Comparison of Spasmolytic Activities of *Piper longum*, *P. sarmentosum* and *Quercus infectoria* Extracts with Loperamide and Verapamil in Rat and Guinea Pig Intestinal Tissues

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**Keywords:** agonist-induced intestinal contraction, antidiarrheal, antispasmodic, isolated ileum, L-type calcium channel blocker, medicinal plant

**Abstract**

Methanolic extracts from dried fruits, roots and nutgalls of *Piper longum*, *P. sarmentosum*, *Quercus infectoria* respectively, were examined for their spasmolytic activities using isolated rat or guinea pig ileums and compared with a reference antidiarrheal drug (loperamide) and an L-type calcium channel blocker (verapamil). Isolated rat ileums were contracted by acetylcholine (ACh, 10 µM) and serotonin (5HT, 3 µM), while guinea pig ileums were contracted by histamine (Hist, 1 µM). Prior application of *P. longum*, *P. sarmentosum* and *Q. infectoria* extracts into the bathing solution, the contraction-induced by ACh, 5HT and Hist were concentration-dependently inhibited with the IC₅₀ of 91, 6, 54 µg/mL; 88, 13, 44 µg/mL and 343, 36, 377 µg/mL, respectively. Loperamide and verapamil exhibited similar pattern of inhibitions with IC₅₀ of 0.61, 0.89, 0.42 µg/mL and 0.44, 0.15, 0.70 µg/mL respectively. All extracts and both drugs also suppressed the contraction-evoked by KCl (30 mM) in rat ileums and caused the rightward shift of concentration-response curves of CaCl₂-induced contractions of guinea pig ileums in a concentration-dependent manner. It is known that the contraction-induced by agonists and KCl are mainly due to calcium influx through L-type calcium channels which opened indirectly and directly by agonist-receptor activation and KCl respectively. Thus, it is concluded that the spasmolytic activities of all extracts may occur mainly through interruption of calcium influx via voltage-gated channels. The order of potency is *P. longum* = *P. sarmentosum* > *Q. infectoria*. However, they are less potent than loperamide and verapamil.

**INTRODUCTION**

The fruits of long pepper (*Piper longum*; Piperaceae), the roots of Cha-plu (Thai name for *Piper sarmentosum*; Piperaceae), and the nutgalls of dyer’s oak (*Quercus infectoria*; Fagaceae) are traditionally used for abdominal pain and as antidiarrheal and antisynergery agents. However, scientific data in support of the claimed curative properties are still lacking. The present study aimed to investigate the effects of the three plant methanolic extracts on the intestinal motility, which might contribute to their antidiarrheal and antispasmodic activities, using isolated rat or guinea pig ileums. The ileums will be induced to contract by acetylcholine (ACh), serotonin (5-HT) or histamine. These substances are chemical mediators released from intestinal mucosa or intramural nerves and have major roles during gastrointestinal disorders (Balazs et al., 1989; Baum et al., 1989; Cassuto et al., 1982; Matinole et al., 1997). The results for the plant extracts were compared with a reference antidiarrheal drug (loperamide) and their calcium channel blocking activities were evaluated by comparison with the standard calcium channel blocker, verapamil.
MATERIALS AND METHODS

Plant Materials and Extractions

Dried fruits of long pepper and nutgalls were purchased from local medicinal plant store; Cha-plu roots were collected in the area of Prince of Songkla University, Hat Yai Campus. They were cleaned with distilled water and allowed to dry under sunlight or at 50°C in a hot air oven. Then, they were pulverized and macerated individually in absolute methanol (ratio of 1 kg of plants/3L methanol). The supernatant was collected after 7 d and evaporated (55°C) under low atmospheric pressure in rotary evaporator. The orange viscous oil (yield 22.8%), dark green semisolid (yield 4.5%) and brown powder (yield 46.7%) were obtained for long pepper fruit, Cha-plu root and nutgall respectively.

Animals and Tissue Preparations

All animals [Wistar rats (250-300 g) and guinea pigs (400-600 g) of either sex] were supplied by the animal house, Faculty of Science, Prince of Songkla University, Hat Yai Campus. They were sacrificed by cervical dislocation and exsanguination. The ileum was removed and put into a petri dish containing 95% O₂ and 5% CO₂ aerated Krebs-Henseleit (Krebs) solution and cut into segments (2 cm long). The ileal segment was then set up in an organ bath filled with 25 mL of 37°C Krebs solution and aerated with 95% O₂ and 5% CO₂. It was loaded with 1 g tension and allowed to equilibrate for 30 min before starting the experiment. The ileal contraction was recorded isometrically with a force FT03 displacement transducer connected to a Grass Model 7H polygraph (Grass International Co., Quincy, MA, USA).

Experimental Procedures

1. Inhibitory Effects on Spasmogen-induced Contractions. After 30 min equilibration period, rat ileum was induced to contract by submaximal concentration of either spasmogens 10⁻⁵ M ACh, or 3x10⁻⁶ M 5-HT. Guinea pig ileum was contracted with 10⁻⁶ M histamine. When stable responses to the spasmogen were obtained, the effects of the test agents (plant extracts, loperamide or verapamil) on the contractions were determined by addition into the bathing solution. Fifteen minutes later, second response to the spasmogen was obtained. This step was then repeated using higher concentrations of test agents. In parallel control experiments, the effects of spasmogen were also performed in the presence of prior addition of equal volume of vehicle.

2. Inhibitory Effects on the KCl (30 mM)-induced Contractions. The rat ileum was induced to contract by KCl 30 mM. After stable responses to KCl were reached, the plant extracts or loperamide were added in the bathing solution. Fifteen minutes later KCl 30 mM was added again. This step was then repeated 2-3 times using higher concentration of the test agents.

3. Inhibitory Effects of the Plant Extracts and Loperamide on CaCl₂-induced Contractions. As described by Reynolds et al. (1984), the isolated segment of guinea pig ileum was set up in an organ bath containing a high K⁺ (KCl 80 mM) and calcium free saline solution. The tissue was allowed to equilibrate for 45 min, during which it was washed every 10 min with the fresh saline solution. Then the cumulative concentration-response relationship of CaCl₂ was obtained by addition into the bathing solution at 2.5 min intervals. The plant extracts or loperamide were added to the bath immediately after washing the tissue and 20 min later, a cumulative dose-response curve of CaCl₂ was obtained. In control experiments, pairs of concentration-response curves of CaCl₂ were performed with the addition of the vehicles.

Drugs and Chemicals

Acetylcholine perchlorate, histamine diphosphate, 5-hydroxytryptamine hydrochloride (serotonin), loperamide hydrochloride and verapamil hydrochloride were purchased from Sigma (St. Louis, USA). Chlorpheniramine maleate for injection was purchased from A.N.B. Laboratory Co., LTD (Bangkok, Thailand). The Krebs-Henseleit
solution had the following composition (mM): NaCl, 118.4; KCl, 4.7; CaCl$_2$, 2.9; NaHCO$_3$, 25; MgSO$_4$.$7$ H$_2$O, 1.2; KH$_2$PO$_4$, 1.2; D-glucose, 11.7; and ascorbic acid, 0.14. The high potassium and calcium free saline solution had the following composition (mM): NaCl, 55.2; KCl, 80; MgCl$_2$, 0.5; NaH$_2$PO$_4$, 0.4; NaHCO$_3$, 12 and D-glucose, 5.6. All chemicals were of analytical grade.

The stock solution (1 g/mL) of nutgall was made by dissolving in distilled water, while (0.1 g/mL) of long pepper fruit or Cha-plu root was prepared by dissolving in 50% dimethyl sulfoxide (DMSO). They were kept at -4°C until use. For each day of experiment, working solutions were freshly diluted from the stock solution (with distilled water or 10% DMSO) to appropriate concentrations.

Statistical Analysis
The results were expressed as mean ± standard error of mean. For each group, the log concentration-response curves were plotted. Regression lines were fitted to the linear portion of the log concentration response curves by method of least squares. IC$_{50}$ values (concentration required to produce 50% of the maximum effect) were determined using PHARM/PCS computer program.

Data were analyzed using analysis of variance (ANOVA). Analysis was performed on the individual IC$_{50}$ values obtained from each concentration-response curve for the test agents. When a significant heterogeneity in their estimate due to treatment was observed, further comparison of individual IC$_{50}$ value was made by Newman-Keuls test (P<0.05).

RESULTS

Effects of Plant Extracts, Loperamide and Verapamil on Rat or Guinea pig Ileal Contraction-induced by Various Spasmogens

1. Effects of Plant Extracts. The methanolic extracts of long pepper fruit, Cha-plu root (0.01-1 mg/mL) and nutgall (0.1-10 mg/mL) exerted inhibitory effects on contractions of rat ileums induced by ACh or 5HT and on guinea pig ileal contraction-induced by histamine in a concentration-dependent manner (Fig. 1). All contractions were completely abolished by the highest concentration of the extract used.

The IC$_{50}$ values of spasmolytic effects of the three plant extracts showed that Cha-plu root was as potent as long pepper fruit and they were about 4-8 times more potent than nutgall in the inhibition of ACh- and histamine-induced ileal contractions (Table 1). In the contraction-induced by 5HT, inhibitions of the three plant extracts were similar.

2. Effects of the Reference Drug, Loperamide. Loperamide (0.3-10 µg/mL) produced similar inhibitory effect to those of the plant extracts. The highest concentration of loperamide (10 µg/mL) completely abolished the contraction-induced by all spasmogens. The results showed that loperamide was a very potent spasmylytic agent and was more potent than long pepper fruit, Cha-plu root and nutgall extracts by 18-187, 15-139, and 96-1,380 times, respectively. The concentration-response lines of loperamide and the three plant extracts did not differ from parallelism (Fig. 1).

3. Effects of the Calcium Antagonist Drug, Verapamil. Verapamil ($10^{-8}$-$10^{-4}$ M or 0.0049-49 µg/mL), caused significant decrease in the contractions of the isolated rat and guinea pig ileum-induced by ACh, 5HT and histamine. The blockade was concentration related and the concentration response curves to the spasmogens were similar for loperamide and the three plant extracts (Fig. 1).

Comparison of the Plant Extracts and Loperamide on Rat Ileal Contraction-Induced by Potassium Chloride

High K$^+$ solution (30 mM) caused biphasic contractions of rat ileum. Initially, a rapidly developing, highly transient phasic component was observed which was followed by a second slower developing and prolonged tonic contraction. Both components of the contractile responses were significantly depressed by the three plants extracts and
loperamide in a concentration-dependent manner.

The potency of the test agents in terms of \( IC_{50} \) and relative potency ratio indicated that all plant extracts are less potent than loperamide (Table 2).

**Comparison of the Plant Extracts, Loperamide and Verapamil on Guinea Pig Ileal Contraction-Induced by CaCl\(_2\)**

In guinea pig ileum preparations contracted with CaCl\(_2\), the extracts of long pepper fruit (0.1-1 mg/mL), Cha-plu root (0.1-1 mg/mL), and nutgall (1-10 mg/mL), loperamide (0.1-1 µg/mL) and verapamil (10\(^{-8}\)-10\(^{-7}\)M or 4.9-49 ng/mL), exhibited rightward shift of concentration-response curves of CaCl\(_2\) (Fig. 2A-E).

**DISCUSSION**

The methanolic extracts of the three plants completely abolished the ileal contraction-induced by all agonists (used as spasmogens), ACh, 5-HT and histamine. Similar results were obtained for loperamide and verapamil. All extracts and loperamide also inhibited both phases of contraction-induced by high K\(^+\) solution of rat ileums.

The agonists, ACh, 5-HT and histamine cause intestinal smooth muscle contractions by activating the muscarinic M\(_3\), histamine H\(_1\) and serotonin 5-HT\(_{2A}\) receptors, respectively, causing increase in intracellular calcium ([Ca\(^{2+}\)]) by releasing of Ca\(^{2+}\) from intracellular stores and thus, muscle contractions (Brown and Robert, 2001; Eglen et al., 1996; Sanders-Bush and Mayer, 2001). Besides the intracellular source, the increase in [Ca\(^{2+}\)] are also due to the influx of Ca\(^{2+}\) via voltage-gated calcium channels. 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 (Carl et al., 1996; Dessey and Godfraind, 1996; Inoue and Isenberg, 1990; Komori et al., 1992; Samueli et al., 1984; Sato et al., 1994). However, it has 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 (another muscarinic agonist), 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 opening and increasing calcium entry through voltage-dependent calcium channels.

The intestinal smooth muscle exposures to high K\(^+\) solution elicits membrane depolarization and thus opens the voltage-dependent L-type calcium channel causing influx of Ca\(^{2+}\) and finally, muscle contraction. These contractions are dependent on extracellular calcium (Godfraind et al., 1986). In guinea pig ileums, exposure to high potassium solution exhibited a biphasic contraction. Both contractions were antagonized by the L-type calcium channel blockers, verapamil (Hurwitz et al., 1980). All plant extracts suppressed both agonist- and KCl-induced contractions of the ileums. Thus, it seems likely that the extracts probably inhibit the influx of extracellular calcium and produce the relaxing effect. This possibility was confirmed by the shifting of concentration-response curves of CaCl\(_2\)-induced contractions of guinea pig ileums to the right. Our results of the two drugs are consistent with the study by Reynolds et al. (1984) who also suggested that calcium channel blocking activity is partly contributed to loperamide’s antimitotility and antidiarrheal activities.

The results of the present studies provide additional information of the three medicinal plants, long pepper, Cha-plu and dyer’s oak nutgall for their spasmolytic activities against ACh, 5HT and histamine, the chemical mediators having a major role in the increased motility of the intestine during diarrhea and gut inflammation. Similar results were obtained for the reference antidiarrheal drug, loperamide and the calcium channel blocker, verapamil. However, the three plant extracts are much less potent than the two reference drugs. The plant extracts also inhibited the contractions evoked by KCl depolarization and behave like verapamil, in blocking the contractile response to.
cumulative increase in concentration of CaCl₂. Therefore, it is speculated that the plant extracts might inhibit the contractions by interrupting the influx of Ca²⁺ probably through voltage-gated L-type calcium channels. These may be possible mechanisms that explain their effects as antidiarrheal agents, antispasmodic and aiding relief of abdominal pain.

ACKNOWLEDGEMENT
The authors thank the Graduate School of Prince of Songkla University for the financial support.

Literature Cited

Tables

Table 1. IC$_{50}$ values (concentration producing 50% of maximum inhibition of spasmogen-induced contractions of rats or guinea pig isolated ileum, n=5) of the three plant methanolic extracts, loperamide and verapamil.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (confidence limits) (µg/mL)</th>
<th>ACh (10 µM)</th>
<th>5HT (3 µM)</th>
<th>Histamine (1 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long pepper fruit</td>
<td></td>
<td>91 (65-130)</td>
<td>6 (2-13)</td>
<td>54 (20-145)</td>
</tr>
<tr>
<td>Cha-plu root</td>
<td></td>
<td>88 (66-117)</td>
<td>13 (7-23)</td>
<td>44 (14-135)</td>
</tr>
<tr>
<td>Nutgall</td>
<td></td>
<td>343 (95-1239)</td>
<td>36 (10-131)</td>
<td>377 (203-701)</td>
</tr>
<tr>
<td>Loperamide</td>
<td></td>
<td>0.61 (0.37-1.02)</td>
<td>0.89 (0.57-1.39)</td>
<td>0.42 (0.20-0.82)</td>
</tr>
<tr>
<td>Verapamil</td>
<td></td>
<td>0.44 (0.16-1.26)</td>
<td>0.15 (0.07-0.30)</td>
<td>0.70 (0.35-1.42)</td>
</tr>
</tbody>
</table>

Table 2. The IC$_{50}$ values of the three plant extracts and loperamide on the inhibitions of phasic and tonic contractions-induced by KCL (30 mM) of rat ileums and the potency ratios (on µg/mL concentration basis) of the plant extracts compared to loperamide (Lop) (n=5).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (confidence limits) (µg/mL)</th>
<th>Potency ratio (Lop:extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phasic contraction</td>
<td>Tonic contraction</td>
</tr>
<tr>
<td>Long pepper fruit</td>
<td>264 (123-563)</td>
<td>406 (253-651)</td>
</tr>
<tr>
<td>Cha-plu root</td>
<td>153 (79-299)</td>
<td>157 (53-463)</td>
</tr>
<tr>
<td>Nutgall</td>
<td>932 (609-1426)</td>
<td>848 (612-1175)</td>
</tr>
<tr>
<td>Loperamide</td>
<td>0.86 (0.61-1.20)</td>
<td>0.88 (0.66-1.18)</td>
</tr>
</tbody>
</table>
**Figures**

**Fig. 1.** Inhibitions of ACh-induced and 5HT-induced contractions of rat ileums and histamine (Hist)-induced contractions of guinea pigs ileums by loperamide, verapamil and the methanolic extracts of Cha-plu root, long pepper fruit and nutgall. Vertical lines represent standard error of means (n=5).

**Fig. 2.** Cumulative concentration-effect curves of calcium chloride-induced contractions of guinea pig ileums by various concentrations of (A) Cha-plu root, (B) long pepper fruit, (C) nutgall extracts, (D) loperamide and (E) verapamil. Vertical lines represent standard error of means (n=5).