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In vitro analysis of *Aegle marmelos* leaf extracts on skin pathogens

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ABSTRACT

The study was undertaken to screen various solvent and aqueous extracts of *Aegle marmelos* leaves display potent *in vitro* antioxidant activity, total phenolics and antimicrobial potential on various skin pathogens in order to explore the potential for future novel antioxidants in food and pharmaceutical formulations and antimicrobial agents. Four extracts showed varying degree of efficacy in each assay. Aqueous extract (486 mg GAE/g dry weight) exhibited the highest total phenolics content followed by methanol which was correlated with total antioxidant activity. Aqueous extract was more effective against *S. saprophyticus*, *S. epidermidis*, *M.luteus* in comparison to other extracts.

INTRODUCTION

The use of plant based drugs dates back to the early Vedic civilizations. Man has always been dependent on the plants for food, shelter, flavors and also for its medicinal properties (Ameenah, 2006). The biologically active compounds present in the plants render them with medicinal characteristics like antimicrobial action of spices against food-borne diseases (Jennifer and Sherman, 1998). Historically, plant based medicines have been used extensively for relief, anti-asthmatic, anti-arrhythmia, astringency quality and wound healing properties. However, the side effects associated with synthetic drugs have now resulted in a visible paradigmatic shift towards plant based drugs. The WHO estimates over 80% of people converting back to the Ayurveda (Jeeva *et al.*, 2009). The free radicals and the other reactive oxidative species are produced in the normal pathological conditions of the body which can lead to structural and functional damage to the DNA, lipids and proteins (Almeida *et al.*, 2012). The herbal medicines have the inherent antioxidant property thereby making them one of the most sought after drugs. The detrimental effects of oxidative stress are lowered by the capability of the plant to produce reactive oxidant species (ROS). The plant extract either in the raw form or its chemical constituents facilitates natural antioxidants that help in lowering down the oxidative stress (Zengin *et al.*, 2011). Synthetic antioxidants have been suspected to be carcinogenic. Considering the limitations associated with synthetic antioxidants, recent trend has now been shifted to use of naturally occurring antioxidants (Siddique *et al.*, 2010). The search for novel natural antioxidants of plants has increased ever since. It is not clear which plant constituents are associated in reducing the risk of chronic diseases, but antioxidants appear to play a major role in the protective effect of plant medicine. Investigations have suggested that plant having polyphenolic compounds such as flavonoids possess antioxidant activity (Cook and Samman, 1996). Evidences have shown a correlation between biological activities of these compounds and their antioxidant activity (Gryglewski *et al.*, 1987). Past decades have witnessed the emergence of new pathogens and rise in the number of multidrug-resistant strains in well-established pathogens during the past decade, resulting in growing public health concern globally. Therefore, new alternatives need to be explored including natural herbal or plant-based extracts with reported antibacterial properties. *Aegle marmelos* (Bael) belongs to the *Rutaceae* family. Leaves, stem, roots and fruits of this plant are used as traditional medicine against various human ailments (Maity *et al.*, 2009). The major bioactive constituents present in the parts of the plant include skimmianine, aegeline, lupeol, cineole, citral, citronellal, cuminaldehyde and marmesin.

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A. marmelos is known to possess a lot of pharmacological properties such as anti-inflammatory, analgesic and antipyretic, wound healing, antineoplastic, radioprotective, chemoprotective, and chemopreventive effects (Arul *et al.,* 2005), antidiabetic (Upadhya *et al.,* 2004), chemoprotective (Singh *et al.,* 2000) and radio-protective (Jagetia *et al.,* 2004). *A. marmelos* is listed as one of the priority plant in National Medicinal Plant Board, India. The aim of this study was to screen various solvent extracts of *A. marmelos* leaves to display potent antioxidant activity *in vitro*, total phenolic and flavonoid contents in order to find possible sources for future novel antioxidants in food and pharmaceutical formulations. Further, role of these compounds for *in vitro* antimicrobial activity was analyzed for bacterial and fungal species with special emphasis on skin pathogens.

MATERIALS AND METHODS

Chemicals and solvents

All chemicals and solvents used were procured from himedia, Qualigens and Sigma. All the chemicals and reagents used were of analytical grade.

Plant material and Extraction

Leaves of *A. marmelos* were collected from locally growing plant from JIIT Noida. Leaves were shade dried at room temperature with occasional shifting and then powdered with a mechanical grinder and stored in an airtight container. Equal weight (33g) of powdered leaves was divided into four parts and dissolved in four respective solvents (190ml), hexane, ethanol, methanol, and water.

For each solvent preparation, each extract was kept on rotary shaker for 4-5 days at room temperature. Extracts were filtered and concentrated using rotary vacuum evaporator (International Scientific Instrument Co. Delhi, India). Extracts were stored in sterile containers for further use.

Antioxidant assays

Total antioxidant capacity

The total antioxidant capacity of the fractions was determined by phosphomolybdate method using ascorbic acid as a standard (Saeed *et al.,* 2012). The assay is based on the reduction of Mo (VI) to Mo (V) by the extracts and fractions and subsequent formation of green phosphate/ Mo (V) complex at acid pH. An aliquot of 0.1 ml of sample solution was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. A typical blank contained 1 ml of the reagent solution and the appropriate volume of the solvent and incubated under the same conditions. Ascorbic acid was used as standard. Total antioxidant capacity is expressed as Ascorbic acid equivalent (AAE). The antioxidant capacity was estimated using following formula:

Antioxidant effect (%) = [(control absorbance–sample absorbance) / (control absorbance)] *100

Total phenolic estimation

The total phenolic content was evaluated using Folin-Ciocalteu method (Singleton *et al.,* 1999). The phenolic compounds in the sample are oxidized by Folin-Ciocalteu reagent at an alkaline pH resulting in a blue-colored molybdenum-tungsten complex. 5ml of 0.2 N of Folin-Ciocalteu along with 4 ml of 7.5% of Na_2CO_3 was added to 100µl of the sample. The mixture was then incubated for three hours at room temperature and total phenolics were determined spectrophotometrically at 765 nm. Gallic acid (1mg/ml) was used a standard and amount of total phenolics was expressed as mg Gallic acid equivalent per gram (mg GAE/gm) dry weight.

Antimicrobial activity by Disc – diffusion assay

The extracts were screened for their antimicrobial potential using disc diffusion assay (Bauer *et al.,* 1996). The bacterial strains *Micrococcus luteus* (MTCC 106), *Staphylococcus epidermidis* (MTCC 435), *Staphylococcus saprophyticus* (MTCC6155), *Pseudomonas putida* (MTCC2445), *Pseudomonas fluorescens* (MTCC 2421), *Bacillus subtilis* (MTCC 1427), *Bacillus licheniformis* (MTCC 1483) were tested for their sensitivity towards each extracts. Sterile discs (2 mm) were infused with 20µl of the extracts on the agar plates. Distilled water was taken as blank and streptomycin (100mg/ml) served as a positive control. The plates were incubated at 37º C for 16 – 18 hours for bacterial cultures for 24-48 hrs. Negative control for each solvent was also taken. The test was carried out in duplicates. Antimicrobial activities were determined by measuring the diameters of zones of inhibition in mm. The means and standard deviations (±SD) of the diameters of zones of growth inhibitions for the treatments are shown in Table 3.

RESULTS AND DISCUSSION

The medicinal properties of Indian medicinal plants have been documents in recent scientific documents primarily due to their antioxidant activities (Chockalingam *et al.,* 2012). Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of therapeutic components in these plants. Phenolic and flavonoids widely distributed in plants have been reported to exert wide range of therapeutic activities such as antioxidant, free radical scavenging activity, anti-inflammatory, antimicrobial, antimutagenic, anticarcinogenic etc. (Soobrattee *et al.,* 2005). There is an increased interest in natural antioxidant extracted from these plants for their suitability as candidates for preventing oxidative damage and for promoting health. Plant phenolics are major group of compounds acting as primary antioxidants or free radical scavengers. It was therefore, logical to determine total phenolic content in various plant extracts. The *A. marmelos* leaves were found to be a good source of several antioxidant components as shown in table 1. The total

antioxidant capacity of *A. marmelos* is observed to have decreased in this pattern: Aqueous>Methanol>Ethanol> Hexane. The total phenolic content of different solvents ranged from 85 to 486 mg GAE/g fresh weight. The total phenolic capacity of *A. marmelos* is observed to have decreased in this pattern: Aqueous > Methanol > Ethanol > Hexane. The aqueous extract exhibited the highest total phenolics content. Extracts with high antioxidant activity showed a high phenolic content which was in well agreement with the previous reports (Okpuzor *et al.,* 2009). Phenolics are also known to have antioxidant activity which are able to terminate free radicals and chelate metal ions capable of catalyzing ROS formation which promote lipid peroxidation (Almeida *et al.,* 2000). This study affirms the *in vitro* antioxidant potential of crude methanol, ethanol, hexane extracts of *A.marmelos* leaves in comparison to standard compounds such as gallic acid and ascorbic acid.

Table. 1: Total phenolics content (mg GAE*/gm fresh weight) and total antioxidant capacity (%).

Plant extracts*	mg GAE*/g dry weight	% antioxidant activity
AE	486	9.95
ME	289.9	6.36
EE	247.8	4.57
HE	85	2.74

**AE-Aqueous extract, EE-Ethanol extract; ME-Methanol extract; HE-hexane extract.*

The antibacterial activity of four extracts was assayed *in vitro* by Disc diffusion assay against seven different bacterial strains. The results revealed that all extracts exhibited antimicrobial activity with different efficacy for different pathogen (table 2). All the extracts exhibited broad spectrum antimicrobial activity with zones of inhibition ranging from 9 to 26 mm against *S. epidermidis*, *S. saprophyticus*, *M. luteus*, *P. fluorescens*, *P. putida*, *B. licheniformis*, *B. subtilis*. Aqueous extract was effective against *S. saprophyticus*, *M.luteus* and *S. epidermidis* where the zones of inhibition were 25mm, 26 mm and 12 mm respectively. Acetone and hexane extract did not show any antibacterial activity against almost all the bacteria studied. Figure 1 showed that Gram-positive bacteria are susceptible to plant extracts more as compared to Gram-negative bacteria as reported previously (Mbwambo and Moshi, 2005; Bartfay *et al.*, 2012). This difference can be attributed to membrane compositions of bacteria. *M. luteus* has been suggested as a standard gram-positive indicator organism for antimicrobial testing (Maher and McClean, 2006).

Table. 2: The antimicrobial effect of *A. marmelos* leaves extracts against Gram- Positive and Gram-Negative Bacteria (Zone of inhibition in millimeters ± SD).

Plant Extracts	*SE*	*SS*	*ML*	*PF*	*BL*	*BS*	*PP*
AE	12 ± 2	25 ± 2	26 ± 2	17 ± 2	ND	10 ± 2	ND
EE	10 ± 2	12 ± 2	12 ± 2	11 ± 2	14±2	10 ± 2	6 ± 2
ME	9 ± 2	ND	ND	14 ± 2	15± 2	ND	5 ± 2
HE	13 ± 2	ND	ND	ND	ND	ND	ND

SE- S. epidermidis; SS- S. saprophyticus; ML- M. luteus, PF- P. fluorescens; BL- B. licheniformis, BS- B. subtilis, PP- P. putida; AE-Aqueous extract, EE-Ethanol extract; ME- Methanol extract; HE-hexane extract. Values are mean ± SEM, n=3.

Taken together, our results and preliminary support from researchers have supported that the plant *A. marmelos* possesses antibacterial properties against a variety of gram-positive and gram-negative bacteria (Kothari *et al.,* 2011). Further, identification and isolation of biologically active compounds playing a role in these activities will be needed. Although, it requires lots of times for isolation and purification of such compounds, it appears beneficial considering the growing public health concern of multidrug-resistant strains of bacteria globally.

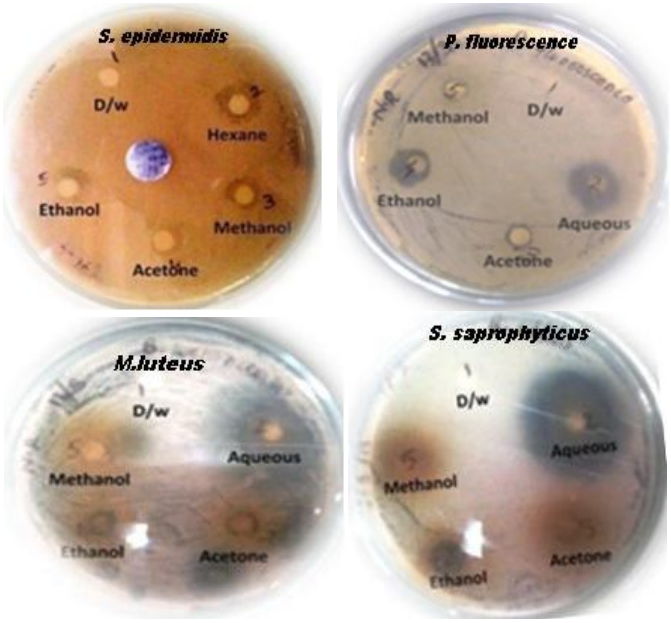

Fig. 1: Antibacterial activities of various *A. marmelos* extracts on various Gram-Positive and Gram-negative Bacteria.

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