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Research Article

ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF IRANIAN BAEL (*AEGLE MARMELOS*) FRUIT AGAINST SOME FOOD PATHOGENS

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ABSTRACT

Aegle marmelos has been widely used in traditional systems of medicine for a variety of diseases. In the present study, fruit of *Aegle marmelos* was evaluated for its antimicrobial and antioxidant activity. Antimicrobial activity was evaluated towards six food pathogenic strains of bacteria and fungi. The results indicated that bael fruit has more antifungal activity in comparison of it's antibacterial properties. Total phenolic content (mg of gallic acid equivalent (GAE)/100 g decoction) was 336.1 ± 8.1. The results of 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity and the antioxidant content of fruit samples were 17.37 ± 2.71 mg/ml and 379.9 ± 28.28 mg ascorbic acid equivalent antioxidant content (AEAC) /100 g, respectively. These results clearly suggest that, the *Aegle marmelos* can rightly be mentioned as a medicinal food additives of considerable interest.

Keywords: Bael fruit, Antioxidant activities, Antimicrobial activities, Food pathogens

INTRODUCTION

Over the last few years, researchers have aimed at identifying and validating plant-derived substances for the treatment of various diseases. Interestingly, it is estimated that more than 25% of modern medicines are directly or indirectly derived from plants. This revival of interest in plant derived drugs is mainly due to the current widespread belief that "green medicine" is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects.[1]

Aegle marmelos, commonly known as Bael, is a spiny tree belonging to the family Rutaceae. It is an indigenous tree found in Iran, India, Myanmar, Pakistan, Bangladesh and most of Southeast Asian countries.[2] The leaves, roots, bark, seeds and fruits are edible and medicinal values. The peel of the fruit which is a very hard shell and green to brown in color depends on ripening stage. The appearance of yellow or orange edible pulp is like a boiled pumpkin, possesses a slightly sweet taste and a characteristic floral, terpene-like aroma, very fragrant and pleasantly flavored. Seeds are surrounded by slimy transparent mucilage. The bael fruit pulp contains many functional and bioactive compounds such as carotenoids, phenolics, alkaloids, coumarins, flavonoids, terpenoids, and other antioxidants which may protect us against chronic diseases. Total dietary fiber found in this fruit can be divided into insoluble dietary and soluble dietary fiber (mucilage and pectin). In addition, it also contains many vitamins and minerals including vitamin C, vitamin A, thiamine, riboflavin, niacin, calcium, and phosphorus.[3] Therefore, bael fruit may indicate that it is one of the important plants used for indigenous traditional medicine. It used for the treatment of diarrhea, intestinal parasites, and dryness of the eyes and alcoholic extracts of the fruits showed hypoglycemic and antidiabetic activity.[4-6]

The uses of bael fruit in aspects of food have many forms in each country. For example, the ripe fruit is consumed fresh and also prepared as nectar, squash, sherbet, jam, marmalade, and cream. Nowadays, the world market for functional foods and nutraceuticals is large and growing. According to the literature, the characteristic of bael fruit in terms of bioactive compounds and characteristic flavor was considered to have a potential for use as functional food and value added processed products.[7]

In the present study, fruit of *Aegle marmelos* was evaluated for its antioxidant and antimicrobial activities against some food pathogens.

MATERIALS AND METHODS

Bacteria and fungi strain, culture conditions, and preparation of inocula

Listeria monocytogenes ATCC 19115, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 9144, *Candida albicans* ATCC 10231, *Aspergillus fumigatus* ATCC 13073, *Aspergillus niger* ATCC 10864 were used in this study that were provided by Iranian research organization for science and technology. A pure culture of the strains were maintained at -20 °C in Brain Heart Infusion broth (Merck) containing 25% glycerol, which was thawed before use. To prepare bacterial and fungi inocula, 0.1 ml of the thawed cultures were transferred to 10 ml of sterile Tryptic Soy Broth (TSB, Merck) and Sabouraud Dextrose Broth (SDB, Merck) and were incubated overnight at 37 and 30 °C, respectively. A Tryptic Soy Agar (TSA, Merck) and Sabouraud Dextrose Agar (SDA, Merck) plates were streaked from these broth and incubated overnight at 37 and 30 °C.

To establish a correlation between the colony forming unit (CFU) ml^{-1} and absorbance of the dilutions at 600 nm, a standard curve was prepared. A single colony from the TSA and SDA plates were inoculated into 10 ml of TSB and SDB and were incubated over night at 37 and 30 °C. A volume of 0.1 ml of the suspensions were transferred to 10 ml of TSB and SDB and were incubated at 37 and 30 °C for about 18 h. The cultures were harvested at the midlog phase, three times pelleted by centrifugation at 3000*g* for 20 min and washed in 0.1% peptone–water. Final cell pellets were resuspended in 10 ml of 0.1% peptone–water. Five twofold serial dilutions were made and their absorbances were measured at 600nm. To determine the $\log_{10}$ $CFUml^{-1}$ corresponding to the absorbance of that same dilution, viable cell counts were performed in duplicate by plating serial dilutions onto TSA and SDA and incubating the plates at 37 and 30 °C for 24 h.

Plant material and preparation of decoction

A crude aqueous extract (decoction) was used for the study since it represents the nearest form to traditional preparations. The decoction was prepared as described by Brijesh, Daswani, Tetali, Antia & Birdi (2009).[8] One g of the powdered dried fruit was boiled in 16 ml double distilled water till the volume reduced to 4 ml. It was centrifuged and filtered through a 0.22 µm membrane before use. To replicate field conditions, each assay was performed with freshly prepared decoction.

The dry weight of the decoction thus obtained was 45.4 mg/ml ± 0.7 mg/ml and 19.8% ± 1.9% (w/w) with respect to the starting dried plant material. The decoction was diluted 1:25 in sterilized distilled water for each experiment.

Determination of MICs, MBCs and MFCs for bacteria and Fungi

The decoction was screened for it's ability to inactivate or inhibit the growth of *Listeria monocytogenes*, *Salmonella typhimorium*, *Staphylococcus aureus, Candida albicans*, *Aspergillus fumigatus* and *Aspergillus niger* using a standard tube dilution technique.[9] Briefly, the experiment was performed by preparing twofold dilution's of

decoction dissolved in sterilized distilled water in a series of tubes containing TSB (for bacteria) and SDB (for fungi). Final concentration ranges of the decoction were 5 mg ml^{-1}–19.5 µg ml^{-1}. Each tube was inoculated with a suspension of the bacterium or fungus to obtain a concentration of 6 log_{10} CFU ml^{-1}. Bacterial or fungi suspension, distilled water and the TSB or SDB were checked separately as control. The inoculated tubes were incubated at 37 °C for 16–18 h (for bacteria) and 30 °C for 15 days (for fungi).

The lowest concentration of decoction at which no visible growth of the bacterium or fungus (no turbidity in the tube) was detected, was defined as the minimum inhibitory concentration (MIC). TSB or SDB tubes showing no visible growth were sub-cultured on TSA and SDA plates and incubated at 37 °C for 24 h and 30 ºC for 5 days to determine whether the organism was inhibited or killed. The lowest concentration of decoction with no growth on the plates, indicating lethality for the bacteria or fungi, was taken as Minimum Bactericidal Concentration (MBC) and Minimal Fungicidal Concentration (MFC), respectively.

All experiments were conducted in triplicate and the results were expressed as mean values.

Estimation of total phenolic content

The Folin–Ciocalteu method (Singleton, Orthofer & Lamuela-Raventos, 1999) was used to determine total phenolic content. 1 g of decotion was diluted to 10 ml with distilled water and filtered through Whatman No. 1 paper. This solution (0.5 ml) was then mixed with 2.5 ml of 0.2 N Folin–Ciocalteu reagent (Merck, Darmstadt, Germany) for 5 min and 2 ml of 75 g/l sodium carbonate (Na2CO3) (Merck, Darmstadt, Germany) was then added. After incubation at room temperature for 2 h, the absorbance of the reaction mixture was measured at 760 nm against a methanol blank (CECIL CE 2041 spectrophotometer 2000 series from CECIL instruments, Cambridge, England). Gallic acid (Merck, Darmstadt, Germany) (0–200 mg/l) was used as standard to produce the calibration curve. The mean of three readings was used and the total phenolic content was expressed in mg of gallic acid equivalents (GAE)/100 g of decoction.[10]

Radical scavenging activity and antioxidant content

The scavenging activity of samples for the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described (Velazquez, Tournier, Mordujovich de Buschiazzo, Saavedra & Schinella, 2003) with some modifications. Decoction sample was dissolved in methanol at a concentration of 2.65–170 mg/ml, and 0.75 ml of the sample was mixed with 1.5 ml of DPPH (Merck, Darmstadt, Germany) in methanol (0.02 mg/ml), with methanol serving as the blank sample. The mixtures were left for 15 min at room temperature and the absorbances then measured at 517 nm. Ascorbic acid (Merck, Darmstadt, Germany) (0–40 mg/l) were used as positive controls. The radical scavenging activity was calculated as follows as: % Inhibition = [(blank absorbance - sample absorbance)/blank absorbance] × 100. The mean of three IC50 (concentration causing 50% inhibition) values of the decoction sample was determined graphically.[11]

The antioxidant content was evaluated as described (Chen, Mehta, Berenbaum, Zangerl & Engeseth, 2000), with some modifications. Decoction sample was dissolved in methanol (0.02 or 0.04 g/ml) and 0.75 ml of it was mixed with 1.5 ml of a 0.02 mg/ml solution of DPPH in methanol. The mixtures were left for 15 min at room temperature and the absorbances then measured (517 nm). The blank sample consisted of 0.75 ml of decoction with 1.5 ml of methanol. The antioxidant content was determined using standard curves for ascorbic acid (0–10 µg/ml). The means of three values were obtained, expressed as mg of ascorbic acid equivalent antioxidant content (AEAC) per 100 g of decoction.[12]

Statistical analysis

All measurements were performed in triplicate. The data are given as mean ± SD. Correlation analysis was performed between the antioxidant activity values. P values less than 0.05 were considered to be statistically significant. SPSS software version 11.5 was used for statistical analysis.

RESULTS

MIC results were 19.5 µg ml^{-1}, 39 µg ml^{-1}, 625 µg ml^{-1} and 1.25 mg ml^{-1} for *Aspergillus niger, Aspergillus fumigatus, Candida albicans and Staphylococcus aureus,* respectively. MFC values of 2.5 mg ml^{-1} and 5 mg ml^{-1} showed against *Aspergillus niger* and *Candida albicans,* respectively. Decoction showed no MIC, MBC or MFC against other studied microorganism.

Total phenolic content (mg of GAE/100 g of decoction) was 336.1 ± 8.1 using the standard curve of gallic acid (R^2 = 0.9986). The results of DPPH radical scavenging activity (IC_{50} values) and the antioxidant content of decoction samples were 17.37 ± 2.71 mg/ml and 379.9 ± 28.28 mg AEAC/100 g (using the standard curves of ascorbic acid, R^2 = 0.9990), respectively. The correlation coefficient between $1/IC_{50}$ and AEAC was r = 0.99. The correlation coefficient between RSA and total phenolics was 0.98 and between AEAC and total phenolic was 0.95.

DISCUSSION

Historically, medicinal plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being. Bael fruit may indicate that it is one of the important plants used for indigenous traditional medicine. It can be used as food additive because of its typical color, flavor, and texture. In the present study, decoction showed more potency against fungi in comparison with food pathogen bacteria. The essential oil of *Aegle marmelos* showed strong fungitoxicity against some storage fungi-causing contamination of foodstuffs. The oil also showed efficacy as aflatoxin suppressor at 500 microL/L as it completely arrested the aflatoxin B_1 production by the toxigenic strains of *Aspergillus flavus*.[2] Brijesh, Daswani, Tetali, Antia & Birdi (2009) exhibited antigiardial and antirotaviral activity for the decoction of *Aegle marmelos* whereas it did not show any antibacterial activity. They suspected that despite not being bactericidal, the antidiarrhoeal effect of this plant is possibly due to its ability to affect other bacterial virulence parameters.[8] Minimum inhibitory concentration and minimum bactericidal concentration were also performed for methanol extract of *Aegle marmelos* leaves towards one of the susceptible organisms, *Serratia marcesens*, it exhibited MIC and MBC values of 200 mg/ml .[13]

Suresh, Senthilkumar & Karthikeyan (2009) studied the leaves and flower extract of *Aegle marmelos* activity against five different clinical pathogens strains such as *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Salmonella typhi* at different concentrations (50, 100 and 200 ppm). In their assay *Escherichia coli* was the most susceptible bacterium, that may be attributed to the presence of Tannins alkaloids, inhibit the growth of microorganisms. The *Aegle marmelos* leaves extract, showed highly active against the microorganisms, *E. coli* followed by *Salmonella typhi, Staphylococcus aureus, Proteus mirabilis, Pseudomonas aeruginosa* in all the concentration. In 200 ppm concentration of leaves methanol extract had no much difference between the organisms. In methanolic extracts of *Aegle marmelos* flowers shows high antimicrobial activity against *Staphylococcus aureus* in all the concentration followed by *Proteus mirabilis, E. coli., Salmonella typi and Pseudomonas aeroginosa*.[6]

Antioxidants are significant in the prevention of human illness and may function as free radical scavengers, complexes of pro-oxidant metals, reducing agents and quencher of singlet oxygen formation.[14] Free radicals posses the ability to reduce the oxidative damage associated with many disease including neurodegenerative diseases, cancer, cardiovascular disease, cataracts and AIDS .[15-17] Antioxidants through their scavenging power are useful for the management of these diseases. Scientific information on antioxidant properties of various plants, particularly those that are less widely used in culinary and medicine, is still rather scarce. Therefore, the assessment of such properties remains an interesting and useful task, particularly for finding new sources for natural antioxidants,

functional foods and neutraceuticals. Synthetic antioxidants like butylated hydroxy anisole (BHA, butylated hydroxyl toluene (BHT), tertiary butylated hydroxy quinone and gallic acid esters have been suspected to be carcinogenic. Hence, strong limitations have been placed on their use and there is a trend to replace them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity.[18] However there is still a need to find out more effective antioxidant having fewer side effects from natural source. It has been found out that plant having polyphenolic compounds such as flavonoids posses antioxidant activity.[19] So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties .[20]

The present study has verified that remedial plants could be good source of antioxidant substances. The high potential of phenolics to scavenge free radicals may be due to many phenolic hydroxyl groups they posses.[21]

The correlation analysis revealed that a correlation exists between total phenolic content and total antioxidant activity. The correlation coefficient was 0.95 indicating a positive relationship between the total phenolic and total antioxidant activity. This result suggests that 95% of the antioxidant activity resulted from the contribution of the phenolic compounds.[22] Different secondary metabolites, such as volatile oils, carotenoids and vitamins may also contribute to the antioxidant capacity, which in this case contributed to approximately 5% of the antioxidant activity.[23]

These findings are in agreement with that reported by Guo, Cao, Sofic & Prior (1997) and Velioglu, Mazza, Gao & Oomah (1998), who found a high correlations between the total antioxidant activities of some fruits and their total phenolic contents.[24,25]

The antioxidant content which is a measure of RSA, was inversely proportional to IC_{50} (r = 0.99). The compounds such as flavonoids, which hold hydroxyls groups, are responsible for the radical scavenging activity in the plants.[26]

The same correlation (r = 0.98) was shown between the RSA results and total phenolic levels, suggesting that phenolic compounds were likely to be contributing to the RSA of this decoction. Others studies on plant extracts have confirmed that such exists.[20]

Siddique, Mujeeb, Najmi & Akram (2010) recorded the comparative antioxidant activity in methanolic extract of the selected parts (leaves, root and stem bark) of *Aegle marmelos*. The total phenol in methanolic extract of the leaves (9.8367 ± 0.0235 mg kg- 1) and in stem extract (7.4693 ± 0.047 mg kg-1) were higher than that in the extracts of root (1.7281 ± 0.049 mg kg-1). The % inhibition of leaf (64.12± 0.01), stem (76.883± 0.03) and root were (64.193± 0.05) in comparison to BHT (65.09± 0.22), ascorbic acid (52.163± 0.02) and rutin (72.686± 0.560) respectively. Free radical scavenging activity of different extracts was evaluated by using DPPH method. The highest free radical scavenging effect was observed in leaves with IC_{50} = 2.096µg ml^{-1}. The effectiveness of radical scavenging activity of leaves extract was about 10 times greater than reference antioxidant butylated hydroxy toluene (BHT).[27]

The study results of Suvimol & Pranee (2008) showed that Thai bael fruit pulps had total phenolic content of 87.34 mg gallic acid equivalent (GAE)/g dry weight (dw).[7] Total phenolics in bael fruits were in the range of traditional Chinese medicinal plants associated with anticancer (2.2-503 mg GAE/g dw), as well as higher than for common fruits and vegetables including kiwifruit, orange, pear, garlic, carrot, and spinach (1.2-10.8 mg GAE/g dw reported by Cai, Luo, Sun, & Corke, 2004).[28] The antioxidant activities obtained from their assay (6.21 µg dw/µg DPPH) were comparable to that of fruits, vegetables, herbs, and chewing plants such as *Diospyros kaki* L., *Garcinia mangostana* L., *Spondias pinnata* Kurz, *Leucaena glauca* Benth, and *Piper betel* Linn. (0.3-7 µg dw/ µg DPPH) which contain high antioxidant activity reported by Maisuthisakul, Suttajit & Pongsawatmanit (2007).[29]

The results of this study can add to the scientific literature and useful application from bael fruit for producers and consumers to be aware of the importance and utilization of this useful resource. Keeping in view the side effects of synthetic fungicides and antioxidants, A. marmelos may be recommended as an antimicrobial and antioxidant of plant origin to enhance the shelf life of stored food commodities by controlling the fungal growth as well as oxidation process. These results are useful for developing and improving the quality of bael fruit cultivar in order to provide more value addition and usefulness from bael fruit.

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