Isolation, characterization and biological activities of terpenoids from *Gunnera perpensa*

Submitted in fulfilment for the Degree of Master of Applied Sciences in Biotechnology in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

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Professor Bharti Odhav

REFERENCE DECLARATION

I, Miss Fitsum K. Mammo (student number: 21449777) and Dr Viresh Mohanlall do hereby declare that in respect of the following dissertation:

Title: **Isolation, characterization and biological evaluation of terpenoids from *Gunnera perpensa*.**

1. As far as we ascertain:

a) no other similar dissertation exists;

2. All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.

Signature of student	**Date**
Signature of supervisor	**Date**
Signature of co-supervisor	**Date**
Signature of co-supervisor	**Date**

DEDICATION

This thesis is dedicated to my mother, Mrs. Aberash Zeleke Kuri and my late father, Mr. Kassa Mammo Abate, who instilled in me the virtues of perseverance and commitment to strive for excellence.

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ABBREVIATIONS

TLC	Thin layer chromatography
PTLC	Preparative Thin-Layer Chromatography
CC	Column chromatography
HPLC	High-performance liquid chromatography
NMR	Nuclear magnetic resonance
DEPT	Distortionless enhancement by polarisation transfer
DPPH	2,2-diphenyl-1-picryhydrazyl
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
UV	Ultra-violet
MEP	Methylerythritol Phosphate
MVA	Mevalonic acid
IPP	Isopentenyl pyrophosphate
DMAPP	Dimethylallyl pyrophosphate
PCT	Pentacyclic Triterpene
OA	Oleanolic acid
UA	Ursolic acid
BA	Betulinic acid
MA	Maslinic acid
DCM	Dichloromethane
S_iO_2	Silica gel
IR	Infra-red
MS	Mass Spectrometry
$CDCl_3$	Deuterated chloroform
DMSO	Dimethyl sulfoxide
TMS	Tetramethylsilane

MIC	Minimum inhibitory concentration
ABTS	2,2’-azino-di [3-ethylbenzthiazoline sulfonate]
ORAC	Oxygen radical absorbance capacity
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PDA	Potato Dextrose Agar
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
RBC	Red blood cell
DMEM	Dulbecco’s Modified Eagle’s Medium
FCS	Fetal Calf Serum
PBS	Phosphate saline buffer
HBV	Hepatitis B virus

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ABSTRACT

Five pentacyclic triterpenes were identified from the leaves and stalks of *Gunnera perpensa*. These were ursolic acid (**I**), 3-β-hydroxyolean-12-en-27-oic acid (**II**) (β-peltoboykinolic acid), 3-β-hydroxyurs-12-en-27-oic acid (**III**) (α-peltoboykinolic acid), 3-α-hydroxyolean-12-en-27-oic acid (**IV**), and 3α-3, 19-dihydroxyurs-12-en-28-oic acid (**V**) (pomolic acid). These compounds were extracted using ethyl acetate and separated by silica gel column chromatography by the aid of TLC. Their structures were elucidated by ^{1}H and ^{13}C NMR spectroscopy and identified by comparing their spectral data with those reported in the literature. High-Performance Liquid Chromatography (HPLC) was used to identify those compounds that were difficult to separate using CC.

Disc diffusion assay was used to evaluate the antimicrobial potentials of the crude extracts and the isolated compounds against bacterial and fungal species. The sickle-cell normalisation and free radical scavenging potentials of the crude extracts and the isolated compounds were done using Emmel's test and DPPH (2, 2 dyphenyl-2-picryl hydrate) photometric assay, respectively. The anticancer potential of the isolated compounds was performed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The antimicrobial results showed that the crude extracts GP/A3 and GP/C4 were highly active against *S. aureus, B. cereus, E. coli* and *P. aeruginosa* with MIC values ranging from 0.75 to 1.5 µg/ml. Significant antibacterial activity was observed with compound **X** against *S. aureus, S. epidermidis* and *E. faecalis*. Neither of the crude extracts nor the isolates showed antifungal activity.The partial fraction (compound **Y**) showed the highest normalisation rate to the sickle-cells with 82.135 % standardisation at 144.93 µg/ml. The crude extracts GP/A2, GP/B3, GP/A3, and GP/B2 exhibited the highest radical scavenging capacity against the free radical 2,2-diphenyl-1-picrylhydrazyl with 96, 95, 94.8, and 94.3 percentage values, respectively.While the free radical scavenging potentials of the isolates were found to be less. The highest cytotoxicity activity was recorded by the compounds **X** and **Y** followed by the moderate activity of

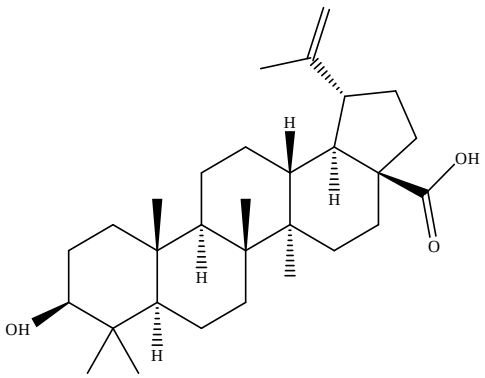
compounds **I**, **II**, **III** and **IV** with equal percentage death values of, 54.58. The tested compounds were found to be more toxic to the MCF-7 than HEK293 cell line.

In conclusion, this is the first study to discover pentacyclic triterpenes from the leaves and stalks of *G. perpensa*. The crude extracts and their isolates together with the two partial fractions (compounds **X** and **Y**) were found to be responsible and add value to the existing known potential of *G. perpensa*. These compounds have shown antimicrobial, anti-sickling, anti-oxidant and anticancer properties. The probability of getting a cure for Sickle Cell Anemia patients from *G. perpensa* is possible through direct use or after chemical modification of the compounds. The high anti-oxidant potential of the crude extracts will serve as a starting point for further studies to identify and characterise undiscovered secondary metabolites. The anticancer properties of the isolated compounds encourage future studies against different cancer cell lines. Moreover, none of the crude extracts and isolated compounds were found to be toxic to brine shrimp larvae which indicate its safety in any human health related applications. It is highly recommended to further purify the partially purified compounds (**X** and **Y)** as they showed high biological activities that can add value to new drug development.

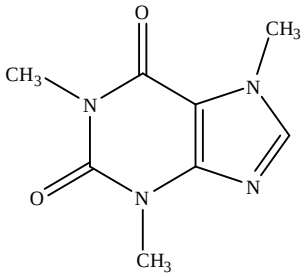
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

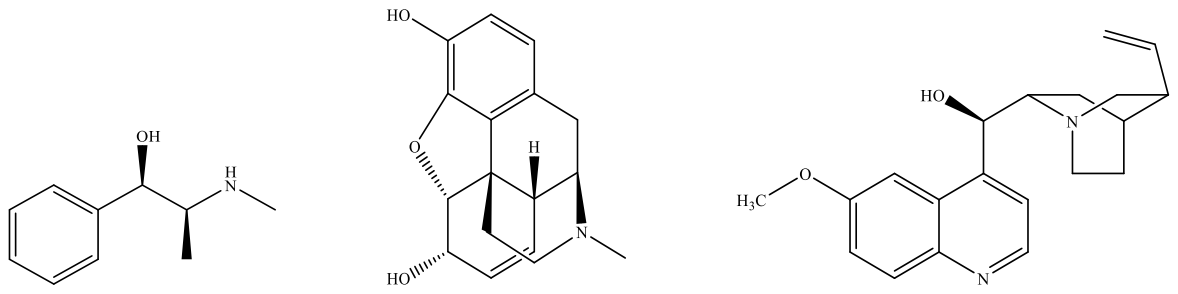
Plants play an important role as natural drugs to alleviate various health problems of humans by contributing herbal medicines to the primary health care systems of rural and remote communities. A significant proportion of the population in South Africa depend on cultural practices and traditional systems of medicine (Mousdale and Coggins 1985; Motsei *et al.* 2003). In addition to the increasing trend in using traditional medicines as a primary health care system, there is a growing interest not only in determining the scientific basis for the plant's usage but also in discovering novel compounds of pharmaceutical value (Jäger, Hutchings and van Staden 1996). Plants are rich in bioactive compounds that have various medicinal properties. With the development of technology, it has been possible to purify and identify secondary plant metabolites which contribute to new drug development (Spigno and De Faveri 2007). These secondary metabolites have been used as the backbone to formulate many of the pharmaceutical drugs in the world market (Chavan *et al.* 2013). Some of the drugs or chemicals that are discovered from plant bioactive compounds include betulinic acid, caffeine, ephedrine, morphine and quinine (Figure 1). In recent times, there is an increase in the spread of diseases caused by pathogens resistant to most conventional drugs (Nguedia and Shey 2014). This resistance is due to the pathogens decreased response to treatment with conventional drugs, and thus, there is a tendency for the disease to reoccur. Moreover, many therapeutic drugs exhibit several side effects and have limited efficacy. Therefore, there is a constant need for new, safer, effective, and multi-targeting drugs.



Betulinic acid



Caffeine



Ephedrine Morphine Quinine

Figure 1: Some of the drugs discovered from plant bioactive compounds.

In this study, the plant of focus was *Gunnera perpensa*. The plant was chosen based on the ethnomedicinal data indicating its traditional application in the treatment of different health problems including: venereal diseases, infertility, endometritis, insect repellant, urinary tract problems, impotence, menstrual pains, and cold (Kaido *et al.* 1997; Brookes and Dutton 2007; Nkomo *et al.* 2010). The plant is also considered to be antioxidant because of the presence of active natural compounds, such as phenolic compounds, that are efficient in scavenging free radicals that can cause many types of health disorders. The plant has been widely used by the South African traditional medicine practitioners, although, little work has been done in identifying its bioactive compounds and investigating their biological properties (Maroyi 2016).

1.1.1. Aim

The aim of this study was to isolate, characterise and screen the bioactive secondary metabolites of *G. perpensa* for their antioxidant, anticancer, anti-sickling and antimicrobial properties, including testing for their toxicity and safe use.

1.1.2. Objectives

The aim was achieved through the following objectives:

1. To collect and prepare polar and non-polar extracts of *G. perpensa* leaves, and stalks.
2. To isolate and purify bioactive secondary metabolites from *G. perpensa* using Thin Layer Chromatography (TLC) and Column Chromatography (CC).
3. To characterise bioactive secondary metabolites of *G. perpensa* using High-Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) spectroscopic techniques (^{1}H, ^{13}C, DEPT, and 2D NMR).
4. To screen the isolated secondary metabolites of *G. perpensa* for their anti-oxidant, anticancer, anti-sickling and antimicrobial properties using DPPH (2, 2 dyphenyl-2-picryl hydrate) photometric assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Emmel's test and agar disc diffusion assay, respectively.
5. To determine the safe use and toxicity of the bioactive secondary metabolites using Brine shrimp assay.

1.2. *Gunnera perpensa*

G. perpensa L. belongs to the family Gunneraceae. The genus *Gunnera* L. was named after a Swedish bishop and botanist Johan Ernst Gunnerus in the eighteenth century (Jarzen 1980; Peter 2010). *Gunnera* L. constitutes a group of herbaceous flowering plants. The genus is one of the six subgenera of its family with approximately 50 species (Brookes and Dutton 2007; Peter 2010; Simelane 2010). Although, it is the only species of its genus to exist in Africa, *Gunnera perpensa* (*G. perpensa*) is an erect, perennial herb that inhabits moderate to heavy rainfall, including marshy areas, sideways of river banks and very limited to high altitudes (Standley and Williams 1963; Jarzen 1980; Fuller and Hickey 2005; Peter 2010). This is in part because of its mutualistic relationship with nitrogen-fixing cyanobacteria (Bergman, Johansson and Soderback 1992; Fuller and Hickey 2005).

The plant is commonly known as “River Pumpkin” because of the round, large pumpkin-like leaves (Figure 2). It has long, thick stalks supporting the leaves and arising from the rhizome. This species is unique from other species of the genus in its thick, tuberous and branching horizontal rhizomes (Wanntorp and Wanntorp 2003). Its flowers are small, wind-pollinated, reddish to brown in colour and found in groups, with nearly a hundred thousand, appearing on a long, thick flowering stem (Figure 2) (Jarzen 1980; Wanntorp and De Craene 2005). The plant is monoecious with unisexual to bisexual flowers (Schindler 1905; Jarzen 1980; De Craene and Wanntorp 2006). In South Africa, the plant is known by its local names: Ugobho (isiZulu), rambola-vhadzimu (Venda), Qobo (Sotho), ‘Iphuzilomlambo’, ‘Uxobo’ (Xosa), Rivierpampoen (Afrikaans) and River pumpkin (Filippich *et al.* 1991)(McGaw *et al.* 2005; Brookes and Dutton 2007).

A

B

C

Figure 2: *Gunnera perpensa* whole plant (A), leaf (B) and Flower (C)

1.2.1. Taxonomy and classification

Table 1: Taxonomic hierarchy of *G. perpensa* L.

Kingdom:	Plantae
Subkingdom:	Viridiplantae
Infrakingdom:	Streptophyta
Superdivision:	Embryophyta
Division:	Tracheophyta
Subdivision:	Spermatophytina
Class:	Magnoliopsida
Superorder:	Myrothamnanae
Order:	Gunnerales
Family:	Gunneraceae
Genus:	Gunnera L.
Species:	*Gunnera perpensa* L.

Gunnera L. with approximately 50 species (Brookes and Dutton 2007; Peter 2010; Simelane 2010) was previously grouped under the family Haloragaceae due to its endosperm rich seeds and vegetative habit. This classification was based on its dimerous and epigynous flowers, endosperm rich seeds and the vegetative habitat of both Gunnera and Haloragaceae (Wanntorp and De Craene 2005; Peter 2010). However, a molecular study by Chase *et al.* (1993) falsified this systematic arrangement based on the results obtained from chloroplast and nuclear gene sequencing. The sequences evidently showed that Gunnera was not closely related to any other genera in the Haloragaceae family. Since then, Gunnera has been placed in the Order Gunnerales and its position as the main branch of eudicots could provide a better evolutionary understanding of core eudicots (Wanntorp and De Craene 2005). The presence of separate vascular strands in the petioles and stems of Gunnera is a characteristic observed in only a few groups of angiosperms. This feature is also an indication of the evolvement of Gunnera L. from its aquatic ancestors. Later revisions on the anatomy, palynology, morphology and embryology of the two

families, Haloragaceae and Gunneraceae, clearly emphasised their differences (Doyle and Scogin 1988; Peter 2010).

Gunnera L. is one of the six subgenera of its family, Gunneraceae. The six subgenera of the family include; Misandra, Gunnera, Pseudogunnera, Panke, Ostenigunnera and Milligania. This traditional classification was based on the biogeography, size and floral pattern of the plants. Misandra consists of three species, which are dioecious, prostrate, stoloniferous herbs, and reside in South America. Pseudogunnera is comprised of stoloniferous and erect plants with panicles of proterandrous flowers and unisexual female flowers at the top and base, respectively. *G. macrophylla* is the only species of this subgenus. It is mainly found in the Philippines, Wallace's line of Java, some Melanesia volcanic islands, South-Eastern Asia and New Guinea. They are monoecious plants with unisexual flowers, occasionally bisexuals (Meijden and vander ; Fuller and Hickey 2005; Peter 2010).

The subgenus Panke has more than 19 species. It is the largest of all the six subgenera (Wanntorp, Wanntorp and Rutishauser 2003; Fuller and Hickey 2005; Wanntorp and De Craene 2005; De Craene and Wanntorp 2006). The species are located on the island of Hawaii, Juan Fernandez Island and South America (Mora-Osejo, Pinto and Ruiz 1984; Doyle 1990). The subgenus lacks stolons and produces large panicles of hermaphroditic, mostly unisexual flowers. The species have erect petioles supporting the large laminae. The subgenus Milligania contains six to eleven small, mat-forming species, which includes the stoloniferous and creeping herbs from New Zealand and one dioecious species from Tasmania (Schindler 1905; Cheeseman 1906; Fuller and Hickey 2005; Peter 2010). Ostenigunnera was included as a subgenus in the year 1933 by Mattfeld (1933). Recent studies on the morphological and molecular basis have shown all the six subgenera are systematically valid (Wanntorp, Wanntorp and Rutishauser 2003).

1.2.2. Geographical Distribution

Geographically, the distribution of the genus *Gunnera* L. has been gradually reduced. According to Jarzen (1980), based on the paleo-geographic and stratigraphic occurrences during the Upper Cretaceous- and Early Tertiary period, the genus *Gunnera* was broadly distributed than it is

today. This report points out that the decrease in rainfall and the seasonal temperature fluctuation increase might be the main reasons for the retreat of *Gunnera* from high latitudes. This is supported by the fact that the species are currently spread in environments of heavy rainfall (Peter 2010).

The species of *Gunnera* L. are currently distributed in different parts of the world, including New Zealand, Mexico, the Philippines, Indonesia, North America, Central and South America, Hawaii, Madagascar and Eastern and Southern Africa (Figure 3) (Mendes 1978; Bergman, Johansson and Soderback 1992; Wanntorp and Wanntorp 2003).

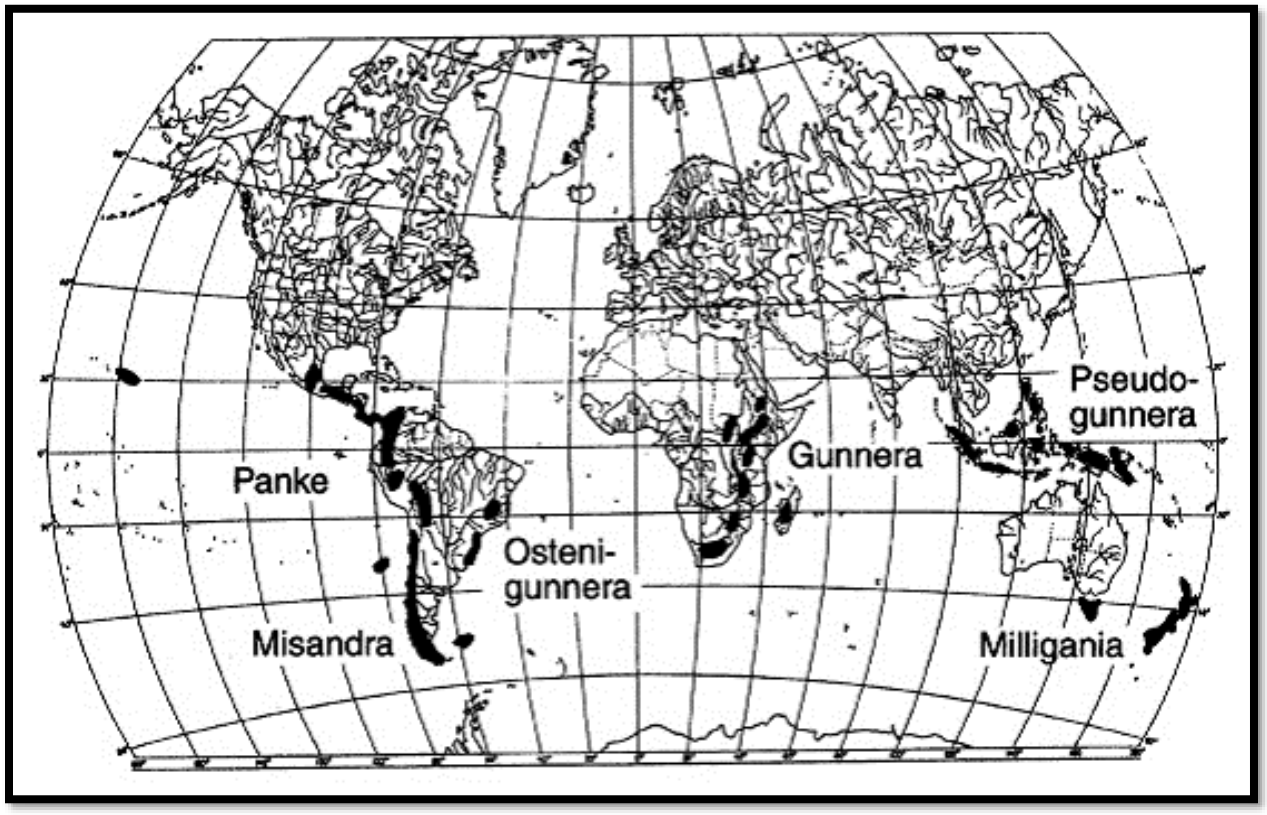


Figure 3: Present distribution (black) of *Gunnera* L. Names of subgenera written near their respective geographical occurrences (Wanntorp and Wanntorp 2003).

Gunnera perpensa is an African species, which is the only species of *Gunnera* L. that is distributed in Africa. The plant is widely spread in tropical Africa from Sudan, Ethiopia, Congo

(D.R.C.), Burundi, Rwanda, Uganda, Kenya, Tanzania, Zimbabwe, and Mozambique, extending to central and eastern areas of southern Africa down to the Western Cape, including Swaziland and Lesotho (Mendes 1978; McGaw *et al.* 2005; Peter 2010). In South Africa, it is found in four provinces; Free State, Eastern Cape, Western Cape and KwaZulu-Natal (Figure 4). The plant is not documented in Botswana or Namibia or the Northern Cape Province of South Africa (Mendes 1978; Bergman, Johansson and Soderback 1992).

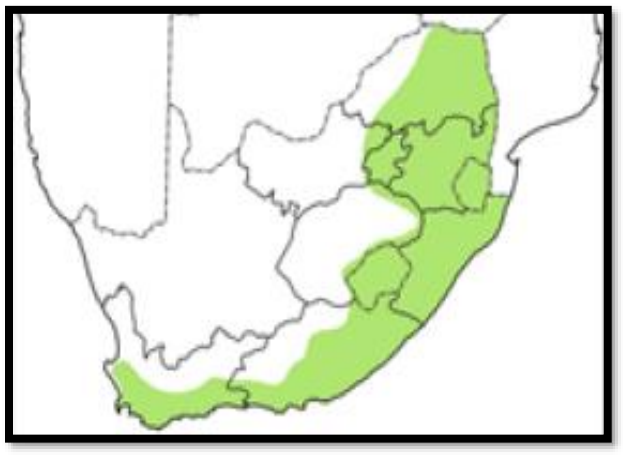


Figure 4: Distribution map (green) of *G. perpensa* in southern Africa (Mendes 1978).

1.2.3. Ethnomedicinal uses

G. perpensa L. is a native South African plant and one of the most valuable medicinal plant species in southern Africa with its leaves, stems, rhizomes and roots widely used as traditional medicine. Inherently, it is used in local traditional medicine systems of the Sotho, Zulu, and Xhosa cultural groups. *G. perpensa* is traditionally used to treat various disorders which are summarised in Table 2.

The leaves, stalks, rhizomes and roots are used to treat various health problems including venereal diseases, infertility, endometritis, urinary tract problems, impotence and cold, and is used as an insect repellant (Brookes and Dutton 2007). The majority of South African women use decoctions of the root for infant development, female fertility, to relieve menstrual pains, to

speed up labour, and to accelerate expulsion of the placenta (Kaido *et al.* 1997; Van Wyk, Oudtshoorn and Gericke 1997; Drewes *et al.* 2005; McGaw *et al.* 2005; Brookes and Dutton 2007; Nkomo *et al.* 2010). Root and leaf extracts are used to dress wounds and treat psoriasis (Grierson and Afolayan 1999). Decoctions are also used in veterinary science to treat endometritis, to induce labour, and to protect animals from tick and insect bites (Hutchings 1996; Brookes and Dutton 2007; Nkomo *et al.* 2010). Traditionally the powdered form of the dried root is boiled in water and given orally to an animal to expel the placenta after calving (Khan *et al.* 2004; Mariotti *et al.* 2014).

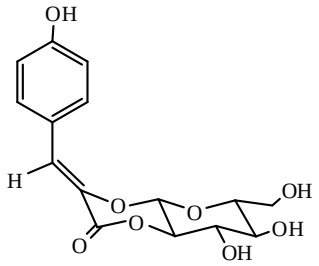
Table 2: Ethnobotanical uses of *G. perpensa* in Zulu, Xhosa and Sotho cultural groups of South Africa.

Medicinal Uses	**Plant Part(s) Used and Preparation**	**References**
Urinary tract	Root decoction	Brookes and Dutton (2007)
Impotence	Root decoction	Brookes and Dutton (2007)
Barrenness	Root decoction	Brookes and Dutton (2007)
Induce labour	Root decoction	Kaido *et al.* (1997), Brookes and Dutton (2007), Khan *et al.* (2004)
Fetal development	Root decoction	Brookes and Dutton (2007), Kaido *et al.* (1997)
Expulsion of placenta	Root decoction	Brookes and Dutton (2007), Kaido *et al.* (1997)
Wound dressing	Root and Rhizome decoction	Brookes and Dutton (2007), Nkomo *et al.* (2010)
Colds	Root decoction	Brookes and Dutton (2007)
Endometritis	Plant Extracts	Brookes and Dutton (2007)
Psoriasis	Rhizome decoction	Nkomo *et al.* (2010)
Dysmenorrhoea	Plant decoction	Nkomo *et al.* (2010)
Rheu	Root decoction	Nkomo *et al.* (2010), Khan *et al.* (2004)
Female infertility	Root decoction	Nkomo *et al.* (2010), Khan *et al.* (2004)
Ethnoveterinary medicine	Root decoction is used to speed up labour	

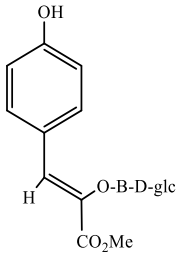
	Root decoction is used to protect animals from tick bites and other parasites	Brookes and Dutton (2007)
	Plant extracts are used to treat Endometritis	
	Root decoction is used to expel the retained placenta	Khan *et al.* (2004)

1.2.4. Phytochemistry

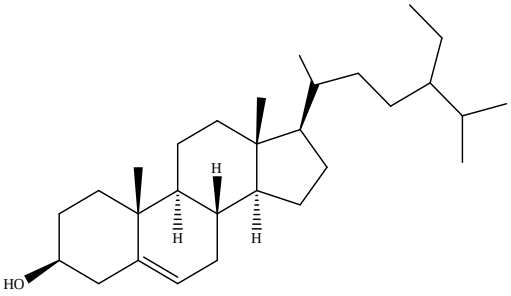
A wide variety of active natural compounds are responsible for the different characteristics of plants such as pigments (quinines and tannins), odours (terpenoids), and flavours (some terpenoids) (Showell *et al.* 2011). Despite its widespread use, there are limited studies on the chemical composition of *G. perpensa*. *G. perpensa* mainly contains tannins, steroids, cardiac glycosides, and flavonoids (Simelane *et al.* 2010). One of the many components which are already known in *G. perpensa* is the phenylpropanoid, Z-venusol (**1**). A phytochemical study by Khan *et al.* (2004) on the aqueous root extract of *G. perpensa*, revealed the presence of Z-venusol (**1**) as a major component. The prolonged exposure of Z-venusol (**1**) to methanol leads to a major compound Z-methyl lespedezate (**2**) mixed with **1**, as an impurity. Other compounds identified from *G. perpensa* include, β-sitosterol (**3**), ellagic acid (**4**), 3,3′,4′-tri-O-methyl ellagic acid (**5**) and 3,3′,4′-tri-O-methyl ellagic acid 4-O-β-D-glucopyranoside (**6**), punicalagin (**7**), p-hydroxybenzaldehyde (**8**), two benzoquinones (2-methyl-6-(3-methyl-2-butenyl)-benzo-1-4-quinone (**9**) and 3-hydroxy-2-methyl-5-(3-methyl-2-butenyl)-benzo-1,4-quinone (**10**)), 6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran (**11**), and phytol (**12**) (Grierson and Afolayan 1999; Drewes *et al.* 2005; Brookes and Dutton 2007; Peter 2010; Chavan *et al.* 2013). The phytochemical structures of these compounds are shown in Figure 5. In addition to the compounds mentioned above, Peter (2010) reported the presence of sucrose and an unidentified sugar. The compound phytol (**12**) was also obtained from the methanol extracts of the aerial parts of the plant. Phytol is a known skin irritant and most likely the reason for the recorded skin irritation nature of the plant (Drewes *et al.* 2005; Peter 2010).



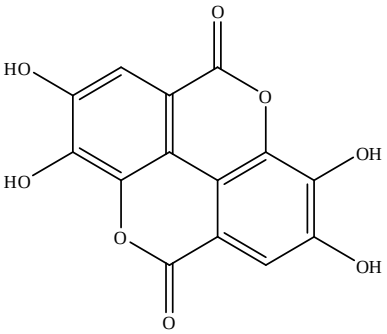
Z-venusol (**1**)



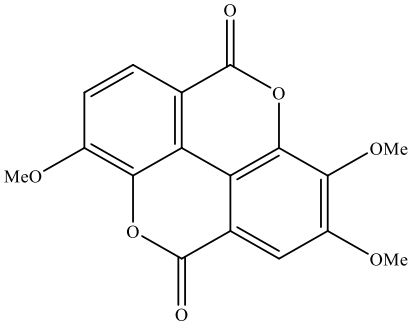
Z-methyl lespedezate (**2**)



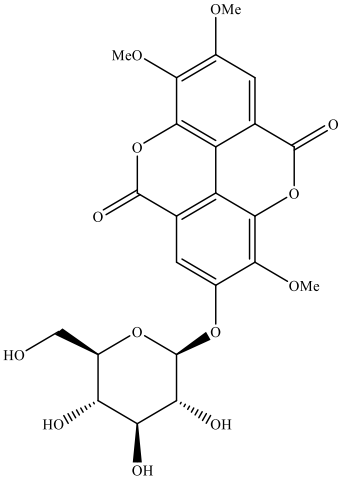
β-sitosterol (**3**)



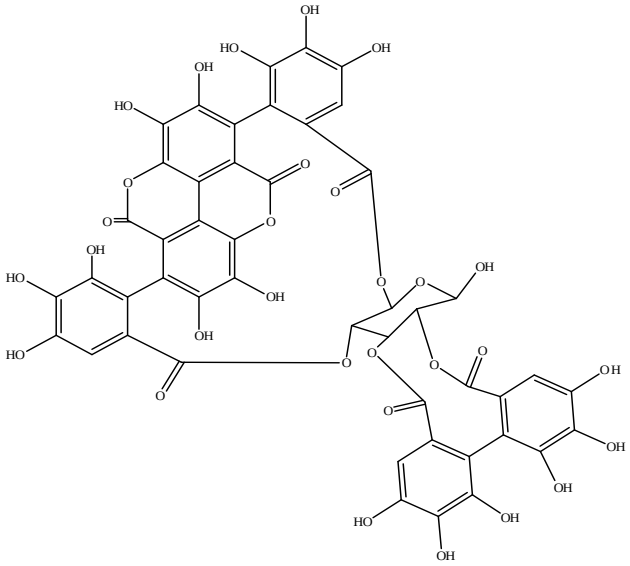
Ellagic acid (**4**)



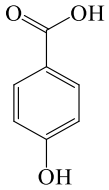
3,3’,4’-tri-O-methyl ellagic acid (**5**)



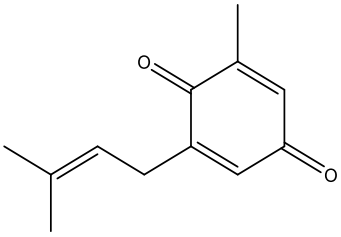
3,3’,4’-tri-O-methyl ellagic acid 4-O-β-D-glucopyranoside (**6**)



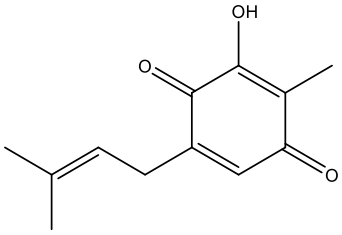
Punicalagin (**7**)



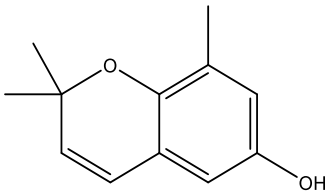
p-hydroxybenzaldehyde (**8**)



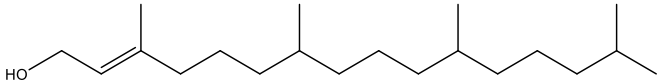
2-methyl-6-(3-methyl-2-butenyl)-benzo-1-4-quinone (**9**)



3-hydroxy-2-methyl-5-(3-methyl-2-butenyl)-benzo-1,4-quinone (**10**)



6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran (**11**)



Phytol (**12**)

Figure 5: Chemical structures of metabolites isolated from *G. perpensa*.

1.2.5. Pharmacology

The crude extracts of *G. perpensa* have been widely studied for their analgesic, antimicrobial and anti-inflammatory properties. The antimicrobial activity of the leaves was found to be higher than that of the stems, followed by the roots (Hutchings 1996; Drewes *et al.* 2005). Grierson and Afolayan (1999) confirmed the traditional wound healing potential of the leaves using the information compiled through general conversations and questionnaires from the Sangomas, herbalists and rural inhabitants in the Eastern Cape Province. According to Steenkamp *et al.* (2004), *G. perpensa* has anti-oxidant and antibacterial properties and contributes to wound healing through fibroblast growth. Buwa and Van Staden (2006) reported that the aqueous, ethanol and ethyl acetate extracts of *G. perpensa* root have antibacterial properties with the highest inhibition by aqueous and ethanol extracts against the tested gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). Several phenolic compounds have been isolated from the root and rhizome of *G. perpensa*. Phenolic compounds can be held responsible for a wide variety of biological activities including antiseptic, antimutagenic, anticancer, anti-haemorrhagic and anti-oxidative properties (Brookes and Dutton 2007). These properties could be helpful during pregnancy and birth, which confirms some of the reported health effects accredited to this species by traditional healers.

Z-venusol

Drewes *et al.* (2005) studied the antibacterial, ileum muscle and uterus muscle contraction potentials of the active compounds isolated from the rhizomes of *G. Perpensa*. These compounds include 1,4-benzoquinones, phenylpropanoid glucoside and Z-venusol (**1**). The results showed that all the compounds have a role in the contraction of both ileum and uterus smooth muscles and also exhibited antibacterial properties. A report by Khan *et al.* (2004) indicated that pure Z-venusol (**1**) did not have a direct effect on uterine smooth muscles contraction. However, it induced spontaneous contractility to some extent after the test substance was flushed from the organ bath. There is an assumption that the degree of activity of Z-venusol (**1**) may be moderated by the presence of other substances in the extract. Prolonged exposure of Z-venusol (**1**) to methanol resulted in the production of Z-methyl lespedezate (**2**). Z-methyl lespedezate (**2**) is common in woody and herbaceous monocotyledons (Harborne 1964). The potassium salt of

lespedezic acid isolated from *Lespedeza cuneata* was proven to be biologically active and responsible for the leaf opening mechanism in night closing plants (Mensor *et al.* 2001).

Phenolic lactones

Phenolic compounds are known to have high anti-oxidant properties (Fauconneau *et al.* 1997; Djeridane *et al.* 2006). Among the many active substances from *G. perpensa*, the majority are phenolic lactones such as ellagic acid (**4**) and its derivatives (3,3′,4′-tri-O-methyl ellagic acid (**5**), 3,3′,4′-tri-O-methyl ellagic acid 4-O-β-D-glucopyranoside (**6**) and punicalagin (**7**)). Ellagic acid (**4**) is a potent anti-oxidant capable of scavenging superoxide and hydroxyl anions. It can also reduce the damaging effect of H_2O_2 in the human body and, most importantly, it can be used as hepato-protective, chemo-protective and an anticancer agent (Do Khac *et al.* 1990; Girish and Pradhan 2008). It is also known for its anti-inflammatory and antimutagenic properties (Loarca-Piña *et al.* 1996; Sgariglia *et al.* 2013). The compound 3,3′,4′-tri-O-methyl ellagic acid (**5**) isolated from *Combretum kraussii* has shown antihaemorrhagic potentials (Do Khac *et al.* 1990). The other ellagic acid (**4**) derivative is punicalagin (**7**) which is the main compound of ellagitannins and a well-known anti-oxidant and anticancer polyphenol (Gil *et al.* 2000; Cerdá *et al.* 2004; Adams *et al.* 2006; Djeridane *et al.* 2006; Ascacio-Valdés *et al.* 2011). Punicalagin is one of the derivatives of ellagic acid (**4**) in which gallagic and ellagic acids (**4**) are connected to a glucose molecule. Therefore, it comprises one molecule of ellagic acid (**4**), one molecule of glucose, and one molecule of gallagic acid. Due to its high anti-oxidant ability, it can be used in the treatment of cardiovascular and neurodegenerative diseases (Cerdá *et al.* 2004; Ascacio-Valdés *et al.* 2011). However, a study conducted by Filippich *et al.* (1991) showed the hepatotoxic property of the compound, though such toxicity had not been reported by other researchers (Castonguay *et al.* 1997).

Quinones

Quinones with 1,4-benzoquinone subunit have been demonstrated with prominent pharmacological uses such as antimalarial, antibiotic, anticoagulant, antineoplastic, antitumor and herbicidal activities (Abraham *et al.* 2011). Drewes *et al.* (2005) were interested in screening

the anti-bacterial potentials of isolated compounds from the aerial parts of *G. perpensa*. Among the compounds assayed, the benzoquinone, 2-methyl-6-(3-methyl-2-butenyl)-benzo-1,4-quinone (**9**) showed effective antimicrobial activity against a range of microorganisms, including *Escherichia coli*, *Klebsiella pneumonia*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus cereus*, *Cryptococcus neoformans* and *Candida albicans*, with the highest activity observed against *Staphylococcus epidermidis*. However, 3-hydroxy-2-methyl-5-(3-methyl-2-butenyl)-benzo-1,4-quinone (**10**) displayed no activity against all microorganisms tested. Another interesting report by Chavan *et al.* (2013), showed that 2-methyl-6-(3-methyl-2-butenyl) benzo-1,4-quinone (**9**), isolated from the Brazilian *G. perpensa* species, has anticancer properties.

β-sitosterol

β-sitosterol (**3**) is the most common phytosterol with anticancer properties, especially for breast, prostate and colon cancers, including tumour growth inhibition and inducing apoptosis. Many studies have described the biological properties of β-sitosterol (**3**) as anti-inflammatory, chemo-preventive and immune-modulating activities (Park *et al.* 2007). It inhibits the hyperproliferation of colonic mucosa, which is the risk factor in the development of colon cancer. It has also inhibited enzymes responsible for testosterone metabolism in normal rat tissues that reduced the development of prostate cancer. Furthermore, it provides protection against cardiovascular diseases through its hypocholesterolemic action (Awad *et al.* 2000). Assmann *et al.* (2006) reported that high concentrations of β-sitosterol (**3**) in the blood increases the severity of heart disease in men that have previously experienced heart attacks.

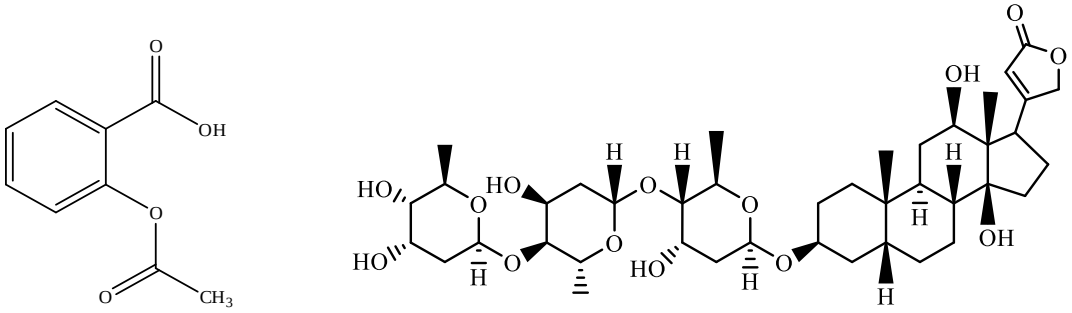
1.3. Plant secondary metabolites

Plants synthesise a vast range of organic compounds that are traditionally classified as primary and secondary metabolites though the precise boundaries between the two groups can, in some instances, be some what blurred. Primary metabolites are phytochemicals that have essential roles associated with photosynthesis, respiration, growth, and development of plants. Other phytochemicals, many of which accumulate in surprisingly high concentrations in some plant

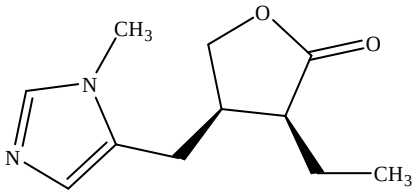
species, are referred to as secondary metabolites (Herrmann 1995; Crozier, Clifford and Ashihara 2008). Plant secondary metabolites (also called natural products) are biological molecules that are produced by a route other than the more predominant macromolecules like polysaccharides, nucleic acids and proteins (Cannell 1998).

Worldwide, there is an increasing trend in using plant-based traditional medicines as a primary health care. There is also a growing interest not only in determining the scientific basis for the plant's usage but also in discovering novel compounds of pharmaceutical value (Jäger, Hutchings and van Staden 1996). Plant secondary metabolites function is now attracting attention as some appear to have a fundamental role in protecting plants from herbivores and microbial infections, as attractants for pollinators and seed-dispersing animals, as allelopathic agents, UV protectants, and signal molecules in the formation of nitrogen-fixing root nodules in legumes (Crozier, Clifford and Ashihara 2008). They also play a major role in the adaptation of plants to their environment and during competition for food and water (Bourgaud *et al.* 2001; Boughalleb *et al.* 2005). These functional properties of secondary metabolites have drawn the attention of biologists including microbiologists, pharmacologists, cellbiologists, biochemists and toxicologists in the 20th century.

The development of spectroscopic techniques and chromatography have made the recovery of natural compounds more possible (Mahato and Kundu 1994; Bourgaud *et al.* 2001). The increase in the techniques involved in the isolation and identification of novel natural compounds from plants together with the diversity of plant species present in South Africa represent a huge source of novel compounds which have the potential to become drugs (Buwa and Van Staden 2006). Many bioactive natural compounds became the focus of research activities of emerging pharmaceutical companies after the discovery of antibiotics (Peter 2010). Some plant based former medicines or drugs include Morphine and Quinine (Figure 1), Aspirin, Digitoxin, and Pilocarpine (Figure 6).



Aspirin Digitoxin



Pilocarpine

Figure 6: Early drugs discovered from plants.

There are more than 100,000 structures of natural compounds present in the plant kingdom (Wink 1988). Recently, increased interests towards the therapeutic values of natural products made biochemists to identify around 40,000 structures in almost one decade (Paiva 2000; D. *et al.* 2011). Natural compounds can be produced either by all tissues or specific tissue or cell of a plant. They can also be accumulated at the site of synthesis or transported to other tissues or cells for storage. Some such as flavonoids, saponins, alkaloids and tannins are stored in the vacuole, while others such as terpenoids are stored in resin ducts, oil cells, cuticles, laticifers and trichomes (Purkayastha and Nath 2006).

1.3.1. Biosynthesis of secondary metabolites

In most cases, the biosynthesis of secondary metabolites takes place at a specific part of the plant. However, only some of them accumulate at the site of synthesis. For example, linamarin (toxin) is synthesised in the leaves of Cassava plant and stored in the roots (Paiva 2000). Based on their biosynthetic origins and chemical structures natural products can be divided into three

major groups; phenolics, terpenoids, and nitrogen-containing compounds (commonly called alkaloids) (Verpoorte 1998; Croteau, Kutchan and Lewis 2000; Lincoln *et al.* 2015). Phenolics are produced through Shikimate and Malonate pathways (Ghasemzadeh and Ghasemzadeh 2011). While terpenoids are produced from Mevalonate (Chappell 1995b) and Methylerythritol Phosphate (MEP) (Holstein and Hohl 2004) pathways.There is no direct or particular pathway for alkaloid synthesis since it is obtained from aliphatic and aromatic amino acids through enzyme catalysts. Alkaloids are defined as compounds comprising nitrogen-containing heterocyclic ring (Paiva 2000). The general pathways of plant secondary metabolites biosynthesis are depicted in Figure 7 (Lincoln *et al.* 2015).

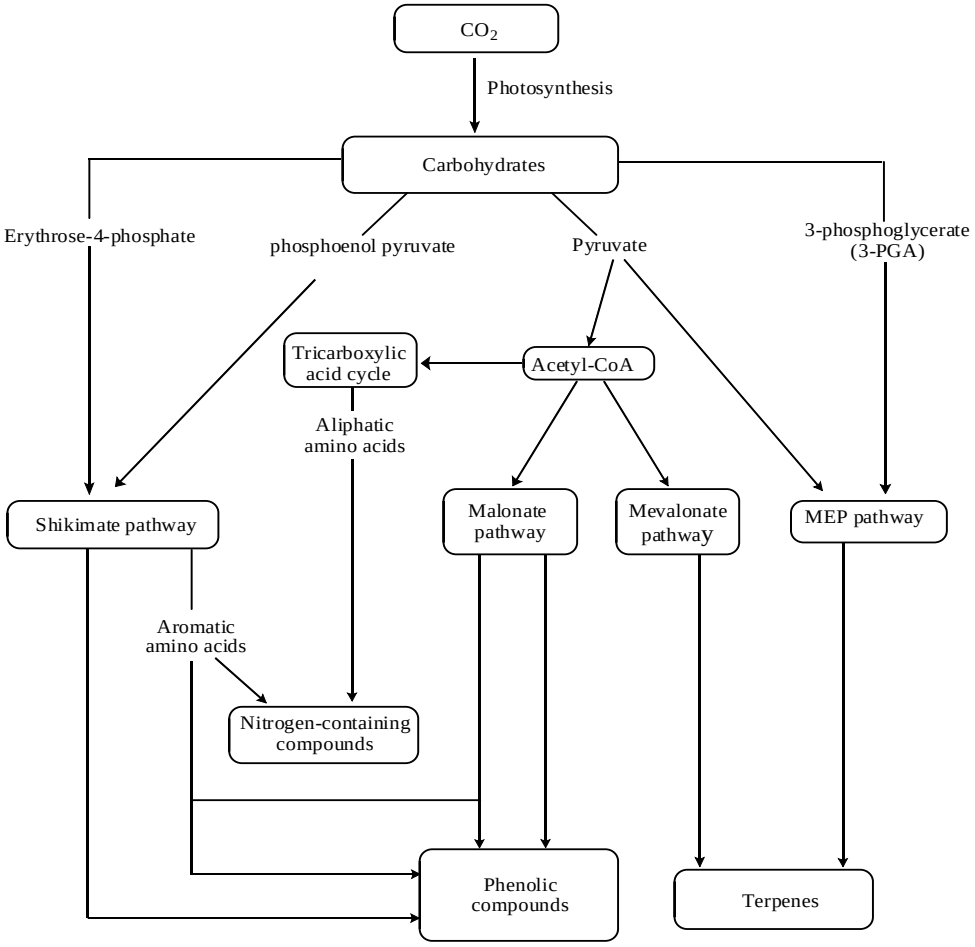


Figure 7: General biosynthesis of plant secondary metabolites (Lincoln *et al.* 2015).

Shikimic acid and Malonic acid pathways:

Although shikimic acid or shikimate was first isolated from *Illicium religiosum*, it can also be produced by micro-organisms. Even though the term 'secondary metabolites' had not then been discovered, the compound was considered as one. Later, shikimate was proved to be one of the mandatory primary metabolites and a precursor to the biosynthesis of folates and essential amino acids (tryptophan, tyrosine, and phenylalanine), benzoic acid, and naphthoquinones. This metabolic route is called shikimic acid or shikimate pathway and metabolites produced from this route are known to be Shikimate metabolites (Mousdale and Coggins 1985; Bentley and Haslam 1990). Malonate pathway is the second route used to synthesise plant phenolics. Unlike shikimate, this pathway is mostly used by bacteria and fungi than in plants (Lincoln *et al.* 2015).

In plants, chorismate is an intermediate in the pathway leading to the biosynthesis of aromatic compounds. The general route of Shikimate pathway in plants is described by Mousdale and Coggins (1985) (Figure 8). The pathway begins with the carbohydrate precursors (erythose-4-phosphate and phosphoenol pyruvate) and ends with the biosynthesis of phenylalanine, tyrosine, tryptophan, amino benzoic acid, and naphthoquinones (Mousdale and Coggins 1985; Herrmann 1995).

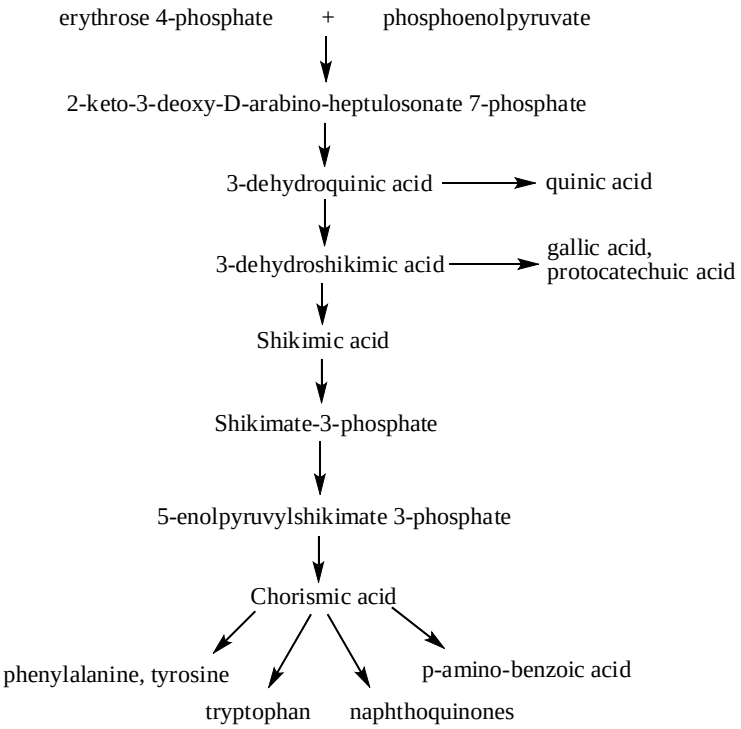


Figure 8: General route of the common Shikimate pathway in plants (Mousdale and Coggins 1985).

Mevalonic acid and Methylerythritol PhosphatePathways:

Mevalonic acid (MVA) and Methylerythritol Phosphate (MEP) pathways are the routes where isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are synthesised (Figure 9). MVA takes place in the cytoplasm where as MEP occurs in the plastid of the plant cell (Vranová, Coman and Gruissem 2013). IPP and DMAPP are five-carbon compounds which act as precursors in the biosynthesis of isoprenes (terpenes) and sterols (Holstein and Hohl 2004; Miziorko 2011). The polymerization of IPP and DMAPP is catalysed by the enzyme prenyl-transferase.

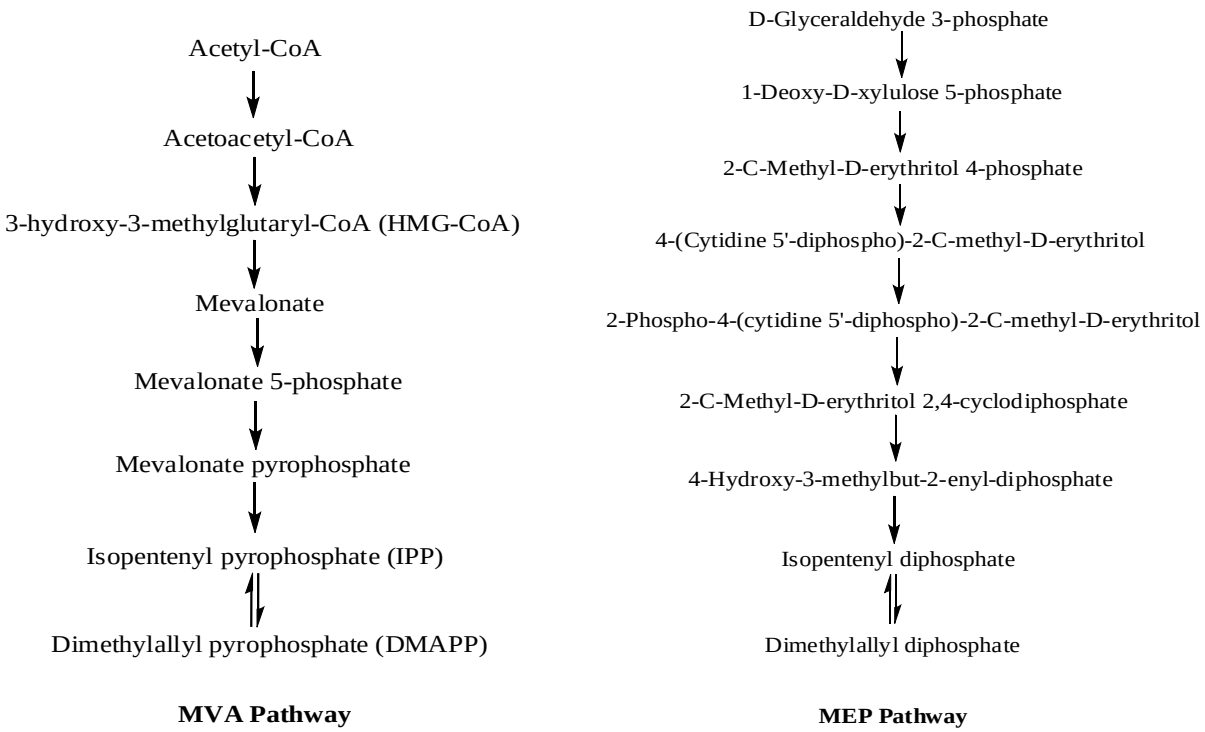


Figure 9: General routes of the common MVA (Miziorko 2011) and MEP (Vranová, Coman and Gruissem 2013) pathways in plants.

The precursors made by the MVA pathway are used for cytosolic isoprenoids (sesquiterpenes, sterols and ubiquinones) synthesis while those made by MEP pathway are used for plastid isoprenoids (monoterpenes, diterpenes, hemiterpenes and carotenoids) synthesis (Eisenreich, Rohdich and Bacher 2001). Isoprenoids include a wide range of primary and secondary metabolites that are important in agriculture, therapy, chemical and other marketable values. Only some algae and higher plants use both pathways to produce isoprenoid precursors. There is promising evidence that in the near future the inhibition of MVA pathway could be the key to novel therapeutic solutions for today's health disorders, including Alzheimer, cancer, and atherosclerosis (Chappell 1995a; Swanson and Hohl 2006; Buhaescu and Izzedine 2007).

1.3.3. Terpenes

Terpenes are defined as a large class of unsaturated hydrocarbons produced by plants, animals and insects. Although the term terpenoid is mostly used interchangeably with terpene, they have slight difference where terpene only contains a chain of carbon atoms with hydrogen while,

terpenoid includes their hydrogenated and oxygenated derivatives (Firn 2010). The name terpene or terpenoid was derived from turpentine, plant species of which the first terpene members were isolated (Croteau, Kutchan and Lewis 2000). The commonly known metabolic pathways of terpene biosynthesis are Mevalonateand MEP pathways. Recently, it has been reported that precursors, IPP and DMAPP can be synthesised through the mevalonate-independent pathway, deoxyxylulose phosphate pathway (Eisenreich, Rohdich and Bacher 2001). Even though terpenoids are synthesised in different organelles of the cell, they are transported to particular secretory structures for storage (Langenheim 1994). Their major functions in a plant include pigments (light-protecting and light-harvesting), part of chlorophyll, membrane modulators, and insect attractants and/or repellents (Eisenreich, Rohdich and Bacher 2001).

Terpenes classification is based on the number of five-carbon (isoprene) units (Figure 10) which are fused head-to-head, head-to-tail and sometimes head-to-middle fusion. The isoprene units, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), are taken as the building blocks of terpenes due toisoprene residue formation after thermal decomposition of terpenes (Kabera *et al.* 2014).

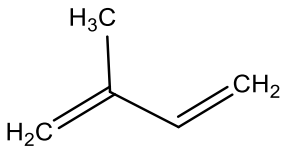


Figure 10: Isoprene unit

The group of terpenes based on the number of carbons and isoprene units include; single isoprene unit (C_5): hemiterpenes, two isoprene units (C_{10}): monoterpenes, three isoprene units (C_{15}): sesquiterpenes, four isoprene units (C_{20}): diterpenes, six isoprene units (C_{30}): triterpenes, eight isoprene units (C_{40}): tetraterpenes and greater number of isoprene units: polyterpenes (Croteau, Kutchan and Lewis 2000). The aforementioned groups of terpenoids further subdivided into five subgroups based on the number of rings present in each group. These subgroups are; acyclic, monocyclic, bicyclic, tricyclic, and tetracyclic for subgroups containing

zero, one, two, three, and four rings, respectively. In this research, triterpenes are discussed in more detail.

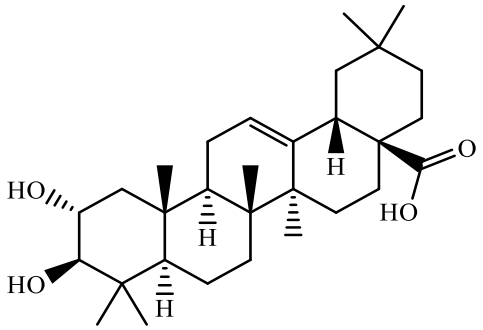
1.3.3.1. Triterpenes

Triterpenes are a group of terpenes with 30 carbon atoms containing two C_{15} chains which are joined head-to-head with the isoprene units linked head-to-tail in each chain (Croteau, Kutchan and Lewis 2000). Triterpenes are abundant in most marine and terrestrial fauna and flora. They occur in the form of ester, glycoside and ether but mostly found in their free forms. Like primary metabolites, some triterpenes have major roles and functions in the growth and development of the plant. For instance, brassinosteroids, are phytohormones with major roles in growth regulation (Lincoln *et al.* 2015). Triterpenes comprise of essential groups of plant bioactive compounds with more than 23,000 structures identified (Langenheim 1994).

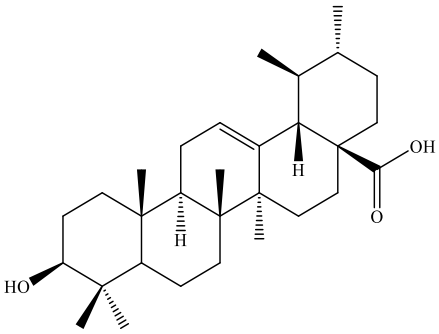
The promising potential of triterpenes has led to an increasing interest in the utilisation of different plants as a source. For example, plant parts like apple peel, birch bark, mistletoe shoots, and rosemary leaves are sources of various triterpenes (Laszczyk 2009). In this group, triterpenes with pentacyclic carbon skeleton, pentacyclic triterpenes (PCTs), are widely studied and comprise of leading groups of compounds. Nowadays, PCTs are attracting considerable attention due to their promising therapeutic potentials (Mahato and Kundu 1994; Babalola and Shode 2013).

1.3.3.2. Pharmacology of Triterpenes

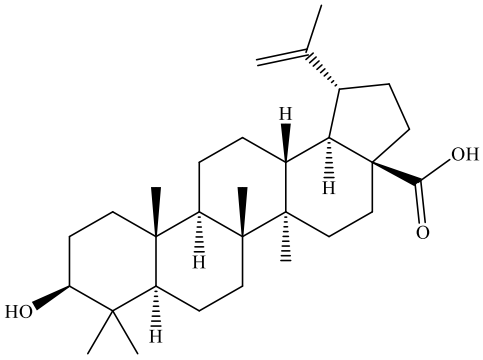
A number of triterpenes from different plant parts and sources have been reported to have both *in vitro* and *in vivo* biological potentials against various human health disorders. For example, oleanolic acid (OA), ursolic acid (UA), betulinic acid (BA), maslinic acid (MA), and lupeol (Figure 11) are among the most common and widely studied triterpenes. Some of the reports on the biological properties of these compounds are described as follows.



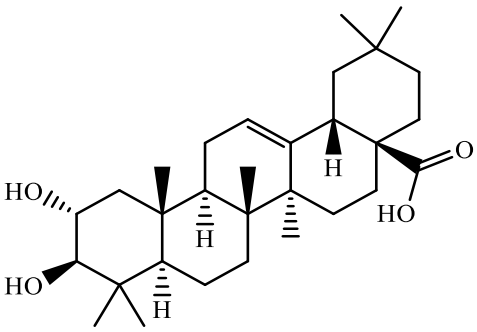
Oleanolic acid



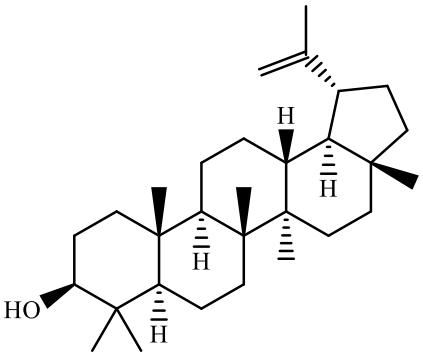
Ursolic acid



Betulinic acid



Maslinic acid



Lupeol

Figure 11: Some common and widely studied pentacyclic triterpenes.

Oleanolic acid is found in most plant species and known for its hepato-protective (Jeong 1999), anti-inflammatory (Singh *et al.* 1992), antitumor (Hsu, Yang and Lin 1997; Gu *et al.* 2006), cytotoxicity (Lee *et al.* 2007; Laszczyk 2009), chemo-protective (Katerere *et al.* 2003), anti-oxidant (Balanehru and Nagarajan 1991; Assimopoulou, Zlatanos and Papageorgiou 2005), anti-

angiogenic (Sohn *et al.* 1995), gastro-protective (Rodríguez, Astudillo and Schmeda-Hirschmann 2003), anti-mycobacterial (Copp and Pearce 2007; Bamuamba *et al.* 2008), anti-HIV (Mengoni *et al.* 2002), antimicrobial (Bhatti and Khera 2014), and cardiovascular (Somova *et al.* 2003; Liu 2005) therapeutic properties. In China, OA has been used as a drug for more than 20 years for the treatment of hepatitis.

A study by Abe *et al.* (2002) showed the complete inhibition of the movement of *Trypanosoma cruzi*, a protozoan parasite, epimastigotes by UA. UA has been suggested to have anti-plasmodial (Simelane *et al.* 2013), antitumor (Ovesna *et al.* 2004; Gu *et al.* 2006), anticancer (Li, Guo and Yang 2002; Laszczyk 2009), chemo-protective (Katerere *et al.* 2003), antimicrobial (Bhatti and Khera 2014), anti-oxidant (Balanehru and Nagarajan 1991; Assimopoulou, Zlatanos and Papageorgiou 2005), anti-angiogenic (Sohn *et al.* 1995), anti-inflammatory (Sohn *et al.* 1995), hepato-protective (Binduja *et al.* 1996), anti-HIV (Rodríguez, Astudillo and Schmeda-Hirschmann 2003), anti-mycobacterial (Copp and Pearce 2007; Bamuamba *et al.* 2008), and cardiovascular therapeutic (Somova *et al.* 2003) properties.

Betulinic acid, a pentacyclic lupane-type triterpene, extracted from Zizyphus species exhibited selective cytotoxicity against human melanoma cell lines (Pisha *et al.* 1995; ltokawa, Hitotsuyanagi and Lee 2011). BA has also been reported to have a chemotherapeutic effect on HIV and cancer (Cichewicz and Kouzi 2004; Fulda 2008). Furthermore, BA has antitumor (Gu *et al.* 2006), antimalarial (Chowdhury *et al.* 2002), anti-sickling (Shode *et al.* 2014), and anti-inflammatory (Sohn *et al.* 1995) properties.

Maselinic acid is proved to have anti-diabetic property through the inhibition of glycogen phosphorylase, which is responsible for glycogen breakdown (Guan *et al.* 2009; Qian *et al.* 2011). It is also among the thirteen triterpenes which were isolated from apple peels and showed effective antiproliferative properties against liver cancer, breast, and colon cell lines (He and Liu 2007). Other reported biological activities of MA include anti-sickling (Shode *et al.* 2014),

Neuroprotective, anti-HIV, anti-oxidative, anti-inflammatory, and anticancer properties (Liu *et al.* 2007; Qian *et al.* 2011).

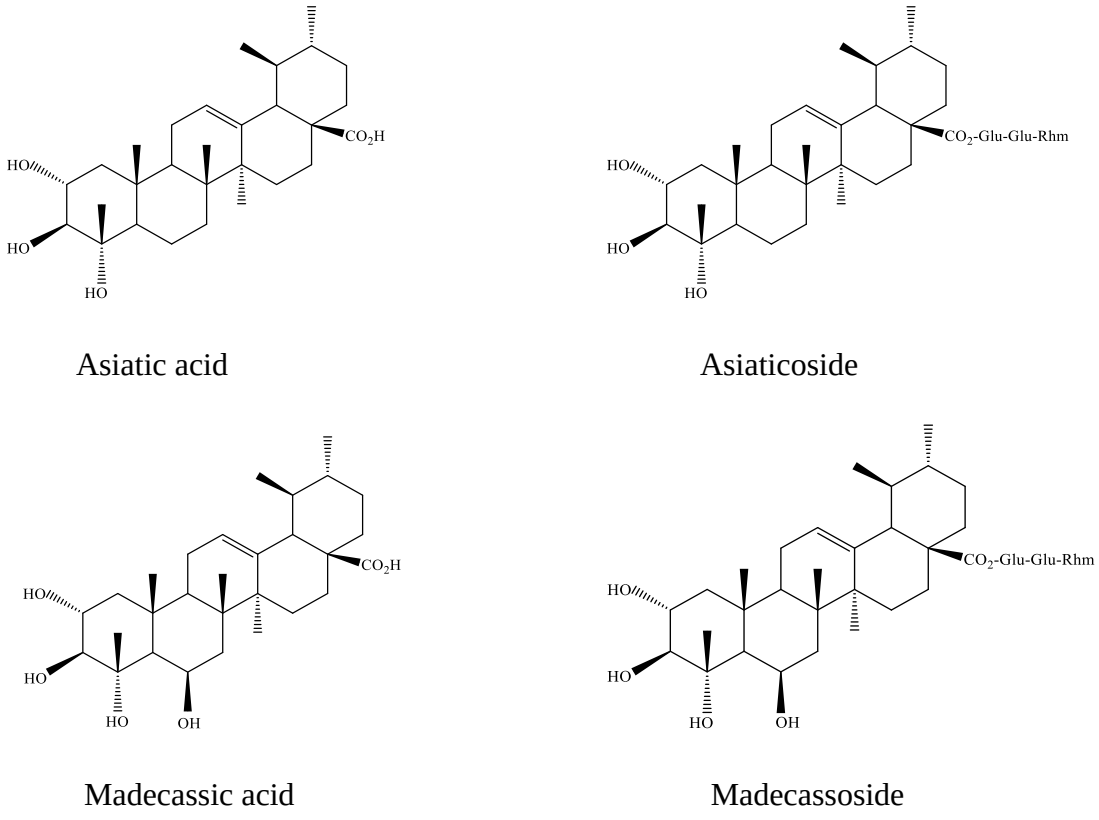
Previous studies revealed the anti-malarial, antiproliferative, antimicrobial, anti-arthritic, anti-mutagenic, antiprotozoal, and anti-inflammatory properties of Lupeol. Lupeol is a dietary triterpene commonly found in fruits such as Mango and Fig (Saleem 2009). The anti-oxidant nature of the compound was studied by Nagaraj, Sunitha and Varalakshmi (2000) through the treatment of rats exposed to Cadmium, potent nephrotoxin. In addition to the aforementioned biological properties, Lupeol has chemopreventive (Saleem 2009), arthritis (Sudhahar, Kumar and Varalakshmi 2006), anti-inflammatory (Geetha and Varalakshmi 2001; Nikiema *et al.* 2001), analgesic (Singh *et al.* 1997), and anticancer properties against various cancer cell lines (Aratanechemuge *et al.* 2004; Saleem *et al.* 2005; Gauthier *et al.* 2006; Saleem *et al.* 2009).

Triterpenes in Food and Cosmetic industries

In the last two decades, food and cosmetic industries are replacing synthetic additives by natural compounds. Due to their high biological activity and low toxicity, most triterpenes are commercially used in cosmetic, food, and beverage industries (Bhatti and Khera 2014). In cosmetic industries, there is an increasing demand for anti-aging creams and skin products commercially. As skin cells grow older their ability to produce new collagen fibres decreases and facilitates ageing. This will make collagen synthesis the main concept in formulating anti-aging skin care products (Moloney *et al.* 1992).

Previous study showed that *Centella asiatica* can be used to treat stretch marks, photo-aging skin, and cellulite (Bylka *et al.* 2013). The bioactive compounds responsible for these properties of *Centella asiatica* include the pentacyclic triterpenes; asiatic acid, asiaticoside, madecassic acid and madecasosside (Figure 12). These bioactive compounds are commonly used in anti-aging and anti-cellulite skin care products in cosmetic industries (Morganti *et al.* 1999; Bylka *et al.* 2014). However, a study by Cho, Gottlieb and Santhanam (1996) showed that BA has six-fold better collagen synthesis stimulation than any of the triterpenes from *Centella asiatica*. This

property of the compound has increased the interest of food and cosmetic companies to use BA and plants proved to contain BA (Cho, Gottlieb and Santhanam 1996).



Asiatic acid Asiaticoside

Madecassic acid Madecassoside

Figure 12: Commercially used pentacyclic triterpenes from *C. asiatica*.

Ursolic acid and Oleanolic acid are among the most commercially utilised natural compounds in the production of health and cosmetic products (Bhatti and Khera 2014). Assimopoulou, Zlatanos and Papageorgiou (2005) suggested that due to the resulted high anti-oxidant properties of UA and OA from vegetable oils and lard, these sources can be used as dispersion medium and anti-oxidants in cosmetic, food supplement, and pharmaceutical preparations. The other most important triterpenes in pharmaceuticals and cosmetic industries are phytosterols. Phytosterols are mostly collected from the processing of cellulose and vegetable oil wastes. They are used to produce lipsticks and creams, and anti-cholesterol additives in cosmetic and food industries, respectively (Fernandes and Cabral 2007; Górnaś, Siger and Segliņa 2013).

CHAPTER 2: EXTRACTION, ISOLATION AND STRUCTURAL ELUCIDATION OF SECONDARY METABOLITES FROM *Gunnera perpensa*

2.1. Introduction

Extraction of the plant material is the most important and first step involved in the isolation and purification of the active compounds from plants. Plant extraction methods could involve the use of fresh or freshly picked and dried plant tissues, even if, the plant materials should be dried in a controlled laboratory environment to prevent the occurrence of chemical changes (Harborne 1998). The other important factor during plant extraction is the solvent used. The solvent used during extraction highly defines the amount and types of compounds to be extracted. For instance, in non-polar solvent extraction, lipids and steroids will be extracted in large quantities whereas, in polar solvent extraction, sugars will be present in large amounts (Verpoorte 1998). Different types of extraction methods have been designed: some target specific compounds, others extract in general. For instance, a report by Al-Yahya *et al.* (1985) stated that the use of the combination of an equal ratio of dichloromethane and methanol extracted lipophilic compounds with antitumor potentials. The extraction of hydrophilic compounds was also reported to be effective using polar solvents such as ethyl acetate, ethanol or methanol (Sasidharan *et al.* 2011). Other studied parameters like extraction time and temperature, and solvent volume and composition can also affect the extraction process (Verpoorte 1998).

Isolation of bioactive compounds from different parts of plants has been performed for decades using various methods (Ley and Baxendale 2002). Among the different separation techniques, chromatographic techniques such as Thin-Layer Chromatography (TLC), Preparative Thin-Layer Chromatography (PTLC), Column Chromatography (CC) and High-Performance Liquid Chromatography (HPLC) have been used in this study. Chromatography is a physical method where mixtures are separated through distribution between stationary and mobile phases (McMurry 2014).

Thin-Layer Chromatography is a method that is used in the separation and purification of compounds from various biological samples such as urine, plant extracts, and blood. It is categorised under liquid chromatography in which glass, plastic or aluminium sheets are layered with fine-particle sorbent stationary phase (Sherma and Fried 2005). Basic TLC method involves the application of sample solution onto the baseline (1 cm away from the edge) of the TLC plate. The plate is developed by capillary movement in a closed developing tank containing the mobile phase (solvent). The developed plate then is removed and the front line (distance travelled by the solvent) is marked and measured to be used to calculate R_f values of the separated compounds. R_f stands for retention factor and is used to quantify the movement of the materials along the plate (Marston 2011). R_f value is equal to the distance travelled by the compound divided by the distance travelled by the solvent. Visualisation of the substances on a developed plate can be done by direct colour observation for colourful compounds, and additional visualisation methods such as ultraviolet (UV) light, iodine vapour, anisaldehyde and sulphuric acid treatments are used for colourless compounds (Andersen 1969).

Preparative Thin-Layer Chromatography is another useful and common separation technique. This method follows similar principles as TLC but can be used to isolate larger quantities of compounds than TLC (milligram to gram) (Colegate and Molyneux 2007). The merit of using PTLC in the laboratory is that it is highly cost effective. The disadvantages of this method include the potentially hazardous recovery of compounds and getting a clear visualisation of compounds (Andersen 1969).

Column Chromatography is also a physical method where a compound is separated from a mixture of compounds. It involves the use of mobile phase (solvents) and stationary phase (solids such as silica gel and alumina). For silica gel column chromatography, depending on the degree of closeness between the components (predetermined by TLC), the ratio of silica to analyte weight ranges within 100:1 to 20:1 (Yanga *et al.* 2012). Each component of the analyte interacts differently with the silica, therefore, their relative speed while passing through the column differs. This technique is more advantageous because of its stationary phase

disposability, and its relatively low cost. The availability of the stationary phase prevents cross-contamination since it avoids recycling (Colegate and Molyneux 2007).

High-Performance Liquid Chromatography is a commonly used analytical technique for the isolation and separation of natural compounds. Generally, HPLC is used for separation of mixtures with less complexity, mostly used at late stages of separation (Cannell 1998). The choice of stationary and mobile phases used while running a particular sample highly affects the extent of chemical separation of the natural products (Sasidharan *et al.* 2011). Purification and identification of compounds is also possible using HPLC. Purification involves the isolation of the target compound from other contaminants using standards. To get a high degree of purification, the chromatographer must choose the best conditions of separation, which includes; suitable mobile phase, detectors, flow rate, and column. On the other hand, identification of natural compounds typically involves the use of high sensitive UV detector as most of the bioactive compounds have some UV absorbance wavelengths (Cannell 1998; Fan *et al.* 2006).

Structural elucidation is fundamental in organic chemistry as it provides the molecular structures of compounds. It is achieved through the use of different spectroscopic techniques such as nuclear magnetic resonance (NMR), X-ray, infra-red (IR), and mass spectrometry (MS) analysis (Koehn and Carter 2005). In this study, NMR techniques were used to structurally identify the isolated compounds of *Gunnera perpensa*. NMR spectroscopy has been used for more than 40 years as a reliable method for the determination of molecular structures (Colegate and Molyneux 2007). This technique provides a way of determining the molecular structure of an organic compound by measuring the chemical environment of the individual nucleus. This is a powerful method for determining the types, numbers and interconnections of nuclei found in a particular organic compound (Martin, Delpuech and Martin 1980; Harborne 1998; Tesso 2005; Colegate and Molyneux 2007).

2.2. Materials and Methods

2.2.1. Collection of plant material

Fresh plant material of *G. perpensa* was collected in April 2015 from Caversham glenn national reserve (-29.8360785, 30.8447608), Durban, KwaZulu-Natal province of South Africa. The plant material was identified by Prof. H. Baijnath (Taxonomist, School of Life & Conservation Sciences, UKZN, Westville Campus) and a voucher specimen of the plant has been preserved in the Ward Herbarium of the School of Life Sciences, Department of Botany, University of KwaZulu-Natal. Healthy plant leaves and stalks (leaves and stalks which were not infected by bacteria, fungi or virus) were separately collected, washed and dried in an oven at 50 ^{o}C for 48 h. Once completely dried, plant materials were ground to a fine powder using a Waring blender. The ground leaf and stalk materials were stored at room temperature in closed containers until required.

2.2.2. Extraction

The extraction of plant materials was done using the method described by (Harborne 1998) with some modifications. The extraction of the leaves and stalks was done with different solvents using different extraction protocols. The first extraction protocol was used for both leaf and stalk plant materials and the second was used only for the extraction of the stalks of *G. perpensa*. These two extraction protocols are discussed in detail below.

2.2.2.1. Extraction of the leaves and stalks (Protocol 1)

One of the standard procedures for isolating major biological constituents from dried and ground plant tissue is to extract the material with polar and nonpolar solvents. In this study, *G. perpensa* leaf and stalk powder (500g each) were macerated in n-hexane for three days (standing) and filtered using Whatman no.1 filter paper. The filtrates were reduced to 20 ml by a rotary evaporator and finally dried by laminar flow vapour extraction in a fume hood to give a total of 10.81 and 1.82 g of the leaf and stalk extracts, respectively. Hexane extraction was done three times (3X) providing 5.47, 3.84 and 1.5 g of the leaf and 0.82, 0.61 and 0.39 g of the stalk for the first, second and third round extracts, respectively. The residue was then macerated in ethyl

acetate (3X) for three days followed by filtration. The filtrate was evaporated under reduced pressure to a viscous extract. The viscous extract was allowed to dry to give 4.12, 4.02 and 2.12 g of the leaf and 2.17, 2.08 and 1.7 g of the stalk for the first, second and third extracts, respectively. The residue was finally extracted with methanol (3X) providing 16.22, 12.2 and 11.4 g of the leaf and 27.19, 24.22 and 20.74 g of the stalk for the first, second and third extracts, respectively. The general extraction method used for the leaves and stalks of *G. perpensa* is represented in Figure 13.

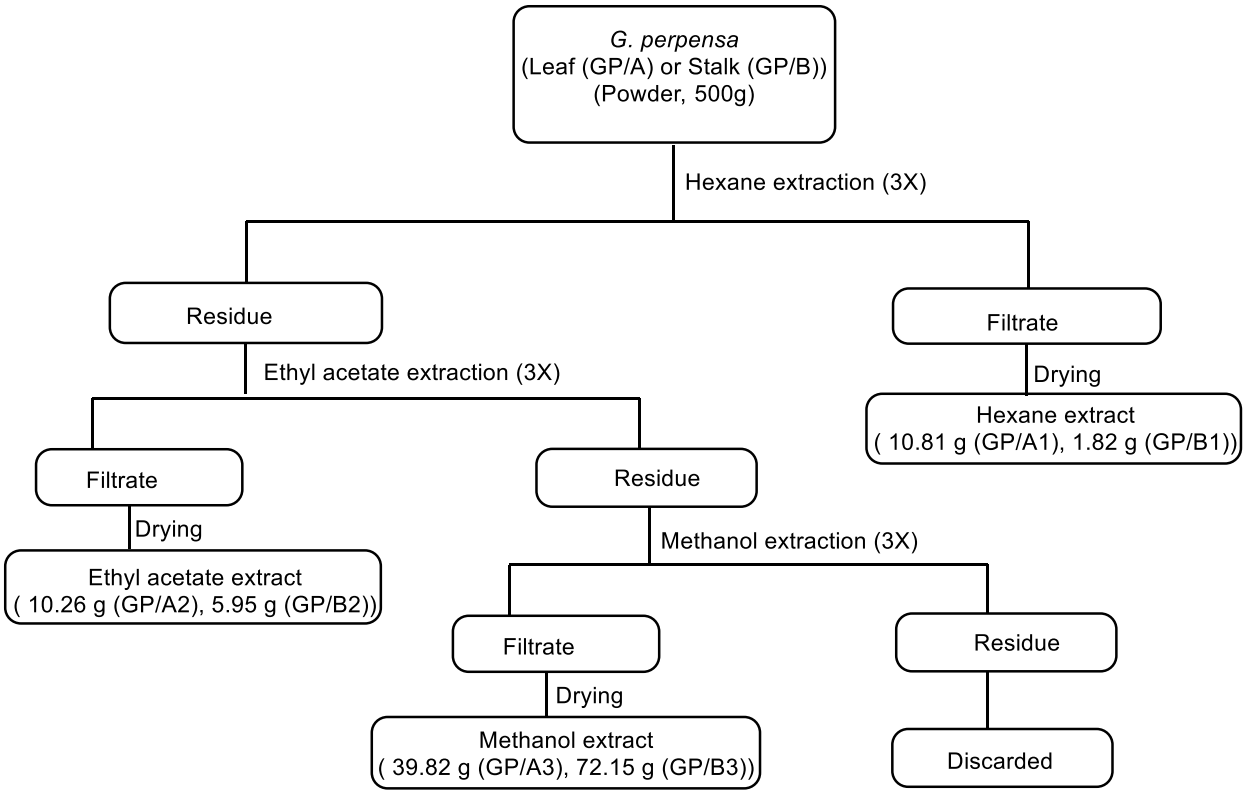


Figure 13: General extraction of the leaves and stalks of *G. perpensa*.

2.2.2.2. Extraction of the stalks (Protocol 2)

The extraction method described by (Harborne 1998) was used with some modification during *G. perpensa* stalk extraction. *G. perpensa* stalk (200 g) was macerated in n-hexane for three days at room temperature and filtered using Whatman no.1 filter paper. The filtrate was then reduced to 20 ml by a rotary evaporator and finally dried by laminar flow vapour extraction in a fume

hood to give a 0.24 g of the stalk extract. The residue was then extracted three times (3X) with ethyl acetate for three days followed by filtration. The filtrates were evaporated under reduced pressure to viscous extracts. The viscous extracts were dried thoroughly to provide 0.96, 0.69 and 0.52 g of the stalk extract for the first, second and third extractions, respectively. The residue (marc) was then macerated in dichloromethane (DCM) for three days and filtrated. The filtrates were dried to give 0.13 g of the stalk extract.The residue from DCM extraction was macerated in acetone (2X) and provided a total of 2.08 g of stalk extract. The residue was finally extracted (2X) with methanol providing 15.33 and 8.53 g of the stalk extract for the first and second extracts, respectively. The general solvent extraction of the stalk of *G. perpensa* is schematically represented in Figure 14.

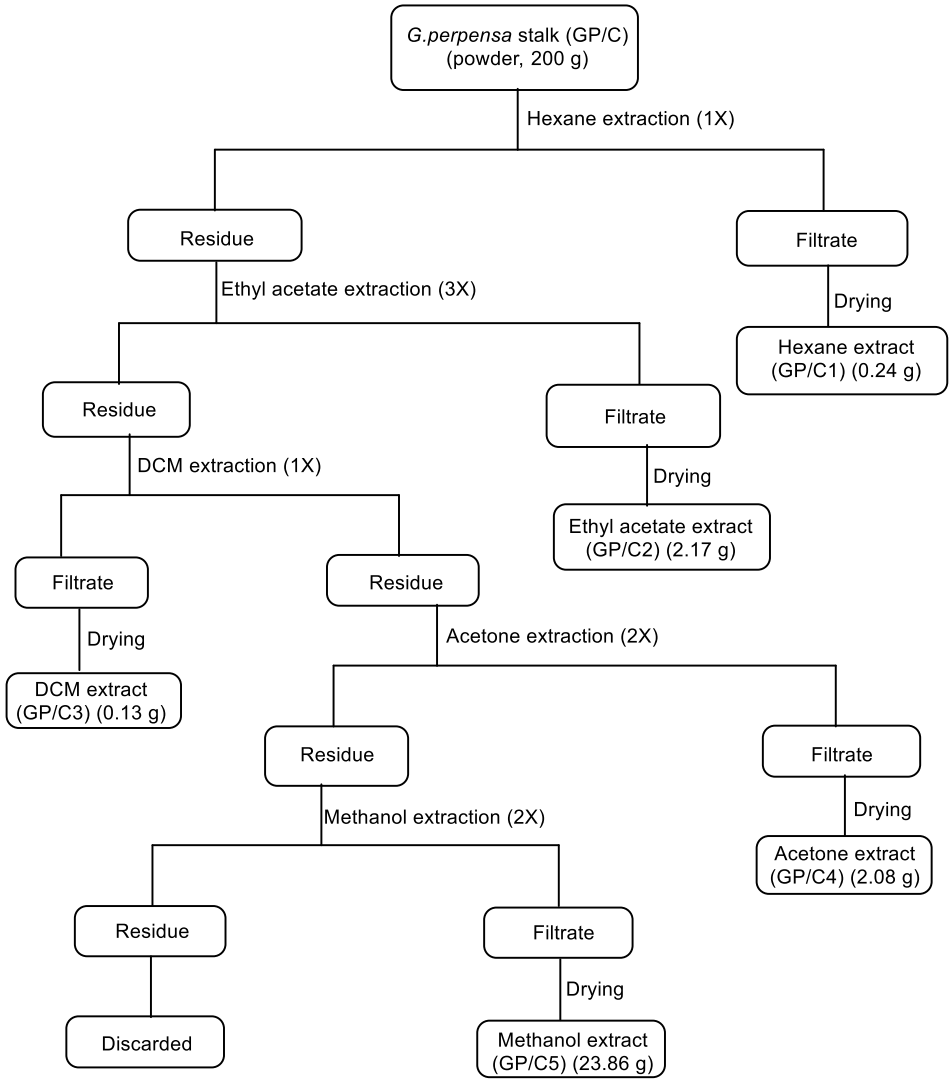


Figure 14: General extraction of the stalks of *G. perpensa*.

2.2.3. Isolation and purification

In this study, the difference in polarity of the active compounds was used as the basic principle of separation. The method described by Harborne (1998) was used with some modifications to separate the secondary metabolites from *G. perpensa*. Fractionation was done using chromatographic techniques; TLC, PTLC, CC, and HPLC (Fair and Kormos 2008). The details of the protocols used during fractionation are explained as follows.

2.2.3.1. Thin Layer Chromatography

TLC was done on silica gel coated aluminium plates (Merck TLC Silica-gel 60 F_{254}). The sample extracts were dissolved in a solvent and spotted on TLC plate 1 cm away from the edge. The plates were developed using different gradients of hexane: ethyl acetate (9-0: 1-10) as eluent. The compounds on the developed plates were visualised using visible and UV light (254 and 366 nm, Camag, Universal UV Lamp TL-600). To further visualise the compounds that were not visible under UV light, the plates were treated with acid (5% sulfuric acid in methanol) and heated for 3-5 minutes at 110^{o}C.

2.2.3.2. Preparative Thin Layer Chromatography

PTLC was used to separate some compounds from less complex combined fractions of column chromatography. It was done using 2mm thick silica gel coated glass plates (ANALTECH, preparative layer with UV 254). Sample solutions were prepared with different concentrations ranging from 20-50 mg/ml depending on the degree of separation of the compounds on normal TLC plates. The plates were developed using a specific mixture of hexane: ethyl acetate based on the best separation observed on TLC plates. The developed plates were visualised using visible light and UV light at two different wavelengths, 254 and 366 nm (Camag, Universal UV Lamp TL-600). To further identify the locations of the UV invisible compounds, a marker aluminium TLC plate was developed together with the glass plate. The marker plate was then treated with acid (5% sulfuric acid in methanol) and heated for 3-5 minutes at 110 ^{o}C to allow for the development of colour. Using the coloured compounds on the marker TLC plate, it was possible to locate the corresponding compounds on the glass PTLC plate. The silica gel with marked compounds was removed and washed with methanol. The solutions were filtered using Whatman No.1 filter paper and the methanol was evaporated from the filtrate to get pure and dry compounds.

2.2.3.3. Column chromatography

A slurry of silica gel 60 (0.063-0.200 mm) was made by mixing the silica gel with the solvent for ten minutes. A specified amount of the extract was dissolved in the same solvent. However, extracts which cannot dissolve in the solvent were mixed with small quantity of silica gel and

applied to the column in powder form. The weight proportion of silica gel to dry plant extract was 30:1. The slurry silica gel was poured into the column and given enough time to pack tightly through a continuous flow of the solvent. The sample solution was applied carefully on top of the silica without disrupting the column. Then, a smaller amount of acid purified sand (0.1-0.3 mm) was applied on top of the sample to prevent disturbance of the top level of the sample during solvent application. More solvent was added to the packed column and allowed to run using gravitational force. The picture showing the setup of the column together with its progress after collecting few fractions is shown in Figure 15.

In this study, the ethyl acetate crude extracts of both the leaves (GP/A2) and the stalks (GP/B2, GP/C2) of *G. perpensa* were subjected to CC. A concentration gradient of hexane/ethyl acetate mixtures (90% - 30% hexane) were used to run the column and 20 ml eluent was collected. The whole process was monitored by TLC and fractions with similar TLC profile were combined. Finally, the column was washed with 100% methanol to give a mixture of very polar fractions that were unable to move with the previous eluents.

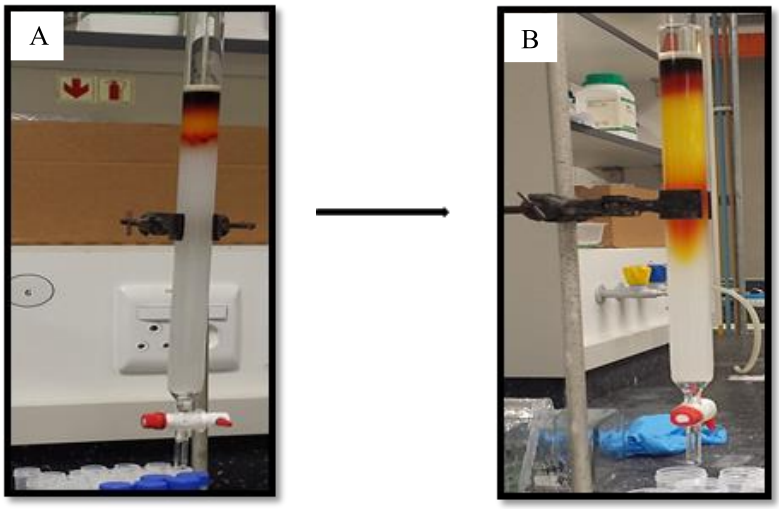


Figure 15: Column Chromatography setup; A: column set up, B: after collection of few fractions.

2.2.3.4. HPLC

HPLC analysis was carried out for the separation of compounds **I** and **II** (chapter 4) with some modifications to the method described by Tian *et al.* (2010). Commercial standards of OA and UA (Sigma-Aldrich, Inc.) were used to identify the compounds. Ten µl of sample and standards were analysed by Shimadzu LC-20AB using SunFire C18 column (150 X 4.6 mm, 5 µm). The mobile phase was prepared from 0.03 M phosphate buffer and methanol with 1:9 buffer to methanol proportion at pH of 3. The analytes were injected at a flow rate of 0.5 ml/min and run time of 30 minutes.

2.2.3.5. Nuclear Magnetic Resonance (1D and 2D NMR) Spectroscopy

The ^{1}H, ^{13}C and 2D NMR spectroscopy were recorded at room temperature either using deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO) as solvents on a Bruker Avance111 400 MHz spectrometer with tetramethylsilane (TMS) as an internal standard. The chemical structures of the isolated compounds (**I-V**) were determined by comparing the assignment of their ^{13}C signals with the literature values of the corresponding known compounds.

2.3. Results

2.3.1. Extraction and isolation of secondary metabolites (Protocol 1)

2.3.1.1. Extraction

The leaves and stalks of *G. perpensa* (powder, 500 g, each) were extracted using hexane, ethyl acetate and methanol. The results showed that the methanol crude extracts of both the leaves (GP/A3) and stalks (GP/B3) had provided the highest percentage recovery yield than the rest of the solvents used (Tables 3 and 4).

Table 3: Percentage recovery yield of *G. perpensa* leaf extracts using three different solvents.

Solvents used	**Extraction Rounds (%)**			**Total Extraction (%)**
	1st Extraction	**2nd Extraction**	**3rd Extraction**	
Hexane	1.1	0.77	0.3	2.17
Ethyl acetate	0.82	0.8	0.42	2.04
Methanol	3.24	2.44	2.28	7.96

Table 4: Percentage recovery yield of *G. perpensa* stalk extracts using three different solvents.

Solvents used	**Extraction Rounds (%)**			**Total Extraction (%)**
	1st Extraction	**2nd Extraction**	**3rd Extraction**	
Hexane	0.16	0.12	0.08	0.36
Ethyl acetate	0.43	0.42	0.34	1.19
Methanol	5.44	4.84	4.15	14.43

As shown in the Tables, the percentage recovery yields of the methanol extracts of the leaves (7.96 %) and stalks (14.43 %) of *G. perpensa* were the highest percentage yields followed by hexane (2.17 %) and ethyl acetate (1.19 %) for the leaves and stalks, respectively. The lowest yield was recorded from the ethyl acetate extraction of the leaves (2.04 %) and hexane extraction of the stalks (0.36 %).

2.3.1.2. Isolation

The TLC analysis of the crude extracts showed that the ethyl acetate extracts of the leaves (GP/A2) and stalks (GP/B2) had several predominant compounds with better separation using different proportions of hexane-ethyl acetate mobile phase. Therefore, the secondary metabolites of GP/A2 and GP/B2 were isolated by column chromatography using hexane/ethyl acetate gradient elution (90% - 40% hexane).

The CC of GP/A2 led to the isolation of compound **I** (see Section 2.3.3) and a mixture of compounds (**X**). A portion of GP/A2 (4 g) was subjected to CC and afforded 354 fractions. Thirty-six initial combined fractions were obtained based on the TLC profile similarities of the fractions. An oily material (**X**) (410 mg) was obtained from the first initial combined fraction. Further attempt to separate these compounds using CC failed. The initially combined fractions that showed white deposit were re-combined after TLC result confirmation. The white amorphous material (70.3 mg) was separated from the supernatant by centrifugation. The TLC analysis of the white material showed one single spot (compound **I**) that showed pink colouration on the TLC plate after acid treatment. The percentage recovery yield of compound **I** and **X** is described in Table 5.

Table 5: Percentage recovery yield of isolated compounds from GP/A2.

Isolated compounds	Weight of isolates (mg)	Percentage Yield
X	410	0.31 %
I	70.3	0.04 %

The CC of GP/B2 (1 g) led to the isolation of mixed compounds (**II**, **III** and **IV)** and compound **V**. The CC afforded 607 fractions (20 ml each) and fifty combined fractions. The combined fractions that showed white deposit were further combined after TLC result confirmation. After combination, the precipitate was separated from the supernatant using centrifugation. A mass of 216 mg white amorphous material was collected after centrifugation. The TLC analysis of the white material showed two very close spots (a mixture of compounds **II**, **III** and **IV**) (R_f = 0.37 and 0.47) that showed pink colouration after acid treatment (Figure 16). However, attempts to separate these compounds using CC failed.

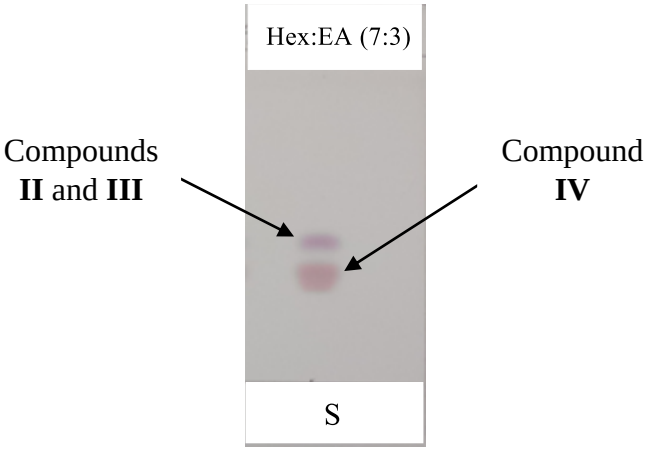


Figure 16: TLC analysis of the white amorphous precipitate from CC of GP/B2.

2.3.2. Extraction and isolation of secondary metabolites (Protocol 2)

2.3.2.1. Extraction

In the second extraction protocol, the extraction of the stalks led to larger amount of methanol extract (GP/C5) than hexane (GP/C1), ethyl acetate (GP/C2), DCM (GP/C3) and acetone (GP/C4) extracts (Figure 14). Even though the extraction frequencies are different for the various solvents used, it is not hard to point out that methanol has shown better extraction yield than the rest of the solvents used during the extraction. As shown in Table 6, the total percentage yield from methanol extraction (11.93 %) is highly incomparable to the others.

Table 6: Percentage recovery yield of *G. perpensa* stalk extracts using five different solvents.

Solvents used	Extraction frequency	Total Extraction
Hexane	1X	0.12
Ethyl acetate	3X	1.09
DCM	1X	0.07
Acetone	2X	1.04
Methanol	2X	11.93

2.3.2.2. Isolation

The TLC analysis of the crude extracts obtained from the second extraction protocol showed that ethyl acetate (GP/C2) contained more compounds than the other extracts. Therefore, GP/C2 (2.17 g) was subjected to CC using hexane/ethyl acetate gradient elution (90% - 30% hexane). The column afforded 664 fractions (20 ml each) and finally, the column was washed with methanol to give a mixture of very polar fractions.

The 664 fractions were combined and gave twenty-nine combined fractions based on their TLC profile similarities. Further TLC analysis based combination resulted in five sub-fractions, namely A1, A2, A3, A4, and A5 (Figure 18) A1, a mixture of non-polar substances, was not investigated further. A2 and A5 on standing, deposited a whitish solid which was separated from the supernatant by centrifugation providing precipitates A2a (266mg) and A5a (160mg), respectively, as well as the corresponding supernatants, A2b and A5b. The precipitate, A2a showed only one spot (a mixture of two compounds **II** and **III**) (see Section 2.3.4) on TLC analysis while A5a showed one major spot (compound **V**) (see Section 2.3.4) and a minor spot. Previously, it was difficult to separate compounds **II**, **III** and **IV** in the CC of GP/B2, however, the compounds were separated in the CC of GP/C2. The separation of compound **IV** was confirmed by TLC analysis comparison of the isolated white amorphous mixture from GP/B2 (**S**) and the separated samples from GP/C2 (**S1** and **S2**) (Figure 17). The presence of compounds **II**

and **III** in A2a was observed in the ^{13}C NMR spectral results and further HPLC analysis was done for confirmation (Figure 19). The percentage recovery yield of the isolated compounds of GP/C2 are described (Table 7).

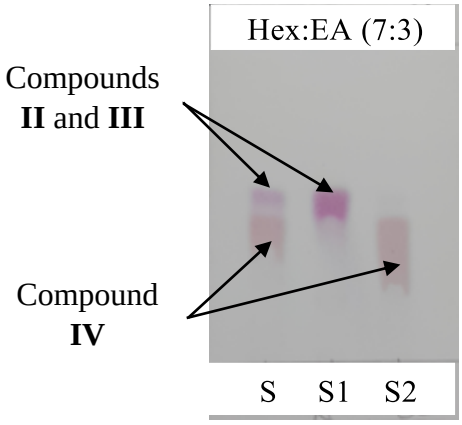


Figure 17: TLC analysis of the isolated white amorphous materials; **S** (GP/B2), **S1** and **S2** (GP/C2).

A3 gave a whitish amorphous solid (a mixture of compounds). The centrifugation of A4 also gave a white amorphous solid with single spot and colouration (compound **IV**) (see Section 2.3.4) on TLC plate. The fractionation summary of GP/C2 is described in Figure 18. The last fraction obtained after washing the column with methanol had less complex compounds and named as compound **Y** in this study.

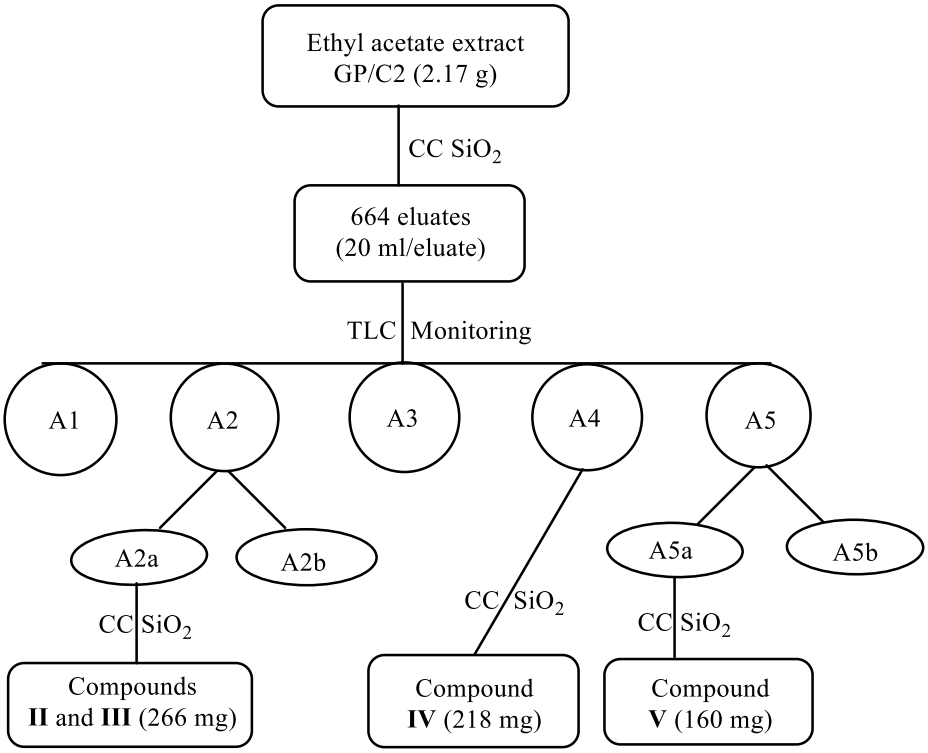


Figure 18: Summary of the fractionation of GP/C2. A1: Non-polar components, A2a: White amorphous solid, A2b: Light green amorphous solid, A3: white amorphous solid, A4: white amorphous solid, A5a: white amorphous solid, A5b: others.

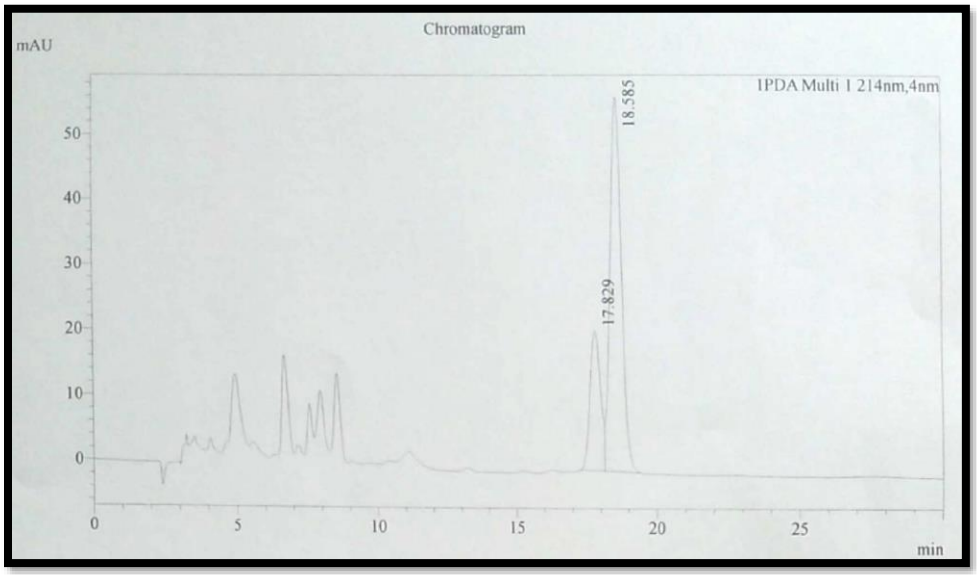


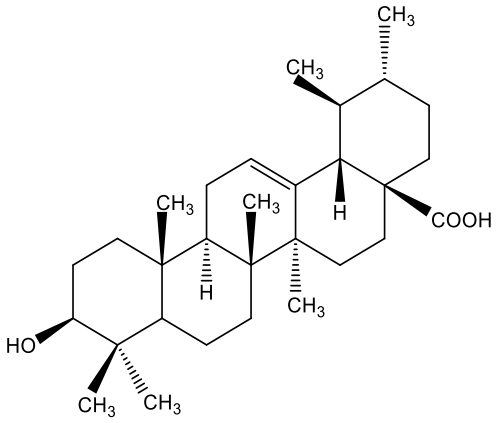
Figure 19: HPLC chromatogram of compounds **II** and **III**.

Table 7: Percentage recovery yield of isolated compounds from GP/C2.

Isolated compounds	Weight of isolates (mg)	Percentage Yield
II and **III**	266	0.133 %
IV	218	0.109 %
V	160	0.08 %

2.3.3. Structural elucidation of isolated compounds from the leaves

The white amorphous solid (compound **I**), obtained from GP/A2, accounted for 0.04 % of the dry weight of the leaves. The ^{13}C NMR data of this compound consisted of chemical shifts that corresponded to the chemical shifts of ursolic acid described by Silva *et al.* (2008) (Table 8). The chemical structure of compound **I** (UA) is shown in Figure 20.



I

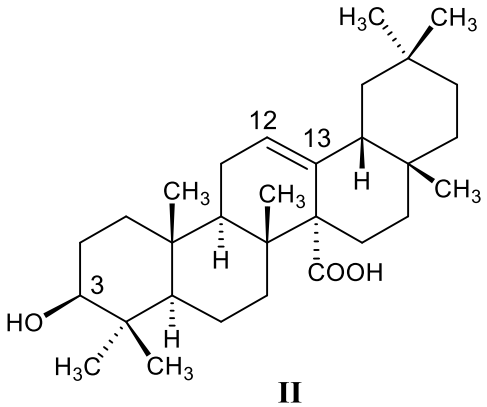
Figure 20: Chemical structure of compound **I**

Table 8: Comparison of ^{13}C NMR spectral data of compound **I** with those of Silva *et al.* (2008).

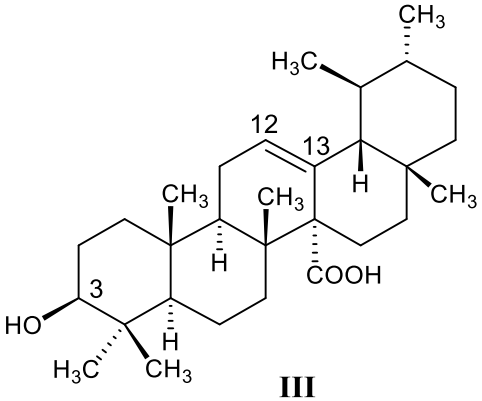
Carbon Position	**(Silva *et al.* 2008)**	**Compound I**
1	38.22	38.18
2	26.79	26.95
3	**76.82**	**76.79**
4	38.35	38.34
5	54.77	54.73
6	17.97	17.96
7	30.77	31.11
8	39.09	39.04
9	47.00	46.97
10	36.51	36.49
11	23.80	23.79
12	**124.54**	**124.54**
13	**138.18**	**138.16**
14	41.62	41.61
15	32.69	32.66
16	46.81	46.79
17	22.82	22.81
18	52.37	52.34
19	38.42	38.39
20	38.48	38.46
21	27.52	27.51
22	36.29	36.49
23	28.23	28.22
24	16.90	16.88
25	16.03	16.05
26	15.18	15.19
27	23.24	23.23
28	**178.23**	**178.26**
29	16.97	16.98
30	21.10	21.04

2.3.4. Structural elucidation of isolated compounds from the stalks

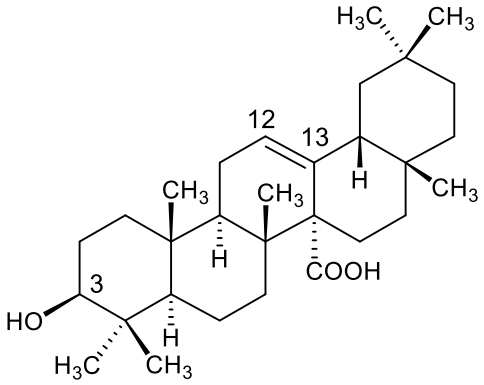
The white amorphous solid (from GP/C2) comprising a mixture of 3β-hydroxyolean-12-en-27-oic acid (compound **II**) and 3β-hydroxyurs-12-en-27-oic acid (compound **III**), accounted for 0.133 % of the dry weight of the stalks. The ^{13}C NMR spectral data result of the mixture consisted of chemical shifts that corresponded to the structures of compounds **II** and **III** is shown in Figure 23. In addition to these chemical shifts of 3β-hydroxyoleanes and 3β-hydroxyursanes, C, CH, CH_2 and CH_3 signals were present but difficult to assign because of overlapping of the signals (Figure 22). The distortionless enhancement by polarisation transfer (DEPT) spectrum of the two compounds is shown in Figure 23. Further proof in support of the assignment of the chemical shifts of the compounds was provided by the spectral properties of β-peltoboykinolic acid and α-peltoboykinolic acid (Figure 21). Furthermore, the carbon chemical shift values of compound **II** were compared to the carbon NMR spectral data of methyl 3β-hydroxyolean-12-en-27-carboxylate with the difference in carbon shift readings between 3α-hydroxyolean-12-en-27-oic acid and methyl 3α-hydroxyolean-12-en-27-carboxylate used as a reference (Chen *et al.* 1983) (Table 9). Although attempts to separate the mixture by repeated column chromatography failed, further separation was performed using High-Performance Liquid Chromatography (HPLC). The HPLC chromatogram result showed two peaks at different retention times 17.829 (compound **II**) and 18.585 (compound **III**) with the UV spectrum ranging from 197-237 and 197-265, respectively (Figure 19).



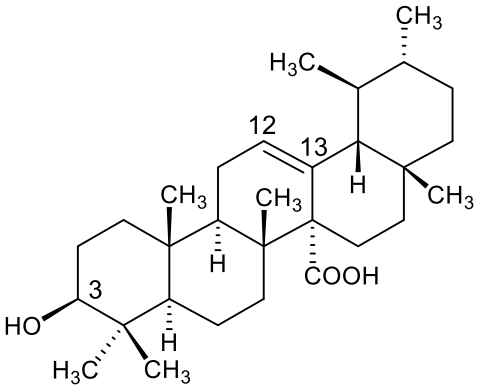
II



III



β–peltoboykinolic acid



α–peltoboykinolic acid

Figure 21: Chemical structures of compounds **II**, **III**, β- and α-peltoboykinolic acids.

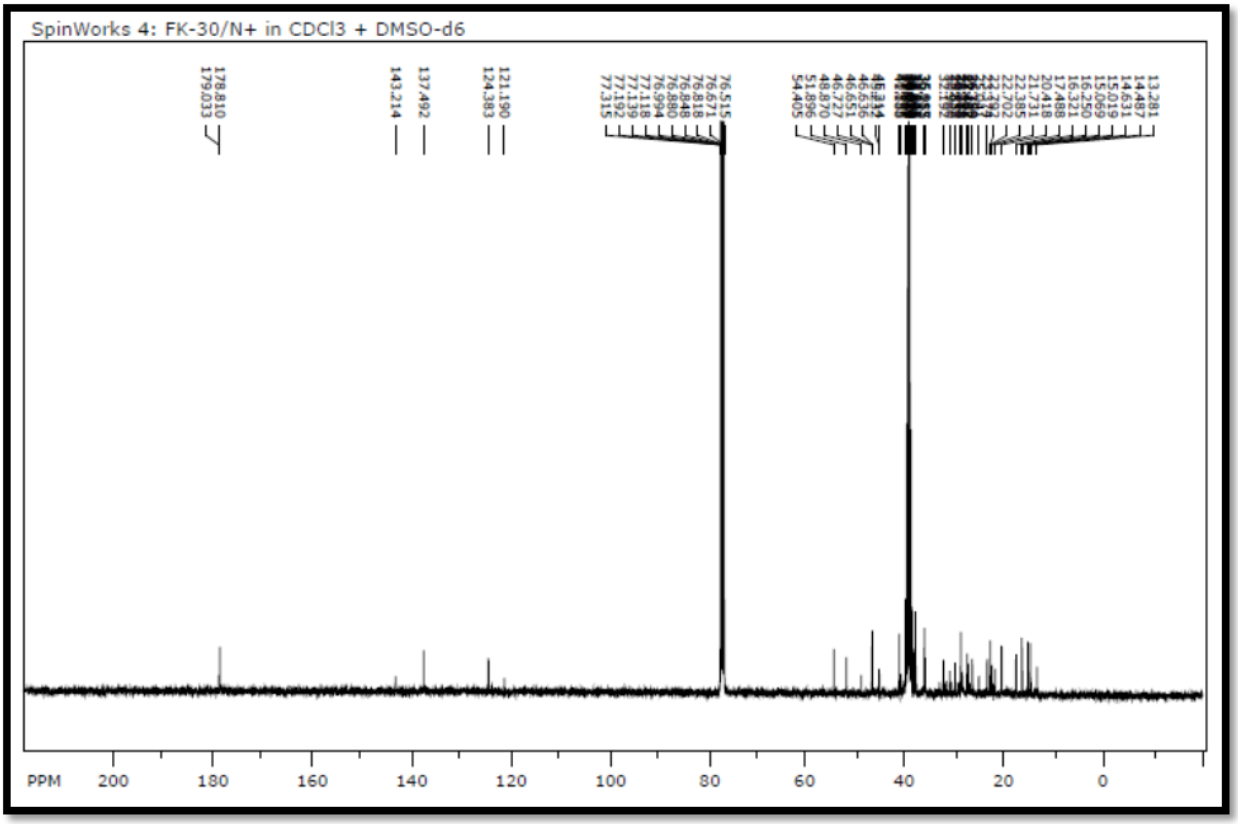


Figure 22: ^{13}C NMR Spectrum of the mixture of compounds **II** and **III**.

Table 9: Comparison of ^{13}C NMR spectral data of compound **II** with those of Chen et al. (1983).

Carbon Position	A	B	C	II
1	36.7	36.7	36.9	36.2
2	27.6	27.6	27.8	27.5
3	**76.4**	**75.8**	**78.8**	**77.5**
4	39.9	40.0	40.2	40.3
5	48.8	49.0	55.5	54.4
6	18.0	18.1	18.3	17.5
7	32.9	33.2	33.5	33.0
8	36.7	37.3	37.3	37.8
9	48.8	49.0	49.2	48.9
10	36.7	37.1	38.8	38.4
11	23.6	22.7	23.0	23.4
12	**126.0**	**125.5**	**125.5**	**124.4**
13	**137.6**	**137.5**	**137.5**	**137.5**
14	56.0	56.1	56.1	54.4
15	22.7	22.3	22.3	22.7
16	26.2	25.3	27.3	26.4
17	32.9	32.9	32.9	33.1
18	47.0	47.4	47.5	46.7
19	44.2	44.0	44.0	45.2
20	31.0	30.9	30.9	31.0
21	34.2	34.3	34.3	33.1
22	36.2	36.5	36.8	36.2
23	28.2	28.2	28.3	28.4
24	22.1	22.8	15.7	15.1
25	15.8	16.3	16.4	16.3
26	18.0	18.1	18.1	17.5
27	**179.0**	**176.0**	**176.1**	**179.0**
28	28.2	28.2	28.2	28.4
29	32.9	33.2	33.2	33.1
30	23.6	23.5	23.5	23.4

Key: Compound **A** (3α-hydroxyolean-12-en-27-oic acid), Compound **B** (methyl 3α-hydroxyolean-12-en-27-carboxylate), Compound **C** (methyl 3β-hydroxyolean-12-en-27-carboxylate), and Compound **II.**

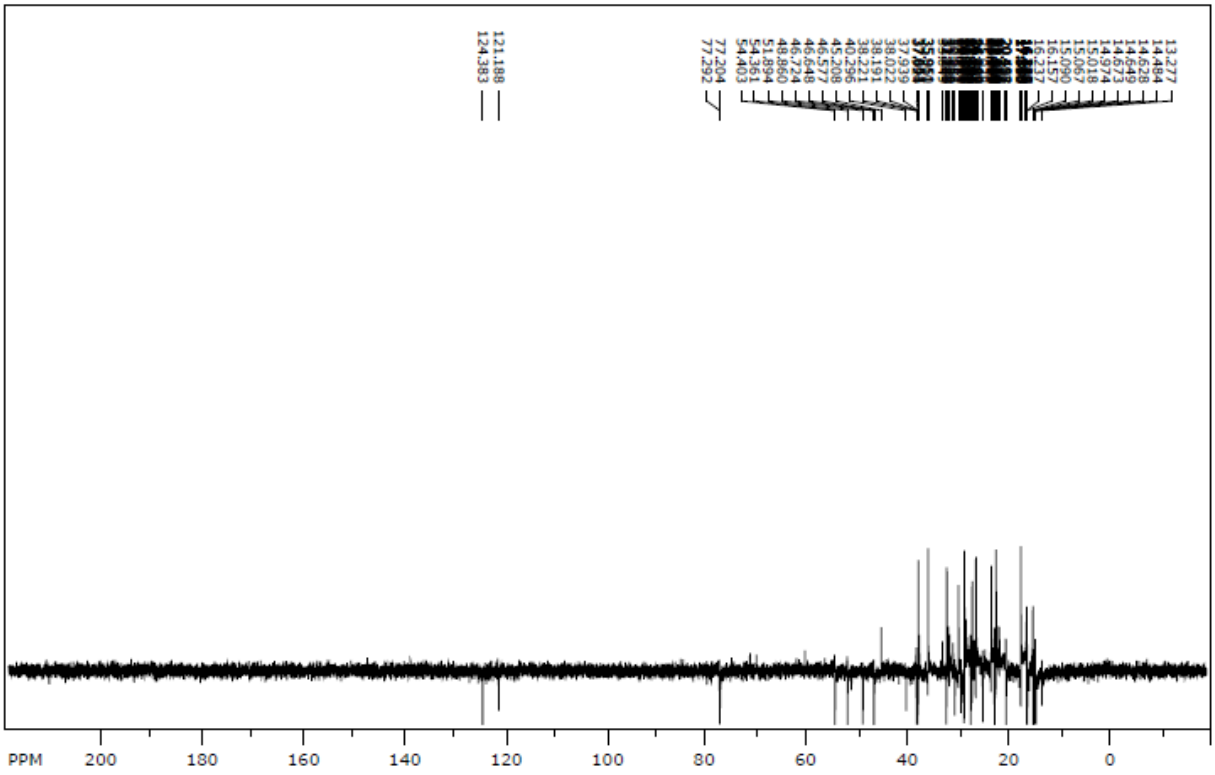


Figure 23: DEPT spectrum of the mixture of compounds **II** and **III**.

Compound **IV** was identified as 3α-hydroxyolean-12-en-27-oic acid by comparing its ^{13}C NMR spectral data with reported literature values by Chen *et al.* (1983) (Table10). Compound **V** was also identified as pomolic acid upon comparing its spectral data (^{1}H and ^{13}C NMR) with literature values of Sidjui *et al.* (2015) and Mahato and Kundu (1994) (Table 11). The chemical structures of compounds **IV** and **V** are described in Figure 24.

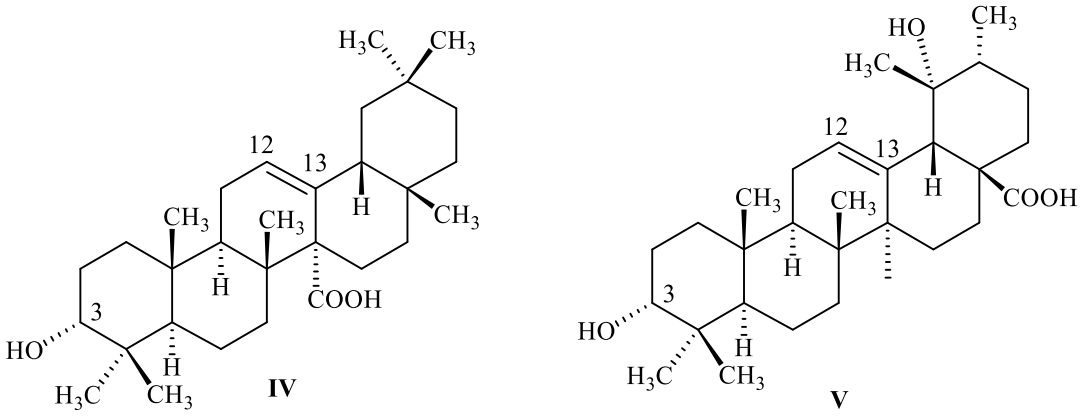


Figure 24: Chemical structures of compounds **IV** and **V**.

Table 10: Comparison of ^{13}C NMR spectral data of compound **IV** with those of Chen et al. (1983).

Carbon Position	Literature	IV
1	36.7	36.7
2	27.6	27.6
3	**76.4**	**76.5**
4	39.9	39.9
5	48.8	46.7
6	18.0	17.6
7	32.9	32.1
8	36.7	36.1
9	48.8	46.6
10	36.7	37.6
11	23.6	23.6
12	**126.0**	**127.5**
13	**137.6**	**137.5**
14	56.0	54.4
15	22.7	22.7
16	26.2	26.3
17	32.9	32.1
18	47.0	46.5
19	44.2	46.2
20	31.0	31.9
21	34.2	36.0
22	36.2	36.0
23	28.2	27.6
24	22.1	22.7
25	15.8	15.9
26	18.0	17.7
27	**179.0**	**179.4**
28	28.2	27.5
29	32.9	32.1
30	23.6	23.6

Key: Literature: Chen *et al.* (1983)

Table 11: Comparison of ^{13}C NMR spectral data of compound **V** with those of Sidjui *et al.* (2015) and Mahato and Kundu (1994).

Carbon Position	Literature 1	Literature 2	Compound V
1	39.5	38.7	38.7
2	28.6	28.0	28.0
3	**78.7**	**78.2**	**78.9**
4	39.9	39.3	39.3
5	56.3	55.8	55.0
6	19.4	18.9	18.4
7	34.1	33.6	32.7
8	40.8	40.3	40.4
9	48.3	47.7	47.3
10	37.8	37.3	37.4
11	24.5	24.0	24.4
12	**128.5**	**128.1**	**128.9**
13	**140.3**	**139.9**	**138.1**
14	42.6	42.1	41.0
15	29.8	29.2	29.2
16	26.9	26.6	26.0
17	48.8	48.2	47.3
18	55.1	54.5	55.0
19	73.2	72.7	73.1
20	42.8	42.3	41.1
21	27.4	27.0	27.3
22	39.0	37.4	37.4
23	17.0	28.7	28.2
24	29.3	16.7	16.1
25	16.0	15.5	15.5
26	17.2	17.1	16.7
27	25.2	24.6	25.4
28	**181.1**	**180.6**	**180.4**
29	27.6	26.8	27.1
30	17.4	16.4	18.4

Key: Literature 1: Sidjui *et al.* (2015), Literature 2: Mahato and Kundu (1994), Compound **V**: Compound of study

2.4. Discussion

The extraction of the leaves and stalks of *G. perpensa* using hexane, ethyl acetate and methanol resulted in the highest percentage yield recovery of methanol for both leaf (GP/A3) and stalk (GP/B3) extracts of the first protocol. The extraction of the stalks from the second extraction protocol, as well led to a greater yield of methanol (GP/C5) than hexane (GP/C1), ethyl acetate (GP/C2), DCM (GP/C3) and acetone (GP/C4) extracts (Figure 14). Even though the extraction frequencies are different for the various solvents used, it is not hard to point out that methanol has shown better extraction yield than the rest of the solvents used during the extraction process. As shown in Table 6, the total percentage yield from methanol extraction (11.93 %) is highly incomparable to the others. This could be due to the potential of methanol to extract both polar and non-polar compounds present in the plant material. A report by Sasidharan *et al.* (2011) confirmed that the use of methanol as a potential extractant for polar and some groups of nonpolar compounds present in a plant material. Therefore, the highest percentage yields recorded by the methanol extracts of the leaves and stalks are rational.

The ^{13}C NMR spectral data of compound **I** showed the characteristic chemical shift values of C-3:76.79, C-12: 124.54, C-13: 138.16, and C-28: 178.26 (Table 8). These values of the spectral data were found to correspond to the values of UA in comparison to literature. Although this is the first report on the isolation of UA from *G. perpensa*, it has been reported that UA has been isolated from other plant species. According to do Nascimento *et al.* (2014), UA has been isolated and identified from the aerial parts of *Sambucus australis*. Gu *et al.* (2006) have also reported the recovery of UA from the whole plant of *Valeriana laxiflora*, and the aerial parts of *Lavandula dentata* and *Tanacetum parthenium*. Jäger *et al.* (2009) and Laszczyk (2009) confirmed the presence of UA in twenty plant species namely: *Arctostaphylos uva-ursi* (leaves), *Coffea Arabica* (leaves), *Cornus mas* (leaves), *Crataegus* (leaves and flowers), *Eucalyptus* (leaves), *Lavandula Angustifolia* (leaves and flowers), *Malus domestica* (fruit peel and pomace), *Melissa Officinalis* (leaves), *Nerium oleander* (leaves), *Olea europeae* (leaves), *Origanum Majorana* (leaves), *Origanum Vulgare* (leaves), *Plantago major* (leaves), *Pyrus communis* (fruit peel), *Rosmarinus Officinalis* (leaves), *Salvia officinalis* (leaves), *Sambucus nigra* (leaves and bark), *Satureja montana* (leaves), *Thymus vulgaris* (leaves), *Verbena Officinalis* (whole plant).

Other researchers also reported the presence of UA in the leaves of other plants. These include; *Catharantus roseus*, *Sambucus Canadensis*, *Vinca minor*, *Thymus broussenettii*, *Thymus willdenowii*, and *Buddleja saligna* (Li, Guo and Yang 2002; Ovesna *et al.* 2004; Singh *et al.* 2016). These reports also showed this compound is abundant in the leaves than other parts of the plants. This is in agreement with our finding of UA from the leaves of *G. perpensa*, but not the stalks.

Oleanane and ursane type triterpenes substituted with acarboxyl groupat the 27-carbon have been identified from various plants and reported to have a wide range of biological properties. In this study, the pentacyclic triterpenoic acids 3β-hydroxyolean-12-en-27-oic acid (compound **II**), 3β-hydroxyurs-12-en-27-oic acid (compound **III**), and 3α-hydroxyolean-12-en-27-oic acid (compound **IV**) were effectively isolated from the stalks of *G. perpensa*. The ^{13}C NMR spectral data of the mixture of compounds **II** and **III** consisted of the characteristic chemical shifts of both compounds. The characteristic chemical shifts for compound **II** were C-3, 77.48, C-12, 124.38, C-13, 137.49, and C-27, 179.03, and chemical shifts for compound **III** were C-3, 77.19, C-12, 121.19, C-13, 143.21, and C-27, 178.81. These chemical shifts were different from the chemical shifts of the corresponding 3β-hydroxyolean-12-en-28-oic acid (OA) and 3β-hydroxyurs-12-en-28-oic acid (UA) (Mahato and Kundu 1994; Martins *et al.* 2013). Further evidence in support of the assignment of the chemical shifts of compounds **II** and **III** was provided by the spectral properties of β-peltoboykinolic acid and α-peltoboykinolic acid by Kim and Kang (1986). Moreover, the carbon chemical shift values of compound **II** were compared to the carbon NMR spectral data of methyl 3β-hydroxyolean-12-en-27-carboxylate with the difference in carbon shift readings between 3α-hydroxyolean-12-en-27-oic acid and methyl 3α-hydroxyolean-12-en-27-carboxylate used as a reference (Table 9). Even though these compounds were isolated for the first time from this plant, the isolation of the compounds from different plants by various researchers has been known. Compound **II** has been reported to present in various parts of different plants including; *Astilbe chinensis* (Sun *et al.* 2003), *Astilbe rivularis* (Han *et al.* 2015), *Saniculiphyllum guangxiense* (Geng *et al.* 2012), *Aceriphyllum rossii* (Han *et al.* 2002), *Astilbe koreana* (Na *et al.* 2006), and *Astilbe myriantha* (Song *et al.* 2011). Compound **III** has been reported to be found in various plants such as*Astilbe koreana* (Na *et al.* 2006), *Astilbe myriantha* (Song *et al.* 2011), and *Astilbe chinensis* (Sun *et al.* 2003).

The characteristic chemical shift values from the ^{13}C NMR spectral data of compound **IV** were C-3:76.5, C-12:127.5, C-13:137.5, and C-27:179.4 (Table 10). These values of the spectral data were found to correspond to the values of 3α-hydroxyolean-12-en-27-oic acid in comparison to literature. Although this is the first report on the isolation of 3α-hydroxyolean-12-en-27-oic acid from *G. perpensa*, it has been reported that this compound has been isolated from other plants. According to Han *et al.* (2002), 3α-hydroxyolean-12-en-27-oic acidhasbeen isolated from *Aceriphyllum rossii*.

The ^{13}C NMR spectral data of compound **V** showed the characteristic chemical shift values of C-3: 78.9, C-12: 128.9, C-13: 138.1, and C-27: 180.4 (Table 11). These values of the spectral data were found to correspond to the values of 3β, 19-Dihydroxyurs-12-en-28-oic acid (pomolic acid) in comparison to literature. Although there is no report on the isolation of pomolic acid from *G. perpensa*, the compound has been isolated from different plant species including; *Licania pittieri* (Estrada *et al.* 2009), *Chrysobalanus icaco* (Fernandes *et al.* 2007), *Rosa woodsi* and *Hyptis capitata* (Kashiwada *et al.* 1998), *Arrabidaea triplinervia* (Leite *et al.* 2006), *Lantana camara* (Siddiqui *et al.* 1995), and *Osmanthus fragrans* (Yoo *et al.* 2013). Although no work has been done on the isolation of secondary metabolites from the leaves and stalks of *G. perpensa*, the abundance of phenolic compounds in the rhizomes and roots of the plant has been reported (Brookes and Dutton 2007).

CHAPTER 3: BIOLOGICAL SCREENING OF EXTRACTS AND SECONDARY METABOLITES OF *Gunnera perpensa*

3.1. Introduction

Phytochemical studies are driven by the need for the discovery of new, safer and inexpensive drugs. The search for new drugs is frequently performed by screening local medicinal plants to develop new drugs to combat various health problems (Webster *et al.* 2008). However, the phytochemical compounds need to be screened and studied for their therapeutic potentials using different biological screening methods. According to Verpoorte (1998), there are two basic screening approaches to discover new drugs and in this study, *G. perpensa* and its isolates were screened to prove the efficacy of *G. perpensa*'s use in traditional medicine. Biological screening is a method that is used as a means of identifying potentially active compounds for their medicinal uses (Choma and Grzelak 2011). The biological assays used in this study will be discussed below.

Infectious diseases from microbial origin contribute to the high morbidity and mortality rates in developing countries (Geyid *et al.* 2005). Recently, there has been an increase in the incidence of infectious diseases due to the micro-organisms decrease response to treatment with conventional antimicrobial agents (Balakumar *et al.* 2011). Plant extracts have been screened for their antibacterial and antifungal properties using diffusion, dilution and bio-autography methods (Choma and Grzelak 2011).

Diffusion Assay: This assay uses diffusion methods which involve the dissemination of an antimicrobial agent from cylinders, hole-plates or discs into a solid medium seeded with a particular microorganism. The susceptibility of the microorganism to the antimicrobial agent is determined by the measurement of the zone of inhibition around the disc (Choma and Grzelak 2011).

Dilution Assay: In this assay, the broth and agar dilution methods are mostly used to determine the minimum inhibitory concentration (MIC) of the antimicrobial agent under study (Wiegand, Hilpert and Hancock 2008). This is because it is possible to estimate the antimicrobial agent concentrations used in the medium. Broth dilution involves introducing the micro-organism to a broth environment with a series of concentrations of the antimicrobial agent. However, agar dilution involves introducing the microorganism onto the agar medium prepared with serially diluted concentrations of the antimicrobial agent (Choma and Grzelak 2011).

Bio-autography Assay: Bio-autography is a technique used to identify compounds with antimicrobial properties. In bio-autography, the biological effects of the compounds can be growth promoting or growth inhibiting. The effects can be determined through the formation of growth zones or inhibition zones (for both growth promotions and inhibitions, respectively) around the compounds visible on a chromatogram (Betina 1973). Bio-autography combines chromatographic separation techniques with the *in situ* bioactivity of compounds, which leads to target-directed separation of the active compounds using the R_f values in the mark TLC as references (Sasidharan *et al.* 2011). A TLC bio-autography approach could be direct, agar overlay (immersion) or contact bio-autography. Among the three approaches, direct bio-autography is widely applied in the antimicrobial screening of natural products (Choma and Grzelak 2011).

Sickling is the process of the changes in the forms of the erythrocytes of individuals into an abnormal sickle shape (Figure 29). This happens due to the erythrocytes ability to change shape in response to reduction of oxygen level. Individuals possessing erythrocytes with sickling potentials are known to have sickle cell disease (Drepanocytosis) (Pauling *et al.* 1949; Tshibangu *et al.* 2014). Due to the extreme destruction of erythrocytes, most of the sickled-cells carriers tend to suffer from severe anaemia and their condition is termed as sickle cell anaemia (Serjeant and Serjeant 1992). Sickle cell disease is a life-long and molecular level disorder which can only be treated through stem cell transplantation from a matched sibling (Pereira *et al.* 2017). Anti-

sickling assays involve direct *in vitro* application of the test compounds on to the sickled cells or direct *in vivo* therapeutic evaluation of previously infected rats (B.O. *et al.* 2015).

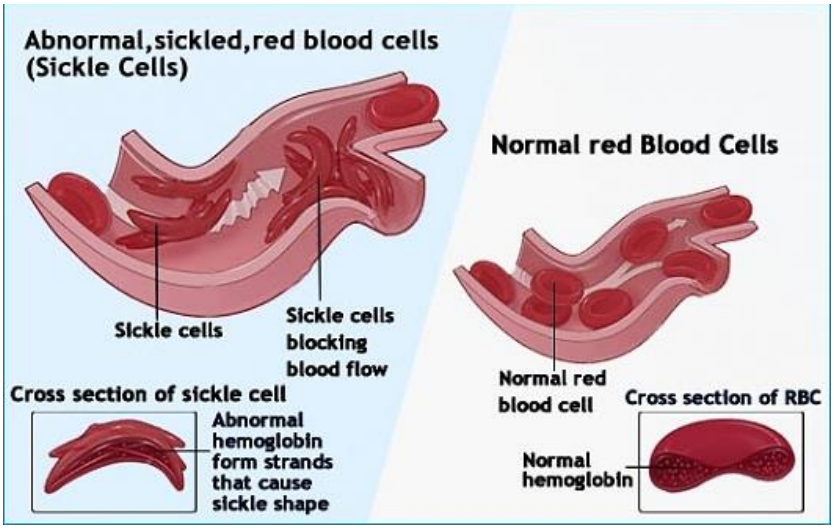


Figure 25: Sickle-cells versus healthy red blood cells (Serjeant and Serjeant 1992).

Free radicals, reactive oxygen and reactive nitrogen intermediates are produced under normal physiological conditions. To neutralise the destruction by free radicals and active intermediates, the body produces anti-oxidants (Rahman *et al.* 2012). An anti-oxidant is a substance that can donate an electron and stabilise a free radical to inhibit or delay cellular damage. Some anti-oxidants are produced naturally by the body, and some are found in foods (Lobo *et al.* 2010). Various anti-oxidant assays have been used in biological screening of natural compounds. These include; DPPH (2,2-diphenyl-1-picryhydrazyl), ABTS (2,2'-azino-di[3-ethylbenzthiazoline sulfonate]), ORAC (oxygen radical absorbance capacity), and Oxidative haemolysis inhibition assays. Previous studies have shown many of the anti-oxidant compounds possess antitumor, anti-inflammatory, anti-mutagenic, anti-carcinogenic, anti-atherosclerotic, antiviral, or antibacterial activities (Mudzwiri 2007).

Cancer is a general term for a disease caused by uncontrolled cell division. This abnormal process can be triggered by viruses, tumour suppressor genes, chemicals, spontaneous

transformation, or chromosomal rearrangements (Reddy, Odhav and Bhoola 2003). Human cancer is mainly caused by environmental carcinogens such as exposure to viruses, chemicals, and radiation (Reddy, Odhav and Bhoola 2003). A number of anticancer drugs have been developed through different screening programs. Although advances in chemotherapy have led to increasing cancer cure rates, little has been achieved for the treatment of colon, ovarian, pancreas, lung, brain, and prostate cancers (Cassady, Baird and Chang 1990). The two widely used techniques in preliminary screening of anticancer agents are MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays. Both methods are colorimetric assays that are used to determine the number of viable cells after the application of the anticancer agent (McCauley, Zivanovic and Skropeta 2013).

Toxicity evaluation of plant extracts and their isolated metabolites is important to secure the safety of consumers (Otang, Grierson and Ndip 2013). Toxicity screening is crucial in extending the use of existing therapeutic substances and in new drug development. The safety evaluation of natural plant products has been done using both *in vivo* and *in vitro* toxicity testing (Williamson, Okpako and Evans 1996). There are various types of toxicity assays based on the areas of application of a particular study. These assays include acute toxicity, mutagenicity, skin sensitisation, repeated dose toxicity, chronic oral toxicity, sub-chronic oral toxicity, carcinogenicity, neurotoxicity, genetic toxicity, embryotoxicity, and one-generation and two-generation reproduction toxicity testing assays (Parasuraman 2011). Since most plant-based bioactive compounds are toxic at higher doses, toxicity screening of the compounds to zoological systems can be an effective general bioassay tool (Wu 2014).

3.2. Materials and Methods

3.2.1. Antimicrobial activity using agar disc diffusion assay

3.2.1.1. Bacteria and Fungi species collection

Equal number of gram-positive and gram-negative bacteria, *Staphylococcus aureus* (DBT*_E), *Enterobacter faecalis* (DBT*_P), *Escherichia coli* (DBT*_L), *Pseudomonas aeruginosa* (DBT*_D), *Staphylococcus epidermidis* (DBT*_S) and *Bacillus cereus* (DBT*_F), and the fungi species, *Penicillium sp*. (DBT*_AC), *Fusarium sp*. (DBT*_AU) and *Aspergillus niger* (DBT*_AE), were obtained from Durban University of Technology, Department of Biotechnology and Food Technology. DBT* indicates a reference for culture collections of Durban University of Technology at Biotechnology and Food Technology Department. These species were preserved in nutrient agar slants at 4^{o}C. Each micro-organism was sub-cultured at an appropriate temperature before susceptibility testing.

3.2.1.2. Antimicrobial Assays

Agar disc diffusion assay: The crude extracts, column fractions and isolates of *G. perpensa* were screened for their antibacterial and antifungal properties using disc diffusion assay described by L. and E.A. (2004). Bacterial and fungal cells were sub-cultured onto Nutrient Agar and Potato Dextrose Agar (PDA), respectively. Fungal cultures were allowed to grow for 48h at 28 ^{o}C until sporulation and bacterial cells for 24 h at 37 ^{o}C. The spores of the fungi were collected in 10 ml distilled water. While bacterial cells were sub-cultured into nutrient broth in centrifuge tubes. The concentration of fungal spores was adjusted to 10^{6} spores/ml using a Neubauer counting chamber, and bacterial cells concentration was adjusted based on McFarland standard (McFarland 1987).

The test compounds (3 mg/ml) were dissolved in dimethyl sulfoxide (DMSO). Ten microliters (10 µl) of each test compound was transferred onto each sterile disc (6 mm) made from Whatman no.1 filter paper. The impregnated discs were allowed to dry and placed onto agar plates pre-inoculated with the bacteria or fungi. The organic solvent, in which the compounds were prepared (DMSO), was used as a negative control while ciprofloxacin and amphotericin B

were used as positive controls of bacteria and fungi, respectively. The plates were incubated for 24 h at 37 ^{o}C for bacteria and 48 h at room temperature for fungi. The experiment was done in triplicate and the average diameter (mm) of the three zones of clearing were recorded. Samples with zones of inhibition measuring greater than or equal to 8 mm (in diam.) were taken as active against the tested micro-organisms.

MIC assay: The minimum inhibitory concentration (MIC) assay was conducted to test samples exhibited zones of inhibition equalled or exceeded 8 mm. Dilution of the test compounds was made with 3 mg/ml, 1.5 mg/ml, 0.75 mg/ml, 0.37 mg/ml, 0.18 mg/ml and 0.09 mg/ml concentrations in DMSO. The lowest concentration at which zone of inhibition was observed was recorded as the MIC value.

3.2.2. Antimicrobial activity using bio-autography

Bio-autography: Direct bioautographic method was used for the crude extracts to guide in the isolation of compounds with antimicrobial properties. The protocol used in this study was the method described by Dewanjee *et al.* (2015) with some modification. A TLC plate was developed for each extract under study. A pure bacterial suspension was prepared in nutrient broth (at McFarland standard concentration) and sprayed over the developed TLC plate. The developed bio-autogram was incubated at 37 ^{o}C for 24 h in a humid environment. Visualisation of bacterial growth was made using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) (Sigma) as an indicator. INT was prepared at a concentration of 2 mg/ml in 70 % ethanol. The solution was sprayed over the bio-autogram and the bio-autogram was placed back in to the incubator for another six h. Then, colourless areas (inhibition zones) around the compounds on the bio-autogram were observed and identified on the marker TLC plate based on their corresponding R_f values.

3.2.2. Anti-sickling activity using Emmel's test

Blood sample collection: Blood samples for anti-sickling assay were taken from young patients from Hospitalier Monkol Health Centre, Kinshasa, D. R. Congo. Patients that did not go through blood transfusion were selected and experiments were performed immediately after blood collection. All the procedures were approved by Biology Department Ethics Committee to meet the ethical requirements. The SS nature of the blood samples was confirmed using electrophoresis on cellulose acetate gel and the samples were stored at 4 ^{o}C for immediate use.

Anti-sickling assay: The anti-sickling property of the isolated compounds were performed using the method described by Courtejoie and Hartaing (1992). Phosphate buffered saline (150 mM) was prepared and used to dilute the blood taken from sickle cell disease patients. An equal volume of 2 % Sodium metabisulphite was mixed with the diluted blood. A drop of the prepared solution was applied on to a microscope slide and covered with a cover slip. The edges of the cover slip were sealed with paraffin to avoid air entrance into the sample. The experiments were carried out in duplicate and computer-assisted image analysis (Motic Images 2000, version 1.3; Motic Chine Group Co LTD) was performed to measure various parameters such as perimeter, radius and area of each red blood cell (RBC). The % normalisation rate was calculated using the equation described in Equation 1. The statistical data were analysed using Microcal Origin 6.1 package software.

$$\% \text{ Normalization rate} = \frac{\text{number of drepanocytes of untreated blood} - \text{number of drepanocytes of treated blood}}{\text{number of drepanocytes of untreated blood}} \text{ X } 100\ \%$$

Equation 1: % Normalization rate formula, drepanocytes: sickled RBCs.

3.2.3. Anti-oxidant activity using DPPH photometric assay

The radical scavenging properties of the crude extracts and the isolated compounds were determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) photometric assay described by Choi *et al.* (2002). A stock solution of 1000 µg/ml of each sample was prepared and diluted to 500, 250, 100, 50 and 10 µg/ml in methanol. The different concentrations of each sample (2.5 ml) was transferred to different test tubes and 1 ml of DPPH solution (0.3 mM in methanol) was added to each test tube. The solutions were allowed to react for 15 min at room temperature. Thereafter, the absorbance of each sample solution was measured using spectrophotometer (Thermo Fisher Scientific Inc.) at 516 nm. The experiments were performed in triplicates and the average of the recorded anti-oxidant values was taken as the mean value. The mean anti-oxidant activity of each sample solution was calculated in percentage using the equation described in Equation 2.

$$\% \text{ Antiradical activity} = \frac{\text{(-) Control absorbance - Sample absorbance}}{\text{(-) Control absorbance}} \text{ X } 100\ \%$$

Equation 2: Antiradical activity calculation.

3.2.4. Anticancer activity using MTT assay

3.2.4.1. Cell lines

The isolated secondary metabolites of *G. perpensa* together with some of the simple fractions from the column were tested against two human cancer cell lines for their anticancer potentials. The cancer cell lines used in this study were human embryonic kidney cells (HEK293) and human breast adenocarcinoma cells (MCF-7) (Separations, South Africa). The cells were stored at -80 ^{o}C in a bio-freezer until further use.

3.2.4.2. Cell regeneration and maintenance

All cell regeneration and culture maintenance were conducted in an aseptic environment using laminar flow cabinet (Scientific Engineering, INC), UV light, and 70 % ethanol (Merck, South Africa). Prior to conducting every experiment, the laminar flow cabinet was sterilised using 70 % ethanol followed by UV radiation for at least 30 min, together with the materials required for a

particular experiment. The cells were allowed to grow in a humidified incubator (SnjidersHepa, United Scientific, South Africa) adjusted at 37 ^{o}C and 5% CO_2 environment.

Cell regeneration

The cancer cell lines stored in a bio-freezer at -80 ^{o}C require to be regenerated and maintained for further anticancer studies. Therefore, when required, the cells were immediately taken from the bio-freezer and defrosted. Defrosting was done by warming the vials containing the cells at 37 ^{o}C in a water bath or by simply rubbing the vials in between the hands. Then, the cells were transferred to two 25 cm^2 tissue culture flasks (Greiner, Germany) containing 20 ml of supplemented Dulbecco's Modified Eagle's Medium (DMEM), Sigma-Aldrich. Supplemented DMEM was prepared with 10 % Fetal Calf Serum (FCS) and 1 % antibiotics (penicillin/ streptomycin), Sigma-Aldrich, Inc. Afterwards, the cells were incubated at 37 ^{o}C in a humidified incubator with 5 % CO_2 environment.

Cell maintenance

The regenerated cell lines were incubated and the supplemented DMEM was replaced with a fresh one until cells became confluent on the side of the flask. The confluent cells were taken and the media was removed. Phosphate saline buffer (PBS, 10 ml) was added to the flask to wash the monolayers of cells. After washing the cells, PBS was removed and 3 ml of trypsin was added and incubated for 4 min. The incubated flask was tapped from the side for few seconds to detach the cells that are still attached to the flask. Cell detachment was confirmed through observation of the flask under the microscope. Supplemented media (10 ml) was added to the flask and mixed very well. Then, 6.5 ml of the solution was transferred to one of the two new flasks and mixed well. Finally, each flask was filled to 20 ml using supplemented media and incubated at 37 ^{o}C. Cell growth and contamination were monitored through the use of an inverted microscope (Nikon, Japan) and daily observation of medium colour and turbidity, respectively.

3.2.4.3. Cell Enumeration

In this study, trypan blue (Bio Whittaker, Wakersville, USA) was used for cell enumeration. Trypan blue is a dye that is used to distinguish between viable and non-viable cells during cell counting. This dye can pass through the membranes of dead or non-viable cells, while, the membranes of living cells block its passage. Therefore, the dye can change the colour of dead cells to blue and make the differentiation and counting of viable cells easy.

Cell enumeration was done for flasks with confluent cell growth. After detaching the cells from the side of the flask using trypsin, 10 ml of supplemented media was added to the flask. The cells with the media (200 µl) were transferred to a small vial and mixed with 25 µl of trypan blue. The dyed cell suspension (5 µl) was then applied to each side of Neubauer hemocytometer and viable cells were counted under a microscope. Cells in the corner squares of the hemocytometer were counted and their mean value was used. The cell density of each well was adjusted to 1.2 x 10^4cells/well. The number of viable cells in the sample was calculated using the formula in Equation 3. The cells were plated into a 48 well plate and incubated overnight at 37 ^oC.

Cells/ml = Average number of cells counted x 10^4 dilution factor

Equation 3: Number of viable cells/ml

3.2.4.4. MTT Assay

In this study, the MTT assay method described by Mosmann (1983) was used with some modifications to measure the cytotoxicity of the isolated compounds of *G.perpensa*. The cells that were incubated overnight in a 48 well plate were taken, the medium was removed and replaced with fresh supplemented medium. Different concentrations of the tested compounds (50, 100, 150, 200, 250, and 300 µg/ml) were then added to each well in triplicate and incubated for 4 h. Thereafter, the medium was removed and replaced with a fresh medium, and the cells were subjected to MTT assay after 48 h of incubation.

At the end of the 48 h incubation, the medium was removed from each well and MTT (5 mg/ml in PBS) was added at a concentration of 10% in a total of 100 µl medium to each well-containing cells. The plate was then incubated for 4 h and the MTT-medium solution was removed from wells. Then, DMSO (100 µl) was added to each well to stop the MTT reaction and dissolve insoluble formazan crystals. Finally, the plate was read at 570 nm using Mindray Plate Reader. The absorbance results were used to calculate the percentage viable cells present in each well (Equation 4).

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \text{ X } 100\%$$

Equation 4: Cell viability formula

3.2.4.5. Cell storage

The media from cell culture flask (80 % confluent) was removed and PBS (10 ml) was applied to the flask to wash the cells. The PBS was removed and trypsin was added to detach the cells as described in cell maintenance. DMEM was added to the flask and cell suspension was transferred to a sterile 50 ml centrifuge tubes. The tubes were centrifuged at 3000 rpm for 5 min in order to separate the cells from the media. After decanting the supernatant/ media, the cells were collected with 2 ml of cryo-protective media (10 % DMSO, 70 % DMEM and 20 % FBS). The solution was transferred to two cryo-tubes (Corning, South Africa) (1 ml each) and kept in thermos flask at -20 ^{o}C prior to storage at -80 ^{o}C in a bio-freezer.

3.2.5. Toxicity evaluation using Brine shrimp assay

The cytotoxicity of the plant extracts and their isolates was evaluated using the Brine shrimp assay with minor modifications to the method described by Meyer *et al.* (1982).

3.2.5.1. Hatching the shrimp eggs

The Brine shrimp (class: *Artemia salina*) eggs were obtained from Natures Petland, Durban, South Africa. The eggs were allowed to hatch in a hatching chamber in an artificial sea water (Brine solution). The brine solution was prepared from 23 g NaCl, 0.7 g KCl, 4 g Na_2SO_4, 1.3 g $CaCl_2.2H_2O$ and 11 g $MgCl_2.6H_2O$ dissolved in 1000 ml of distilled water. The eggs were left in the chamber for 48 h under constant aeration and light source until most of the larvae (nauplii) emerge from their eggs. The hatched larvae were fed on yeast suspension prepared from yeast extract powder (3 mg) and brine solution (5 ml). The active larvae were collected and transferred to a smaller glass jar filled with a fresh Brine solution.

3.2.5.2. Bioassay

The test samples were dissolved in DMSO at different concentrations (100, 500 and 1000 µg/ml) and 100 µl of each concentration was added to each well of a 12-well plate containing 4.9 ml of brine solution. Using a glass capillary 10 larvae were transferred to each well filled with 5 ml of brine solution and test sample (in triplicate). The larvae were fed on 3 drops of yeast suspension in each well and the plate was left for 24 h under continuous light source at room temperature. Finally, the mean value of the dead larvae was counted and the percentage death was calculated.

3.3. Results

3.3.1. Agar disc diffusion assay

3.3.1.1. Antibacterial

The results from the disc diffusion screening of crude extracts (Table 12) and the isolated compounds (Table 13) showed activity against some of the tested micro-organisms. The highest bactericidal activity was recorded by the crude extracts GP/A3 and GP/C4. GP/A3 was highly active against *S. aureus*, *E. coli* and *P. aeruginosa* with MIC values of 0.75, 0.375 and 0.75 µg/ml, respectively. While, GP/C4 was highly active against *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa* with MIC values ranging from 0.75 to 1.5 µg/ml. The highest inhibition zone was observed by GP/C4 against *S. aureus* followed by the inhibition by GP/C4 against *P. aeruginosa* and GP/A3 against *S. aureus*.

The isolated compounds from *G. perpensa* leaves and stalks showed slight antibacterial activity with better activity recorded from compound **X** against *S. aureus*, *S. epidermidis* and *E. faecalis* (Table 13). Compound **IV** showed slight activity against the tested *S. aureus* strain. The negative control (DMSO) showed no inhibition while the positive control (ciprofloxacin) showed inhibition zones ranging from 30 to 44 mm against both gram positive and negative bacteria. Generally, the recorded antibacterial activities of the crude extracts were greater than the isolated compounds.

Table 12: Antibacterial results of crude leaf and stalk extracts of *G. perpensa*.

Crude extracts	Micro-organisms and the corresponding zones of inhibition (mm)					
	S. aureus	*B. cereus*	*S. epidermidis*	*E. coli*	*E. faecalis*	*P. aeruginosa*
GP/A1	6.5 ± 0.0	na	7 ± 0.0	na	na	6.5 ± 0.0
GP/A2	7.0± 0.0	na	na	na	6.5 ± 0.0	na
GP/A3	12.0± 0.0	na	6.5± 0.0	11.0 ± 0.0	6.5 ± 0.0	11.3± 0.6
GP/C1	na	na	na	7.0 ± 0.0	na	na
GP/C2	na	na	na	na	na	na
GP/C3	na	na	na	na	7.3 ± 0.6	na
GP/C4	15.5 ± 0.7	11.0± 0.0	7.5± 0.0	11.0± 0.3	na	12.0± 0.0
GP/C5	na	na	na	7.3 ± 0.6	7.7 ± 0.3	na
+ve control	33	30	43	31	33	44

Key: na - no activity, zones of inhibitions are presented as mean ± SD.

Table 13: Antibacterial results of compounds isolated from the leaves and stalks of *G. perpensa*.

Isolated compounds	Micro-organisms and the corresponding zones of inhibition (mm)					
	S. aureus	*B. cereus*	*S. epidermidis*	*E. coli*	*E. faecalis*	*P. aeruginosa*
I	7 ± 0.0	na	na	6.5 ± 0.0	6.2 ± 0.3	na
X	7.7 ± 0.6	na	7.5 ± 0.0	7.0 ± 0.3	7.5±0.5	na
IIand**III**	na	na	na	na	na	na
IV	6.5 ± 0.8	na	na	na	na	na
V	na	na	na	na	na	na
+ve control	33	30	43	31	33	44

Key: na – no activity, zone of inhibitions are presented as mean ± SD.

3.3.1.2. Antifungal

The disc diffusion results of both the crude extracts and the isolated compounds showed no antifungal activities against the tested fungi species. The negative control (DMSO) showed no inhibition while the positive control (Amphotericin B) showed inhibition zones ranging from 12 to 15 mm (in diam.) against the fungal species studied.

3.3.2. Bio-autography assay

The bio-autography results for the crude extract GP/C2 revealed cleared areas of bacterial growth inhibition with potential susceptibility with *S. aureus* and slight susceptibility with *E. coli*. The chromatograph under UV light (366 nm), after acid treatment (H_2SO_4 in methanol) and the bio-autography results of GP/C2 against *E. coli* and *S. aureus*, are described in Figures 26 and 27, respectively.

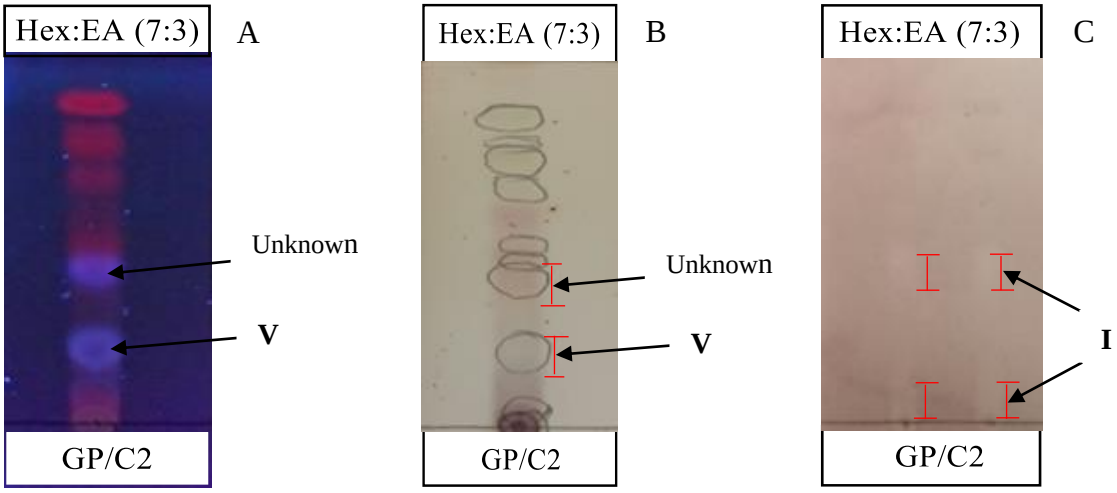


Figure 26: Bio-autography of GP/C2 against *E.coli* using 7:3 (Hexane: Ethyl acetate) as eluent. A: Observation under UV light (366 nm), B: H_2SO_4 in methanol treatment, C: Bio-autography result, **V**: compound **V**, **I**: Clear zones

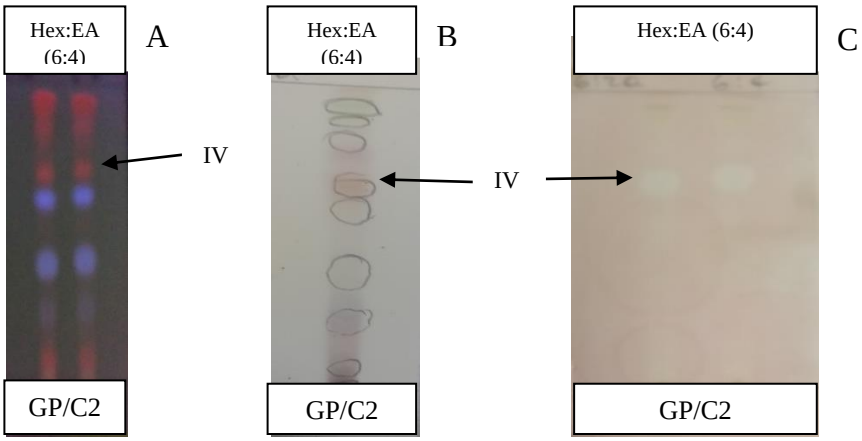


Figure 27: Bio-autography of GP/C2 against *S. aureus* using 6:4 (Hexane: ethyl acetate) as eluent. A: Observation under UV light (366 nm), B: H_2SO_4 in methanol treatment, C: Bio-autography result, **IV**: compound **IV**

3.3.2. Anti-sickling, Emmel's test

The results of the anti-sickling assay showed that compounds **X** and **Y** have the potential to reverse the tested sickled red blood cells to normal. The highest anti-sickling property was observed from compound **Y** with 82.135 % standardisation at a sample concentration of 144.93 µg/ml and above. This was followed by the activity of compound **X** which was 40.23 % standardisation at a concentration of 105.84 µg/ml and above. The phenotypic optical micrograph results of the sickled RBCs treated with 31.25 µg/ml (A) and 250 µg/ml (B) of compound **Y** are shown in Figure 28, and the normalisation graph of compounds **X** and **Y** is described in Figure 29. The rest of the isolated compounds of *G. perpensa* did not show anti-sickling properties.

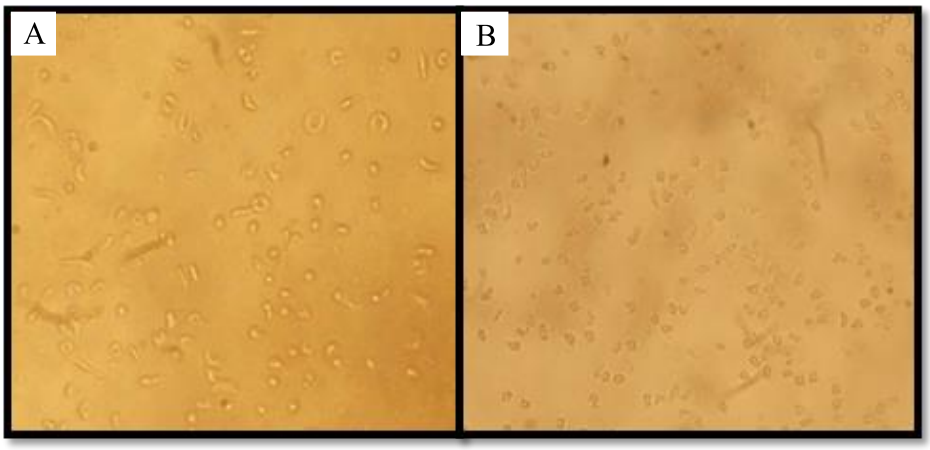


Figure 28: Pictures of sickled RBCs treated with compound **Y**; A:31.25 µg/ml, B:250 µg/ml

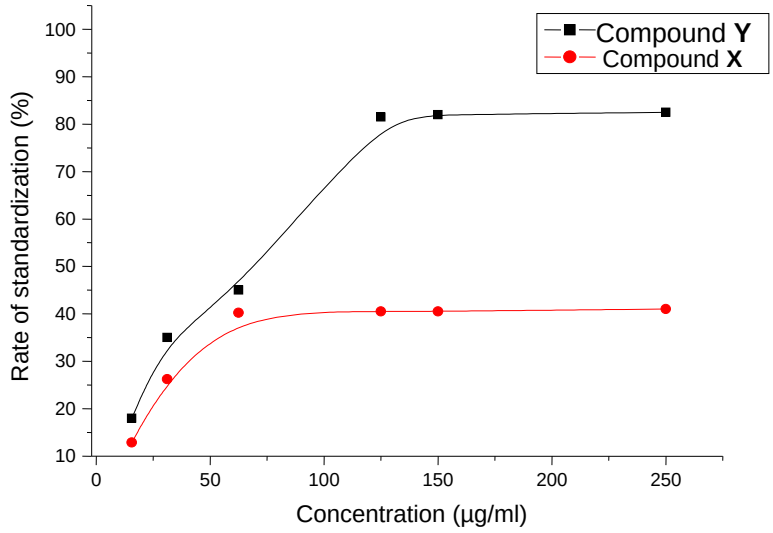


Figure 29: Normalisation graph of compounds **X** and **Y**.

3.3.3. DPPH radical scavenging capacity

The results of the free radical scavenging capacity of the crude extracts of *G. perpensa* exhibited that the extracts GP/A2, GP/B3, GP/A3, and GP/B2 have the highest radical scavenging activity against the stable free radical DPPH with 96, 95, 94.8, and 94.3 percentage values, respectively (Table 14). The DPPH scavenging activity of the isolated compounds is described in Table 15. The anti-oxidant activity of both the extracts and isolated compounds were additionally evaluated based on the degree of colour change of DPPH, which is violet in methanol. The anti-oxidant result of the ethyl acetate crude extract of the leaves (GP/A2) and the methanol crude extract of the stalks (GP/B3) are shown in Figure 30 below. The recorded anti-oxidant properties of the isolated compounds were found to be very weak.

Table 14: DPPH radical scavenging activity results of crude extracts of *G. perpensa*.

Crude Extracts	Antiradical scavenging activity (%)				
	1000 µg/ml	**500 µg/ml**	**250 µg/ml**	**100 µg/ml**	**50 µg/ml**
GP/A1	24	14.6	14	0.775	-2.2
GP/A2	96	89.7	70.8	39	20
GP/A3	94.8	94.3	91.7	90.4	89
GP/B1	32.9	20.7	6.8	4	1.4
GP/B2	94.3	93	90.4	48.6	33.1
GP/B3	95	93.9	93.3	91.5	91.1

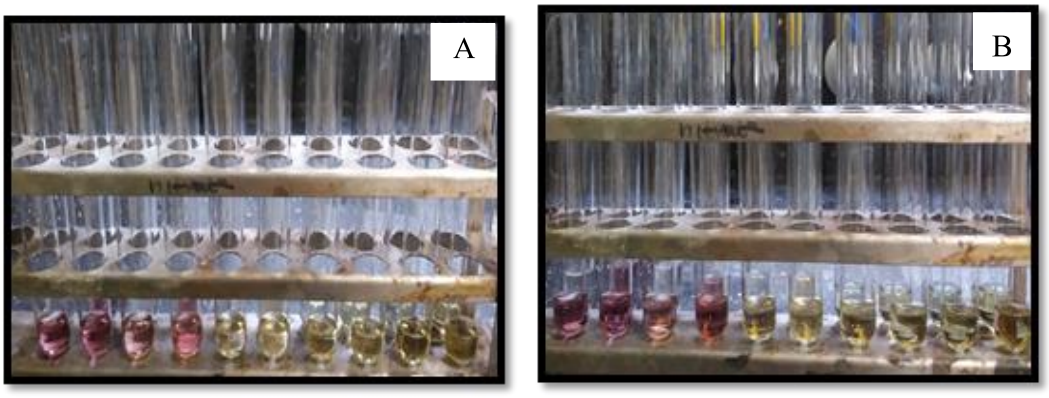


Figure 30: DPPH colour change anti-oxidant results; A: GP/A2, B: GP/B3 with concentrations ranging from low (left) to high (right).

Table 15: DPPH radical scavenging activity results for the isolated compounds of *G. perpensa*.

Isolated Compounds	Antiradical scavenging activity (%)				
	1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml
I	26.3	30.6	29	29.5	23.3
II & III	25.2	12.7	16.5	31.2	27.6
IV	33.5	14.5	11.2	20.2	3.5
V	38	26	12.8	12.8	8.8
X	16.8	14.6	8.5	5.1	10.1
Y	38.8	46.1	61	68.3	57.7

3.3.4. MTT assay

The MTT assay results of the isolated compounds of *G. perpensa* against the tested cancer cell lines (HEK293 and MCF-7) displayed moderate to low cytotoxicity. Potential cytotoxicity was observed for compounds **I**, **II**, **III** and **IV** against the HEK293 cell line. These compounds exhibited more than half percentage death of the tested HEK293 cells (Figure 31). Compounds **V**, **X** and **Y** showed weak anticancer potential.

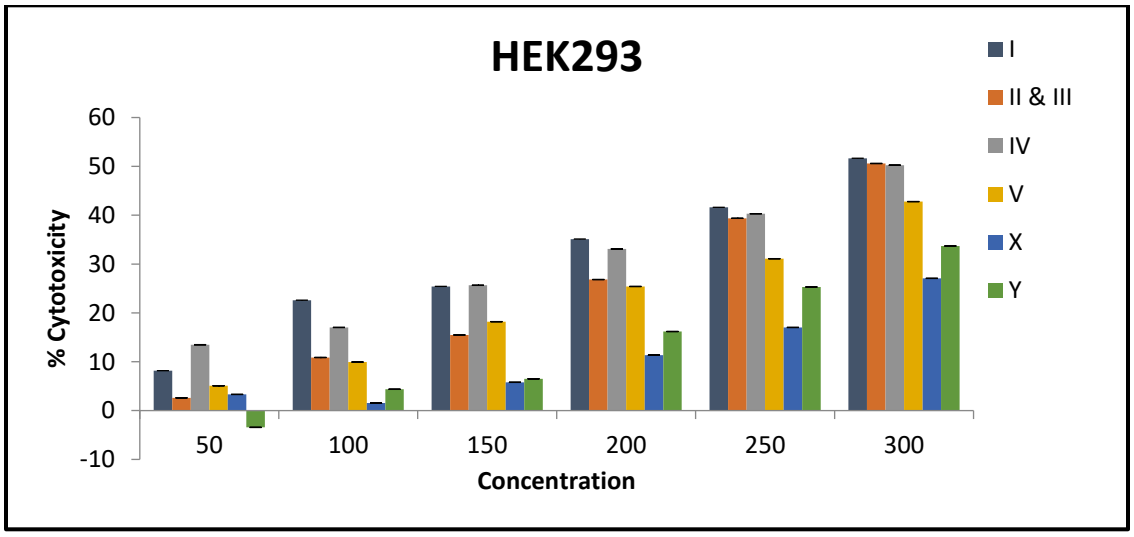


Figure 31: Percentage cytotoxicity results of the isolated compounds of *G. perpensa* against HEK293 cell line.Values are expressed as mean ± SD.

The isolated compounds were found to give more cytotoxicity potential against the tested MCF-7 cancer cell line. The highest cytotoxicity potential was recorded by compounds **X** and **Y** followed by moderate activity of compounds **I**, **II**, **III** and **IV** with an equal percentage value of death, 54.58 (Figure 32). From these results, it is clear that the isolated compounds of *G. perpensa* have highest cytotoxicity potentials to MCF-7 than HEK293 cell line.

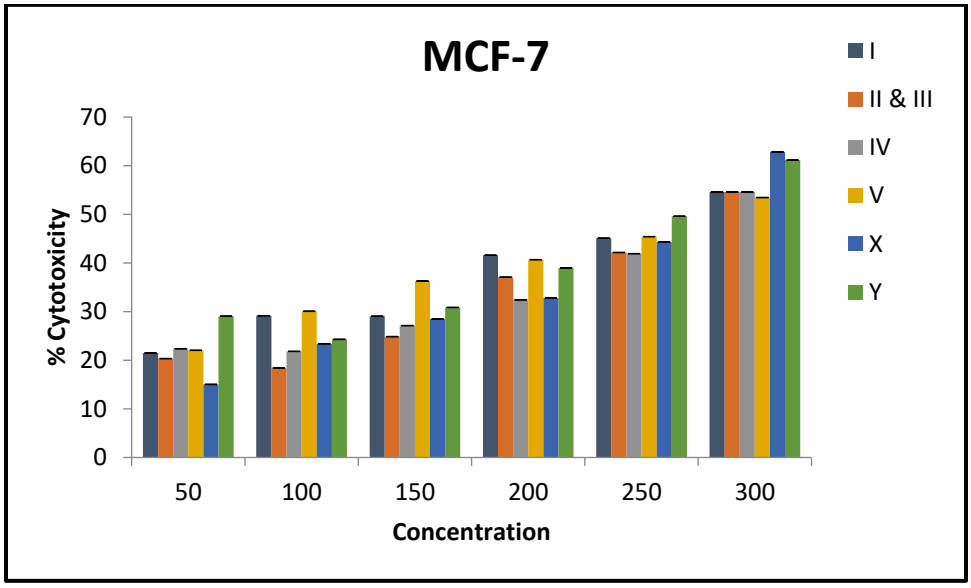


Figure 32: Percentage cytotoxicity results of the isolated compounds of *G. perpensa* against MCF-7 cell line. Values are expressed as mean ± SD.

3.3.5. Biosafety assay

The brine shrimp lethality assay results of both the crude extracts and isolated compounds of *G. perpensa* showed that they are not lethal since the recorded shrimp larvae death is much lower than 50 %. The lethality results of the crude extracts and isolated compounds are described in Table 16 and 17, respectively.

Table 16: Brine shrimp lethality assay death results of extracts of *G. perpensa*.

Crude extracts	**Concentrations (µg/ml)**		
	100	500	1000
GP/A1	0	0	0
GP/A2	0	0	0.3 ± 0.5
GP/A3	0	0	1.3 ± 0.5
GP/B1	0	0	1.3 ± 0.5
GP/B2	0	0	1 ± 0.5
GP/B3	0	0	1.3 ± 0.5
+ve control	0	0	0

Values are described as average ± SD.

Table 17: Brine shrimp lethality assay death results of the isolates from *G. perpensa*.

Isolated compounds	**Concentrations (µg/ml)**		
	100	500	1000
I	0	0	0
II & III	0	0	0.6 ± 0.5
IV	0	0	0
V	0	0	0.6 ± 0.5
X	0	0	0
Y	0	0	0
+ve control	0	0	0

Values are described as average ± SD.

3.4. Discussion

The antibacterial results of the crude extracts (Table 12) and the isolated compounds (Table 13) showed that some of the crude extracts and compounds were active against the tested bacterial strains. The highest bactericidal activity was recorded by the methanol leaf extract (GP/A3) and acetone extract of the stalk (GP/C4). The methanol leaf extract was highly active against *S. aureus*, *E. coli* and *P. aeruginosa* with MIC values of 0.75, 0.375 and 0.75 µg/ml, respectively. While, the acetone stalk extract was highly active against *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa* with MIC values ranging from 0.75 to 1.5 µg/ml. There is little work done on the aerial parts of *G. perpensa*. The only literature we found at the time of this study agrees with the results recorded by Drewes *et al.* (2005). A study by Drewes *et al.* (2005) stated that the methanol extract of the leaves was found to be highly active than the extracts of the stalks and the roots of *G. perpensa*. Unlike the aerial parts, much work has been done on the crude extracts and some isolated compounds of the root. Steenkamp et al. (2004) reported that the methanol extract of *G. perpensa* root has shown efficient antibacterial property against *S. aureus*, *Streptococcus pyogenes* and *E. coli*. While the aqueous extract showed activity against *S. aureus* and *Streptococcus pyogenes*. Buwa and Van Staden (2006) has also reported that the ethanol root extract has shown effective inhibitory potential against *E. coli* and *K. pneumoniae*, while the aqueous extract was active against all the tested bacteria including *B. subtilis* and *S. aureus*. A report by McGaw, Jäger and Van Staden (2000) stated that the ethanol and aqueous crude extracts of *G. perpensa* root showed slight antibacterial activity against *S. aureus* but showed no activity against *E. coli*, *Klebsiella pnuemoniae* and *Bacillus subtilis*. Moreover, it has been reported that the hexane extract of the root showed no antibacterial activity against the aforementioned bacterial species (Nigam *et al.* 2009). While, the methanol and aqueous leaf extracts showed activity against the studied ten bacterial species; including *B. cereus*, *E. coli*, *S. aureus*, and *E. faecalis* (Chigor 2014).

The isolated compounds from *G. perpensa* leaves and stalks showed slight antibacterial activity with better activity recorded from **X** against *S. aureus*, *S. epidermidis* and *E. faecalis*. Compound **IV** (3α-hydroxyolean-12-en-27-oic acid) showed slight activity against the tested *S. aureus*

strain. This result is in agreement with the result reported by Zheng *et al.* (2008) where 3α-hydroxyolean-12-en-27-oic acid showed weak activity against different strains of *S. aureus*.

Although compound **I** in this study showed only a slight activity against *S. aureus*, *E. coli* and *E. faecalis*, it has been reported that this compound from other plant sources has shown potential antimicrobial activities (see Section 1.3.3.2). The results of compounds **II** and **IV** were also in agreement with a report by Zheng *et al.* (2008) where 3β-hydroxyolean-12-en-27-oic acid and 3α-hydroxyolean-12-en-27-oic acid showed very weak antimicrobial activity against the tested strains of bacteria. These results showed that the isolated pentacyclic triterpenoic acids have higher antibacterial potential against the studied gram-positive bacteria than the gram-negative bacteria. An observation made by Pacheco *et al.* (2012) demonstrated that the bactericidal potential of triterpenes is highly influenced by their chemical structures. The presence of an oxygenated group (such as carbonyl and carboxyl groups) at the 3-carbon position was also suggested as a possible reason for the recorded bactericidal potentials of the triterpenes. The recorded activities of the crude extracts were greater than the isolated compounds and this could be due to the presence of other non-isolated compounds within the crude extracts (Verpoorte 1998).

The disc diffusion results of both the crude extracts and the isolated compounds showed no antifungal activities against the tested fungi species. The negative control (DMSO) showed no inhibition while the positive control (Amphotericin B) showed inhibition zones ranging from12 to 15 mm (in diam.) against the fungal species studied. The antifungal results from this study are in agreement with the report by Buwa and Van Staden (2006) and Ndhlala *et al.* (2009). Buwa and Van Staden (2006) studied the antifungal activity of the ethanol, ethyl acetate and aqueous extracts of *G. perpensa* root which resulted in no inhibition against the tested fungi species. Thus, the results from most of the plant extracts showed some level of antibacterial potential. This is supported by previous observations that plant extracts have weak antimicrobial potential against plant pathogenic fungi than bacteria (Leven *et al.* 1979; Naqvi, Khan and Vohora 1991).

Although the isolated pentacyclic triterpenes showed weak antimicrobial activities, previous studies indicated that the site of action for terpene hydrocarbons is the cell membrane of microorganisms. According to Sikkema, De Bont and Poolman (1994), the physical and functional natures of the studied artificial membranes were affected by the action of the terpenes, limonene, γ-terpinene, β-pinene and α-pinene. Other terpenoids were able to affect micro-organisms by distorting microbial oxidative phosphorylation and oxygen uptake (G. *et al.* 1999). However, the weak antimicrobial activity of the isolated triterpenes in this study could be due to the test method used for screening. This is a problem since disc diffusion assay is dependent on the diffusion ability of the test agent through agar, and the water solubility of the test agent. Because the water solubility of different extracts differs based on the solvent used during extraction (L. and E.A. 2004). Moreover, there was an observation made by Nazzaro *et al.* (2013) which indicated that terpenes are known for their low or no antimicrobial properties.

The bio-autography results for the crude extract GP/C2 revealed cleared areas of bacterial growth inhibition with potential susceptibility with *S. aureus* and slight susceptibility with *E. coli*. This result is supported by a study where compound **IV** showed slight antibacterial activities against different *S. aureus* strains (Zheng *et al.* 2008).

The results of the anti-sickling assay showed that the partial fractions **X** and **Y** have the potential to reverse the tested sickled red blood cells to normal shaped red blood cells. The highest anti-sickling property was observed from compound **Y** with 82.135 % standardisation at a minimum sample concentration of 144.93 µg/ml. This was followed by the activity of **X** which was 40.23 % standardisation at the lowest concentration of 105.84 µg/ml (Figure 29). The anti-sickle result of compound **I** in this study contradicts with literature. According to Tshilanda *et al.* (2015), a mixture with UA as a major compound has shown effective anti-sickling properties. Based on the result of our study, there is a possibility for the activity of UA reported in the mentioned report to be due to other impurities found in the mixture.

The anti-oxidant results of the crude extracts of *G. perpensa* exhibited that the ethyl acetate extract of the leaves (GP/A2), methanol extract of the leaves (GP/A3), ethyl acetate (GP/B2) and methanol (GP/B3) extracts of the stalks have the highest antiradical scavenging activities against the stable free radical DPPH (Table 14). This result is in agreement with previous studies made by other researchers. A study by Steenkamp *et al.* (2004) demonstrated the significant anti-oxidant properties of the aqueous and methanol extracts of *G. perpensa* root. A strong antiradical scavenging activity of the methanol extract of *G. perpensa* to DPPH and ABTS (3-ethylbenzothiazoline-6-sulfonate) was also reported by (Simelane *et al.* 2010). The weak activity of compound **I** to DPPH in this study is in contradiction with previous studies reported on UA isolated from other plant sources (Assimopoulou, Zlatanos and Papageorgiou 2005).

In MTT assay, the determination of viable cells is centered on the measurement of the activity of mitochondrial dehydrogenase within living cells. The enzyme, dehydrogenase, leads to the reduction of the reagents to form colored formazan (McCauley, Zivanovic and Skropeta 2013). In this study, the results of the isolated compounds of *G. perpensa* against HEK293 and MCF-7 cancer cell lines displayed moderate to low cytotoxicity. Potential cytotoxicity was observed for compounds **I**, **II**, **III** and **IV**, and weak anticancer potential were observed for compounds **V**, **X** and **Y** against HEK293 cell line. The isolated compounds were found to be more cytotoxic to the tested MCF-7 than HEK293 cell line. The highest cytotoxicity potential was recorded by **X** and **Y** followed by moderate activity of compounds **I**, **II**, **III** and **IV** (Figure 32).

Previous studies showed that compound **I** (UA) has a wide range of biological properties. It has been reported that this compound has therapeutic potential for a range of cancer problems through induction of apoptosis, growth inhibition, cytotoxicity and proliferation suppression. For the reported studies, the cancer cell lines were taken from different body organs including; bladder (Zheng *et al.* 2013), cervix (Li *et al.* 2014), breast (Wang, Ren and Xi 2012), gastric (Kim and Moon 2015), liver (Liu *et al.* 2014), lung (Liu *et al.* 2013), skin (Manu and Kuttan 2008), pancreas (Li, Liang and Yang 2012), thyroid (Bonaccorsi *et al.* 2008), prostate (Shin, Kim and Park 2012), ovary (Song *et al.* 2012), colon (Li, Guo and Yang 2002), and lymph nodes (Lauthier *et al.* 2000).

The anticancer results of compounds **II** (3β-hydroxyolean-12-en-27-oic acid) and **III (**3β-hydroxyurs-12-en-27-oic acid) were in agreement with previous works done by other researchers. According to Wang *et al.* (2013), compound **II** had shown high antitumor activity through apoptosis of cells, cell arrest in G2/M phase, and invasion and metastasis suppression of cells. It was also responsible for upregulation and downregulation of nine and eight genes, respectively. Arisawa *et al.* (1992) reported that this compound in combination with OK-432 and mitomycin enhanced the antitumor potential of the compounds against mouse melanoma in C57BL/6 mice. Compounds **II** and **III** have also been reported for their potent cytotoxic effect against different types of cancer cell lines (Sun, Zheng and Tu 2006).

A report by Zheng *et al.* (2006) described that the carboxyl group attachment at the 27-carbon made 3β-hydroxyurs-12-en-27-oic acid to have higher anticancer potential against human cervical squamous carcinoma (HeLa) cells than UA. Moreover, the compound 3β-hydroxyolean-12-en-27-oic acid has been reported to be highly effective against HeLa and human colorectal carcinoma (COLO-205) cells than OA (Tu, Sun and Ye 2006). A group of triterpenes with the carboxyl group attached to the 27-carbon, including compounds **II** and **IV**, were reported to have significant cytotoxicity potential against HL-60 and K562 cell lines (Lee *et al.* 2007). On the other hand, a study by Geng *et al.* (2012) showed that compound **II** has no anti-hepatitis B virus (HBV) activities against the HBeAg and HBsAg secretion, and Hep G 2.2.15 cell line HBVDNA replication. Compound **V** (Pomolic acid) discovered from the fruits of *Licania tomentosa* and the leaves of *Chrysobalanus icaco*was found to have effective anticancer property against the K562 cell line. The high anti-HIV potential of compound **V** (Pomolic acid) isolated from the leaves of *Rosa woodsii* and *Hyptis capitata*was also reported by Kashiwada *et al.* (1998). Furthermore, a study by (Estrada *et al.* 2009) demonstrated that pomolic acid has platelet anti-aggregating potential to aggregates induced by ADP and Epinephrine (Estrada *et al.* 2009). The compound also induced apoptosis in human ovarian adenocarcinoma (SK-OV-3) cells through the death receptor-induced extrinsic and mitochondrial-mediated intrinsic pathways (Yoo *et al.* 2013).

The safety bioassay results of all the tested crude extracts and isolated compounds showed that the samples are biologically safe to be used at the concentrations studied. This result is in agreement with previous studies of the isolated compounds from various plant sources (Sultana 2011). In this study, the death percentage of a brine shrimp (*Artemia salina*) larvae was used to evaluate the killing capacity of the test sample (Wu 2014). Apart from its use in screening cytotoxicity, this method is used as an indicator of other biological properties like fungicidal, antitumor, and insecticidal potentials. This method is also crucial in guiding water quality tests, bioactive compounds isolation, and fungal toxins detection (Mclaughlin, Rogers and Anderson 1998; Karchesy *et al.* 2016).

CHAPTER 4: GENERAL CONCLUSION

G. perpensa is an indigenous perennial plant and the only species of its genus found in South Africa (Fuller and Hickey 2005; Peter 2010). It is used by traditional health practitioners of South Africa to treat various human ailments; including venereal diseases, infertility, endometritis, insect repellant, urinary tract problems, impotence, menstrual pains, and cold (Kaido *et al.* 1997; Brookes and Dutton 2007; Nkomo *et al.* 2010). Even if the plant is widely used in the traditional medicine system of South Africa, little work has been done in the identification and biological screening of the secondary metabolites of the plant (Brookes and Dutton 2007; Chavan *et al.* 2013). Thus, this study was undertaken to isolate, characterize and biologically screen the secondary metabolites of *G. perpensa* leaves and stalks.

Extraction of the plant material is the most important and first step involved in the isolation and purification of the active compounds from plants. In this study, the two extraction protocols used have shown that methanol is the best extractant with higher percentage yield for both leaves and stalks of *G. perpensa* than the other solvents used in this study. This supports the common use of methanol for the extraction of bioactive compounds from plants in many studies.

Crude extract selection for further purification was based on the results from the TLC profiles of the sample extracts. The TLC profiles indicated that the ethyl acetate extracts of both the leaves and stalks have the highest number of natural compounds. These extracts were subjected to CC for further isolation and purification of the secondary metabolites. HPLC was also used for compounds that were difficult to separate with CC. Structural elucidation of the isolated compounds was done by comparison of the resulted NMR spectral data with literature. Fractionation of the ethyl acetate crude extracts from the leaves and stalks of *G. perpensa* gave five isolates (**I-V**). One compound (**I**) was obtained from the leaves and four compounds (**II-V**) were obtained from the stalks. Since these extraction methods have not been used to separate metabolites from *G. perpensa*, it is possible that the extraction protocols used in this study contributed to the isolation of the above compounds.

Among the solvents used in this study, acetone, methanol and ethyl acetate were found to be the best solvents to extract the active compounds with antibacterial potentials. The antimicrobial screening results of the crude extracts of *G. perpensa* revealed the potential of the plant's leaves and stalks to be used as antibacterial agents. This is in agreement with the traditional use of *G. perpensa* as dressing for wounds (Grierson and Afolayan 1999). Methanol and ethyl acetate crude extracts of both the leaves and stalks showed the highest anti-oxidant activities. These results will serve as a starting point for further studies to identify and characterize undiscovered secondary metabolites, since the presence of this property gives a hint for other possible biological activities. The anticancer activities of the isolated compounds were high against MCF-7 than HEK293 cell line. This indicates the cytotoxicity of the compounds is cell line dependent, we suggest further studies of the compounds against different cancer cell lines is required. It is highly recommended to further purify the partially purified compounds (**X** and **Y)** as they showed high biological activities that can add value to new drug development. Moreover, none of the crude extracts and isolated compounds was found to be toxic to brine shrimp larvae.

In general, the biological screening of the crude extracts and isolated compounds revealed the potential of the plant to be used in cosmetic and pharmaceutical industries. The high anti-sickling property of the plant together with its antioxidant property can be further studied and applied in medications designed to treat and cure patients suffering from sickle cell disease and its consequences. With all these biological activities while devoid of noticeable toxicity, *G. perpensa* will attract more phytochemical exploration in the future to create more medicinal applications.

CHAPTER 5: REFERENCES

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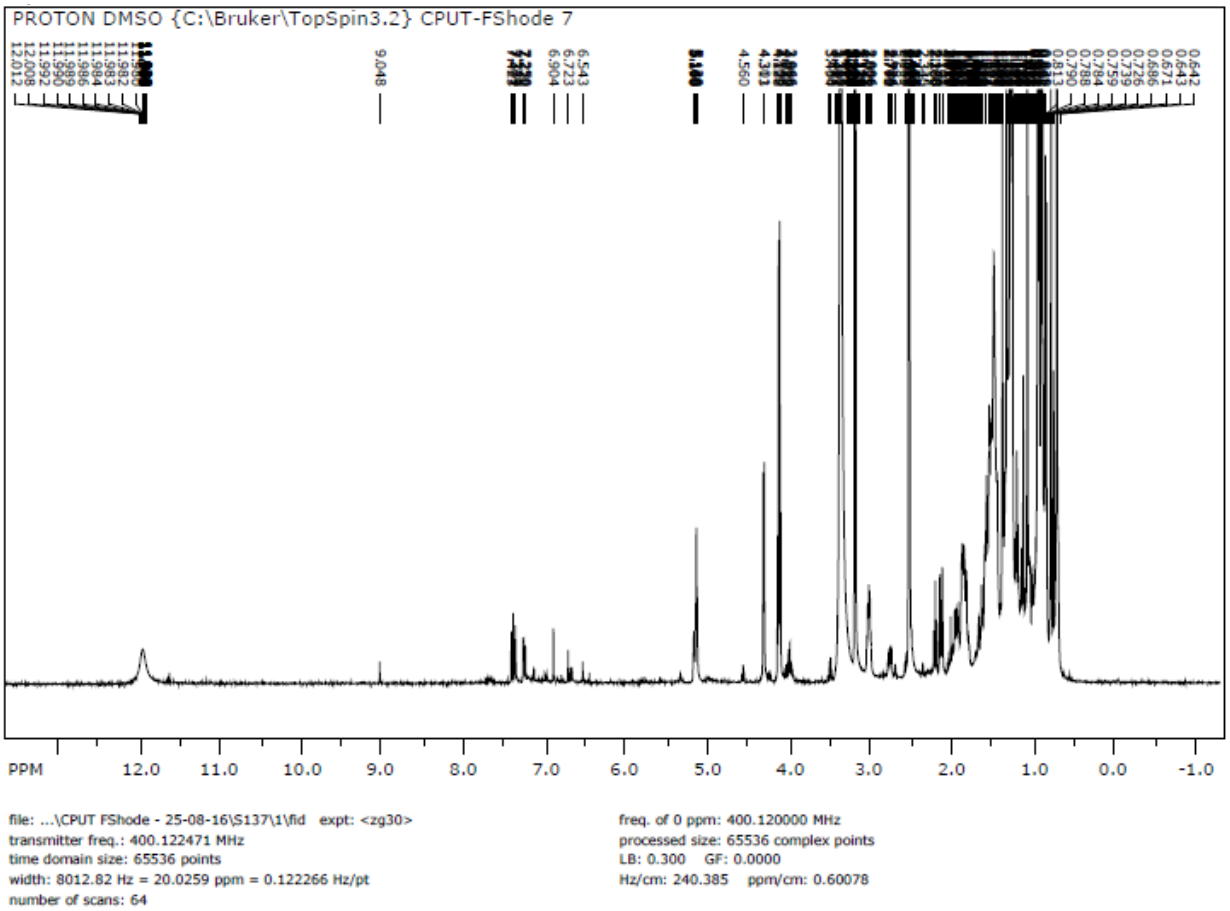
APPENDIX I

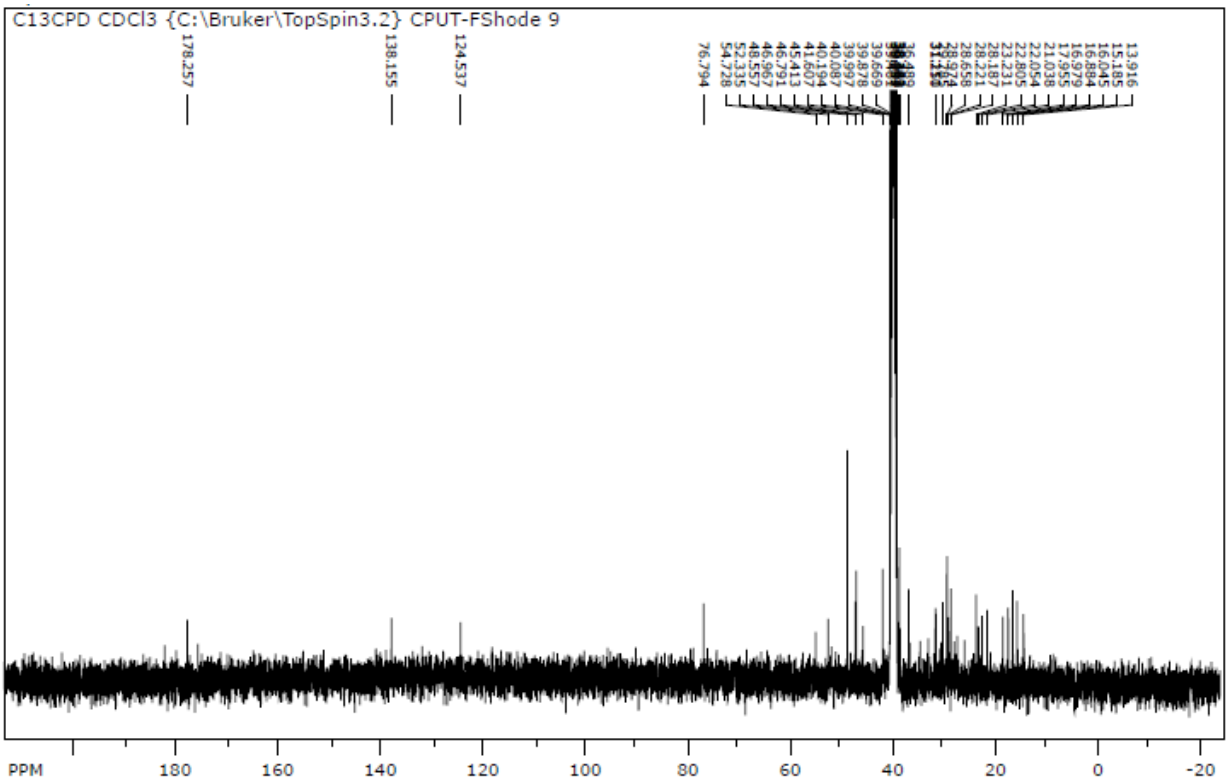
^{1}H and ^{13}C NMR spectral data of the isolated compounds (**I-V**) and the DEPT of Compounds **II** & **III**.

Compound **I/ 34W2**

^{1}H NMR



^{13}C NMR



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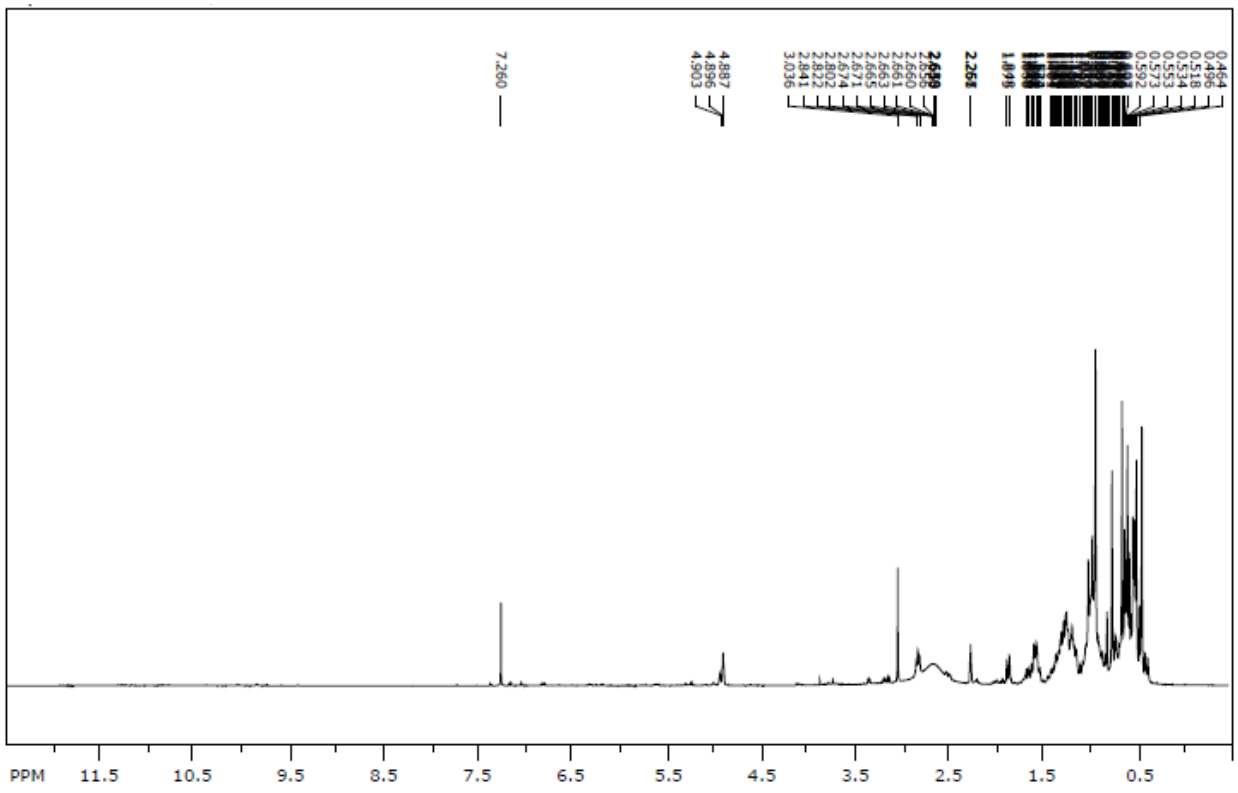
Compounds **II** and **III/ 30/N+**

^{1}H NMR



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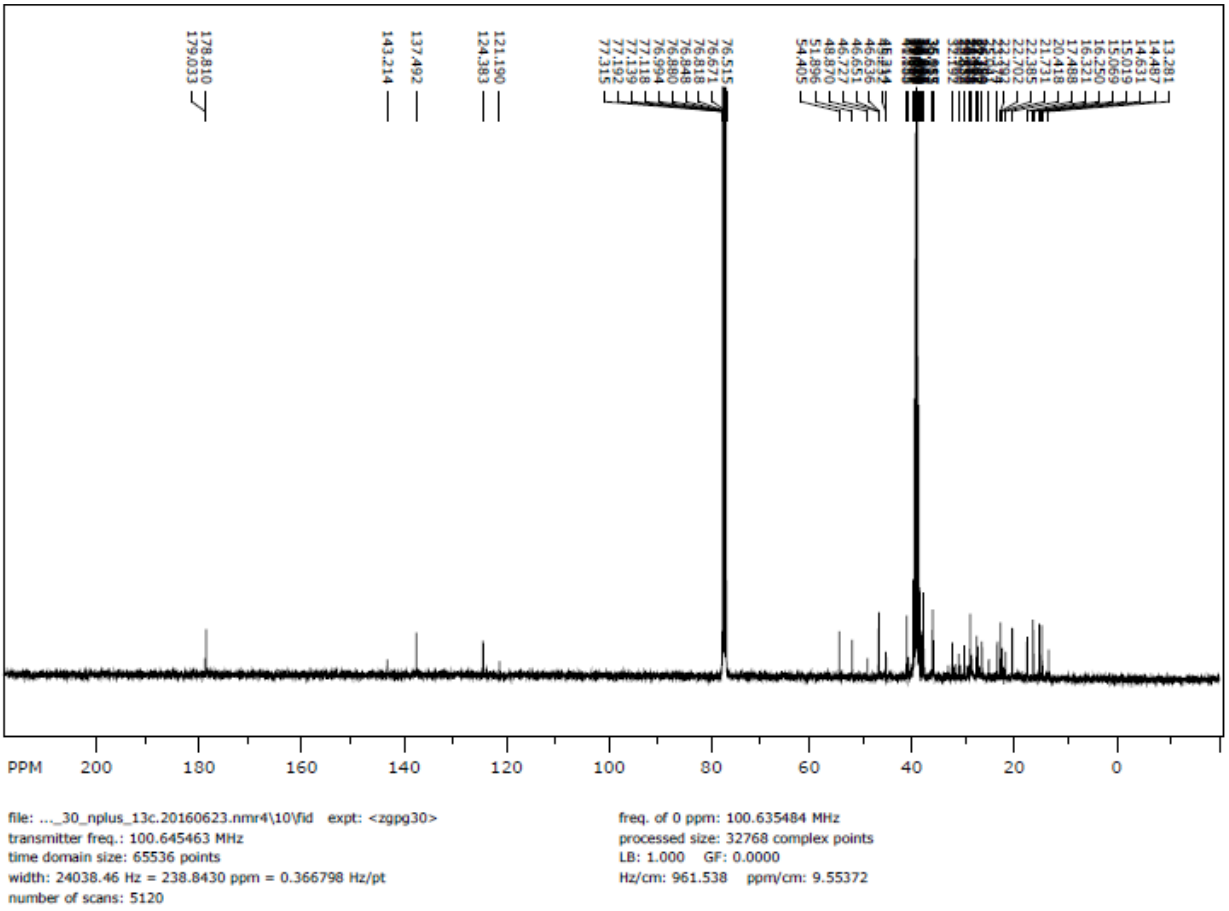
Hz/cm: 208.333 ppm/cm: 0.52054

^{13}C NMR



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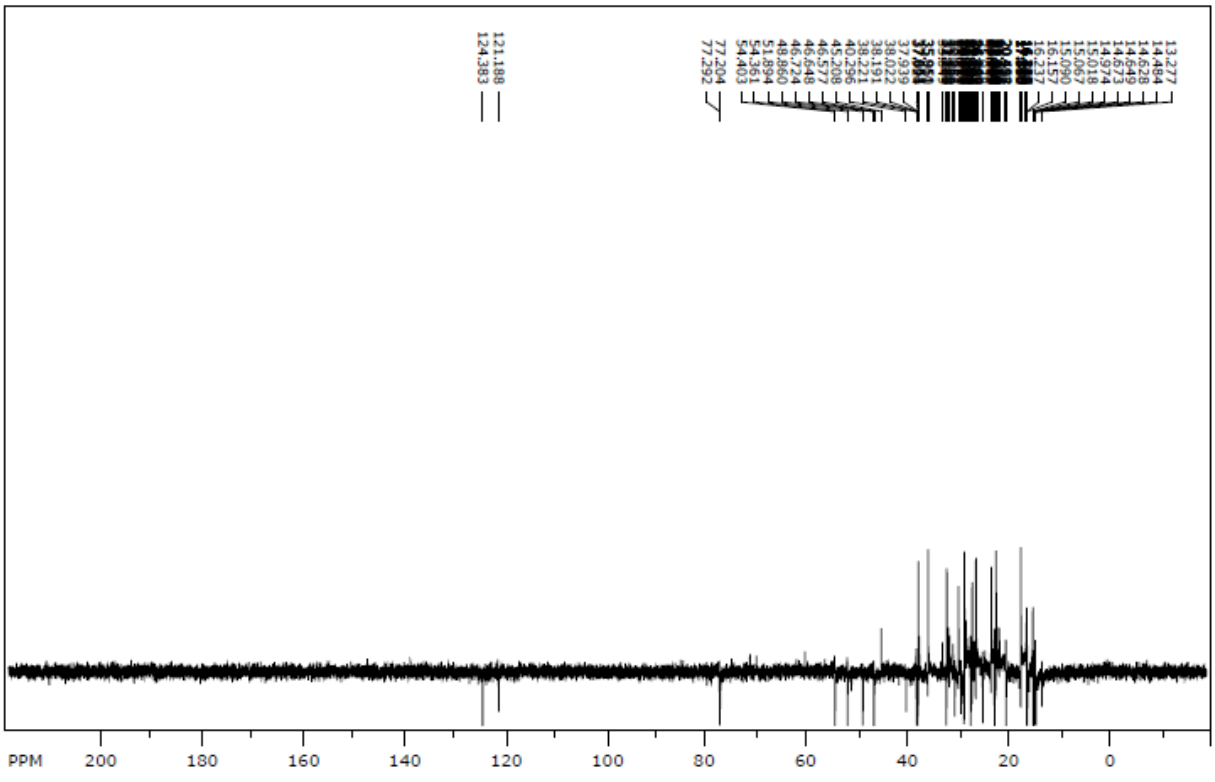
DEPT



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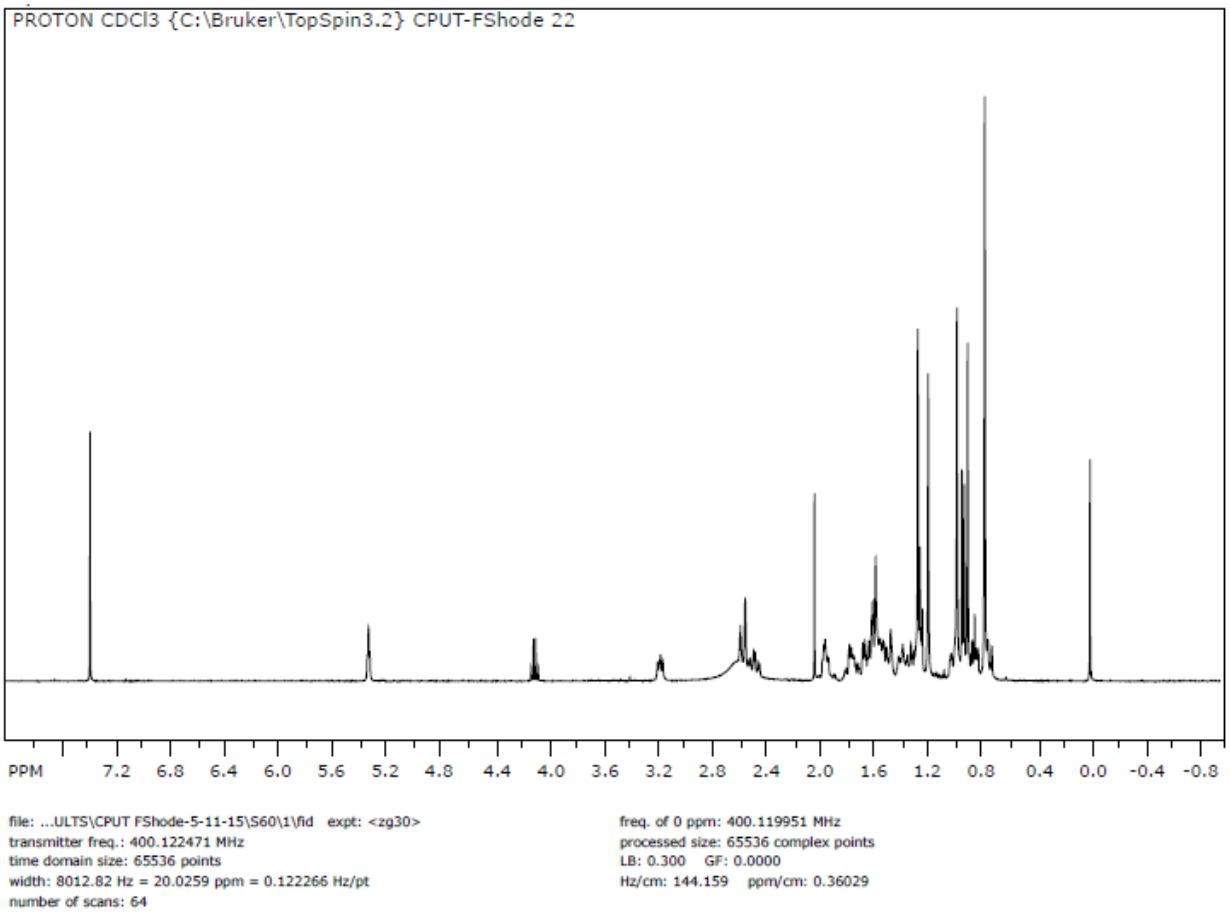
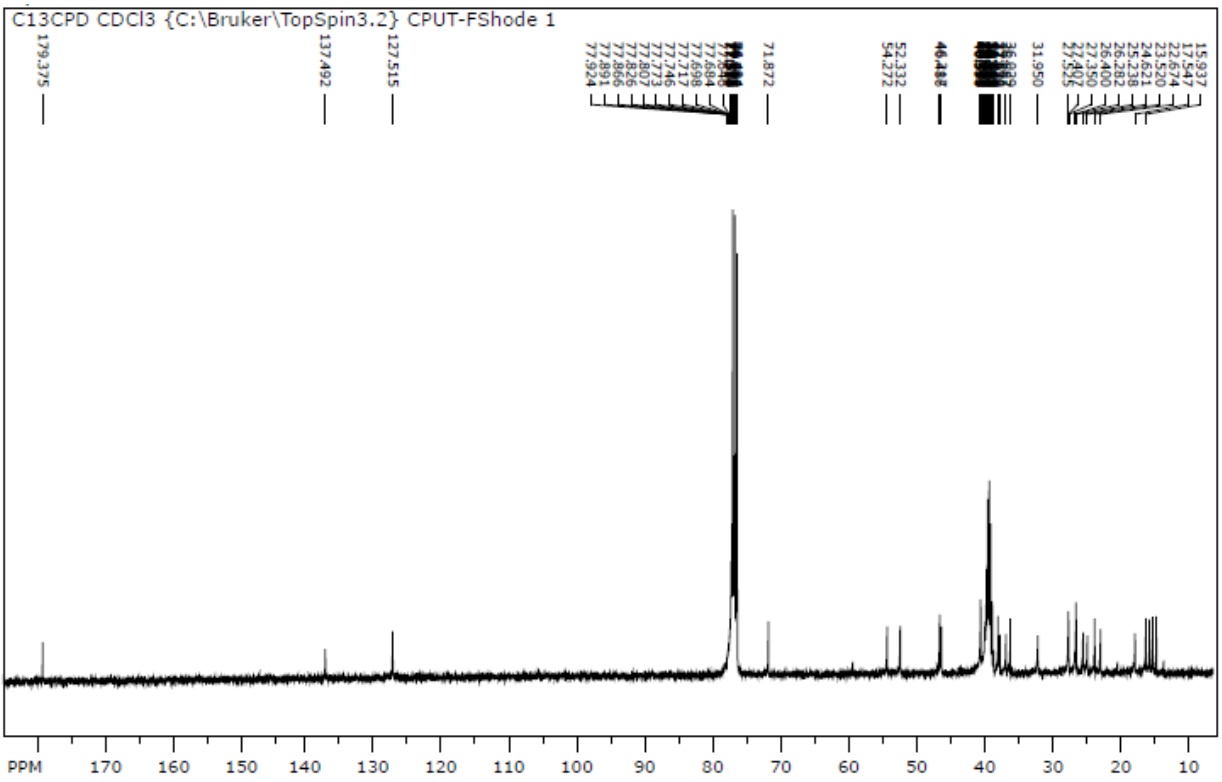
Compound **IV/ 30/Q+**

^{1}H NMR



^{13}C NMR



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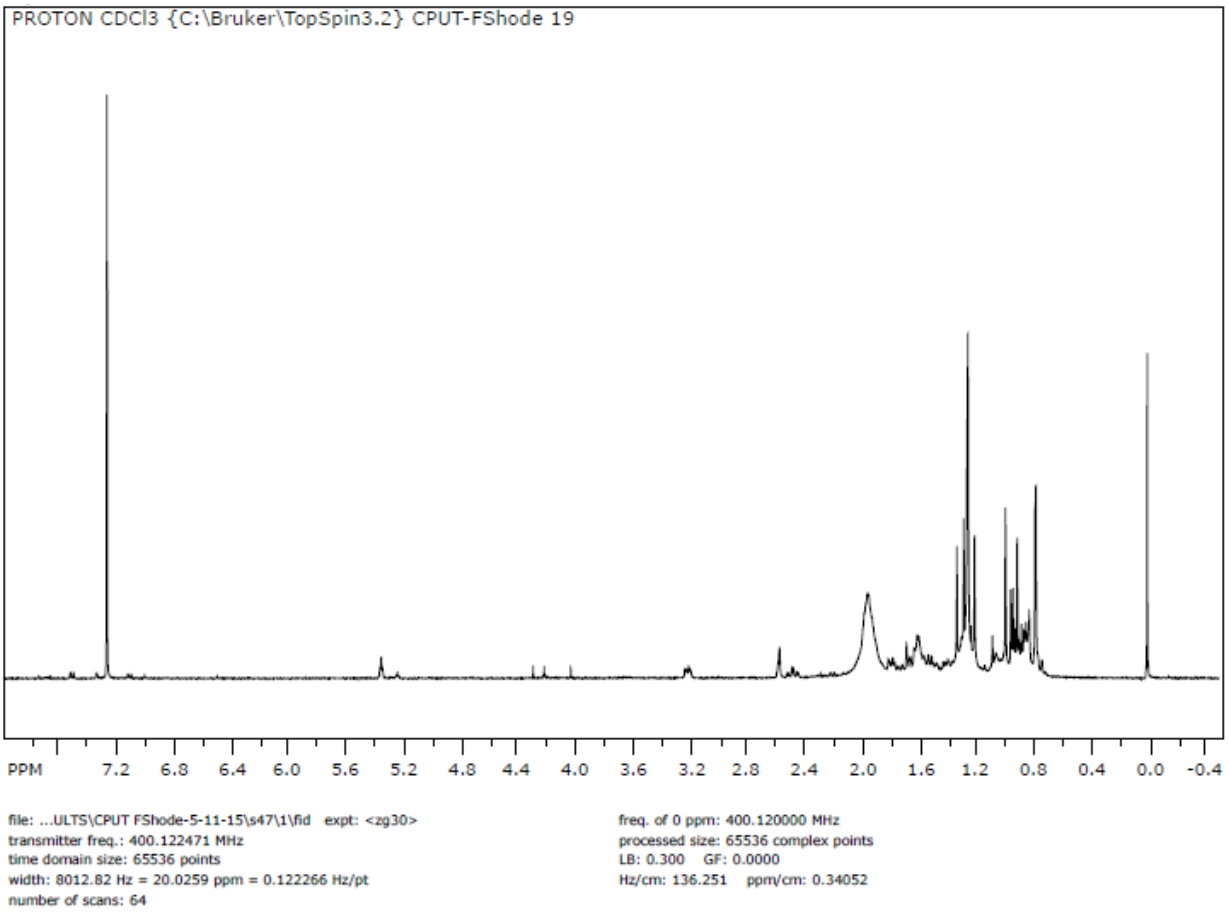
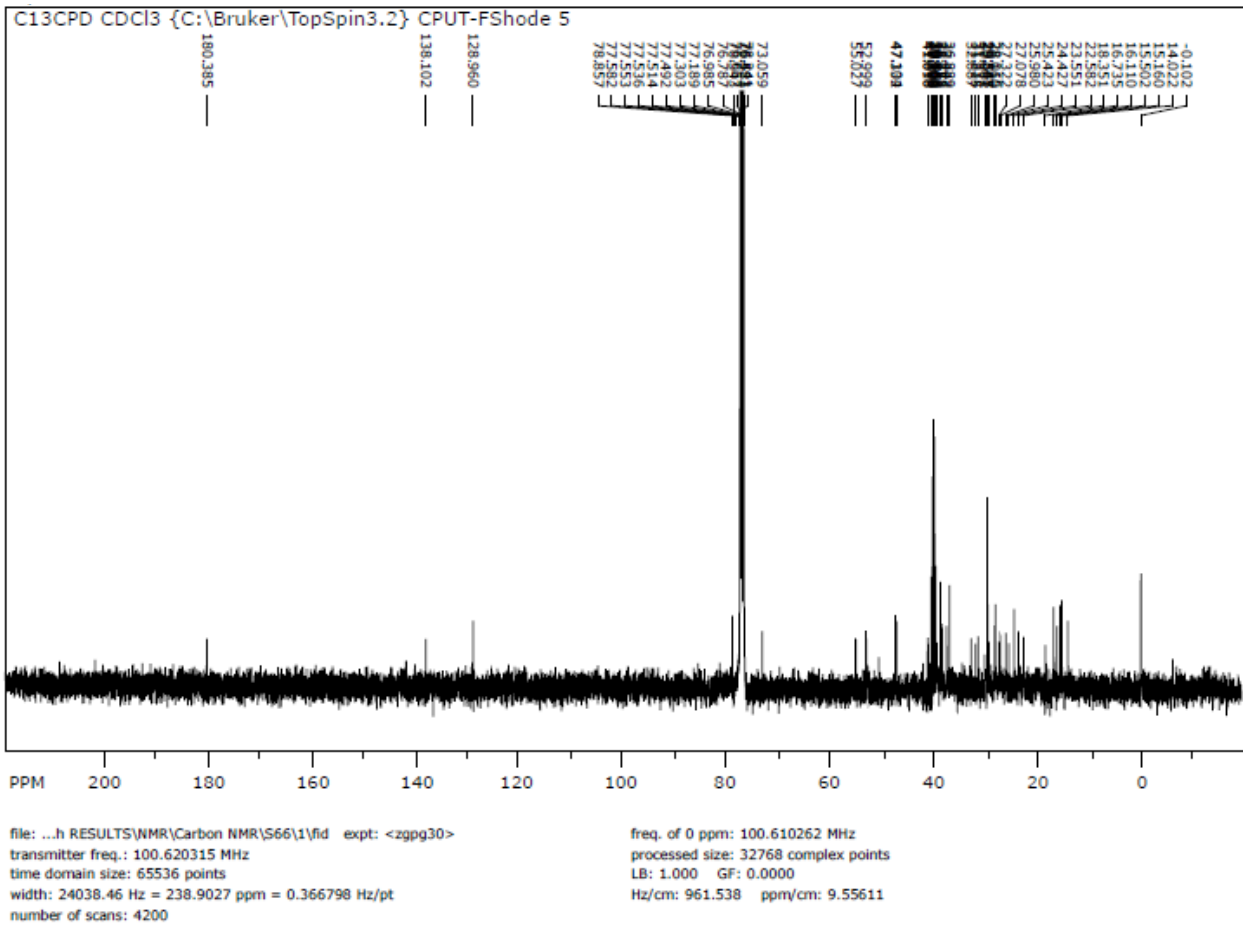
Compound **V/ 11G**

^{1}H NMR



^{13}C NMR



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processed size: 32768 complex points
LB: 1.000 GF: 0.0000
Hz/cm: 961.538 ppm/cm: 9.55611

APPENDIX II: PUBLICATIONS

African Journal of Science, Technology, Innovation and Development, 2016
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Routledge
Taylor & Francis Group

Gunnera perpensa L.: A multi-use ethnomedicinal plant species in South Africa

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Gunnera perpensa L. is a medicinal plant used in many parts of South Africa to treat several human ailments. *G. perpensa* is inherited from and linked to the Siswati, Changana, Sotho, Venda, Shona, Tsonga, and Zulu traditional healing systems, particularly in South African provinces where the plant species is still found in the wild. *G. perpensa* is declining considerably throughout its natural habitat due to over-exploitation of the plant for its medicinal uses. This review aims to gather the fragmented information on the past and present ethnomedicinal uses, phytochemistry and pharmacological properties of *G. perpensa*.

Keywords: ethnobotanical uses, *Gunnera perpensa*, phytochemistry, pharmacological properties, southern Africa, traditional medicine

Introduction

The genus *Gunnera* L. (family Gunneraceae) was named after a Swedish bishop and botanist Johan Ernst Gunnerus in the eighteenth century (Jarzen 1980; Peter 2010). *Gunnera* L. constitutes a group of herbaceous flowering plants. The genus is one of the six subgenera of its family with approximately 50 species (Brookes and Dutton 2007; Peter 2010; Simelane 2010). Due to its endosperm rich seeds and vegetative habit, the genus was previously grouped under the family Haloragaceae. Later revisions on the anatomy, palynology, morphology and embryology of the two families, Haloragaceae and Gunneraceae, clearly emphasized their differences (Peter 2010).

The six subgenera of the family Gunneraceae include; Misandra, Gunnera, Pseudogunnera, Panke, Ostenigunnera and Milligania. This traditional classification was based on the biogeography, size and floral pattern of the plants. Misandra consists of three species, which are dioecious, prostrate and stoloniferous herbs, and reside in South America. Pseudogunnera is comprised of stoloniferous and erect plants with panicles of proterandrous flowers and unisexual female flowers at the top and base, respectively. *G. macrophylla* is the only species of this subgenus. It is mainly found in the Philippines, Wallace's line of Java, some Melanesia volcanic islands, South-Eastern Asia and New Guinea. They are monoecious plants with unisexual flowers, occasionally bisexuals (Meijden 1975; Fuller and Hickey 2005; Peter 2010). The subgenus Panke has more than 19 species. It is the largest of all the six subgenera (Wanntorp and Wanntorp 2003; Fuller and Hickey 2005; Wanntorp and De Craene 2005; De Craene and Wanntorp 2006). The species are located on the island of Hawaii, Juan Fernandez Island and South America (Mora-Osejo, Pinto, and Ruiz 1984; Doyle 1990). The subgenus lacks stolons and produces large panicles of hermaphroditic, mostly unisexual flowers. The species have erect petioles supporting the large laminae. The subgenus Milligania contains six to 11 small, mat-forming species, which includes the stoloniferous and creeping herbs from New Zealand and one dioecious species from Tasmania (Schindler 1905; Cheeseman 1925; Fuller and Hickey 2005; Peter 2010). Ostenigunnera was included as a subgenus in the year 1933 by Mattfeld (1933). Recent studies on the morphological and molecular basis have shown all the six subgenera are systematically valid (Wanntorp and Wanntorp 2003).

The majority of *Gunnera* L. species mainly occur in the Southern Hemisphere (Wanntorp and Wanntorp 2003; Brookes and Dutton 2007; Mariotti et al. 2014). *Gunnera perpensa* is one of the many species of *Gunnera* L. and it resides only in Africa. The plant is known for its medicinal values in different countries, including South Africa. This review provides an overview of the traditional uses, phytochemistry and pharmacological properties of *G. perpensa*. Moreover, the review attempts to discuss literature related to the traditional uses, pharmacological properties, phytochemistry and biological activities of *G. perpensa* (Table 1).

Taxonomy and description

According to traditional morphological studies, *Gunnera* used to be under the family of Haloragaceae. This classification was based on its dimerous and epigynous flowers, endosperm rich seeds and the vegetative habitat of both *Gunnera* and Haloragaceae (Wanntorp and De Craene 2005; Peter 2010). However, a molecular study by Chase et al. (1993) falsified this systematic arrangement based on the results obtained from chloroplast and nuclear gene sequencing. The sequences evidently showed that *Gunnera* was not closely related to any other genera in the Haloragaceae family. Since then, *Gunnera* has been placed in the Order Gunnerales and its position as the main branch of eudicots could provide better evolutionary understanding of core eudicots (Wanntorp and De Craene 2005). The presence of separate vascular strands in the petioles and stems of *Gunnera* is a characteristic observed in only few groups of angiosperms. This feature is also an indication of the evolvement of *Gunnera* L. from its aquatic ancestors (Peter 2010) (Figure 1).

Table 1: Taxonomic hierarchy of *G. perpensa* L.

Kingdom:	Plantae
Subkingdom:	Viridiplantae
Infrakingdom:	Streptophyta
Superdivision:	Embryophyta
Division:	Tracheophyta
Subdivision:	Spermatophytina
Class:	Magnoliopsida
Superorder:	Myrothamnanae
Order:	Gunnerales
Family:	Gunneraceae
Genus:	Gunnera L.
Species:	*Gunnera perpensa* L.

G. perpensa is an erect, perennial herb that inhabits moderate to heavy rainfall areas, including marshy areas, and sideways of river banks and very limited high altitudes (Standley and Williams 1963; Jarzen 1980; Fuller and Hickey 2005; Peter 2010). This is in part because of its mutualistic relationship with nitrogen-fixing Cyanobacteria (Bergman, Johansson, and Soderback 1992; Fuller and Hickey 2005).

The plant is most commonly known as 'River pumpkin' because of its round, large pumpkin-like leaves. It has long, thick stalks supporting the leaves and arising from the rhizome. This species is unique among other species of the genus in its thick, tuberous and branching horizontal rhizomes (Wanntorp and Wanntorp 2003). The flowers are small, wind-pollinated, reddish to brown in colour and found in groups, with nearly a hundred thousand, appearing on a long, thick flowering stem (Jarzen 1980; Wanntorp and De Craene 2005). The plant is monoecious with unisexual to bisexual flowers (Jarzen 1980; Schindler 1905; De Craene and Wanntorp 2006). In South Africa, the plant is known by its local names: *Ugobho* (isiZulu), *Rambola-vhadzimu* (Venda), *Qobo* (Sotho), *Iphuzilomlambo* or *Uxobo* (isiXhosa), *Rivierpampoen* (Afrikaans) and River pumpkin (Filippich et al. 1991; McGaw et al. 2005; Brookes and Dutton 2007).

Geographical distribution

The geographic distribution of the genus *Gunnera* has been gradually reduced. According to Jarzen (1980), based on the paleo-geographic and stratigraphic occurrences during the Upper Cretaceous and Early Tertiary period, the genus *Gunnera* was more broadly distributed than it is today. This report points out that the decrease in rainfall and the seasonal temperature fluctuation increase might be the main reasons for the retreat of *Gunnera* from high latitudes. This is supported by the fact that the species are currently spread in environments of heavy rainfall (Peter 2010).

The species of *Gunnera* are currently distributed in different parts of the world, including New Zealand, Mexico, the Philippines, Indonesia, North America, Central and South America, Hawaii, Madagascar and eastern and southern Africa (Mendes 1978; Bergman, Johansson, and Soderback 1992; Wanntorp and Wanntorp 2003) (Figure 2).

Gunnera perpensa is an African species, which is the only species of *Gunnera* L. that is distributed in Africa. The plant is widely spread in tropical Africa from

Figure 1: *Gunnera perpensa*: (a) whole plant (b) rhizomes and roots (Mendes 1978) (c) flowers and leaves.

Figure 2: Present distribution (black) of *Gunnera* L. Names of subgenera written near their respective geographical occurrences (Wanntorp and Wanntorp 2003).

Sudan, Ethiopia, DRC (E.D.R. Congo), Burundi, Rwanda, Uganda, Kenya, Tanzania, Zimbabwe and Mozambique, extending to central and eastern areas of southern Africa down to the Western Cape, including Swaziland and Lesotho (Mendes 1978; McGaw et al. 2005; Peter 2010). In South Africa, it is found in four provinces; Free State, Eastern Cape, Western Cape and KwaZulu-Natal. The plant is not documented in Botswana or Namibia or in the Northern Cape Province of South Africa (Mendes 1978; Bergman, Johansson, and Soderback 1992) (Figure 3).

Review procedure

Information presented in this paper was collected from different sources found online. Electronic databases such as Science Direct, ISI Web of Science, Google Scholar, Scopus and MEDLINE were used to look for literature. The reviewed online sources include scientific studies published in articles, theses, books, book chapters, journals and abstracts. Potential literature sources were found by searching for the terms and/or phrases like taxonomic hierarchy, traditional medicine, folk medicine, folkloric uses, indigenous medicine, ethno-medicine, ethnobotany, economic uses, horticultural uses, cultural uses, phytochemistry, active compounds, biological activities and pharmacological properties of *G. perpensa* (Maroyi 2013).

Figure 3: Distribution map (green) of *G. perpensa* in southern Africa (Mendes 1978).

Ethnomedicinal uses

G. perpensa L. is a native South African plant and one of the most valuable medicinal plant species in southern Africa with its leaves, stems, rhizomes and roots widely used as traditional medicine. Inherently, it is used in local traditional medicine systems of the Sotho, Zulu and Xhosa cultural groups. *G. perpensa* is traditionally used to treat numerous disorders which are summarized in Table 2.

The leaves, stems, rhizomes and roots are used to treat various health problems including: venereal diseases, infertility, endometritis, urinary tract problems, impotence and cold, and is used as an insect repellant (Brookes and Dutton 2007). The majority of South African women use decoctions of the root for good infant development, female fertility, to relieve menstrual pains, to speed up labour and to accelerate expulsion of the placenta (Kaido et al. 1997; Van Wyk, van Oudtshoorn, and Gericke 1997; McGaw et al. 2005; Drewes et al. 2005; Brookes and Dutton 2007; Nkomo et al. 2010). Root and leaf extracts are used to dress wounds and treat psoriasis (Grierson and Afolayan 1999). Decoctions are also used in veterinary science to treat endometritis, to induce labour and to protect animals from tick and insect bites (Hutchings 1996; Brookes and Dutton 2007; Nkomo et al. 2010). Traditionally the powdered form of the dried root is boiled in water and given orally to an animal to expel the placenta after calving (Khan et al. 2004; Mariotti et al. 2014).

Phytochemistry

A wide variety of active natural compounds are responsible for the different characteristics of plants such as pigments (quinines and tannins), odours (terpenoids) and flavours (some terpenoids) (Showell et al. 2011). Despite its widespread use, there are limited studies on the chemical composition of *G. perpensa*. *G. perpensa* mainly contains tannins, steroids, cardiac glycosides and flavonoids (Simelane 2010). One of the different components which is already known in *G. perpensa* is the phenylpropanoid, Z-venusol (**1**). A phytochemical study by Khan et al. (2004) on the aqueous root extract of *G. perpensa*, revealed the presence of Z-venusol (**1**) as a major component. The prolonged exposure of Z-venusol (**1**) to methanol leads to a major compound Z-methyl lespedezate (**2**) mixed with **1**, as impurity. Other compounds identified from *G. perpensa* include, β-sitosterol (**3**), ellagic acid (**4**), 3,3′,4′-tri-*O*-methyl ellagic acid (**5**) and 3,3′,4′-tri-*O*-methyl ellagic acid 4-*O*-β-D-glucopyranoside (**6**), punicalagin (**7**), p-hydroxybenzaldehyde (**8**), (Figure 4) two benzoquinones (2-methyl-6-(3-methyl-2-butenyl)-benzo-1-4-quinone and 3-hydroxy-2-methyl-5-(3-methyl-2-butenyl) benzo-1,4-quinone), 6-hydroxy-8-methyl-2,2-dimethyl-2H-benzopyran, and phytol (Grierson and Afolayan 1999; Drewes et al. 2005; Brookes and Dutton 2007; Chavan et al. 2013; Peter 2010). In addition to the above-mentioned compounds, Peter (2010) reported the presence of sucrose and an unidentified sugar. The compound *trans*-phyt-2-enol was also obtained from the methanol extracts of the aerial parts of the plant. Phytol is a known skin irritant and most likely the reason for

Table 2: Ethnobotanical uses of *G. perpensa* in Zulu, Xhosa and Sotho cultural groups of South Africa.

Use Medicinal uses	Plant part(s) used and preparation	References
Urinary tract	Root decoction	Brookes and Dutton (2007)
Impotence	Root decoction	Brookes and Dutton (2007)
Barrenness	Root decoction	Brookes and Dutton (2007)
Induce labour	Root decoction	Kaido et al. (1997), Brookes and Dutton (2007), Khan et al. (2004)
Foetal development	Root decoction	Brookes and Dutton (2007), Kaido et al. (1997)
Expulsion of placenta	Root decoction	Brookes and Dutton (2007), Kaido et al. (1997)
Wound dressing	Root and Rhizome decoction	Brookes and Dutton (2007), Nkomo et al. (2010)
Colds	Root decoction	Brookes and Dutton (2007)
Endometritis	Plant Extracts	Brookes and Dutton (2007)
Psoriasis	Rhizome decoction	Nkomo et al. (2010)
Dysmenorrhoea	Plant decoction	Nkomo et al. (2010)
Rheu	Root decoction	Nkomo et al. (2010), Khan et al. (2004)
Female infertility	Root decoction	Nkomo et al. (2010), Khan et al. (2004)
Other uses		
Ethnoveterinary medicine	Root decoction is used to speed up labour	Brookes and Dutton (2007)
	Root decoction is used to protect animals from tick bites and other parasites	
	Plant Extracts is used to treat Endometritis	
	Root decoction to expel the retained placenta	Khan et al. (2004)

the recorded skin irritation nature of the plant (Drewes et al. 2005; Peter 2010).

Pharmacology

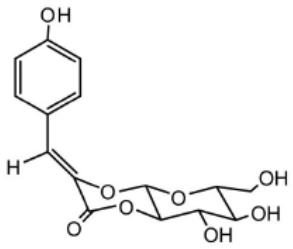
The crude extracts of *G. perpensa* have been widely studied for their analgesic, antimicrobial and anti-inflammatory properties. The antimicrobial activity of the leaves were found to be higher than that of the stems, followed by the roots (Hutchings 1996; Drewes et al. 2005). Grierson and Afolayan (1999) confirmed the traditional wound healing potential of the leaves using the information compiled through general conversations and questionnaires from the *Sangomas*, herbalists and rural inhabitants in the Eastern Cape Province. According to Steenkamp et al. (2004), *G. perpensa* has antioxidant properties and antibacterial properties, and contributes to wound healing through fibroblast growth. Buwa and Van Staden (2006) reported that the aqueous, ethanol and ethyl acetate extracts of *G. perpensa* root have antibacterial properties with highest inhibition by aqueous and ethanol extracts against the tested gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). A number of phenolic compounds have been isolated from the stem, root and rhizome of *G. perpensa*. Phenolic compounds can be held responsible for a wide variety of biological activities including antiseptic, antimutagenic, anticancer, antihaemorrhagic and antioxidative properties (Brookes and Dutton 2007). These properties could be helpful during pregnancy and birth, which confirms some of the reported health effects accredited to this species by traditional healers.

Z-venusol

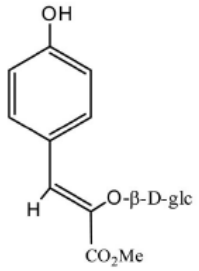
Drewes et al. (2005) studied the antibacterial, ileum muscle and uterus muscle contraction potentials of the isolated active compounds 1,4-benzoquinones, phenylpropanoid glucoside and Z-venusol (**1**)a. The results showed all the compounds have a role in the contraction of both ileum and uterus smooth muscles and also exhibited antibacterial properties (Mariotti et al. 2014). A report by Khan et al. (2004) showed that pure Z-venusol (**1**) does not have a direct effect on uterine smooth muscles contraction. However, it induced spontaneous contractility to some extent after the test substance was flushed from the organ bath. There is an assumption that the degree of activity of Z-venusol (**1**) may be moderated by the presence of other substances in the extract. Prolonged exposure of Z-venusol (**1**) to methanol results in the production of Z-methyl lespedezate (**2**). Z-methyl lespedezate (**2**) is common in woody and herbaceous monocotyledons (Harborne 1964). The potassium salt of lespedezic acid isolated from *Lespedeza cuneata* was proven to be biologically active and responsible for the leaf opening mechanism in night closing plants (Mensor et al. 2001).

Phenolic lactones

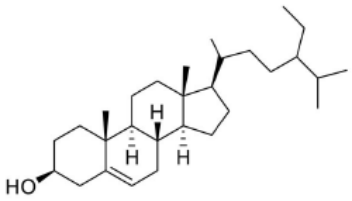
Phenolic compounds are known to have high anti-oxidant properties (Fauconneau et al. 1997; Djeridane et al. 2006). Among the many active substances from *G. perpensa*, the majority are phenolic lactones such as ellagic acid (**4**) and its derivatives (3,3′,4′-tri-*O*-methyl ellagic acid (**5**), 3,3′,4′-tri-*O*-methyl ellagic acid 4-*O*-β-D-glucopyranoside (**6**) and punicalagin (**7**)). Ellagic acid (**4**) is a strong antioxidant capable of scavenging superoxide and hydroxyl anions. It can also reduce the damaging effect of H_2O_2 inside the human body and, most importantly, it can be used as hepato-protective, chemo-protective and an anticancer agent (Khac et al. 1990; Girish and Pradhan 2008). It is also known for its anti-inflammatory and antimutagenic properties (Loarca-Piña et al. 1996; Sgariglia et al. 2013). The compound 3,3′,4′-tri-*O*-methyl ellagic acid (**5**) isolated from *Combretum kraussii* has shown antihaemorrhagic potentials (Khac et al. 1990). The other ellagic acid (**4**) derivative is punicalagin (**7**) which is the main compound



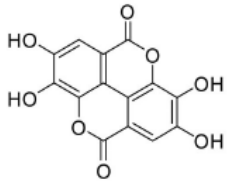
Z-venusol (**1**)



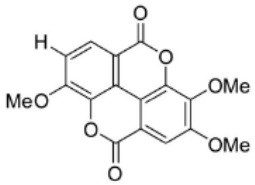
Z-methyl lespedezate (**2**)



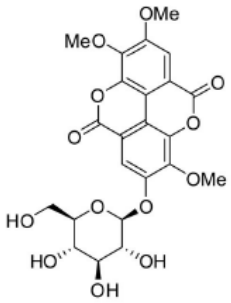
β-sitosterol (**3**)



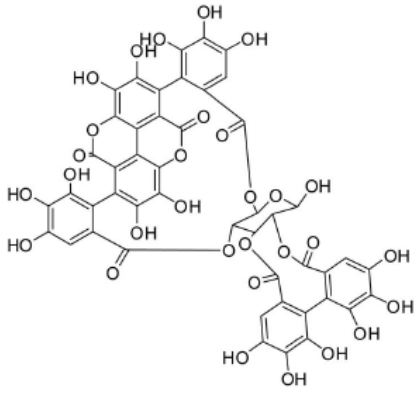
Ellagic acid (**4**)



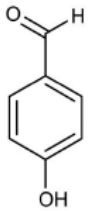
3,3',4'-tri-O-methyl ellagic acid (**5**)



3,3',4'-tri-O-methyl ellagic acid 4-O-β-D-glucopyranoside (**6**)



Punicalagin (**7**)



p-hydroxybenzaldehyde (**8**)

Figure 4: Phytochemical structures of metabolites isolated from *Gunnera perpensa*.

of ellagitannins and a well-known anti-oxidant and anticancer polyphenol (Gil et al. 2000; Cerdá et al. 2004; Adams et al. 2006; Djeridane et al. 2006; Ascacio-Valdés et al. 2011). It is one of the derivatives of ellagic acid (**4**) in which gallagic and ellagic acids (**4**) are connected to a glucose molecule. Therefore, it comprises one molecule of ellagic acid (**4**), one molecule of glucose and one molecule of gallagic acid. Due to its high anti-oxidant ability, it can be used in the treatment of cardiovascular and neurodegenerative diseases (Cerdá et al. 2004; Ascacio-Valdés et al. 2011). However, a study conducted by Filippich et al. (1991) showed the

hepatotoxic property of the compound, though such toxicity had not been reported by other researchers (Castonguay et al. 1997).

Quinones

Quinones with 1,4-benzoquinone subunit has been demonstrated with prominent pharmacological uses such as antimalarial, antibiotic, anticoagulant, antineoplastic, antitumor and herbicidal activity (Abraham et al. 2011). Drewes et al. (2005) was interested in screening the antibacterial potentials of isolated compounds from the aerial parts of *G. perpensa*. Among the compounds, the benzoquinone 2-methyl-6-(3-methyl-2-butenyl)-benzo-1,4-quinone showed effective antimicrobial activity against a range of microorganisms, including *Escherichia coli*, *Klebsiella pneumonia*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *staphylococcus aureus*, *Bacillus cereus*, *Cryptococcus neoformans* and *Candida albicans*, with the highest activity observed against *Staphylococcus epidermidis*. However, 3-hydroxy-2-methyl-5-(3-methyl-2-butenyl) benzo-1,4-quinone displayed no activity against all microorganisms tested. Another interesting report by Chavan et al. (2013), showed that 2-methyl-6-(3-methyl-2-butenyl)benzo-1,4-quinone, isolated from the Brazilian *G. perpensa* species, has anticancer properties.

β-sitosterol

β-sitosterol (**3**) is the most common phytosterol with anticancer properties, especially for breast, prostate and colon cancers, including tumour growth inhibition and inducing apoptosis. Many studies have described the biological properties of β-sitosterol (**3**) as anti-inflammatory, chemopreventive and immune-modulating activities (Park et al. 2007). It inhibits the hyperproliferation of colonic mucosa, which is the risk factor in the development of colon cancer. It has also inhibited enzymes responsible for testosterone metabolism in normal rat tissues that reduced the development of prostate cancer. Furthermore, it provides protection against cardiovascular diseases through its hypocholesterolemic action (Awad et al. 2000). Assmann et al. (2006) reported that high concentrations of β-sitosterol (**3**) in blood increases the severity of heart disease in men that have previously experienced heart attacks.

Conclusions

G. perpensa is a multi-purpose South African ethnomedicinal plant. The popular utilization of *G. perpensa* by many South African cultural groups has diminished the wild stock of the plant. Therefore, there is a need for cultivation and conservation of the plant by the general public and the government to meet the high local demand. The diverse chemical constituents found in *G. perpensa* are further motivations to carry out more pharmaceutical exploration of the plant. Additionally, efforts must be made to standardize and formulate phytomedicines from *G. perpensa* to compete with and substitute currently used synthetic drugs.

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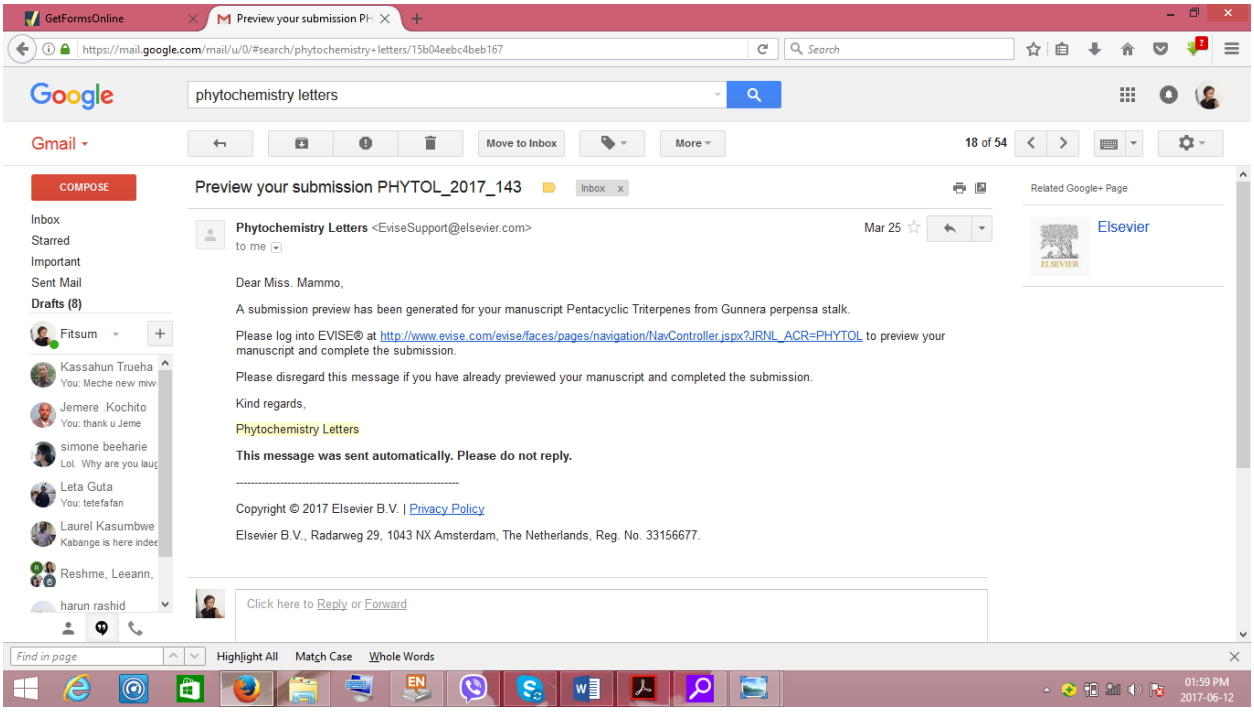
APPENDIX III: SUBMISSION



AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Dr Viresh Mohanlall**, **Prof. Francis O. Shode** and **Prof. Bharti Odhav**.

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Student's signature