

CHAPTER EIGHT

Motility modulation potential of *Bauhinia galpinii* and *Combretum vendae* phenolic-enriched leaf extracts on isolated rat ileum

8.0. Introduction

Gastrointestinal tract (GIT) used the smooth muscle of the mucosal lining enriched with an enteric neural network to regulate propulsive transport and mixing of food material directionally through the digestive systems (Wood, 2004). The neural network initiates and coordinates secretion and absorption across the intestinal lumen as well (Bohn and Raehal, 2006). The enteric neurons function independent of the central nervous system (CNS), therefore referred to as enteric nervous system (ENS). Enteric nervous system controls the motility and contractility of the GIT as its rate and intensity of contraction regulates the absorption of fluid, and expulsion of solid material. Therefore ENS exhibit significant role in GIT disorders such as diarrhoea and constipation through these means. Neurotransmitters such as acetylcholine (ACh), serotonin (5-hydroxytryptamine (5-HT)), substance P, histamine and opioids are the important chemical mediators in contractile regulatory actions of ENS (Farthing, 2002). The activities of the neurotransmitters in the intestine are coordinated by a large number of receptors and sub-receptors. Some of the receptors have been proved to play essential roles in GIT disorders such as peristaltic colonic motility, diarrhoeal and constipation diseases.

Some of the diarrhoea aetiologies such as infectious pathogens or their toxins, inflammatory mediators and oxidation by-products targets to control the peristaltic colonic movement by manipulating the ENS, and also control fluid and electrolyte movement across the intestinal mucosa (Guttman and Finlay, 2008). The modulations in the quantity of the neurotransmitters or the activity of the receptors can have enormous effects on intestinal motility and contraction. The process may help in regulating absorption or secretion of fluid and electrolyte by the intestine; hence provide relief against GIT disorders including diarrhoea and constipation diseases (Sikander *et al.*, 2009).

Enteric nervous system presents an attractive potential target for pharmacological intervention in diarrhoea. The use of agonists and antagonist that target these ENS hormone receptors are routinely used clinically to modulate intestinal motility, absorption and secretion. Antispasmodic or antimotility (atropine, clonidine and deodorized tincture opium), and antisecretory agents (racecadotril, octreotide) are used to treat or prevent smooth muscle contraction and control intestinal secretion, thus alleviating many symptoms of GIT disorder including diarrhoea. However, prolonged uses of these drugs are often associated with some side effects such as dry mouth and urinary retention for antimuscarinic drugs, headache, nausea, vomiting and constipation for calcium blockers. Several medicinal plants are used by different traditional cultures across the world in alleviating GIT disorders clinically manifesting as diarrhoea without reported cases of adverse effects. These provide the rationale in continuous search for safer and efficient drugs from plant phytochemicals that might target a specific receptor.

In South Africa and other developing countries treatment of gastrointestinal disorders such as diarrhoea with medicinal plants are particularly common in rural areas. The antidiarrhoea activities of medicinal plant extracts can be exhibited through spasmolytic effects (intestinal smooth muscle relaxation), delay gastrointestinal transit, suppress gut motility, stimulate water absorption or reduce electrolyte secretion. In contrast, the mechanism of actions of medicinal plants used in constipation include spasmogenic effects (intestinal smooth muscle contraction), rapid gastrointestinal transit, activated gut motility, suppressed water absorption or increase electrolyte secretion (Gilani *et al*, 2005a). All these effects are related to the regulation of ENS motility and contractility. However, scientific evaluations of the therapeutic claims as well as mechanisms of action are still unreported for many of the antidiarrhoeal plants used in traditional medicine. The aim of this study therefore is to evaluate motility regulatory potentials and determine possible mechanism of action of phenolic-enriched leaf of *Bauhinia galpinii* and *Combretum vendae* as antidiarrhoea medicinal plants on isolated rat ileum.

8.1. Drugs and reagents

Acetylcholine hydrochloride (Ach), serotonin (5-HT), nicotine, Histamine, Prostaglandin E₂ (PGE₂), Prostaglandin F_{2α} (PGF_{2α}), N^G-nitro-L-arginine methyl ester (L-NAME), Carbachol, Pilocarpine, Cyclopiazonic acid, Dimethylsulphoxide (DMSO), Sodium chloride (NaCl), Potassium chloride (KCl), Calcium chloride (CaCl₂), Sodium bicarbonate (NaHCO₃), Magnesium sulphate (MgSO₄), Potassium hydrogen phosphate (KH₂PO₄), Glucose, and carbogen

8.2. Animals

Male Wistar rats (250-300 g) obtained from University of Pretoria Biological Research Centre (UPBRC), Faculty of Veterinary Science, Onderstepoort, Pretoria were used. All animals were housed under standard environmental conditions and provided with food and water ad libitum. All the procedures were in accordance with the guidelines for use of experimental animals established by the Animal Use and Care Committee (AUCC), University of Pretoria based on specification in the South African National Standard (SAN 10386-2008). The approval of ethical committee at Faculty of Veterinary Science, University of Pretoria was obtained before the start of the work. The project was also approved by Faculty of Veterinary Science, University of Pretoria research committee (UP-RESCOM) with approval number of V027-10.

8.2.1. Isolated ileum preparation

The animal was humanely sacrificed with inhalation of isoflurane and dissected immediately. The ileum was removed and placed in carbogenated (95% O₂ and 5% CO₂) Krebs solution with the following composition (g/l): NaCl, 6.94; KCl, 0.354; KH₂PO₄, 0.163; NaHCO₃, 2.1; MgSO₄, 0.370; CaCl₂, 0.367; glucose, 2.07 and pH 7.4.

The intestinal content was removed by washing with Krebs's solution and the mesenteric constituents were eliminated. Longitudinal segments (1.5–2.0 cm) obtained from the distal ileum were placed in a 50 mL thermostatically controlled (37°C) organ bath containing Krebs solution gassed with carbogen. The preparations were connected to an isotonic transducer (load 0.5 g) in such a way as to record contractions mainly from the longitudinal axis and allowed to equilibrate for 60 min before the start of experiment: contractions were recorded using Bioscience transducers.

8.3. Contractility test

8.3.1. Spasmogenic assays

The crude extracts was prepared in stock solution of 20 mg/ml in DMSO and cumulatively added to the organ bath from concentration of 10, 25, 50, 100, 250, 500, 750 and 1000 µg/ml. The effective concentration of DMSO in the waterbath was less than 5% in all the experiments. Effect of the extracts on spontaneous motility of the ileal preparations were monitored at 20 min contact time for each concentration and cumulative dose-dependent curves for the extracts were determined to measure stimulatory effects.

8.3.2. Spasmolytic assays

8.3.2.1. Effects on acetylcholine-induced contraction

Acetylcholine hydrogen chloride was added to the organ bath cumulatively in the absence of test extracts at concentration ranging between 0.01-1.00 µg/ml in water. The process was repeated with addition of ACH (0.01-1.00 µg/ml) after 20 min pre-incubation of the isolated ileum with the extracts (10, 25, 50, 100, 250, 500, 750 and 1000 µg/ml).

8.3.2.2. Effects on Serotonin-induced contraction

Serotonin was added to the organ bath cumulatively in the absence of test extracts at concentration ranging between 0.001 - 0.1 µg/ml. The process was repeated with addition of 5-HT after 20 min pre-incubation of the isolated ileum with the extracts (10, 25, 50, 100, 250, 500, 750 and 1000 µg/ml).

8.3.2.3. Effects on K-induced contraction

The isolated ileum preparation was washed with K⁺ free Krebs's solution (composition (g/l): NaCl, 6.94; KCl, 0.354; KH₂PO₄, 0.163; NaHCO₃, 2.1; MgSO₄, 0.370; CaCl₂, 0.367; glucose, 2.07 and pH 7.4) for 20 min after equilibration and incubated with the extracts for 20 min. Thereafter, KCl solution (100 µl) was added cumulatively.

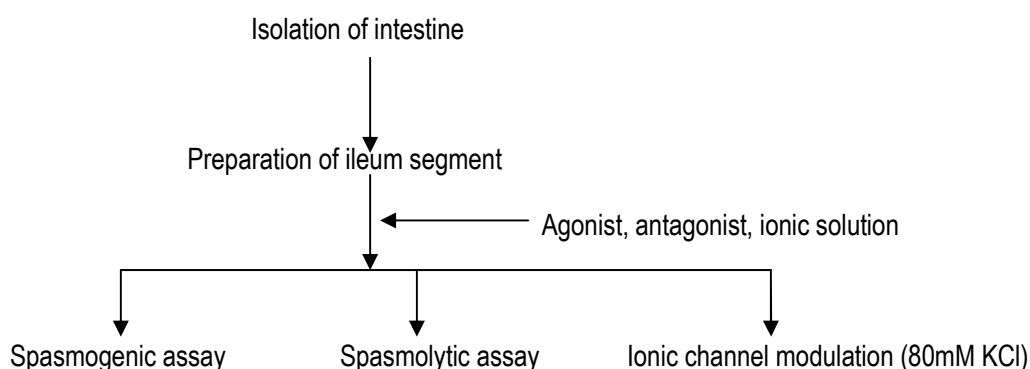


Fig. 8.1. Schematic presentation of the contractility assay

8.4 Data analysis

The inhibition of ileum contraction by test sample was normalized and expressed as a percentage of mean \pm SEM from 3-4 experiments of the references responses induced by acetylcholine (10 μ g/ml), other spasmogens, receptor agonists and antagonists using the following formula:

$$\% \text{ Inhibition} = [A_C - A_T/A_C] \times 100$$

Where A_C is the amplitude (cm) of the ileum contraction induced by the agonists and antagonists in the absence of the test sample; A_T is the amplitude (cm) of the ileum contraction by the agonists and antagonists in the presence of the test sample. The changes in EC_{50} will be used to compare the effect of the extracts using an ANOVA.

8.5. Results.

8.5.1. Effect of *B. galpinii* crude extract on isolated rat ileum

The 70% acetone extract which should have high concentration of phenolics of *B. galpinii* (10 - 1000 μ g/ml) stimulate spontaneous contraction of the rat ileum as shown in Fig 8.2 with EC_{50} value of 27.85 μ g/ml. Maximum contraction (E_{max}) of 44 mm was obtained at 200 μ g/ml and additional doses causes suboptimal response but increase duration of response caused an irreversible spasm at the maximum dosage of 1000 μ g/ml. Repeated administration of the extract at maximum dosage (1000 μ g/ml) caused exhaustion of the ileum.

Effects of the extract on acetylcholine, serotonin, K^+ induced contractions and acetylcholine in the presence of atropine (acetylcholine non-specific muscarinic receptors antagonist) indicated dual mechanisms of being an agonist (prokinetic) and an antagonist (relaxant) agent. The extract also exhibited additive contractility activity with acetylcholine and agonistic tendency to serotonin-induced contraction of the isolated rat ileum (Fig 8.3 and 8.4).

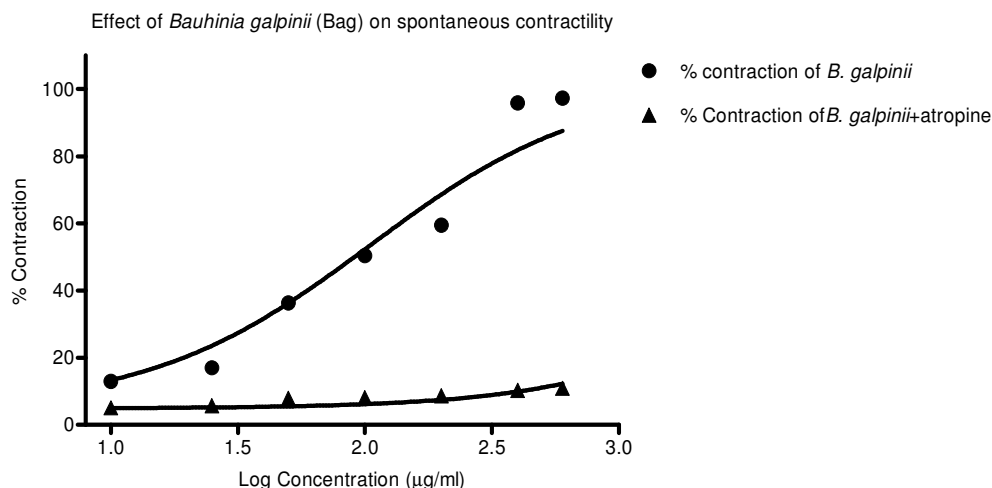


Fig.8.2: Stimulatory effect of 70% acetone leaf extract of *B. galpinii* on spontaneous contractility of isolated rat ileum and the antagonised effect of atropine.

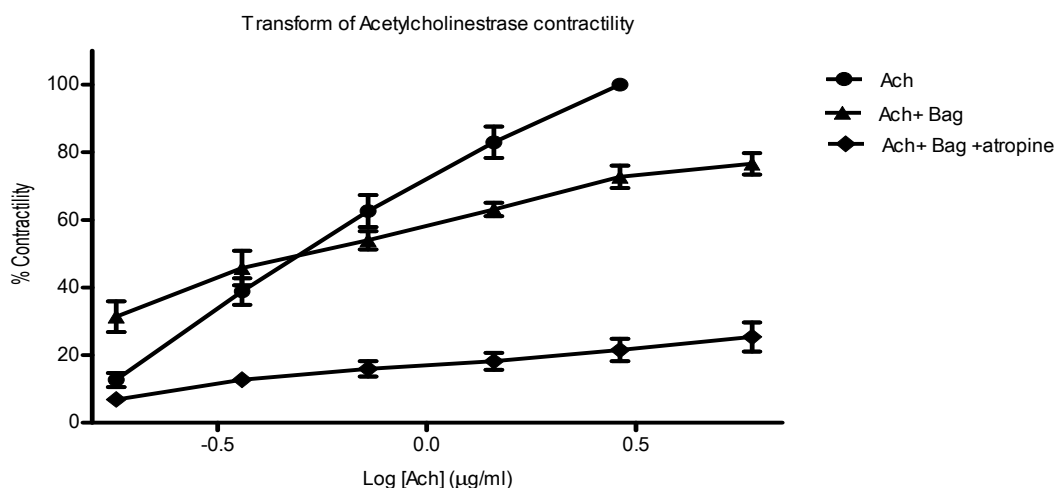


Fig. 8.3. Effect of 70% acetone leaf extract of *B. galpinii* (200 µg/ml) on the acetylcholine cumulative concentration-effect curves in the presence and absence of atropine

From the concentration-response curve (CRC) for acetylcholine-induced contraction, the EC_{50} value in the absence of *B. galpinii* was 0.033 µg/ml and the EC_{50} in the presence of *B. galpinii* was 0.049 µg/ml. The stimulation of spontaneous contraction and agonistic effects on acetylcholine-induced contraction were partially abolished by atropine (Fig 8.3). In the CRC for serotonin-induced contraction, the EC_{50} value in the absence of *B. galpinii* was 0.0025 µg/ml and the EC_{50} in the presence of *B. galpinii* was 0.0014 µg/ml. In contrast, the *B. galpinii* extract resulted in a concentration-dependent spasmolytic effect (antagonist) on K^+ -induced contraction of the isolated rat ileum (Fig. 8.5) with maximum effect (E_{max}) of 40.66 ± 5.13 mm at concentration of 200 µg/ml.

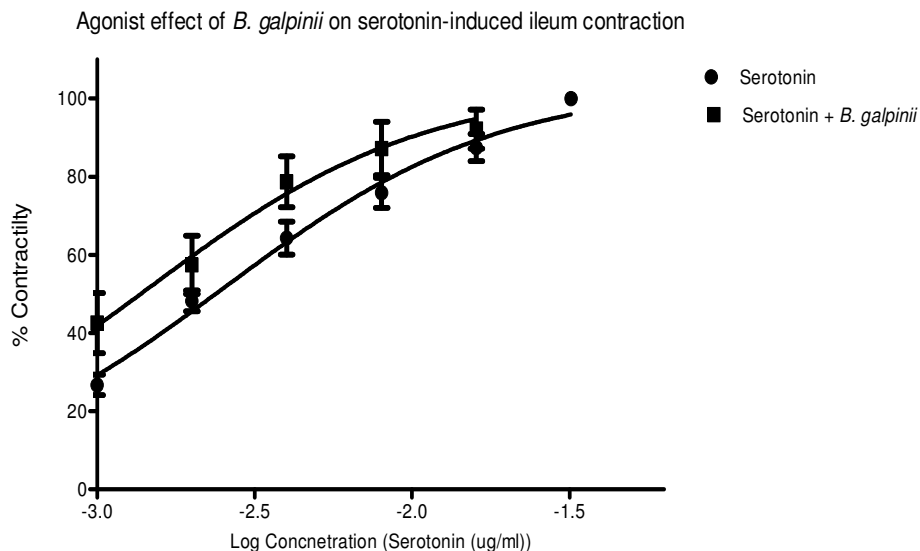


Fig. 8.4. Agonised effect of 70% acetone leaf extract of *B. galpinii* (200 $\mu\text{g/ml}$) on serotonin induced-contraction on rat isolated ileum.

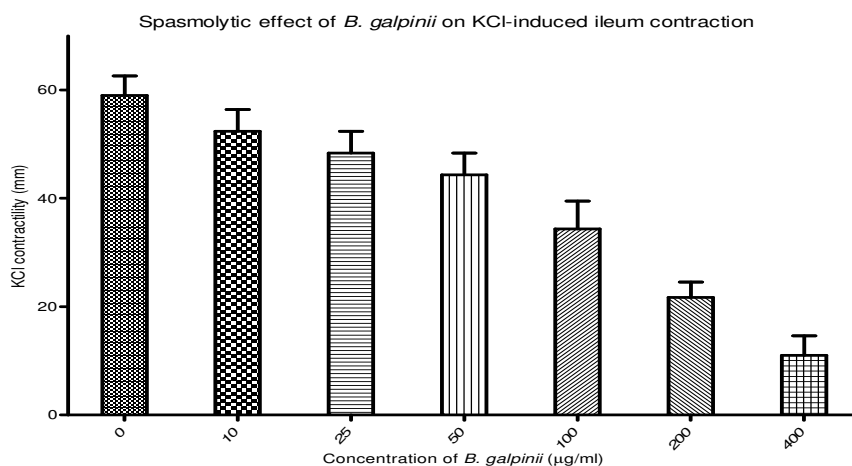


Fig. 8.5. Relaxant effect of 70% acetone leaf extract of *B. galpinii* on KCl induced contractility of isolated rat ileum

8.5.2. Effect of *C. vendae* crude extract on isolated rat ileum

The phenolic-enriched extract leaf extract of *C. vendae* do not stimulate spontaneous contraction (spasmogenic) of the isolated rat ileum, we therefore conclude that the extract have spasmolytic potential. The crude extract of *C. vendae* exhibited concentration-dependent spasmolytic effect on acetylcholine-induced contraction with EC_{50} values of 0.037, 0.027, 0.117, 0.365, and 0.396 $\mu\text{g/ml}$ at the concentration of 0, 100, 200, 400, and 600 $\mu\text{g/ml}$ of *C. vendae* in the organ bath (Fig 8.6) and concentration-dependent spasmolytic effect on serotonin-induced contraction of isolated rat ileum with EC_{50} value of 0.0017, 0.0044 and 0.012 $\mu\text{g/ml}$ at the concentration of 0, 100, 200 $\mu\text{g/ml}$ of *C. vendae* in the organ bath respectively (Fig 8.7). Equivalent volume of the solvent (5% DMSO) used in dissolving the extract had no effect on the spontaneous contraction or on 5-HT-induced contraction.

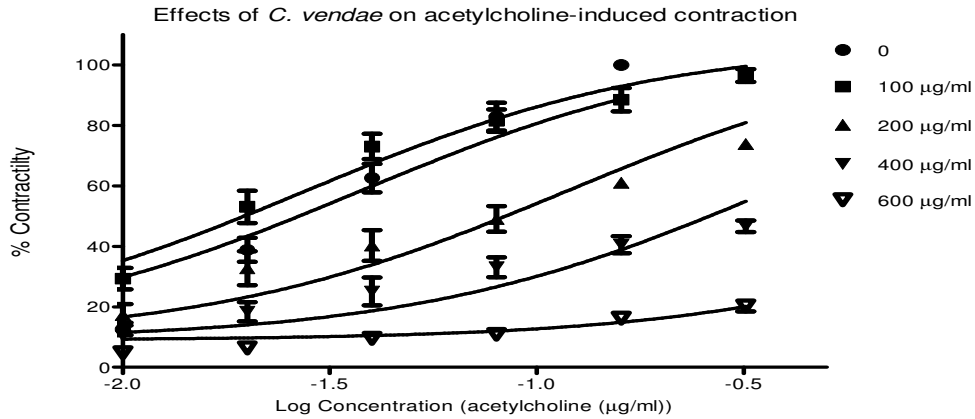


Fig.8.6. Spasmolytic effect of 70% acetone leaf extract of *C. vendae* on Ach-induced contractility of isolated rat ileum

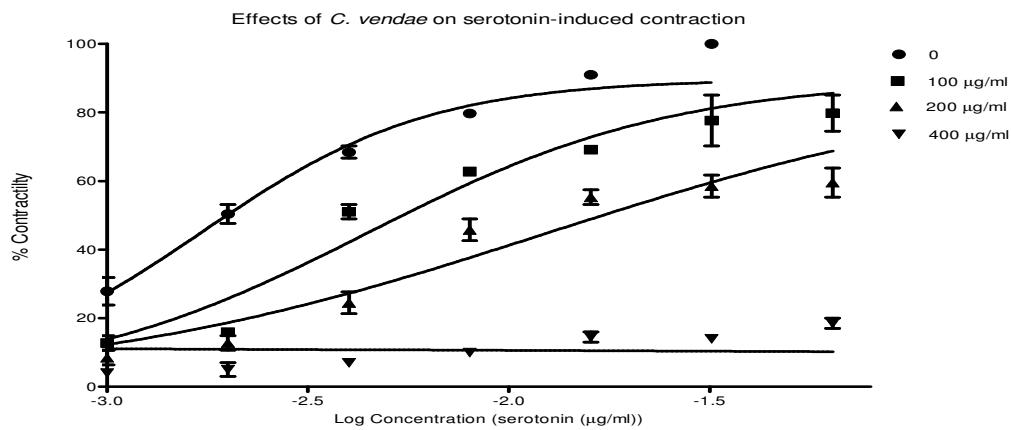


Fig.8.7. Relaxant effect of 70% acetone leaf extract of *C. vendae* on 5-HT-induced contractility of isolated rat ileum

Addition of depolarised KCl solution (80mM) caused sustained contractions which were inhibited by *C. vendae* phenolic enriched leaf extracts in concentration-dependent response (Fig. 8.8). Therefore, agent that inhibits contraction induced by depolarised KCl solution is considered to be a calcium channel blocker (Godfraind et al., 1986). The spasmolytic effects were reversible and the spontaneous contraction returned to normal after washing three times with kerbs' solution.

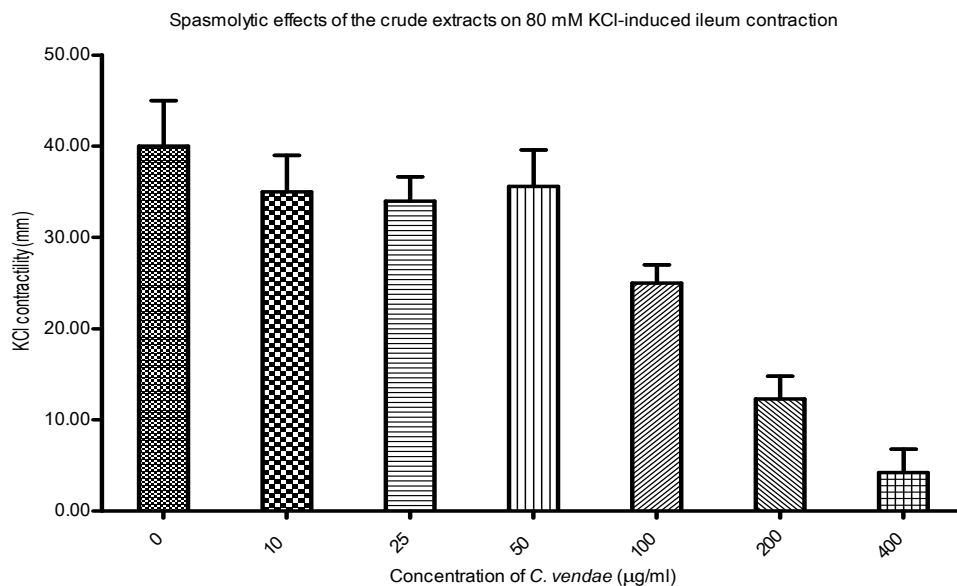


Fig. 8.8. Spasmolytic effect of the *C. vendae* on the depolarised KCl-induced isolated rat ileum contractions

8.6. Discussion

Gastrointestinal motor tone is modulated through multiple physiological mediators which include neurotransmitters, inflammatory mediators and oxidative metabolites (Hoogerwerf and Pasricha, 2006). The release of these chemical modulators in GIT causes stimulatory effect mediated through an ultimate increase in cytosolic Ca^{2+} (Burks, 1987). Drug substances with ability to block or alter any of the above pathways or with non-receptor specific inhibitory action such as Ca^{2+} antagonists could be considered to be effective as therapeutic agent in hyperactive or hypoactive GIT disorders. These are important in control or alleviating diseases such as diarrhoea, constipation, emesis and dyspepsia. To study the pharmacology and possible mechanism of smooth muscle excitatory or inhibitory effect of drugs and medicinal plant extracts isolated tissue preparations of laboratory animal are usually used for *in vitro* assays.

Acetylcholine (ACh) is a neurotransmitter released by the parasympathetic nervous system mediating its action in the GIT by stimulation of nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). In the GIT, five subtypes of the muscarinic receptors, namely M_1 , M_2 , M_3 , M_4 and M_5 have been identified (Tobin *et al.* 2009). However, M_2 and M_3 receptors play some essential roles in the smooth muscle contraction/relaxation of GIT (Matsui *et al.*, 2002; Takeuchi *et al.*, 2005; Unno *et al.*, 2005). Through this mechanism, acetylcholine plays a critical physiological role in regulating the peristaltic movements of the GIT (Brown and Taylor, 1996). The possible mechanisms responsible for contractility mediating action of drugs including medicinal plant extracts may include one or combinations of:

- Stimulation/inhibition of ACh release from the cholinergic nerve endings.
- Stimulation/inhibition of acetylcholinesterase (AChE) enzyme at the neuro-effector junction.

- Direct activation/inactivation of the muscarinic receptors of all smooth muscles, including those of GIT.

The effects of serotonin in the ENS are complex and diverse including modulation of smooth muscle function (promoting both contraction and relaxation), potent intestinal secretagogue (predominantly pro-secretory) and responses to visceral pain. Serotonin (5-HT) receptors found within the ENS and motor neurones of the GIT include 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆ and 5-HT₇. However, only 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄ and 5-HT₇ receptor subtypes are known to affect GIT motor functionality.

The 5-HT₃ and 5-HT₄ receptors are the most studied subtypes with the regards to physiological function and histological distribution in GIT (Chetty *et al.*, 2006; Cellek *et al.*, 2006). The 5-HT₃ receptor induces a rapid depolarization of the myenteric neuron through enhancing ACh release (Kim, 2009), while 5-HT₄ receptor expressed in the nerve terminal facilitates the releases of neurotransmitters including ACh, substance P and vasoactive intestinal peptides (Kim, 2009; Wouters *et al.* 2007). These cellular events of 5-HT lead to an up-stream regulation enhancing the excitatory activity of GIT smooth muscles through mediating the ACh release. Serotonin is involved in cholera toxin-and bile salt-induced fluid and electrolyte secretion by activating the ENS.

Contractions of all smooth muscles, including those of GIT depend on the presence of Ca²⁺. Increase and decrease in intracellular free Ca²⁺ are the principal mechanisms that initiate contraction and relaxation respectively in smooth muscle (Sanders, 2001). Agonists-induced contractions are related to the release of intracellular Ca²⁺ from sarcoplasmic stores and extracellular Ca²⁺ influx through L-type channels (Makhlouf, 1994). Therefore smooth muscle relaxations can be effected by antispasmodic drugs through the inhibition of Ca²⁺ entry or release into the cells. Exposure of smooth muscle cells to high concentration of K⁺ (>30 mM) stimulate contractions through opening of voltage-dependent L-type Ca²⁺ channels and influx of extracellular Ca²⁺ (Bolton, 1979; Godfraind *et al.*, 1986).

The results obtained in this work indicated that phenolic-enriched crude leaf extract of *B. galpinii* contracted the rat ileum dose-dependently and its initial contractile phase was partially blocked by atropine, a naturally occurring alkaloid and a well known non-selective muscarinic receptor antagonists. Atropine competes with ACh and other muscarinic agonists for a common binding site on the muscarinic receptor. This result shows the involvement of cholinergic muscarinic receptors alongside with other stimulatory receptors exhibiting initial contraction by *B. galpinii* on isolated rat ileum.

The phenolic-enriched crude leaf extract of *B. galpinii* also exhibited dose-dependent stimulating activity on serotonin-induced contraction of isolated rat ileum. The spasmogenic effects of the extract on ileum longitudinal muscle may be direct serotogenic activation of 5-HT receptor pathways or through the enhanced release of other neurotransmitters without serotogenic potential.

Addition of KCl (80 mM) caused sustained contractions which were inhibited by *B. galpinii*. Therefore the inhibitory effect of the crude extract of *B. galpinii* against K⁺-induced contractions can be as result of the blockade

of Ca²⁺ channels. Thus it can be concluded that *B. galpinii* has a dual-mechanism of action (prokinetic and relaxant) on gastro-intestinal motility, depending on the prevalent patho-physiological condition. The *B. galpinii* 70% acetone leaf extract can therefore be clinically relevant as therapeutic agent in diarrhoea and constipation which are both diseases with aetiology based on motility disturbances to a large extent.

Fumaria indica crude extract also has dual-spasmogenic and spasmolytic effects on isolated organs (Gilani *et al.*, 2005a). The aqueous-ethanolic extract (80% ethanol) of the aerial parts of *Hibiscus rosasinensis* Linn (Malvaceae) contains spasmogenic and spasmolytic constituents mediating their effect through cholinergic receptors activation and blockade of Ca²⁺ influx, respectively (Gilani *et al.*, 2005b). Crude aqueous leaf extracts of *Morinda morindoides* (Baker) Milne-Redh (Rubiaceae) agonise spontaneous contractility of isolated rat ileum (Cimanga *et al.*, 2010). The petroleum ether soluble fraction and the crude saponin constituents of the extract are responsible for the spasmogenic activities. The spasmogenic and spasmolytic effect of a particular medicinal plant extract on the isolated ileum depends on predominant phytochemical constituents. Phenolic compounds exhibit spasmolytic activity while saponins are responsible for the spasmogenic activities of many plant extract preparation. From the phytochemical analysis of the extract of *B. galpinii*, the extract contains high content of phenolics. However, the result obtained in this study indicated that the crude extract of *B. galpinii* also contains other active ingredients with spasmodic effect higher than the anticholinergic effect of the phenolics.

C. vendae extract did not stimulate spontaneous contractility of the rat ileum. Further investigation of its effects on ACh-induced contraction led to a concentration-dependent inhibitory activity against ACh contraction of the rat ileum. Anti-tractility effects of *C. vendae* against ACh-induced contraction are similar to atropine indicating that the extract may be acting via nAChR or mAChR.

Addition of KCl (80 mM) caused sustained contractions which were inhibited by both *B. galpinii* and *C. vendae* phenolic enriched leaf extracts in concentration-dependent response. Agents that inhibit contraction induced by KCl are considered to be a calcium channel blocker (Godfraind *et al.*, 1986). The spasmolytic effects were reversible and the spontaneous contraction returned to normal after washing three times with Ca²⁺ free-krebs solution.

The results indicate that *C. vendae* extract is capable of mediating spasmolytic effects on isolated rat ileum through multiple inhibitions of a wide range of contractile stimuli, such as neurotransmitters (acetylcholine and serotonin) and high potassium (depolarizing stimulus). This suggests that the ileum relaxant effects of the extract are not specific to a type of receptor but rather due to either general receptor inactivation or membrane depolarization. Muscarinic receptor antagonists, 5-HT receptor antagonist and Calcium channel blockers of the L-type are known to be effective as antispasmodic, anti-motility and antidiarrhoeal agents (Lee *et al.*, 1997; Brown and Taylor, 2006; Pasricha, 2006). Hence, the presence of multiple acting spasmolytic activities in the plant extract might be contributing towards its effectiveness in diarrhoea and abdominal spasm. The isolated triterpenoids such as ursolic acid, maslinic acid, corosolic acid, asiatic acid and arjunolic acid from the plant also

have good antimicrobial activity and the stilbenoid glycosides such as combretastatin B5-O-2'- β -D-glucopyranoside and combretastatin B1-O-2'- β -D-glucopyranoside has good antioxidant activity. Such activities of the plant could account for additional benefits providing a wider cover for its use in diarrhoea of different aetiologies. This is also in accordance with the general understanding that plants contain multiple active constituents with effect enhancing activities ([Gilani and Rahman, 2005](#)).

8.7. Conclusion

The result indicated the *B. galpinii* have dual activities with the capacity of acting as prokinetic and spasmolytic agent while *C. vendae* acts as spasmolytes against the three spasmogens used to induce contraction of the ileum. Further studies aiming to identify the targeted receptor subtype and the type of interaction with muscarinic receptors as well as the identification of the main active principle are needed.

The results indicate that there is a scientific rationale for using extracts of these plant species to treat diarrhoea in humans or animals. In the next section some of the antimicrobial and anti-oxidant compounds present in these extracts will be isolated and characterized.

CHAPTER NINE

Isolation and characterization of antimicrobial and antioxidant compounds from *Bauhinia galpinii* and *Combretum vendae*

9.0. Introduction

One of the cardinal objectives in medicinal plant research and development is identification, isolation and characterization of the bioactive components present in an extract. Medicinal plant extracts are inherently complex mixture of diverse chemical components. Separation of the active components of plant phytochemicals from the inactive components are categorized into three parts: extraction, purification and chromatography. Extraction and purification involved sample preparations schematically represented in Fig. 9.1 and Fig. 9.2. Various chromatographic methods are available for qualitative and quantitative (TLC fingerprint, high performance liquid chromatography (HPLC) fingerprints) as well as for isolations (open column chromatography (OCC), vacuum liquid chromatography (VLC), HPLC, high-speed counter-current chromatography (HSCCC), gas-liquid chromatography (GLC) and/or gel permeation chromatography (GPC)). The principles of separation are based on molecular size, adsorption to the stationary phase, polarity and solubility in the mobile phase.

Structural information on isolated compounds are usually obtained from different spectroscopic techniques namely: nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS), and to a lesser extent infrared spectroscopy (IR) and ultraviolet-visible spectroscopy (UV-visible). The characteristic features of each NMR experiments are summarized in Table 9.1.

Table 9.1 NMR experiments commonly applied for natural product structural elucidation (Simpson *et al.*, 2011)

NMR experiment	Information/interpretation
Proton NMR (1D ^1H NMR)	Quantitative overview of the distribution of protons in a sample.
Carbon-13 NMR (1D ^{13}C NMR)	Can provide a quantitative overview of the carbon distribution.
Distortionless enhancement through polarization transfer (DEPT) (1D ^{13}C NMR)	Separate the carbon of a compound into primary (CH_3), secondary (CH_2), Tertiary (CH) and quaternary (C) spectra.
^1H - ^1H Correlation spectroscopy (COSY) (2D ^1H NMR)	Connectivity information of protons on adjacent carbons. Cross-peaks connect the chemical shifts of protons that are coupled. Symmetrical cross peaks appear around a central diagonal.
^1H - ^{13}C Heteronuclear single quantum correlation (HSQC) and heteronuclear multiple quantum correlation (HMQC) (2D ^1H - ^{13}C NMR)	^1H - ^{13}C 1 bond correlation. Cross peaks represent carbon chemical shifts in one dimension and proton chemical shifts in the other dimension.
^1H - ^{13}C Heteronuclear bond multiple correlation (HMBC) (2D ^1H - ^{13}C NMR)	^1H - ^{13}C 2–4 bond correlations. Quaternary carbons are observed. Connectivity information is read as vertical lines.

9.1.1. Column chromatography

Isolation of such compounds is usually carried out by open column chromatography under gravitation force using silica gel, sephadex, polyamides or reverse phase (RP) mode on C8 or C18-bonded silica gel stationary phase. The separation of individual compounds from the complex extract mixture is based on the compound characteristic ability for the stationary phase in the column relative to the polarity of the mobile phase. Changing the polarity (gradient elution) of the mobile phase will allowed all target compounds to elute in a sequential manner. The chromatographic process should be rapid, do not lead to decomposition of compounds, material loss, or formation of artefacts.

Open column chromatography is simple, cheap and universally practiced despite some obvious disadvantage of method being slow and often produces irreversible adsorption of sample onto the stationary phase. The method is also encumbered with large sample and solvent requirement. The bioactive compounds of interests in this project are non volatile.

9.1.2. Mass spectrometry

Mass spectrometry (MS) is an important physico-chemical tool applied for structural elucidation of compounds from natural products including medicinal plants. The fundamental principle of MS is the use of different physical means for sample ionization and separation of the ions generated based on their mass (m) to charge (z) ratio (m/z) (Rijke *et al.*, 2006). The ionization techniques available include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), electron ionization (EI), chemical ionization (CI), fast atom bombardment (FAB), and matrix-assisted laser desorption ionization (MALDI) (Rijke *et al.*, 2006). Mass spectrometry has high sensitivity with detection limit of femtogram compared to NMR with sensitivity limit of nanogram range and above (Simpson *et al.*, 2011). The high sensitivity and the flexibility for hyphenation with other chromatographic technique made MS a versatile analytical instrument.

9.2. Materials and Methods

9.2.1. Preparation of plant extracts

The extraction and fractionation protocol was followed as described in Chapter 3. The schematic diagrams of the extraction, fractionation and isolation processes for *Combretum vendae* and *Bauhinia galpinii* are presented in Fig 9.1 and 9.2.

9.2.2. Bioautography

The bioautography against bacteria (*E. coli* and *S. aureus*) protocol were followed as described in chapter 4 while the TLC-DPPH antioxidant assay were carried out as described in chapter 5.

9.2.3. Isolation of bioactive terpenoids from *Combretum vendae*

The n-Hexane and ethyl acetate fractions showed one and two clear zone(s) of microbial growth inhibition respectively. The two fractions were subjected to gravitational column chromatography on silica gel

(2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of hexane: ethyl acetate starting with 100% n-Hexane, 99: 1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7 and finally 90:10 as mobile phase. Schematic representation of the isolation procedure is presented in Fig 9.1.

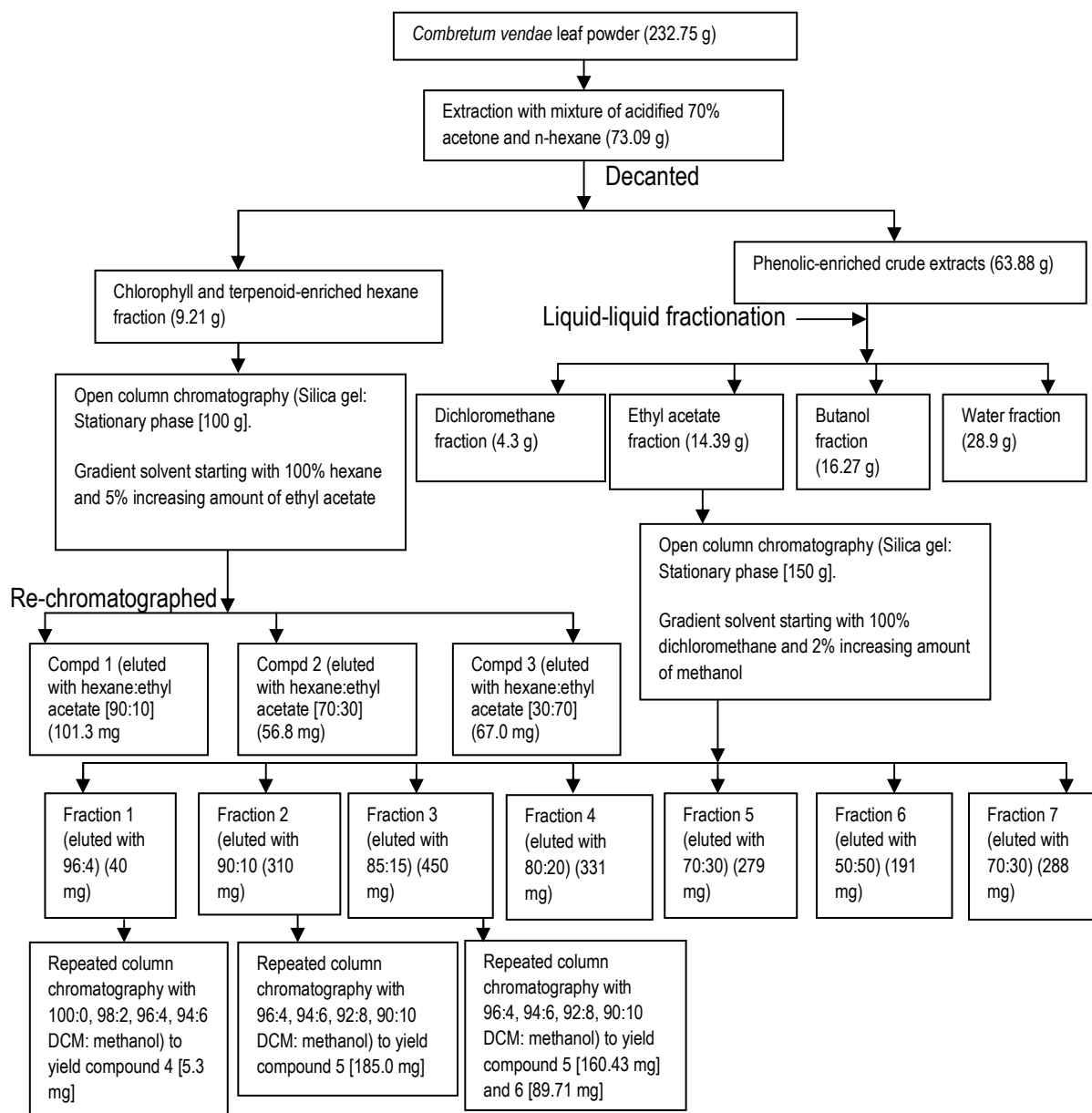


Fig. 9.1. Extraction, fractionation and isolation of bioactive compounds from the leaf extract of *Combretum vendae*

The dried fractions were reconstituted (10 mg/ml) and 10 µl of the aliquot spotted on TLC. Three mixtures of n-Hexane: ethyl acetate (90:10; 95:5 and 98:2) was used to develop the plates. The fractions with R_f corresponding to the R_f values of bioautography assay were combined. The purification of the compounds was achieved by repeated column chromatography until single spot was obtained for each compound using three different mobile phases to develop the TLC.

9.2.4. Isolation of bioactive phenolics from *Combretum vendae*

The ethyl acetate, n-Butanol and residual water fractions exhibited good antimicrobial and antioxidant activities. In TLC-DPPH assay four clear zones of antioxidant components were observed in ethyl acetate fraction. The fraction was subjected to open column chromatography under gravitational force (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of dichloromethane: methanol starting with 100% dichloromethane, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50 as mobile phase. The eluents were monitored with DPPH-TLC antioxidant assay and vanillin/H₂SO₄ spray reagent. The fractions with the same compounds were combined and subjected to further cleaning by re-chromatography until single spots were obtained on TLC chromatogram using three different mobile phases. Schematic representation of the isolation procedure is presented in Fig 9.1.

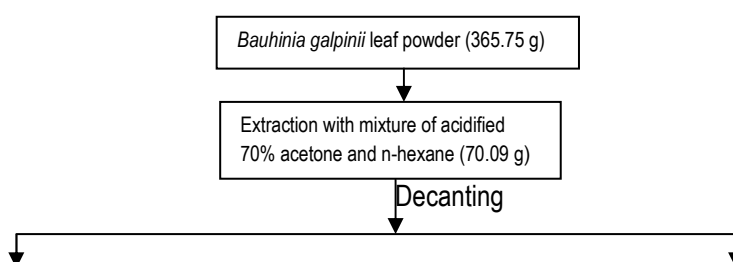
9.3. Isolation of compounds from *B. galpinii*

9.3.1. Isolation of bioactive terpenoid from *Bauhinia galpinii*

The n-Hexane fractions showed one clear zone of microbial growth inhibition and was subjected to open column chromatography under gravity on silica gel (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the solvent mixture of hexane: ethyl acetate starting with 100% n-Hexane, 98:2, 96:4, 94:6, 92:8 and finally 90:10 as mobile phase. The eluent were monitored using TLC and vanillin/H₂SO₄ spray. The fractions containing the target compound was combined and the chromatography process was repeated until a single spot using three mixtures of n-Hexane: ethyl acetate (90:10; 95:5 and 98:2) as mobile phases for TLC chromatogram was obtained. Schematic representation of the isolation procedure is presented in Fig 9.2.

9.3.2. Isolation of bioactive phenolics from *Bauhinia galpinii*

The ethyl acetate, n-Butanol and residual water fractions exhibited good antimicrobial and antioxidant activities. In TLC-DPPH assay four clear zones of antioxidant components were observed in ethyl acetate and butanol fractions. The fractions were subjected to open column chromatography under gravitational force (2.5cm×73cm using 150 g silica, particle size 0.063–0.200 nm, Merck 70–230 mesh ASTM) using the acidified solvent mixture of dichloromethane: methanol starting with 100% dichloromethane, 98:2, 96:4, 94:6, 92:8, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50 as mobile phase. The eluents were monitored with DPPH-TLC antioxidant assay and vanillin/H₂SO₄ spray reagent. The fractions with the same compounds were combined and subjected to further cleaning by re-chromatography using silica gel or Sephadex L20 until single spots were obtained on TLC chromatogram using three different mobile phases. Schematic representation of the isolation procedure is presented in Fig 9.2.



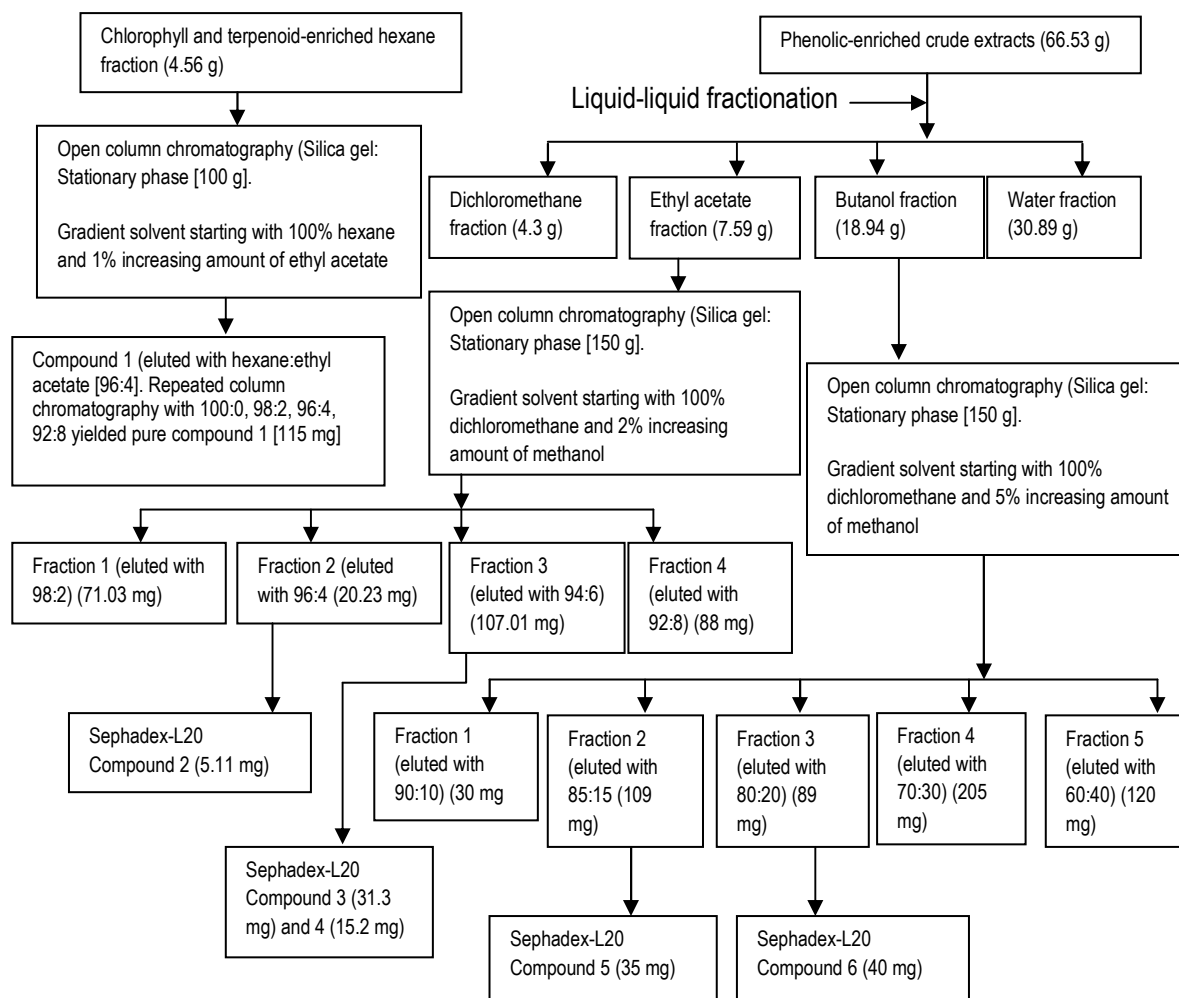


Fig. 9.2. Extraction, fractionation and isolation of bioactive compounds from the leaf extract of *Bauhinia galpinii*

9.4 Characterization of the compounds

9.4.1 NMR spectroscopy

One dimensional (1D) (^1H and ^{13}C) and two dimensional (2D) NMR spectra (^1H - ^1H COSY, HMQC and HMBC) NMR spectra were recorded on a Varian-NMR-vnmrs 600 spectrometer with tetramethylsilane (TSM) as internal standard. Standard pulse sequences were used for homo- and heteronuclear correlation experiments. ^1H NMR spectra were measured at 599 MHz whereas ^{13}C NMR spectra were run at 150 MHz. Multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. All NMR experiments were performed at constant temperature (27 °C) using software supplied by the manufacturer, employing deuteriochloroform, deuteriomethanol, or deuteriodimethylsulphoxide as solvent on the basis of solubility of the sample and literature data.

9.4.2 Mass Spectroscopy:

Electrospray ionization mass spectrometric analyses (negative and positive mode) were carried out to obtain the molecular weight and fragmentations patterns of the isolated compound(s) using TOF mass spectrometer (WATERS HPLC).

9.4.3 UV spectroscopy:

The UV-spectrum of the isolated bioactive compound(s) was recorded using Agilent 1200 UV-Visible spectrophotometer.

9.5 Results

9.5.1 Identification of the chemical structures of isolated compounds from *Combretum vendae*

The n-Hexane and ethyl acetate fractions from the acidified 70% acetone leaf extract of *Combretum vendae* A. E. van Wyk through bioassay guided fractionation were repeatedly subjected to gravity column chromatography to yield one pure, mixture of four position isomer antimicrobial triterpenoids and two stilbene glucopyranoside. The structures of the compounds were determined by extensive NMR techniques and chemical methods mainly by 1D NMR (^1H , ^{13}C and DEPT) and 2D NMR (HSQC, HMBC and COSY), ESIMS, UV-visible spectra and by comparison with the literature data.

Compound 1 was obtained as an amorphous white powder. Detailed analyses of the 1D and 2D NMR spectra indicated the presence of 30 carbons which revealed 7 methyl, 9 methylene, 6 methane, 6 quaternary, 1 carboxylic acid at δ_{C} 178.9, an olefinic broad triplet proton at δ_{H} 5.25 (H-12) coupled to a carbon at δ_{C} 125.4, a quaternary carbon at δ 138.8.0 (C-13) and a β -18 proton at δ_{H} 2.05 characteristic of signal of an ursol-12-ene skeleton (Appendix 9.1). The Comparison of these NMR data with the literature confirms the compound as **ursol-12-en-28-oic acid (ursolic acid)** (Mahato and Kundu, 1994).

Compound 2 and 3 were obtained as position isomeric mixture; the TLC fingerprint indicated unresolved single spot with three different mobile phases. However, the 1D and 2D NMR spectra exhibited chemical shift characteristic of both olean-12-ene and ursol-12-ene. ^{13}C NMR (DMSO): δ 179.7(C-28, olean), 178.0 (C-28, urs), 145.08 (C-13, olean), 122.72 (C-12, olean), 139.42 (C-13, urs), 125.55 (C-12, urs), 41 (C-18, olean), 53 (C-18, urs), 27.97 (C-19, olean), 39.07 (C-19, urs) and 46.13 (C-20, olean), 39.02 (C-20, urs) (Appendix 9.2). Comparing the data with the literature values, the two compounds in the mixture were identified to be **corosolic acid and maslinic acid** (Mahato and Kundu, 1994)

Compound 4 and 5 were also obtained as position isomeric mixture. The spectra 1D and 2D were similar to the spectra of compound 2 and 3 except the presence of additional hydroxyl at C-24) (appendix 9.3). From the correlation data and literature values, compound 4 and 5 were identified to be **asiaitic acid and arjunolic acid** respectively (Mahato and Kundu, 1994).

Compound 6 was obtained as an amorphous powder from the ethyl acetate fraction. The proton NMR spectrum shows signal for apigenin: δ_{H} 6.19 (d, $J=2.0$ Hz, H-6), 6.38 (d, $J=2.0$ Hz, H-8), 7.2 (d, $J=2.1$ Hz, H-3), 6.50 (d, $J=8.9$ Hz, H-3' and H-5'), and 7.30 (d, $J=2.1$ Hz, 8.9, 2H' and H-6').

Compound 7 was obtained as creamy glass-like solid mass and deduced to have molecular formula $\text{C}_{23}\text{H}_{29}\text{O}_{11}$ with molecular weight 481 by EIMS. The ^{13}C -NMR spectrum indicated 17 carbon signals due to aglycone and six carbon signals of a glycoside group. The signal consist twelve aromatic signals, three methoxy and two aliphatic (Appendix 5). The ^1H -NMR spectra revealed the presence of two aromatic benzylic rings. The ^1H detected heteronuclear multiple-bond connectivity (HMBC) spectrum indicated long-range correlations from ^1H -1a' (δ_{H} 2.9, 3.0) to C-1' (δ_{C} 128.55), C-2' (δ_{C} 144.35), C-6' (δ_{C} 118.95), C-1a (δ_{C} 36.96) and C-1 (δ_{C} 132.72). Long range correlation observed between ^1H -1a (δ_{H} 2.7) and C-1 (δ_{C} 132.72, C-2 (δ_{C} 106.16), C-6 (δ_{C} 106.16), C-1a' (δ_{C} 31.73) and C-1' (δ_{C} 128.55). Additional long-range correlation were observed between ^1H -2, 6 (δ_{H} 6.5) and C-1 (δ_{C} 132.72), C-3, C-5 (δ_{C} 148.16), C-4 (δ_{C} 133.79), C-1a (δ_{C} 36.96); ^1H -5' (δ_{H} 6.7) to C-1' (δ_{C} 128.55), C-3' (δ_{C} 139.71), C-6' (δ_{C} 118.95), C-4' (δ_{C} 147.26) ; and ^1H -6' (δ_{H} 6.6) to C-1a' (δ_{C} 31.73), C-3' (δ_{C} 139.71), C-4' (δ_{C} 147.26). Long-range correlation also observed between methoxyl signal (δ_{H} 3.7) and the C-3, C-5 (δ_{C} 148.16), C-4' (δ_{C} 147.26) (appendix 9.4). Based on these data, the aglycone of compound 6 was determined to be stilbenes derivative. The signals for anomeric proton and carbon (δ_{H} 4.5 and δ_{C} 106.26) indicated the presence of a sugar moiety. The long-range correlation in the HMBC experiment between the anomeric proton signal (δ_{H} 4.5) of the β -D-glucopyranosyl group and the C-2' signal (δ_{C} 144.35) confirmed the position of the attachment of the glycopyranosyl moiety on the phenolic ring. The structure of compound 6 was determined to be **combretastatin B5-O-2'- β -D-glucopyranoside**. The EIMS, 1D (^1H and ^{13}C , DEPT), and 2D (HSQC, HMBC, COSY) data correlate with literature information on the compound (Pettit *et al.*, 1985).

Compound 8 had molecular weight of 496 and the molecular formula was deduced to be $\text{C}_{24}\text{H}_{32}\text{O}_{11}$ by EIMS. The compound differs from compound 6 by the presence of extra methyl group. This was confirmed by 1D and 2D NMR spectra. From HSQC one additional methoxyl signal (δ_{H} 3.6 and δ_{C} 59.69) having long range correlation C-4 (δ_{C} 135) (appendix 9.5). The long range correlation from HMBC experiment between ^1H -4 proton of the glycopyranosyl moiety (δ_{H} 3.7) and the C-2' signal (δ_{C} 143.78) confirmed the position of the structure of compound 7 to be **combretastatin B1-O-2'- β -D-glucopyranoside**. The EIMS, 1D (^1H and ^{13}C , DEPT), and 2D (HSQC, HMBC, COSY) data correlate with literature information on the compound (Schwickard *et al.*, 2000).

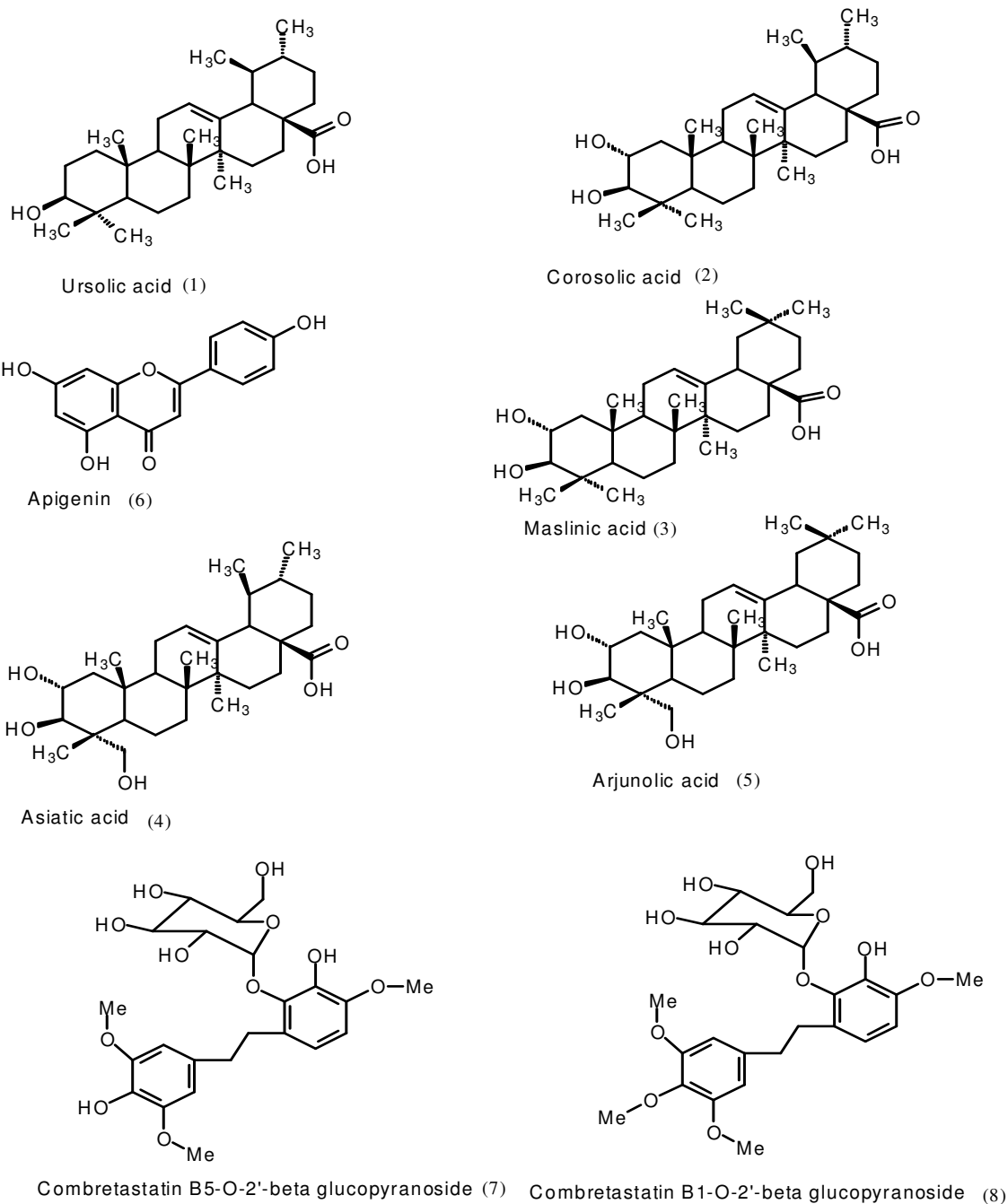


Fig 9.3 Chemical structures of isolated bioactive compounds from 70% acetone leaf extract of *Combretum vendae*

9.5.2 Antimicrobial assay of isolated compound from *C. vendae*

Antimicrobial activities of the isolated compounds against standard and clinical isolate pathogens expressed as MIC are presented in Table 9.1. Some of the compounds exhibited good microbial growth inhibitory potential worthy of pharmacological considerations with MIC ranging from 3.9-31 $\mu\text{g/ml}$.

Table 9.2: Minimum inhibitory concentration ($\mu\text{g/ml}$) of the isolated compounds from the leaf extract of *Combretum vendae*

Microorganisms	Compound 1	Compounds 2 and 3	Compounds 4 and 5	Compound 7	Compound 8
<i>E. coli</i>	62	250	250	250	250
<i>E. faecalis</i>	31	31	31	31	31
<i>S. aureus</i>	62	125	125	125	62
<i>P. aeruginosa</i>	125	125	62	250	250
<i>C. albicans</i> (M0825)	15	7.8	7.8	31	31
<i>C. albicans</i> (M0824)	62	62	31	125	125
<i>C. albicans</i> (1051604)	3.9	3.9	3.9	3.9	3.9
<i>C. albicans</i> (1051608)	7.8	7.8	7.8	15	15
<i>C. albicans</i> (ATCC 10231)	7.8	15	7.8	31	31
<i>C. neoformans</i>	15	15	15	125	125
<i>A. fumigatus</i>	31	31	31	250	250

1: Ursolic acid, 2 and 3: mixture of maslinic and corosolic acid, 4 and 5: mixture of asiatic and arjulongic acid, 6: combretastatin B5-O-2'- β -D-glucopyranoside. 7: combretastatin B1-O-2'- β -D-glucopyranoside. *C. albicans* (M0824), *C. albicans* (M0825), *C. albicans* (1051604), *C. albicans* (1051608) are clinical isolate obtained from National Health Laboratory Service, Pretoria, South Africa.

9.5.3 Identification of the isolated bioactive compounds from *Bauhinia galpinii*

The combine TLC fingerprint, TLC-DPPH assay and bioactivity guided fractionation of acidified 70% acetone leaf extract of *B. galpinii* detected four major flavonoids from ethyl acetate fraction with antioxidant activity. Bioautography against fungal and bacterial pathogens revealed two microbial inhibitory growth spot in Hexane fraction and one in DCM fraction respectively. The bioactive compounds were isolated from each fraction using open column chromatography with silica gel as stationary phase. The phenolics compounds were further purified using Sephadex L-20 as stationary phase and acetone/methanol (50:50) as mobile phase at a rate of 2ml/5min. The chemical structures of the compounds were determined by detailed nuclear magnetic resonance (NMR) techniques including the one dimensional (1D) NMR (proton (^1H), carbon-13 (^{13}C) and distortion enhancement DEPT) and two dimensional (2D) NMR (HSQC, HMBC and COSY). Mass spectrometry and the fragmentation patterns of the compounds were extensively used for the structural elucidation.

Compound 9 obtained as white amorphous powder from hexane fraction was characterized by ^1H NMR spectra (in CDCl_3), ^{13}C -NMR spectra (in CDCl_3), HSQC, HMBC, DEPT and COSY. The ^1H NMR spectrum of 9 showed a one-proton doublet at δ_{H} 5.33 (J^{H} 5.5 Hz) assigned to a vinylic H-6 proton. A one-proton broad multiplet at δ_{H} 3.46 with half-width of 18.5 Hz was attributed to carbinol H-3 proton. Two three-proton broad signals at δ_{H} 0.67 and 1.01 were attributed to a tertiary C-18 and C-19 methyl protons. A six-proton broad signal at δ_{H} 0.83 was associated with C-29 and C-20 methyl protons. Two three proton doublets at δ_{H} 0.91 (J^{H} 6.2 Hz) and 0.85 (J^{H} 6.3 Hz) were due to secondary C-26 and C-27 methyl protons. The remaining methylene and methine protons appeared between δ_{H} 2.50 and 1.27. The presence of all the methyl signals in the range δ_{H} 1.01–0.67 suggested that all these functionalities were located on the saturated carbons. The ^{13}C NMR spectrum of 9 exhibited signals for vinylic carbons at δ_{C} 140.97 (C-5) and 121.96 (C-6). The two carbinol signals appeared at δ_{C} 72.04 (C-3) and

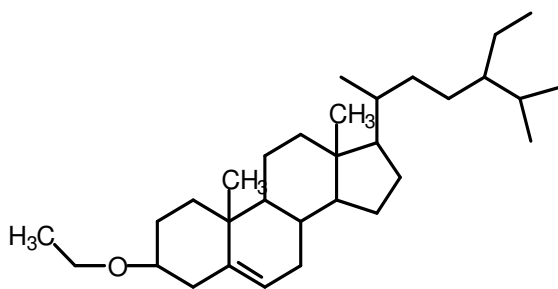
76.86 (CH₃ CH₂O-) respectively. The carbon signals in the upfield region at δ_C 12.20, 21.30, 19.25, 20.04, 19.62, 12.08 and 19.00 were associated with the methyl functionalities. The remaining methylene and methine carbon resonated between δ_C 56.99 and 23.29. The ¹H–¹H COSY spectrum of 9 showed correlations of H-6 with H2-7 and H-8; H-3 with H2-2, H2-4 (appendix 9.6). The ¹H and ¹³C NMR spectral data of steroidal nucleus of 9 were compared with related steroidal constituents (Alam *et al.*, 1994). On the basis of the foregoing discussion, the structure of 9 was elucidated as 3 β -ethoxy stigmast-5-en-ol (3 β -ethoxy sitosterol).

Compound 10 and 11 was isolated from the ethyl acetate fraction as a yellow powder by repeated gravitational column chromatography on silica gel and Sephadex L-20 stationary phases. The ¹H NMR spectrum in deuterated methanol of 10 showed signals for **quercetin**: δ_H 6.19 (d, J=2.0 Hz, H-6), 6.38 (d, J=2.0 Hz, H-8), 7.57 (d, J=2.1 Hz, H-2', δ_C), 6.86 (d, J=8.9 Hz, H-5', δ_C), and 7.56 (d, J=2.1 Hz, 8.9, H-6', δ_C). Carbon 13 NMR (100 MHz, in ppm, methanol-d₄) shows 15 signals and the data (appendix 6) correlated well with literature for 3, 5, 7, 3', 4'-pentahydroxyflavone (Quercetin) (Said *et al.*, 2009)

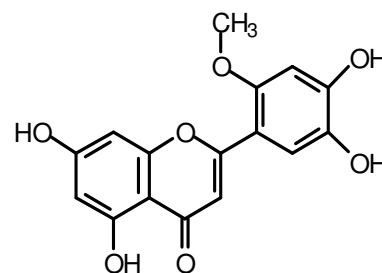
The ¹H NMR of compound 11 in deuterated methanol at 599.74MHz revealed the presence of myricetin aglycone: δ_H 7.383 (s, H-2',6', δ_C 107.09) for the B phenyl ring, and 6.369 (d, H-8, δ_C 92.95), 6.338 (d, H-8, δ_C 92.95), 6.172 (d, H-6, δ_C 97.80) and 6.170 (d, H-6, δ_C 97.80) for the meta substituted A phenyl ring. The structure was confirmed by ¹³C-NMR which showed 15 signals without methoxy or glycoside substituent and 2D correlation (HSQC and HMBC) (appendix 9.7). The data correlated well with literature data for **3, 5, 7, 3', 4', 5'-hexahydroxyflavone (myricetin)** (Said *et al.*, 2009)

The NMR spectrum of compound 12 was typical for a flavone with one meta-substituted and para-substituted phenolic moiety. The UV spectrum showed λ_{max} 261.27 and 369.27 nm which compared favourably with 263 and 367 nm reported by Abdurrahman and Moon, 2007 for isoetin moiety. The compound was assigned molecular formula C₁₆H₁₀O₇ with the aid of a peak observed in the ESI-MS experiment at 315.054. The carbon chemical shifts were assigned by the combination ¹H-¹³C HSQC and long range couplings in the ¹H-¹³C HMBC experiments. The ¹H-NMR spectrum was indicative of five aromatic protons, one methoxyl group. The ¹H and ¹³C NMR spectra data correlated with those of isoetin flavone moiety (Abdurrahman and Moon, 2007; Pauli and Junior, 1995; Voirin *et al.*, 1975) (Table 1). The ¹H-¹³C HSQC correlations were used to assign signal at δ_H 7.19, 6.27 and 6.40 to the protons at C-3 (s, δ_C 108.85), C-6 (d, J = δ_C 99.97) and C-8 (d, J = δ_C 94.86) positions of A ring, and the signal at δ_H 6.65 and 7.38 to the proton at C-3' (s, δ_C 101.36) and C-6' (s, δ_C 114.45) positions of B ring of the isoetin moiety respectively. The long range coupling in the HMBC presented in appendix 9.10 also supported the isoetin flavone moiety. The methoxy protons signal at δ_H 3.8 was correlated with the quaternary carbons (C-2') at δ_C 153.28 indicating the attachment of methoxy group at the carbon (appendix 9.8). From all the correlations, compound 13 was determined to be a new flavone named as **5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether)** or 5, 7, 2' 5' tetrahydroxy-4'-methoxyflavone (isoetin 4'-methyl ether)

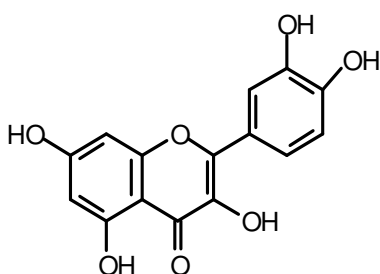
Compound 13 and 14 obtained as yellow powder respectively. UV spectra in MeOH showed λ_{\max} of 203.27, 255.27, 355.27 nm for compound 14, and 207.27, 257, 354.27 nm for compound 13 respectively. The ESI-MS peaks of compound 13 and compound 14 in negative mode, were observed at m/z 463 [M-H] - and m/z 479 [M-H] - respectively. The molecular ion of compound 13 was 16 mass units smaller than that of compound 14, which corresponds to the difference in the number of hydroxyl groups on the B-ring of the flavonol aglycone. The ^1H NMR spectrum in deuterated methanol of 13 showed signals for quercetin: δ_{H} 6.19 (d, $J=2.0$ Hz, H-6, δ_{C} 99.17), 6.38 (d, $J=2.0$ Hz, H-8, δ_{C} 94.09), 7.57 (d, $J=2.1$ Hz, H-2', δ_{C}), 6.86 (d, $J=8.9$ Hz, H-5', δ_{C}), and 7.56 (d, $J=2.1$ Hz, 8.9, H-6', δ_{C}) and anomeric protons at δ_{H} 5.192 (s, δ_{C} 104.14) and 5.22 (s, δ_{C} 104.14) characteristic of galactopyranose. The ^1H NMR of 14 in deuterated methanol at 599.74MHz revealed the presence of myricetin aglycone with five aromatic proton: δ_{H} 6.19 (s, H-6, δ_{C} 98.48), 6.38 (s, H-8, δ_{C} 93.25), 7.37 (s, H-2', H-6', δ_{C} 108.52) and similar anomeric protons to those in 13 (appendix 9.9). The ^{13}C NMR and HMBC, HSQC spectra in deuterated methanol at 150MHz of 13 and 14 were very similar, except for the signals corresponding to the flavonol aglycone (appendix 9.8). The spectral data and correlation with literature information revealed the two compounds to be [quercetin-3-O- \$\beta\$ -galactopyranoside](#) (Rayyan *et al.*, 2004; Yan *et al.*, 2002) and [myricetin-3-O- \$\beta\$ -galactopyranoside](#) (Yan *et al.*, 2002) respectively.



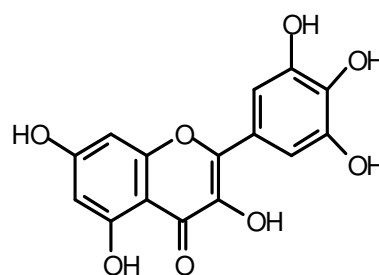
3 beta-O-ethyl sitosterol (9)



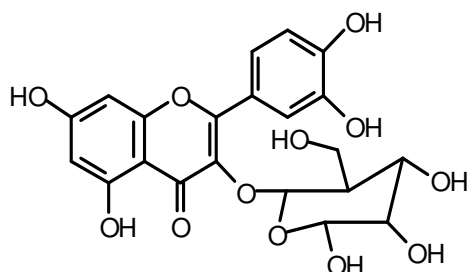
5, 7, 4', 5' tetrahydroxy-2-methoxyflavone
(Isoetin 2'-methyl ether) (12)



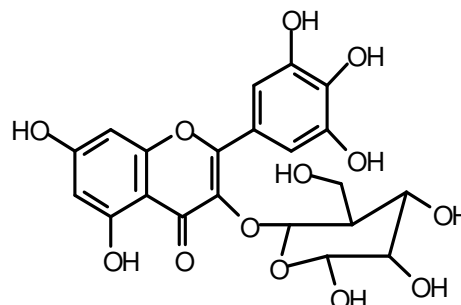
3, 5, 7, 3' 4' pentahydroxyflavone
(Quercetin) (10)



3, 5, 7, 3' 4', 5' Hexahydroxyflavone
(Myricetin) (11)



Quercetin-3-O-galactopyranose (13)



Myricetin-3-O-galactopyranose (14)

Fig. 9.4. Chemical structure of bioactive compound isolated from the leaf extract of *bauhinia galpinii*

9.5.4. Antimicrobial assay of isolated compounds from *B. galpinii*

Antimicrobial potential of the isolated compounds against some diarrhoeal pathogens and organisms of other important infectious diseases are presented in Table 9.2 as minimum inhibitory concentration (MIC) ($\mu\text{g/ml}$).

Table 9.3. Minimum inhibitory concentration ($\mu\text{g/ml}$) of the isolated compounds from the leaf extract of *Bauhinia galpinii* and positive control

Microorganisms	9	10	11	13	14	Gentamicin	Amphoteric B

E.coli	125	31	31	31	62	15.5	-
<i>E. faecalis</i>	31	31	7.8	62	62	7.75	-
<i>S. aureus</i>	62	62	15	31	31	3.87	-
<i>P. aeruginosa</i>	125	62	15	15	15	3.87	-
<i>C. albicans</i> (M0825)	15	3.9	3.9	31	31	-	3.9
<i>C. albicans</i> (M0824)	31	31	31	31	31	-	3.9
<i>C. albicans</i> (1051604)	3.9	3.9	3.9	3.9	3.9	-	0.8
<i>C. albicans</i> (1051608)	62	3.9	3.9	15	15	-	1.93
<i>C. albicans</i> (ATCC 10231)	62	3.9	3.9	31	31	-	1.93
<i>C. neoformans</i>	125	31	31	125	125	-	7.75
<i>A. fumigatus</i>	125	31	31	125	125	-	7.75

1: 3 β -ethoxyl sitosterol; 2 Quercetin; 3 Myricetin, 4 Quercetin-3-O- β -galactopyranose; 5 Myricetin- 3-O- β -galactopyranose. *C. albicans* (M0824), *C. albicans* (M0825), *C. albicans* (1051604), *C. albicans* (1051608) are clinical isolate obtained from National Health Laboratory Service, Pretoria, South Africa.

9.6. Discussion

9.6.1. Bioactive compounds from *Combretum vendae*

Bioactivity guided investigation of *Combretum vendae* afforded ursolic acid ((3 β)-3-hydroxyurs-12-en-28-oic acid), mixture of asiatic and arjunolic acid, mixture of maslinic and corosolic acid, apigenin, Combretastatin B5-2'-O- β -glucopyranoside and Combretastatin B1-2'-O- β -glucopyranoside all exhibiting broad based microbial growth inhibitory potentials.

Ursolic acid and its derivatives biosynthetically derived from the cyclization of squalene (and other triterpene acids) have been extensively studied as pharmacological active molecules in many *in vitro* and *in vivo* studies. Some of the biological activities include antioxidant, hepatoprotective, anti-inflammatory, anticancer, anti-HIV, vasorelaxant (Aguirre-Crespo *et al.*, 2006) and antidiabetic activities. Several mechanisms have been proposed to explain its anti-inflammatory activity, including inhibition of secretory PLA₂ enzymes, IL-1 β secretion, iNOS and COX-2. Ursolic acid have no antispasmodic effect (Estrada-Soto *et al.*, 2007), however the broad base antimicrobial activities (3.9-125 μ g/ml) obtained in this work and other reports of antioxidant, anti-inflammatory and antidiabetic provide pharmacological bases for further investigation of the compound as anti-diarrhoeal agent.

Biological activities such as α -glucosidase inhibition of arjunolic acid (18.63 \pm 0.32 μ g/ml), asiatic acid (30.03 \pm 0.41 μ g/ml), maslinic acid (5.52 \pm 0.19 μ g/ml) and corosolic acid (3.53 \pm 0.27 μ g/ml) isolated from *Lagerstroemia speciosa* were reported. The α -glucosidase inhibition of these compounds shows their antidiabetic and antiadhesion potential against microbial pathogens both of which are also important in anti-diarrhoea therapy. However the compounds have no α -amylase inhibitory activities (Hou *et al.*, 2009). Mixture of arjunolic acid and asiatic acid isolated from *Combretum nicholsonii* have antifungal activities with MIC of 0.2-1.5 μ g/ml. However, antimicrobial activities obtained in the work is slightly higher for the mixture (3.9-250 μ g/ml), the difference in result may be due to experimental variable such as concentrations of the culture media, composition of the

mixture, incubation time and strains. These results indicate that individual compound or the mixtures have pharmacological potentiality against infectious pathogens.

Apigenin have been isolated previously from the acetone leaf extract of *C. vendae*. The antibacterial activity of the compound was evaluated (Eloff *et al.*, 2008).

Combretastatin B5-2'-O- β -glucopyranoside have been isolated previously from the seed of *Combretum kraussii*. The aglycone moiety of this compound was isolated from the acetone leaf extract of *Combretum woodii* reported to have antibacterial activity. This is the first report on the isolation of the compound from the leaf extract and its antimicrobial activities.

Combretastatin B1-2'-O- β -glucopyranoside have been previously isolated from the seeds of *Combretum kraussii* (Pettit *et al.*, 1987) and wood bark of *Combretum erythrophyllum* (Schwikkard *et al.*, 2000). The stilbenes have been reported to have cytotoxic activity with effect on tubulin polymerization, the primary protein component of microtubules in cancer hence the potential of the compound as anticancer drug is being explored. The compound has been evaluated for selective inhibitory activity against the DNA-damaging repair-deficient strain of *Saccharomyces cerevisiae* deficient in the RAD52 recombination repair gene and exhibited no activity while the derivative with unsaturated bond at 1aC- 1a'C (combretastatin A1-2'-O- β -glucopyranoside) was active (Schwikkard *et al.*, 2000). This indicated the importance of the unsaturated bond in the structure-activity relationship for cytotoxicity effects. However, there is no literature report on other biological activity potential such as antimicrobial, antioxidant, anti-inflammatory and antidiarrhoea. This is the first report on the isolation of the compound from the leaf extract and its antimicrobial activities. The non cytotoxic effect of the compound against the cancer cells lines (Schwikkard *et al.*, 2000) indicates that the compound can be exploited for other biological activities.

9.6.2. Bioactive compounds from *Bauhinia galpinii*

Isoetin (5, 7, 2' 4' 5'-pentahydroxyl flavone) and its various derivatives are rare compounds formed by insertion of a 2' hydroxyl group into luteolin to give characteristic yellow pigments in some plant part (Lattanzio *et al.*, 2006). 5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) is a new compound (unfortunately the quantity obtained was not enough for bioassays). A related compound 5, 7, 2' 4' tetrahydroxy-5'-methoxyflavone (isoetin 5'-methyl ether) isolate from *Trihosanthes kirilowii* (Cucurbitaceae) was reported to be cytotoxic against human lung cell line A549 (IC₅₀ 0.92 μ g/ml), human melanoma Sk-Mel-2 (IC₅₀ 8.0 μ g/ml), and mouse melanoma B16F1 cell lines (IC₅₀ 7.23 μ g/ml). High cytotoxicity (IC₅₀ 2.5 μ g/ml) of the acetone root extract of *B. galpinii* against Vero cell lines has been reported (Samie *et al.*, 2009). Isolation of more 5, 7, 4' 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) from *B. galpinii* for further studies on cytotoxicity effect important.

Quercetin and myricetin are the common flavonol present in dietary and constitute the active component of medicinal plant with characteristic hydroxyl substitutions at the 3, 5, 7, 3', 4' (Quercetin), and 3, 5, 7, 3', 4' and 5'

(myricetin) positions of flavone ring. The compounds occur in nature mostly as glycoside with D-glucose, L-rhamnose, D-galactose or arabinose rather than free aglycone. Biological effects of phenolic compounds depend on their bioavailability which is determined by the lipophilicity of each molecule. The mechanisms involved in digestion and absorption of phenolic compound is complex but passive transports through the membrane have been proposed. The glycosides and methylated phenolics are not readily absorbed in native form but need to be hydrolysed by intestinal enzymes or colonic microflora to aglycone before absorption.

Quercetin and myricetin alongside their galactopyranoside derivatives were isolated from the acidified 70% acetone leaf extract of *Bauhinia galpinii*. Quercetin galactopyranose and myricetin galactopyranose were previously isolated from this plant and their antioxidant activity and cytotoxicity was evaluated (Aderogba *et al.*, 2007).

A wide range of biological activities related to diarrhoeal pathogenesis including antimicrobial (Naz *et al.*, 2007), anti-inflammatory and spasmolytic due to their antioxidant and/or free radical scavenging (Aderogba *et al.*, 2007) as well as ability to interfere with several enzymatic pathways have been reported for the compounds. Quercetin and myricetin are active against microorganisms of the genera *Bacillus*, *Corynebacterium*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, and against *Escherichia coli* and *Vibrio cholerae* (Naz *et al.*, 2007).

Both compounds also have protective and promotive effects on intestinal TJ barrier function through interaction with intracellular signaling molecules, tyrosine kinases and protein kinase C δ (PKC δ) (Suzuki and Hara, 2010). The intercellular TJs are the major determinant of the intestinal physical barrier regulating the paracellular movement of ions, solute, and water through the intestinal epithelium. Impaired intestinal TJs functions are involved in several intestinal and metabolic diseases, such as diarrhoea, inflammatory bowel disease and food allergy (Suzuki and Hara, 2010). Myricetin inhibits the generation of MDA a cytotoxic by-product of lipid peroxidation of arachidonic acid liberated from membrane phospholipids (Robak *et al.*, 1986).

Myricetin has potential as an antiviral agent by its ability to inhibit the reverse transcriptase from Moloney murine leukaemia virus, Rauscher murine leukaemia virus and human immunodeficiency virus (Ono *et al.*, 1990). Myricetin also has antidiabetic activity with ability to stimulate lipogenesis and enhanced glucose uptake into adipocytes. The mechanisms postulated include changes in lipid-protein interaction or increase membrane fluidity. Myricetin inhibits the intraluminal accumulation of fluid and prevent diarrhoea induced by castor oil.

9.7. Conclusion

All the isolated compounds from the two plant species have biological activities with relevance against one or more diarrhoeal pathophysiology. The antimicrobial of some of the compounds are worthy of pharmacological consideration. In order to exploit the full potentials of these compounds some *in vitro* and *in vivo* studies are required to determine the mechanism of action.

CHAPTER TEN

General conclusion and future prospects

10. Introduction

Diarrhoea is one of the major health challenges facing the world and especially developing countries. The problem is aggravated due to the increasing number of immunocompromised people infected by HIV, with associated opportunistic infections and other health complications manifesting as diarrhoeal symptoms. The emergence of more virulent strains resulting from drug resistant pathogens and the apparent side effects of some conventional drugs currently in use is also serious concerns in diarrhoeal control and management. In animal production, diarrhoeal outbreaks usually cause serious economic losses due to reduced productivity, cost of treatment, lower level of reproduction and increased mortality.

However, the success of oral rehydration therapy in reducing mortality and lack of commercial interest in drugs for developing countries has slowed the progress in the development of novel agents for treating diarrhoeal diseases. Therefore, there is an urgent need for new therapeutic drugs or herbal products with lower cost, high efficacy and little or no side effects. Plants and plant preparations have been used ethnopharmacologically in treating diarrhoea successfully, although their efficacies, mechanisms of action and safety have generally not been proven scientifically. Thus, the overall aim of this project was to systematically determine the efficacy, mode of action and safety of some plants used traditionally in South African traditional medicine as diarrhoea therapy.

The following objectives were identified to attain this aim:

- ❖ To conduct comprehensive literature works on diarrhoeal aetiologies and mechanisms and, medicinal plants use for treating diarrhoea symptoms in Southern Africa.
- ❖ To determine the phenolic compositions of the crude extract.
- ❖ To evaluate the effects of some selected medicinal plant species against pathogenic microbes known to induce diarrhoea.
- ❖ To determine the antioxidative properties of the selected plants using various standard protocols.
- ❖ To determine the anti-inflammatory potentials of the selected plants using various standard protocols.
- ❖ To evaluate the toxicity risk of the crude extracts.
- ❖ To determine the intestinal motility modulatory effects of the most promising extracts on the contractility process of the isolated rat ileum induced by spasmogens and ion channels activators.
- ❖ To isolate and characterize the component(s) that exhibit antimicrobial and antioxidant properties from the most promising extracts.

The achieved objectives of the study are outlined as follows:

10.1. Identification of diarrhoeal pathogenesis and medicinal plants used as therapeutic agents

The data generated from the literature work indicate that diarrhoea has a number of pathogenesis such as microbial infection, chronic inflammation, oxidative injury to intestinal mucosal lining, and deranged intestinal motility. The mechanisms involved include one or combination of ionic and water secretion into the lumen and reduced absorption of fluid from the intestine. The compendium of the medicinal plants used as antidiarrhoeal agents (254 species) in Southern Africa also revealed the diarrhoeal challenges and the wide acceptability of medicinal for cure.

10.2. Antimicrobial evaluation of the extracts against infectious pathogens

The results obtained by the antimicrobial screening indicate the presence of many compounds with potent antibacterial and antifungal activity against the standard strains of microbes responsible for infectious diarrhoea and other important infectious diseases in humans and animals. The significant inhibitory activity exhibited by the water fraction of the *C. vendae* against *S. aureus* can be considered as important for the traditional use of this plant, where water is the main extractant available. Generally, the results revealed that the antimicrobial potential of the extracts are potentiated in the hexane and dichloromethane fractions. Future investigation of the potent extracts and fractions against resistant and virulent pathogens might indicate new mechanisms for the growth inhibition of the microorganisms.

10.3. Antioxidant evaluation of the extracts

The crude extracts and the polar fractions of ethyl acetate and butanol had significant radical scavenging activity, through hydrogen and proton donation mechanisms. These activities are ascribed to the presence of large quantity of phenolics. In view of the oxidative stress in the pathogenesis of diarrhoea through tissue injury by ROS/RNS involvement in lipid peroxidation, exacerbation of inflammatory processes and some of the reactive species serving as secretagogues, the strong antioxidant activity could indicate the presence of compounds with potentially important mechanisms of pharmacological relevance in reducing the deleterious effects of the oxidative species in diarrhoea. *In vitro* results however, cannot be literally translated into *in vivo* situation due the problem of bioavailability, absorption and possible metabolic transformations of the bioactive compounds in the intestine. Further research are needed to verify using other models with different mechanisms against substrates which are generated in human or animal cells as well as *in vivo* studies to evaluate their efficacy and safety.

10.4. Anti-inflammatory potential of the extracts

Inflammation is regarded as the hallmark of many diseases aetiology and the significant mediators are eicosanoids (prostaglandins, prostacyclin) from cyclooxygenase (COX) and leukotrienes from lipoxygenase (LOX) pathways. These two enzymes are the target for modulating the inflammatory process. The result obtained indicated that the polar extracts of *Bauhinia*, *Carissa*, and *Syzygium* species used for COX inhibitory assay were

active against COX-1 with no activity against COX-2 while the *Combretum* species were inactive against both enzymes. COX-1 selective inhibitors are considered to cause GIT injury while selective COX-2 inhibitors are more beneficial against inflammatory processes, therefore these plant polar extracts should be used with caution because possible intestinal injury. Most of the plant extracts however, had good LOX inhibitory activity. Current research on anti-inflammatory agent focus on dual activity as COX and LOX inhibitors since both pathways uses the same substrate. If one pathway is closed down, more substrate will be available to the other unperturbed pathway, thus increasing its products and consequently promoting some other inflammatory mechanisms. Additional work is required to determine the fraction(s) in which the active component is present and the probable mechanisms of action. Since the polar extract are not active against COX-2, the non-polar extracts or fractions still have to be tested. The plant extracts also have to be tested against other inflammatory biomarkers and mediators including an *in vivo* studies using laboratory animal model.

10.5. Toxicity risk of the extracts

The toxicity risk assessment using MTT assay (Mosmann, 1983) using Vero African green monkey kidney cell lines indicated that the extracts of *Combretum* species except *C. bracteosum* were highly toxic. The other extracts have varying degree of toxicity with *Ozoroa mucronata* being the least toxic. These results are important because they show that there are risks of toxicity with an inappropriate use of some of these extracts as therapeutics for any ailments except perhaps cancer. The toxicity of medicinal plants depends on many factors such as the plant part used, and the solvents used as extractant which are determinant of the compositional characteristics and biological activity of extracts. Most of the highly toxic extracts also contain a high quantity of hydrolysable tannin. Poor handling of raw or processed materials may produce exogenous toxic contaminants not inherent as plant phytochemicals. It should be kept in mind that the results of cytotoxicity testing may vary considerably depending on the cell type used, the initial cell density to which the extracts are exposed, and the duration of exposure. Vero cells were selected as these are readily available and are commonly used in cytotoxicity tests. In this study, a low cell density was used and the cells were exposed for a long time, 5 days (McGaw et al., 2007). Hence, relatively low LC₅₀ values were obtained and differences in cytotoxicity between the extracts were maximised. Further work is needed to test the extracts, fractions and subfractions against other cell lines and, also to conduct acute and chronic toxicity assays with a view of determining the toxic constituents present in the plants.

It is a pity that one of the two plant species selected for in depth work *C. vendae* had a high cellular toxicity. In future studies toxicity of extracts should be investigated at an early stage.

10.6 Motility modulatory effects of *Bauhinia galpinii* and *Combretum vendae*

Considering the wide ethnopharmacological use of *B. galpinii* and *C. vendae* against GIT disorders and their excellent activity in some of the preliminary screening, the two plants were chosen for motility modulatory assays despite the toxicity potential of *C. vendae*. This was with the view that the toxic component(s) will be determined

and separated from the other active components. The data generated by the study indicate that *B. galpinii* has a dual-mechanism of action (prokinetic and relaxant) on gastro-intestinal motility while *C. vendae* extracts exhibited spasmolytic (relaxant) effects on isolated rat ileum through multiple mechanisms. These results were important as they indicate that *B. galpinii* extract can clinically be relevant as therapeutic agent in diarrhoea and constipation which are both diseases with aetiology based on motility disturbances to a large extent while the presence of multiple acting spasmolytic activities in the *C. vendae* extract might be contributing towards its effectiveness in diarrhoea and abdominal spasm therapy. Further work are needed for the identification of the specific ENS receptors through which these extract acts as well as the phytochemical compounds responsible for their activities.

10.7. Isolation and characterisation of antimicrobial compounds

Bioassay-guided protocols for antibacterial and antioxidant activity were adopted for the identification and isolation of 14 compounds (8 from *C. vendae* and 6 from *B. galpinii*) using open column chromatography with silica gel and Sephadex LH 20 as stationary phases. However, some of the compounds are mixtures of position isomers which are extremely difficult to separate. The compounds were characterised as ursol-12-en-28-oic acid (ursolic acid), a mixture of corosolic acid and maslinic acid, and a mixture of asiatic acid and arjunolic acid, two stilbenoid glycosides (combretastatin B5-O-2'- β -D-glucopyranoside and combretastatin B1-O-2'- β -D-glucopyranoside) and one flavone (apigenin) from the *Combretum vendae*.

One phytosterol (β -3 ethoxy sitosterol), one new flavone (5, 7, 4', 5' tetrahydroxy-2'-methoxyflavone (isoetin 2'-methyl ether) or 5, 7, 2', 5' tetrahydroxy-4'-methoxyflavone (isoetin 4'-methyl ether)), two known flavonols (3, 5, 7, 3', 4'-pentahydroxyflavone (Quercetin) and 3, 5, 7, 3', 4', 5'-hexahydroxyflavone (myricetin)) and their galactoside derivatives (quercetin-3-O- β -galactopyranoside and myricetin-3-O- β -galactopyranoside) were isolated from *Bauhinia galpinii*.

The results from this study indicate that medicinal plants used in ethnopharmacology are reservoirs of bioactive compounds. Some of the medicinal plants may serve as potential sources of novel active compounds or lead molecule for synthesis of more potent drugs. There is also a distinct possibility of developing plants extracts that could be used by poor rural people or sophisticated herbal medicines from some of the species investigated in this study. The information gained from this work provides a baseline study for other scientist to explore other medicinal plant species in depth with possible commercial application.