



## BACTERICIDAL EFFECT OF *AEGLE MARMELLOS* EXTRACTS ON PATHOGENIC BACTERIA

R. K. Sujatha<sup>1</sup> and \*S. Rajan<sup>2</sup>

<sup>1</sup>Department of Microbiology, Srimad Andavan Arts and Science College, Thiruvanaikoil, Tiruchirappalli – 620 005, Tamil Nadu, India.

<sup>2</sup>Department of Microbiology, M. R. Government Arts College, Mannargudi – 614 001, Tamil Nadu, India.

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### \*Correspondence for

#### Author:

**Dr. S. Rajan,**

Department of Microbiology,  
M. R. Government Arts  
College, Mannargudi – 614  
001, Tamil Nadu, India.

### ABSTRACT

Pathogenic microorganisms are effectively controlled by making use of modern combined antibiotics. Improper and overuse of antibiotics cause various side effects and creates resistance among bacterial pathogens. To overcome these problems peoples turned towards the use of medicinal plants as therapeutic agents. *Aegle marmelos* fruit pulp is an efficient traditional phytomedicine used for the treatment of various infections. Antibacterial activity of aqueous and ethanolic extract of *Aegle marmelos* fruit pulp s were determined using disc diffusion, modified agar dilution methods. MIC, MBC, Percentage of inhibition and IC<sub>50</sub> were calculated using modified drug dilution method. The results showed that the fruit pulp extracts of *A. marmelos*

have antimicrobial activity against *E. coli*, *Salmonella*, *Shigella*, *Klebsiella*, *Pseudomonas*, *Staphylococcus aureus*, *Streptococcus pyogenes*. Ethanolic extracts showed the highest inhibition zone of 16.0±1.0mm diameter against *Pseudomonas aeruginosa*. The plant extracts were shown to have a best MIC at 150±050µg/ml for ethanolic extract and 216.6±028.8 µg/ml for aqueous extract. Percentage of inhibition created by the extracts against the pathogens varied from 47.6% to 92. 6% 2ith best activity against *Escherichia coli* by both the extracts. Effective IC<sub>50</sub> exhibited by ethanolic extract 112.9 µg/ml concentrations against *E. coli*.

**Keywords:** Bacterial pathogens, MIC, MBC, Antibacterial activity, IC<sub>50</sub>, Percentage inhibition, *Aegle marmelos*, Fruit pulp.

## INTRODUCTION

Novel drugs are made from Medicinal plants. They are the valuable ingredients in traditional systems of medicine and modern medicines. They are considered as a nutraceuticals, food supplements, bioactive principles and lead compounds in synthetic drugs (Ncube *et al.*, 2008). Medicinal plants are utilized as therapeutic agents since time immemorial in both organized system of medicine like siddha, ayurvedha etc., and unorganized system of medicinal system like tribal, folk medicine. India is rich heritage of traditional knowledge. Indian medicinal plants are considered as a vast source of pharmaceutically active components. Despite of the various advancements in modern medicine, the prevalence of infectious disease and development of multidrug resistance among human pathogens, haphazard use of synthetic drugs and side effects created by the modern medicine drives the need to screen medicinal plants for novel bioactive compounds as they are biodegradable, safe and have fewer side effects (Prusti *et al.*, 2008). *Aegle marmelos* (L.) Corr., belongs to the family Rutaceae and is popularly known as Bael tree (Gamble, 1935 and Matthew, 1983). Traditional medical practitioner's uses fruit pulp as an astringent, digestive and stomachic and also used for the treatment of diarrhoea and dysentery (Bakhru, 1997). The fresh juice of the leaves is taken with honey as a laxative and febrifuge; it is used in asthmatic complaints. The fruit is used as a remedy for diarrhoea. Beverages prepared with fruit pulp are used to relieve body heat (Jain and Sastry, 1979). The present study is performed to check antibacterial activity of *Aegle marmelos* fruit pulp against multidrug resistant pathogens. Though few studies are available on the pharmacological activities of *Aegle marmelos* fruit pulp, there is no specific study on the antibacterial activities of fruit pulp of this plant. Hence in this study, antibacterial activity of *Aegle marmelos* fruit pulp was screened using routine disc diffusion method as well as using modified drug dilution method for MIC, MBC, percentage inhibition and IC<sub>50</sub> calculation.

## MATERIALS AND METHODS

### Preparation of plant material

*Aegle marmelos* fruit pulp was collected from the temple gardens of Thiruvanaikoil, Tiruchirappalli, Tamilnadu, India. The fruits were washed thoroughly with water and cut the fruits as half using sterile sharp scalpel. Fruit pulp was collected from the fruit using scoop and air dried at room temperature for five days. After drying, the fruit pulps were ground into powder and then sieved using a sieve. Five hundred grams of powdered plant were transferred into airtight containers and stored at room temperature.

**Extraction of the crude extracts from fruit pulp powder.**

Plant active components were extracted using the cold extraction method (Fransworth, 1988). Water and ethanol were used for the extraction. pure ethanol and sterile distilled water were added 50g portions of the fruit pulp powder in sterile conical flasks individually and allowed to soak at room temperature for 48 hours. A shaker set at 120 rpm was used to improve extraction of phyto-chemicals. The filtrate was obtained by means of a vacuum filter pump. Filtering was repeated three times with same plant material until the solution was clear. The filtrate was evaporated in a weighed flask, with a water bath set at 40°C. A small proportion of dry extracts was stored for phyto-chemical analysis. Remaining portion of the extracts was used for antibacterial assay. Extracts were reconstituted by re-dissolving in DMSO. The final filtrates were filter-sterilized by using syringe filter with a pore size of 0.45µm. Sterile extracts obtained were stored separately in labelled, sterile capped bottles, in a refrigerator at 4°C.

**Determination of antibacterial activity**

Antimicrobial activity was performed by standard methods like the disc diffusion method on Mueller Hinton agar (Bauer *et al.*, 1966) and MIC, MBC were calculated using modified drug dilution methods (Kowser and Fatena, 2009). Cells used for antibacterial assays were harvested at log phase while they are most active.

**Preparation of inoculum**

Direct colony suspension method was used to make a suspension of bacteria. Three to four colonies from overnight grown (18 hours) bacteria were suspended in saline using a sterile loop. The turbidity standard was shaken vigorously before use, and used to make a visual comparison with the density of the suspension against a white background with black lines. Density of the suspension was adjusted to 0.5 Mcfarland either by adding sterile saline. The standardized culture was used within 15 minutes of preparation for sensitivity tests.

**Disc diffusion test**

Antibacterial activities of the extracts were tested on Mueller-Hinton agar by disc diffusion method. The inoculum was spread evenly over the entire surface by swabbing in three directions using sterile cotton swab. Inoculated plates were allowed to dry for ten minutes before depositing the discs (Anusha *et al.*, 2009). Sterile paper disc having a diameter of 6 mm, were impregnated with different concentrations of extracts 50µg/disc, 100µg/disc, 200µg/disc and 250µg/disc. Paper discs were placed on the agar plate using sterile forceps.

Six filter paper discs were placed on each plate and were placed at the same distance from each other and the edge, to prevent overlapping of inhibition zones. Sensitivity discs were pressed with forceps to make complete contacts with the surface of the medium. Plates were kept at room temperature for 30 minutes (pre-diffusion time), inverted and incubated at 37°C for 24 hours, in an aerobic atmosphere. A Hi Antibiotic zone scale was used to mark the diameter of the zone. The experiment was repeated three times for each extract and the mean diameter was taken. Oxytetracycline discs were used as a positive control and DMSO impregnated discs were used as negative control (Anonymous, 1999).

#### **Assessment of MIC, MBC and IC<sub>50</sub>**

It was performed by making use of the method of Kowser and Fatena, 2009 with few modifications.

#### **Preparation of Turbidity standard for inoculum**

Inoculum for the assay of MIC and MBC were prepared at 0.5 level of Mc farland standard. The approximate cell density corresponding to 0.5 Mc Farland is  $1 \times 10^8$  CFU/ml.

#### **Inoculum preparation**

Overnight Mueller Hinton broth cultures of uropathogenic *E. coli* was prepared. The culture was adjusted to obtain turbidity comparable to that of the turbidity of Mc Farland 0.5 standard and then further diluted to 1: 40 in Mueller Hinton broth. The inoculums thus prepared expected to obtain  $10^3$ CFU/ml.

#### **Determination of MIC**

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. A MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. Various concentrations of the extracts, antibiotics were prepares as illustrated below. Fourteen tubes were placed in a rack and labeled each 1 through 11, one tube marked as B (blank), one tube was labeled as AC (Antibiotic Control) and other tube was labeled as G.C (Growth Control). Different volumes of Mueller Hinton broth was added in each test tube as described in table 4.3 and plugged with cotton. All the tubes were sterilized at 121°C for 15 minutes. 100µl of 0.1% antibiotic solution was added to test tube A.C. 100µl of plant extract from stock was added to the tube no. 1 and mix properly. Similarly the volume of extracts ranges from 90µl to 5µl for the tube no.2 through 11 was

added. The tube G.C received no extracts and was served as a growth control. A.C labeled test tube was served as an antibiotic control. Each tube was inoculated (including the growth control except blank and antibiotic control) with 100 $\mu$ l of the culture of respective organism. All the tubes were incubated at 37°C for 24 hours. The tubes were examined for visible growth (cloudy) and was recorded visible growth as (+) and no growth as (-). The concentration at which no visible growth was described as the MIC of the extract.

### Determination of MBC

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium completely. It can be determined from broth dilution of minimum inhibitory concentration (MIC) tests by sub culturing to agar plates that do not contain the test agent. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by  $\geq 99.9\%$ . Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC. Select MIC tubes for determining MBC based on the visible growth. Last two non-visible tube to the 1<sup>st</sup> two visible tube were selected to screen MBC. 1ml of culture (grown in Mueller hinton Broth) from MIC tube were serially diluted upto 1:1000 dilution and inoculated 1:100 and 1:1000 diluted samples into Mueller Hinton agar containing Petri plate by spread plate technique. GC tube containing culture was serially diluted upto  $10^{-8}$  and plated  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$  diluted materials on respective properly labeled nutrient agar plate to check total viable count of the initial inoculums used to determine percentage inhibition. The number of bacterial colonies in each plate were counted properly and recorded. The dilution at which counted no colonies were considered as MBC of the extract.

### Determination of % inhibition

It is a calculation of inhibitory effect of extracts at particular concentration by making use of total viable count value of GC tube and dilution tubes. It was calculated by making use of the following formula.

$$\frac{(\text{Number of colonies in tube GC} - \text{Number of colonies in dilution tube})}{\text{Number of colonies in tube GC}} \times 100$$

### Determination of IC<sub>50</sub>

According to the FDA, IC<sub>50</sub> represents the concentration of a drug that is required for 50% inhibition *In Vitro*. It is obtained from the % inhibition and the concentration of extract used. IC<sub>50</sub> was calculated by using the formula.

$$\frac{\text{Concentration of Extract}}{\% \text{ inhibition}} \times 50$$

## RESULTS AND DISCUSSIONS

The aqueous and ethanolic fruit pulp extracts of *A. marmelos* showed prominent antibacterial activity against all the test pathogens. Both extracts inhibited the growth of pathogens effectively and the inhibition zones ranged from 11.5±0.7mm to 16±1mm at 250 µg/ disc concentrations (Table 1). Ethanolic extract produced best activity against *Pseudomonas aeruginosa* (16±1), followed by *Salmonella typhi* (15.6±0.57), *Shigella* sp., (15.3±2.08), *Streptococcus pyogenes* (13.6±1.52), *Escherichia coli* (12.3±2.08), *Klebsiella* sp. (12±2.82) and *Staphylococcus aureus* (11.5±0.7). Aqueous extract yielded 13.33±1.15mm zone of inhibition against *Escherichia coli*. Lower activity was noted against *Streptococcus pyogenes* (11.5±2.12). by aqueous extract. Ethanolic extracts of *Aegle marmelos* gives lower effect against *Staphylococcus aureus* (11.5±0.7mm). There was a significant difference in mean zone of inhibition between positive control and plant extracts (P < 0.05). There was no zone of inhibition around discs of Negative control. To determine the extent of antibacterial activity, the extracts were subjected to MIC assay. Our antibacterial activity study also supported by Mohan *et al.*, (2005). They reported that the aqueous, acetone and petroleum ether extract of *A. marmelos* were found to be effective against *B. coagulans*, *B. subtilis*, *B. thuringiensis*, *P.aeruginosa* and *S. aureus*. They analysed the antibacterial activity only by making use of gram positive bacteria. The present findings support the applicability of *A. marmelos* in traditional system for its claimed uses and can be recommended by the scientific community as an accessible alternative to synthetic antibiotics.

Modified drug dilution method was adopted to assess the MIC and MBC of the extracts. Table 2 describes the MIC and MBC values of the extracts against all the test pathogens. Ethanolic extract completely arrested the growth of *E. coli* at 150.0±050.0 µg/ml concentration, which was evidenced in our MBC report also. Concentration of extracts required to kill the pathogens (MBC) completely was higher than to inhibit the growth of bacteria (MIC). MIC values of ethanolic extract ranges from 150.0±050.0 to 333.3±076.4 µg/ml concentration where as MBC ranges from 150.0±050.0 to 383.3±028.9 µg/ml

concentrations. Aqueous extract also showed good MIC and MBC performances but at higher concentrations when compared to ethanolic extract. Bactericidal concentration required to kill 100% bacteria ranges from  $316.6 \pm 076.4$  to  $616.6 \pm 028.9$   $\mu\text{g/ml}$ . Best bactericidal activity of ethanolic and aqueous extract was found to be higher against *Escherichia coli*. Aqueous extract exhibited lower MBC against *Salmonella typhi* whereas ethanolic extract yielded lower activity against *Shigella sp.*

Percentage inhibition and  $\text{IC}_{50}$  value were calculated by making use of MBC value. Percentage of inhibition created by aqueous extract ranged from 46.3% to 92.6% at 200  $\mu\text{g/ml}$  concentration. Ethanolic extract produced 88.6% inhibition against *Escherichia coli* and its percentage of inhibition ranged from 55% to 88.6%. *E.coli* and *Staphylococcus aureus* were best inhibited by aqueous extract whereas other test organisms were best inhibited by ethanolic extract (Figure 1). More than 80% inhibition was noted against *Escherichia coli* (92.6% for aqueous extract, 88.6% Ethanolic extract), *Pseudomonas aeruginosa* by ethanolic extract (83%) and *Klebsiella sp.* by both the extracts (81% for aqueous extract, 87.6% Ethanolic extract).

$\text{IC}_{50}$  values exhibited by the extracts were depicted in figure 2. It clearly indicated that ethanolic extract yielded best results when compared to aqueous extract.  $\text{IC}_{50}$  value of ethanolic extract ranges from 112.9  $\mu\text{g/ml}$  to 176  $\mu\text{g/ml}$ , similarly  $\text{IC}_{50}$  value for aqueous extract ranges from 162.3  $\mu\text{g/ml}$  to 326.8  $\mu\text{g/ml}$ . Best  $\text{IC}_{50}$  value for both the extracts were noted against *E. coli*. The half maximal inhibitory concentration ( $\text{IC}_{50}$ ) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance is needed to inhibit a given biological process by half. According to the FDA,  $\text{IC}_{50}$  represents the concentration of a drug that is required for 50% inhibition in vitro. This is the first of this kind of study that evaluated  $\text{IC}_{50}$  values exhibited by the plant extracts along with percentage of inhibition.

This present study exhibited good antimicrobial activity, which could be due to phytochemicals available in the extracts. One of our previous studies indicated the presence of steroids, terpenoids, flavonoids, phenolic compounds in both extracts (Rajan *et al.*, 2011). This report also indicated that tannins were only present in ethanolic extracts along with other reported chemicals. This also evidenced by Rajeswari Prabha and Ramachandramurty (2013). Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they

have remarkable activity in cancer prevention and are thought to be responsible for coagulating the wall proteins of pathogenic organisms. Thus, *A. marmelos* ethanolic extract containing this compound may serve as a potential source of bioactive compounds in the treatment of infectious diseases. Flavonoids have been shown to exhibit their actions through effects on membrane permeability and by inhibition of membrane bound enzymes such as the ATPase and phospholipase (Li *et al.*, 2003). They also serve as health promoting compounds as a result of their anion radicals (Hausteen, 1983).

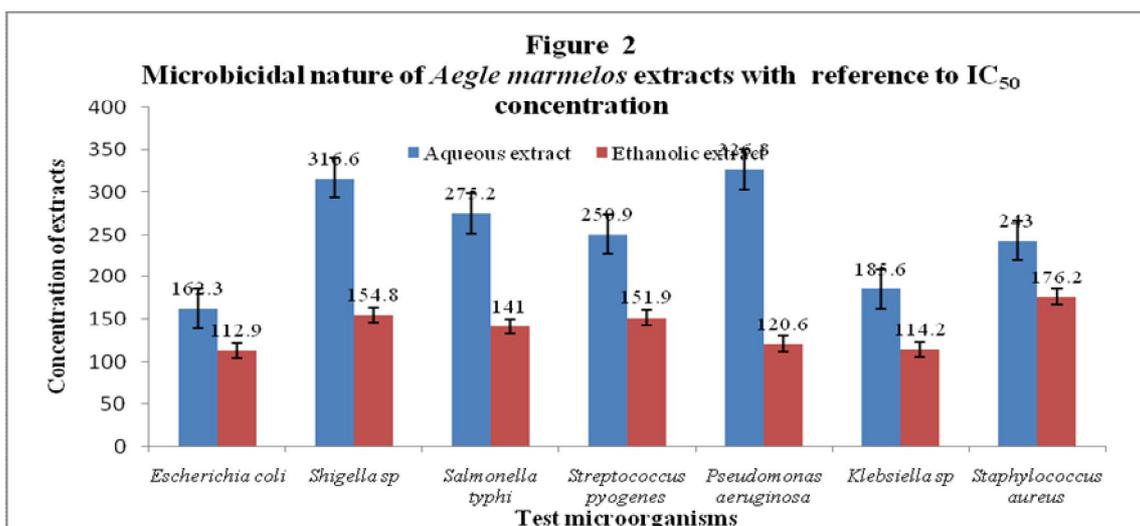
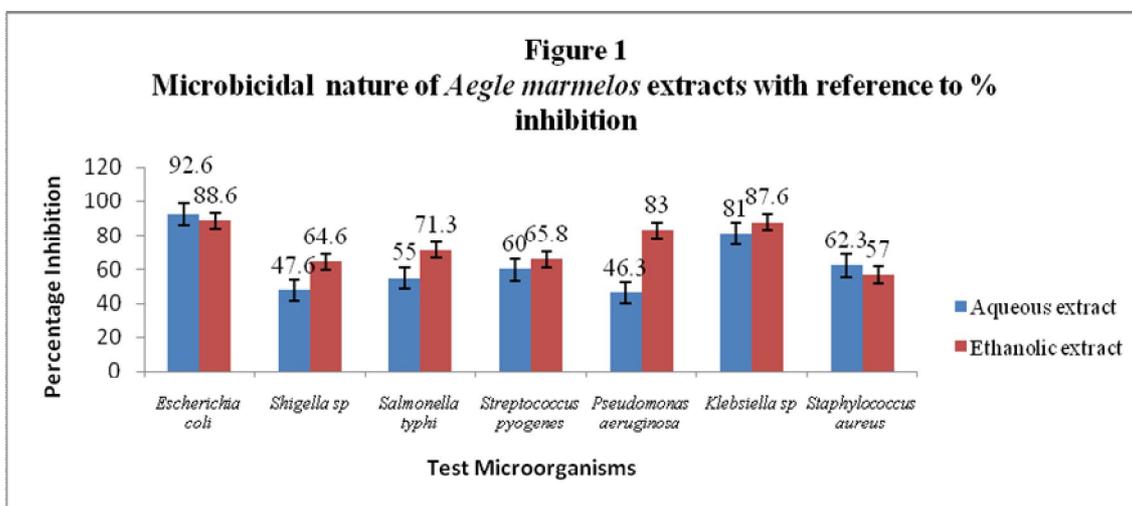
This result may suggest that all extracts possess compounds with antimicrobial properties which can be used as antimicrobial agents in new drugs for therapy of infectious diseases in human. Extracts had an inhibition zone diameter between 10mm to 16mm, which was higher than to a standard antibiotic, hence we suggest their effectiveness as antimicrobials from the plant. The active components in the crude extract may be acting synergistically to produce antimicrobial effects (Elloff, 1998).

**Table 1. Antibacterial nature of Aqueous and ethanolic extracts of *Aegle marmelos* at 250µg/ disc concentration**

1. S. No	2. Test Organism	3. Aqueous extract	4. Ethanolic extract
1	<i>Escherichia coli</i>	13.33±1.15	12.3±2.08
2	<i>Shigella sp</i>	12.66±1.52	15.3±2.08
3	<i>Salmonella typhi</i>	11.60±0.57	15.6±0.57
4	<i>Streptococcus pyogenes</i>	11.50±2.12	13.6±1.52
5	<i>Pseudomonas aeruginosa</i>	12.60±2.30	16.0±1.00
6	<i>Klebsiella sp</i>	12.60±2.08	12.0±2.82
7	<i>Staphylococcus aureus</i>	12.60±0.57	11.5±0.70
Negative control : Dimethyl sulfoxide - Zone of Inhibition - Nil			
Positive control : Oxytetracycline 30µg/disc – Zone of Inhibition ranges from 11.5mm - 16 mm			

**Table 2. Minimal inhibitory and Minimal Bactericidal nature of *Aegle marmelos* extracts**

S. No	Test Organism	Aqueous Extract		Ethanollic extract	
		MIC ( $\mu\text{g/ml}$ )	MBC ( $\mu\text{g/ml}$ )	MIC ( $\mu\text{g/ml}$ )	MBC ( $\mu\text{g/ml}$ )
1	<i>Escherichia coli</i>	216.6 $\pm$ 028.8	316.6 $\pm$ 076.4	150.0 $\pm$ 050.0	150.0 $\pm$ 050.0
2	<i>Shigella sp</i>	300.0 $\pm$ 050.0	433.3 $\pm$ 076.4	333.3 $\pm$ 076.4	366.6 $\pm$ 076.4
3	<i>Salmonella typhi</i>	416.6 $\pm$ 028.8	616.6 $\pm$ 028.9	266.6 $\pm$ 076.4	383.3 $\pm$ 028.9
4	<i>Streptococcus pyogenes</i>	366.6 $\pm$ 125.8	500.0 $\pm$ 000.0	250.0 $\pm$ 050.0	316.6 $\pm$ 028.9
5	<i>Pseudomonas aeruginosa</i>	350.0 $\pm$ 132.3	516.6 $\pm$ 028.9	233.0 $\pm$ 104.1	316.6 $\pm$ 076.4
6	<i>Klebsiella sp</i>	450.0 $\pm$ 050.0	483.3 $\pm$ 076.4	283.3 $\pm$ 028.7	350.0 $\pm$ 050.0
7	<i>Staphylococcus aureus</i>	383.3 $\pm$ 028.9	416.6 $\pm$ 028.9	250.0 $\pm$ 050.0	250.0 $\pm$ 050.0



Most of the identified components with antimicrobial activity extracted from plants are aromatic or saturated organic compounds which are more soluble in polar solvents such as water and organic solvents. However water extracts were less potent. This can be attributed to the presence of water-soluble compounds such as polysaccharides and polypeptides, which are commonly more effective as inhibitors of pathogen adsorption and have no real impact as antimicrobial agents (Ncube *et al.*, 2007). The antibacterial activity demonstrated by water extract provides the scientific bases for the use of water extracts in traditional treatment of diseases. There are also reports in literature that organic solvent is a better solvent for consistent extraction of antimicrobial substances for medicinal plants (Elloff, 1998). This may be attributed to two reason, firstly, the nature and potentiality of biologically active components (alkaloids, steroids, flavonoids, essential oils, biterpenoids), which could be enhanced in the presence of methanol. Secondly, the stronger extraction capacity of methanol could have produced greater number or amount of active constituents responsible for antibacterial activity (Jeyachandran *et al.*, 2010). This is also proved in our study in which ethanolic extracts exhibited the highest antibacterial activity against all pathogens tested.

## CONCLUSIONS

The Fruit pulp extracts of *Aegle marmelos* were found to have good antibacterial activity against bacterial pathogens. This was evidenced in disc diffusion method, MIC, MBC, Percentage inhibition and IC<sub>50</sub> value determination.

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