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Anti-microbial activity of different solvent extracts of dried flowers of *Aegle marmelos*

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Abstract
Aegle marmelos has been used to treat various illnesses of human beings in traditional and folk medicine for thousands of years. The aim of the present study is to validate the *in vitro* antibacterial activity of different extracts of dried flowers of *A. marmelos*. Antibacterial activity of aqueous, methanol, acetone and hexane extracts from flowers of *A. marmelos* was evaluated against two clinically important pathogenic bacteria *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) using agar well diffusion method and broth dilution method. Among the extracts tested, the highest zone of inhibition against both bacterial strains was exhibited by aqueous extract. All the extracts exhibited a significant antibacterial activity against *E. coli* compared to respective negative control. Hexane extract did not exhibit a significant antibacterial activity against *S. aureus*, while all the other extracts exhibited a significant activity compared to respective negative control. Gentamycin was used as the positive control and the antibacterial-activity exhibited by the test extracts was not comparable to that of Gentamycin. The minimum inhibitory concentrations confirmed the results obtained by agar diffusion method. The present study indicated the ability of the flower extracts of *A. marmelos* to inhibit the growth of both Gram negative and Gram positive bacteria, which is an indication of the broad spectrum antibacterial activity. Thus flower of *A. marmelos* could be a potential source for the development of novel antibacterial agents.

Keywords: *Aegle marmelos*, antibacterial activity, minimum inhibitory concentration, zone of inhibition

Introduction
Plants and plant products have been used to treat the physical and mental illnesses of human beings in ayurveda, traditional and folk medicine for thousands of years. The medicinal properties of different plants used in traditional medicinal systems have been validated through scientific research during past decade. These studies revealed that medicinal effects of plant products are resulted from the synergistic activity of the chemical compounds present in them. Mainly the secondary metabolites such as alkaloids, steroids, tannins, phenol compounds, etc are studied in depth and found that they are capable of altering biochemical and physiological reactions in the consumer's body [1, 2].

Currently due to inappropriate usage of antibiotics, many virulent bacteria developed resistance against the antibiotics used to eradicate them. Therefore introduction of new treatment remedies against bacterial infections has become a global requirement. In addition, the associated adverse effects of the synthetic antibiotics also leads to the need of new therapeutic strategies against bacterial infections. The medicinally active herbs have been recognized as rich sources of biologically active compounds [3, 4]. Hence recently the attention of scientists have directed towards the investigation of medicinally active compound from plants.

Aegle marmelos is a small deciduous plant belongs to family Rutaceae, which found in many Asian countries including Sri Lanka, India, Burma, Bangladesh, Egypt, Malaysia, Myanmar, Pakistan, Thailand and Indo-China. Almost every part of the *A. marmelos* tree including root, bark, leaf, flower and fruits is used in traditional medicinal systems and folk medicine to treat various diseases like diarrhea and dysentery, diabetes, cancers, constipation, respiratory infections nervous system diseases, etc [4, 5, 6]. The extensive studies on different parts of *A. marmelos* revealed that the plant possess different pharmacological properties like anti-inflammatory, antipyretic, analgesic, antioxidant, anti-diabetic, anti-diarrheal, anti-proliferative, etc. Phytochemical analysis of the different parts of the plant indicated that it contains various classes of bioactive compounds including alkaloids, tannins, steroids, coumarins and essential oils [7]. Although many parts of the plant have been studied extensively, the scientific validation of flower extracts is limited. The hot water extract of the dried flowers of the *A. marmelos* is a popular beverage in Sri Lanka with soothing and calming effects. The hypoglycaemic and anti-inflammatory activities of water and ethanolic extracts

and the bioactivity fractionation of the ethanolic extracts of the dried flowers of the *Aegle marmelos* have been established [7, 8]. In present investigation an attempt has been taken to assess the antibacterial activities of aqueous, methanol, acetone and hexane extracts of the flowers of *Aegle marmelos* against two clinically important pathogens, *E. coli* and *S. aureus.*

2. Materials and method

2.1. Collection of plant material

The fallen flowers were collected and dried in open air until a constant weight was obtained. The plant material was identified by Prof. P. Tissera, Professor of Botany, Department of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka in comparison to the herbarium specimens of the Department. A voucher specimen (USJP FMS 6/2010) has been deposited at the herbarium of the Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.

2.2. Extract preparation

The dried flowers were powdered using an electric blender. The powder (13.55 g each) was subjected to extraction with 70 mL of distilled water, methanol, acetone and hexane separately for 7 days. Each extract was filtered through a Whatmann filter paper and concentrated. They were stored in refrigerator (4 ^{0}C) until use.

2.3. Test microorganisms

Pathogenic strains of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were obtained from Medical Research Institute, Colombo 08, Sri Lanka and were maintained on Nutrient agar slant at 4 ^{0}C for further experiments.

2.4. Preparation of inoculum

Culture plates were prepared for two organisms separately. Bacterial colonies of each organism were added to saline water to prepare suspensions equivalent to 0.5 McFarland standard.

2.5. Agar well diffusion method

Nutrient agar plate was inoculated with (200 µl) each microbial suspension by spreading over the entire surface uniformly. Four wells with a diameter of 8 mm were punched aseptically in each nutrient agar plate. Four wells of each agar plate were filled with 100 µL of two dilutions (100 mg/mL and 50 mg/mL) of each extract, positive and negative controls. Gentamycin (1 µg/mL) was used as the positive control, whereas distilled water, methanol, acetone or hexane was used as the negative control for respective extracts. This procedure was performed in triplicates for both microbial suspensions of *E. coli* and *S. aureus*. Then all agar plates were incubated at 37 ^{0}C for 24 hours and zone of inhibition was measured using a venire caliper.

2.6. Determination of minimum inhibitory concentration (MIC)

A dilution series (100 mg/mL, 50 mg/mL and 25 mg/mL) of each test extract was prepared separately. One mL of bacterial suspension was added to 150 mL of nutrient agar broth (1:150 ratio) to prepare a microbial broth of each organism with required bacterial concentration. Then the microbial broth (1 mL) was added to the tubes containing each concentration of test extract (1 mL). This procedure was repeated for three times for each test extract. All the tubes were incubated at 37 ^{0}C for 24 hours. After 24 hours, turbidity of each mixture was observed visually and measured spectrophotometrically. The lowest concentration of each extract that inhibit the growth of the microorganism was designated as the minimum inhibitory concentration (MIC).

2.7. Analysis of results

The results were given as mean ± SEM. Data analysis was performed by SPSS version 21.0. Statistical comparisons were made using Duncan's new multiple range test. Significance was set at $P < 0.05$.

3. Results

3.1. Zone of Inhibition

Different extracts of flowers of *A. marmelos* was tested for *in vitro* anti-bacterial activity using Agar well diffusion method. The zone of inhibition of each extract against *E. coli* is presented in Table 1. Among the extractions tested 100 mg/mL of aqueous extract exhibited the highest zone of inhibition for *E. coli* and it showed a significant inhibition compared to negative control ($P < 0.01$). Methanol and hexane extracts (100 mg/mL) showed the second highest zone of inhibition against *E. coli* compared to ($P < 0.05$) respective negative controls. Besides, 100 mg/mL acetone extract exhibited the least zone of inhibition which was not significant with respect to the negative control ($P > 0.05$).

The 50 mg/mL concentration of aqueous, methanol and hexane extracts also exhibited a significant inhibition against *E. coli* compared to the respective negative control ($P < 0.05$).

Table 1: Zone of inhibition of the aqueous, methanol, acetone and hexane extracts of the flowers of *A. marmelos* against *E. coli*

Diameter of the zone of inhibition in mm (Mean ± SEM)				
Extracts	**100mg/mL**	**50mg/mL**	**Negative control**	**Gentamycin 1µg/mL**
Aqueous	17.67 ± 0.34**/ ^{c}	15.33 ± 0.33**/ ^{c}	8.00 ± 0.02	37.00 ± 0.34***
Methanol	13.67 ± 0.37*/ ^{c}	11.00 ± 0.06*/ ^{c}	8.33 ± 0.31	32.67 ± 0.36***
Acetone	10.67 ± 0.35 ^{c}	10.00 ± 0.23 ^{c}	8.67 ± 0.34	34.33 ± 0.31***
Hexane	12.67 ± 0.33*/ ^{c}	10.33 ± 0.33*/ ^{c}	8.67 ± 0.33	36.67 ± 0.33***

Significance compared to negative control P<0.05*, P<0.01**, P<0.001***
Significance compared to positive control P<0.05^a, P<0.01^b, P<0.001^c

As indicated in Table 2, the highest zone of inhibition against *S. aureus* was exhibited by the 100 mg/mL concentration of aqueous extract which is a significant inhibition compared to the negative control ($P < 0.01$). The 100 mg/mL concentration of methanol ($P < 0.01$) and acetone ($P < 0.05$) extracts also exhibited significant inhibition against *S. aureus*. Although 50 mg/mL aqueous and methanol extracts showed a significant inhibition compared to respective negative control ($P < 0.05$),

the same concentration of acetone extract did not show a significant inhibition (P > 0.05). Both concentrations of the hexane extract did not exhibit significant inhibition against *S. aureus* (P > 0.05).

Gentamycin, the positive control showed the largest zones of inhibition against both *E. coli* (P<0.001) and *S. aureus* (P<0.001) compared to the negative controls used in this study. Further, all extracts showed a significant difference of inhibition compared to Gentamycin (P > 0.05). This result indicated that although the tested extracts possess *in vitro* antibacterial activity against *E. coli* and *S. aureus*, the activity is not comparable with the reference drug, Gentamycin.

Table 2: Zone of inhibition of the aqueous, methanol, acetone and hexane extracts of the flowers of *Aegle marmelos* against *S. aureus*

Diameter for the zone of inhibition in mm (Mean ± SEM)				
Extracts	**100 mg/mL**	**50 mg/mL**	**Negative Control**	**Gentamycin 1 µg/mL**
Aqueous	15.33 ± 0.67**/ [a]	11.67 ± 0.32*/ [a]	8.33 ± 0.31	33.33 ± 0.35***
Methanol	13.33 ± 0.35**/ [b]	10.33 ± 0.33*/ [b]	8.00 ± 0.33	32.67 ± 0.33***
Acetone	10.67 ± 0.33*/ [b]	10.00 ± 0.58 [b]	9.00 ± 0.05	31.33 ± 0.67***
Hexane	8.33 ± 0.31 [c]	8.67 ± 0.31 [c]	8.00 ± 0.09	35.33 ± 0.31***

Significance compared to negative control P<0.05*, P<0.01**, P<0.001***

Significance compared to positive control P<0.05[a], P<0.01[b], P<0.001

All four tested extracts showed a higher inhibition against *E. coli* than *S. aureus*.

3.2. Minimum inhibitory concentration (MIC)

The MIC value for both *S. aureus* and *E. coli* was 100 mg/mL for water, methanol, acetone and hexane extracts.

4. Discussion

People used to believe that the plants had healing potential against the diseases even before they discovered the existence of the microbe in the world [9]. They also believed that green medicines are healthier and safer than the synthetic drugs. The use of medicinal plants is becoming more popular throughout the world due to their minimum side effects. The previous studies indicated that the microorganisms develop a lower resistance towards the plant products than towards the synthetic drugs. Hence, there was a dramatical increase in the usage of medicinal plants during past decade to fulfill the medicinal needs of mankind [10]. Therefore scientific validation of medicinal properties of commonly using herbs is recommended by WHO [11]. The present study investigated the *in vitro* antibacterial activity of the aqueous, methanol, acetone and hexane extracts of the flowers of *Aegle marmelos*.

E. coli is the most common Gram negative bacteria in fecal flora and the most common cause of urinary tract infection in humans [12]. *S. aureus* is a gram positive bacteria, which are a major cause of hospital-acquired infections and is more difficult to eliminate as their emerging resistance to many antibiotics [13]. Therefore discovery of novel compounds act against theses bacteria is becoming more concern.

The antibacterial activity of aqueous, acetone and hexane extracts of flowers of *A. marmelos* is studied for the first time. The results of the study revealed that the aqueous, methanol, acetone and hexane extracts exhibit a significant antibacterial activity against *E. coli* whereas aqueous, methanol and acetone extracts possess antibacterial activity against *S. aureus.* Suresh *et al.* [2009] also reported *in vitro* antibacterial activity of methanolic flower extract of the *A. marmelos* against selected Gram negative and positive bacteria including *E. coli* and *S. aureus.* Although methanol is considered as the best solvent for the *in vitro* antibacterial investigations of the plant extracts [14], present study indicated that aqueous extract of the *A. marmelos* is having the highest antibacterial activity against both tested bacteria. Interestingly, the water extract of the dried flowers of the *A. marmelos* is a traditional drink in Sri Lanka, which is consumed by rural population instead of black tea [8]. Apart from that, traditional practitioners commonly use water as a medium to extract plant material when preparing herbal remadies [14]. Thus, the anti-bacterial activity exhibited by the flower extracts scientifically validates the usage of water extract of flowers as a berevage as well as a medication.

The results of the present study revealed that the dried flower extract of the *A. marmelos* possess relatively higher antibacterial activity against *E. coli* compared to *S. aureus* despite of the type of the extract used. This might be due to the structural difference between the cell wall of the Gram positive and Gram negative bacteria as bacterial cell wall is a target for the antibacterial agents. Gram positive bacteria have a thick peptidoglycan layer and lipothilic acids, a class of amphiles in their cell wall. In Gram negative bacteria, there is a thin peptidoglycan layer with an additional layer called outer membrane consists of phospholipids and lipopolysaccharides [15]. Due to these structural differences, the bioactive compounds present in different test extracts may be able to penetrate the Gram negative cell wall readily than the Gram positive cell wall.

All four extracts exhibited a significant antibacterial activity against *E. coli*, whereas, the aqueous, methanol and acetone extracts expressed antibacterial activity against *S. aureus*. But hexane extract failed to exhibit a significant antibacterial activity against both bacterial strains. The bioactivities exhibited by plant materials are due to presence of compounds with different chemical properties. According to the results of the present study, aqueous extract may contained the highest amount of bioactive compounds which extert the antimicrobial effect. Comparatively, other extract may contain lesser amount of active compounds or compounds with different chemical properties. The results indicate that the hexan extract did not contain compounds which are active againt test organisms. The varetiy of the bioactive compounds contain in the extract depends on the chemical properties of the solvent.

It was predicted that the phyto- constituents which are the secondary metabolites of the plants serve as a major defense mechanism of the plants against the microbes [16, 17]. The phytochemical profile of the *A. marmelos* flowers ethanolic extracts showed the presence of tannins, saponins, alkaloids, flavonoids, phenols, coumarins and quinones [7]. Tannins is a group of polymeric phenolic compound which extert the antibacterial activity via inactivating microbial adhesins, enzymes and cell envelop transport proteins[17]. Antibacterial activity of the alkaloids is mediated via interactions with the bacterial DNA and also flavonoids, phenols and saponins mediate the antibacterial activity via disrupting the functional integrity of the bacterial cell wall and the cell membrane [16,

[17]. Thus antibacterial activity of the different extracts of *A. marmelos* flowers may be exhibited via single or multiple mechanisms.

The results of the present study indicated that the flower of *A. marmelos* possess a promising anti-bacterial activity against *E. coli* as well as *S. aureus*. Further studies on identification and purification of active ingredients of the flower extracts may leads to discovery of novel compounds with potent anti-microbial activity. The findings of the present study scientifically validated the antimicrobial effect of flowers of *A. marmelos* and confirmed it's medicinal value. Therefore these findings added a new value to the traditional beverage, the hot water extract of dried flowers of *A. marmelos*.

5. Conclusion

In conclusion, this study, for the first time, showed *in vitro* antibacterial potential of the flowers of *A. marmelos*. There is a strong possibility to develop a novel, safe and cheap natural antibacterial agent(s) from the flower *A. marmelos*. Future research focusing on higher concentrations of different flower extracts is recommended for better understanding of the antibacterial effect of the flower extracts of *A. marmelos*.

6. References

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