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Antimicrobial screening and phytochemical analysis of *Aegle marmelos* against enteric pathogens

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Abstract: Aqueous and solvent extracts of leaves collected from *Aegle marmelos* plant leaves were screened against enteric pathogens such as *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. This study initiated with isolation of test pathogens from enteric patients. Three pathogens were isolated from 50 patients and used as test pathogens. Ethyl acetate and acetone extract of *Aegle marmelos* showed antibacterial activity against test pathogens. Phytochemical study of *Aegle marmelos* shows presence of steroid, carbohydrate, alkaloid, phenolic compounds, saponins, xanthoprotein, tannins and flavonoids in ethyl acetate fraction. The study proves that compounds from *Aegle marmelos* will be a good source for diarrhea causing organisms.
Keywords: Antimicrobial, *Aegle mermelos,* Diarrhea.

Introduction

Diarrhoea is the third leading cause of morbidity and mortality in developing countries especially among young children. Diarrhoeal disease is a major contributory factor to malnutrition. Recurrent diarrhea coupled with inadequate feeding results in impaired body defense mechanisms. For infants and children in India, diarrhoea is one of the leading causes of death. Sixty-eight percentage of the diarrheal disease occurs in young children and it accounts for 17 % of death in children's below 5 years. In HIV infected persons diarrhoea can be prolonged severe and life threatening[1]. Diarrhoea is mainly transmitted through faecal by oral route. It is caused by a wide variety of bacterial, viral and protozoan pathogens excreted in the faeces of humans and animals. *Escherichia coli, Salmonella* spp., *Shigella* spp., *Campylobacter jejuni, Vibrio cholerae, Rotavirus, Norovirus, Giardia lamblia, Cryptosporidium* spp., and *Entamoeba histolytica*[2] are the major causative agent of diarrhoea. Majority of diarrhoel disease is caused by bacterial pathogens in developing countries while virus and protozoa tend to cause diarrhoea in developed countries.

In many parts of the world there is a rich tradition in the use of herbal medicine for the treatment of infectious diseases. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants[3, 4, 5, 6].

Microorganism like *E.coli, Salmonella, Shigella, Camphylobacter, Yersinia, Aeromonas, Plesimonas* etc., are involved in the process of diarrhea. Several plant species have been reported in controlling various human pathogens[7].

Aegle marmelos is a medicinal plant which is a fairly large, deciduous and glabrous tree with auxillary spines and usually trifoliate leaves belonging to the family, Rutaceae[8]. *Aegle marmelos* is commonly known as

vilvam in Tamil, bael in Hindi, sripal or bilwa in Sanskrit and bael tree in English. It is claimed to be useful in treating pain, fever, inflammation, respiratory disorders, cardiac disorders, dysentery and diarrhoea.The leaves extract of *Aegle marmelos* consist of tannins, skimmianin, essential oil (mainly caryophyllene, cineole, citral, citronellal, d-limonene and eugenol), sterols and/or triterpenoids, including lupeol. Essential oil obtained from leaves was found to possess broad spectrum anti bacterial and anti fungal activity[9]. Based on enteric diseases and importance of medicinal plant, the study was aimed to test the antimicrobial potentials of *Aegle marmelos* against enteric pathogens.

Materials and Methods

Collection of sample

Retrospective study was undertaken for a period of 6 months. Total of 50 cases, suspected with acute gastroenteritis admitted in KMC hospital, Tiruchirappalli, Tamil Nadu was subjected to microbial investigation at Department of Microbiology. Stool sample was collected from all the patients and transferred to laboratory with help of transferred medium.

Microscopic examination

The stool samples were examined under microscope for the presence of parasites by iodine wet mount method. The presence of protozoa and helminthes were identified by their morphology.

Isolation of Microorganisms

Escherichia coli, Salmonella spp., and Shigella spp.

Based upon the suspecting microorganisms, the mediums were prepared. The sample from the transferred medium was inoculated into Hektoen enteric agar, Xylose-lysine deoxycholate agar and Salmonella Shigella agar for isolation of *E.coli, Salmonella*, and Shigella bacteria. To differentiate *Salmonella* spp., from *Shigella* spp., the sample was inoculated into Rajhans medium. All the plates were incubated at 37 °C for 24–48hours.

Vibrio species and Aeromonas species

The sample also inoculated into Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar for the isolation of *Vibrio* species, Ampicillin blood agar for isolation of *Aeromonas* species. The inoculated plates were incubated at 37 °C for 24–48hours.

Campylobacter spp. and Yersinia enterocolitica

The stool sample was inoculated on Campy blood agar plates and incubated under microaerophilic condition for 2 days. Colony formation in the medium was subjected to biochemical tests to confirm *Camplylobacter* spp., The Stool specimen was directly streaked on Yersinia selective medium and incubated at 37 °C for 24 h. The plates were observed for dark pink colonies after overnight incubation for the isolation of *Yersinina enterocolitica*

Antibiotic sensitivity test

All the isolated pathogens were analysed for antibiotic sensitivity test by Kirby Bauer method. About 18 h old bacterial culture was prepared and inoculated into Mueller Hinton Agar (MHA) plates. Standard antibiotics disc were placed on MHA plates inoculated with test bacterial strains. All the plates were incubated at 37 °C for 24 h.

Collection of plant leaves

The plant *Aegle marmelos* leaves were collected from in around Tiruchirrapalli. The freshly collected leaves were washed and dried in shade at room temperature for 10–15 days. The dried leaves were used for powdering by using mortar and pestle. The larger plant debris was removed and powdered leaves were used for extraction for antimicrobial compounds.

Extraction of crude compounds

Aqueous extract

One gram of fresh leaves were taken and washed with sterile distilled water. The leaves were crushed by using mortar and pestle. The crushed leaf paste was mixed with 20 ml of sterile distilled water in 50 ml beaker. The aqueous leaf mixture was covered with aluminium foil and kept at room temperature for 24 hrs.

Solvent extract

Five different solvents such as methanol, chloroform, ethyl acetate, dichloromethane and acetone were used for the extraction of antimicrobial compounds from leaf powder. One gram of dried plant powder was taken in a 50 ml beaker and 20 ml of methanol was added into it. This content was mixed well and the beaker was covered with aluminium foil and kept for extraction at room temperature for 24 h. The procedure was adopted for remaining solvents.

Antibacterial activity of crude extracts

Aqueous extracts

The antimicrobial activity of mangrove aqueous extracts was studied by well diffusion method using MHA plates. About 18 hours old bacterial culture was prepared and inoculated into MHA plates. 5 mm diameter well was cut on plates. Each 10µl of aqueous plant extracts were added in wells using micropipette. Ten micro liter sterile distilled water was used as a control well. All the plates were incubated at 37 °C for 24 h and plates were observed for zone of inhibition[6].

Solvent extracts

The antimicrobial activity of solvent extracts was studied by disc diffusion method using MHA plates. About 18 h old bacterial cultures were inoculated into MHA plates. 0.25 mg of crude extracts were added into sterile filter paper disc (5 mm diameter) and allowed to dry at room temperature for few minutes. Crude plant extract impregnated discs were placed on MHA plates inoculated with test bacterial strains. Sterile empty disc was used as a control. All the plates were incubated at 37 °C for 24 h. After incubation the plates were observed for zone of inhibition.

Phytochemical study

Methanol extract was refluxed with 2N HCL in methanol. This concentration was saponified with 5 % KOH in ethanol. Solvent was removed by evaporation under reduced pressure and diluted with water. Then the mixture was extracted several times with chloroform. The extracted compound was dissolved in chloroform and analyzed for the presence of flavanoids, alkaloids, terprnoids, saponins, tannins, amino acids, anthraquinone, steroids, glycosides and reducing sugar.

Result and Discussion

Microscopic examination and gross examination of stool specimen revealed, absence of adult worms in all the 50 samples and 14 % of the sample showed positive to Entamoeba cyst. The prevalence of diarrhea with reference to age showed that bacterial incidence was higher than any other microbial etiology. Incident rate of diarrhea is higher among children who are 2–5 years of age (Table 1).

Table 1: Incidence of diarrhoea with reference to age

Age	No. of samples	Microbial etiology			
		Bacteria	Protozoans	Nematodes	Combination
0–1 year	13	13	Nil	Nil	Nil
2–5 years	17	14	3	Nil	Nil
6–10 years	10	5	2	Nil	1
11–15 years	10	4	3	Nil	1

Isolation of pathogens

Totally three genus were isolated from stool samples. All the test pathogens were identified by cultural characteristics and biochemical analysis and confirmed by Bergey's manual of systematic bacteriology.

Selective and differential culturing results showed that 50 % of the infection was due to *E.coli* followed by *Shigella* 24 % and *Salmonella* 26 %. This result correlates with study of Paniagua et al. 1997 in which 38 % of the diarrhoel episode is caused by *E.coli*[10]. The isolated *E. coli*, *Salmonella* spp., and *Shigella* spp., used as test pathogens.

Antibiotic sensitivity test

Antibiotic sensitivity pattern of enteric isolates shows an alarming rate of increasing resistant properties of the isolates. This result highly correlates with the result of Agarwal et al.[11] The sensitivity pattern of isolated test pathogens was represented in Table 2.

Table 2: Antibiotic sensitivity assay of enteric isolates

S.No.	Antibiotics	Quantity (mcg)	Percentage of resistance		
			E.coli	***Salmonella* spp.**	***Shigella* spp.**
1	Amikacin	30	S	S	S
3	Azithromycin	30	I	S	S
3	Vancomycin	30	S	S	S
4	Chloramphenicol	30	R	R	R
5	Amoxyclav	30	R	I	S
6	Kanamycin	30	R	R	R
7	Erythromycin	30	R	I	S
8	Tetracycline	30	R	R	R
9	Sreptomycin	30	S	R	R
10	Ampicillin	30	I	I	R

R- Resistant; I-Intermediate; S-Sensitive

Antimicrobial activity of Crude extract

Antimicrobial activity of plant *Aegle marmelos* aqueous extract does not showed any of activity against test pathogens (*E. coli*, *Salmonella* spp., and *Shigella* spp.).

In this present study, solvents such as methanol, chloroform, ethyl acetate, dichloromethane and acetone were tested for extraction of crude compound. Among the various solvents tested, the crude compounds were extracted only in ethyl acetate and acetone but not in other solvents. Solvent extracts of *Aegle marmelos* showed antibacterial activity with average zone of inhibition from 10 to 15 mm (Table 3). *Aegle marmelos* grown as a tree has shown to have the antimicrobial activity due to the seed oil as their component[12].

Table 3: Antibacterial activity of *Aegle Marmelos* crude extract

S.No.	Extract	Zone of inhibition in mm		
		E. coli	***Salmonella* spp.**	***Shigella* spp.**
1	Aqueous	–	–	–
2	Hexane	–	–	–
4	Chloroform	–	–	–
5	Ethyl acetate	15	12	11
6	Methanol	–	–	–
7	Acetone	13	–	12

Phytochemical analysis of *Aegle marmelos*

Aegle marmelos showed positive results for the presence of steroid, carbohydrate, alkaloid, phenolic compounds, saponins, xanthoprotein, tannins and flavonoids in ethyl acetate fraction. The other extract also

shows some of these compounds in acetone and chloroform (Table 4). Tannins, phenolics and flavonoids contributed for the antibacterial property of the plant. This is similar to the effect observed by Randir et al.[13]

Table 4: Phytochemical analysis of *Aegle marmelos*

Extracts	Steriod	Triterpenoids	Reducing sugar	Carbohydrates	Alkoloids	Phenol	Saponins	Xanthoproteins	Tanins	Flavanoids
Hexane	+	–	–	–	–	+	+	–	+	+
Benzene	–	–	–	–	–	+	+	–	+	+
Chloroform	–	–	–	+	–	+	+	–	+	+
Ethyl acetate	–	–	–	+	+	+	+	–	+	+
Methanol	–	–	–	+	–	+	+	–	+	+
Acetone	+	–	–	–	–	+	+	–	+	+
Aqueous	+	–	–	+	–	+	+	+	+	+
Water	+	–	–	+	–	+	+	–	+	+

+ ——> Present; – ——> Absent

Conclusion

E.coli, Salmonella spp., and *Shigella* spp., were isolated in this study, among the three isolates *E.coli* shows highest predominance (50 %). *Aegle marmelos* are traditional medicinal plants used for the treatment of diarhoea. The findings of the present study conclude that *Aegle marmelos* plants will be a potential source for production of bioactive compounds against enteric organisms like *E. coli, Salmonella* spp., and *Shigella* spp. Further studies such as purification, chemical characterization and structure elucidation of active compounds from the plant are in progress.

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