

Full Length Research Paper

***In vitro* gastric H⁺/K⁺-ATPase, anti-*Helicobacter pylori*, anti-muscarinic and anti-oxidant, activities of a gastroprotective purified fraction of *Phyllanthus emblica* fruit**

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***Phyllanthus emblica* fruit (PE) is a potent anti-ulcer and gastroprotective plant. Its most purified fraction (PE-AqE 47) has also been presented anti-ulcerogenic activities in stress-induced, indomethacin- and histamine-induced gastric ulcers in the albino rats. PE-AqE 47 was tested therefore, for its gastric H⁺/K⁺-ATPase, anti-*Helicobacter pylori*, anti-muscarinic and anti-oxidant activities (*In vitro*), presently. Purified fraction exerted no effect on gastric H⁺/K⁺-ATPase activity and did not interact with muscarinic receptors. The experiments revealed that it inhibited the *H. pylori* growth while it's anti-secretory, anti-ulcer and gastroprotective effects might be caused due to its potent anti-oxidant (IC₅₀: 3.39±0.08 µg/ml) characteristics. Therefore, PE-AqE 47 may prove an effective gastroprotective tool.**

Key words: *Phyllanthus emblica* fruit, anti-secretory, anti-ulcer, gastroprotective, anti-*Helicobacter pylori*, anti-oxidant.

INTRODUCTION

The plants of the genus *Phyllanthus* (Euphorbiaceae) are widely distributed throughout tropical and subtropical countries (Brusotti et al., 2011). They have been used in China, India, Pakistan, Philippines, Nigeria, East and West Africa, the Caribbean, and Central and South America by traditional medicinal practitioners for the treatment of different type of diseases (Ishtiaq et al., 2007; Krishnaveni and Mirunalini, 2010). Several therapeutic properties such as antipyretic, antibacterial, antiparasitic, antinociceptive, and antiviral activities have been attributed to this genus (Mythilypriya et al., 2007; Habib et al., 2011). *Phyllanthus emblica* (*amla*) has been known in Ayurvedic medicine for its tonifying,

antiageing and immune enhancing properties as it provides a superior source of vitamin C (Saini et al., 2008; Charoenteeraboon et al., 2011). Each *amla* fruit contains up to 700 mg of vitamin C. *Amla* has been particularly indicated for anaemia, asthma, bleeding gums, diabetes, cold, chronic lung disease, hyperlipidemia, yeast infections, scurvy and cancer (Saini et al., 2008; Majeed et al., 2009).

In our previous report, it was shown that the aqueous extract of *P. emblica* fruit (PE) protected rats against indomethacin-induced gastric ulcers while its ethyl acetate fraction (PE-AqE) and purified fraction (PE-AqE 47) also antagonized the gastric effects induced by

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indomethacin (Akhtar et al., 2004). It was further reported that PE-AqE 47 remained unable to antagonize the histamine-induced gastric changes in secretion volume, pH, acid-output and pepsin activity but it attenuated potently the indomethacin, hypothermia and restraint-induced gastric effects. The gastric lesion formation and reduction in gastric mucus secretions induced by histamine as well as indomethacin and hypothermic-restraint stress were also antagonized highly significantly ($P < 0.001$) with the pre-treatment of PE-AqE 47. However, the gastroprotective mechanism of PE-AqE 47 remained unknown (Akhtar et al., 2004). Therefore the present study was planned.

MATERIALS AND METHODS

All materials/chemicals were of analytical grade which were used as received from Beacham-GSK Pharmaceuticals, USA (Amoxicillin), Abbott Laboratories, USA (Clarithromycin), Novartis Healthcare Pvt. Ltd., Germany (Omeprazole), BDH Chemicals Ltd, Poole, UK (Methanol, Atropine, Ethyl cellulose, Butanol, Chloroform, Ethyl acetate, N, N-dimethyl formamide, Pipes-Tris buffer), E-Merck, Germany (Microaerophilic kit, Brain-heart-infusion broth, Valinomycin and ATP) and Fluka-Switzerland (Silica-gel fluorescent plates, Potassium chloride, Magnesium chloride, Acridine orange, L-ascorbic acid and 1, 1-diphenyl-2-picryl-hydrazyl).

Preparation of purified fraction of *P. emblica* fruit

P. emblica fruit was purchased locally from herbal dealer in Bahawalpur, Pakistan. The plant material was authenticated and compared with its standard in the herbarium maintained in the Department of Botany, University of Agriculture, Faisalabad, Pakistan. A specimen (PE Ph. 103) of this drug was preserved in the Pharmacology Laboratory, Department of Pharmacy, the Islamia University of Bahawalpur, Pakistan. Aqueous extract of *P. emblica* fruits (5.0 g) was fractionated in butanol, chloroform and ethyl acetate soluble fractions (Kit-Lam et al., 1998). Ethyl acetate (47% in water) fraction (324 mg) was purified following chromatography (silica gel G-plates, 10 to 40 μ m) method described previously (Sharma et al., 2013).

Test animals

Male wistar rats (100 \pm 10 g) were kept under controlled conditions (temperature, 26 \pm 2°C; humidity, 50 \pm 5%; 12 h light/dark cycle) (Tasduq et al., 2005). The animals were maintained on a standard diet, prepared by the Department of Nutrition, University of Agriculture, Faisalabad-Pakistan. Male albino rabbits used were also kept under similar conditions. They were fed on a special rabbit feed (Department of Nutrition, University of Agriculture, Faisalabad-Pakistan) and green fodder. Normal tap water was allowed *ad libitum* (Hulig et al., 2012). The conduct of experiments and the procedure of sacrifice (under ether) were approved by the Ethics Committee of the Experimental Animal Care Society, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur-Pakistan.

Determination of proton pump inhibition

Gastric membranes containing H⁺, K⁺-ATPase were prepared from

rat gastric mucosae according to the modified method of Beil et al. (1988). The proton transport in membrane vesicles were monitored by the fluorescence quenching of a weak base, acridine orange as described by Lee and Forte (1978). Gastric membrane protein (60 μ g) was suspended in 2.0 ml samples containing 2 mmol/L of MgCl₂, 150 mmol/L of KCl, 0.2 mmol/L of ATP, 10 mmol/L of Pipes-Tris buffer (pH 7.0) and 5 μ mol/L of acridine orange. Test fraction (2.0 mg) or Omeprazole (2.0 mg) was added separately to the suspensions before proton uptakes were initiated by addition of ATP and 50 μ g of Valinomycin (Beil et al., 1988).

Determination of anti-*Helicobacter pylori* activity

PE-AqE 47 (20 μ g/ml, 200 μ g/ml or 2.0 mg/ml), Amoxicillin 100 mg/ml and Clarithromycin 100 mg/ml in sterile N, N-dimethyl formamide (DMF) were tested for anti-*H. pylori* activity according to the methods of Beil et al. (1995) and Fabry et al. (1995). Test substance was added to 5.0 ml of Brain Heart Infusion broth supplemented by 7.5% heat inactivated calf serum separately. The tubes were inoculated with *H. pylori* strain (approximately 1.5 \times 10⁵ CFU/ml) which were obtained from biopsies specimens of gastric ulcer patients (Pathology Department, BVH-Hospital, Bahawalpur-Pakistan) and identified by a competent microbiologist (Gardazi Clinical Laboratory, Bahawalpur-Pakistan). Specimens were preserved in Microbiology Laboratory, Department of Pharmacy, the Islamia University of Bahawalpur, Pakistan. Inoculated tubes were incubated under micro-aerobic conditions of 5% O₂, 15% CO₂ and 80% N₂ at 37°C for 72 h. Turbidity of Brain Heart Infusion broth under trials was read at 600 nm against the blank (Belagihalli and Dharmesh, 2012).

Determination of effect on isolated rabbit's ileum

Segments of rabbit's ileum (2.5 to 3.0 cm long) were suspended in a 10 ml organ bath containing Tyrode's solution at 37°C and aerated continuously with 95% O₂ and 5% CO₂. The contractions were recorded isometrically. Contractile responses of PE-AqE 47 (20 μ g to 2.0 mg) were compared to the normal movements with/without pre-treatments of antagonist (atropine sulphate, 3.0 mg) according to the modified method of Jaiswal and Mohan (2012) and Karl et al. (1998).

Determination of DPPH anti-oxidative activities

1, 1-Diphenyl-2-picrylhydrazyl, Sigma-Aldrich (DPPH) scavenging activity was determined according to the method described by Basu et al. (2012). Different concentrations (2.5, 5.0 and 10 μ g/ml in methanol) of the test extract and standard; Ascorbic acid (Fluka) were prepared separately. DPPH solution (1.0 ml, 0.3 M) was added to 2.5 ml solution of extract and standard after 20 min incubation period at room temperature in the dark, the absorbance of the resulting mixture was read at 518 nm. The percentage antioxidant activity (AA%) was calculated using the expression:

$$AA\% = 100 - [(Abs\ sample/nAbs\ control) \times 100]$$

The absorbance of the control (nAbs) which was prepared by adding methanol (1.0 ml) to the extract solution (2.5 ml) without DPPH, while the positive control was prepared by adding 1.0 ml of DPPH solutions to 2.5 ml of ascorbic acid (Habiba et al., 2010).

Statistical analysis

The data was analyzed statistically using student-*t* test. *P* value

Table 1. *In vitro* anti-*Helicobacter pylori* activity of aqueous-ethyl acetate fraction of *Phyllanthus emblica* fruit (PE-AqE 47).

S/N	Treatment	Concentration	Turbidity due to <i>H. pylori</i> growth Mean \pm SEM
1	DMF (Blank)	-	10.00 \pm 0.00
2	<i>H. pylori</i> (Inoculants)	10 ⁷ cfu	2.613 \pm 0.01**
3	Amoxicillin	2.0 mg/ml	1.968 \pm 0.06**
4	Clarithromycin	2.0 mg/ml	1.863 \pm 0.02**
5	Famotidine	2.0 mg/ml	2.614 \pm 0.01
6	Omeprazole	2.0 mg/ml	2.615 \pm 0.01
7	PE-AqE 47	20 μ g/ml	1.630 \pm 0.05**
8	PE-AqE 47	200 μ g/ml	1.331 \pm 0.04**
9	PE-AqE 47	2.0 mg/ml	1.234 \pm 0.03**

PE-AqE 47: Aqueous-ethyl acetate fraction of *Phyllanthus emblica* fruit. Inoculant (*H. pylori*): compared with Blank * P < 0.05; ** P < 0.001. Test drugs: compared with Inoculant (*H. pylori*) * P < 0.05; ** P < 0.001. All the other values are N.S. (P > 0.05) compared with Inoculant (*H. pylori*). Mean \pm SEM = Mean values \pm Standard error of means of eight (*In vitro*) experiments.

Table 2. Free radical scavenging activity of aqueous-ethyl acetate fraction of *Phyllanthus emblica* fruit (PE-AqE 47).

S/N	Treatment	Concentration (μ g/ml)	Inhibition percent (Mean \pm SEM)	IC ₅₀ (Mean \pm SEM)
1	Ascorbic acid ^a	2.5	8.2 \pm 0.14	
2	Ascorbic acid ^c	5.0	17.3 \pm 1.07	6.4 \pm 0.31 μ g/ml
3	Ascorbic acid ^e	10.0	75.9 \pm 5.32	
4	PE-AqE 47 ^b	2.5	34.9 \pm 2.63**	
5	PE-AqE 47 ^d	5.0	78.4 \pm 6.65**	3.39 \pm 0.08 μ g/ml
6	PE-AqE 47 ^f	10.0	98.2 \pm 6.85*	

PE-AqE 47: Aqueous-ethyl acetate purified fraction of *Phyllanthus emblica* fruit. ^bCompared with ^a, ^dcompared with ^c and ^fcompared with ^e, *P < 0.05; **P < 0.001. Mean \pm SEM = Mean values \pm Standard error of means of eight experiments.

<0.05 was considered statistically significant.

(P < 0.001) decline in the *H. pylori* growth (Table 1).

RESULTS

Proton pump inhibitory effect of purified fraction of *P. emblica* fruit

Figure 1 showed fluorescence spectrum of PE-AqE 47 (2.0 mg/L) in the absence/presence of increasing ATP concentration (50 to 200 μ mol/L). Fluorescence of PE-AqE 47 was not altered by different concentrations of ATP used. However, a reference drug, Omeprazole (2.0 mg/L) inhibited H⁺, K⁺-ATPase activity.

Anti-*H. pylori* activities of purified fraction of *P. emblica* fruit

PE-AqE 47 inhibited the growth of *H. pylori* high significantly (P < 0.001) in a dose-dependent manner. Famotidine, a reference drug, did not show any change in *H. pylori* growth. However, the reference antibiotics, Amoxicillin and Clarithromycin caused the high significant

Effects of purified fraction of *P. emblica* fruit on isolated rabbit's ileum

PE-AqE 47 remained unable to modify the spontaneous contractile activity of rabbit's ileum. Increasing doses (20 μ g to 2.0 mg) of the fraction could not antagonize the effects of atropine (3 mg) on spontaneous movements of isolated rabbit ileum (Figure 2).

DPPH anti-oxidative effect of purified fraction of *P. emblica* fruit

PE-AqE 47 showed free radical-scavenging effect in a dose-dependent manner on DPPH assay. This effect was highly significant (P < 0.001) with all the doses tested (20 μ g to 2.0 mg/ml) of the purified fraction of powdered *P. emblica* fruit. IC₅₀ of the substance and ascorbic acid were observed, 3.39 \pm 0.08 μ g/ml and 6.4 \pm 0.31 μ g/ml, respectively (Table 2).

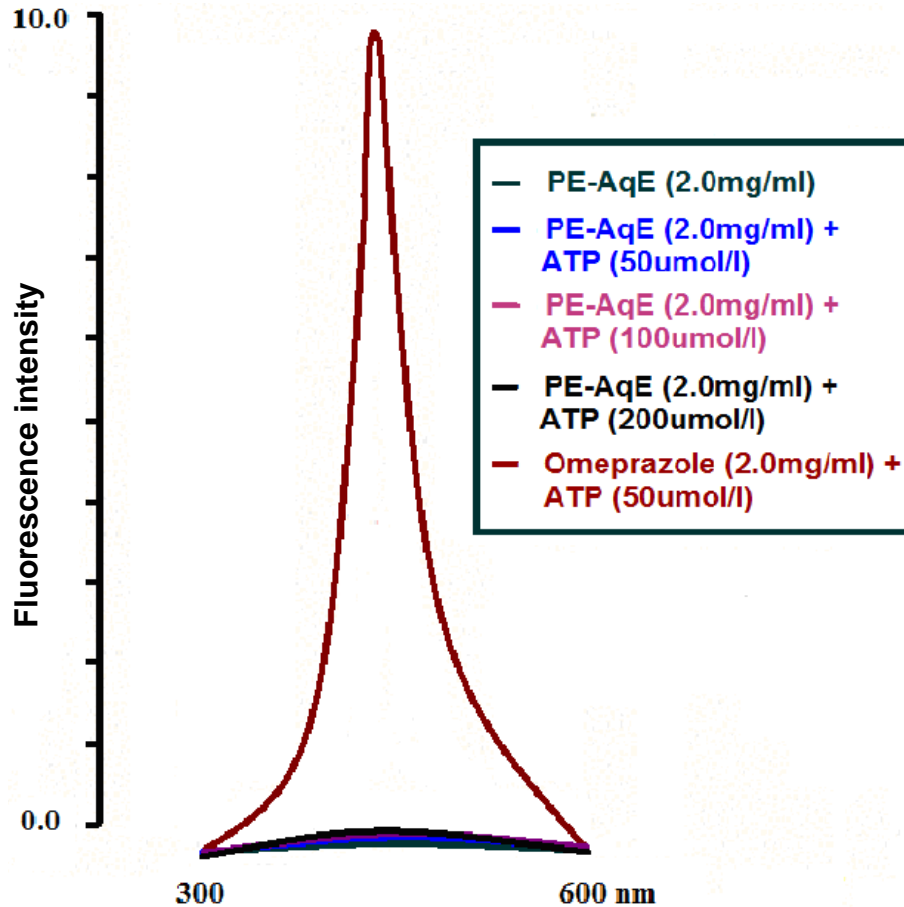


Figure 1. Fluorescence changes of aqueous-ethyl acetate fraction of *Phyllanthus emblica* fruit (PE-AqE 47). Aqueous-ethyl acetate fraction of *P. emblica* fruit (PE-AqE 47, 2.0 mg/ml) did not cause any change in fluorescence intensity with the increasing concentration of ATP (50 to 200 $\mu\text{mol/L}$) while Omeprazole (2.0 mg/ml) exhibited a marked change in fluorescence intensity in the presence of ATP (50 $\mu\text{mol/L}$).

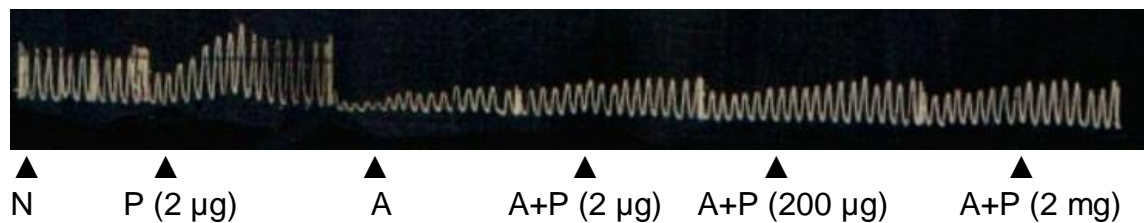


Figure 2. Muscarinic effect of aqueous-ethyl acetate fraction of *Phyllanthus emblica* fruit (PE-AqE 47). A representative tracing showing effect of increasing concentration (20 $\mu\text{g/ml}$ to 2.0 mg/ml) of aqueous-ethyl acetate fraction of *P. emblica* fruit (PE-AqE 47) in the presence/absence of atropine sulphate on spontaneous movements of isolated rabbit ileum. Triangles indicating the time of addition of various drugs to the tissue organ bath containing Tyrode solution (10 ml). N=Normal/Control; P=Aqueous-ethyl acetate fraction of *Phyllanthus emblica* fruit; A=Atropine sulphate (3.0 mg).

DISCUSSION

Among various causes of gastric ulceration, lesions caused by *H. pylori* infection, stress and those due to the

use of non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be mediated largely through the generation of reactive oxygen species (ROS), especially the hydroxyl radical (Prabha et al., 2011). Stress-induced

gastric lesions are mainly caused by oxidative damage due to hydroxyl radicals (OH^{\cdot}) (Sener et al., 2005). While ROS mediate apoptosis (Zhou et al., 2001). Earlier, it has been reported that most purified fraction of *P. emblica* (PE-AqE 47) have exerted significant anti-ulcer activity (Akhtar et al., 2004). It has further been reported that PE-AqE 47 remains unable to antagonize the histamine-induced gastric changes in gastric secretion volume, pH, acid-output and pepsin activity, but it attenuates successfully the indomethacin, hypothermia and restraint-induced gastric effects. Further, the gastric lesion formation and reduction in gastric mucus secretions by histamine as well as indomethacin and hypothermic-restraint stress are also blocked highly significantly ($P < 0.001$) with the pre-treatment of PE-AqE 47.

Additionally, the fluorescence changes observed in the absence/presence of increasing ATP concentrations (Figure 1) indicated that the fraction remained unable to inhibit the gastric proton pump (H^+ , K^+ -ATPase). The present study further revealed that PE-AqE 47 did not alter the spontaneous movements of rabbit's ileum in the presence/absence of atropine (Figure 2). Thus it may be speculated that cholinergic system and H^+ , K^+ -ATPase inhibition might not be involved in the anti-ulcerogenic mechanism of test fraction. Data pointing out that PE-AqE 47 might exert its anti-ulcer effect through its cytoprotective action and might not through the histaminic pathway as proposed by Sairam et al. (2001). The results further suggesting that PE-AqE 47 might produce anti-ulcerogenic effect due to its anti-oxidant activity (Table 2). Many research workers have reported the anti-oxidant activity of PE and its ethyl acetate extract (Kumaran and Karunakaran, 2006; Kusirisin et al., 2009). Our finding is consistent with Bandyopadhyay et al. (2001), Provinciali et al. (2002) and Takeuchi et al. (2001). It has been reported that oxygen free radicals serve as second messengers in pro-inflammatory signal transduction pathways (Moon et al., 2000). The oxygen active species, such as $\text{O}_2^{\cdot-}$, H_2O_2 , HO^{\cdot} and lipid radicals, such as ROO^{\cdot} , RO^{\cdot} and hydroperoxides, are generated during lipid peroxidation and metabolism (Kwiecien et al., 2001). Anti-oxidants have been proved useful in all the disorders in which up-regulation of inflammatory response are implicated (Zhou et al., 2001). Therefore, oxidative damage to endothelial cell results in nitric oxide (NO) shortage which might be inhibited due to possible free-radical scavenging activity exhibited by PE-AqE 47 (Tsuchiya et al., 2002). This is in agreement with Sala et al. (2002), Chaudhuri (2002) and Kumaran and Karunakaran (2006). Additionally, PE has indicated a rich source of vitamin C. Anti-oxidant activity of the fraction might be caused due to the presence of vitamin C. Further, vitamin C has also reported anti-*H. pylori* (Aditi and Graham, 2012).

The data indicates highly significant *in vitro* anti-*H. pylori* activity of the test fraction (Table 1). PE-AqE 47 showed a dose-dependent anti-*H. pylori* inhibition. *H. pylori* has been indicated a major causative agent of gastric

ulcer. It weakens the protective mucous coating of the stomach and duodenum and allows acid to get through to the sensitive lining beneath. Both acid and bacteria irritate the lining and cause gastric ulcer (Aditi and Graham, 2012; Seo et al., 2002). It has been indicated that infection caused by *H. pylori* is mediated largely through the generation of reactive oxygen species, especially the hydroxyl radicals (Akyon et al., 2002).

Conclusion

Therefore, potent *in vitro* anti-oxidant and anti-*H. pylori* activity of PE-AqE 47 might ultimately prove it to be an ideal and curative anti-ulcer tool (Sun-Young et al., 2008).

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