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Phytochemical and antimicrobial activity of fruit pulp of aegle marmelos

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ABSTRACT

The present investigation was carried out to evaluate the phytochemical constituents and antibacterial activities of fruit pulps of Aegle marmelos (Linn.) Correa. The phytochemical screening of the crude extract revealed the presence of Reducing Sugars, Saponins, Tannis, Flavonoids and Phenols. Further the total phenolic and flavonoid content was also estimated. Besides, the crude extract was tested for antimicrobial activity against two gram positive strains of Staphylococcus aureus (ATCC 29213, ATCC 700699) at different concentrations of 10, 50, 100, 250 and 500ug/ml at different time span of 3hrs. It was found that a concentration range 50-100ug/ml of the ethanolic extracts was effective in inhibiting the growth of bacterial strain Staphylococcus aureus ATCC 29213. 250ug/ml was effective for aqueous extract and 500ug/ml concentration was effective for petroleum ether extracts in inhibiting the growth of the above strain. When the similar study was carried out using other strain, Staphylococcus aureus ATCC 700699, it was found that 100ug/ml of ethanolic extract, 250ug/ml and 500ug/ml of petroleum ether was effective in inhibiting the growth of bacteria,where as the concentration of aqueous extracts taken were ineffective against the growth of the bacteria. The broad spectrum antimicrobial activity of fruit pulp extracts of Aegle marmelos was indicated by the inhibition of growth of the bacteria, which in turn in future, might lead to further study for the development of novel bioactive antimicrobial agents.

Key words: *Aegle marmelos*, Medicinal plants, Phytochemical Screening, Antibacterial activity

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INTRODUCTION

India is a treasure trove of aromatic and medicinal plants. Today medicinal plants play a major role as pillar of traditional healthcare systems of medicine in many developing countries [1]. Since from the ancient times, different drugs have been formulated using the bioactive compounds present in these medicinal plants. More than 60% of the world's population depends on phytomedicines derived from these medicinal plants for primary health care needs [2]. The Phytoconstituents from these medicinal plants serve as lead compounds in the modern era in drug discovery and design [3]. The most important phytoconstituents which are biologically active in plants are Tannins, Flavonoids, Alkaloids and Phenolic compounds [4]. A correlation needs to be established in between the phytoconstituents and pharmacologically bioactive compounds present in these medicinal plants in order to solve and cure many diseases prevailing in the present scenario [5].

Plants are considered as the bank of effective chemotherapeutants. The biological screening of the compounds present in these medicinal plants proved their activity in treating diseases like dysentery, diarrhoea, stomach pain, dyspepsia [6]. In the past few years several plant species have been assessed and evaluated for their antimicrobial activity. The present study targets on a biologically and pharmacologically active plant that shows several medicinal properties.

Aegle marmelos (Linn) Correa commonly known as Bael belongs to family Rutaceae, having cosmopolitan distribution present throughout the deciduous forests of India. The therapeutic value of the plant has been referred

by almost all the ancient Ayurvedic treatises like *Siddha, Unani, Sushruta Samhita* and *Charaka Samhita* etc [7]. Bael tree is a medium sized deciduous tree with unusual branches surrounded by aromatic trifoliate leaves, sweet scented and greenish-white flowers [8]. Studies on Bael fruit shows that it consists of fiber 2.9 per cent, fat 0.3 per cent, protein 1.8 per cent, moisture 61.5 per cent, minerals 1.7 per cent, carbohydrates 31.8 per cent per 100 grams of edible portion and the estimated calorific value found to be 137 [9].

In the past, many researchers has evaluated the pharmaceutical importance of different parts of *Aegle marmelos* plant and found that the plant possesses natural healing capacity in curing many ailments along with anti-inflammatory, antipyretic, anti-diarrhoeal, anti-diabetic, analgesic, antimicrobial, radio protective, anticancer and antiviral properties [10-13]. The blessed nature of Bael plant can be recognized by analyzing its therapeutic uses, such as for treatment of Swollen Joints, High Blood Pressure, Asthma, Fractures, Dyspepsia, Healing of Wounds, Stomach pain, Vomiting, Brain Typhoid Troubles during Pregnancy, Diabetics, Anaemia, Jaundice, Diarrhoea and Healthy Mind [14]. The Bael fruit pulp contains many functional, biological and pharmacological active compounds such as alkaloids, coumarins, flavonoids , carotenoids, terpenoids , phenolics and antioxidants which helps us in protecting against various chronic diseases [15].

Thus, considering the uses and owing to the significance of this important phytochemicals in the present scenario, this study aimed at evaluating the phytochemical potential and antibacterial activity of *Aegle marmelos* aqueous, petroleum ether and ethanolic fruit pulp extracts.

EXPERIMENTAL SECTION

Collection of Plant materials

The plant materials (Bilva fruits) were obtained from the trees growing in Puttaparthi Mandal situated to the south of Anantapur town in the Sri Sathya Sai taluk of Anantapur district, Andhra Pradesh, India and the institute campus. The fruits were collected from the villages of Puttaparti, Gangireddipalli, Ammagondapalem, Pedapalli, Nidimamidi, Neredukonda, Gajaulapalli. These were marked one to ten respectively. Fruits were shade dried naturally for a period of thirty to forty-fives days. After drying, fruits pulp was scraped using knife and was powdered and stored in tight containers for various experiments. The solvents and chemicals of analytical grade used in the experimental setup were purchased from Sigma Aldrich Co., Ltd (Steinheim, Germany) from Sigma Chemical Co., Ltd (St.Louis, MO, USA).

Preparation of plant extracts

Three solvents (water, ethanol, petroleum ether) were used. The plant material (fruit pulp powder) was put in a conical flask and the solvent (double distilled water) was added till the material submerged. Then the flasks were kept for shaking at 145 rpm for a period of twenty four hours at room temperature in an orbital shaker. At the end of twenty four hours, the content was filtered using whatman paper-grade 1 and the solvent thus obtained was stirred in refrigerator by covering with aluminum foil. The material which was left in the filter paper was kept again for extracting by adding solvent newly and the whole process was carried out thrice. Then the material which was left in filter paper was kept for extraction by adding solvent (ethanol) and then with petroleum ether. The procedure was followed three times for each solvent and a gradation was maintained. Then the solvents (fractions) thus obtained was further processed to remove the solvent. In case of aqueous extracts lyophilizer was used to remove excess water. Ethanol and Petroleum ether were air dried at room temperature. The compound thus obtained was stored in vials and are stored at 4^0 C till further use.

Qualitative Phytochemical Analysis

The Plant extracts of fruit pulp of Aegle marmelos were tested for the presence of reducing sugars, saponins, tannins, flavonoids (phenolic compounds) by following the methods given by D Venkatesan et al [16].

Test for reducing sugars

2 ml of crude plant extract was taken in test tubes and 5ml of Distill water was added. The sample was filtered using whatman paper. The filtrate was boiled with 4 drops of Fehling solution A followed by Fehling's solution B for 2 minutes. An orange red precipitate was observed which indicated the presence of reducing sugars.

Tests for Saponins

0.2 gm of plant extract was taken in test tubes and 5 ml of distilled water was added to each and heated to boil. Occurrence of frothing (appearance of creamy mass of small bubbles) in the test tubes indicated the presence of Saponin.

Tests for Tannin
To 2ml of each plant extract, a few drops of 10% lead acetate were added. The appearance of white precipitate indicated the presence of tannins in the sample.

Test for Flavonoids
2ml of plant extracts were taken in separate test tubes and diluted Sodium hydroxide was added followed by addition of diluted Hydrochloride. Yellow solutions were observed turning colorless which indicated the presence of flavonoids.

Test for phenols
1ml of aqueous /alcoholic solution was taken in test tubes and 3-4 drops of ferric chloride reagent was added. Violet color was obtained indicating the presence of phenols in the test samples.

Quantitative Estimation of Phytochemicals
Determination of Total phenolic content in fruit pulp
Total phenol estimation can be carried out with help of the Folin-Ciocalteau reagent as described by MG Rajanandh et al [17]. 10mg of plant extract (aqueous and ethanolic) was taken in a conical flask and 10mL of methanol was added to get a concentration of 1mg/mL solution. Suitable volume of the above plant extract containing solution was transferred into a 10mL standard flask. The color development was carried out as that for standard calibration curve. The absorbance of the test solution was measured at 765nm against reagent blank. Total phenolic concentration in the test sample was quantified by extrapolation from the calibration graph. Total phenol values are expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a common reference compound. It was expressed as µg/ml.

Estimation of total flavonoid content
Flavonoid concentration was measured using Aluminium chloride colorimetric method where Quercetin was used as a standard by following procedure by MG Rajanandh et al [17]. 25mg of aqueous and 30 mg of ethanol plant extracts were weighed and dissolved in 25ml and 30ml of methanol respectively to get a concentration range of 1mg/ml. A 10ml standard flask was taken and required volume of above solution was added and colour change was observed. At 415nm the absorbance was taken Using HITACHI 2000 UV/VIS spectrophotometer. The concentration of total Flavonoids present in the sample was determined by extrapolation and was expressed as ug/ml.

Anti Microbial Assay
Micro-organisms
The Gram positive bacteria Staphylocaccus aureus was used for anti-microbial activity of different extracts of fruit pulp of Bilva. In the present study different strains like ATCC 29213, ATCC 700699 (Staphylococcus aureus subsp. aureus Rosenbach) were obtained from National Collection of Industrial Microorganism (NCIM) Pune, India and grown at 37°C overnight on Luria-Bertani (LB) broth.

Preparation of Bacterial Suspension Culture
On the day before experiment two sterile culture tubes were taken to laminar air flow chamber. 20ul of the bacterial strain ATCC 700699 from the glycerol stock was inoculated in to culture tubes containing autoclaved LB broth and left in orbital shaker at 150rpm over night at temperature 37^0C for bacterial growth. Next day morning sub culturing of the bacterial strain was done in 100ml LB broth by inoculating overnight culture(1.5ml of culture was transferred into flasks containing fresh broth) in laminar air flow chamber. Next the inoculated fresh broth with bacteria strain was left in orbital shaker at 150rpm, temperature 37^0C for 1 to 2 hours to get an absorbance of 0.6nm. After the incubation period the turbidity was observed. These bacterial cell suspension cultures were used later for the antimicrobial studies. 21 test tubes with LB broth (4.5ml) in each were taken and labeled from 1-21. In first 2 test tubes bacterial strain ATCC 700699 was taken. In next two test tubes control antibiotic ampicillin (Anti A) was added. Then test tube no 5-6, antibiotic kanamycin (Anti B) was added. Next tubes onwards along with broth, bacterial culture, 10ug/ml of different fruit pulp extracts were added. It was done in triplicates. After that the increasing concentrations of different plant extracts (fruit pulp) were added in triplicates. After the addition of maximum concentration of 500ug/ml, the test tubes were in a test tube stand. All these activity were done inside laminar air flow chamber. Then the absorbances of the samples were taken at 600nm with the help of HITACHI 2000 UV-VIS spectrophotometer. The test was done in similar way for bacterial strain ATCC 29213 *(Staphylocaccus aureus*) to see the effect against aqueous, Ethanolic and petroleum extracts of *Aegle marmelos* fruit pulp.

RESULTS AND DISCUSSION

Phytochemical analysis

Plants play an important role in having beneficial therapeutic effects in traditional Indian system of medicine. Studies on ethnomedicinal plants are gaining consensus in recent years in India and Abroad. Thus, in the present study, three different extracts (aqueous, ethanol, petroleum ether) of fruit pulp of *Aegle marmelos* plant growing in Puttaparthi Mandal were subjected to both qualitative and quantitative phytochemical analysis to explore its anti-microbial activity for its medicinal applications.

The fruits were collected from different places of Puttaparthi Mandal, were assigned numbers from 1 to 10 respectively and the percentage yields of various fruit pulp extracts were tabulated (Table1) and the yield of extraction (%) of various fruit pulp extracts are depicted (figure 1).

Table 1: Plant samples with yield of extraction (%)

Name of the Place fruits collected	Serial no Assigned for the experiment	Aqueous extract (%)	Ethanolic extract (%)	Petroleum ether extract (%)
Prasanthi Nilayam	1	15.94	3.45	0.44
Pedapalli	**2**	**25.15**	**8.96**	**0.19**
Ammagondapalem	3	7.92	2.74	0.27
Gangireddipalli	4	15.04	3.92	2.1
Nidimamidi	5	16.89	2.36	1.29
Neredukonda	6	11.02	3.59	0.67
Gajaulapall	**7**	**39.07**	**2.93**	**0.38**
Puttaparthi	**8**	**20.62**	**4.24**	**1.96**
Gangireddipalli	**9**	**26.21**	**1.74**	**0.3**
Ammagondapalem	**10**	**27.67**	**1.38**	**0.75**

Figure 1: Percentage yield of extraction of fruit pulp of Bilva

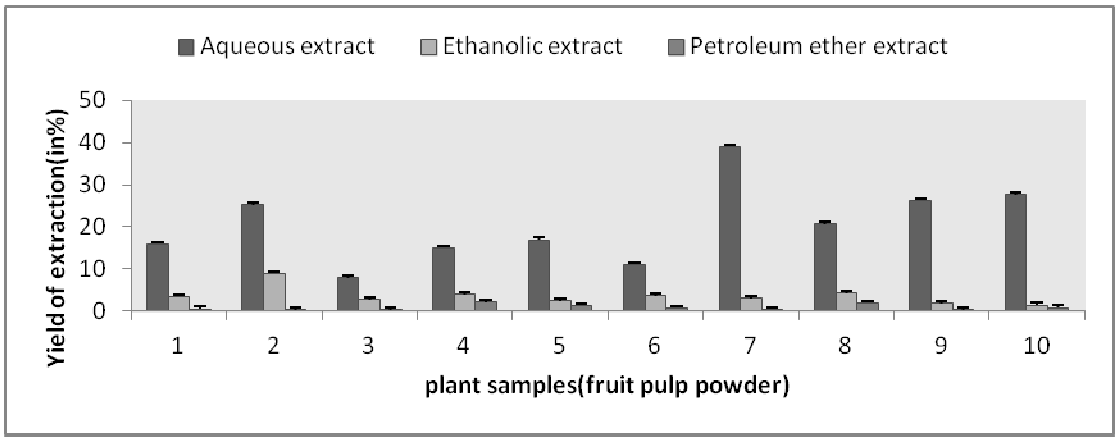


Table 2: Qualitative phytochemical analysis of different extract of fruit pulp

		Samples				
Extractions	**Compound tested**	**2**	**7**	**8**	**9**	**10**
Aqueous	Reducing Sugar	+	-	+	-	-
Aqueous	Tannins	+	+	+	+	+
Aqueous	Saponins	+	+	+	+	+
Aqueous	Flavonoids	+	+	+	+	+
Aqueous	Phenols	+	+	+	-	+
Ethanolic	Reducing Sugar	+	+	+	+	+
Ethanolic	Tannins	+	+	+	+	+
Ethanolic	Saponins	+	+	+	+	+
Ethanolic	Flavonoids	+	+	-	+	+
Ethanolic	Phenols	+	+	+	+	+
Petroleum Ether	Reducing Sugar	-	-	-	-	-
Petroleum Ether	Tannins	-	-	-	-	-
Petroleum Ether	Saponins	-	-	-	-	-
Petroleum Ether	Flavonoids	-	-	-	-	-
Petroleum Ether	Phenols	-	-	-	-	-

+ Presence of constituent, - Absence of constituent

Among the above mentioned plants in table1, those having the maximum yield of extraction are highlighted (the fruits from the areas of Pedapalli (sample 2), Gajaulapalli (sample 7), Puttaparthi (sample 8), Gangireddipalli (sample 9), and Ammagondapalem village (sample 10)) and were subsequently taken for qualitative phytochemical analysis.

Phytochemical analysis showed the presence of reducing sugars, flavonoids, saponins, tannins, phenols in aqueous, ethanolic extracts and these biochemicals were absent in petroleum ether extracts as shown in the table 2.These results were in consonance with the studies carried out by various researchers, TS Dhanraj et al, S Rajan et al, MS Baliga et al, P Kaur et al, Amit Pandey et al and K Sudharameshwari et al [18-23].

From the above plant species the fruits collected from puttaparthi village (sample 8) was taken for further analysis (quantitative phytochemical analysis and antimicrobial activity) as the percentage yield in three extraction taken were found to be more as comparatively to other plant species. The amount of phenolic compounds in the Ethanolic extracts of *Aegle marmelos* was found to be 15.588 ug per mg of extract. Similarly the total amount of phenolic compounds in the aqueous extract of *Aegle marmelos* was found to be 10.509 ug per mg of extract as given in the table 3.

Table 3: Total phenolics contents in the aqueous and ethanolic extracts of fruit pulp

Fruit pulp extracts(samples)	Concentration of phenolics (ug/mg)
Ethanolic extract	15.588
Aqueous extract	10.509

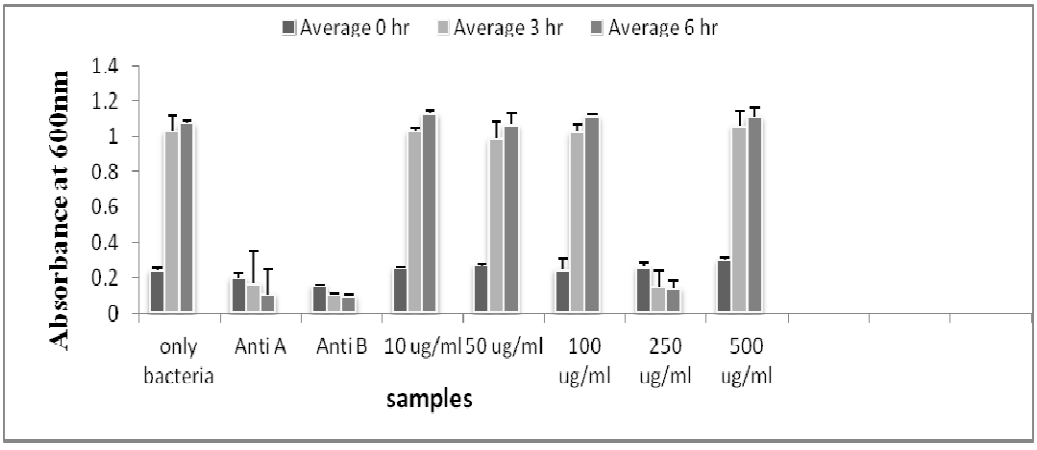
The amount of Flavonoids in the Ethanolic extracts of *Aegle marmelos* was found to be 32.305ug per mg of extract. Similarly the total amount of flavonoids compounds in the aqueous extract of *Aegle marmelos* was found to be 6.388ug per mg of extract as given in the table 4.

Table 4: Total Flavonoids content in the extracts of fruit pulp

Fruit pulp extracts(samples)	Concentration of phenolics (ug/mg)
Ethanolic extract	32.305
Aqueous extract	6.388

In the present study, the amounts of flavonoids and phenolic compounds were found more in ethanolic extracts than the aqueous extracts which show a similar correlation observed by the studies of S Rajan et al [19] and HR Gheisari et al [24]. The presence of above mentioned biochemical compounds which are generally the secondary metabolites in the plants have significant role of medicinal properties for curing various ailments.

Figure 2: Antibacterial activity of Aqueous extracts samples against strain ATCC 29213 (Staphylococcus aureus)

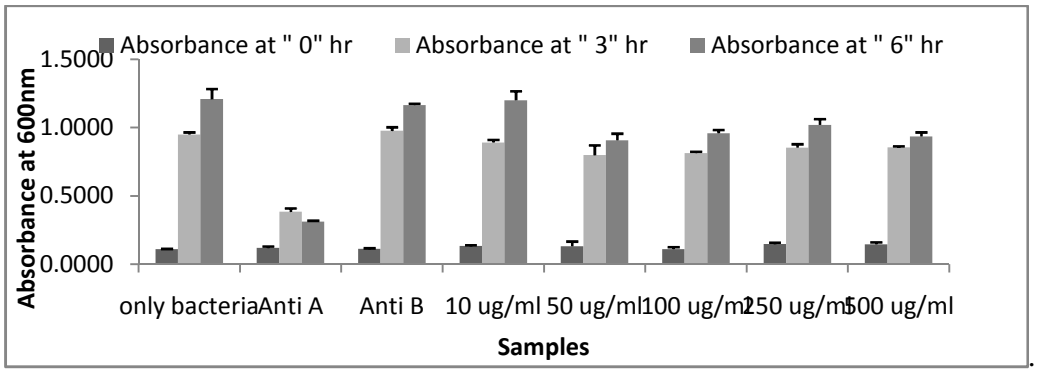


Antimicrobial activity

In the present study three extracts of *Aegle marmelos* fruit pulp was used to carry out the antimicrobial activity of gram positive bacteria *Staphylococcus aureus.* Two different strains of this bacterium were used and their effects on extracts were studied.

The aqueous extract carried out using ATCC 29213 strain revealed that at 250ug/ml concentration, it has the potential to inhibit the bacterial growth, whereas with other strain ATCC700699, it was found that the concentration of extracts taken were ineffective against the growth of the bacteria as represented in the figure 2 &3.

Figure 3: Antibacterial activity of Aqueous extracts samples against strain ATCC 700699 (Staphylococcus aureus)



In case of petroleum ether extract, the concentration of 500ug/ml was effective in inhibiting the growth of strain ATCC29213 and Concentration of 250ug/ml and 500ug/ml was effective in inhibiting the growth of strain ATCC 700699 as shown in the figure 4&5.

Figure 4: Antibacterial activity of petroleum ether extracts against strain ATCC 29213 (Staphylococcus aureus)

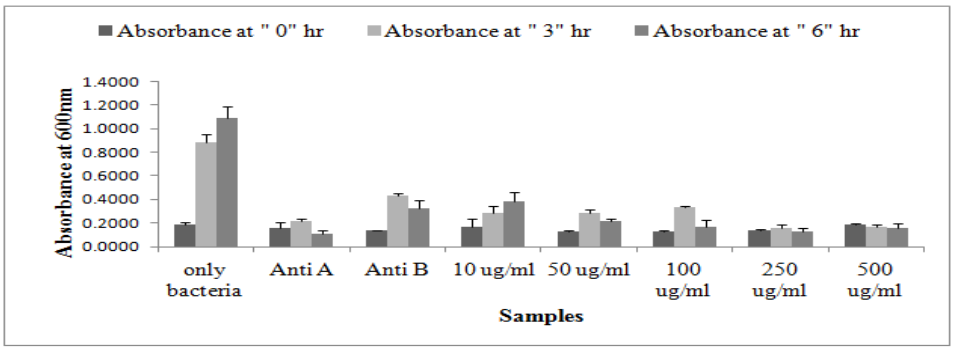
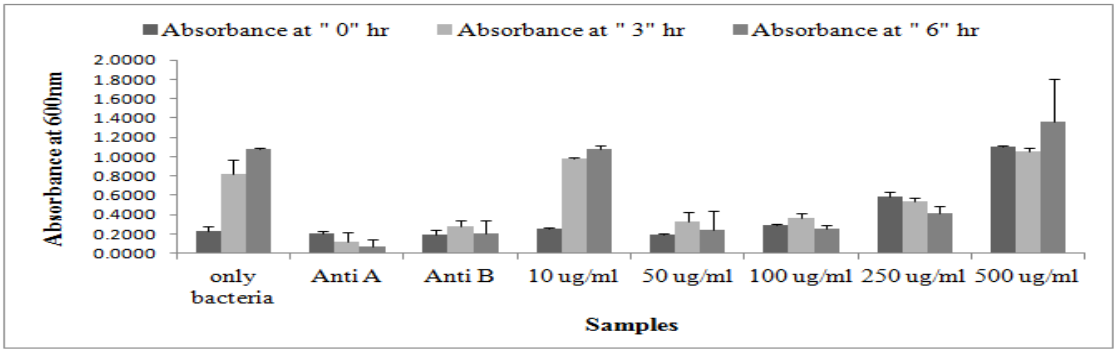


Figure 5: Antibacterial activity of petroleum ether extracts against strain ATCC 700699 (Staphylococcus aureus)



When ethanolic extracts were used on strain ATCC29213, it was found that concentration of 50-100ug/ml is effective in inhibiting the growth and concentration of 100ug/ml was effective in inhibiting the growth of bacterial strain ATCC 700699 as given below in the figure 6&7. The results were in accordance with similar studies carried out by VB Lambole et al [25].

Figure 6: Antibacterial activity of ethanolic extracts against strain ATCC 29213 (Staphylococcus aureus)

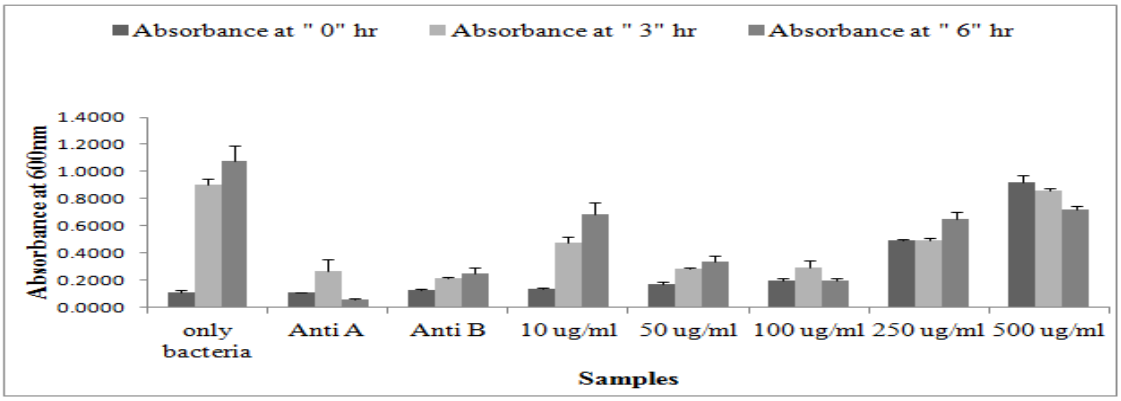
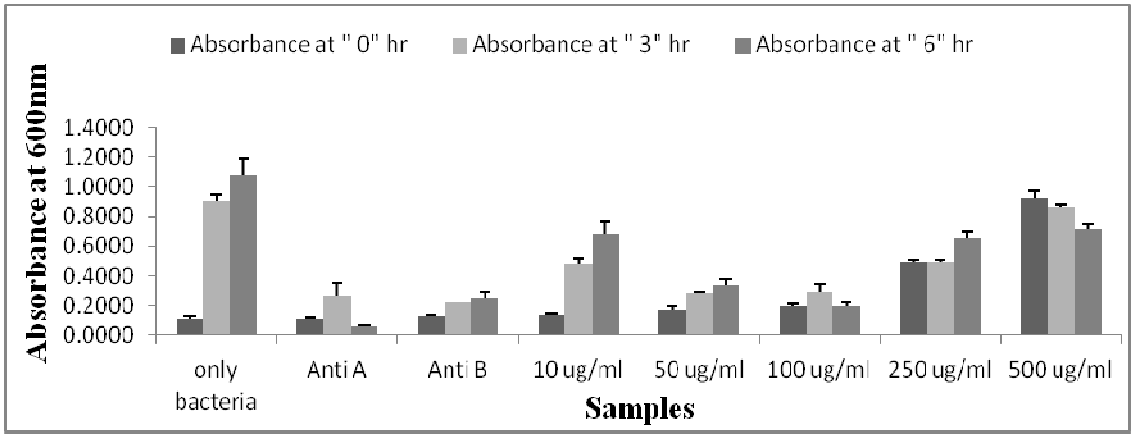


Figure 7: Antibacterial activity of ethanolic extracts against strain ATCC 700699 (Staphylococcus aureus)



C Rajasekaran [26] used Chloroform, Ethanol, Petroleum ether, Dichloromethane and Aqueous extract for the study on selected Gram negative and Gram positive bacteria. He found that ethanol and chloroform extracts were more effective than other of solvents, more or less similar to the present study. A study by D Venkatesan [16] using only ethanolic extracts on bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* revealed that *Bacillus subtilis* showed maximum zone of inhibition when treated with crude ethanolic extracts which also were observed in the present study that concentration of 50-100ug/ml is effective in inhibiting the growth of bacterial strain ATCC29213. K Sudharameshwari et al [23] found out that *Aegle marmelos* showed maximum zone of inhibition against P.aeruginosa and S.aureus in etanolic extracts which is in consonance with the present study. Using Ethanolic, Methanolic, Ethyl acetate and Hot water extract from leaves, fruits and peels of Aegle marmelos, MIC value was determined by broth dilution method by Amit Pandey [22] as done in the present study. The methanolic and hot water extracts showed least antibacterial activity as compared to ethanolic and ethyl acetate extracts. The MIC values were obtained 1.98 mg/ml in ethanolic and ethyl acetate extract of fruits against S. aureus and 11.90mg/ml in methanolic extract against P. aeruginosa. Hence from the previous study and present study demonstrates that the ethanolic extraction of the fruit pulp can be use as a potential antimicrobial active compound in future studies.

CONCLUSION

This present study evaluated the presence of phytoconstituents such as reducing sugars, saponins, tannins, flavonoids (phenolic compounds) in the ethanolic extract and aquaeous extracts fruit pulp of *Aegle marmelos* which were responsible for its antimicrobial activity. These compounds exhibited a zone of inhibition against *Staphylococcus aureus*, when compared with the control drug penicillin. From this study it was found that the effectiveness of ethanolic extract against both bacterial strains of *Staphylococcus aureus* was more or less equal to the treatment of control antibiotic ampicilin. As the treatment of control drug ampicilin was found similar to the ethanolic extract of the fruit pulp of *Aegle marmelos* in inhibiting the growth of pathogenic bacterial strains of *Staphylococcus aureus*, this can be attribute to its pharmaceutical important to human beings and in future can be used as an important tool while formulating drugs and ointments for curing different ailments.

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