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Antimicrobial activity of *Aegle marmelos* leaves extracts in different solvents

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Abstract
Aegle marmelos (L.) Correa (Family-Rutaceae) is an important medicinal herb, used for antimicrobial testing against five selected pathogenic Microorganisms *viz*., *Salmonella typhi* MTCC3216**,** *Escherichia coli* MTCC40**,** *Pseudomonas aeruginosa* MTCC2581**,** *Proteus vulgaris* MTCC428**,** *Staphylococcus aureus* MTCC3160. Cold and hot extraction methods were used to extract *A. marmelos* leaves in solvents such as ethanol and petroleum ether. Thus, four extracts were prepared. For control petroleum ether, ethanol, and Ampicilin (in petroleum ether and ethanol) were used. Disk Diffusion Method and Broth Dilution Method were used to determine the Zone of Inhibition and Minimum Inhibitory Concentration, respectively. The antibacterial impact of these two approaches was found against four microorganisms *S. typhi* MTCC3216**,** *E. coli* MTCC40**,** *P. vulgaris* MTCC428**,** *S. aureus* MTCC3160.

Keywords: antimicrobial activity, *Aegle marmelos*, clinical pathogens, disc diffusion, serial dilution

Introduction
Aegle marmelos (L.) Correa Family-Rutaceae (*A. marmelos*) is commonly known as Bael and "Bilipatra" in Gujarati. Its Medicinal properties have been described in the ancient medical treatise in Sanskrit, "Charak-Samhita [1]. The stem, bark, root, leaves, and fruit of this tree, at all stages of maturity, have therapeutic properties and have been used as traditional medicine for a long time. Traditional medicine is a prevalent practise in India because of its vast range of pharmacological activities [2]. Traditional medicine is used in primary health care in both developed and developing countries. Many commonly used medications are expensive or difficult to obtain, and the emergence of resistance is a major impediment to their continued use. This circumstance forced scientists to look for new, low-cost medications that can act for longer periods of time before resistance develops [3]. The main aim of this research work was to screen antimicrobial potency of extracts from leaves of *A. marmelos* in different solvents (petroleum ether, ethanol) by cold and hot extraction method.

Material and Methods
Collection of Selected Plant
Fresh leaves of *Aegle marmelos* (L.) Correa Family-Rutaceae were randomly taken from the "temple of Bhagvan Shive" in hamlet Bela, Gujarat, India. Dr. M. H. Parabia, Department of Biosciences, South Gujarat University, Surat, confirmed the taxonomic identity of this plant.Fresh leaves were collected and washed under running tap water and then dried in oven at 37 ˚C and then homogenized to fine powder individually.

Preparation *Aegle marmelos* Extracts
For extract preparation, 10gm of fine powder of leaves of *A. Marmelos* were extracted in 250 ml of petroleum ether and ethanol separately using Soxhlet apparatus at 70 ˚C and 80 ˚C respectively up to four cycles. Solvents were evaporated in oven to make final concentration 40mg/ml. Stock solution of antibiotic-amphicilin also prepared at same concentration. For cold extraction 10gm of fine powder of leaves of *A. Marmelos* was extracted in 250 ml of petroleum ether and ethanol separately using BOD bottle at room temperature and 150 rpm for 48 hours. Whatman filter paper No. 1 was used to filter the extracts. In the oven, the solvent is evaporated to a final concentration of 40 mg/ml. These stock solutions were stored at 4 ºC in air tight bottles for further studies.

Determination of antimicrobial activity
The antibacterial activity was tested on a variety of bacteria *viz*., *Salmonella typhi* MTCC3216, *Escherichia coli* MTCC40, *Pseudomonas aeruginosa* MTCC2581, *Proteus vulgaris* MTCC428, *Staphylococcus aureus* MTCC3160 procured from Microbial type culture collection, Chandigarh. The agar disc diffusion technique described by Kirby-Bauer [4] was used for determining antimicrobial activity. The culture of test organism (optical Density 0.22 at 600nm, approximately 10^5 CFU/ml) was prepared. 0.2ml of it was spread on nutrient agar plates. Discs prepared by impregnating 5µl of extract/solvent/amphicilin using micropipette and dried it in oven, were applied on same nutrient agar plate. The plates were held at 4 °C for 15 minutes to allow for diffusion before being incubated at 37 °C overnight. The zone of inhibition against the test organisms was used to assess antimicrobial activity.

Determination of minimum inhibitory concentration (MIC)
The minimum inhibitory concentration (MIC) was determined using the two-fold broth dilution method [5]. The MIC of each extract that inhibited the growth of one or more bacteria was determined. The dilutions were made in nutritional broth in decreasing concentration order. The dilutions were prepared in nutrient broth the decreasing order of concentration (2000µg, 1000µg, 500µg, 250µg, 125µg, 62.50µg, 31.25µg) of extracts/antibiotic/solvent.

From the inoculums 10µl of each culture was inoculated separately in each set so that final concentration of microorganism in tubes became 10^6cells/ml. The MIC was determined by taking the maximum dilution of each extract that corresponded to the test organism that showed no observable growth. (the growth was compared with positive as well negative controls).

Results and Disscusion

For test organisms such as *Salmonella typhi* MTCC3216, *Escherichia coli* MTCC40, *Pseudomonas aeruginosa* MTCC2581, *Proteus vulgaris* MTCC428, and *Staphylococcus aureus* MTCC3160, the disc diffusion method was used to screen antibacterial activity and the two-fold broth dilution method was used to determine MIC values.

Results of Determination of Zone of Inhibition (ZOI)

The ZOI of extracts in petroleum ether and ethanol of 600 µg/ml concentrations against the test organisms are shown Table: 1. provides the measured ZOI of the extracts of leaves of *A. marmelos* with different solvents against five bacteria.

Table 1: Results of determination of zone of inhibition

Sr. No.	Extracts	Zone of Inhibition (mm)				
		E. coli	*S. typhi*	*S. aureus*	*P. vulgaris*	*Ps. arugenosa*
1.	P	0	0	0	0	0
2.	AP	14	20	14	22	0
3.	LPH	16	22	16	22	0
4.	LPC	16	22	16	22	0
5.	E	0	0	0	0	0
6.	AE	16	14	12	24	0
7.	LEH	16	24	18	28	0
8.	LEC	16	22	18	28	0

Note: For full form of extract column refer the abbreviation. EC: *Escherichia coli* MTCC40, ST*: Salmonella typhi* MTCC3216, PA*: Pseudomonas aeruginosa* MTCC2581, PV: *Proteus vulgaris* MTCC428, SA: *Staphylococcus aureus* MTCC3160. P: Petroleum ether; AP: Ampicilin in Petroleum ether; LPC: Leaves in Petroleum ether by Cold extraction; LPH: Leaves extract in Petroleum ether by Hot extraction; E: Ethanol; AE: Ampicilin in Ethanol; LEH: Leaves extract in Ethanol by Hot extraction; LEC: Leaves extract in Ethanol by Cold extraction.

Except for *P. aeruginosa*, which is resistant to leaf extract, each plant extract showed antibacterial efficacy against *E. coli*, *S. typhi*, *S. aureus*, and *P. vulgaris*. Each extract was very effective against *P. vulgaris* among all five organisms and it was also seen that ethanol extract was more effective than the petroleum ether extract against *P. vulgaris*. Among four susceptible stains *E. coli* and *S. typhi* had less susceptibility towards the petroleum ether extracts, while among them *S. aureus* was less susceptible towards ethanol extracts. For *P. vulgaris* and *S. typhi* both extracts (petroleum ether and ethanol) were very effective and less effective in case of *E. coli* and *S. aureus*. It was seen that the all extract has more effect than reference antibiotics and control-petroleum ether and ethanol with respective pathogenic microorganism.

Results of Determination of MIC

After evaluating the values of ZOI of extracts, each positive extracts was taken for MIC test by two fold broth dilution method. MIC of ethanol extract and petroleum ether extract of leaves were tested against *E. coli*, *P. vulgaris*, *S. aureus*, *and S. typhi.* The test organisms were inoculated in various concentrations of plant extracts i.e. 2000µg/ml, 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, and 31.25µg/ml.

Table 2: Results of MIC for *E. coli* MTCC40

Sr. No.	Extracts	Dilution of Extracts (µg/ml)						
		2000	1000	500	250	125	62.50	31.25
1.	P	+	+	+	+	+	+	+
2.	AP	-	-	-	+	+	+	+
3.	LPH	-	-	-	+	+	+	+
4.	LPC	-	-	-	+	+	+	+
5.	E	+	+	+	+	+	+	+
6.	AE	-	-	-	+	+	+	+
7.	LEH	-	-	-	+	+	+	+
8.	LEC	-	-	-	+	+	+	+

Note: Abbreviation: P: Petroleum ether; AP: Ampicilin in Petroleum ether; LPC: Leaves in Petroleum ether by Cold extraction; LPH: Leaves extract in Petroleum ether by Hot extraction; E: Ethanol; AE: Ampicilin in Ethanol; LEH: Leaves extract in Ethanol by Hot extraction; LEC: Leaves extract in Ethanol by Cold extraction. '+': presence of growth '-': absence of growth.

Table 3: Results of MIC for *S. typhi* MTCC3216.

Sr. No.	Extracts	Dilution of Extracts (µg/ml)						
		2000	1000	500	250	125	62.50	31.25
1.	P	+	+	+	+	+	+	+
2.	AP	-	-	-	+	+	+	+
3.	LPH	-	-	-	-	+	+	+
4.	LPC	-	-	-	-	+	+	+
5.	E	+	+	+	+	+	+	+
6.	AE	-	-	-	-	+	+	+
7.	LEH	-	-	-	-	+	+	+
8.	LEC	-	-	-	-	+	+	+

Note: abbreviation → P: Petroleum ether; AP: Ampicilin in Petroleum ether; LPC: Leaves in Petroleum ether by Cold extraction; LPH: Leaves extract in Petroleum ether by Hot extraction; E: Ethanol; AE: Ampicilin in Ethanol; LEH: Leaves extract in Ethanol by Hot extraction; LEC: Leaves extract in Ethanol by Cold extraction. '+': presence of growth '-': absence of growth.

Table 4: Results of MIC for *S. aureus* MTCC2581

Sr. No.	Extracts	Dilution of Extracts (µg/ml)						
		2000	1000	500	250	125	62.50	31.25
1.	P	+	+	+	+	+	+	+
2.	AP	-	-	+	+	+	+	+
3.	LPH	-	-	-	+	+	+	+
4.	LPC	-	-	-	+	+	+	+
5.	E	+	+	+	+	+	+	+
6.	AE	-	-	-	+	+	+	+
7.	LEH	-	-	-	+	+	+	+
8.	LEC	-	-	-	+	+	+	+

Note: abbreviation: P: Petroleum ether; AP: Ampicilin in Petroleum ether; LPC: Leaves in Petroleum ether by Cold extraction; BPH: Leaves extract in Petroleum ether by Hot extraction; E: Ethanol; AE: Ampicilin in Ethanol; LEH: Leaves extract in Ethanol by Hot extraction; LEC: Leaves extract in Ethanol by Cold extraction. '+': presence of growth '-': absence of growth.

Table 5: Results of MIC for *P. vulgaris* MTCC428.

Sr. No.	Extracts	Dilution of Extracts (µg/ml)						
		2000	1000	500	250	125	62.50	31.25
1.	P	+	+	+	+	+	+	+
2.	AP	-	-	-	+	+	+	+
3.	LPH	-	-	-	+	+	+	+
4.	LPC	-	-	-	+	+	+	+
5.	E	+	+	+	+	+	+	+
6.	AE	-	-	-	+	+	+	+
7.	LEH	-	-	-	-	-	+	+
8.	LEC	-	-	-	-	-	+	+

Note: abbreviation: P: Petroleum ether; AP: Ampicilin in Petroleum ether; LPC: Leaves in Petroleum ether by Cold extraction; BPH: Leaves extract in Petroleum ether by Hot extraction; E: Ethanol; AE: Ampicilin in Ethanol; LEH: Leaves extract in Ethanol by Hot extraction; LEC: Leaves extract in Ethanol by Cold extraction. '+': presence of growth '-': absence of growth

The growth of *E. coli* MTCC40 in leaves extract of *A. marmelos* in petroleum ether and ethanol, Ampicilin was seen below the concentrations of 500µg/ml concentrations. So, 500µg/ml is MIC of leaves extract of *A. marmelos* in petroleum ether and ethanol (Table 2).
Similarly, the growth of *S. typhi* MTCC3216 in leaves extract of *A. marmelos* in petroleum ether and ethanol was seen below the concentrations of 250µg/ml concentration. So, 250µg/ml is MIC of leaves extract of *A. marmelos* in petroleum ether and ethanol (Table 3).
In the same way MIC of each extracts for *S. aureus* is 500µg/ml (Table 4). And MIC of ethanol extracts for *P. vulgaris* is 125µg/ml and MIC of Petroleum ether extracts for *P. vulgaris* is 500µg/ml (Table 5).

Summary

For test organisms such as *Salmonella typhi* MTCC3216, *Escherichia coli* MTCC40, *Pseudomonas aeruginosa* MTCC2581, *Proteus vulgaris* MTCC428, and *Staphylococcus aureus* MTCC3160, the disc diffusion method was used to screen antibacterial activity and the two-fold broth dilution method was used to determine MIC values. The antibacterial impact of these two approaches was found against four microorganisms *S. typhi* MTCC3216**,** *E. coli* MTCC40**,** *P. vulgaris* MTCC428**,** *S. aureus* MTCC3160. Except for *P. aeruginosa*, which is resistant to leaf extract, each plant extract showed antibacterial efficacy against all selected bacteria. It was seen that the all extract has more effect than reference antibiotics and control-petroleum ether and ethanol with respective pathogenic microorganism.

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