International Journal of Microbiological Research 3 (3): 216-220, 2012
ISSN 2079-2093

DOI: 10.5829/idosi.ijmr.2012.3.3.66161

In-vitro Anti-*Helicobacter Pylori* Activity of *Emblica officinalis*

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Abstract: *Helicobacter pylori*, a Gram negative microaerophilic bacterium is a major etiological agent in duodenal, peptic and gastric ulcers. Antibiotic resistance has become a global concern. Phytochemicals are the plant-derived naturally occurring compounds that showed antimicrobial activity. The fruits of *Emblica officinalis* commonly known as amla, is highly valued in traditional Indian system of medicine. In the present study, phytochemical estimation (phenolic, flavonoid and carotenoid), anti-H. pylori activities of various extracts (petroleum ether, ethanol and aqueous) of *E. officinalis* determined by Kirby-Bauer's disk diffusion and minimum inhibitory concentration (MIC) were carried out. The ethanolic extract of *E. officinalis* found to possess high phytochemical content followed by aqueous and petroleum ether extracts. Further, *H. pylori* is found to be susceptible for ethanolic and aqueous extracts of *E. officinalis* from 100 and 750µg/disc respectively. In MIC, *H. pylori* was susceptible at a concentration of 10µg/mL ethanolic and 1000µg/mL aqueous extract. No susceptibility against petroleum ether extract of *E. officinalis* was observed with the studied concentration using Kirby-Bauer's disk diffusion and MIC methods. The present study revealed that the ethanolic and aqueous extracts of *E. officinalis* show anti-*H. pylori* activity. Among the studied extracts, ethanol is found to be the best solvent for the extraction of phytochemicals in *E. officinalis*.

Key words: Helicobacter Pylori · Emblica Officinalis · Kirby-Bauer's Disk Diffusion · Minimum Inhibitory Concentration

INTRODUCTION

Helicobacter pylori is a spiral-shaped, Gram-negative microaerophilic rod considered as the etiological agent of peptic ulcers and gastritis and is associated with mucosa-associated lymphoid tissue lymphoma and gastric cancer. *H. pylori* infects over 50% of the world population [1-3]. In most of the developing countries the prevalence of *H. pylori* exceeds 50% by 5 years of age and by adulthood, infection rates exceed 90%. [1] The prevalence of *H. pylori* has been shown to be higher in some extragastric disease like coronary heart disease, chronic renal failure, diarrheal diseases in children and hepatic encephalopathy [2]. The treatment of *H. pylori* infection depends on the combination of antibiotics and acid suppression drugs, with efficiency only up to 80%. Unexpectedly, about 20% of the patients infected with *H. pylori* remain unimproved or reinfected due to the incomplete eradication or antibiotics resistance [3] In addition, the side effects of using multi-antibiotics are obvious, such as antibiotic associated diarrhea and enteric dysbacteriosis. Thus attention has been drawn to seek for any alternative method which can eradicate or inhibit *H. pylori* infection efficiently without any side effects.

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There has been a worldwide move towards the use of traditional medicines because of its efficacy and being free from serious toxic effects [4]. Several studies reported, that many natural plants extracts have anti-*H. pylori* activity [5, 6]. The fruit of *Emblica officinalis* commonly known as amla is highly valued in traditional Indian medicine [7]. The fruit of *E. officinalis* is hepatoprotective, anti tumor [8], antioxidant [9] and antiulcerogenic [10]. *E. officinalis* showed antibiotic activity against a wide range of bacteria [11]. Therefore the present study aimed to evaluate phytochemical and antibacterial potential of various extracts of *E. officinalis* against *H. pylori* isolates from human using the conventional Kirby-Bauer's disk diffusion and minimum inhibitory concentration methods.

MATERIALS AND METHODS

Plant Material: *E. officinalis* fruits were collected from the Anna Siddha College, Chennai, Tamil Nadu, India and taxonomically identified by the Department of Botany, University of Madras, Chennai, India.

Extract Preparation: Seedless fruits of *E. officinalis* were air dried, crudely grained and subjected to extraction by using different solvents in their increasing order of polarity viz., petroleum ether, methanol and aqueous extracts by soaking the plant material in the respective solvents overnight at room temperature. The content present in the respective solvents were subjected to reflux below the boiling point for 6-8 hrs in order to extract the compounds into the solvent [12]. Each extract was vacuum filtered and the filtrates were concentrated by vacuum distillation. The concentrated extracts from petroleum and ethanol solvents were incubated at 37°C to facilitate complete evaporation of the volatile solvent leaving behind the dried extract of *E. officinalis*. Dried extracts were reconstituted in dimethylsulfoxide (DMSO) and stored in the dark at 4°C.

Isolation and Estimation of Phenolics: Isolation and estimation of phenolics were performed using protocols suggested by Price *et al*. [13]. The absorbance was measured at 720 nm using quercetin as a standard. The total phenolics were expressed as µg/g of extract.

Isolation and Estimation of Flavonoids: The isolation and estimation of flavonoids was carried out using protocols suggested by Harborne [14] and Lamaison and Carnat [15]. The absorbance was measured at 430 nm.

Isolation and Estimation of Carotenoids: Extraction and estimation of carotenoids were performed by protocols suggested by Narayanaswamy and Palanisami [16]. Carotenoids were expressed as µg/g sample.

Isolation of Test Organism: The biopsy specimen of gastric mucosa from the patient with upper gastrointestinal (UGI) symptoms were obtained at Stanley Medical College Hospital, Chennai, Tamil Nadu, India after obtaining a written consent from the patient. The biopsies were placed directly into transport medium brain heart infusion (BHI) supplemented with 20% glycerol, within 2 hrs gastric mucosa was homogenized and suspended in 500µl brain heart infusion broth containing 10% sheep red blood cells and 100µl was inoculated onto Brucella chocolate agar. The inoculated plates were incubated in a microaerophilic environment-(CO_2 incubator, Nauire Inc, USA with 12% CO_2) at 37°C and observed for growth from 3rd day onwards. The isolate was confirmed to be *H. pylori* based on its colony morphology viz., small, translucent grey colonies with convex elevation and an entire edge, Giemsa and Gram's staining to demonstrate slight to moderately thick rod forms interspersed with curved or even horseshoe-shaped cells and Biochemical tests [17].

Disk Diffusion Analysis: Sterile blank diffusion disks were placed into labeled trays for each type of extracts. A 10% extract was prepared with both distilled water and ethanol. This preparation was processed to prepare disks with various concentrations by saturating with 5µ1 of the individual petroleum ether or ethanol or aqueous extracts. The positive control discs were prepared by saturating blank discs in either petroleum ether or ethanol or sterile distilled water and allowing all the solvent to evaporate. Discs with 10µ1 DMSO and metronidazole (5µg/disc) were placed as negative and positive controls, respectively. The standardized inoculum, l-2xl0^7CFU/mL (0.5 McFarland standards) was lawn cultured on the surface of sterile Muller-Hinton blood agar plates using sterile swab [18] and plates were incubated at 37°C for 72hrs in an incubator with a 12% CO_2 atmosphere (Nuaire Inc, USA). The antibacterial activity of each extract against *H. pylori* was quantified by determining average diameter of the zone of inhibition around the paper discs in millimeters. The tests were performed twice and average diameters of zones were calculated.

Minimum Inhibitory Concentration (MIC): MIC values were determined using broth dilution method [19]. The extracts, sterilized by 0.45mm Millipore filters and inoculums were added to MH broth medium. Serial 10-fold dilutions were made that furnished a concentration range from 0.01-1000µg/mL for each extract. Two control tubes include antibiotic control (containing the growth medium without extract) and organism control (containing growth medium, physiological saline and the inoculum). The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes were regarded as MIC [20].

RESULTS

The petroleum ether, ethanol and aqueous extract yields of *E. officinalis* were 8, 19 and 12% respectively.

Phytochemical Content: The result of various phytochemicals content present in *E. officinalis* extracts were given in table 1. The estimation of phytochemical content has revealed that one gram of crude petroleum ether extract of *E. officinalis* contains 4040µg of phenolic, 322µg of flavonoids and 108µg of carotenoids, while ethanolic extract contains 9342µg of phenolic, 718µg of flavonoids and 286µg of carotenoids and aqueous extract contains 9510µg of phenolic, 680µg of flavonoids and 190µg of carotenoids.

Disk Diffusion Analysis: No zone of clearance was observed by the solvents-only control disc and petroleum ether extract disc of *E. officinalis* at the concentration up to 1000µg/disc (Table 2). The ethanolic extract of *E. officinalis* showed zone of clearance measuring less than 3mm against the *H. pylori* at the concentration of 100µg/disc and maximum of greater than 8mm at 1000µg/disc. Aqueous extract of *E. officinalis* produced zone of clearance of less than 3mm only at the concentration of 750µg/disc and greater than 3-8mm at 1000µg/disc.

Minimum Inhibitory Concentration (MIC): In MIC, *H. pylori* was sensitive at the concentration of 10µg/mL ethanolic and 1000µg/mL aqueous extract of *E. officinalis* and no sensitivity was observed with the studied concentration of petroleum ether extract of *E. officinalis* against *H. pylori* (Table 3).

Table 1: The phytochemicals (Phenolics, Flavonoids and Carotenoids) in petroleum ether, ethanol and aqueous extracts of *E. officinalis*

Extract	Phenolics	Flavonoids (µg/g)	Carotenoids
Petroleum ether	4040	322	108
Ethanol	9342	718	286
Aqueous	9510	680	190

Table 2: Anti-H. pylori activity of petroleum ether, ethanolic and aqueous extracts of *E. officinalis* by Kirby-Bauer's disk diffusion

	Concentration of extract (µg/ disc)								
Extract	10	25	50	75	100	250	500	750	1000
EOP	-	-	-	-	-	-	-	-	-
EOE	-	-	-	-	+	++	+++	+++	+++
EOA	-	-	-	-	-	-	-	+	++
Solvents	-	-	-	-	-	-	-	-	-

-, No Zone of inhibition; +, Zone of inhibition less than 3mm; ++, Zone of inhibition 3-8mm; +++, Zone of inhibition greater than 8mm; EOP-petroleum ether extract; EOE-ethanolic extract; EOA-aqueous extract.

Table 3: Minimum inhibitory concentration of petroleum ether, ethanol and aqueous extracts (0.01-1000µg/mL) of *E. officinalis* against H. pylori

Extract	MIC
EOP	R
EOE	10
EOA	1000

MIC values are expressed as µg/mL: R, no inhibition even at the highest tested concentration. EOP-petroleum ether extract; EOE-ethanolic extract; EOA-aqueous extract

DISCUSSION

The present study investigated the anti-*H. pylori* effect of petroleum ether, ethanolic and aqueous extracts of *E. officinalis* and its phytochemical content. In this study, gastric biopsy samples were obtained from patients presenting with gastro duodenal complications. *H. pylori* was isolated from the specimens using standard microbiology procedures and isolates subjected to various extracts (petroleum ether, ethanol and aqueous) of *E. officinalis* for susceptibility. The selection of crude extracts for screening programs has the potential of being more successful in initial steps than the screening of pure compounds isolated from natural products [21]. Hence in the present study petroleum ether, ethanol and aqueous crude extracts of *E. officinalis* was studied. Phytochemical studies revealed that the presence of phenolic, flavonoids and carotenoids which have been reported to have antibacterial activities [22, 23]. Among the studied extracts ethanolic extracts of *E. officinalis* were observed to be the most active against *H. pylori*, compared with the other solvents used in this study (table 2 and 3). This might be due to the fact that the active compounds against *H. pylori* may be less polar/semi-polar making ethanol an ideal solvent [24]. In addition, most of the antibiotic compounds already identified in herbs are reported as aromatic or saturated organic molecules, making ethanol an ideal solvent [25]. The mechanisms behind the anti *H. pylori* activity are complex to understand and could be attributed to either inhibiting the cell division or damaging the cell wall or ability to intercalate with DNA [26], which however require to be further investigated in detail.

In-vitro studies of various crude extracts of *E. officinalis* has demonstrated anti-*H. pylori* effect. *E. officinalis* may provide novel or lead compounds, which could become template for the synthesis of new anti-*H. pylori* drugs of inexpensive, safe and effective treatment. Our major focus in future studies is to isolate and characterize bioactive compounds from *E. officinalis* against *H. pylori*.

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