3
Semi-upright	Slightly brownish green	Green	2.0	2.0	4.3	18.3	1.6
Upright	Brownish green	Green	2.6	2.6	-	25	2.4
Semi-upright	Purplish brown	Green	1.8	1.8	12.8	28.5	1.7
Upright	Brownish green	Green	1.1	1.2	-	24.9	0.8
Semi-upright	Brown	Dark green	1.6	1.7	11	18.5	1.2
Semi-upright	Slightly brownish green	Light green	2.5	2.3	12.4	22.5	2.2
Semi-upright	Brownish green	Light green	2.5	2.4	12.6	20.7	2.1
Upright	Slightly brownish green	Dark green	2.5	2.5	14.9	23	2.2

Upright	Slightly brownish green	Green	2.0	1.9	12.9	25.8	1.8
Upright	Slightly brownish green	Dark green	2.2	2.2	12.2	23.7	2.0
Upright	Slightly brownish green	Light green	2.4	2.5	12.1	21.6	2.1
Semi-upright	Brownish green	Dark green	2.1	1.9	11.5	26.9	1.8
Prostrate	Brownish green	Dark green	2.9	2.9	7.0	26.3	2.6

2.5 Traditional and current uses

P. sidoides is traditionally used as medicine for the treatment of infections including bacterial infections of respiratory tract like tuberculosis and coughs (van, Wyk *et. al*., 1997) flu, colds and chest troubles and is effective for bronchitis in children and adults (Helmstadter, 1996; Kolodziej, 2002). The main clinical effects stemming from the use of EPs7630® has been to reduce the seriousness and duration of upper respiratory tract infections in children and adults, with negligible toxic side effects. It is used for the treatment of infections such as fever, sore throat, fatigue and weakness. Infusions of the tuber are used to treat dysentery and diarrhea (Kayser *et al*, 1998; Watt and Breyer-Brandwijk, 1962). Commercial products are developed, like Umckaloabo® (**Figure 2.3**), and are taken for treatment in the form of pills and syrups. The pulverized root is administered orally with fresh milk to treat tuberculosis as a traditional Xhosa medicine (Bladt 1974, 1977).

Other ailments that are treated include diarrhoea, gonorrhea, stomachache, colic, dysentery (Matsiliza and Barker, 2001; Lewu *et al.,* 2007). The local people of the Eastern Cape in South Africa use the plant to treat dysentery in cattle and it is a remedy for worms in calves (Lewu *et al*., 2007 and Smith, 1966). Fresh harvested leaves of the plant are used for the treatment of wounds in humans and livestock (Batten and

Bokelmann, 1966). The aerial parts of these Pelargonium species are also employed as wound healing agents (Kolodziej, 2000).

Figure 2.3 Examples of *P. sidoides* product available on the commercial market

2.6 Chemical composition

Different chemical constituents have been identified in the extracts of *Pelargonium sidoides*. Phytochemical investigations have shown that the main constituents of *P. sidoides* are phenolic acids (e.g. gallic acid and its methyl ester), proanthocyanidins and several coumarins [Kolodziej (2007, Kolodziej and Kiderlen (2007), Latte *et. al*, 2000].

P. sidoides contains more coumarins than any other *Pelargonium* species (Kolodziej, 2007). Coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. Coumarins owe their class name to 'coumarou', the vernacular name of the tonka bean (*Dipteryx odorata* Wild. Fabaceae), from which coumarin itself was isolated in 1820 (Bruneton, 1999). The characteristic constituents of *Pelargonium* species include a remarkable series of simple coumarins as regards the high degree of aromatic functionalization including hydroxyl and methoxyl groups (Kayser and Kolodziej, 1995). These coumarin derivatives and umckalin are known to be useful marker compounds for *P. sidoides*, as they appear to be absent in *P. reniforme* (Brendler and van Wyk, 2008). In addition, there is much divergence in concentration, with generally significantly higher yields of coumarins in *P. sidoides*.

These extracts from *P. sidoides* are unsaturated aromatic lactones of which they have medicinal properties. Coumarins are used as medicines in strictly controlled dosage forms, as if it is taken in relatively larger quantities it can cause internal bleeding. Examples of coumarins include Scopoletin , 6,7,8-Trihydroxycoumarin, Esculin, 6,8-Dihydroxy-7-methoxycoumarin, Umckalin, 7-Acetoxy-5,6-dimethoxycoumarin, 5,6,7-Trimethoxycoumarin, 6,8-Dihydroxy-5,7-dimethoxycoumarin , Artelin. According to European Pharmacopoeia, *Pelargonium* root has to contain not less than 2.0% of tannins expressed as pyrogallol. The method of identification of European Pharmacopoeia is thin layer chromatography of methanol root extract, but HPLC fingerprint analysis of *Pelargonium* extract already achieved (Bladt and Wagner, 1988).

Comprehensive chemical studies of underground and aerial parts of *P. sidoides* were done by Kolodziej in 2007. From these studies the medicinal properties were ascribed to at least eight different coumarins, of which umckalin (Figure 2.4) and 5,6,7-methoxycoumarin are known to be useful marker compounds (Kayser and Kolodziej, 1994; Kayser and Kolodziej, 1995; Kayser and Kolodziej, 1997; Kolodziej and Kayser, 1998 and Kayser *et al*., 2001). Umckalin, 5,6,7- trimethoxycoumarin and other coumarins were known to be useful marker compounds for *P. sidoides*, as they appear to be absent in *P. reniforme*.

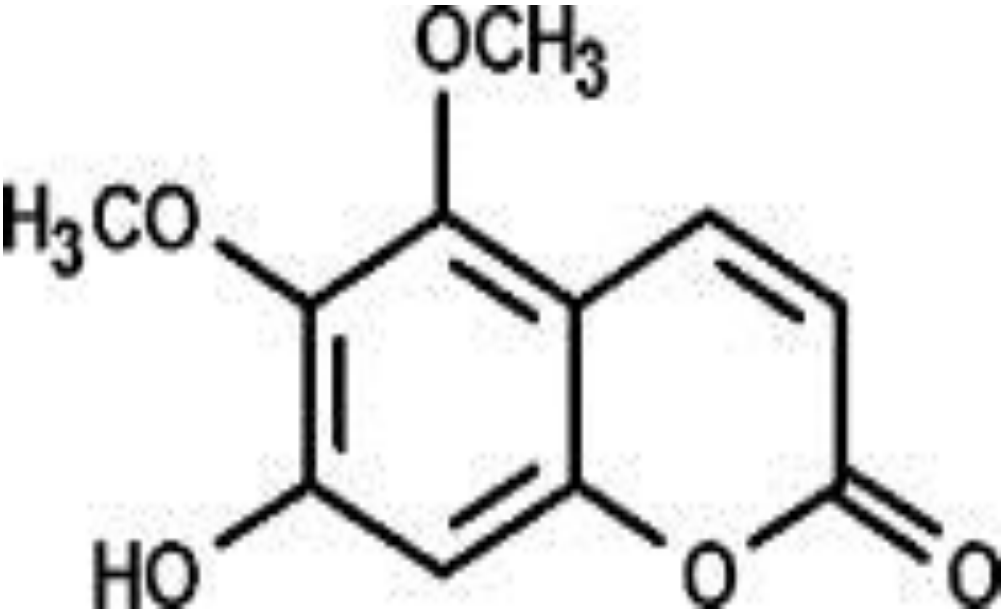


Figure 2.4 Structure of umckalin (7-hydroxy-5, 6-dimethoxycoumarin)

2.7 Pharmacological activities

Previous reports have shown that, crude extracts of *P. sidoides* exhibit anti-bacterial activity and have been used across the world as a source for newly developed medicines. It has been reported to be effective against the pathogens *Escherichia coli*, *S. aureus*, *Shigella flexineri* and *Salmonella.* These organisms are causative microorganisms for diarrhea, tuberculosis, coughs, gonorrhea (Bladt, 1974; Watt and Breyer-Brandwijk, 1962).

Literature indicates that these bacterial organisms are susceptible to plant extracts. Gram-negative bacteria have been found to be less susceptible to plant extracts (Lewu *et al*., 2006). Recent reports of the biological effects of coumarins have been reported. Roots extracts from *P. sidoides* were reported to have antimicrobial activity against a wide variety of human pathogens (Lewu *et al.*, 2007a and Kayser and Kolodziej, 2001). These examples reflect that *P. sidoides* extracts are effective against bacterial infections. General literature reviews of other plants related to P. *sidoides* suggest that no data on their biological activity and their phytochemicals are available.

2.7.1 Antidiarrheal assay of medicinal plants

Pathogenic intestinal infections are the major cause of diarrhoea and the repetitive outbreaks of Cholera. Shigella and other diarrhoea-genic micro-organisms cause obstacles in the management of the diseases (Mathabe *et al*., 2006: WHO 2009). Diarrhoea causes malnutrition as food is not absorbed well in the gastrointestinal tract and this comes with loss of appetite. In the third world countries this is very problematic as these regions already face multiple malnutrition ailments (WHO, 2009). Another danger caused by diarrhoea is dehydration and the loss of electrolyte, these two causes cause mortality rates of diarrhea to be high, especially in infants. Diarrhoea treatment for populations has proven to be costly forcing poor sanitised, resource-poor communities to seek ulternative and cheaper forms of health treatment. The predominant form of this treatment is the use of traditional medicines, provided by tradition healers. The World Health Organisation acknowledges the fact that

approximately 80% of populations in the third world countries use traditional medicinal remedies as source of primary health care (WHO, 1996).

The WHO's diarrhoeal disease control program also incorporate, aspects of traditional medicinal practices in combating the disease as it is understood that the use of these medicines is very common practices globally (Syder and Merson, 1982; Abdulkarim *et al*., 2005).

A study by Parimala *et al*., (2002) was undertaken to evaluate the effect of a methanol extract of the entire plant Cleome viscosa L. for its anti-diarrheal potential against some of the experimental models of diarrhea in rats. The plant showed significant inhibitory activity against castor-oil-induced diarrhea in rats. The results obtained establish the efficacy and substantiate the folklore claim as an anti- diarrheal agent.

Shoba and Thomas (2001) undertook a study to evaluate the effect of aqueous and methanolic plant extracts of *Acorus calamus, Pongamia glabra, Aegle marmelos* and *Strychnos nux-vomica* root bark for their antidiarrhoeal potential against castor-oil induced diarrhoea in mice. The methanolic plant extracts were effective against castor-oil induced diarrhoea. The methanolic plant extracts significantly reduced induction time of diarrhoea and total weight of the faeces. The results obtained establish the efficacy of these plant extracts as antidiarrhoeal agents.

In certain countries such as RSA traditional healers are so effective in that they have existed since before the first Dutch colonists in the 17th century and today have flourished beyond the modern doctors. RSA is regarded as to have vast and undocumented knowledge in treating various mental and health disorders. Very little scientific research has been done to verify many of their effective findings.

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Description of study areas

The study was conducted in two places that vary in climatic conditions namely at the University of Zululand orchard (28^0 51′ S 31^0 50′E) and at the Agricultural Research Council-Roodeplaat, Pretoria (**Figure 3.1**). University of Zululand orchard unit is located in the northern part of KwaZulu Natal in South Africa near Empangeni. The daytime maximum peak temperature ranges from 26^{0}C to 34^{0}C during January to March, dropping to 19^{0}C to 28^{0}C from June to August. The annual rainfall is about 1009 mm. Roodeplaat is situated approximately 30 km north east of Pretoria (Panagos *et al.* 1998). The area is situated between 25˚20`-25˚40` south latitudes and between longitudes of 28˚17`-28˚25` east. The average annual rainfall is 646 mm. The minimum and maximum temperatures are 20˚C and 29˚C and 2˚C and 16˚C in January and July respectively. The phytochemical evaluation was done at Agricultural Research Council-Roodeplaat.

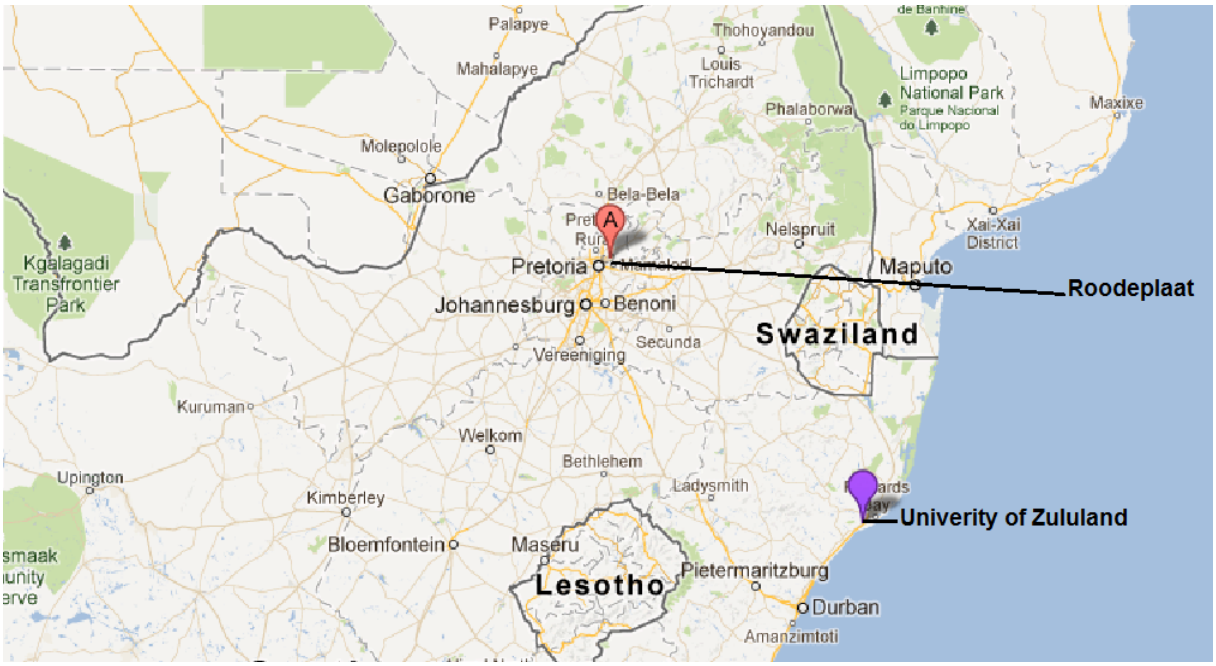


Figure 3.1 A map of the University of Zululand and Roodeplaat-VOPI (AfriGIS (Pty) Ltd, 2013).

3.2 Primary materials

The following materials were used in the various studies of *P. sidoides*

3.2.1 Morphological characterisation

- Seedlings obtained from ARC-VOPI
- Tags
- Ruler
- Recording sheets

3.2.2 Chemical analysis

- plant material (University of Zululand)
- oven for drying
- grinder (Polychem supplies)
- weighing scale
- foil
- spatula
- distilled water
- orbital shaker
- rotary vacuum evaporator (Polychem supplies)
- methanol
- filter papers
- Micro pipettes (Eppendorf)
- Dimethylsulphide (DMSO)
- Buchner funnel
- standards (umckalin, esculin and scopoletin) for TLC
- Fridge
- TLC plates
- TLC bank
- Spectrophotometer (Polychem supplies)
- HPLC system well equipped

3.2 3 Antimicrobial assay

- Platform shaker (labcon)
- Autoclave (RexMed)
- Whatman No. 1 filter papers
- Bacterial strains used in this study consisted of reference strains identified and obtained in the University of Zululand: (*Escherichia coli*, *Staphylococcus aureus, Shigella flexineri* and *Salmonella* KZN
- Agar plates
- paper disc
- glass beads
- Standard antibiotics [Ampicillin (30µg) and Cloxaccilin (30µg)]
- 96 well microplates (Costar)
- Micro pipette (Eppendorf)
- P-Iodonitrotetrazolium violet
- incubator (Scientific)
- Nutrient agar
- weighing balance (Kern pls)

3.2.4 Antidiarrheal activity

- metabolic cages
- CMC
- Rats (Sprague-Dawley)
- castor oil B.P.
- pellet and water
- water bottles
- Syringes (Sigma-Aldrich)

3.3 Morphological characterisation study

3.3.1 Plant establishment and management

About 350 seedlings of *P. sidoides* were obtained from ARC-Roodeplaat. These seedlings from ARC were kept in a nursery for three weeks after which they were transplanted in October 2010. Additional seedlings were further raised at the nursery of the orchard unit of the University of Zululand. The seedlings were transplanted in open field that has been ploughed and leveled. There were 10 rows of 21 m length consisting of 30 plants per row. Intra-spacing between adjacent rows was 70 cm, and inter-row spacing was planted 70 cm.

3.3.2 Agronomic practices

All the recommended agronomic practices were followed when raising the plants in the field. These included irrigation, weeding and chemical sprays against insect pests and diseases and harvesting procedures. The research plot was kept weed free for the duration of the study. Hand-hoeing method was used to control weeds. No fertiliser was applied to the plants. Plants were irrigated as and when needed except during rainy days**.**

3.3.3 Morphological characterization

Morphological description was done according to descriptors for Bambara ground nut described by IPGRI *et al*., 2000. As plants grew, the morphological characteristics were identified and the plants of the same morphological characteristics were grouped by the statisticians using and the groups were marked with tags. The following traits were measured for each accession in the field: Plant spread, leaf length, leaf breadth, leaf petiole length, length of inflorescence, petiole length, sepal length, number of leaves per plant, flower colour, leaf colour and leaf shape and leaf margin. The plants were harvested at 16 months in its second flowering stage. Plants were harvested according to the groupings made.

3.3.4 Accession number

This number served as a unique identifier for each plant and was assigned when an accession was entered into the collection. Once assigned this number was not reassigned to another accession in the collection. Even if an accession was lost, its assigned number was never re-used.

3.4.5 Number of leaves

These were recorded after first and second flowering stage and average numbers of all accessions were taken.

3.4.6 Petiole length [cm]

These were recorded 10 weeks after planting; average length of three leaves at the fourth node of five healthy plants.

3.4.7 Plant spread [cm]

Recorded after planting and the widest length between two opposite points.

3.4.8 Plant height [cm]

The plant height was measured from the ground level (at the base of the plant) to the tip of the highest point, including the terminal leaflet, recorded in all accessions.

3.4.9 Leaf length [cm]

Recorded from the point part at one end of the leaf to the point where the leaf joins the stalk at the other end.

3.4.10 Leaf breadth [cm]

Recorded from tip to tip at the widest part of the leaf.

3.4.11 Flower colour

Recorded by characterised by purple colour and pink colour flowers.

3.4 Harvesting of plant material (leaves & roots)

Healthy, disease free leaves and roots of *P. sidoides* (**Figure 2.1**) were harvested. Freshly harvested, leaves and roots with high moisture (55-90%) were chopped into small pieces and dried in an oven in the Department of Agriculture Laboratory (University of Zululand) at a temperature of 60˚ C for 5 days, so as to prevent microbial infestation, which will in turn deteriorate active components of the crude sample. Dried materials were milled into a powder (2 mm mesh) in the Department of Biochemistry and Microbiology.

3.4.1 Extraction procedure

Nine morphotypes were selected for phytochemical screening. The reason for selected morphotypes was that there were many of them in the field and there was enough material to perform phytochemical screening. Twenty grams of the powdered material (roots and leaves) were separately weighed and extracted in 300 ml methanol twice. The extracts were put in the VELP VORTEX mixer with a magnetic stirring for 20 minutes at a room temperature of 25ºC. Crude extract was filtered with MN 615 No. 1 filter paper. The extracts were dried using Stuart rotary evaporator. The crude extracts (0.5 g) were further dissolved in 2 ml of methanol and shaken with a VORTEX mixer for 15 minutes and then filtered (**Figure 3.2**.)

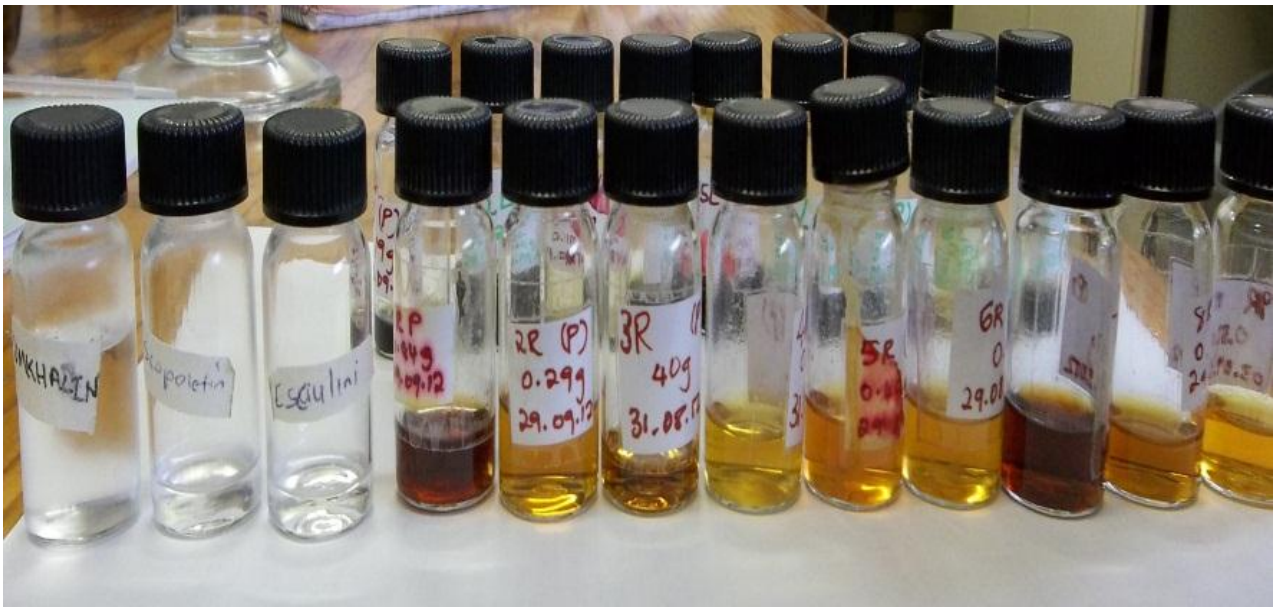


Figure 3.2 Photograph showing filtered *P. sidoides* extract used for TLC and HPLC. The first three from left is standards (umckalin, scopoletin and esculin) and the rest are extracts of different morphotype groups of *P. sidoides*.

3.4.2 Thin Layer Chromatography

A thin layer chromatography (TLC) test was done to analyse the root and leaf extracts for chemical compounds using the European pharmacopoeia of 2008. A mobile phase which consisted of water, methanol and ethyl acetate (10:14:76) was used and the extracts were applied in 10 µl as bands. For further detection, 1 mg of umckalin, 1 mg of scopoletin and 2 mg of esculin were dissolved in 20 ml of methanol, for use as standards. The plates were dried before they were visualised by the UV Lamp (**Figure 3.3**). Examination was done under ultraviolet light at 365 nm.

Figure 3.3 Photograph showing ultraviolet lamp for examination of the TLC plates

3.4.3 High Performance Liquid Chromatography methodology

Following the positive TLC of plant extracts, a High Performance Liquid Chromatography (HPLC) based assay was developed for the quantification of umckalin, esculin and scopoletin concentrations in methanolic *P. sidoides* root and leaf extracts. The developed assay was then used in subsequent experiments to assess the variation of root and leaf umckalin, esculin and scopoletin concentrations between different morphotype groups.

This chemical analysis was carried out on a HPLC system (**Figure 3.4**) equipped with a quaternary pump (2LC-10 AD), photodiode (PDA) UV detector SPD-10A (V), Refractive index (RI) detector and auto sampler (SIL-10AD) (LC Solution, Shimadzu, Japan). The chromatographic separation was achieved on a Luna 5µ C18 (2) 100 A 150 x 4.60 mm column, equilibrated with MeOH: H_2O (9:1). Ten milligram of crude extract was dissolved into two millilitre of the HPLC grade methanol and 1 mg/ml was transferred into vials and inserted in the auto sampler. The chromatographic separation was performed at 25 min at a flow rate of 1 ml/min with a mobile phase composed of water and methanol. The multigradient solvent system used was gradually changed to water: methanol (9:1) (Isocratic elution) using a wavelength of 260 nm.

Figure 3.4 Photograph showing the equipment set of the HPLC

3.5 Plant extraction method for antimicrobial and antidiarrheal assay

Dried powdered plant materials were extracted with methanol for antibacterial assay. Forty grams of each plant sample were mixed with 100ml of the solvent. The mixture was left in a shaking water bath at 150 rpm for 24 hours at a temperature of 25ºC. The extracts were filtered through Whatman No. 1 filter paper. The extracts were further concentrated to dryness under reduced pressure at 37°C, using a rotary evaporator (Laborota 4000-efficient, Heildolph, Germany). The yields resulting from the different extracts were weighed and recorded. Each extract was re-suspended in 10% DMSO to make a 50 mg/ml stock solution. This was diluted to the required concentrations (5 mg ml^{-1} and 10 mg/ml). The extracts were then stored at 4ºC until used for antibacterial tests. For antidiarrheal assay, plant extracts were suspended in 0.5 g/ml of the CMC solution.

3.5.2 Bacterial strains

Microorganisms used in the determination of the antimicrobial activity of different plant extracts were obtained from Microbiology and Biochemistry Laboratory, University of Zululand. Bacterial cultures included were Gram-positive (*Staphylococcus aureus* ATCC 6538) and three Gram-negatives (*Escherichia coli ATCC 8739, Shigella flexineri KZN and Salmonella spp*). The chosen strains were used in the study because they were available and these pathogens are primarily responsible for respiratory tract infections, gastro-intestinal disorders (diarrhea) and the immunomodulatory potential of the product provide the rational basis for its current therapeutic use. All the bacterial cultures were maintained on the Muller-Hilton agar medium. Bacterial cultures were prepared by transferring three colonies into a tube containing 20 ml nutrient broth and grown overnight at 37°C. Then, the inoculum was used for antimicrobial activity.

3.5.1 Disc diffusion assay

The antimicrobial tests were performed using disk diffusion method (Salie *et al*., 1996). Agar plates were prepared using Mueller-Hinton (MH) agar. About 20 ml of nutrient agar medium was poured in the sterilised petri dishes and allowed to solidify. Microbial strains were evenly spread onto the surface of the agar plates using sterile bent glass rod spreader. Ten microliters of methanol extracts (5 mg/ml and 10 mg/ml) were added in each well. Petri dishes containing 10µl of DMSO per well served as a negative control. The standard antibiotics, Ampicillin (30 µg) and Cloxaccilin (30 µg) were used as positive controls. The agar plates were then covered and incubated at 37°C for 24 hours. The plates were observed for the inhibition of microbial growth that was indicated by a clear zone around the well **(Figure 3.10B)**. The zones size of inhibition of microbial activity was measured in terms of the average diameter of the inhibition zone. Where there was no zone of inhibition, it was concluded that there was no activity. Each of the plant extracts were tested in triplicates so as to allow for statistical analysis.

3.5.1.3 Minimal inhibitory concentration

A technique by Eloff (1998) of micro-dilution using 96 well micro plates was used in order to obtain MIC values of crude extracts of plant that were showing high inhibition zones. The plant root crude extracts were tested against the following microorganisms:

Escherichia coli ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Shigella flexineri* KZN and *Salmonella spp*. Methanolic plant extract of 5 mg/ml and 10 mg/ml, was serially diluted so as to obtain 2.5 mg/ml starting concentration in the first well. An equal volume of 100µl fresh bacterial cultures were added into well. Micro-plates were then covered with lids and incubated at 37°C overnight.

P-Iodonitrotetrazolium violet (Sigma) reagent (0.5 g/l) was used to indicate the presence of no microbial growth and microbial growth. Where the wells show pink colour, that indicated that there was bacterial growth and where the wells show colorless, that indicated, that there was no inhibition in each well. The lowest concentration of the extract that inhibited the microbial growth after incubation was taken as the minimal inhibitory concentration. Only root extracts that showed high antimicrobial activity with inhibition zone (>15.25 mm) were tested for MIC.

3.5.1.4 Minimal bacterium concentration

The minimum bactericidal concentration (MBC) which is defined as the lowest concentration of the sample at which inoculated bacterial strains are completely killed was confirmed by re-inoculating 10 µl of each culture medium from the micro-titer plates, which were used for MIC, on nutrient agar plates and incubated at 37^0C for 24hours.

3.6 Antidiarrheal activity

3.6.1 Ethical consideration

The experimental procedures including the administration of schedule medicinal substances and assurance of safety for the project were approved by the Research Animal Ethics committee of the University of Zululand (See **Appendix 4**).

3.6.2 Animals used for antidiarrheal activity

The rats were collected from the animal research unit of the Department of Biochemistry and Microbiology for conducting the experiment. The rats, *Sprague-Dawley* (**Figure 3.5**), of either gender (adults±200 g) were used in the experiments**.** Rats were kept in

supervision under standard environmental conditions. Standard pellet and water was given to the rats. All animals were acclimatised to laboratory conditions for five days before performing an actual experiment. Animals were divided into control and test groups of eight each. Animals were placed in metabolic cages, the floor of which was lined with blotting paper. Evaluation of antidiarrheal activity in the study followed method of Mukherjee *et al*., 1998.

Figure 3.5 Photograph showing the *Sprague-Dawley* used for antidiarrheal activity of *P. sidoides* extract.

3.7 Castor oil-induced diarrhoea in rats

Rats were fasted for 18 hours before starting the experiment. For this study rats were divided into males and females. The experimental groups, Group I (control) received distilled water, Group II received plant extract at a dose of 100 mg/kg, Group III received plant extract at a dose of 200 mg/kg and Group IV received plant extract at a dose of 400 mg/kg. Each of the plant extracts was administered orally to each group of the experimental animals. After 1 hr. of treatment, each animal was fed with 1 ml of castor oil, orally. The rats were observed for defecation overnight. The total defecates for each animal was weighed and means obtained for each group for comparison.

3.8 Statistical analyses

3.8.1 Morphological characterization

Microsoft Excel package was used to group accessions according to their qualitative characters namely leaf size, leaf colour, leaf margin and flower colour. Patterns of morphological variation similarity and differences were analyzed by multivariate statistical method using SPSS statistical package. Principal Component Analysis (PCA) was used to analyze patterns of individuals in order to visualise possible differences among populations. The eigenvectors resulting from PCA and the standardized discriminant function coefficients resulting from DFA were used to identify the characteristics that most significantly contributed to classifying individuals. Cluster analysis was performed to examine the morphological similarity between the morphotypes studied. The purpose was to visualize possible differences between populations of the University of Zululand plants, which would be possibly related to the environmental differences existing within the populations. Cluster analyses included the mean values of the quantitative characters.

3.8.2 Identification of coumarins

The analyses were performed with the use of HPLC. An enhanced version was used for data collection and conversation to chromatographs. All programmes of the chemo-metrics resolution method were decoded. Identification was based on HPLC retention data and chromatographs. The component (Umckalin) content was identified by comparison of their retention times and micro Milli Absorbance (mAU) fragmentation patterns on the computer library built up using pure umckalin and confirmed by comparison of their retention indices.

3.8.2.1 Linearity

Concentration of response curves were analysed to give the concentration of the available umckalin by the linear gradient equation. The calibration curve was drawn for each isolate using eighteen calibration standards in the concentration range from 1mg/mL and was found to be linear. Linearity equation was expressed as $y = mx+c$

where, '*y*' is area in milli absorbance units, '*x*' is concentration in g/mL and 'c' was 0. The regression analysis was performed using Agilent chem-station software. Calibration curve was drawn with concentration on *x*-axis and peak areas on *y*-axis.

3.8.2.2 Method validation

The reproducibility of the retention time of the bioactive isolates under optimum HPLC conditions was investigated by doing repeated injections of a mixture of the standards at a concentration of 1 mg/ml. The good reproducibility in retention time indicated that this method is accurate, robust and would probably be reliable for screening the active bioactive constituents in the plant sample.

3.8.2.3 Supporting Information

Concentration-response curves for umckalin are available as Supporting Information (See APPENDIX 2).

3.8.2.4 HPLC (Quantitative)

A standard curve of umckalin dissolved in methanol (1mg) was also created so that the effects of using methanol as an extracting solvent could be assessed. The linearity of the standard curves confirmed that the developed method could be used to accurately determine the concentration of umckalin in methanolic root and leaf extracts of different morphotypes.

3.8.3 Antimicrobial assay

The mean and standard error of the mean of the three experiments (antimicrobial assay) were determined. Statistical analyses of the differences between mean values obtained for experimental groups were calculated using Microsoft Excel program, 2002 and Origin 6.0 for IC_{50}. Data were subjected to one way analysis of variance (ANOVA). P values ≤0.05 were regarded as significant and P values ≤0.01 were considered as very significant.

3.8.4 Antidiarrheal assay

The means of excretes were recorded after *Sprague-Dawley* were induced with castor oil. Diarrheal excretes were measured in each cage and the results were presented in graphs.

CHAPTER 4

4. RESULTS

4.1 Morphological characterization

The morphological parameters were recorded at the flowering stage using recording sheets (**See Appendix 1**). Among the accessions, nine phenotypes were distinguished on the basis of leaf colour, leaf shape, leaf margin, stem length, inflorescence length and flower colour. The null hypothesis that there was no significant ($p<0.05$) morphological difference within the accessions of *P. sidoides* established at the orchard of the University of Zululand was rejected.

4.1.1 Morphological Characterisation

The Microsoft Excel programme grouped the accessions according to their qualitative characters namely leaf shape, leaf color, leaf margin and flower colour **(Table 4.1)**. The examined *P. sidoides* plants showed great variability for each observed characteristic and some did not show variability. The leaf margins and leaf bases of the P. *sidoides* did not show any variability. The leaves showed different shapes, sizes, colours and the plants differed in height and leafing **(Table 4.1 and 4.2)**. The accessions with the longer leaf stalk (greater than ten cm) were more abundant in the field compared to the accessions with shorter leaf stalks (with a length less than ten). Most of the accessions studied produced many bigger leaves with the length greater than six centimeter. Considering all the collected data it was possible to identify morphotypes with similar phenotypic characters that could be considered as a homogeneous group. In this way, fourteen groups were distinguished **(Figure 4.1 - 4.4)**.

Table 4.1 Qualitative traits of the accessions of *P. sidoides.* Different morphotypes selected for analysis indicated as G1-G14

Flower colour	Leaf stalk	leaf colour	Leaf margin	Bigger/ broad leaf	Smaller/ narrow leaf	Grand Total
Pink	Long	deep green	curled up	1		1
			uncurled	4	1	5
		deep green Total		5	1	6
		light green	curled up	3		3
			uncurled	1		1
		light green Total		4		4
	long Total			9	1	10
	Short	deep green	uncurled	2	1	3
		deep green Total		2	1	3
		light green	uncurled	2		2
		light green Total		2		2
	short Total			4	1	5
pink Total				13	2	15 **(G16)**
Purple	Long	deep green	curled up	15 **(G1)**	4**(G2)**	19
			uncurled	43**(G3)**	14**(G4)**	57
		deep green Total		58	18	76
		light green	curled up	18**(G5)**	2**(G6)**	20
			uncurled	27**(G7)**	7**(G8)**	34
		light green Total		45	9	54
	long Total			103	27	130
	Short	deep green	curled up	7**(G9)**	1	8
			uncurled	16**(G10)**	3**(G11)**	19
		deep green Total		23	4	27
		light green	curled up	4(**G12)**	1 **(14)**	5
			uncurled	16(**G13)**	**3 (G15)**	19
		light green Total		20	4	24
	short Total			43	8	51
purple Total				146	35	181
Grand Total				159	37	196

G-group

Most of the accessions studied had uncurled leaves while some had curled leaves (**Figure 4.2**; **Table 4.1**). Most of the accessions produced bigger leaves while other plants produced smaller leaves (Table 4.1). Two distinguishing flower colours (**Figure 4.4**) in the field were observed. Many of the accessions had deep purple flowers and a few accessions had pinkish stalks with pink flowers. The occurrence of different flower colours among the studied accessions may be due to association of *Pelargonium sidoides* with its close relative *Pelargonium reniforme* (with pink colour) found growing in the same habitat (See photographs in Appendix A(i) and (ii)).

Figure 4.1 Photograph showing the leaf margin (entire and wavy leaf margin) of *P. sidoides*

Figure 4.2 Photograph showing the curled and uncurled leaves of *P. sidoides*

Figure 4.3 Photograph showing the deep green and light green leaves of *P. sidoides*

Figure 4.4 showing the plants with Purple flowers of P. sidoides and plants with pink stalks with flowers P. reniforme

4.1.1 Quantitative traits

The plants differed in several agronomic characteristics such as plant height, plant spread, inflorescence length, stem length and number of leaves. Some of these can be advantageous for commercial exploitation of such traits. **Table 4.2** shows the coefficient of variation, standard deviation, mean, maximum and minimum values obtained from quantitative traits measured in the accessions assessed.

Table 4.2 Descriptive statistics of the quantitative traits measured on the accessions of P. sidoides established at the University of Zululand orchard unit

Traits	Minimum	Maximum	Mean	Std. Deviation	Coefficient of Variation
Plant spread	15.0	37.0	29.432	4.6087	21.240
Plant height	31.0	62.0	55.179	6.0777	36.939
Leaf Length	2.0	4.0	2.883	0.6086	0.370
Leaf Breadth	2.0	4.2	2.905	0.6163	0.380
Inflorescence Length	24.0	53.3	42.334	5.7456	33.012
Number of Leaves/plant	22	48	38.97	4.844	23.468
Stem Length	6.5	18.0	12.864	2.8323	8.022

The phenotypic correlations between traits (**Table 4.3**) showed that there is a highly significant correlation between plant height and length of inflorescence (r = 0.878) and between plant spread and number of leaves per plant (r=0.839). Plant height was also positively correlated with plant spread (r = 0.440), stem length (r = 0.335) and number of leaves per plant (r = 0.387). A positive correlation was also observed between plant spread, inflorescence length (r = 0.29) and stem length (r = 0.319). The only other significant correlation was between number of leaves and length of inflorescence (r = 0.238).

Table 4.3 Simple correlation between the morphological traits in accessions of P. sidoides traits

Trait	Plant spread	Plant height	Leaf length	Leaf breadth	length of inflorescence	# of leaves/ plant	Stem length
Plant spread	1.000						
Plant height	0.440*	1.000					
Leaf length	-0.091	-.052	1.000				
Leaf breadth	0.112	-.011	0.034	1.000			
length of inflorescence	0.290*	0.878**	-0.014	0.048	1.000		
# of leaves/ plant	0.839**	0.387*	-0.051	0.179	0.238*	1.000	
Stem length	0.319*	0.335*	-0.068	-0.138	-0.150	0.312	1.000

**. Correlation is significant at the 0.01 level (2-tailed)

*. Correlation is significant at the 0.05 level (2-tailed)

Eigen vectors resulting from PCAs (Table 4.4) show that the most important characters in the PC are plant spread, plant height and length of inflorescence. The PCA revealed that the first three principal components exhibited Eigenvalues greater than 1 and explained 74.170% of the total variability, contributing to the morphological variation of the accessions established at the University of Zululand. According to the PCA, plant spread, plant height and length of inflorescence are the most important for classifying accessions within the populations. The quantitative variability exhibited indicates a good possibility for finding desirable traits to meet demands of researchers.

Table 4.4 Illustrating the first three principal components showing Eigenvalue greater than 1 and their contributions to the variability among accessions of P. sidoides studied

Component	Total	% of Variance	Cumulative%	Total	% of Variance
Plant spread	2.670	38.149	38.149	2.670	38.149
Plant height	1.358	19.403	57.552	1.358	19.403
Length of inflorescence	1.159	16.564	74.116	1.159	16.564
Number of leaves/plant	0.974	13.914	88.030		
Stem length	0.679	9.702	97.731		
Leaf length	0.156	2.228	99.959		
Leaf breadth	0.003	0.041	100.000		

4.1. 2 A dendogram of Cluster Analysis of *P. sidoides* and *P. reniforme* accessions

The dendogram of cluster analysis (Figure 4.5) was able to group the morphotypes into two major groups with each group having two sub-groups. Morphotypes encircled together are most similar, and formed a distinct sub group. Morphotypes of Group 1(long stalk, deep green, curled up and bigger leaves) Morphotypes Group 2 (long stalk, deep green ,uncurled and bigger leaves), Group 3 (long stalk, deep green, curled up and smaller leaves) and Group 4 (long stalk, deep green, uncurled and smaller leaves) and morphotypes Group 13 (short stalk, light green , uncurled and smaller leaves), and Group 12 (short stalk, light green, uncurled and bigger leaves) and Group 14 (short stalk, light green curled up and smaller leaves) and Group 15 (short stalk, light green, uncurled, smaller leaves) formed the most dissimilar subgroups whilst accessions Group 5 (long stalk, light green, curled and bigger leaves), Group 7 (long stalk, light green, uncurled and bigger leaves) and Group 6 (long stalk, light green,

curled and smaller leaves) and accessions Group 8 (long stalk, light green, uncurled and bigger leaves , Group 11 (short stalk, deep green, uncurled and smaller leaves), Group 9 (short stalk, deep green, curled and bigger leaves) and Group 10 (short stalk, deep green, uncurled and bigger leaves) being located in the intermediate area were the most similar. Morphotypes, Group 16 (*P. reniforme*) out lied from all the other groups.

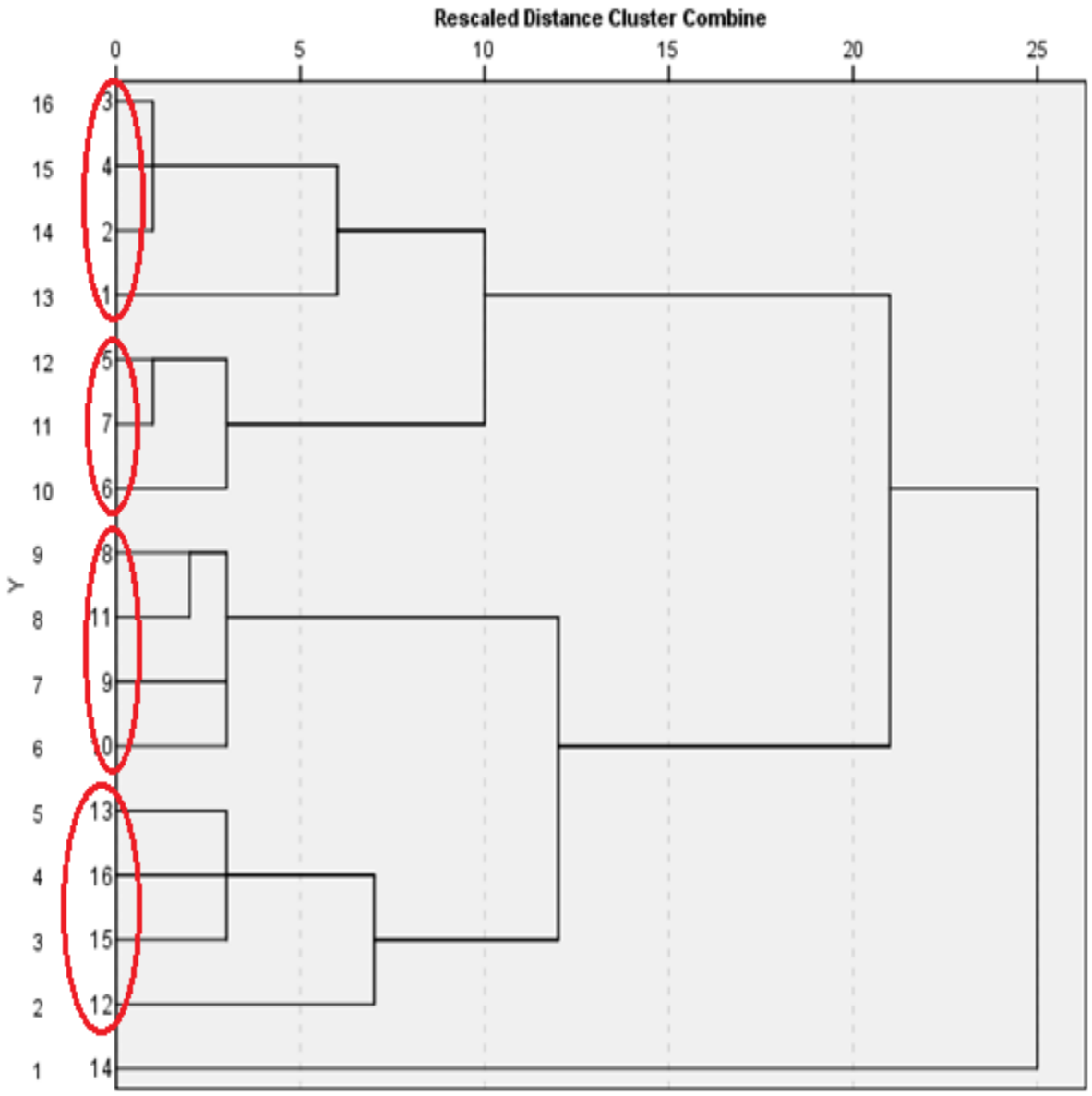
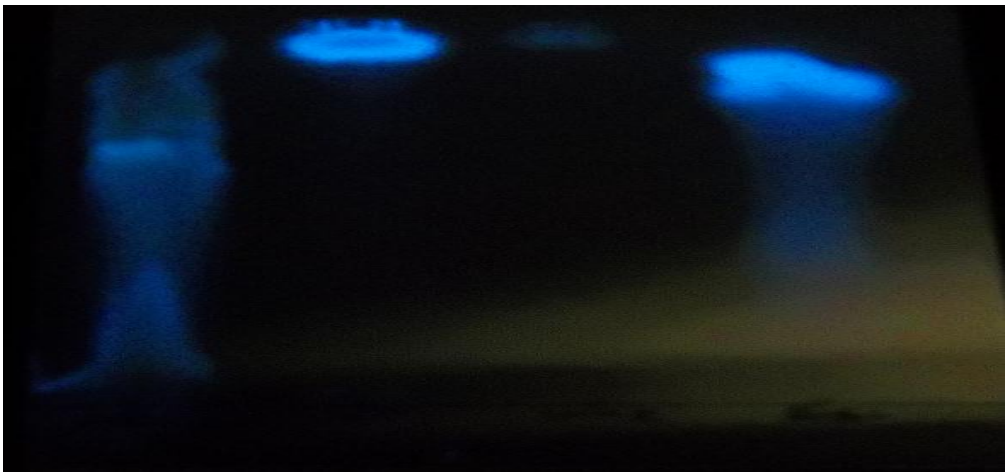


Figure 4.5 Dendrogram of cluster analysis of *P. sidoides and P. reniforme* accessions. The quantitative phenogram (Dendrogram) obtained from the cluster analysis is presented.

4.2 Coumarin analyses

TLC plates (Figures 4.6 – 4.8) of fractions obtained from the standards to confirm umckalin with the mobile phase consisting of Methanol: Water: Ethyl acetate (10:14:76) examined under Spetroline ultra violet light at 365nm. Esculin, Umckalin and Scopoletin

standard results is shown in **Figure 4.6**. TLC was performed for qualitative identification of the coumarins in the *P. sidoides* plants and the results confirmed the presence of standards within the plant extracts; however the fingerprint for the leaves was different from the roots. The TLC plates showed that the roots contained esculin, scopoletin and umckalin (coumarin derivatives), whilst the leaves contain only esculin and umckalin. The TLC plates showed clear and visible fluorescent for the root morphotypes compared to the TLC plates of the leaves. Within the root there seem to be no difference and no differences were observed within the leaves as well.



Esculin **Umckalin** **Scopoletin**

Figure 4.6 TLC plate of fractions obtained from the standards to confirm umckalin with Methanol: Water: Ethyl acetate (10:14:76) **examined under Spetroline ultra violet light at 365nm. Esculin, Umckalin and Scopoletin**

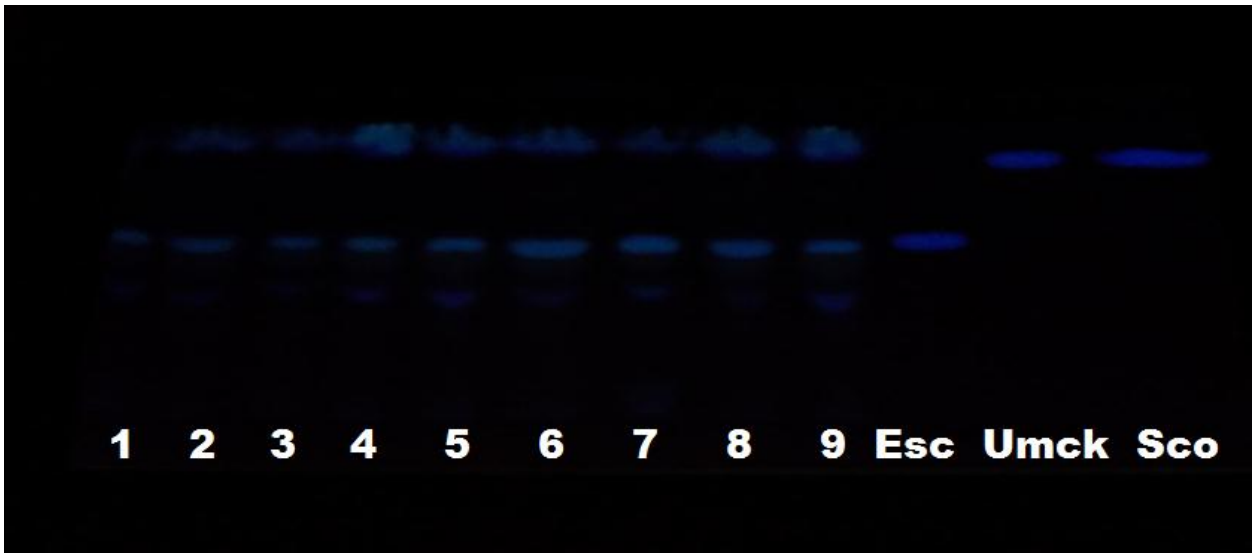


Figure 4.7 TLC plate of fractions obtained from *P. sidoides* leaves extracts with Methanol: Water: Ethyl acetate (10:14:76) examined under Spetroline ultra violet light at 365nm. Number 1-9 shows groups of different morphotypes groups, Escu is for esculin, Umcka is for umckalin and Scop is for Scopoletin. 1. LDCS 2.LDUB 3. LDUs 4. LlCB 5. LlUB 6. SDCB 7. SDUB 8. SLCB 9. SlUB . **L**-long stalks **S**-short stalks **D**-deep green leaves **l**-light green **C**-curled **U**-uncurled leaves **B**-bigger leaves **s**-smaller leaves

Figure 4.8 TLC plate of fractions obtained from *P. sidoides* root extracts with Methanol: Water: Ethyl acetate (10:14:76) examined under Spetroline ultra violet light at 365nm. Number 1-9 shows groups of different morphotypes groups, Esc is for esculin, Umck is for umckalin and Sco is for Scopoletin. 1. LDCS 2.LDUB 3. LDUs 4. LlCB 5. LlUB 6. SDCB 7. SDUB 8. SLCB 9. SlUB: L-long stalks S-short stalks D-deep green leaves l-light green C-curled U-uncurled leaves B-bigger leaves s-smaller leaves

4.2.1 HPLC (Qualitative)

The HPLC results showed the same profile on all roots samples regardless of the morphological variations that had been observed (See chromatograms in Appendix B). The chromatograms were eluting at same retention at time of 1.55 minutes (Figure 4.9). The peaks represent different compounds in the samples. Umckalin was detected from both the leaves and the roots samples. The chemical profiles of all the leaf samples are similar to each other but they slightly differ from that of the roots. The difference is apparently to concentrations of other compounds within the samples. There are some peaks that are visible on the leaves samples but not on the roots samples (**See chromatograms in Appendix B**). The HPLC results confirmed that the morphological variations observed in *P. sidoides* do not affect its chemical profile. The HPLC results also confirmed the TLC results because TLC results showed that Umckalin is visible on the roots samples and in the leaves.

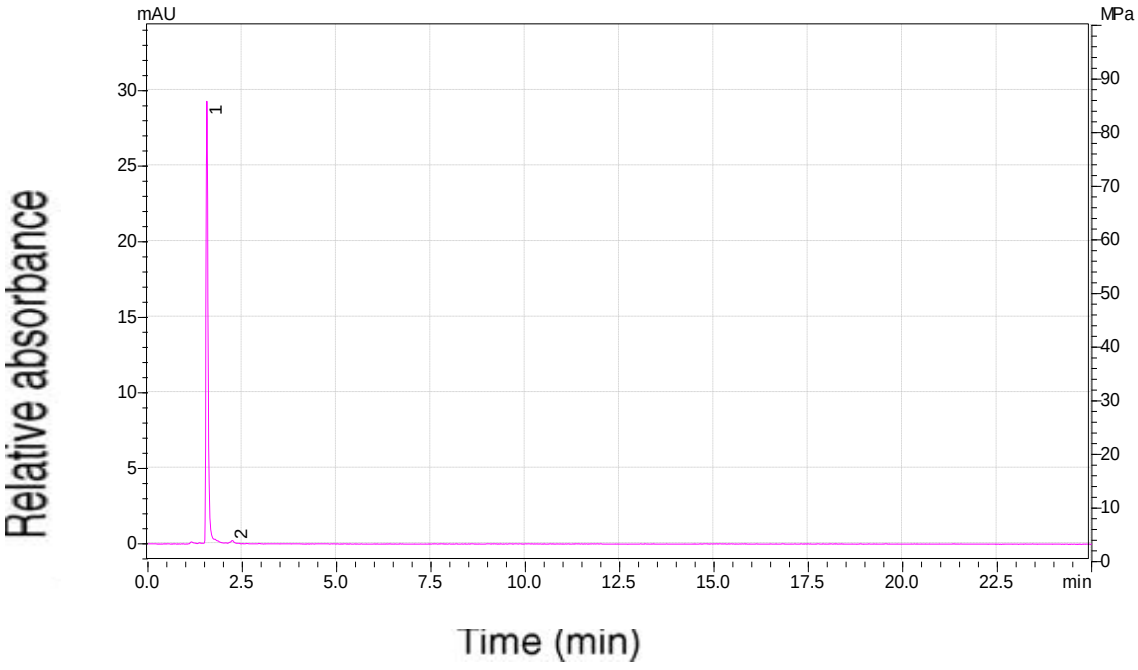


Figure 4.9 Retention times (RT) of umckalin standard absorption peaks of known concentration, in methanol, using reversed phase HPLC, on Luna 5µ C18 (2) 100 A 500x 4.60 column, equilibrated with MEOH: H_2O (9:1)

The yield concentration of umckalin in the leaves (**Table 4.6**) in all groups of morphotypes showed considerable differences compared to the root **(Table 4.7)**. Short stalk, Deep green, Uncurled and Bigger leaves (3.90mg/ml) and Short stalk, Deep green, Curled and Bigger leaves (3.53mg/ml) showed higher concentration of umckalin compared to the other groups of accessions with different morphotypes

Table 4.6 Umckalin content in the leaves of P. sidoides morphotypes.

Morphological characteristic	y-values, {mAU}	Conc, mg/ml
G1-LDCS	30.5	1.0
G2-LDUB	4.5	0.15
G3-LDUs	30	1.02
G4-LlCB	70	2.37
G5-LlUB	65	2.20
G6-SDCB	145	3.53
G7-SDUB	143	3.90
G8-SLCB	18	0.61
G9-SlUB	98	3.32

1. LDCs 2.LDUB 3. LDUs 4. LlCB 5. LlUB 6. SDCB 7. SDUB 8. SLCB 9. SlUB
L-long stalks S-short stalks D-deep green leaves l-light green C-curled U-uncurled leaves B-bigger leaves s-smaller leaves

Umckalin was identified using HPLC in the roots *P. sidoides* morphotypes. Groups of accessions of *P. sidoides* showed considerable variability in the production of morphological traits and phytochemical content. The recorded traits are presented in (Table 4.1). Among groups analyzed (Table 4.7), morphotypes with Long stalk, deep green, Uncurled and Smaller leaves (13,90mg/ml) were the most variable characteristics of accession. Beside accessions (Long stalk, Deep green, Uncurled and Smaller leaves) other groups of morphotypes (Long stalk, deep green Curled and Bigger leaves 9.38 and Long stalk, deep green, Uncurled and bigger leaves

9.49 mg/ml) showed good results. The Short stalk, light green, Uncurled Bigger leaves had the lowest concentration of Umckalin (4.41mg/m).

Table 4.7 Umckalin content in the roots of P. sidoides morphotypes.

Morphological characteristic	y-values, {mAU}	Conc, mg/ml
G1-LDCS	290	9.83
G2-LDUB	280	9.49
G3-LDUS	410	13.90
G4-LlCB	145	4.92
G5-LlUB	280	9.49
G6-SDCB	104	4.92
G7-SDUB	115	4.85
G8-SLCB	200	6.78
G9-SlUB	130	4.41

1. LDCS 2.LDUB 3. LDUs 4. LlCB 5. LlUB 6. SDCB 7. SDUB 8. SLCB 9. SlUB

L-long stalks **S**-short stalks **D**-deep green leaves **l**-light green **C**-curled **U**-uncurled leaves **B**-bigger leaves **s**-smaller leaves

4.3 Antimicrobial assay

Crude extracts used in this study were obtained from the leaves and roots of *P. sidoides.* The extractions were carried out using methanol solvent. The results showed that methanolic extracts of *P. sidoides* roots exhibited antimicrobial activities against the test bacterial strains (Figure 4.10A). The results of the general screening for antimicrobial activity are shown in Table 4.8 and Table 4.9. In this study, the highest inhibition was exhibited by the concentration of 5mg/ml methanol *P. sidoides* root extracts against *S. aureus*, the lowest inhibition was exhibited by the concentration of 10mg/ml methanol *P. sidoides* leaf extract against *Salmonella spp* KZN. The results of the study showed that root extracts from *P sidoides* morphotypes have a broad spectrum antimicrobial activity. Extracts from the leaves were less inhibitory to the growth of the tested microbial strains. The results for roots compare favorably with the standard antibiotics.

Table 4.8 Antimicrobial activity of methanolic extracts of leaves of P. sidoides

Parameter	5mg/ml	10mg/ml	Ampicillin	Cloxaccilin
1 Ss	10.75±3.10	9.0±2.60	19.0±5.48*	20.0±5.77*
Ec	11±3.18	12.50±3.61	19.5±5.63*	18.75±5.41*
Sf	11±3.18	10.25±2.96	18.5±5.34*	20.75±5.99*
Sa	11.5±3.32	11±3.18	19.75±5.70*	19.75±5.70*
2. Ss	11.5±3.32	9.5±2.74	19.0±5.48*	20.0±5.77*
Ec	11.25±5.25	10.75±3.10	19.5±5.63*	18.75±5.41*
Sf	11.5±3.32	10.75±3.10	18.5±5.34*	20.75±5.99*
Sa	11.25±3.5	11.5±3.32	19.75±5.70*	19.75±5.70*
3. Ss	12.0±3.46	8.0±2.31	19.0±5.48*	20.0±5.77*
Ec	10.75±3.10	9.5±2.74	19.5±5.63*	18.75±5.41*
Sf	9.25±2.67	10.25±2.96	18.5±5.34*	20.75±5.99*
Sa	10.75±3.10	9.25±2.67	19.75±5.70*	19.75±5.70*
4. Ss	12.0±3.46	11.0±3.18	19.0±5.48*	20.0±5.77*
Ec	11.25±3.25	11.5±3.32	19.5±5.63*	18.75±5.41*
Sf	10.75±3.10	10.5±3.03	18.5±5.34*	20.75±5.99*
Sa	10.5±3.03	10.75±3.10	19.75±5.70*	19.75±5.70*
5. Ss	11.5±3.32	10.0±2.89	19.0±5.48*	20.0±5.77*
Ec	11.5±5.25	10.25±2.96	19.5±5.63*	18.75±5.41*
Sf	10.5±3.03	10.25±2.96	18.5±5.34*	20.75±5.99*
Sa	10.25±2.96	10.5±3.03	19.75±5.70*	19.75±5.70*
6. Ss	11.0±3.18	11.5±3.32	19.0±5.48*	20.0±5.77*
Ec	11.25±3.25	10.5±3.03	19.5±5.63*	18.75±5.41*
Sf	11.25±5.25	12.0±3.46	18.5±5.34*	20.75±5.99*
Sa	11.5±5.25	11.25±3.25	19.75±5.7*	19.75±5.7*

The results were presented as means (mean±SEM) of percentage growth inhibition of duplicates. Values with * were significantly different at P<0.05 compared to the standard antibiotics (Ampicillin and Cloxacillin) according to the LSD. **1-6** represent groups 1. SDCB, 2.LDUB, 3.LDUS 4.LlUB 5.SLCB, 6.LDCB, **L**-long stalks **S**-short stalks **D**-deep green leaves **l**-light green **C**-curled **U**-uncurled leaves **B**-bigger leaves **s**-smaller leaves. Ss- Salmonella sp, Ec- *Escherichia coli* ATCC 8739, *Sf- Shigella flexineri* KZN, *Sa- Staphylococcus aureus* ATCC 6538

Table 4.9 Antimicrobial activity of methanolic extracts of roots of P. sidoides

Parameter	5mg/ml	10mg/ml	Ampicillin	Cloxaccilin
1 Ss	18.75±5.41	15.0±4.33	19.0±5.48*	20.0±5.77*
Ec	19.75±5.70	16.75±3.10	19.5±5.63*	18.75±5.41*
Sf	17.25±4.98	19.0±5.48	18.5±5.34*	20.75±5.99*
Sa	19.75±5.70	15.75±4.55	19.75±5.70*	19.75±5.70*
2. Ss	20.0±5.77	18.25±5.27	19.0±5.48*	20.0±5.77*
Ec	19.5±5.63	16.75±4.84	19.5±5.63*	18.75±5.41*
Sf	18±5.20	16.25±4.69	18.50±5.34*	20.75±5.99*
Sa	19.25±5.56	17±4.91	19.75±5.70*	19.75±5.70*
3. *Ss*	18.5±5.34	15.5±4.48	19.0±5.48*	20.0±5.77*
Ec	19.0±5.48	17.0±4.90	19.5±5.63*	18.75±5.41*
Sf	17.75±5.12	15.75±4.55	18.5±5.34*	20.75±5.99*
Sa	18.5±5.34	16.5±4.76	19.75±5.70*	19.75±5.70*
4. *Ss*	17.75±5.12	15.75±4.55	19.0±5.48*	20.0±5.77*
Ec	19.25±5.56	16.0±4.62	19.5±5.63*	18.75±5.40*
Sf	18.25±5.41	15.25±4.40	18.5±5.34*	20.75±5.99*
Sa	18.75±5.41	15.25±4.40	19.75±5.70*	19.75±5.70*
5. *Ss*	19.25±5.56	16.0±4.62	19.0±5.48*	20.0±5.77*
Ec	19.75±5.70	17.0±4.91	19.5±5.63*	18.75±5.41*
Sf	18.75±5.41	17.0±4.91	18.5±5.34*	20.75±5.99*
Sa	18.5±5.34	16.0±4.62	19.75±5.70*	19.75±5.70*
6. *Ss*	19.5±5.48	17.5±5.05	19.0±5.48*	20.0±5.77*
Ec	20.5±5.63	17.25±4.10	19.5±5.63*	18.75±5.41*
Sf	19.5±5.34	18.25±5.27	8.5±5.34*	20.75±5.99*
Sa	19.75±5.70	17.75±5.12	19.75±5.70*	19.75±5.70*

The results were presented as means (mean±SEM) of percentage growth inhibition of duplicates. Values with * were significantly different at P<0.05 compared to the standard antibiotics (Ampicillin and Cloxacillin) according to the LSD. **1-6** represent groups 1. SDCB, 2.LDUB, 3.LDUS 4.LlUB 5.SLCB, 6.LDCB, **L**-long stalks **S**-short stalks **D**-deep green leaves **l**-light green **C**-curled **U**-uncurled leaves **B**-bigger leaves **s**-smaller leaves. Ss- Salmonella sp, Ec- *Escherichia coli* ATCC 8739, *Sf- Shigella flexineri* KZN, *Sa- Staphylococcus aureus* ATCC 6538

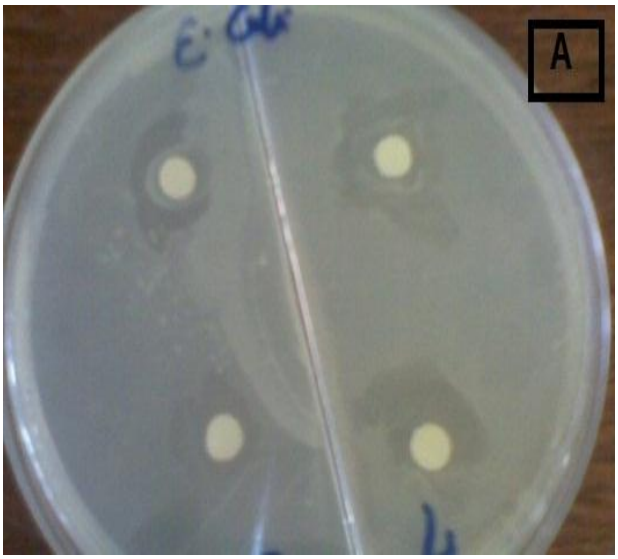


Figure 4.10 (A) Photographs showing the inhibition zones

Minimum inhibitory concentration

The results of the minimum inhibitory concentration of methanolic extracts of *P. sidoides* morphotypes are shown in Table 4.10 and Table 4.11. All the extracts demonstrated activity against the gram positive and gram negative bacteria tested in this study. The leaf extracts showed more activity against gram negative bacteria, *E. coli* compared *S. flexineri* with lowest concentration of 1.5 mg/ml. The MIC of the leaves ranged from 1.5 to 5mg/ml whereas MICs of the roots ranged from 3.5 to 8mg/ml. All the plants showed activity against the bacterial strains.

Table 4.10 Minimum inhibitory concentrations from leaves plant extracts of selected morphotypes of P. sidoides

Bacterial sp.	**Gram +/-**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**
Salmonella spp.	**+**	5	5	5	2.5	5	5
***Escherichia coli* ATCC 8739**	**-**	1.5	1.5	2.5	2.5	5	2.5
***Shigella flexineri* KZN**	**-**	5	5	5	1.5	1.5	2.5
***Staphylococcus aureus* ATCC 6538**	**-**	5	2.5	5	2.5	5	5

Table 4.11 Minimum inhibitory concentrations from roots plant extracts of selected morphotypes of P. sidoides

Bacterial sp.	**Gram +/-**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**
Salmonella spp.	**+**	7	5	5	5.5	7.5	8
***Escherichia coli* ATCC 8739**	**-**	4.5	4.5	5.5	3.5	5	3.5
***Shigella flexineri* KZN**	**-**	5	6.5	5	4.5	5.5	5
***Staphylococcus aureus* ATCC 6538**	**-**	8	2.5	7.5	2.5	5	7

The minimum bactericidal concentration (MBC)

The MBC of leaves and roots was <10 mg/ml.

4.4 Antidiarrheal activity

The general condition of the animals, such as alertness, movement and activity were normal. There was neither sedation nor excitation. In all rats, there were no differences in the effects of the plant extracts based on their morphotypic characters. Evaporated methanolic extract dissolved in CMC of *P. sidoides* morphotypes showed similar effects in cases conducted. In the castor oil induced diarrhea experiment, the rats that did not receive the *P*. *sidoides* plant extracts showed typical diarrheal signs, stools were too wet. *P. sidoides* extracts inhibited castor oil induced diarrhea in *Sprague-Dawley* rats at doses of 100, 200 and 400mg/kg. The weight of feacal pellets and extracts treated groups showed lower diarrheal severity than control rats. **Figure 4.11- 4.14** shows that within four hours after castor oil administration, rats treated with *P. sidoides* extract (400mg/kg) had less severe diarrhea in comparison with the control rats.

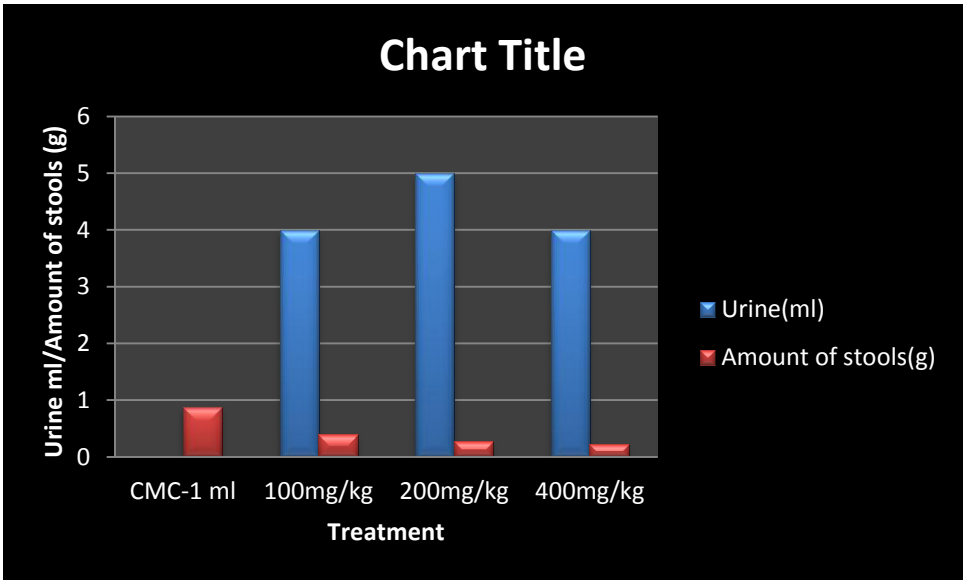


Figure 4.11 The effect of methanolic extract of morphotypes LDCB of *P. sidoides* on mean weights of wet feacal pellets of *Sprague-Dawley* rats with castor oil induced diarrhea. (LDCB = **L**-long stalks **D**-deep green leaves **C**-curled leaves **B**-bigger leaves).

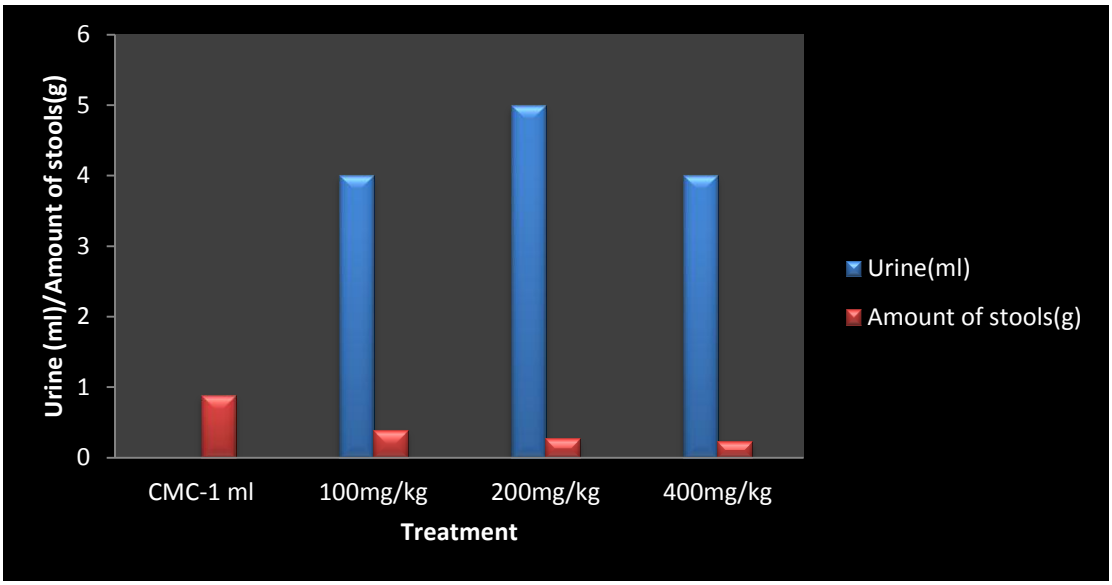


Figure 4.12 The effect of methanolic extract of morphotype LDUS of *P. sidoides* on mean weights of wet feacal pellets of *Sprague-Dawley* rats with castor oil induced diarrhea. (LDUS = **L**-long stalks **D**-deep green leaves **U**-uncurled leaves **s**-smaller leaves)

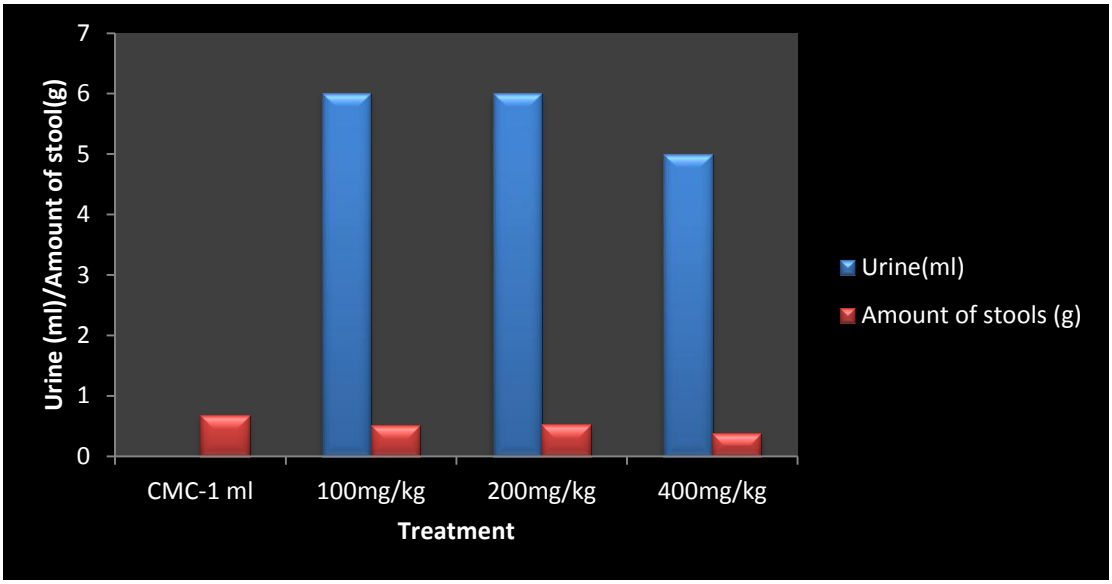


Figure 4.13 The effect of methanolic extract of the morphotype LDUB of *P. sidoides* on mean weights of wet feacal pellets of *Sprague-Dawley* rats with castor oil induced diarrhea. (LDUB = **L**-long stalks **D**-deep green leaves **U**-uncurled leaves **B**-bigger leaves)

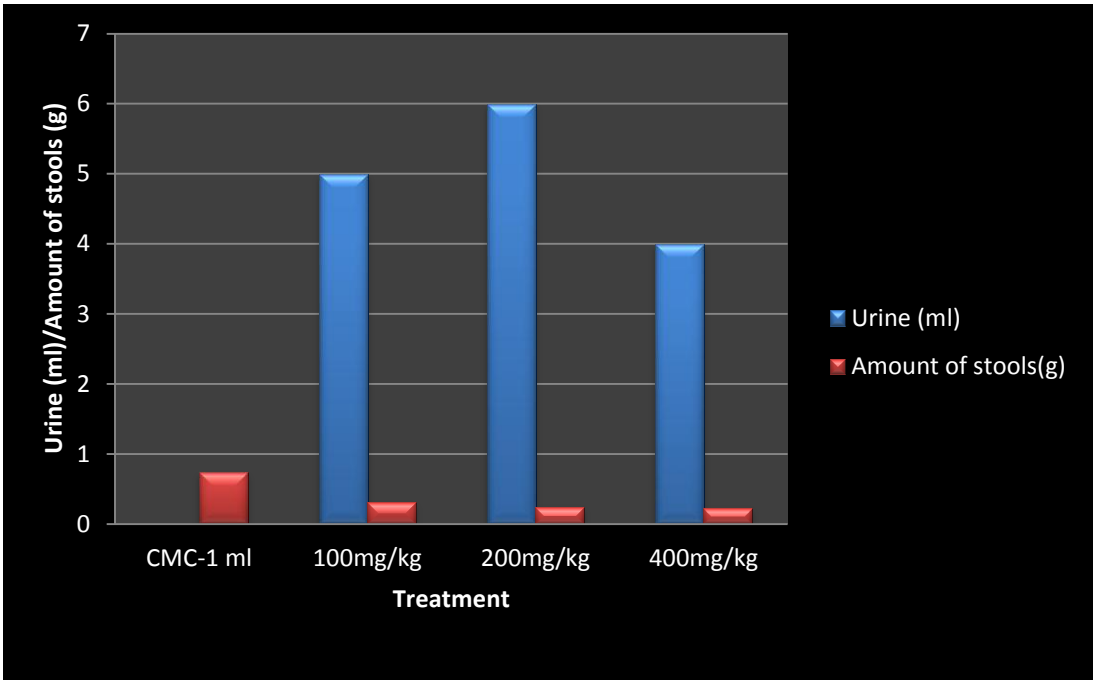


Figure 4.14 The effect of methanolic extract of the morphotypes LIUB of *P.sidoides* on mean weights of wet feacal pellets of *Sprague-Dawley* rats with castor oil induced diarrhea. (LIUB = **L**-long stalks **I**-light green **U**-uncurled leaves **B**-bigger leaves)

CHAPTER 5

5. DISCUSSION

Classification and quantification of morphological variation is vital for researchers, plant breeders, germplasm curators and farmers. Before the objective of plant breeding is pursued in any species, basic information on existing morphological variability in the cultivated species and their wild relatives is essential (Adebola and Morakinyo, 2006). Morphological traits analysis is convenient for simple, fast, preliminary and inexpensive accessions identifications and can be used as a general method for evaluating genetic diversity among accessions.

In medicinal plant cultivation, the use of plant breeding techniques and selecting stock with high bioactivity can provide plants that are genetically uniform, high-yielding, and that produce a less variable final product (WHO *et al*., 1993). Selecting high-yielding stock is possible as the yield and composition of secondary metabolites of certain medicinally important plants has been found to vary within and between populations (Van Wyk *et al*., 1997; Wills and Stuart, 1999; Homer *et al*., 2000; Binns *et al*., 2002; Kim *et al*., 2006).

There are two interlinked options that would promote the conservation of wild *P. sidoides* populations for future use, to the benefit of people and the biodiversity of the natural environment. The first option would be to develop and implement sustainable harvesting practices for wild populations. The second would be to effectively cultivate *P. sidoides* plants in order to supply local and international demand, thus reducing the harvest of roots from the wild (WHO *et al.*, 1993). A combination of good cultivation and wild harvest practices may provide for the sustainable harvest of *P.sidoides* in the future.

The high variation among quantitative characters measured in studied germplasms indicated that, a good possibility exists of finding desirable traits to meet the demands of both researchers and farmers interested in the development of promising *cultivars of P. sidoides*. The encouragement of vegetative propagation of *P. sidoides* could serve as a viable option for the *ex situ* conservation of this plant.

The possibility of propagating *P. sidoides* through the use of its vegetative aerial parts also exists. Aerial parts of the plant are thrown away and wasted during the collection of the roots for medicinal use and trade. Lewu *et al*., (2006) conducted a study determine the appropriate plant part and the minimum length suitable for the clonal propagation of *P. sidoides*. The results revealed that the 6 cm vine length exhibited best results and petioles generally showed high potential for vegetative propagation in terms of rooting ability and survival rates.

Observations from the study have shown likely variations among germplasms collected from ARC-VOPI nursery, where the morphotypes with red and deep purple petals resulted. In some areas of the Eastern Cape *P. sidoides* is found growing together with a similar looking Pelargonium, P. reniforme. The two species differ mainly in flower colour, the latter having light pink petals while the former has deep purple petals. When harvesting takes place in the non-flowering season in areas where both species occur, harvesters are unable to distinguish between the two species and harvesting of both species may occur (Newton 2004; Lewu 2007; White 2007).

The principal component analysis revealed that the first three principal components of which were plant spread, plant height and length of inflorescence exhibited Eigenvalues greater than 1 and explained 74.170% of the total variability, contributing the entire variable to the morphological variation of the accessions established at the University of Zululand. The eigenvectors resulting from PCA can be used to identify the characteristics that most significantly contribute to classify individuals (Sheath and Sokal, 1973; Van Hintum 1995; Lewu *et al*., 2007). Lewu *et al*., 2007 studied morphological diversity among accessions of *P. sidoides* in the Eastern Cape. Some of the accessions produced large number of leaves but the numbers of leaves were few in the plants and some accessions produced many smaller leaves per plant. Some accessions had a peltate leaf shape while some had peltately-digitate leaf shape. Their study revealed that accessions exhibit high production of foliage and could be cultivated by clonal propagation to meet the increasing demand for the species in order to take pressure off wild stock.

Comprehensive chemical studies of underground and aerial parts of *P. sidoides* were done by Kolodziej in 2007. From these studies the medicinal properties are ascribed to at least eight different coumarins, of which umckalin and 5,6,7-methoxycoumarin are known to be useful marker compounds (Kayser and Kolodziej, 1994; Kayser and Kolodziej, 1995; Kayser and Kolodziej, 1997; Kolodziej and Kayser, 1998 and Kayser *et al*., 2001).

The differential synthesis of umckalin by plants cultivated under the same conditions but originating from different areas suggests a genetic rather than an environmental control of production. Furthermore, the genetic control of umckalin production may be influenced by plant adaptation to the climate of the site of origin.

Morphological variation can lead to morphological differences. This is supported by a study conducted by Paim *et al.* (2010) reported, differences in morphological characteristics resulting in differences in phytochemical content in the species *Calendula officinalis.* They reported that plants with tubular flowers and a yellow centre (TYC), and tubular flowers with brown centre (TBC) produced higher levels of flavonoids (1.41 and 1.44% respectively) when compared to ligulate flowers with a yellow centre (LYC) and ligulate flowers with a brown centre (LBC) (0.89 and 0.95% respectively).

Another example where morphological variation resulted difference in chemical content is the work done on *Halophila ovalis* (McMillan 1983) known for its antioxidant properties showed drastic variations in flavonoids compounds from accessions with different morphological characteristics. Plants with larger leaves and or more pairs of pairs of cross veins lacked these compounds. The production of sulfated flavonoids has not been shown to be environmentally controlled in *H. ovalis.* Plants with cross veins in the blades produced sulfated flavonoids, but plants with smaller veins and or fewer did not produced sulfated flavonoids.

Kolodziej *et al*. (2003) reported that extracts of *P. sidoides* showed significant activity against multi-resistant *Staphylococcus aureus* strains. They also presented evidence to suggest that *P. sidoides* extracts possess antimycobacterial activity as claimed in the traditional use for the treatment of tuberculosis. These pathogens are primarily responsible for numerous respiratory tract infections. The crude *P. sidoides* extracts were found to be moderately active against the tested bacteria. Methanol extracts of *P. sidoides* were investigated for antimicrobial activity against 10 bacterial (*S. aureus, M. kristinae, S. pyogenes, E. coli, S. pooni, P. aeruginosa, K. pneumonia)* by Lewu *et al*. (2006a).

Extracts obtained from roots demonstrated a high significant activity against all the bacteria tested compared with the leaves extracts in this study. Results obtained revealed that the methanol extracts show the presence of coumarins in both leaves and roots of *P. sidoides*. In the study conducted by Lewu *et al*., (2006) both the shoot and the roots of *P. sidoides* generally had antimicrobial properties. There was no significant observable difference between the MIC of extracts from the aerial and underground parts. The result suggests that compounds identified in the roots of the plant may be similarly present in the leaves but with different concentrations of the active compounds.

In the present study, methanol was used as an organic solvent for extraction as in the previous studies on *P. sidoides*, its extracts showed significant antimicrobial activity against the pathogens causing microbial infections (Ahmad *et al*., 1998; Lewu *et al*., 2006; Adewusi and Afolayan, 2009; Ozblige *et al*., 2010;) and water was excluded for extraction as previous reports by (Meyer and Afolayan, 1995; Masika and Afolayan, 2002) suggested that water extracts give little inhibition or no significant effect against bacterial species tested.

In this present study, the methanolic extracts of *P. sidoides* demonstrated antidiarrheal activity against experimentally induced diarrhea of *Sprague-Dawley* rats. At doses of 200-400 mg/kg, the plant extract inhibited castor oil induced diarrhea. The obtained results validate the traditional usage of *P. sidoides* in the management and treatment of diarrhea. The ability of the plant extract of this medicinal plant is a characteristic to the

phytochemical present in the plants as supported by the literature (Longanga *et al*., 2000 Boominathan *et al*., 2005 and Dahiru *et al*., 2006 , and

Previous studies have shown antidiarrheal properties of plants are due to phytochemicals (Longanga *et al*., 2000) and these phytochemicals (tannins, flavonoids and alkaloids, triterpenes and saponins) found in the roots of *P. sidoides* which may be responsible for the mechanism of action of *P. sidoides* against antidiarrheal activity. Many plant compounds found in the medicinal plants are known to possess antispasmodic effects, gastrointestinal transit and reduce electrolyte secretion (Palombo, 2006).

The results presented here offer favorable prospects for the commercial cultivation of *P*. *sidoides* to supply the international and local markets. Plants with high root umckalin concentrations can be selected for further production. The results of the study also provide evidence for the potential of plant part substitution of *P. sidoides*, though the MIC of morphotypes leaves were not as high as in roots extracts but the leaves extracts also indicated activity. The substitution of roots by leaves would go a long way towards the conservation of P. *sidoides*. When leaves are harvested the plant remains intact to continue with regeneration thereby ensuring the survival of the species. The use of roots cause the destruction of plants resulting in the present problems with unsustainable harvesting already facing P. *sidoides*. However, further investigations and clinical trials need to be performed before this substitute can replace root extracts in the market. In their studies comparing the phytochemical content and biological activity of various parts of four important and threatened South African medicinal plants, Zschocke *et al*. (2000) and Lewu *et al*., (2006) strongly recommended plant part substitution.

Several pharmacological investigations have been conducted to determine efficacy. The demand is increasing year by year and this situation warrants further scientific research to develop both agricultural and medical uses. Research on medicinal plants should be focused primarily on species whose pharmaceutical activities have already been demonstrated.

CHAPTER SIX

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The following conclusions can be drawn from this study:

1. The accessions of P. *sidoides* evaluated in this study showed considerable and significant differences in morphological traits.

2. All the morphotypes of P. *sidoides* used in this study proved to have phytochemical activity and contained coumarins and other active compounds.

3. Construction and investigation of phytochemical fingerprints of *P. sidoides* morphotypes by means of thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) revealed the existence of umckalin between the morphotypes of *P. sidoides.*

4. The variants utilised in this study seem to have similar compounds and could be all utilized in future research on cultivation practices. Morphotypes with long stalk, deep green, uncurled and smaller leaves were the most variable characteristics of accession. Accessions with long stalk, deep green, uncurled and smaller leaves, morphotypes with long stalk, deep green curled and bigger leaves and long stalk, deep green, uncurled and bigger leaves showed good results of umckalin content compared to the other morphotypes and that is why they can be further used for propagation programmes. The morphological variation of *P. sidoides* plants showed differences in the MIC, but the roots extracts were more active than the leaves' extracts.

5. The results indicate that the nine selected and tested morphotypes of *P. sidoides* did not differ in terms of their activity and can thus all be used since they possess anti-microbial activities. *P. sidoides* may be a potential source of antimicrobial compounds which could be used to treat infections caused by these pathogens.

6. Roots extracts of the four the *P. sidoides* morphotypes tested had anti-diarrhoeal effect in *Sprague-Dawley* rats.

.6.2 Recommendations for future studies

The following recommendations are suggested based on the results of this study:

1. Further studies on the efficacy and concentrations of leaf extracts need to be conducted urgently. If leaves can substitute for roots in the extraction of umckalin and other important compounds, this would help in conservation efforts by preventing the digging up of plants.

2. Genetic differences and the age of the plant material can also be a cause of species phytochemical content variation. Further research is necessary so as to determine the extent of the influence of these other factors.

3. When the scope of this present work is extended, it will be obligatory to confirm and analyze the identity of other key phytochemical (other types of coumarins) by isolation procedures.

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LIST OF APPENDICES

APPENDIX A

I. Morphological characterization recording sheet

Accession #	Plant spread	Plant height	Leaf length	Leaf breadth	length of inflorescence	# of leaves/ plant	Stem length
8	31	50	2.5	2	37.8	32	12.2
18	33	56	3	3	44	44	12
20	22	53	2	2	39	36	14
25	29	61	3	3	45.8	38	15.2
49	27.5	58	4	2	44.6	41	13.4
71	30	54	3	3	42.5	34	11.5
117	28	55.5	2.3	3	41.5	39	14
144	32	53	3	2.54	39.5	40	13.5
170	27	56	2.4	3	40.9	38	15.1
198	31	52	3	2.5	39.5	40	12.5
199	30	57	3.1	3	41.7	35	15.3
218	32	60.5	2.5	3	46.2	40	14.3
238	26	51.3	3	2	35.3	33	16
173	19	54.5	4	2.5	41	36	13.5
178	17	54.3	3	3	39.8	29	14.5
3	15	31	2.5	3	24	31	7
4	22	34	3	2	25.9	31	8.1
10	20	33	3.2	3	26.5	28	6.5
22	18	33.5	3	2	25.5	30	8
83	22	31.6	4	3	24.1	31	7.5
110	17	32	2	4	24.5	25	7.5
208	15	33	3	3	25	28	8
5	31	56	3	3	43.8	42	12.2

11	26	54	4	4.2	42	38	12
16	31.5	58	2	2.3	44	40	14
29	30	56	3	2	40.8	42	15.2
52	31	52	4	2	38.6	39	13.4
62	25	57	3	2.5	45.5	36	11.5
73	28	60.5	3	3	46.5	38	14
101	30.4	58	4	2	44.5	41	13.5
105	29	55.5	3	2	40.4	34	15.1
112	28	59	2.5	3	46.5	39	12.5
121	34	60.5	3	2	45.2	40	15.3
125	30	53	2.5	3	38.7	38	14.3
131	31	61	3	3	45	40	16
155	33	58	3.5	3.5	45.5	46	12.5
157	32	54	2	2	40.5	42	13.5
164	23	56	2	2	41.5	38	14.5
167	29.5	52	2.5	2.5	36	40	16
187	24	57	3	3	44.5	35	12.5
209	30.5	60.5	2.5	2	49	40	11.5
210	23	53	3	3	38.7	33	14.3
224	34	61	2	2	48.7	43	12.3
231	27	58	2	3	45.5	38	12.5
2	33	53	3	2.5	36	43	17
6	29	61	2.5	2	46.5	39	14.5
9	32	58	4	2	42	44	16
17	27	54	2	2.5	41.5	39	12.5
19	30.4	56	3	3	42.8	40	13.2
24	26	52	4	3	37	38	15
33	32.5	60.5	3	4	45.3	46	15.2
37	30	53	3	3	36	42	17
46	26	61	4	3	47.6	38	13.4

47	33	58	3	4	45.5	40	12.5
48	28	54	2.5	3	40	38	14
53	30.5	56	3	2.5	42.5	42	13.5
54	29	52	2.5	3	37	38	15
56	30.2	58	3	2.5	45.5	40	12.5
57	27	56	3	3	42	35	14
66	28	52	2.5	4	38.5	38	13.5
69	30.2	57	3	3	40	42	17
95	27	60.5	2.5	3	46	39	14.5
97	35	58	3	4	45.5	44	12.5
99	28	55.5	3.5	3	41.5	39	14
108	33	59	2	2.5	44.7	40	14.3
115	30	60.5	2	3	44.5	38	16
116	30.2	53	2.5	2.5	40.5	41	12.5
118	36	61	3	3	48	46	13
119	34.5	58	2.5	4	42	42	16
128	30	54	4	2	42	38	12
132	34	56	2	3	40.7	40	15.3
134	28	52	3	4	37.7	35	14.3
138	27.5	57	3.5	3	41	38	16
141	34	54	2	3	41.5	42	12.5
145	30	56	2	4	42.5	39	13.5
150	32	52	2.5	3	40.5	40	11.5
153	27	60.5	4	2.5	45.5	39	15
154	36	53	2	3	39	48	14
159	32	61	3	2.5	45	43	16
161	30.5	58	4	3	45.5	41	12.5
168	26	54	2.5	2.5	40.5	34	13.5
171	28	56	3	3	41	39	15
174	33	52	2.5	2	36.7	38	15.3

176	30	58	4	3	42	42	16
177	34	56	2	4	43.8	46	12.2
184	30.5	52	3	3	40	42	12
185	32.5	57	3.5	3	43	45	14
186	30	60.5	3	4	45.3	42	15.2
191	29	58	3	3	45	39	13
196	30.2	55.5	4	2.5	44	40	11.5
197	28	61	3	3	46	39	15
204	32.5	56	2.5	2.5	42.5	48	13.5
213	30.4	52	3	3	36.9	43	15.1
215	30.2	57	2.5	4	44.5	41	12.5
216	28.5	56	3	2	40.7	38	15.3
219	33	52	4	3	40	41	12
220	35	57	2.5	4	41	46	16
229	32	54	3	3	41.5	42	12.5
233	30	56	2.5	3	43	38	13
234	32.5	52	4	4	38.6	40	13.4
235	28	60.5	2	3	49	35	11.5
236	29.5	53	3	2.5	39	38	14
237	36	55	2.5	3	40	44	15
26	28	58	3	4	43	36	15
38	34.5	55.5	2.5	3	38.5	40	17
50	27	61	3	2.5	45.8	35	15.2
51	27.5	56	2.5	3	42	38	14
61	34	52	3	2.5	36.5	44	15.5
100	27	57	3.5	3	43.5	36	13.5
102	32	56	2	4	40	41	16
104	29	62	2	3	46.7	39	15.3
113	30.5	56	2.5	2.5	38	40	18
122	29.5	52	4	3	35.7	39	16.3

124	35	60.5	2	2	43.5	48	17
126	30.5	61	3	3	44.5	43	16.5
127	32	58	4	4	46	41	12
130	31	54	3	3	38	38	16
142	30.2	56	3	3	40.5	41	15.5
148	33	52	4	4	40.5	46	11.5
156	28	58	3	3	45	42	13
158	29	56	2.5	2.5	40	38	16
179	34	52	3	3	37.5	40	14.5
193	26	57	2.5	3	43.5	35	13.5
195	28	56	3	2.5	42	32	14
200	30	52	3	3	37	36	15
202	31.5	60.5	4	2	48	35	12.5
203	32	61	3	3	45	41	16
211	28	58	2.5	4	45	38	13
212	30.5	54	3	3	41.5	41	12.5
232	36	56	2	3	41	46	15
14	19.5	54.5	2	2.5	50	22	8
27	18	58	4	3.5	50.8	24	9.2
28	20	60	3	2.5	51.7	26	8.3
60	37	53	3	3.2	45	44	8
68	29	61	2.5	3	51.8	36	9.2
85	28	58	3	4	50.7	38	7.3
103	33	54	2.5	3	45.5	41	8.5
111	27.5	58	3	2.5	50.5	34	7.5
143	27	56	4	4	47	39	9
149	30	52	3	3	44.8	38	7.2
165	30.5	57	2.3	2.5	48.5	42	8.5
189	34	60.5	3	3	51.5	46	9
194	32	58	2.4	2	49.8	42	8.2

217	33	55.5	3	3	48	45	7.5
225	30.5	59	2	4	51	42	8
228	28	60.5	3	3	53.3	39	7.2

P. sidoides with purple flowers plant in the field

P. reniforme with pink flowers plant in the field

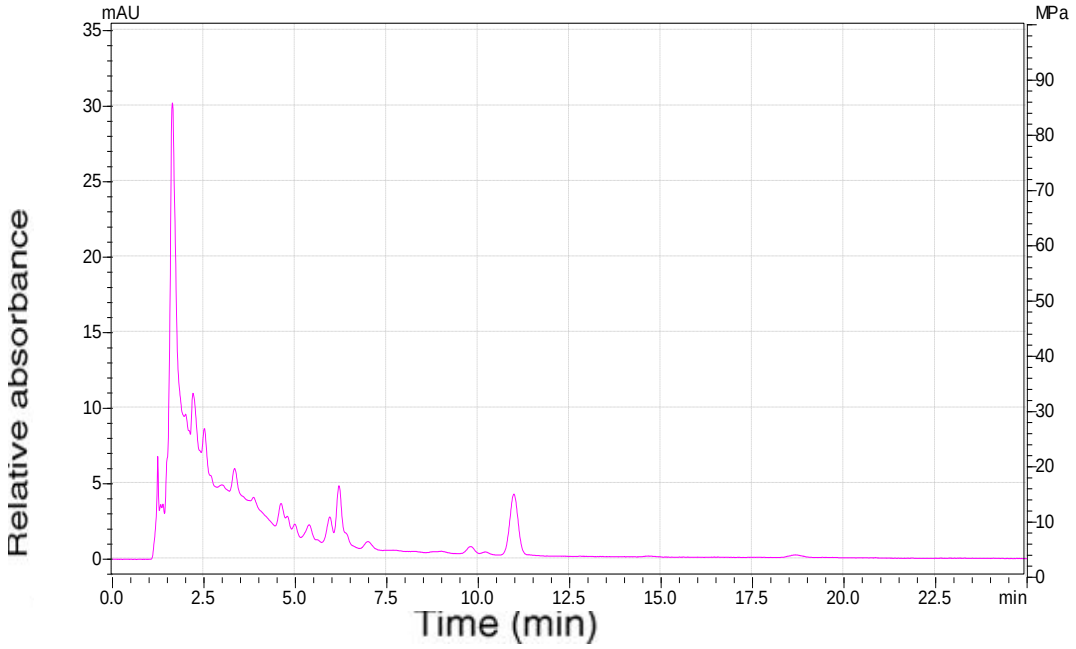
APPENDIX B

II. Chromatographs

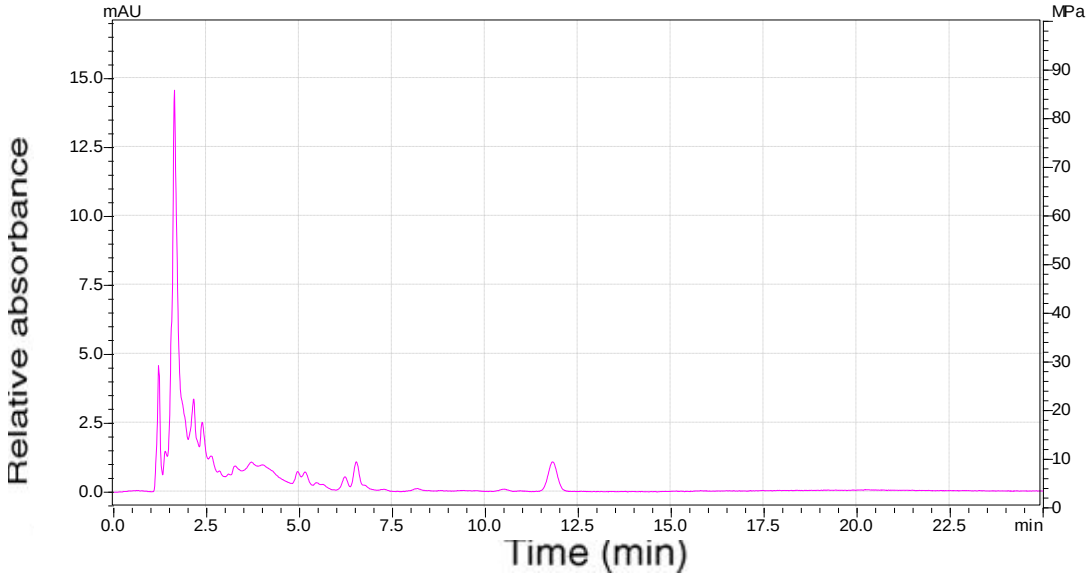
Umckalin

Pelargonium sidoides leaves

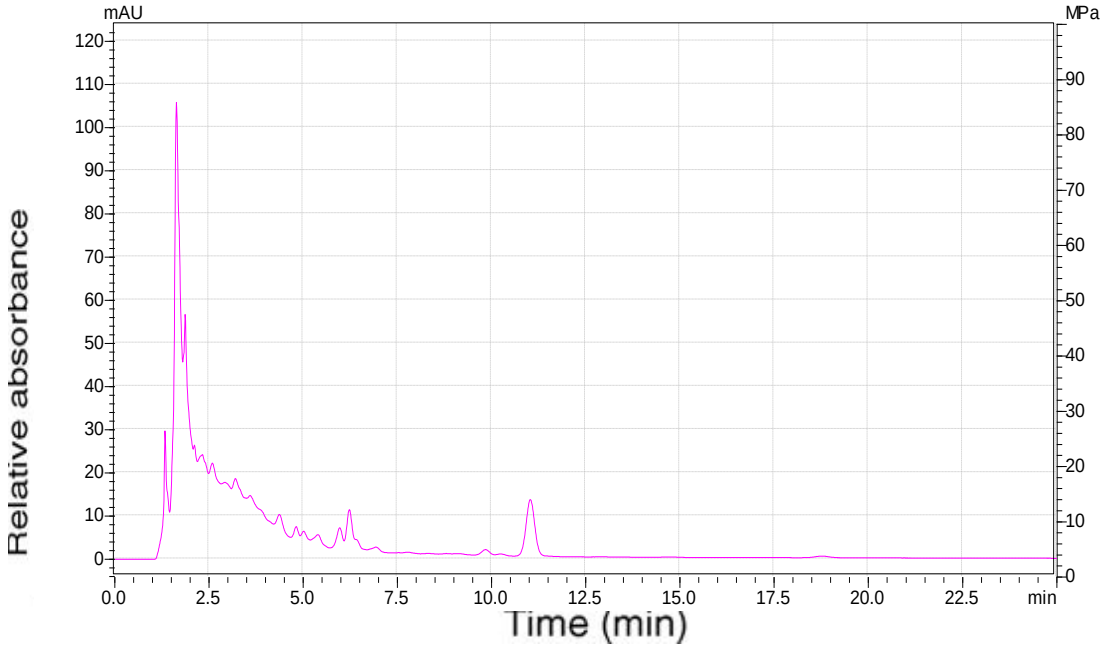
(a) 1L



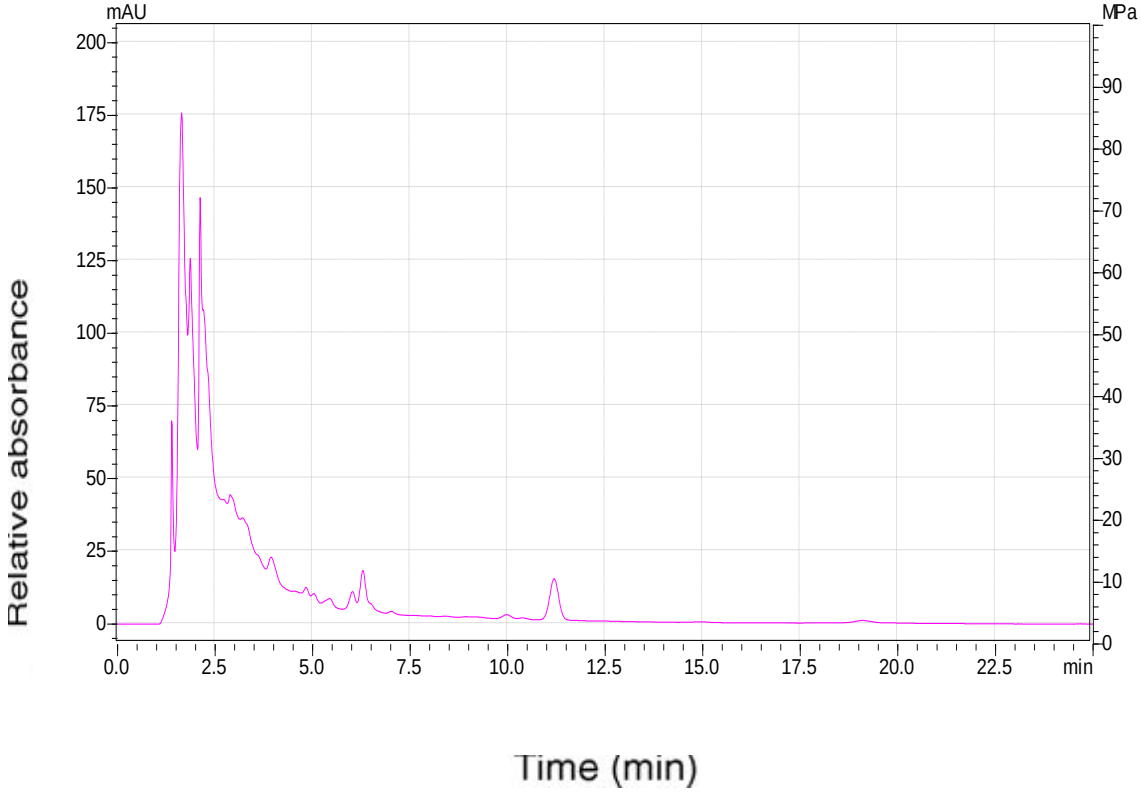
(b) 2L



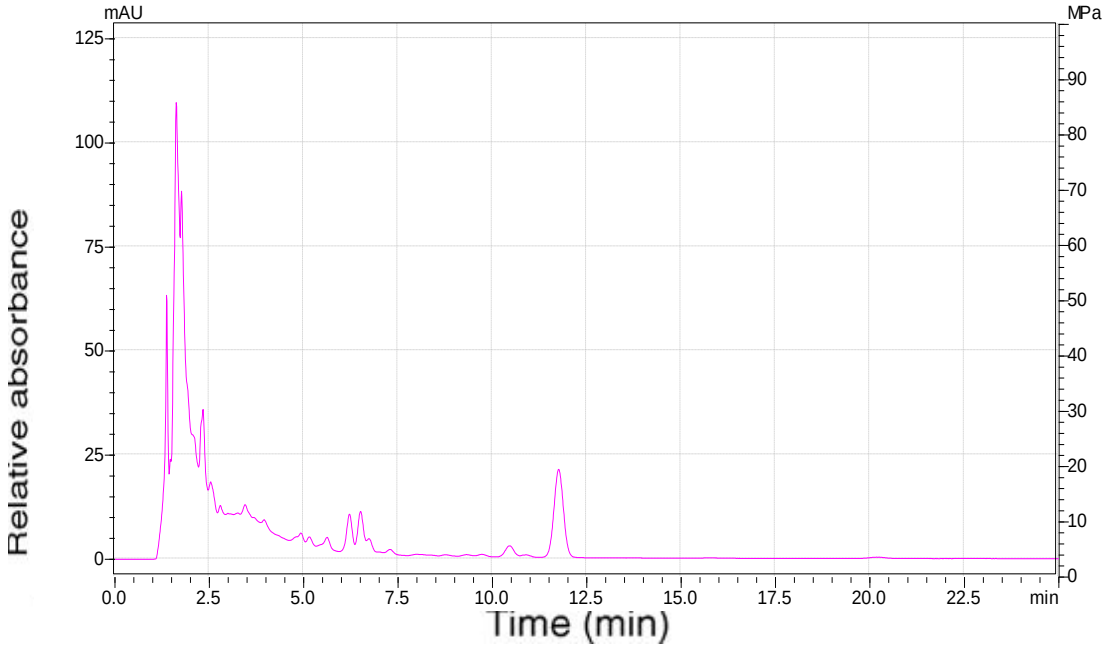
(C)3L


mAU
MPa
Relative absorbance
Time (min)
min

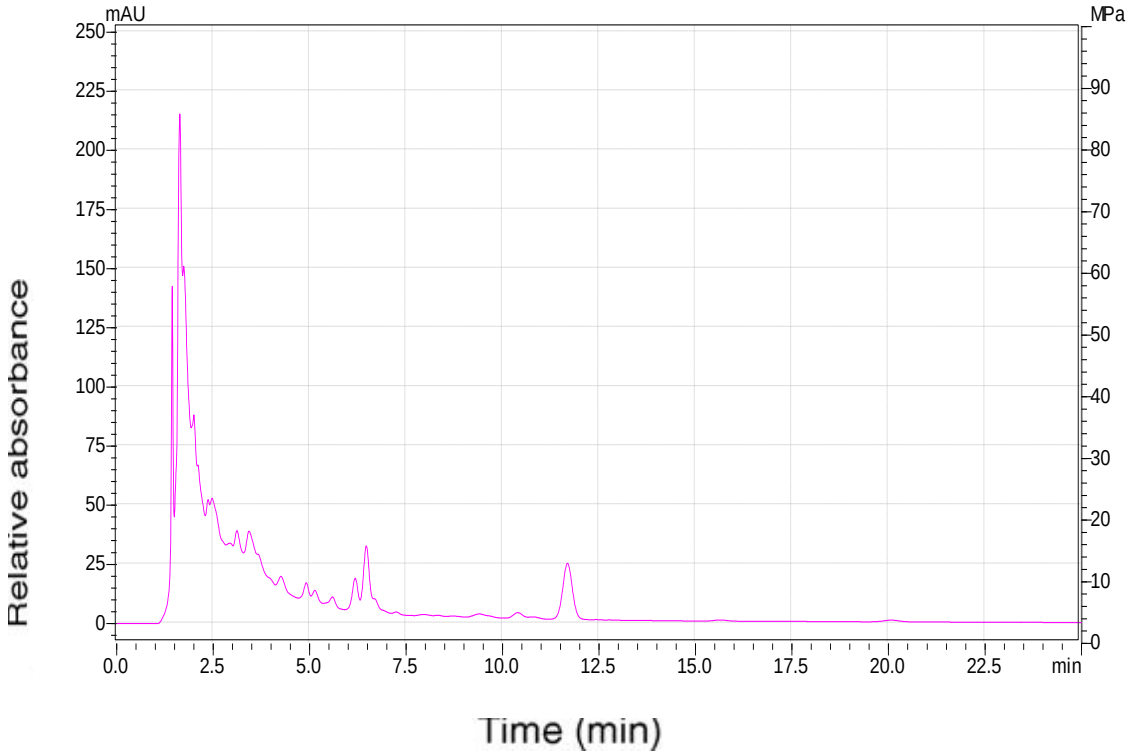
(c) 4L


mAU
MPa
Relative absorbance
Time (min)
min

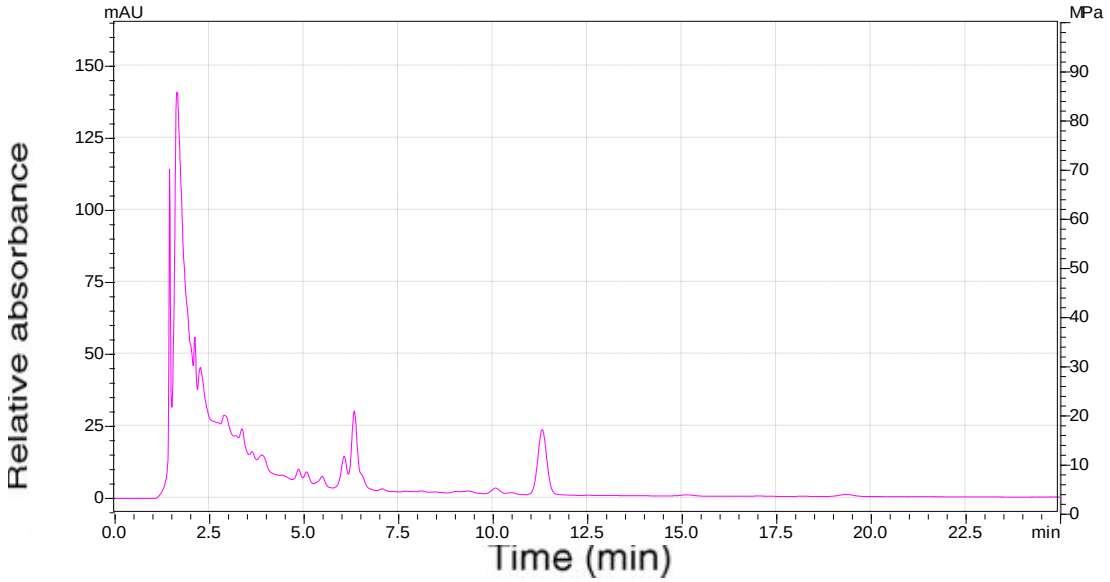
(E) 5L


mAU
MPa
125
100
75
50
25
0
90
80
70
60
50
40
30
20
10
0
Relative absorbance
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min
Time (min)

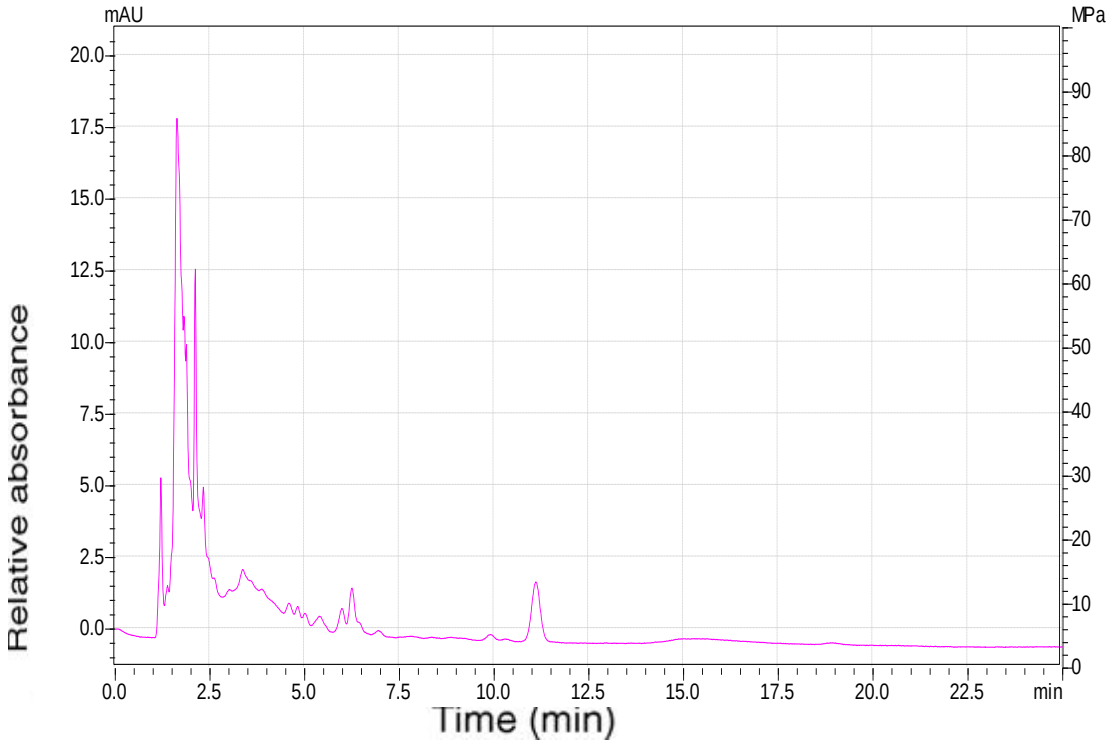
(F) 6L


mAU
MPa
250
225
200
175
150
125
100
75
50
25
0
90
80
70
60
50
40
30
20
10
0
Relative absorbance
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min
Time (min)

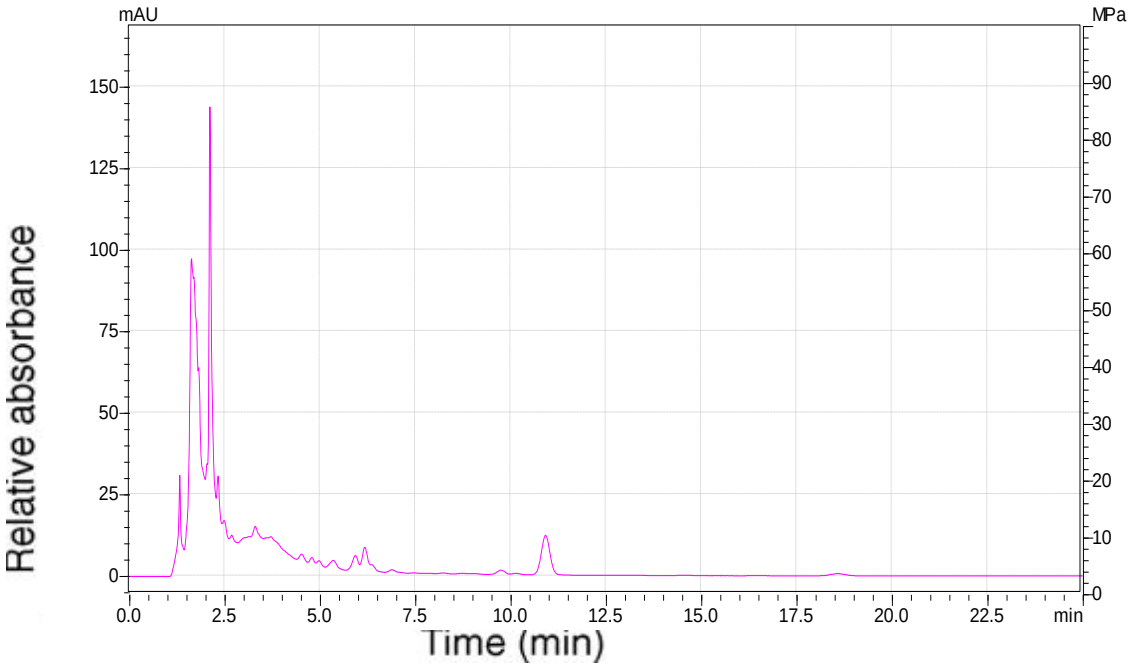
(G) 7L


mAU
MPa
Relative absorbance
Time (min)
min

(H) 8L

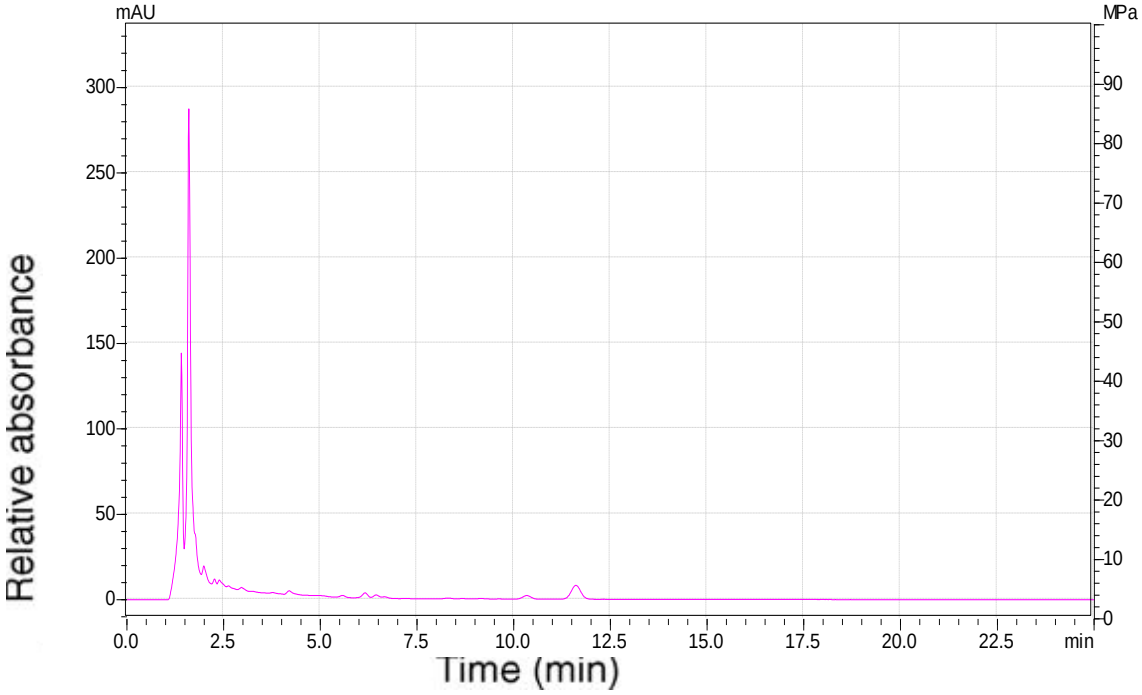

mAU
MPa
Relative absorbance
Time (min)
min

(I) 9L

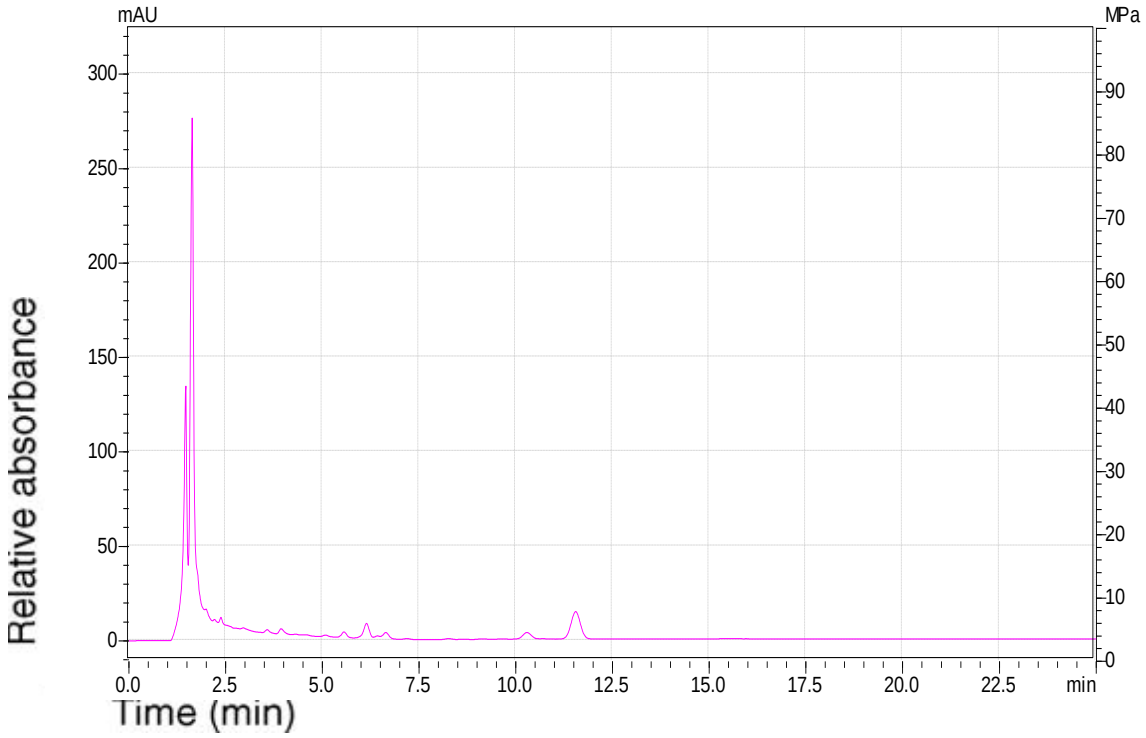


***Pelargonium sidoides* roots HPLC chromatographs**

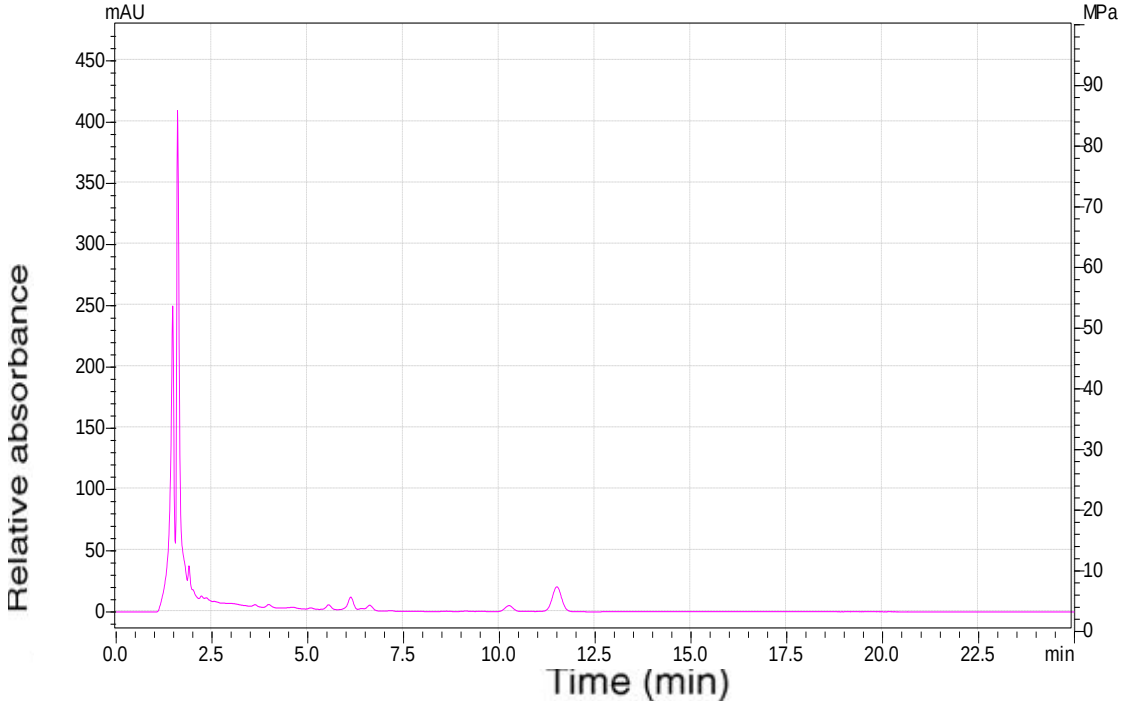
(J) 1R



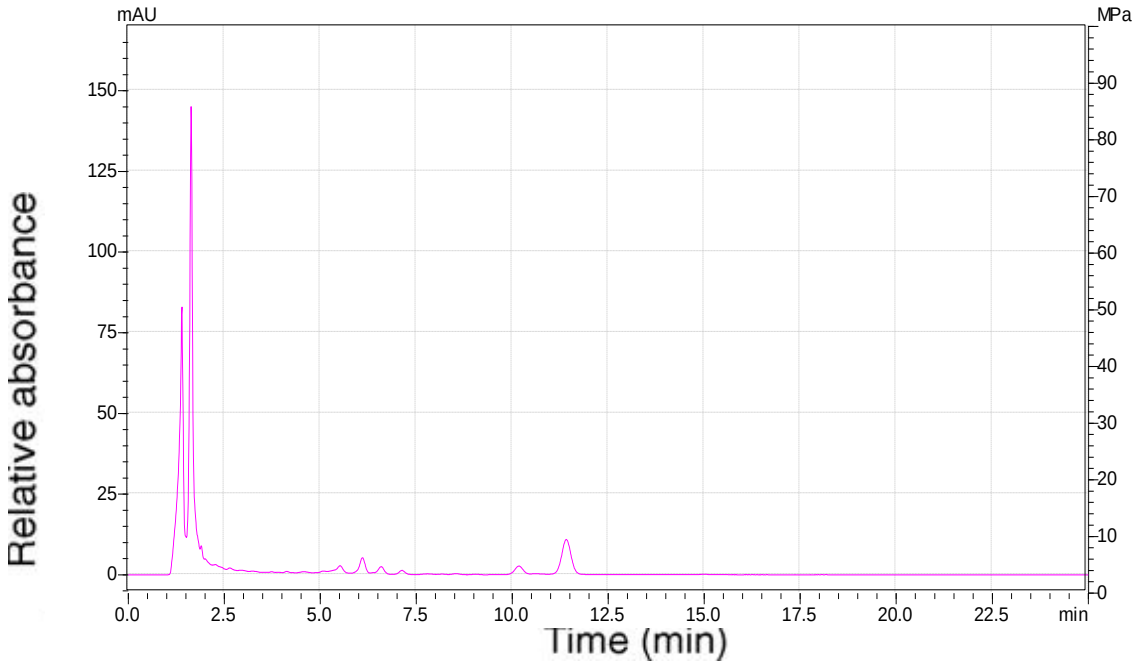
(K) 2R


mAU
MPa
Relative absorbance
Time (min)
min

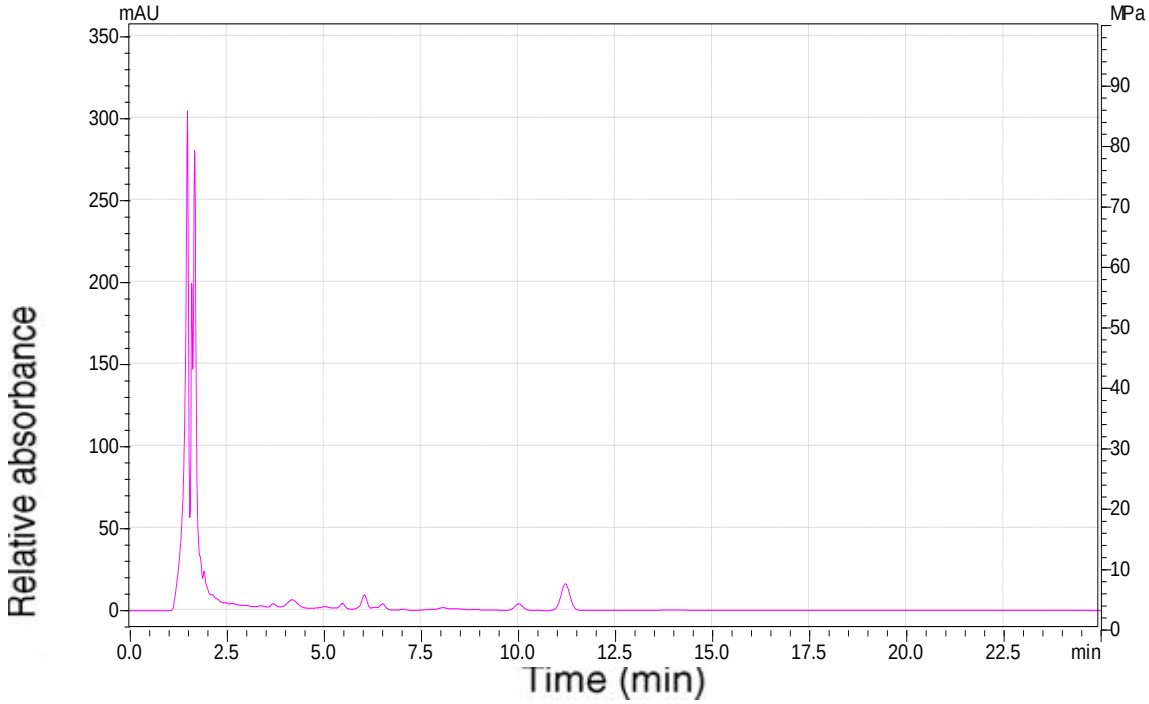
(L) 3R


mAU
MPa
Relative absorbance
Time (min)
min

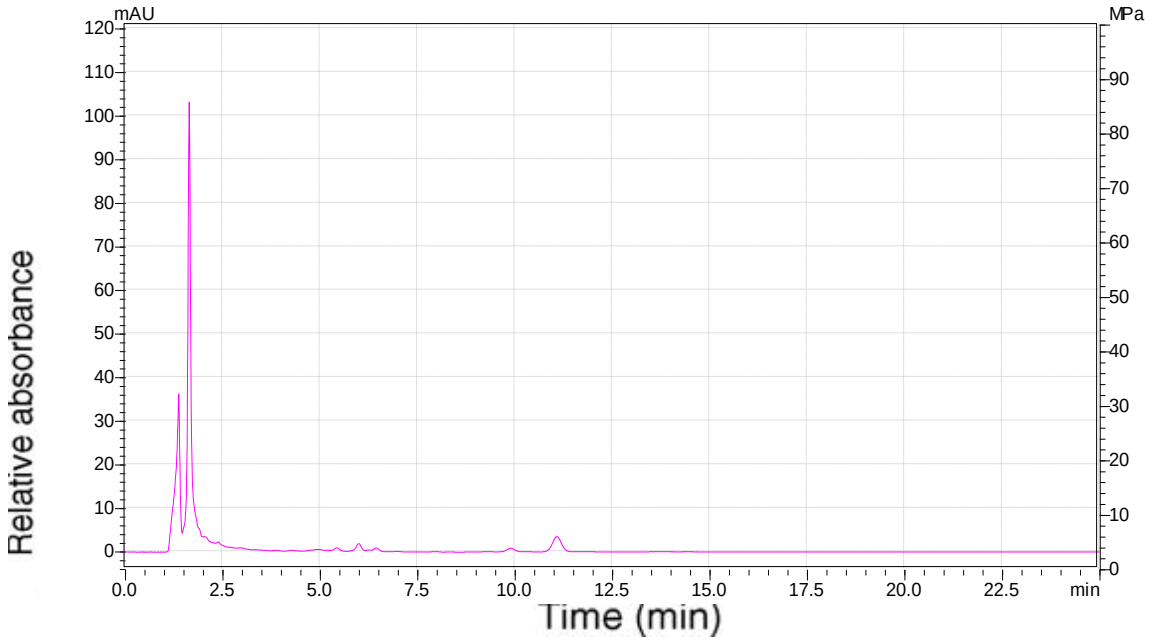
(M) 4R


mAU
MPa
Relative absorbance
Time (min)
min

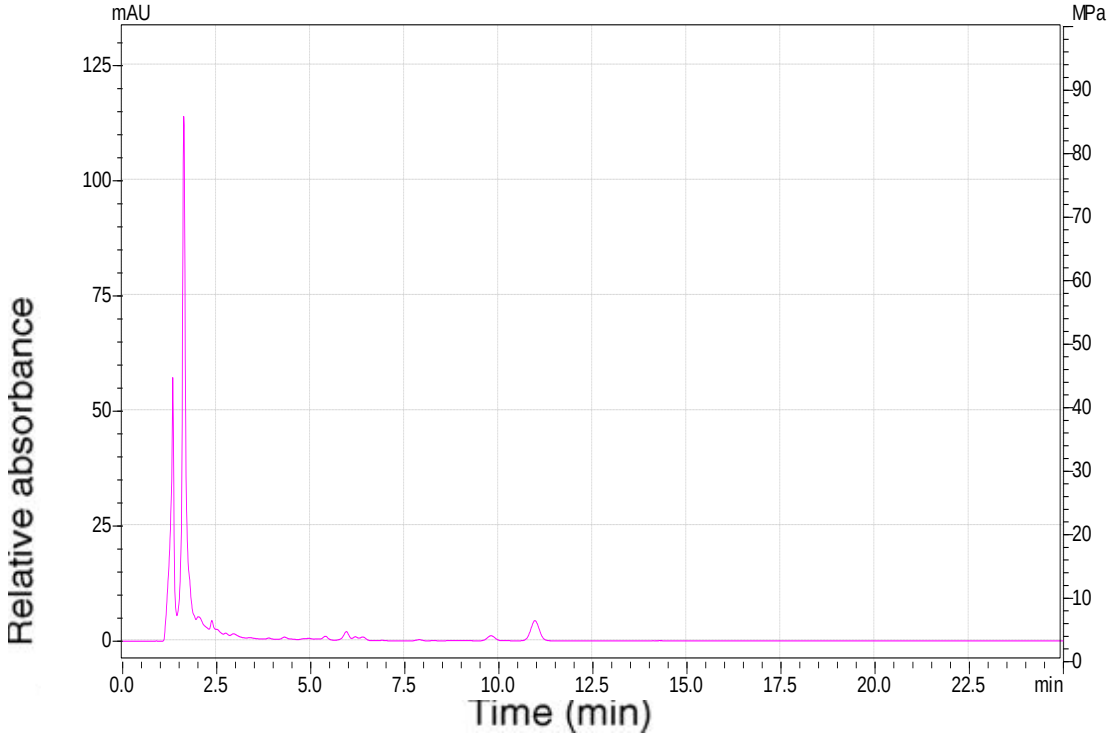
(N) 5R


mAU
MPa
Relative absorbance
Time (min)
min

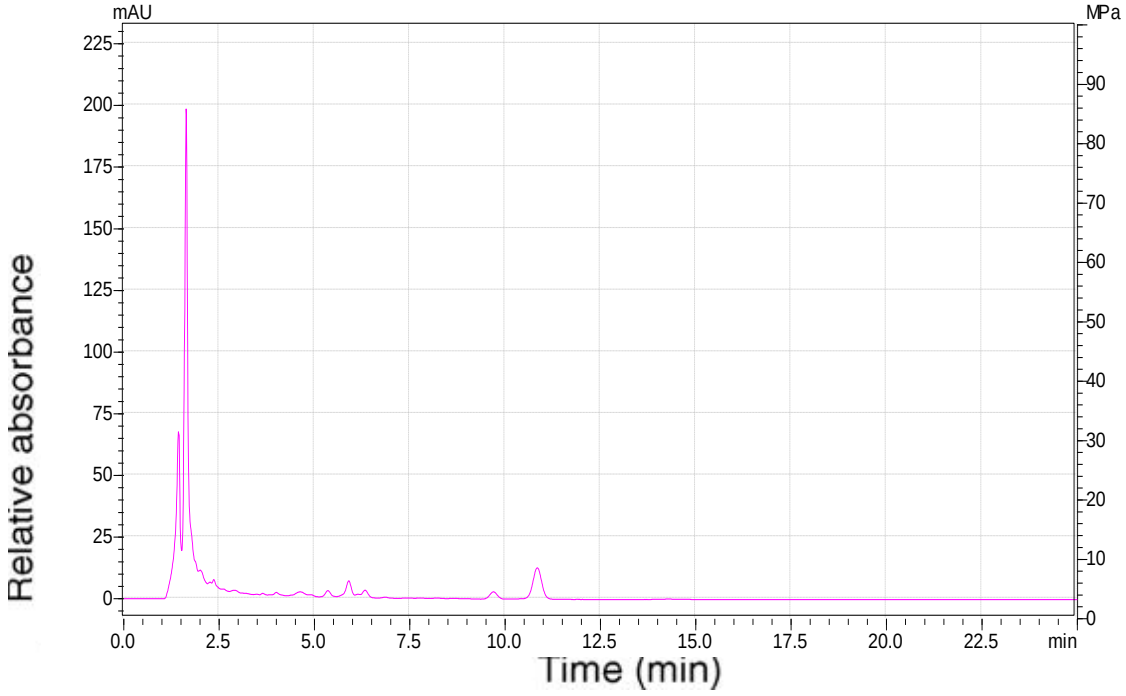
(O) 6R


mAU
MPa
Relative absorbance
Time (min)
min

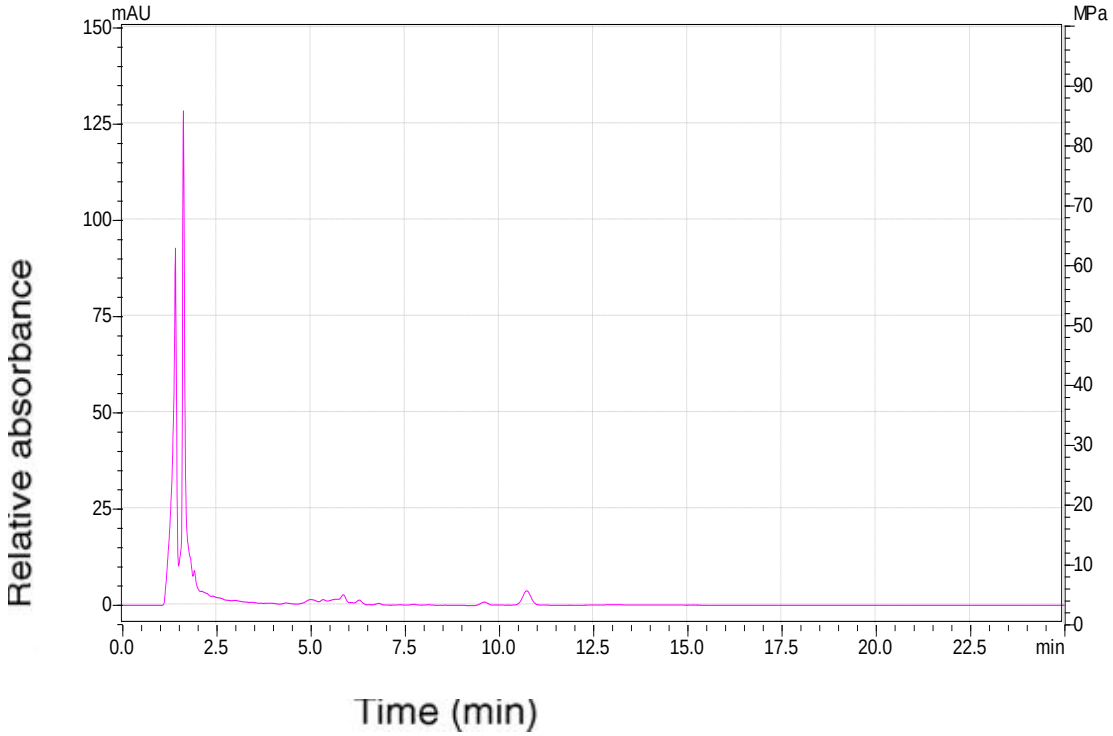
(P) 7R


mAU
MPa
Relative absorbance
Time (min)
min

(Q) 8R


mAU
MPa
225
200
175
150
125
100
75
50
25
0
90
80
70
60
50
40
30
20
10
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min
Relative absorbance
Time (min)

(R) 9R


mAU
MPa
150
125
100
75
50
25
0
90
80
70
60
50
40
30
20
10
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
min
Relative absorbance
Time (min)

APPENDIX C

LIST OF CHEMICALS AND REAGENTS

- Dimethylsulfoxide
- Nutrient broth (Oxoid)
- P-iodotetrazolium

EQUIPMENT

- Rotor evaporator (Heidolph—Laborota 4000)
- Spectrophotometer (Spekol 1300)
- Incubator (Lab-com)

ANTIMICROBIAL ACTIVITY

DAY 1:

- Autoclave distilled water, nutrient broth, nutrient broth in culture flasks, pipette tips.
- Prepare cultures of bacteria and incubate overnight at 37°C.

DAY 2:

A technique by Eloff (1998) of micro-dilution using 96 well micro plates was used in order to obtain MIC values of crude extracts of roots that were showing high inhibition zones. The plant root crude extracts were tested against the following microorganisms: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Shigella flexineri* KZN and *Salmonella spp*. Methanolic plant extract of 5 mg ml^{-1} and 10 mg ml^{-1}, was serially diluted so as to obtain 2.5 mg ml^{-1} starting concentration in the first well. An equal volume of 100µl fresh bacterial cultures were added into well. Cover the micro-plate and incubate overnight at 37°C.

DAY 3:

- Make up the P-Iodonitrotetrazolium (INT) solution (0.2mg/ml)
- Add 10µl of INT to all wells
- Incubate at 37°C for 10-30 minutes
- INT is reduced to a pinkish coloured product and a colorless well indicates inhibition in that well.

Preparation of the nutrient agar

- Suspended 31g in 1 litre of distilled water
- Boil whilst stirring until complete dissolved
- Autoclave at 121^0C for 15 minutes
- Cool again to 45^0C to 50^0C
- Mixed well and poured on plates

Preparation of 10% DMSO

- 100ml glass beaker
- Put 10ml of DMSO
- Refill with distilled water

Preparation of 70% methanol

- 100ml glass beaker
- Put 70ml of methanol
- Refill with distilled water

1. WHITE, A.G. 2005. MSc student, Botany Department, Rhodes University, Grahamstown, South Africa. Personal observations.

APPENDIX D

ETHICAL CLEARANCE CERTIFICATE

Ethics Committee
Faculty of Science and Agriculture
University of Zululand
C/O Dr L Vivier
Department of Zoology
University of Zululand
Private Bag 1001
KwaDlangezwa
3886
Tel: 035 – 902 6741
Email: lvivier@pan.uzulu.ac.za

20 June 2012

To whom it may concern

ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that **PG Mthiyane (Student no. 20050010)** are registered for an **MSc Degree** in the **Department of Agriculture,** Faculty of Science and Agriculture, at the University of Zululand, and in accordance with appropriate rules, submitted a research project proposal to the Ethics Committee of the Faculty of Science and Agriculture. The research project these researchers will investigate is titled: **Chemo-Morphological Characterization And Antimicrobial And Antidiarrhoeal Activity Of Pelargonium Sidoides Accessions In Northern Kwazulu Natal**. Based on the research protocol stipulated, the Ethics Committee of the Faculty of Science and Agriculture could find no reason from an ethical standpoint to reject the proposed research. Provisional ethical clearance is granted pending approval by the UZREC.

Yours sincerely

Dr L Vivier
Chairperson
Ethics Committee
Faculty of Science and Agriculture
University of Zululand