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EVALUATION OF ANTIBACTERIAL ACTIVITY OF *PHYLLANTHUS EMBLICA* L. LEAVES EXTRACTS AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

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ABSTRACT

The antibacterial activity of *Phyllanthus emblica L.* flower extracts in different solvents (methanolic, ethanolic and aqueous) were studied against seven different bacterial strains, including four gram-positive (*Micrococcus luteus*, *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus* sp.) and three gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*). In the present study, aqueous extracts gave the highest yield percentage as compared to methanol as well as ethanol. The extracts were tested for the presence of antibacterial activity by agar well diffusion assay (AWDA) method and the patterns of inhibition varied with the solvent and the tested organisms. The results showed that *M. luteus* among the gram-positive and *E. coli* among the gram-negative were highly susceptible as compared to other tested organisms. The methanolic extract of *P. emblica* was the most effective as the widest inhibitory zone was observed as compared to the ethanolic and aqueous extract. Screening of crude extract showed notable minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at concentrations range of 12.50 to 100 mg/ml. The remarkable antibacterial activity of the *P. emblica* leaves extracts against tested gram-positive and gram-negative bacteria suggests that there is a scientific basis for its utilization as antibacterial agents in designing and developing new drugs.

KEY WORDS: Antibacterial; *Phyllanthus emblica*; Solvents; Zone of Inhibition.

INTRODUCTION

A dramatic increase in microbial antibiotic resistance developed over the last forty years in both the agriculture and medical sectors, thus forced the researchers to develop a new antimicrobial drug which is not based on the synthetic agents for controlling pathogenic species.[1-4] Thus, demand has increased for less harmful and environmentally friendly natural products, especially that produced by plants, which are sources of novel bioactive substances. In developing countries about 80% of the population utilizes medicinal plants for the treatment of infectious/ various diseases.[5]

Plants contain many biologically active compounds which have potential for development as medicinal agents. Herbal medicines have already formed the basis of therapeutic use in the developing countries, but of recent, there has been an increase in the use of herbal medicines in the developing world too.[6]

Plants provide an alternative strategy in the search for new drugs. There is a rich abundance of plants reputed in traditional medicine to possess protective and therapeutic properties.[7] It is likely that plants will continue to be a valuable source of new molecules which may, after possible chemical manipulation, provide new and improved drugs.[8] Bacterial resistance to antibiotics represents a serious problem for clinicians and the pharmaceutical industry and great efforts are being made to reverse this trend, and one of them is the widespread screening of medicinal plants from the traditional system of medicine hoping to get some newer, safer, and more effective agents that can be used to fight infectious diseases.[9]

Phyllanthus emblica L. commonly known as Indian gooseberry or amla belongs to Euphorbiaceae family (Figure 1). It is native of tropical and subtropical parts of Asia, including India, China, and Thailand. The leaves are simple and light green in color closely attached along the branches. The fruits are spherical, smooth, and light greenish-yellow in color. It has high content of vitamin C and constitute of phyllemblin, gallic acid, ascorbic acid, tannins etc..[10] Due to the presence of polyphenol and tannin in *P. emblica* fruit the vitamin C is highly stable. It is useful in treating diabetes, asthma, jaundice, cough, inflammation, etc. It is highly valued in Indian traditional medicines.[11]

P. emblica also has antioxidant, antibacterial, and antimicrobial properties. Bioactive molecules or phytochemicals are found to be useful for treating various diseases. Antibacterial compounds from plants appear to have a potential approach to contain antibiotic

resistance and can help in managing disease. The antimicrobial properties of the plant can be of great significance in therapeutic treatments.[12]

Figure 1: Photo of *P. emblica* L. plant

Hence, the present study was initiated to evaluate the antibacterial activity of methanolic, ethanolic and aqueous extracts of *P. emblica* leaves against gram-positive as well as gram-negative bacterial strains.

MATERIALS AND METHODS

Sources of bacterial strains

Seven bacterial strains, including both Gram-positive as well as Gram-negative obtained from M.D. University, Rohtak, Haryana and Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology, Chandigarh. The bacterial strains include *Bacillius subtilis*, *Micrococcus luteus* (MTCC106), *Staphylococcus aureus* (MTCC6908), *Streptococcus* sp. (MTCC9724), *Escherichia coli* DH5α, *Pseudomonas aeroginosa* (MTCC4673) and *Salmonella typhimurium* (MTCC3224) have been selected for the present study.

Culture of bacterial strains

The bacterial strains were propagated in the nutrient broth medium (5g/l peptone, 3g/l beef extract, 5g/l NaCl, and pH 7.0) incubated for 18hr at a respective growing temperature. Slants were prepared from the separated colonies of bacteria, stored at 4°C temperature and sub-cultured in a nutrient broth medium before testing the antibacterial activity. The media were purchased from Hi-media, Mumbai, India.

Preparation of plant material

The collected samples were thoroughly washed under tap water, dried in the shade for one month and then ground into coarse powdered with the help of mortar and pestle. These powders were stored in airtight brown bottles at 4°C until needed for future use.

Extraction of plant material (Maceration)

The shade dried 100 gm coarse powdered of leaves was immersed in 200 ml of different solvents (methanol, ethanol and aqueous) contained in 500 ml sterile conical flasks and covered with cotton wool separately. It was placed aside with intermittent shaking for one week. They were first filtered with double layered muslin cloth and then through Whatman No. 1 filter paper, and the march was discarded. The filtrate was subjected to evaporation by treating at 40°C in an oven to obtain a dried extract. The dried extract was stored at 4°C until used for further study.[13, 14]

Yield percentage of solvent extracts

After complete drying, the yield of the each extraction was measured separately and the extraction efficiency was quantified by determining the weight each of the extracts and the percentage yield was calculated as the dry weight of coarse plant material/ dry weight of extracted material x 100.[15]

Antibacterial activity by agar well diffusion assay (AWDA) method

The antibacterial activity of crude solvent extracts (methanol, ethanol and aqueous) of *P. emblica* leaves against gram-positive as well as gram-negative bacterial strains were evaluated by agar well diffusion assay (AWDA) method.[15, 16] For this, a well (6mm diameter) was made with the help of a borer in cooled nutrient agar plate, overlaid with soft agar (5ml), seeded with a target strain (~10^6 cfu/ml). Aliquots of the test compound (100µl) were introduced into the well and the plates were incubated for overnight at 37ºC. The diameters of the inhibition zones were measured in millimeters (mm). For each bacterial strain, the dissolving solvent 10% DMSO and streptomycin (50 µg/ml) were used as negative and positive controls respectively. To test the antibacterial activity of all extracts was dissolved in 10% DMSO solvent to make a final concentration 200 mg/ml.

Determination of minimum inhibitory concentration (MIC)

MIC values were determined by using the Broth dilution method.[17] Briefly, 1.0 ml of the reconstituted extract solution at a concentration of 200 mg/ml was added to another test tube

containing 1 ml of sterile broth so as to obtain a concentration of 100 mg/ml. 1.0 ml of this dilution was transferred to another test tube till the 7th test tube was reached. The 8th test tube did not contain any extract, but a solution of pure solvent and served as negative control. Then 1ml of 18hr grown cultures of each of bacterial strains, adjusted at ~ $1x10^6$ cfu/ml was put into each tube and thoroughly mixed in a vortex mixer. The tubes were incubated at 37°C for 18hr and observed for growth in the form of turbidity. The test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC value.

Determination of minimum bactericidal concentration (MBC)

The MBC values were determined by removing 100 µl of bacterial suspension from the MIC positive tube as well as one above and one below the same tube, on nutrient agar plates and incubated at 37°C for 18hr. After incubation, the plates were examined for colony growth and MBC's were recorded.[18,19]

Statistical analysis

The experiment was carried out in three independent sets, each consisting of 3 replicates. Values shown here represent mean ± standard error of the mean (SEM).

RESULTS

In the present study, after complete drying, the percentage yield of plant leaves extracts with the used different solvents was measured separately and quantified the efficiency of extraction. The results of the present investigation, aqueous extraction gave the highest percentage yield (15.41%) followed by methanol (12.26%) and ethanol (10.52%) shown in Table 1.

Table 1: Percentage yield of *P. emblica* plant leaves extracts in different solvents

Solvent	Percentage yield of extracts (gms)		
	Weight of dry powder	Weight of dry extracts	Percentage yield
Methanol	100	12.26	12.26
Ethanol	100	10.52	10.52
Aqueous	100	15.41	15.41

P. emblica plant leaves extracts with various solvents (methanol, ethanol and aqueous) showed inhibitory activity against all the tested seven bacterial strains as shown in Figure 2.

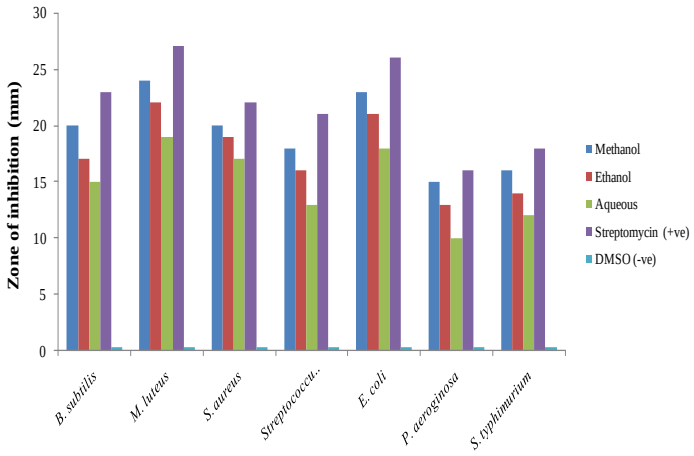

Figure 2: Antibacterial activity of *P. emblica* leaves extracts

The maximum zone of inhibition was recorded for the methanol extracts against *M. luteus* (24), *B. subtilis* (20), *S. aureus* (20), *Streptococcus* sp. (18), *E. coli* (23), *P. aeroginosa* (15) and *S. typhimurium* (16). Ethanol extracts showed against *M. luteus* (22), *B. subtilis* (17), *S. aureus* (19), *Streptococcus* sp. (16), *E. coli* (21), *P. aeroginosa* (13) and *S. typhimurium* (14). Similarly, aqueous extracts offered an inhibitory zone against *B. subtilis* (15), *M. luteus* (19), *S. aureus* (17), and *Streptococcus* sp. (13), *E. coli* (18), *P. aeroginosa* (10) and *S. typhimurium* (12). Streptomycin used as a positive control exhibited higher activity than using different solvents extract with the zone of inhibition against *B. subtilis* (23), *M. luteus* (27), *S. aureus* (22), *Streptococcus* sp. (21), and *E. coli* (26), *P. aeroginosa* (16), and *S. typhimurium* (18), while DMSO doesn't showed activity.

The MIC values were determined for methanol, ethanol and aqueous leaves extracts by broth dilution method, shown in Figure 3.

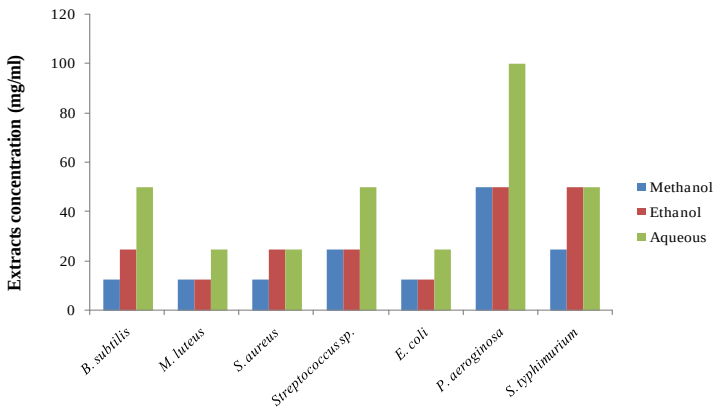

Figure 3: Minimum inhibitory concentration (MIC) (mg/ml) values of *P. emblica* leaves extracts

Methanol extracts exhibited the MIC values 12.5mg/ml against *B. subtilis*, *M. luteus*, *S. aureus* and *E. coli*; 25mg/ml against *Streptococcus* sp. and *S. typhimurium*; 50mg/ml against only one bacteria *P. aerogenosa*. Ethanol extract showed 12.5mg/ml against *M. luteus* and *E. coli*; 25mg/ml against *B. subtilis*, *S. aureus* and *Streptococcus* sp.; 50mg/ml against *S. typhimurium* and *P. aerogenosa*. Similarly, samples of aqueous extracts possessed 25mg/ml against *M. luteus*, *S. aureus* and *E. coli*; 50mg/ml against *B. subtilis*, *Streptococcus* sp. and *S. typhimurium*; 100mg/ml against *P. aerogenosa*.

The MBC values were determined for methanol, ethanol and aqueous leaves extracts against bacterial strains by broth dilution method as shown in Figure 4. Methanol extracts showed MBC values 12.5mg/ml against *M. luteus* and *E. coli*; 25mg/ml against *B. subtilis*, *S. aureus* and *Streptococcus* sp.; 50mg/ml against *P. aerogenosa* and *S. typhimurium*. Ethanolic extract exhibited 25mg/ml against *B. subtilis*, *M. luteus*, *S. aureus* and *E. coli*; 50mg/ml against *Streptococcus* sp., *S. typhimurium* and *P. aerogenosa*. Similarly, aqueous extracts possessed MBC values 25mg/ml against *M. luteus*, *S. aureus* and *E. coli*; 50mg/ml against *B. subtilis*, *Streptococcus* sp.; 100mg/ml against *P. aerogenosa* and *S. typhimurium*.

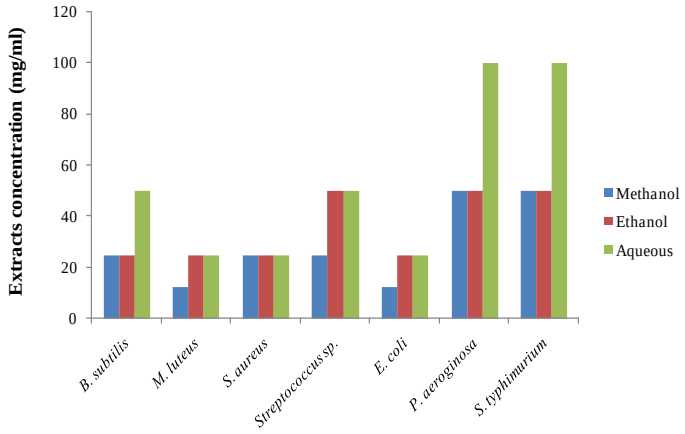

Figure 4: Minimum bactericidal concentration (MBC) (mg/ml) values of *P. emblica* leaves extracts

DISCUSSION

The plant possesses the different phytochemicals like alkaloids, tannins, phenolics, saponins, cardiac glycoside, terpenes and flavonoids. Some workers have also attributed to their observed antimicrobial effect of plant extracts to the presence of these secondary metabolites.[20] The antimicrobial activities of alkaloids and flavonoids have also been reported.[21,22] Tannins are important in herbal medicine in treating wounds and to arrests

bleedings.[23] Phyto-constituents such as saponins and phenolic compounds have also been reported to inhibit the growth of some bacteria. Infectious diseases caused by bacteria, fungi, viruses and parasites are a major threat to public health in developing countries due to unavailability and high cost of medicines.[24]

There are many literatures reporting the ethno-medicinal values of *P. emblica*, but there is little scientific proof for further using this plant commercially or in a more effective form. For this, the yield of extraction was calculated because the crude plant extracts are generally a mixture of active and non-active compounds. A number of medicinal plants described in Ayurveda still need to be testified, according to the modern parameters to ensure their activity and efficacy. Drugs used in Ayurveda are mostly prepared by extraction with water. Therefore healers may not be able to extract all the active compound(s).[25] The percentage yield of medicinal plant extracts which contain the bioactive metabolites vary considerably with plant species and the method or solvent used for extraction. Also, factors like age of the plant and the polarity of the solvent used may have affected the percentage yield.[26] In the present study, an aqueous solvent extract gave the highest yield of extraction followed by methanol and ethanol.

Similarly, in order to evaluate the antibacterial potential of crude leaves extracts of *P. emblica* against gram-positive and gram-negative bacterial strains by the AWDA method. In the present study, all the extracts exhibited excellent antibacterial activity against all the tested bacterial strains, including both gram-positive as well as gram-negative with varying degrees. The aqueous fruit extracts of *E. officinalis* showed the antibacterial activity against all the five tested bacterial strains namely *Bacillus* sp., *Lactobacillus* sp., *Pseudomonas* sp., *Proteus* sp., and *Streptococcus* sp..[27] In another study suggested that the ethanol and acetone extracts of *E. officinalis* possess antibacterial activity.[28] One of the study revealed that the plants contained bioactive agents which are connected with antimicrobial properties in plants. The crude extract of *E. officinalis* showed notable MIC and MBC at concentrations of 12.50-50 and 200-300 mg/ml, respectively.[29] However, in the present investigation, MIC values of methanol as well as ethanol extract from 12.5-50 mg/ml; aqueous extract 25-100 mg/ml was calculated against the tested bacterial strains. Similarly, MBC values were determined for methanol 12.5-50 mg/ml; ethanol 25-50 mg/ml; and aqueous 25-100 mg/ml against the bacteria.

CONCLUSION

The results obtained from this work revealed that the plants contained bioactive agents which are connected with antibacterial properties in plants. From the above study, *P. emblica* leaves extract with different solvents (methanol, ethanol and aqueous) showed remarkable antibacterial activity against the tested four gram-positive (*M. luteus*, *B. subtilis*, *S. aureus* and *Streptococcus* sp.) as well as three gram-negative bacteria (*E. coli*, *P. aeruginosa* and *S. typhimurium*). Based on the result of this study it can be said that *P. emblica* leaves extracts are an effective antibacterial agent that can be used in folk medicine and will be a good source to treat and control many diseases. These findings could also be of commercial interest to both pharmaceutical companies and research institutes in designing and developing new drugs. Further research has to be conducted on the activity of the crude extracts against a wider range of bacteria and fungi.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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