January 2011

The antidiarrheal and spasmolytic activities of Phyllanthus emblica are mediated through dual blockade of muscarinic receptors and Ca(2+) channels

Malik Hassan Mehmood  
_Aga Khan University_

Hasan Salman Siddiqi  
_Aga Khan University_

Anwarul Hassan Gilani  
_Aga Khan University_

Follow this and additional works at: http://ecommons.aku.edu/pakistan_fhs_mc_bbs

Part of the Natural Products Chemistry and Pharmacognosy Commons

Recommended Citation

Available at: http://ecommons.aku.edu/pakistan_fhs_mc_bbs/140
The antidiarrheal and spasmolytic activities of *Phyllanthus emblica* are mediated through dual blockade of muscarinic receptors and Ca$^{2+}$ channels

Malik Hassan Mehmood, Hasan Salman Siddiqi, Anwarul Hassan Gilani

Keywords:
- *Phyllanthus emblica*
- Antidiarrheal
- Anticholinergic
- Ca$^{2+}$ antagonist
- Euphorbiaceae
- Antispasmodic

**Aim of the study:** This study was aimed at providing the possible mechanisms for the medicinal use of *Phyllanthus emblica* in diarrhea.

**Materials and methods:** The in vivo studies were conducted in mice, while isolated rabbit jejunum and guinea-pig ileum were used for the in vitro experiments.

**Results:** The crude extract of *Phyllanthus emblica* (Pe.Cr), which tested positive for alkaloids, tannins, terpenes, flavonoids, sterols and coumarins, caused inhibition of castor oil-induced diarrhea and intestinal fluid accumulation in mice at 500–700 mg/kg. In isolated rabbit jejunum, Pe.Cr relaxed carbachol (CCh) and K$^{+}$ (80 mM)-induced contractions, in a pattern similar to that of dicyclomine. The preincubation of guinea-pig ileum with Pe.Cr (0.3 mg/mL), caused a rightward parallel shift in the concentration–response curves (CRCs) of acetylcholine without suppression of the maximum response. While at the next higher concentration (1 mg/mL), it produced a non-parallel rightward shift with suppression of the maximum response, similar to that of dicyclomine. The activity-directed fractions of Pe.Cr showed a combination of Ca$^{2+}$ antagonist and anticholinergic like components in all fractions but with varying potency.

**Conclusion:** These results indicate that the *Phyllanthus emblica* fruit extract possesses antidiarrheal and spasmolytic activities, mediated possibly through dual blockade of muscarinic receptors and Ca$^{2+}$ channels, thus explaining its medicinal use in diarrhea.

© 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Diarrheal diseases are the major causes of illness and death all over the world. Around 88% of diarrheal-related deaths are caused due to inadequate sanitation and poor hygiene (Kosek et al., 2003). Diarrhea is considered as one of the leading causes of growth retardation and death in infants (Petri et al., 2008). In developing countries, the death rate due to diarrhea is high (1.5–2 million/year) amongst the children under five years of age (WHO, 2009). Despite the availability of several remedies to treat diarrhea including botanicals and chemical agents, yet there is a great need for the evaluation of newer, economical and cost-effective agents to meet the challenges of upcoming era regarding disease burden. According to World Health Organization, the use of traditional remedies in healthcare system is increasing day by day (Bodeker et al., 2006; Hollenberg et al., 2008). It is well accepted that herbal remedies are relatively safe, affordable and easily accessible to layman, when compared with that of chemical drugs. Moreover, the plant remedies or naturally sourced products are known to contain synergistic and/or side effects neutralizing potentials, and usually offer their pharmacological actions mediated through multiple pathways (Gilani and Rahman, 2005).

*Phyllanthus emblica* Linn. (Syn. *Embla officinalis* Gaertn.), a member of family Euphorbiaceae, locally known as “amla” is one of the valuable medicinal herbs, used in gastrointestinal disorders (Usmanghani et al., 1997). *Phyllanthus emblica* is very healthful and serves as a good dietary source of vitamin C, amino acids and minerals. All parts of this plant are commonly used in various illnesses, especially its fruit is popular in different traditional systems of medicine (Ayurvedic, Greco-Arab, Chinese) for its usefulness in diarrhea and dysentery, in addition to its other health benefits.
Phyllanthus emblica is one of the three components of a popular Greco-Arab herbal compound preparation, known as Trifla, the other two constituents include Terminalia chebula and Terminalia bellirica (Nadkarni, 1986). Trifla is also known for its chemopreventive and antioxidant activities (Deep et al., 2005; Sandhya et al., 2006).

Phyllanthus emblica is reported to contain ellagitannins, such as, phyllanemblins A–F (Zhang et al., 2001), flavonoids, such as, kaempferol-3-O-α-L-(6′-methyl)-rhamnopyranoside and kaempferol-3-0-α-L-(6′-ethyl)-rhamnopyranoside (Zhang et al., 2001a) and apigenin glucoside (El-Desouky et al., 2008). Its roots are reported to contain norsesquiterpenoid glycosides, such as, phyllaemblicins A–C, E and F, and phyllaemblic acid, a methyl ester (Liu et al., 2009). The leaves, bark and fruits of Phyllanthus emblica are found rich in phenolic contents (Bajpai et al., 2005; Chalise et al., 2010). The plant has also been reported to possess gallic acid, chebulinic acid, quercetin, corilagin and isoorientin (Habib-ur-Rehman et al., 2007). Furthermore, the presence of β-caryophyllene, β-bourbonene, 1-octen-3-ol, thymol and methyleugenol has been documented from its essential oils with antimicrobial activity (Liu et al., 2009a).

In a recent review article, the fruit of Phyllanthus emblica has been described to possess numerous pharmacological activities, such as, gastroprotective, antiulcerogenic, hypolipidemic, and antidiabetic activities (Krishnaveni and Mirunalini, 2010). Moreover, it has also been reported to possess antioxidant (Rao et al., 2005; Reddy et al., 2009), antiaging (Datta et al., 2009), chondroprotective (Sumantran et al., 2008), chemoprotective (Sultana et al., 2008), anti-inflammatory (Sharma et al., 2003; Nicolas et al., 2008), hepatoprotective (Panchabhai et al., 2008), antibacterial (Saeed and Tariq, 2007), antipyretic and analgesic (Perianayagam et al., 2004) activities. Despite the availability of various studies on different aspects of Phyllanthus emblica and a preliminary report showing its efficacy in diarrhea (Perianayagam et al., 2005), it has not been widely studied for the possible mode(s) of action using both the in vivo and in vitro assays, for its medicinal use in hyper-active gut disorders. In this study, we provide evidence that the plant exhibits antidiarreal and antispasmodic activities, mediated possibly through the dual blockade of muscarinic receptors and Ca2+ channels. Moreover, the activity-guided fractionation of the crude extract was carried out, showing that the biologically active constituents were widely distributed in the organic and aqueous fractions.

2. Materials and methods

2.1. Plant material and preparation of the crude extract

The dried fruits of Phyllanthus emblica were purchased from a local market (Jouria Bazaar) of Karachi, Pakistan in June, 2007. A specimen has been preserved at the herbarium of the Natural Product Research Division, Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, with voucher # Pe-F-06-07-100. For preparation of the crude extract of plant material, a previously described method was followed (Mehmood and Gilani, 2010). Plant material was soaked in 70% methanol for 3 days and filtered through muslin cloth and Whatman (Maidstone, UK) No. 1 filter paper simultaneously. This procedure was repeated three times, all the filtrates were pooled and evaporated on a rotary evaporator (Buchii model RE-111, Switzerland) under reduced pressure to get a thick blackish paste like mass, the crude extract of Phyllanthus emblica (Pe.Cr), yielding 7–8% (wt/wt). The extract was collected and diluted were made in distilled water/saline for experimentation.

2.2. Fractionation of the crude extract

Activity-directed fractionation of the crude extract was carried out following standard phytochemical procedures using different organic solvents (Williamson et al., 1998). Approximately 20 g of Pe.Cr was dissolved in 200 mL of distilled water. An equal volume of petroleum spirit was added into it and was shaken vigorously in a separating funnel. The mixture was separated in two layers by a separating funnel without shaking. The petroleum spirit layer (upper) was removed. The extraction with petroleum spirit was repeated two times more. All of the petroleum spirit layers were combined and subjected to evaporation to get the petroleum spirit fraction (Pe.Pet). The lower separated layer was taken into a separating funnel; chloroform was added into it, and separated by following the previous procedure. The lower layer in this case was of chloroform, which was separated and the procedure was repeated two times. The separated chloroform layers were pooled and evaporated to obtain the chloroform fraction (Pe.CHCl3). The remaining layer (upper in this case) was again taken; ethyl acetate was added into it, separated and subjected to evaporation, resulting in the ethyl acetate fraction (Pe.ET.Ac). The remaining lower layer was collected and concentrated to obtain the aqueous fraction (Pe.Aq). The organic fractions were dissolved in 5% DMSO, while the aqueous fraction was dissolved in distilled water/saline. The vehicle used for solubility was found inactive in all experiments.

2.3. Phytochemical analysis

Phytochemical screening of the crude extract of Phyllanthus emblica and its fractions was performed qualitatively for the presence of alkaloids, flavonoids, tannins, coumarins, sterols, terpenes and saponins according to the standard methods (Evans, 2006). Briefly, alkaloids were detected by treating the plant material with Dragendorff’s reagent, resulting in the appearance of a precipitate at the bottom of the test tube. The plant material was deemed positive for flavonoids when it gave a yellow color with AlCl3 reagent, and for tannins when a green or black color was produced with aqueous FeCl3. The observation of yellow fluorescence under ultraviolet light on examination of the filter paper previously exposed to vapors from the boiling plant material indicated the presence of coumarins. For the detection of sterols and terpenes, plant material was treated with petroleum ether and subsequently extracted with CHCl3. The gradual appearance of green to pink (for sterols) and pink to purple colors (for terpenes) was noted after the treatment of CHCl3 layer with acetic anhydride and concentrated HCl in succession. Lastly, the presence of saponins was based on the appearance of froth upon vigorous shaking of diluted samples.

2.4. Drugs and chemicals

Potassium chloride (KCl), loperamide hydrochloride, dicyclomine, carbamylcholine (CCh), nifedipine, acetylcholine chloride (ACh), atropine sulphate, some components of physiological salt solution; calcium chloride (CaCl2), glucose (C6H12O6), magnesium chloride (MgCl2), sodium chloride (NaCl), ethylendiamine-tetraacetic acid (EDTA), aluminum chloride (AlCl3), ammonium hydroxide (NH4OH), Dragendorf’s reagent and ferric chloride (FeCl3) were purchased from Sigma–Aldrich Chemicals Company, St. Louis, MO, USA. Sodium bicarbonate (NaHCO3), sodium dihydrogen phosphate (Na2HPO4) and some solvents like benzene, chloroform, hydrochloric acid and petroleum ether were obtained from E. Merck KGa (Darmstadt, Germany). Castor oil was purchased from Karachi Chemical Industries (Pvt.) Ltd. F/25 S.I.T.E., Karachi (Pakistan). All the chemicals used were of analytical grade and dissolved in distilled water/saline except nifedipine which was dissolved in 5% DMSO and 3% Tween-80. The vehicle used for solubility
was found inert in the in vitro experiments. Stock solutions of all the chemicals were made fresh on the day of experiment.

2.5. Animals

BALB/c mice (weighing 20–25 g), local breed rabbits (weighing 1–1.5 kg) and guinea-pigs (weighing 400–600 g) of either sex were housed at the animal house of Aga Khan University under a controlled environment (23–25 °C). The animals were kept in plastic cages (47 × 34 × 18) with sawdust (changed at every 48 h) and fasted for 24 h following the start of the experiment but routinely were given tap water and standard diet. Experiments were performed with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996) and approved by the Ethical Committee of Aga Khan University.

2.6. In vivo experiments

2.6.1. Castor oil-induced diarrhea

To assess the antidiarrheal activity, a previously described method was followed (Borrelli et al., 2006). Mice (weighing 20–25 g; n = 30) of either sex were fasted for 24 h before starting the experiment. The animals were housed in individual cages and divided into six groups (n = 5 each). The first group received saline in vehicle (10 mL/kg, p.o.) and served as the negative control. The next two groups received different doses (500 and 700 mg/kg) of the plant extract. Further groups received loperamide (10 mg/kg) or dicyclomine (50 and 100 mg/kg), acting as the positive control. One hour after the treatment, each animal received castor oil (10 mL/kg, p.o.) through a feeding needle. At 6th hour after dosing the castor oil, the individual mouse cages were inspected (by an observer not knowing about the particular treatment) for the presence of unformed water fecal pellets; their absence was recorded as a positive result, indicating protection from diarrhea at that time.

2.6.2. Intestinal fluid accumulation

By following an earlier method (Gilani et al., 2005), overnight-fasted mice (weighing 20–25 g; n = 30) of either sex were housed in cages in equally divided five groups (n = 6 each). The first two groups received saline in solubilizing vehicle (10 mL/kg, p.o.) and acted as the negative control. The animals in the third group were treated with loperamide (10 mg/kg), intraperitoneally, using a detachable U-100 insulin syringe with 25G × 1 in. (0.50 mm × 25 mm) needle, as the positive control. The groups number four and five were treated with increasing doses of the plant extract (500 and 1000 mg/kg, i.p.). One hour after the treatment, each animal received castor oil (10 mL/kg) orally except the animals of group one. All the animals were sacrificed 30 min later by cervical dislocation and whole intestine was isolated out and weighed with care, not allowing any intestinal fluid to leak out. The results were expressed as (Pf/Pm) × 1000 where, Pf is the weight of the intestine and Pm is the weight (in g) of the animal.

A preload of 1 g was applied to each tissue, while intestinal contractions were measured using isotonic transducer 50–6360 (Harvard Apparatus, Holliston, MA, USA) coupled with student oscillograph (Harvard Apparatus)/powerLab model ML-845, data acquisition system (ADInstruments, Sydney, Australia) (Gilani et al., 2005a). Each tissue was allowed to equilibrate for at least 30 min before the addition of any drug. Afterwards, each tissue was stabilized with submaximal concentration of acetylcholine (ACh, 0.3 μM). In order to observe the spasmodytic effect of the test substance on induced contractions, high K+ (80 mM) and carbachol (CCh, 1 μM) were used as spasmodyic agents, which were used to determine the different inhibitory mechanisms, such as, Ca2+- antagonist and anticholinergic. The test material was added in a cumulative fashion to obtain the concentration-dependent inhibitory responses. The relaxation of the tissue preparation was expressed as percentage of the control contraction mediated by the added spasmogen.

High K+ (80 mM) was used for the assessment of Ca2+-channel blocking (CCB) activity. It depolarizes the smooth muscle preparations and is known to produce myo-contractions through opening of the voltage-dependent Ca2+ channels, thus allowing influx of extracellular Ca2+ causing contractile effect (Karaki et al., 1997). A substance causing inhibition of high K+-induced contraction is considered as blocker of Ca2+ influx through L-type Ca2+ channels (Godfraind et al., 1986). To further confirm the CCB activity of the test material, the tissue was allowed to stabilize in normal Tyrode's solution, which was then replaced with Ca2+-free Tyrode's solution containing EDTA (0.1 mM) for 30 min, in order to chelate the Ca2+ from the environment and the tissues (Farre et al., 1991). This solution was further replaced with K+-rich and Ca2+-free Tyrode's solution with the following constitution (mM): KCl 50, NaCl 91.04, MgCl2 1.05, NaHCO3 11.90, NaH2PO4 0.42, glucose 5.55 and EDTA 0.1. After an incubation period of 30 min in normal Tyrode's solution, the concentration–response curves (CRCs) of Ca2+ were constructed. When the control CRCs of Ca2+ were found super-imposable (usually after two cycles), the tissue was preincubated with different concentrations of the test substance for 60 min, to note the presence of possible CCB-like activity.

2.7. In vitro experiments

2.7.1. Rabbit jejunum

Rabbits (weighing 1–1.5 kg; n = 20) of local breed and either sex, starved for 24 h, were humanely sacrificed by cervical dislocation and the ileum was isolated out (Mehmood and Gilani, 2010). The tissues were prepared, equilibrated and stabilized by following the above mentioned procedure in Section 2.7.1. The contractile responses in the tissues were recorded using similar isotonic transducers as used for rabbit jejunum. After getting the constant responses of the submaximal concentrations of ACh, the CRCs of ACh were constructed in the absence and presence of the test material for the assessment of the anticholinergic and/or Ca2+-antagonist like activities (Gilani et al., 2008; Khan and Gilani, 2009).

2.8. Acute toxicity testing

Mice (weighing 20–25 g; n = 40) of either sex were divided into different groups of 10 animals each. The test was performed using increasing doses of the test material (3, 5 and 10 g/kg) given orally, in 10 mL/kg saline to different animals serving as the test groups. Another group of mice was administered saline (10 mL/kg) as the negative control. The animal were allowed food and water ad libitum and kept under regular observation for 6 h to observe their pilo erection, changes in exploratory behavior and blindness, while lethality was monitored up to 24 h.
2.9. Statistical analysis

The data expressed as mean ± SEM values (n = number of experiments) and the median inhibitory concentrations (IC50) values with 95% confidence intervals. Chi-square ($\chi^2$) test was applied to differentiate the results in antidiarrheal activity assay. $p < 0.05$ was considered significantly different using one-way analysis of variance and/or unpaired t-test to compare the results of antisecretory assay. The concentration–response curves (CRCs) were analyzed by non-linear regression and two-way ANOVA followed by Bonferroni’s post-test correction was used for multiple comparisons and statistical analysis were performed using GraphPAD program (GraphPAD, San Diego, California, USA).

3. Results

3.1. Phytochemical analysis

The crude extract of *Phyllanthus emblica* and its resultant fractions (Pe.Pet, Pe.CHCl3, Pe.EtAc and Pe.Aq) was found to contain alkaloids, flavonoids, tannins, coumarins, sterols, terpenes and saponins. However, the froth formation (saponins contents) with Pe.Aq < Pe.Pet < Pe.CHCl3 was higher when compared with those of Pe.CHCl3 and Pe.EtAc. Similarly, a critical observation showed that the treatment of Pe.CHCl3 and Pe.EtAc solutions with AlCl3 produced an intense yellow color, indicating the presence of flavonoids, when compared with the parent extract and other fractions.

3.2. In vivo experiments

3.2.1. Effect on castor oil-induced diarrhea in mice

Pe.Cr showed protection from castor oil-induced diarrhea in mice at a dose range of 500–700 mg/kg. The negative control treatment (saline) did not protect the animals from diarrhea, while the animals in groups pretreated with increasing doses of the plant extract showed 40 and 80% protection at 500 and 700 mg/kg, respectively. The positive control, loperamide exhibited 100% protection at the dose of 10 mg/kg, while dicyclomine showed 40 and 100% protection, at 50 and 100 mg/kg, respectively (Table 1).

<table>
<thead>
<tr>
<th>Treatment (p.o.), dose (mg/kg)</th>
<th>No. of mice with diarrhea</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (10 mL/kg) + castor oil</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>Pe.Cr + castor oil</td>
<td>700 = 10</td>
<td>3’/5</td>
</tr>
<tr>
<td>Loperamide + castor oil</td>
<td>10 = 10</td>
<td>0’’/5</td>
</tr>
<tr>
<td>Dicyclomine + castor oil</td>
<td>50 = 10</td>
<td>3’/5</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. saline + castor oil treated group ($\chi^2$-test). ** p < 0.01 vs. saline + castor oil treated group ($\chi^2$-test).

3.2.2. Intestinal fluid accumulation

In the enteropooling assay, castor oil caused a marked ($p < 0.001$) increase of fluid accumulation in the small intestine of mice with $\left(P_i/P_m\right)$ x 1000 value of 159 ± 4.6 as compared with that of the saline treated group $\left[P_i/P_m\right] \times 1000$ value of 100 ± 2.9. Loperamide significantly reduced ($p < 0.001$) the fluid accumulation with $\left[P_i/P_m\right] \times 1000$ value of 72 ± 4.0 at the dose of 10 mg/kg, Pe.Cr showed a dose-dependent reduction in castor oil-induced fluid accumulation at 500 and 700 mg/kg with respective ($P_i/P_m$) x 1000 values of 127 ± 2.9 and 85 ± 1.9 (Fig. 1). All ($P_i/P_m$) x 1000 values are expressed as mean ± SEM (n = 6).

3.3. In vitro experiments

3.3.1. Effect of the crude extract on rabbit jejunum

When tested on CCh (1 μM) and high K+ (80 mM)-induced contractions, Pe.Cr inhibited both of the induced contractions with IC50 values of 0.09 mg/mL (95% confidence interval, 0.05–0.14; n = 6) and 1.38 mg/mL (1.06–1.79; n = 5), respectively, being around 10 times more potent against CCh (Fig. 2A). Dicyclomine was also more potent against CCh than high K+ with respective IC50 values of 0.33 μM (0.26–0.42; n = 5) and 2.75 μM (2.19–3.46; n = 4), as seen in Fig. 2B. Nifedipine was more potent against high K+ than CCh with respective IC50 values of 0.04 μM (0.03–0.04; n = 5) and 0.39 μM (0.34–0.45; n = 4) (Fig. 2C), while atropine inhibited only the CCh contractions with IC50 value of 1 nM (0.9–1.0; n = 6) (Fig. 2D).

3.3.2. Effect of the crude extract on guinea-pig ileum

Guinea-pig ileum is known to be a quiescent preparation and is considered suitable to construct CRCs for the stimulant effect. The CRCs of ACh were constructed with a rightward parallel shift in the control CRCs of ACh (maximum contractile response of ACh was taken 100%), without suppression ($p > 0.05$) of the maximum contractile response (95.0 ± 5.2% vs.100%) (mean ± SEM, n = 4), a typical characteristic of anticholinergic drug. Whereas, at the next tested concentration (1 mg/mL), Pe.Cr produced a non-parallel shift with a significant suppres-
Fig. 2. Concentration–response inhibitory effect of (A) the crude extract of *Phyllanthus emblica* (Pe.Cr), (B) dicyclomine, (C) nifedipine and (D) atropine against K⁺ (80 mM) or carbamylcholine (CCh, 1 μM)-induced contractions in isolated rabbit jejunum preparations. Symbols represent mean ± SEM, values (n = 5–6).

sion (p < 0.001) of the maximum response (43.4 ± 8.2% vs. 100%) (n = 5) (Fig. 3A). Similarly, dicyclomine shifted the CRCs of ACh to the right in a parallel manner without suppression (p > 0.05) of the maximum response (94.4 ± 5.4%) (n = 6) at 0.03 μM, while at the concentration of 0.1 μM, it caused a rightward non-parallel shift in the ACh CRCs with a marked suppression (p < 0.001) of the maximum effect (58.2 ± 4.7%) (n = 6) (Fig. 3B). Nifedipine showed a non-parallel rightward shift in the ACh CRCs to the right with significant suppression (p < 0.001) of the maximum response at both tested concentrations of 0.01 μM (66.4 ± 5.9%) (n = 5), and 0.03 μM (30 ± 7.9%) (n = 6) (Fig. 3C), while atropine caused a parallel rightward shift without suppression (p > 0.05) of the maximum response of ACh at both tested concentrations of 0.003 and 0.01 μM (Fig. 3D).

3.3.3. The CCB activity of Pe.Cr, and the spasmolytic effect of its fractions on rabbit jejunum

The CCB activity was confirmed when pretreatment of the tissue with the plant extract caused a rightward shift in the CRCs of Ca²⁺ with a significant suppression (p < 0.01) of the maximum response of Ca²⁺ (taken as 100%) to a level of 71.4 ± 11.7% (n = 4) and 32.2 ± 4.3% (n = 4), at 1 and 3 mg/mL, respectively (Fig. 4A). Dicyclomine also produced a rightward shift in the CRCs of Ca²⁺ and attenuated (p < 0.01) its maximum response to 83.7 ± 4.8% (n = 6) and 46.6 ± 7.5% (n = 5), at tested concentrations of 3 and 10 μM, respectively (Fig. 4B). Similarly, nifedipine shifted the CRCs of Ca²⁺ to the right with a significant suppression (p < 0.001) of the maximum effect to a level of 64.8 ± 9.3% (n = 4) and 40 ± 5.1% (n = 5) at 0.03 and 0.1 μM, respectively (Fig. 4C).

Amongst the resultant fractions of the plant extract, the petroleum fraction (Pe.Pet) completely relaxed high K⁺-induced contractions at lower concentration with IC₅₀ value of 0.12 mg/mL (0.09–0.16; n = 5), but was less effective against CCh-induced contractions with maximum effect reaching 60 ± 4.4% (n = 5) at the highest tested concentration of 10 mg/mL (Fig. 5A). Similarly, the chloroform fraction (Pe.CHCl₃) was more potent against high K⁺ with IC₅₀ value of 0.02 mg/mL (0.01–0.03; n = 5) than CCh-induced contractions with IC₅₀ value of 0.24 mg/mL (0.09–0.59; n = 3) (Fig. 5B). The ethyl acetate fraction (Pe.EtAc) was more potent against CCh with IC₅₀ value of 0.08 mg/mL (0.05–0.10; n = 4) than high K⁺-induced contractions with IC₅₀ value of 0.34 mg/mL (0.27–0.43; n = 4) (Fig. 5C). The aqueous fraction (Pe.Aq) completely inhibited CCh-induced contractions at lower concentrations with IC₅₀ value of 0.34 mg/mL (0.23–0.51; n = 6). While, it partially relaxed high K⁺-induced contractions with maximum relaxant effect reaching only 54.3 ± 3.6% at 10 mg/mL (Fig. 5D), indicating that the petroleum and chloroform fractions were selective against high K⁺, while the ethyl acetate and aqueous fractions were found potent against CCh-induced contractions.

3.4. Acute toxicity test

Three different groups of mice were administered Pe.Cr, in graded doses of 3, 5 and 10 g/kg, respectively. The animals were kept under observation for the change in behavior or death up to 24 h following the plant extract administration. The extract administration neither caused any significant change in the behaviors nor the death of animal(s) in all the test groups.
4. Discussion

In an attempt to validate the medicinal use of the dried fruit of *Phyllanthus emblica* in gastrointestinal disorders, such as diarrhea, this study was carried out to assess the antidiarrheal and antisecretory effects of the plant extract in mice, and the possible underlying mechanism was evaluated using isolated tissue preparations. In castor oil-induced diarrhea and intestinal fluid accumulation models, Pe.Cr showed antidiarrheal and antisecretory activities at 500–700 mg/kg, similar to loperamide, a standard antidiarrheal agent (Reynolds et al., 1984), or dicyclomine, a known spasmolytic drug acting through dual inhibition of muscarinic receptors and Ca\(^{2+}\) channels (McGrath et al., 1964; Downie et al., 1977). Castor oil increases intestinal fluid contents and causes diarrhea indirectly through recinolic acid formation, which changes the electrolytes and water transport and generates enormous contractions in transverse and distal colon (Iwao and Terada, 1962). The observed antidiarrheal and antisecretory effects of the crude extract following oral administration appear to be mediated through the presence of gastrointestinal relaxant component(s) in *Phyllanthus emblica*.

Gastrointestinal motor tone is regulated through multiple physiological mediators, such as, acetylcholine, histamine, substance P, cholecystokinin, prostaglandins and 5-hydroxytryptamine (Hoogerwerf and Pasricha, 2006). The release of these chemicals in gut causes stimulatory effect mediated through an ultimate increase in cytosolic Ca\(^{2+}\) (Burks, 1987) and a substance with ability to block any of the above pathways or with non-specific inhibitory action like that of Ca\(^{2+}\) antagonists, is considered to be effective in hyperactive gut disorders. To study the possible mechanism of gastrointestinal inhibitory effect, isolated tissue preparations of rabbit jejunum and guinea pig ileum were used. In jejunum, the plant extract completely reversed CCh and high K\(^{+}\)-induced contractions, being more potent against CCh, similar to dicyclomine, while nifedipine, a known Ca\(^{2+}\) antagonist was more potent against K\(^{+}\)-induced contractions, a typical characteristic of Ca\(^{2+}\) antagonist (Fleckenstein, 1977). These data suggest that the relaxant effect of the plant extract is primarily mediated through muscarinic receptors with Ca\(^{2+}\) channel blocking effect observed at slightly higher concentrations, similar to dicyclomine. K\(^{+}\) at concentration high than 30 mM (high K\(^{+}\)) is known to cause smooth muscle contractions through opening of voltage-dependent L-type Ca\(^{2+}\) channels, thus allowing inward movement of extracellular Ca\(^{2+}\) (Bolton, 1979). Thus, a substance that inhibits high K\(^{+}\)-provoked contractions is considered as a blocker of Ca\(^{2+}\) influx (Godfraind et al., 1986). The Ca\(^{2+}\) antagonist effect of the extract was confirmed further in jejunum, when it shifted the CRCs of Ca\(^{2+}\) to the right with suppression of the maximum effect at both tested concentrations, similar to nifedipine or dicyclomine.
The dual inhibitory mode involving anticholinergic and Ca\textsuperscript{2+} channel blockade of the plant extract was also evaluated separately by constructing the CRCs of ACh in guinea-pig ileum, a quiescent preparation. This preparation is considered useful to quantify contractile responses of an agonist in the presence of an inhibitor, thus allowing exploration of the nature of antispasmodic effect (Gilani et al., 1997). The plant extract at a low concentration (0.3 mg/mL) caused a rightward parallel shift in the ACh curves without suppressing the maximum response, a characteristic of a specific antagonist, like atropine (Brown and Taylor, 2006). Whereas, it caused a non-parallel shift with suppression of the maximum response at higher concentration (1 mg/mL), indicating the co-existence of an additional non-competitive inhibitory effect (Eglan and Whiting, 1987) as observed with Ca\textsuperscript{2+} antagonists (Cejalvo et al., 1993). Dicyclomine also shifted the ACh curves in a manner similar to that of the plant extract, while nifedipine resulted in a rightward but non-parallel shift with suppression of the maximum contractile effect at both tested concentrations, a typical characteristic of a non-competitive inhibitor (Farre et al., 1991). This confirmed that the presence of spasmylytic effect in *Phyllanthus emblica* is mediated through dual inhibition of muscarinic receptors and Ca\textsuperscript{2+} channels. Antimuscarinic drugs and CCBs are known to be effective as antispasmodic, anti-motility and antidiarrheal (Lee et al., 1997; Brown and Taylor, 2006; Pasricha, 2006). Hence, the presence of dually acting spasmylytic activity in the plant extract might be contributing towards its effectiveness in diarrhea and abdominal spasm, in addition to the reported antidiarrheal activity related with inhibition of prostaglandins synthesis (Perianayagam et al., 2005).

*Phyllanthus emblica* is an essential component of a popular herbal formulation, known as *Trifla* in the Unani (Greco-Arab) system of medicine. Interestingly, *T. bellerica*, another component of this formula has recently been shown to possess antispasmodic effect with similar mode of action (Gilani et al., 2008a). The plant has also been shown to possess antibacterial activity (Saeed and Tariq, 2007), while some of its norsesquiterpenoid glycosides are reported to have antiviral effects (Liu et al., 2009). Such activities of the plant could account additional benefit providing a wider cover for its use in diarrhea of different etiologies.

The observed antidiarrheal activity of *Phyllanthus emblica* fruit extract in two species, such as, in rats, mediated through inhibition of prostaglandins action (Perianayagam et al., 2005) and in mice through antimuscarinic and Ca\textsuperscript{2+} antagonist effects, provides a sound mechanistic base for its effectiveness in diarrhea. This is also in accordance with the general understanding that plants contain multiple active constituents with effect enhancing activities (Gilani and Rahman, 2005).

Preliminary experiments on the activity-directed fractionation of Pe.Cr revealed that the Ca\textsuperscript{2+} antagonist and anticholinergic like components were distributed in all, the organic and aqueous fractions. The fractions of *Phyllanthus emblica* possessing Ca\textsuperscript{2+} antagonist like constituent(s) were found with the following order of potency Pe.CHCl\textsubscript{3} > Pe.Pet = Pe.EtAc > Pe.Cr > Pe.Aq.
whereas, the order of anticholinergic potency was as, Pe.EtAc > Pe.Cr ≥ Pe.CHCl3 > Pe.Aq > Pe.Pet. However, a greater potency of Pe.CHCl3 and Pe.EtAc in spasmylic effects could be corroborated with the findings of phytochemical analysis of these fractions, showing an indication for the presence of higher contents of flavonoids. Whereas, the lower potency of Pe.Aq and Pe.Pet in exhibiting spasmylic actions might be due the presence of high levels of saponins, which are known to be spasmogenic in nature (Akah et al., 1997), thus offering a hindrance in the relaxant effects. Our observation about the presence of different levels of phytochemical constituents and their influence on the activity profile of Phyllanthus emblica is also in line with couple of studies done earlier on this plant (Kumaran and Karunakaran, 2006; Mouming, 2006). Overall, the presence of different phytochemical classes in Phyllanthus emblica such as, alkaloids, flavonoids, tannins, terpenes and sesquiterpenes possessing spasmylic and antidiarrheal activities (Di Carlo et al., 1993; Borrelli et al., 2004; Capasso et al., 2004; Palombo, 2006), might be contributing towards its antidiarrheal, antisecretory and spasmylic actions, acting through multiple target sites.

5. Conclusion

This study shows that the crude extract of Phyllanthus emblica possesses antidiarrheal, antisecretory and antispasmodic effects mediated through dual blockade of muscarinic receptors and Ca	extsuperscript{2+} channels. Thus, this study provides a sound mechanistic support to its medicinal use in hyperactive gastrointestinal disorders like diarrhea, and is a step forward towards the evidence-based use of phytomedicine.

Acknowledgement

The study was carried out with the financial support from the Higher Education Commission, Government of Pakistan, under the scheme of Distinguished National Professor research allowance.

References


