CHAPTER 5

DISCUSSION

The traditional systems of medicine have a crucial role to play in the development of novel drug. However, many medicinal plants are used, particularly in developing countries, without regard to therapeutic, phytochemical and toxicological evaluation. It is thereby expedient to direct research efforts towards the development of novel remedies from traditional drugs. In the present study, methanolic extracts of 3 medicinal plants, *P. longum*, *P. sarmentosum* and *Q. infectoria*, used to alleviate bloody diarrhea in folk medicine, were evaluated for its efficacy to treat the cause of this symptom. Although the cause of bloody diarrhea was not determined or recorded in the folk medicine, amebiasis caused by *E. histolytica* infestation is very likely to be the cause. Caecal amoebiasis in mice was therefore selected to be a model to test their effects. This study also extended our investigation to evaluate whether the antidiarrheal activity of those medicinal plants was generated by their effects to reduce intestinal motility.

The results from the present study demonstrated that methanolic extracts from selected medicinal plants are effective against *E. histolytica* in mice as evaluated by the number of mice cured and the reduction of severity of the mice caecal content and caecal wall lesions in comparison to the untreated mice. The anti-amoebic effects of all extracts are clearly dose-dependent. Most of the published data on medicinal plants against *E. histolytica in vivo* is based on the rat model (Sohni *et al.*, 1995; Ghoshal *et al.*, 1996). Our study shows for the first time that the selected medicinal plant also reduces the severity of caecum due to *E. histolytica* infection in mice.

The pooled controls of 20 mice were all positive for amoebae at the time of sacrifice. This amoebic infection generally produced score of caecal content and caecal wall ranging between 2 and 3 with the average of 2.55

and 2.40, respectively. This indicates the virulence of the strain of *E. histolytica* used in this study. Although, this strain was originally isolated from human bloody stool diarrhea, it was still infective in mice. It is generally known that axenic strain of *E. histolytica* becomes non-invasive after prolonged cultivation *in vitro* (Phillips *et al.*, 1972; Phillips, 1973). We found that the amoebae isolated from the control mice infected with this strain of *E. histolytica* was still virulence and could be used subsequently. In the present study, mice treated with metronidazole at a concentration of 125 mg/kg per day for 5 days were successfully cured from amoebiasis, confirming that this strain of *E. histolytica* was still sensitive to this drug. Our results on the efficacy of metronidazole were similar to the studies of several investigators whose studies on caecal amoebiasis were performed, both in rats and mice models (Bhopale *et al.*, 1995; Sohni *et al.*, 1995; Ghoshal *et al.*, 1996).

The extract from *P. longum* appeared to be the most effective at a concentration of 1000 mg/kg per day, as this dose cleared all *E. histolytica* from the intestine of mice on the day of examination. This is comparable to metronidazole at the dose 125 mg/kg per day. Although treatment with extract from *P. longum* at a concentration of less than 1000 mg/kg per day did not cure all animals, the caecal content and caecal wall of these mice appeared normal indicating the effectiveness of the extract against the parasites. The use of this extract to treat amoebiasis may at least help in reducing severity occurred in the intestine. Our finding from this study on the effect of *P. longum* on *E. histolytica* are consistent with those previously reported that an ethanol extract of *P. longum* at a concentration of 1000 mg/ml per day can cure 90% of rats infected with *E. histolytica* (Ghoshal *et al.*, 1996). Although *P. longum* is effective for the treatment of amoebiasis in rodents, the mode of actions of *P. longum* extract against *E. histolytica* is unknown. An *in vitro* study showed that allicin from freshly crushed garlic inhibited the activity of cysteine proteinases, an important contributor to

amoebic virulence (Ankri *et al.*, 1997). In addition, piperine which is widely known to be a major constituent of *P. longum* is not effective as an amoebicide *in vitro* (Ghoshal *et al.*, 1996). Further investigations are therefore needed to identify an active compound of this extract and to determine whether the alteration of the enzyme activity is the target mode of action of this extract.

The methanolic extracts from *P. sarmentosum* and *Q. infectoria* appeared to be effective against caecal amoebiasis in mice in this study. However, the effect of these extracts on the amoebiasis seem to be much less potent than that of *P. longum.* It is unlikely therefore that their antidysenteric activity occur solely from the antiamoebic activity. Their mode of actions on the treatment of dysentery might be the result of activities performed in concert which have yet to be determined. We therefore study further to test whether these extracts contained antidiarrheal activity which could be a mode of action of each extract.

In the present study, the methanolic extracts of the three plants, *P. longum, P. sarmentosum and Q. infectoria* completely abolished the contraction-induced by acetylcholine and serotonin of rat ileums and those induced by histamine of guinea-pig ileums. Similar results were obtained for their corresponding receptor antagonists (atropine, cyproheptadine and chlorpheniramine, respectively), the reference antidiarrheal drug, loperamide and the L-type calcium channel blocker, verapamil. The order of potency (on μg/ml concentration basis) in the inhibition of acetylcholine-induced rat ileum contractions were atropine > loperamide = verapamil > *P. Sarmentosum* = *P. longum* > *Q. infectoria.* The same order of potency was obtained for suppression of histamine-induced guinea-pig ileum contraction in which the histamine-H_1 receptor antagonist, chlorpheniramine was the most potent. Slightly different potency was found for the inhibition of serotonin-induced rat ileum contractions. The order of potency was cyproheptadine > loperamide, verapamil > *P. Sarmentosum* = *P. longum* = *Q.*

infectoria. Dimethyl sulfoxide (DMSO) which used as a solvent for dissolving *P. longum* and *P. sarmentosum* had no effect on the contractivity of rat and guinea-pig ileums induced by all spasmogens used in this study. Similar to the agonist-induced contractions, the three plant extracts and loperamide also depressed both phases (phasic and tonic) of contraction-induced by high K^+ solution on rat ileums in the concentration-dependent manners. The order of potency is similar to their inhibitory effects on agonists-induced contractions, *i.e.* loperamide > *P. Sarmentiosum* = *P. longum* > *Q. infectoria.*

The agonists, ACh, 5-HT and histamine cause intestinal smooth muscle contraction by acting on the muscarinic M_3, histamine H_1 and serotonin 5-HT_{2A} receptors, respectively. All these receptors are G-protein coupled, when stimulated they activate G_q and IP_3/DAG system which cause intracellular release of calcium ion and muscle contraction (Hoyer *et al.*, 1994, Caulfield and Birdsall, 1998, Brown & Robert, II, 2001, and Sanders-Bush & Mayer, 2001). In our stuidies, the contractions were dose-dependently inhibited by the corresponding receptor antagonists.

Besides the increase in the intracellular calcium release, there were reports showing that the increase in $[Ca^{+2}]_i$ by the agonists were also due to the influx of calcium through voltage-gated calcium channel. In addition, there have been suggested that the release of stored calcium in smooth muscle such as the longitudinal muscle of the guinea-pig small intestine is only important for contractions to near maximally effective concentration of carbachol, and then only briefly during initial tension development (Blackwood and Bolton, 1991; Brading and Snedden, 1980). Pacaud and Bolton (1991) suggested that calcium released from stores did not directly determine tension but did so indirectly by potentiating receptor-operated channel.

Reviewed by Makhhlouf and Murthy (1997) provides evidences that there are differences in the sources of Ca^{2+} responsible for the initial phases of agonist-induced contractions between circular and longitudinal muscles. The initial Ca^{2+} transient in circular muscle cells is not affected by Ca^{2+} channel blocker or withdrawal of Ca^{2+} from the medium (Grider and Makhlouf, 1988; Murthy *et al.*, 1991). Depletion of Ca^{2+} stores with the sarcoplasmic Ca^{2+}/ATPase inhibitor, thapsigargin, abolish the agonist-induced increase in $[Ca^{2+}]_i$, confirming the initial step in Ca^{2+} mobilization is the release of Ca^{2+} from sarcoplasmic store (Kuemmerle *et al.*, 1995). However, Ca^{2+} release in intestinal longitudinal muscle exhibit an obligatory dependence on an initial step involving Ca^{2+} influx into the cell. The agonist-induced increase in $[Ca^{2+}]_i$, Ca^{2+} release from sarcoplasmic stores and initial contraction are abolished in Ca^{2+}-free medium or in the presence of Ca^{2+} channel blocker (Grider and Makhlouf, 1988; Murthy *et al.*, 1991; Kuemmerle *et al.*, 1995). The calcium channels are opened indirectly by membrane depolarization caused by the agonist-induced inward current by opening of nonselective cation channels or blocking of delayed rectified potassium channels or activation of chloride channels (Samueli *et al.*, 1984; Inou and Isenberg, 1990; Komori *et al.*, 1992; Sato *et al.*, 1994; Carl *et al.*, 1995; Dessy & Godfraind, 1996).

The Ca^{2+} mobilization in the longitudinal muscle involves a cascade initiated by agonist-induced transient activation of phospholipase A_2 (PLA_2) by pertussis toxin-sensitive (PTX) G-protein and formation of arachinodic acid (AA), AA-depolarization of the plasma membrane and opening of voltage-sensitive Ca^{2+} channels. The influx of Ca^{2+}-induces Ca^{2+} release by activating sarcoplasmic ryanodine receptor/Ca^{2+} channels and stimulates cADPR formation which enhances Ca^{2+}-induced Ca^{2+} release (Makhlouf and Murthy, 1997). The effect of AA is not exerted directly on plasmalemma Ca^{2+} channels but involves activation of Cl^- channels resulting in depolarization of and opening of voltage-sensitive Ca^{2+} channels

(Kuemmerle and Makhlouf, 1994). However, others presented evidences that the agonist-induced membrane depolarization were due to the opening of nonselective cation channels (Inou and Isenberg, 1990, Pacaud and Bolton, 1991; Komori *et al.*, 1992) or the suppressing of Ca^{2+}-activated K^+ current (Sim *et al.*, 1985; Cole *et al.*, 1989, Carl *et al.*, 1995; Faber, 2003). Inou and Isenberg (1990) and Pacaud and Bolton (1992) reported that the increase in calcium influx through votage-gated calcium channel caused by ACh and carbachol in the guinea-pig ileum were not directly, but via a membrane depolarization (Inoue and Isenberg, 1990; Pacaud & Bolton, 1991). The depolarization resulted from agonist-induced inward currents by opening of nonselective cation channels. The nonselective cation channels were gated by muscarinic receptor activation via a pertussis toxin-sensitive G-protein (Inoue & Isenberg 1990; reviewed by Bolton *et al.*, 1999) which was in contrast to the PTX-insensitive G-protein involved in the release of Ca^{+2} store (Komori *et al.*, 1992). However, Inoue & Isenberg (1990) also demonstrated that without ACh, micromolar calcium concentration were unable to activate the nonselective cation channels. Thus, an increase in calcium concentration alone is insufficient for the channel activation. Similarly, Komori *et al.*, (1992) using single smooth muscle cells of the longitudinal muscle layer of guinea-pig ileum, suggested that stimulation of histamine receptor caused release of Ca^{+2} from storage sites and activation of cationic channels which may be mediated via a PTX-insensitive and sensitive G-protein, respectively.

Although Makhlouf and Murthy (1997) presented evidences that only the initial transient contraction was Ca^{2+}-dependent but the sustained phase was mediated by Ca^{2+}-independent pathway. The latter phase of contraction is mediated by a Ca^{2+}-independent isoform of PKC, PKCε. The pattern of PKC activation parallels that of DAG formation, with a sustained phase coinciding with that of sustained muscle contraction. The mechanism whereby PKC regulate sustained contraction has not been defined. It is

possible that PKC regulate the MLC-dependent slowly cycling crossbridges. Alternatively, actin-binding proteins (e.g., caldesmon and/or calponin) may be involved that are initiating interaction of actin and myosin (Horowitz *et al.*, 1995; reviewed by Makhlouf and Murthy, 1997). But there are some works demonstrated that both phases of contraction were Ca^{2+}-dependent (Brading and Snedden, 1980; Morel *et al.*, 1987; Sato *et al.*, 1994; Dessy and Godfraind, 1996). Sato *et al.*, (1994) showed that both the initial and sustained phases of ACh (1μM)-induced-contraction of canine colonic circular muscle were abolished or reduced by nicardipine (1μM) or verapramil (10μM), while the increase in $[Ca^{2+}]_i$ was reduced by about 30%. Thus, they suggested that the influx through voltage-dependent Ca^{2+} channels and release of Ca^{2+} from intracellular stores contribute to the regulation of $[Ca^{2+}]_i$ by ACh. Dessy and Godfrained (1996) also reported that the phasic and tonic contractions and the increase in $[Ca^{2+}]_i$ produced by histamine (10μM), in guinea-pig ileum longitudinal muscle were abolished by the L-type calcium channel blockers, nimodipine (1 μM) and D600 (10μM).

The intestinal smooth muscle, as in other varieties of smooth muscle, exposure to high potassium solution elicits membrane depolarization and thus opens the volatge-dependent (L-type) calcium channel which causing influx of calcium ion and finally, muscle contraction. This contraction is dependent on extracellular calcium (reviewed by Godfraind *et al.*, 1986). It has also been reported that in guinea-pig ileum, exposure to high potassium solution exhibited a biphasic contraction. Initially, a rapidly developing, highly transient phasic component was observed which was followed by a second slower developing and prolonged tonic contraction. Both these components were antagonized by the L-type calcium channel blockers, nifedepine (Triggle *et al.*, 1979) and verapamil (Hurwitz *et al.*, 1980; Franskish, 1983).

In the present study, the preparation of isolated segment of the rat or guinea-pig ileum preparations were set up to record the contractions of the longitudinal muscle (Costa and Furness, 1979). All plant extracts suppressed both agonist- and KCl-induced contractions of the ileums. Accordingly, it seems likely that the extracts probably inhibit the influx of extracellular calcium and produce the relaxing effect. This possibility was confirmed by our further experiment showing that all extracts significantly shifted the concentration-response curves of $CaCl_2$-induced contractions of guinea-pig ileums to the right. Similar results were obtained for loperamide and verapamil. Our results of the two drugs are consistent with the study by Reynolds *et al.*, (1984); the authors also suggested that calcium channel blocking activity is partly contributed to loperamide's antimotility and antidiarrheal activities. In addition, Yagasaki *et al.,* (1978), demonstated the inhibition of acetylcholine and prostaglandin release from guinea-pig ileum by loperamide, an effect that could not be reversed by naloxone. In Ca^{2+}-free solution, loperamide and verapamil had no influence of PGE_1-induced contraction, but markedly inhibited Ca^{2+}-induced contraction (Honda *et al.*, 1994).

Thus, the results of our studies provide additional information of the three medicinal plants, *P. longum, P. sarmentosum* and *Q. infectoria* for their spasmolytic activities against acetylcholine, histamine and serotonin, the chemical mediators which have a major roles in the increasing motility of the intestine during diarrhea and gut inflammation (Cassuto *et al.*, 1982; Balazs *et al.*, 1989; Baum *et al.*, 1989; Matinole *et al.*, 1997). The similar results were obtained for the reference antidiarrheal drug, loperamide and the receptor antagonists. However, the three plant extracts are much less potent. The order of potency was receptor antagonist > loperamide > *P. longum* = *P. sarmentosum* ≥ *Q. infectoria.* The plant extracts and loperamide also relaxed the contractions evoked by KCl depolarization. Moreover, they also behaves like verapamil, the L-type calcium channel blocker, in blocking

the contractile response to cumulative concentration of $CaCl_2$. Therefore, it is suggested that the three plant extracts might inhibit the contractions by blocking the influx of Ca^{+2} probably through voltage-gated L-type calcium channels. These could be a possible mechanism that explains their effects as antidiarrheal agent, antispasmodic and aiding relief of abdominal pain.

In addition to their antimotility effects, the plant extracts might inhibit secretion of water and electrolyte of the intestinal mucosa. Because calcium regulated the balance between absorption and secretion across the intestinal mucosa (Berridge, 1984). Low intracellular calcium levels favor absorption, whereas a rise in intracellular calcium promote secretion (Ilundain and Naftalin, 1979; Donowitz, 1983; Berridge, 1984). The epithelial cells of the crypts of the small intestine are stimulated to secrete water and electrolytes by elevation of cAMP and intracellular Ca^{2+} concentrations (Berne *et al.*, 1998). Acetylcholine, serotonin, substant P and histamine elicit intestinal secretion of water and electrolytes by increasing the concentration of the intracellular Ca^{2+} concentrations and thus may contribute to diarrhea in certain pathogenic conditions, e.g. infectious diarrhea and diarrhea in ulcerative colitis (Cassuto *et al.*, 1982; Balaz *et al.*, 1989; Baum *et al.*, 1989; Kirchgessner *et al.*, 1992; Berne *et al.*, 1998b). Accordingly, modulation of calcium influx in the mucosal may play a role in antidiarrheal action. Chang *et al.*, (1984) have shown that loperamide also prevents the influx of calcium into epithelial cell. Therefore, it is likely that the plant methanolic extract might also possess the antisecretory effects, by inhibition of the influx of Ca^{+2} into the intestinal epithelial cell, which can contribute to their antidiarrheal effects.

Piperine, a major alkaloid of the fruit of *P. longum* has been reported to inhibit contraction-evoked by transmural nerve stimulation of guinea-pig ileum (Cole, 1985) and possessed antidiarrheal activity in mice (Bajad *et al.*, 2001). Thus, it might be possible that piperine may provide the relaxing effect in our studies. However, the constituent of the root of *P. sarmentosum*

which related to the intestinal activity has not been examined, so future study should be performed. The major constituent of the gall of *Q. infectoria* is tannic acid (Tyler *et al.*, 1977). Because of its astringent effect, it might provide a nonspecific blocking effect by interfering with the receptor or ion channel of the mucosa or muscle fiber membrane. Plants with astringent properties are particularly valued to treat diarrhea and dysentery (Heinrich *et al.*, 1992). Since tannin can precipitate the proteins of the enterocytes, reducing the peristaltic movements and the intestinal secretions. The layers formed by the precipitate of proteins on the mucosal surface of the enterocytes also inhibit the development of micro-organisms, thus explaining the antiseptic action of the tannins which also contribute to the treatment of diarrhea (reviewed by Almeida *et al.*, 1995).

In conclusion, these studies provide scientific data support the uses of these three medicinal plants in the treatment of some common gastrointestinal disorders, i.e. diarrhea, abdominal pain and dysentery in folk medicine. In addition, the methanolic extract of *P. longum* fruits appeared to be the most effective among the 3 plant extracts to treat caecal amoebiasis in mice. Its mode of action could be from antiamoebic effect as well as antidiarrheal activity. The methanolic extract of *P. sarmentosum* root and *Q. infectoria* nut gall seem to be much less potent than that of *P. longum* to treat caecal amoebiasis in mice. It is unlikely therefore that these extracts could be used successfully for the treatment of amoebic dysentery. Their use in folk medicine to treat bloody diarrhea could occur mostly from their antidiarrheal activity rather than amoebicidal activity. Their antidiarrheal activities should be further examined in experimental animal as well as their toxicities.