# Chapter Seven

## Cytotoxicity evaluation of the crude extracts against Vero African green monkey kidney cell lines

### 7.0. Introduction

Medicinal plants are assumed to be non-toxic and regarded safe due to their natural origin and long use in traditional medicine to treat various forms of diseases (Chen *et al.*, 2011; Fennell *et al.*, 2004). Medicinal plant preparations are administered with the hope of promoting health and treating various diseases such as infections, colds, inflammation, GIT disorders, insomnia, depression, heart diseases, diabetes, cancer, acquired immunodeficiency syndrome, and liver diseases has increased in recent times (Chen *et al.*, 2011). However, scientific studies on efficacy and safety of some medicinal plants indicated that there are many phytochemicals that have cytotoxic, genotoxic, and carcinogenic effects when used chronically (Ernst, 2004; Rietjens *et al.*, 2005). It should also be kept in mind that if a different extractant is used, the safety ascribed to traditional use based mainly on aqueous extracts may not be relevant at all.

The adverse effects of medicinal plant use arise due to organ toxicity, adulteration, contamination, contents of heavy metals, herb–drug interactions, poor quality control and inherent poisonous phytochemical (Jordan *et al.*, 2010). Some medicinal plant phytochemicals are associated with toxicities of the heart, liver, blood, kidney, central nervous system, gastrointestinal disorder such as diarrhoea, and less frequently carcinogenesis (Jordan *et al.*, 2010). In the formal herbal industry the toxicity problems of medicinal plants are attributable to insufficient quality assurance and non-compliance to the standards of Good Manufacturing Practise (Palombo, 2006). Furthermore, the problem is complicated by adulteration of herbal remedies by surreptitious addition of synthetic drugs and other potentially toxic compounds such as other botanicals, microorganisms, toxins, pesticides, and fumigants (Palombo, 2006).

More importantly, if herbal medicines are used with prescription drugs especially those with narrow therapeutic indices it can result in potential harmful herb–drug interactions that cause altered drug response and toxicity (Chen *et al.*, 2011). The fact that herbal medicines contain many compounds (active and non-active); the large number of pharmacologically active compounds also increases the chance of herb-drug interaction (Palombo, 2006). Like synthetic drugs, herbal bioactive compounds can also undergo Phase I and Phase II enzymatic transformations to form nontoxic metabolites which are excreted through the faeces and urine. However, the production of reactive and potentially toxic metabolites is feasible with associated toxicity implications (Chen *et al.*, 2011).

With the current emphasis on research and development of medicinal plant worldwide, it is important to have some information regarding the toxicity potential and efficacy of plants utilized ethnobotanically to treat ailments.

As part of ethnopharmacological studies of medicinal plant available literature should be searched for known toxic properties of plants of interest before embarking on biological activity studies. However, where toxic effects are unavailable, the inclusion of cytotoxicity and other toxicity protocols in the study are useful in detecting potential toxicity. This strategy is applicable when screening plant extracts or isolated natural products for some other biological activities such as anti-infectious, anti-inflammatory, antioxidant, antidiarrhoea and anti-parasitic property. The aim of this work was to determine the potential risk of the crude phenolic-enriched extracts by evaluating the cytotoxicity using Vero cell lines.

## 7.1. Materials and Methods

### 7.1.1. Preparation of plant extract

The plant extracts were prepared as described in section 3.6.3. The dried sample were reconstituted in 70% acetone at the concentration of 1.0 mg/ml (3 ml) and from it a serial dilution of the concentration range of 1.0 to 0.001 mg/ml were made on the 96 well tissue culture plate.

### 7.1.2. Cytotoxicity assay against Vero cell

Cytotoxicity of the extract was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] assay (Mosmann, 1983) using Vero African green monkey kidney cell lines. The cells were cultured in Minimal Essential Medium (MEM) Earle's Base, supplemented with 20 mM L-glutamine, 16.5 mM $NaHCO_3$ supplemented with 0.1% gentamicin and 5% foetal calf serum. Confluent monolayer culture suspensions of the cells were seeded into 96-well tissue culture microtitre plate at a density of $0.5\times10^3$ cells per well and incubated for 24 h.at temperature of 37°C in a 5.0% $CO_2$ incubator. The cells were washed with cultured media and extract (1.0, 0.1, 0.001 mg/ml), positive control (berberine at concentrations of 100, 10, 1.0, 0.1 µg/ml) were added and incubated for 5 days. The cells were observed using inverted microscope to check for cytopathic effect from the extract. The cells proliferation and viability was examined by addition of 30 µl of a 5 mg/ml solution of MTT in PBS to each well and incubated for another 4 h.at 37°C. The medium was carefully removed from the wells without disturbing the MTT concentrate and washed twice with PBS. The liquid was aspirated from the cells and 50 µl of DMSO was added to each well to dissolve the crystallized MTT formazan. The amount of reduced MTT was measured as absorbance at 570 nm using microtitre plate reader. The result expressed as a percentage of the control cells and $IC_{50}$ was calculated

Dose response curves were obtained by plotting the percentage growth of cells versus log concentration of the compound. The $LC_{50}$ (50% inhibitory concentration) values were calculated from a non-linear regression model (best fit curve) of sigmoidal dose-response curve (variable) and computed using GraphPad Prism 5.04 (Graphpad, USA).

## 7.2. Results

The cytotoxicity of phenolic-enriched crude leaf extracts of the 19 medicinal plants used ethnopharmacologically in treating diarrhoea and other GIT disorders is presented in Table 7.1. The results indicate that the extracts had varying degrees of toxicity to Vero cell lines with $LC_{50}$ ranging from 3.51±2.03 to 741.90±44.22 µg/ml. The most cytotoxic extract was *Combretum woodii* (3.51±2.03 µg/ml) followed by *Combretum vendae* (5.70±1.25 µg/ml) while the least cytotoxic extract was *O. mucronata* (741.90±44.22 µg/ml) followed by *Maytenus procumbens* (187.71±19.92 µg/ml).

Table 7.1 The $LD_{50}$ of the cytotoxicity assay of some medicinal plants used in South African traditional medicine to treat diarrhoea and related ailments

| Plant species | $LD_{50}$ (µg/ml) |
|---|---|
| *Bauhinia bowkeri* | 17.90±2.56 |
| *Bauhinia galpinii* | 35.68±2.15 |
| *Bauhinia petersiana* | 40.68±18.13 |
| *Bauhinia variegata* | 76.37±7.50 |
| *Combretum bracteosum* | 48.81±6.15 |
| *Combretum padoides* | 9.03±0.20 |
| *Combretum vendae* | 5.70±1.25 |
| *Combretum woodii* | 3.51±2.03 |
| *Euclea crispa* | 31.61±4.04 |
| *Euclea natalensis* | 26.99±4.48 |
| *Maytenus peduncularis* | 89.41±16.37 |
| *Maytenus procumbens* | 187.71±19.92 |
| *Maytenus senegalensis* | 87.62±3.03 |
| *Maytenus undata* | 99.17±11.88 |
| *Ozoroa mucronata* | 741.90±44.22 |
| *Ozoroa paniculosa* | 16.58±1.85 |
| *Searsia leptodictya* | 25.09±2.40 |
| *Searsia pendulina* | 22.30±2.42 |
| *Searsia pentheri* | 50.62±4.30 |

## 7.3. Discussion

For medicinal plant extracts to be useful in clinical application, the preparation must be selectively toxic to the targeted organism or interfere directly with specific reaction pathway without a major effect on the host cell or interference with normal physiological pathways. In categorization of crude extract safety, $IC_{50}$ value of 20 µg/ml and below were considered to be cytotoxic in an in vitro assay according to US National Cancer Institute (NCI) plant screening program (Kuete *et al.*, 2011) following incubation for more than 48 h. Some of the phenolic-rich crude leaf extract of the medicinal plants tested in this study are relatively toxic compared to the positive berberine control.

The cellular toxicity effects of the crude extracts were evaluated by MTT-formazan viability assay. Cellular viability and proliferation are considered to be an important functional characteristic of healthy growing cells. Increase in cell viability indicate cell proliferation, while decrease in cell viability indicate cell death as a result of either toxic effects of the test extracts or sub optimal culture conditions. With the cell viability of the negative control (DMSO) at the highest concentration of 1000 µg/ml under the same experimental condition, the latter postulate is eliminated. Therefore, all the phenolic-enriched extracts of the medicinal plants tested may be suggested to be safe for use in treating diarrhoea if the dosage is below the cytotoxic level. Although, *Ozoroa paniculosa* (16.58±1.85 µg/ml), *Searsia pendulina* (22.30±2.42 µg/ml), *Searsia leptodictya* (25.09±2.40 µg/ml) and *Euclea natalensis* (26.99±4.48 µg/ml) are within the defined cytotoxicity range, therefore the use of these extracts in traditional medicine need to be monitored carefully. It is also important to note that no report of toxicity has been recorded for the traditional use of these plant extracts. One should however remember that cellular toxicity does not necessarily equate to whole animal toxicity due to possibly interactions in the gut and bioavailability issues.

*C. woodii* acetone extracts have however been reported to be toxic in an *in vivo* test as anticoccidial in poultry at concentration of 160 mg/kg (Naidoo *et al*, 2008). Furthermore, several cytotoxic and anti-tumour derivative of stilbenoids such as Combretastin A and Combretastatin B5 ($IC_{50}$ value of 10 µg/ml) have been isolated from the genus *Combretum*.

Toxicity is usually encountered due to irrational use causing accumulation of potentially toxic constituents or interactions between herbal medicinal products and conventional therapies. Indicative observations of toxicity is alterations of one of the clinical signs such as diarrhoea, weight loss, agitation, hispid hair, convulsions, tremors, dyspnoea among other) and mortality (Caparroz-Assef *et al.*, 2005).

Some medicinal plant metabolites can cause GIT toxicity. The mechanism of action can be primarily irritative or cytotoxic in nature resulting in an initial release of mucus from goblet cells, hypersecretion from crypt cells, and

maladsorption causing diarrhoea and emesis. Administration of high dose of some phytochemicals can cause effects such as necrosis, haemorrhage, and even ulceration on the GIT. Medicinal plant toxins can have additional toxicity or more directly life-threatening effects on other organ system.

### 7.4. Conclusion

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. *In vivo* acute toxicity studies may be necessary to establish the safety level of the extracts as *in vitro* assay results not necessarily translate to *in vivo* activity. Long term effect of the use of the extracts such as mutagenicity and genotoxicity also need to be determined.

In vivo animal studies are frequently very expensive and requires much work to establish changes in enzyme concentrations or histological evaluation of toxicity. It is also possible to do *ex vivo* studies using isolated organs. In the next chapter some *ex vivo* studies will be described to investigate the possible mechanism of activity of two selected species.

# 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 evaluated motility regulatory potentials and determined 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 $E_2$ ($PGE_2$), Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), $N^G$-nitro-l-arginine methyl ester (L-NAME), Carbachol, Pilocarpine, , Cyclopiazonic acid, Dimethylsulphoxide (DMSO), Sodium chloride (NaCl), Potassium chloride (KCl), Calcium chloride ($CaCl_2$), Sodium bicarbonate ($NaHCO_3$), Magnesium sulphate ($MgSO_4$), Potassium hydrogen phosphate ($KH_2PO_4$), 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% $O_2$ and 5% $CO_2$) Krebs solution with the following composition (g/l): NaCl, 6.94; KCl, 0.354; $KH_2PO_4$, 0.163; $NaHCO_3$, 2.1; $MgSO_4$, 0.370; $CaCl_2$, 0.367; glucose, 2.07 and pH 7.4. The intestinal content was removed by washing with Kreb'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 Kreb's solution (composition (g/l): NaCl, 6.94; KCl, 0.354; $KH_2PO_4$, 0.163; $NaHCO_3$, 2.1; $MgSO_4$, 0.370; $CaCl_2$, 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.

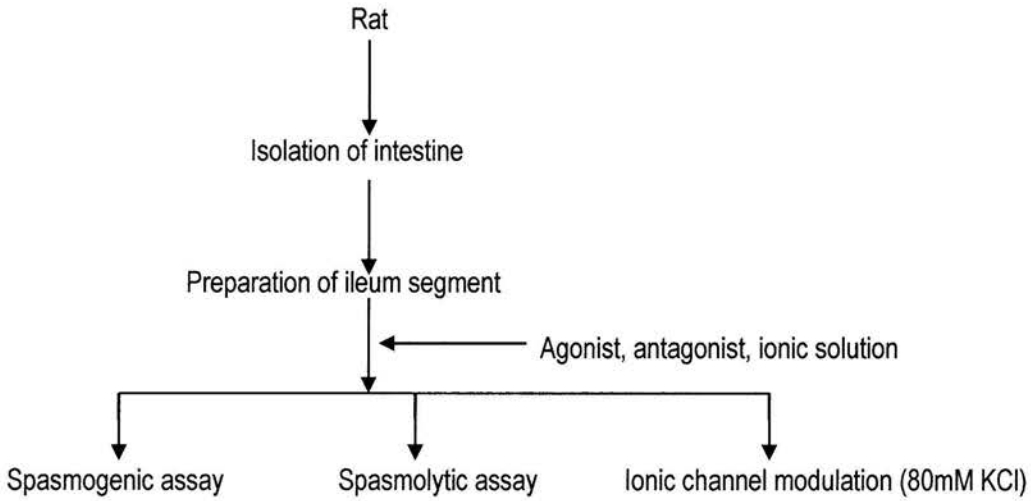


Fig. 8.1. Schematic presentation of the contractility assay

**8.4 Data analysis**

The inhibition of ileum contraction by test sample was normalized and expressed as a percentage of mean± SEM from 3-4 experiments of the references responses induced by acetylcholine (10 µg/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 µg/ml) stimulate spontaneous contraction of the rat ileum as shown in Fig 8.2 with $EC_{50}$ value of 27.85 µg/ml. Maximum contraction ($E_{max}$) of 44 mm was obtained at 200 µg/ml and additional doses causes suboptimal response but

increase duration of response caused an irreversible spasm at the maximum dosage of 1000 µg/ml. Repeated administration of the extract at maximum dosage (1000 µg/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).

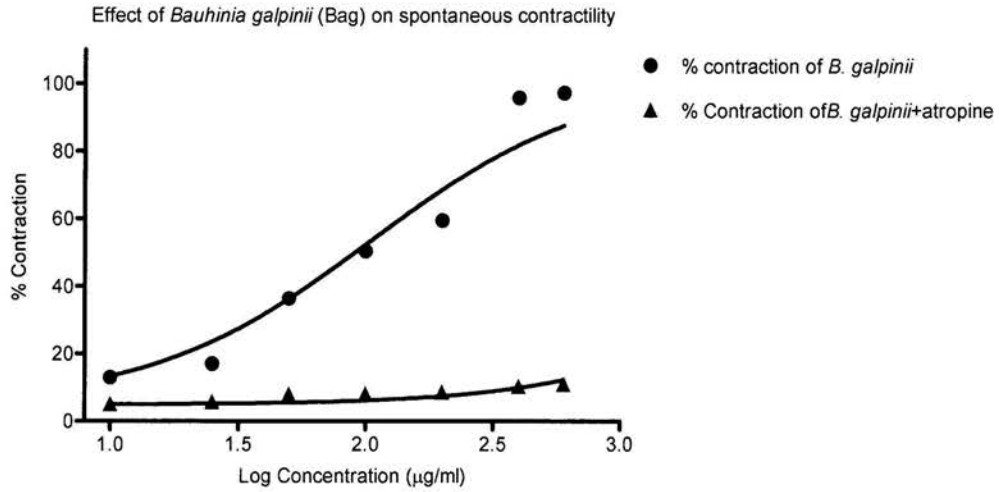


Fig.8.2: Stimulatory effect of 70% acetone leaf extract of *B. galpinii* on spontaneous contractility of isolated rat ileum and the antagonised effect of atropine.

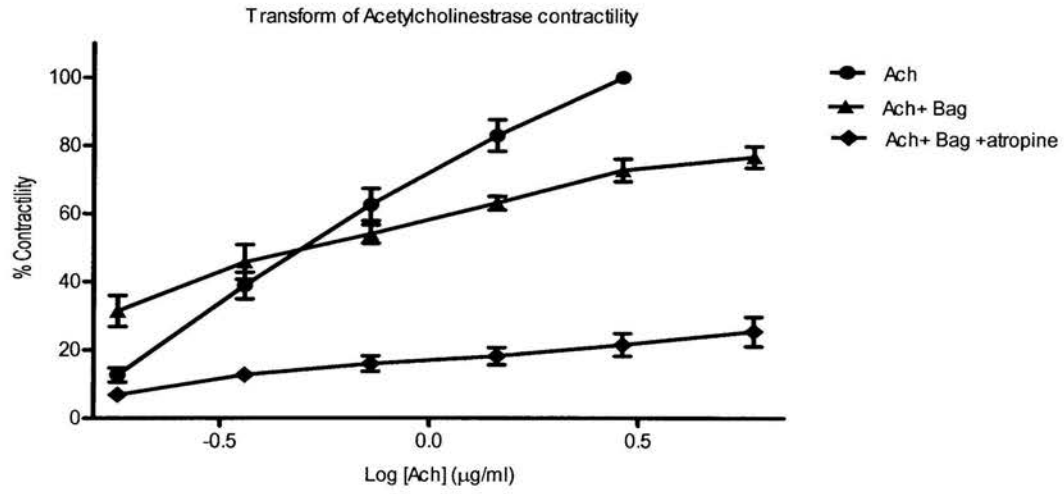


Fig. 8.3. Effect of 70% acetone leaf extract of *B. galpinii* (200 µg/ml) on the acetylcholine cumulative concentration-effect curves in the presence and absence of atropine

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.

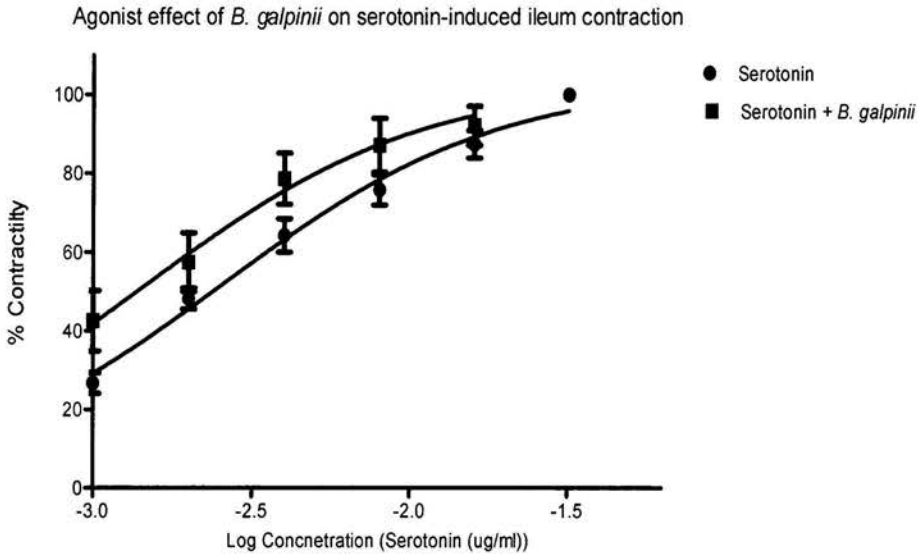


Fig. 8.4. Agonised effect of 70% acetone leaf extract of *B. galpinii* (200 µg/ml) on serotonin induced-contraction on rat isolated ileum.

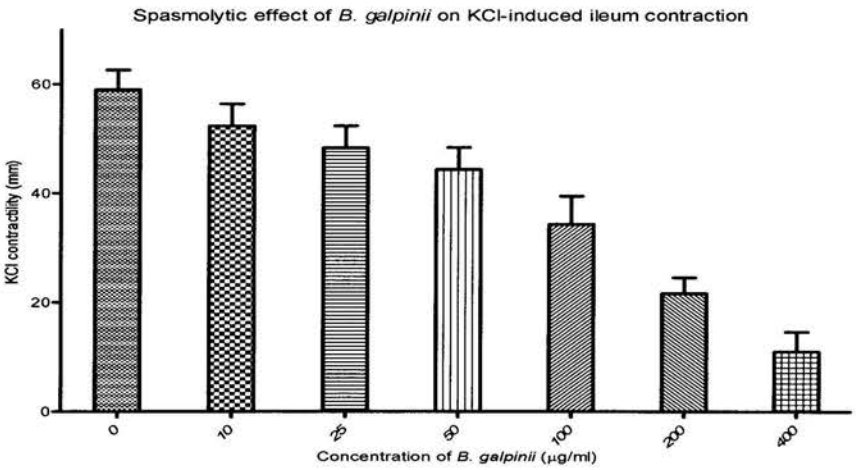


Fig. 8.5. Relaxant effect of 70% acetone leaf extract of *B. galpinii* on KCl induced contractility of isolated rat ileum

### 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 µg/ml at the concentration of 0, 100, 200, 400, and 600 µg/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 µg/ml at the concentration of 0, 100, 200 µg/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.

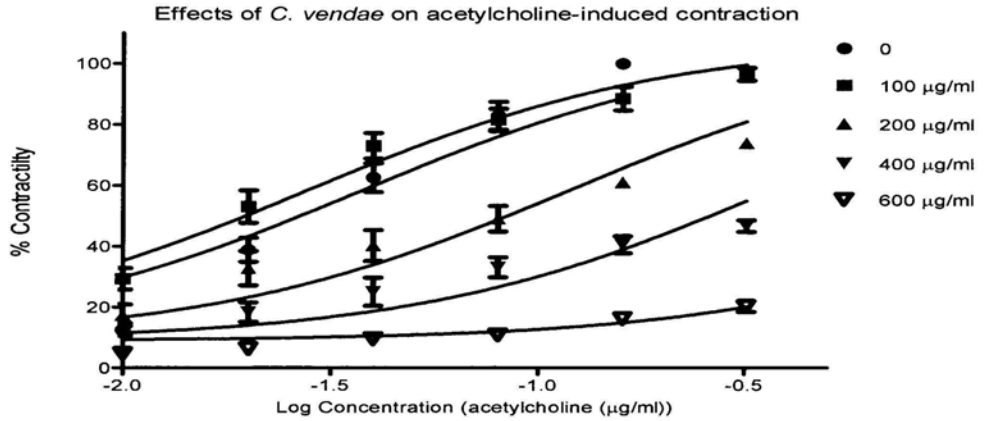


Fig.8.6. Spasmolytic effect of 70% acetone leaf extract of *C. vendae* on Ach-induced contractility of isolated rat ileum

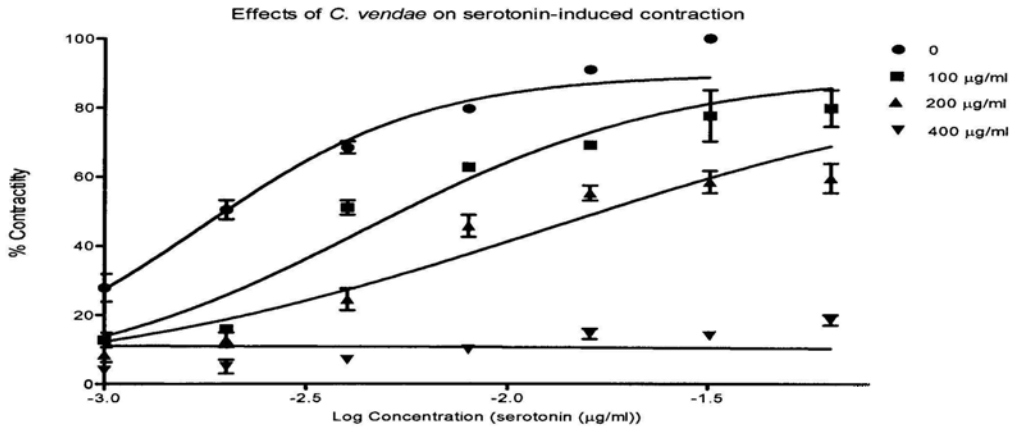


Fig.8.7. Relaxant effect of 70% acetone leaf extract of *C. vendae* on 5-HT-induced contractility of isolated rat ileum

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.

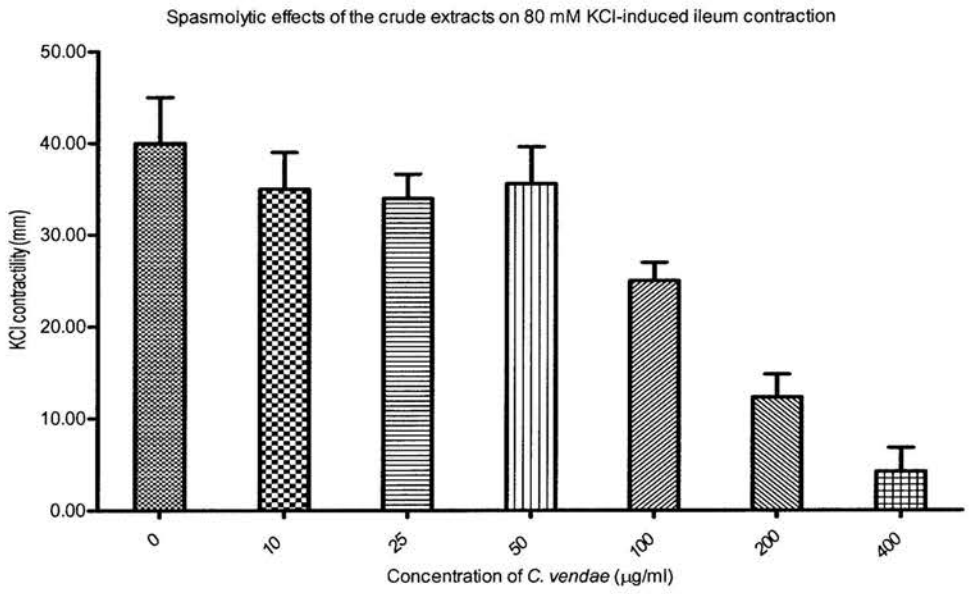


Fig. 8.8. Spasmolytic effect of the C. vendae on the depolarised KCl-induced isolated rat ileum contractions

## 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-$HT_1$, 5-$HT_2$, 5-$HT_3$, 5-$HT_4$, 5-$HT_5$, 5-$HT_6$ and 5-$HT_7$. However, only 5-$HT_1$, 5-$HT_2$, 5-$HT_3$, 5-$HT_4$ and 5-$HT_7$ receptor subtypes are known to affect GIT motor functionality.

The 5-$HT_3$ and 5-$HT_4$ 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-$HT_3$ receptor induces a rapid depolarization of the mesenteric neuron through enhancing ACh release (Kim, 2009), while 5-$HT_4$ 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 $Ca^{2+}$. Increase and decrease in intracellular free $Ca^{2+}$ 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 $Ca^{2+}$ from sarcoplasmic stores and extracellular $Ca^{2+}$ influx through L-type channels (Makhlouf, 1994). Therefore smooth muscle relaxations can be effected by antispasmodic drugs through the inhibition of $Ca^{2+}$ 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 $Ca^{2+}$ channels and influx of extracellular $Ca^{2+}$ (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 well-known non-selective muscarinic receptor antagonists. Atropine competes with Ache 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 erotogenic activation of 5-HT receptor pathways or through the enhanced release of other neurotransmitters without erotogenic 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 $Ca^{2+}$ 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 $Ca^{2+}$ 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-contractility 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 $Ca^{2+}$ 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.