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Full Length Research Paper

Anti-*Helicobacter pylori* and antioxidant properties of *Emblica officinalis* pulp extract: A potential source for therapeutic use against gastric ulcer

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We report a novel property of ethanolic extract of *Emblica officinalis* (amla) fruit pulp in inhibiting the growth of *Helicobacter pylori in-vitro*. In this study, three standard laboratory strains, and five clinical isolates of *H. pylori* showed sensitivity towards amla extract with minimum inhibitory concentration values ranging from 0.91 to 1.87 µg/µl. Out of five clinical isolates two were resistant to clarithromycin. The anti-*H. pylori* bioactive spot with retention factor (Rf) value 0.16 in toluene: choloroform: acetone (40:25:35) has been separated using thin layer chromatography followed by contact bioautography. The bioactive spot appears to be essential oil and/or phenolics. The extract contained 20.55 mg gallic acid equivalent/gm of dry weight of extract. Total reducing power was estimated to be 75.8 mg ascorbic acid equivalent per gram of dry weight of extract. Antioxidant activity was checked by 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). Trolox Equivalent Antioxidant Capacity (TEAC) was 175.76 mg/gm of dry weight of extract when checked by ABTS assay. Thus total phenolics, flavanoids, reducing power and the antioxidant properties of amla is very well retained in the ethanolic extract and makes it suitable for a therapeutic use against *H. pylori* infection and gastric ulcer.

Key words: Amla, phenolics, flavonoids, contact bioautography, clarithromycin.

INTRODUCTION

Helicobacter pylori is a genetically diverse microaerophilic gastric pathogen with the extraordinary ability to establish infection in human stomach that can last for years or decades, despite immune and inflammatory responses. In developing countries, 70-90% of the population carries *H. pylori* and develop persistent inflammation in their stomach, and overall around half of all people worldwide are infected with this organism (Graham, 1997). As virulence markers of *H. pylori* are not associated with diseases in all the geographical regions, eradication of *H. pylori,* if infection is detected, provides the most effective treatment for *H. pylori*-associated diseases. Numerous studies in Europe, America and East Asia have established that *H. pylori* eradication from infected ulcer patients generally results in cure of peptic ulcer disease (Forbes et al., 1994; Hopkins et al., 1996).

Currently, the most preferred *H. pylori* eradication therapy (triple-therapy) employ, one proton pump inhibitor and two antibiotics (Bytzer and O'Morain 2005). However, such multiple therapy regimens have not been very successful in clinical practice, since the overuse or rather misuse of antibacterial agents have resulted in the emergence of antibiotic-resistant strains which is the main cause of treatment failure apart from adverse side effects (Bytzer and O'Morain 2005;Cameron et al., 2004).

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Increasing complications in the conventional triple-therapies stimulate an urgent need to develop new nonsynthetic antibacterial agents against *H. pylori*-infection that are safe, highly effective and have specific cellular targets (Colombo and Bosisio 1996; Falcao et al., 2008). Furthermore, several studies using extracts of traditional medicinal plants from several parts of the world have assessed the *in vitro* susceptibility of *H. pylori* indicating the possibility of finding a cure from natural sources (Bhamarapravati et al., 2003; Castillo-Juarez et al., 2009; Cwikla et al., 2010; Mahady et al., 2005; Nostro et al., 2005; Zaidi et al., 2009)

It has also been recognized that plant polyphenols are an important class of defensive antioxidants (Cai et al., 2003; Cotelle et al., 1996; Zheng and Wang 2001) reported to block the generation of carcinogenic precursors (Webb and McCullough 2005) and proved to be an anti-*H. pylori* and associated gastric carcinogenesis (Toyoda et al., 2007). Antioxidants are capable of protecting from DNA damaging free radicals, which are generated by various factors, including chronic *H. pylori* infection and thus anti-*H. pylori* coupled with antioxidant activity in turn lowers the risk of gastric cancer (Akyon, 2002). Amla is highly valued in the Indian traditional medicine system (Scartezzini et al., 2006). The dried fruits of amla in Unani and Ayurvedic medicine has been reported for the treatment of haemorrhage, diarrhoea and dysentery (Krishnaveni and Mirunalini 2010). Preclinical studies carried out in the past three decades have validated many traditional uses of amla having gastroprotective, antiulcerogenic and antibacterial properties (Khan et al., 2002; Krishnaveni and Mirunalini 2010). In addition, the amla fruit pulp is reported to have properties like diuretic (Bobbarala et al., 2009) adaptogenic (Rege et al., 1999), hepatoprotective (Jeena et al., 1999; Jose and Kuttan 2000), antitumorous (Jose et al., 2001), and hypocholestrolemic (Kim et al., 2005). We report here the novel anti-*H. pylori* activity of ethanolic extract of amla fruit pulp against three reference strains and five clinical isolates of *H. pylori.* In addition, we also report here the total phenol content, total flavonoid content, total reducing power and antioxidant properties of amla fruit pulp extract.

MATERIALS AND METHODS

Preparation of plant extracts

Amla fruit was collected from Bharmar village district Kangra in Himachal Pradesh, India. After removing the seeds, it was washed with deionized water and dried in oven at 35-40ºC for 4-5 days till the weight became constant. Plant materials were regularly examined to check for any fungal growth or rotting. Dried amla pulp weighing 15 g was pounded and soaked in 50 ml of absolute ethanol and kept in 250 ml sterile conical flasks at 37ºC with shaking at 120 rpm for 24 h. The content was filtered through Whatman No. 1 paper and followed by sterilized through 0.22 µ membrane. The filtrates obtained were stored separately in sterile glass vials at 4ºC until use.

Preparation of bacterial inoculum

Three reference strains of *H. pylori* SS1 (Taxon identifier 102617), 26695 (*H. pylori* ATCC 700392) , J99 (Taxon identifier 85963) and five clinical isolates 154(1A), 225(1A), 216(1A), 33 and 39A were used in this study and their identification was carried out using the standard biochemical tests (Itoh et al., 1987). *H. pylori* strains were grown and maintained as described earlier (Chattopadhyay et al., 2004).

Anti-*H. pylori* activity assay of amla extracts

Kirby Bauer method was used for preliminary screening of plant extracts effective against *H. pylori* strains (Bauer et al., 1966). Sterile Whatman paper disks (6 mm in diameter) were soaked with different amount of plant extracts and placed on the inoculated plates with 1.2 $X10^9$ colony forming unit (CFU) of *H. pylori*. The plates were kept under observation for 2 days at 37°C under microaerophillic conditions (5% O_2, 10% CO_2 and 85% N_2). All experiments were performed in triplicates. Minimum inhibitory concentration (MIC) was calculated based on the minimum amount of plant extract exhibiting hair line growth inhibition around the antibiotic containing disc. Pure ethanol was used as a negative control and clarithromycin as positive control against the sensitive strains.

TLC, contact bioautography and identification of chemical nature of bioactive component

TLC separation followed by contact-bioautography to determine bioactivity was performed as established by us previously (Mehrotra et al., 2010). In brief, precoated TLC sheet (Silica gel 60 F^{254} nm) was spotted with 50 µl of amla pulp extract. TLC were run in toluene: chloroform: acetone (40:25:35) and methanol: formic acid (1:1) for first dimension (1D) and second dimension (2D) respectively. After drying, for contact bioautography the chromatogram was placed with face down onto the inoculated agar plate to enable diffusion. The plate was incubated at 37°C, for 24 h and the zone of inhibition was measured. Rf value of the bioactive spot was measured as the ratio of mobility of center of bioactive spot against the bacteria to the total distance travelled by the solvent front. Presence of alkaloid, phenolics and essential oils on TLC was tested as described earlier (Pascual et al., 2002).

Determination of phenolic content, anti-oxidant activity and reducing properties of the plant extract

Total phenolic and flavonoid contents were determined in terms of standards gallic acid and rutin respectively as described earlier (Zou et al., 2004).

RESULTS

Anti *-H. pyloric* activity of amla extract

The amla pulp extract was found to be very effective against all the tested strains of *H. pylori* grown in brain-

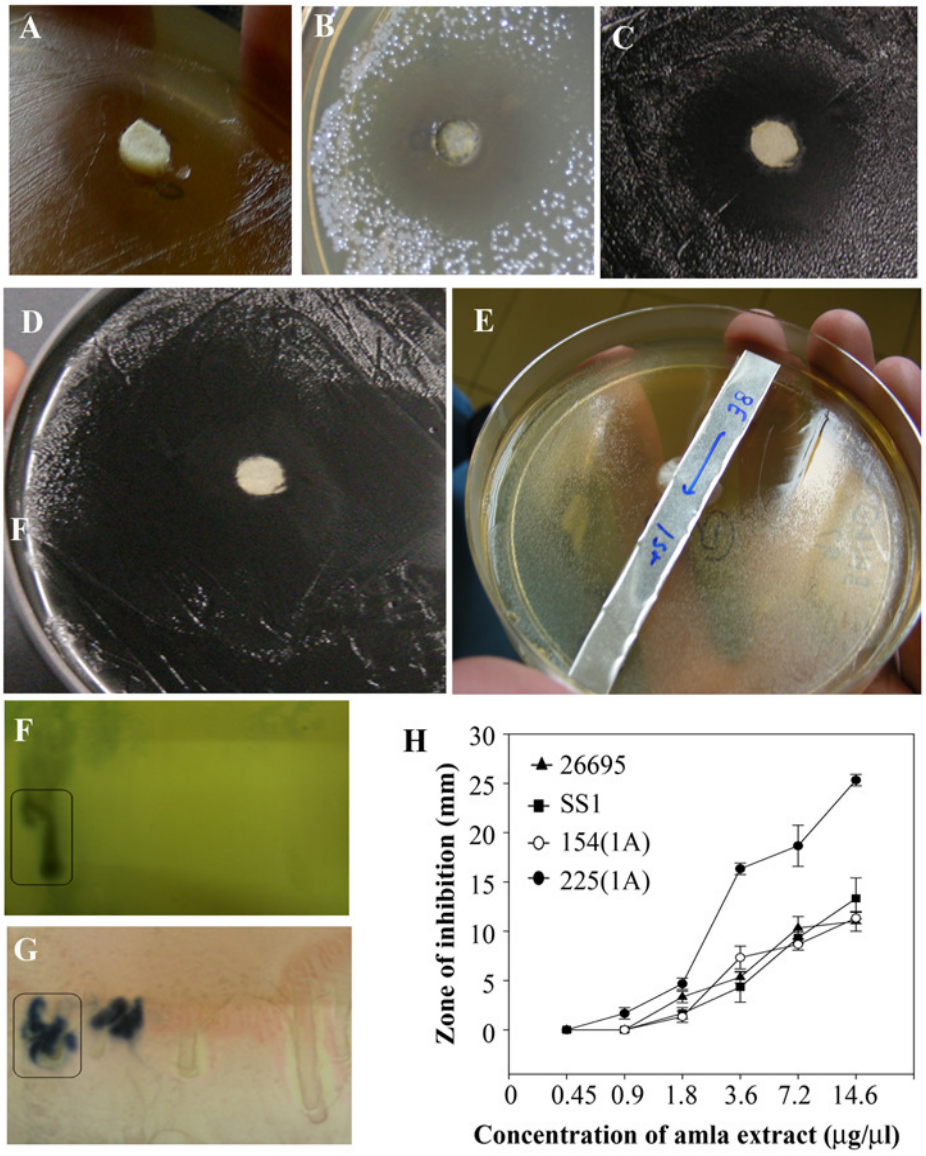

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Figure 1. Antibiotic activity of amla pulp extract against *H. pylori* strains. A, strain 26695; B, strain J99; C, strain 33A; D, 39A, ; E, contact bioautography of TLC strip against strain 26695; F, phosphomolybdic acid spray to detect essential oil on TLC plate; G, ferric chloride spray to detect tanins on TLC plate; F &G- rectangles indicate the spots on TLC having anti-*H. pylori* activity. H, MIC against indicated strains. Strains 33A and 39A are two clarithromycin resistant clinical isolates.

heart-infusion-agar plate (Figure 1 A, B) including the two strains that were resistant to clarithromycin (Figure 1C, D). MIC was calculated as the minimum concentration of amla extract required to produce hair-line growth inhibition in the disc-diffusion assay. Determination of MIC was conducted using two strains each from reference and clinical strains. As shown in figure (Figure 1 C) the MIC of amla extract was 0.91 µg against 225(1A) strain, 1.87 µg against 26695, 154 (1A) and SS1 strains of *H. pylori*.

TLC separation and determination of chemical nature of bioactive component

The crude ethanolic extract of amla was resolved by TLC. Solvents with toluene: chloroform: acetone = 40:25:35 for 1D TLC followed by methanol: formic acid (1:1) for 2D TLC was found to be best for separation of compounds without compromising the bioactivity against *H. pylori.* Contact bioautography with cut strips of TLC plates identified only one spot of Rf value 0.16 in 1D

solvent and 0.46 in 2D that showed bioactivity against all the strains of *H. pylori.* A representative contact bioautography of amla extract after 1D-TLC against *H. pylori* strain 26695 is shown in Figure 1D. The chemical nature of the bioactive spot was identified by developing the bioactive spot with different chemical sprays in 2D-TLC plates. When treated with phosphomolybdic acid the bioactive spot turned blue indicating that the spot contain essential oils (Figure 1E). However, when the plate was sprayed with 3% ferric chloride the spot turned dark blue (Figure 1F) indicating the bioactive spot contained phenolics. The bioactive spot was negative for alkaloid as it did not stain after spraying with dragendorff reagent. Thus the bioactive spot appears to contain either phenolics and/or essential oil. Further experiments are required to determine exact nature of the bioactive components.

Antioxidants and reducing power content of amla extract

Amla is reported be an excellent source of antioxidants (Bhattacharya et al., 1999). Combination of antioxidant activities and anti -*H. pylori* activity are ideal way of treating the infection. Thus we measured the antioxidant contents and the total reducing power of the ethanolic extract of amla pulp that showed anti-*H. pylori* activity. Total phenolic content in the crude extract of amla pulp was 20.55 mg gallic acid equivalent /gm (y=0.005x, r^2 = 0.9989) of dry weight of extract. Total flavonoid content was measured and it was 7.57 mg rutin equivalents per gram (y= 0.0022 x, r^2 =0.9996) of dry weight of amla pulp extract with reference to standard curve and total reducing power estimated to be 75.8 mg ascorbic acid equivalents per gram (y= 0.0055 x, r^2 =0.9975) of dry weight of amla pulp extract with reference to standard curve obtained from ascorbic acid. Due to the complex nature of phytochemicals, it was important to employ commonly accepted assays to evaluate the antioxidant activity of plant extract. Numerous antioxidant methods have been developed to evaluate antioxidant activity. Of these, total antioxidant activity, reducing power, DPPH assay are most commonly accepted assays to evaluate antioxidant activity (Frankel and Meyer 2000; Sanchez 2002). In this study antioxidant activity was determined by three different assays namely, ABTS, DPPH and FRAP. All the three assays were calibrated with the water soluble α-tocopherol analogue, Trolox (Figure S1). It was observed that ethanolic extract of amla pulp had the highest Trolox Equivalent Antioxidant Capacity (TEAC) of 175.76 mg/gm of dry weight of extract by ABTS assay with reference to standard curve (y= 0.0013 x, r^2 =0.9975) and of 124.6 mg/gm of dry weight of extract by FRAP assay with reference to standard curve (y= 0.0019 x, r^2 =0.9996) and 58.06 mg/gm of dry weight of extract by DPPH assay with reference to standard curve (y= 0.001 x, r^2 =0.9992). The results of these assays are summarized in Table 1 and standard curves are shown in supplementary figure (Figure S1).

DISCUSSION

Eradication of *H. pylori* is almost impossible even by administrating 'triple therapy', which is a combination of proton pump inhibitor and antibiotics. Geographical differences shows variation at the genetic level as well as in clinical manifestation of the disease which makes the treatment of the disease more complicated. One of the major reasons for the *H. pylori* treatment failure is the development of drug resistance. In India, approximately 85% of clinical strains of Kolkata are resistant to 8 mg/l metronidazole (Datta et al., 2005). Similarly, 91 and 96% of *H. pylori* strains from Mumbai and Hyderabad respectively showed resistance to clarithromycin; 73% of Mumbai and 80% strains of Hyderabad are resistant to amoxycillin (Abraham et al., 1997; Thyagarajan et al., 2003). Frequent emergence of antibiotic resistance in microbial pathogens encourages the use of natural agents as alternative therapies. Application of antibiotic coupled with antioxidant treatment may be very useful in controlling the pathogen growth and associated inflammation. This study was undertaken to check anti-*H. pylori* activity of amla extract and to test the retention of antioxidant properties in the extract. Antimicrobial properties of amla have already been evaluated against various bacterial pathogens like *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *Micrococccus varians*, *Micrococccus lylae*, *Micrococccus roseus*, *Micrococccus halobius*, *Micrococccus sedenterius*, *Bacillus subtilis*, *Bacillus megaterium* and *Candida albicans* (Saeed and Tariq 2007). However, at present there is no report about the effect of amla against *H. pylori*, even though, the fruit pulp of amla is known to contain high anti-oxidant properties. The clinical strains 225 (1A) and 216 (1A) used in this paper were resistant to furazolidone and metronidazole and 154 (1A) was sensitive to furazolidone but resistant to metronidazole and amoxicillin (Datta et al., 2005). Our results indicate the *anti-H. pylori* activity of amla extract against all the tested clinical and laboratory strains that were differentially resistant to furazolidone, metronidazole and clarithromycin. The MIC value of amla extract against these strains varied between 0.91 to 1.87 µg/ µl of dry extract indicating it to be extremely potential to control the growth of this deadly pathogen. The variation in sensitivity observed may be due to the difference in the genotype of these strains as reported earlier (Chattopadhyay et al., 2004).

Earlier we had shown that ethanolic extract of amla pulp contained two bioactive spots in TLC against

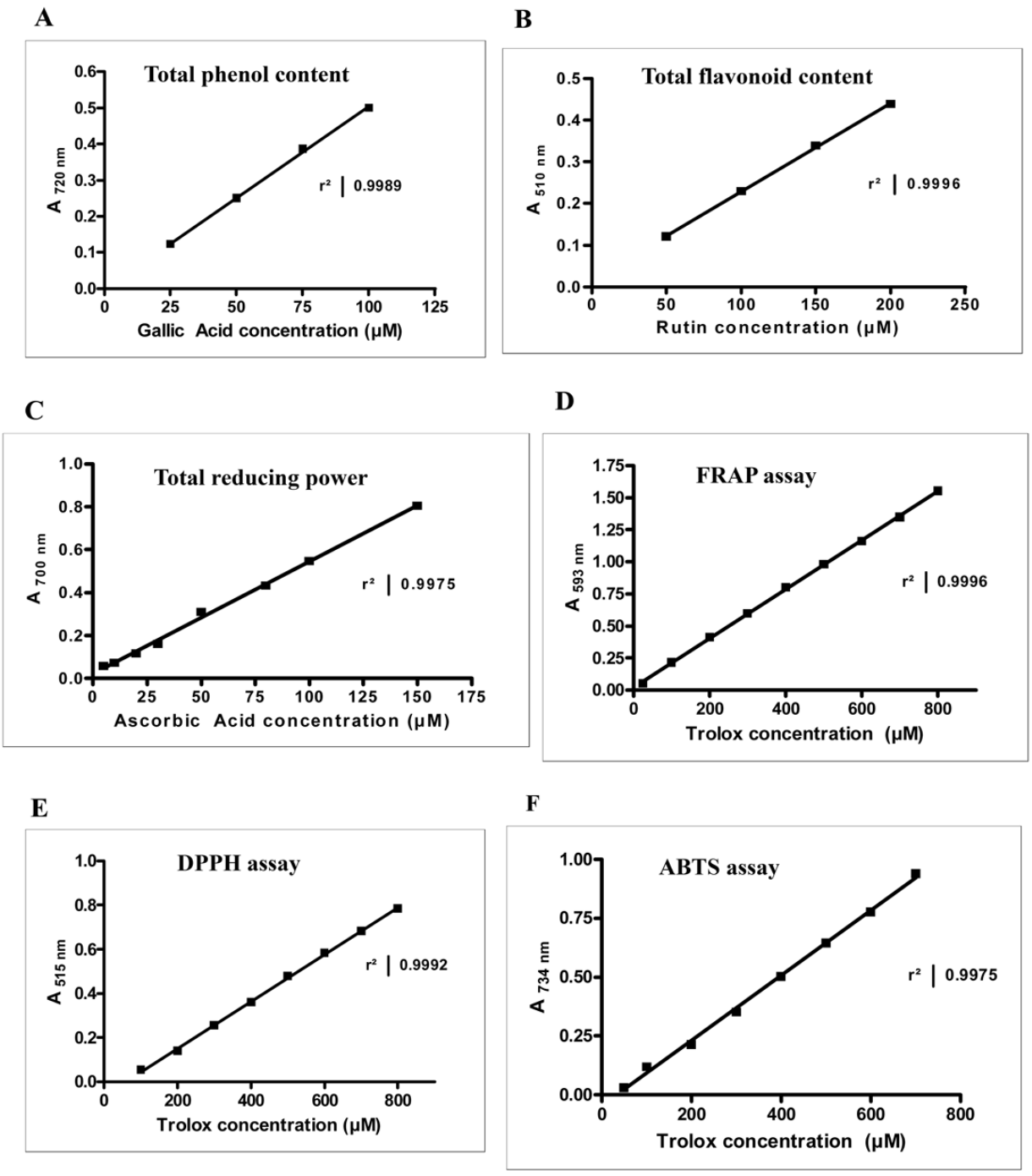

Figure S1. Standard curves for phenolics, flavonoids, antioxidants and reducing power assay. Standard curves obtained by using the specific substrates as mentioned in X-axis in each graph.

methicllin resistant *S. aureus, Pseudomonas aeruginosa, Vibrio cholera,* that moved with Rf values of 0.13 and 0.8 (Mehrotra et al., 2010). In the present study the anti-*H. pylori* activity was obtained only at Rf value of 0.16 in TLC with same solvent system. Thus it can be assumed that the faster moving band that was shown earlier is not having anti-*H. pylori* activity and it is possible that the slower moving band is the compound having broad-spectrum antibiotic activity.

In addition to the antibiotic activities, the extract also retained the antioxidant properties in terms of total phenolics, flavonoid contents and as well as in terms of

Table 1. Total phenolic, flavonoid content and antioxidant properties of ethanolic extract of amla.

Name	Content (mg/g) of dry weight of extract
Phenolics	20.55±1.45
Flavonoid	7.57±0.12
Total Reducing power	75.8±1.5
FRAP	124.6±5.53
ABTS	175.76±10
DPPH	58.06±6.19

the reducing power. This study provides the clear indications of the possibilities of exploiting amla extract as potential source for therapeutic use against gastric ulcer as well as the possibilities of purifying anti-*H. pylori* component from amla fruit pulp.

Conclusion

Ethanolic extract of *E. officinalis* is highly effective in controlling growth of *H. pylori in vitro* with MIC ranging from 0.91 to 1.87 µg/ µl. The TLC separation followed by detection spray indicates that the bioactive spot is having mixed properties of both phenolics and essential oils. The extract also retained high level of antioxidant properties that makes it suitable for therapeutic use against gastric ulcer.

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