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EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF UNRIPE AND HALF RIPE *AEGLE MARMELOS* CORR. FRUITS

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ABSTRACT

This study was undertaken to examine the antioxidant and antimicrobial activity of methanolic extract of *Aegle marmelos* unripe or half ripe fruits. The antioxidant activity was done by using DPPH free radical scavenging assay. The IC_{50} (The concentration of sample required to scavenge 50% of DPPH free radical) was calculated by plotting graph between % inhibition vs concentration. The ascorbic acid was used as standard antioxidant in comparison to methanolic extract of *Aegle marmelos*. The IC_{50} value of extract and ascorbic acid was found to be 62.59 µg/ml and 2.80 µg/ml. The antimicrobial activity was performed using two strains of bacteria *E. coli* and *S. aureus*. The minimum inhibitory concentration (MIC) of the extract was taken out against two strains and was compared with the inhibition of standard drug tetracycline. From the results it may be concluded that the methanolic extract of *Aegle marmelos* has significant activity against *E. coli* and *S. aureus*, the average inhibition of the test drug against *E. coli* is 0.43 which is comparable with the inhibition of tetracycline, 0.954. Again the average inhibition of test drug was found to be 0.49 which was again comparable with the standard, which showed its median inhibition of 0.965 against *S. aureus*.

The antioxidant and antimicrobial activities found in *Aegle marmelos* may be associated with their main phytochemical compounds like flavonoids, phenols and tannins. This gives the support that the fruit can be used as the medicinal plant to treat free radical damages in cells and could also be used as medicinal supplement which could we used with other drugs to give synergistic effect, as it showed sufficient antibacterial activity at high concentration as compared to standard drug tetracycline.

Keywords: flavonoids, phenols and tannins.

INTRODUCTION

Natural products offer an untold diversity of chemical structures. These natural compounds often serve as lead molecules whose activities can be enhanced by manipulation through combinations with chemicals and by synthetic chemistry. An important source of natural products, plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids[1]. In order to promote the use of medicinal plants as potential sources of antimicrobial compounds, it is pertinent to thoroughly explore their composition and activity and thus validate their use[2].The effectiveness of phytochemicals in the treatment of various diseases may recline in their antioxidant effects[3]. Antioxidants are important in the prevention of human diseases. Antioxidant compounds may function as free radical scavengers, complexing agents for pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation[4,5,6]. Antioxidants are often used in oils and fatty foods to retard their auto-oxidation; therefore, the importance of search for natural antioxidants has greatly increased in the recent years[7]. In recent years, multiple drug/ chemical resistance in both human and plant pathogenic micro organism has been developed due to indiscriminate use of synthetic drugs. This drives the need to screen medicinal plants for novel bioactive compounds as they are biodegradable, safe and have fewer side effects [8].

Aegle marmelos (L.) Correa ex Roxb., locally know as ‘bael’, is indigenous to India, grows wild throughout the deciduous forests of central and southern parts; it is considered highly religious since it is used extensively to worship gods specially for worshiping Lord Siva. It is a spinous, small or medium sized tree, having alternate, tri-foliate leaf, white scented flowers and bears berry (amphisaraca) type of fruits[9,10]. All parts of this tree, viz. root, leaf, trunk, fruit and seed are useful in several ailments like diabetes[11], diarrhoea, cancer, ulcers, etc and is one such herbal source which is rich in bio active compounds having oxygen scavenging activity. The phytochemical screening of different extracts of fruit extract revealed that

methanolic extract of fruit pulp contains maximum amount of functional and bioactive compounds such as carotenoids, phenolics, alkaloids, coumarins, flavonoids, terpenoids, and other antioxidants which may protect us against chronic diseases. In addition, it also contains many vitamins and minerals including vitamin C, vitamin A, thiamine, riboflavin, niacin, calcium, and phosphorus [12,13].

Hence the present study was design to evaluate antioxidant activity of *A. marmelos* and to compare the IC_{50} of *A. marmelos* with IC_{50} of Ascorbic acid (Standard Antioxidant) and the study was also directed to investigate the antibacterial activity of methanolic extracts of *Aegle marmelos* against *E. coli* and *S. aureus* as the literature review and phytochemical investigation have established the presence of tannins, phenols, flavonoids and coumarins which can be effective against antimicrobial infections.

MATERIALS AND METHODS

Plant material

The fruits were collected from the local market of Delhi. The drug was authentified as *Aegle marmelos* (L.) Correa ex Roxb. by Dr. H. B. Singh (Taxonomist), National Institute of Science Communication and Information Resources, NISCAIR, New Delhi. The reference no is NISCAIR/RHMD/Consult/-2010-11/1509/107. A voucher specimen is preserved in the NISCAIR department of New Delhi.

ANTIOXIDANT ACTIVITY

Preparation of plant material

The pulp of the ripe and unripe fruit of *Aegle marmelos* was chopped into pieces and dried in sun light. The completely dried fruits were powdered and 15 g of dried fruit powder was taken and extraction was carried out in soxhlet apparatus. The extract was concentrated by evaporating the methanol and stored at 4°C.

Preparation of reagents

The 500µM solution of DPPH was prepared by dissolving 23 mg of DPPH in 100 ml of methanol. TRIS [2-amino-2 (hydroxy methyl) propane 1-3di-ol] buffer (pH 7.4) was prepared by adding 0.605g of TRIS buffer in 30 ml of water and adding 0.33 ml of concentrated hydrochloric acid, diluted to 100 ml with distilled water. TRIS buffer prevents the sudden pH change during the preparation of test dilutions [14, 15].

Preparation of reference standard solution

Various dilutions of ascorbic acid were made of concentrations 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6 & 4.0 µg per 0.5 ml of methanolic solution of ascorbic acid.

Preparation of sample solution and dilutions

Prepare the stock solution by dissolving 20mg of *A. marmelos* extract in methanol and make up the volume to 10 ml with methanol. Prepare the initial dilutions from stock solution using volume 0.02 ml (8µg / 5ml), 0.04 ml (16µg / 5ml), 0.06 ml (24µg / 5ml), 0.08 ml (32µg / 5ml), 0.10 ml (40µg / 5ml), 0.12 ml (48µg / 5ml), 0.14 ml (56µg / 5ml), 0.16 ml (64µg / 5ml), 0.18 ml (72µg / 5ml) and 0.20 ml (80µg / 5ml). The volume make up was done in methanol (Mangla *et al* 2010). The final concentrations used for taking the absorbance are 8µg, 16µg, 24µg, 32µg, 40µg, 48µg, 56µg, 64µg, 72µg, and 80µg per ml.

Measurement Of *In Vitro* Antioxidant Activity

The antioxidant activity of the methanolic extract of *Aegle marmelos* was determined by using a method based on the reduction of methanolic solution of colored-free radical 1,1-di phenyl-1-2-picryl hydrazyl (DPPH). The radical scavenging activity of tested sample was expressed as an inhibition percentage. Ascorbic acid was used as reference standard.

In 5 ml volumetric flasks added 1 ml of DPPH solution, 0.5 ml of TRIS Buffer and 0.5 ml of final dilutions of different concentrations range prepared from *A. marmelos* methanolic extract stock solution and made up the volume to 5 ml with methanol. In same way, prepare the control dilutions of DPPH, replacing 0.5 ml of prepared dilutions (the drug solution under investigation) with methanol. The absorbances of all the dilutions were taken after 30 minutes at λ max 517nm using methanol as blank.

9.12 Statistical Analysis

The percentage inhibition was calculated using:

$$\text{Percent Inhibition} = \frac{(Ac - As)}{A_C} \times 100$$

Where,

A_C is absorbance of control,

As is the absorbance of sample

IC_{50} value (a concentration at 50% inhibition) was determined from the curve between percentage inhibition and concentration.

All determinations were done in triplicate and the IC_{50} value was calculated by using the equation of line [16].

ANTIMICROBIAL ACTIVITY

Bacterial strains Used for Determination of Antibacterial Activity

Escherichia coli and *Staphylococcus aureus* strains of bacteria were used for the study and were maintained in solid nutrient agar media at 4° temperature in refrigerator.

Preparation of Media

Nutrient Broth

Nutrient Broth of following composition was used for culturing and sub-culturing of *E. Coli* and *S. aureus.*

Peptone	1.0 %
Sodium Chloride	0.5 %
Beef Extract	1.0 %
Distilled Water	q.s.

Media was prepared in distilled water to produce 100 ml by dissolving the given amount of ingredients to it. The pH was adjusted to 7.2 ± 0.2 and then sterilized for 20 minutes at 15 lb pressure in an autoclave.

Solid Media Nutrient Agar Media

Beef extract	3g
Peptone	5g
Agar	20g
NaCl	8g
Distilled water	up to 1000ml

Hydrate the agar with distilled water; dissolve by boiling with continuous stirring. Add weighed amount of other ingredients to it, adjusting the volume to 1000 ml. If necessary, adjust the pH 7.2 ± 0.2. The prepared media is poured in a suitable conical flask and a cotton plug was tight fitted to its mouth and wrapped with a brown sheet of paper. Sterilization was done for 20 minutes at 15 lb pressure in an autoclave. Finally, media was allowed to cool to 45°C - 50°C prior to use.

Preparation of Inoculum

Bacterial preserves (*E. coli* and *S. aureus*) were cultured and sub cultured to obtain pure colonies in a following manner.

- One loopful (2mm dia) of each bacterial_suspension was inoculated in 5 ml of nutrient broth and all test tubes were incubated at 37°C for 24 hrs.
- The overnight grown nutrient broth culture of each test organism was used for streaking over solid nutrient agar plates and subjected to incubation at 37° C for 24 hrs and same process was repeated until pure isolated colonies were obtained.
- From these isolated colonies fresh sterile nutrient broth and media were re-incubated at 37° C for 24 hrs. These nutrient broth cultures served as inoculum for determination of anti-bacterial activities of the extract.

Preparation of Standards

For the preparation of standard solution, pure tetracycline, 10 mg (tetracycline IP) was taken and a stock solution of 10 mg/ 10 ml was prepared in 0.1M HCl. This stock solution was further used to prepare dilutions of standards of concentrations 1.5 µg/ml, 2 µg/ml, 2.5 µg/ml, 3 µg/ml and 3.5 µg/ml. Each concentration was assigned a code S_1, S_2, S_3, S_4 and S_5 respectively. All the dilutions were prepared in 0.1M HCl and the final dilution was done with distilled water [14].

Preparation of Test Solution

For the preparation of test solution, methanolic extract 100 mg was taken and a stock solution of 100 mg/ 10 ml was prepared in 0.1M HCl. This stock solution was further used to prepare dilution of concentration 2350 µg/ml which was determined after taking minimum inhibitory concentration. The dilutions were prepared in 0.1M HCl and the final dilution was done with distilled water [14].

ASSAY METHOD

Preparation of Agar Plates for standards of *E. coli* and *S. aureus*

- For the preparation of agar plates, 12 petri plates of diameter 10 cm were washed, dried, neatly wrapped in suitable size brown paper, tied with a thread and sterilized at 180° C for 45 min. After sterilization, wrapped plates were taken in the aseptic room and were unwrapped under Laminar Air Flow Bench (LAFB) prior to use. Before use, the bench of the laminar air flow bench was sterilized with cotton drenched in methanol.

- The sterilized solid nutrient agar media was also unwrapped under LAFB and 1 ml of cultured innoculum (prepared by pouring little amount of single strain innoculum in 100 ml of water) of *E. coli* was poured in each plate. Then after this, 100ml of nutrient agar media was poured in each plate to fill the plate to 1/3rd of its height and was shaken gently to evenly distribute the innoculum throughout the nutrient media, taking care that no air bubble enters it and was left to get solidified.
- When the plates got solidified, sterilized test tube of 6 mm dia was used to make cups in the agar plates. Each plate was bored to get equidistant 6 cups of 6mm dia. Now, three plates were prepared for each concentration. Each plate contained 3 cups filled with 0.4 ml of standard mean S_3 and standard S_1, S_2, S_4 and S_5 respectively. So that the inhibition of each concentration could be compared with median concentration.
- After preparation of plates, each plate was kept in incubator at 35°C for 24 hrs.
- The given procedure was repeated for preparation of agar plates for standard curve against *S. aureus*.

Preparation of Agar Plates for Test Drug Against *E. coli* and *S. aureus*

- For the preparation of agar plates for test drug, 3 petri plates of diameter 10 cm were washed, dried, neatly wrapped in suitable size brown paper, tied with a thread and sterilized at 180° C for 45 min. After sterilization, wrapped plates were taken in the aseptic room and were unwrapped under Laminar Air Flow Bench (LAFB) prior to use. Before use, the bench of the laminar air flow bench was sterilized with cotton drenched in methanol.
- The sterilized solid nutrient agar media was also unwrapped under LAFB and 1 ml of cultured innoculum (prepared by pouring little amount of single strain innoculum in 100 ml of water) of *E. coli* was poured in each plate. Then after this, 100ml of nutrient agar media was poured in each plate to fill the plate to 1/3rd of its height and was shaken gently to evenly distribute the innoculum throughout the nutrient media, taking care that no air bubble enters it and was left to get solidified.
- Minimum inhibitory concentration of methanolic fruit extract was taken out so as to get the idea of concentrations of test drug which are to be compared with standard drug tetracycline.
- When the plates got solidified, sterilized test tube of 6 mm dia was used to make cups in the agar plates. Each plate was bored to get equidistant 6 cups of 6mm dia. Now, three plates were prepared for each concentration. Each plate contained 3 cups filled with 0.4

ml of standard mean S_3 and a test drug respectively. So that the inhibition of each concentration could be compared with the median concentration.

- After preparation of plates, each plate was kept in incubator at 35°C for 24 hrs.
- The given procedure was repeated for preparation of test agar plates against *S. aureus*.

Calculation of Inhibition Zones

After 24 hrs of incubation of plates, each plate was examined under sufficient light and the area around the cups were growth of bacteria was inhibited was marked with a marker and the diameter was measured with the help of a scale in centimetres for both standard and test drug and for both the strains. After taking inhibition zone of each concentration, correction factor is calculated by deducting the S_3 inhibition in each concentration from total inhibition of S_3 in all 12 plates. While incubation takes place, the bacteria respire and release moisture, which gets settled on the plates and gets adsorbed in nutrient media forming a whitish zone around the cups in form of patches. These moisture patches should not be confused with the zone of inhibition while calculating the inhibitions.

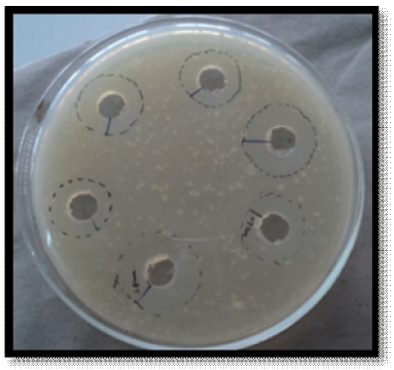

Fig. 1: Inhibition zone of tetracycline against *E. coli*

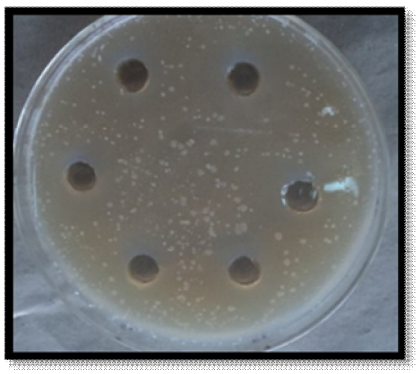

Fig. 2: Inhibition zone of tetracycline against *S. aureus*

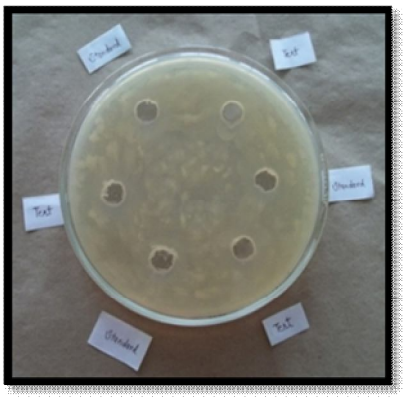

Fig. 3: Inhibition of *A. marmelos* against *E. coli*

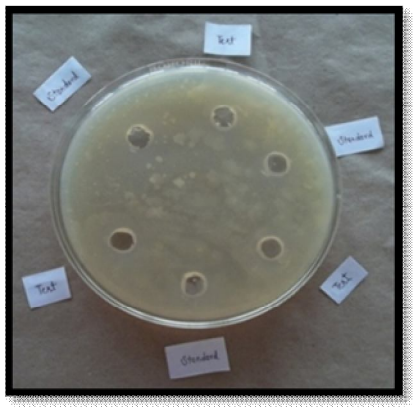

Fig. 4: Inhibition of *A. marmelos* against *S. aureus*

RESULTS AND DISCUSSION

Antioxidant Activity

The Methanolic extract of *A. marmelos* tested for *in vitro* antioxidant activity using DPPH showed good free radical scavenging activity, as evidenced by IC_{50} values. (Fig. 6) depicted that the IC_{50} value of *A. marmelos* extract was 62.59, whereas ascorbic acid used as a standard showed an IC_{50} of 2.08 µg (Fig. 5).

The absorbance of sample (methanolic extract of *A. marmelos*) and standard (Ascorbic acid) were taken in triplicate. With the increase in concentration, there was the decrease in absorbance values and increase in percent inhibition of the drug. The calculated percentage inhibition has shown with the help of tables. Table no. 1 represents the values of absorbance and percentage inhibition of Ascorbic Acid (standard antioxidant) and the table no. 2 represents the values of absorbance and percentage inhibition of methanolic extract of *A. marmelos* test solution.

Table 1: Values of absorbance and percentage inhibition with increase in concentration of methanolic solution of Ascorbic Acid (standard antioxidant)

Concentration (µg/ml)	Absorbance	Percentage Inhibition
0.4	1.674 ± 0.012	16.75
0.8	1.542 ± 0.057	23.32
1.2	1.462 ± 0.033	27.29
1.6	1.368 ± 0.022	31.97
2.0	1.255 + 0.036	37.59
2.4	1.173 + 0.035	41.67
2.8	0.997 ± 0.063	50.42
3.2	0.832 ± 0.110	58.62
3.6	0.763 ± 0.102	62.05
4.0	0.692 ± 0.011	65.58

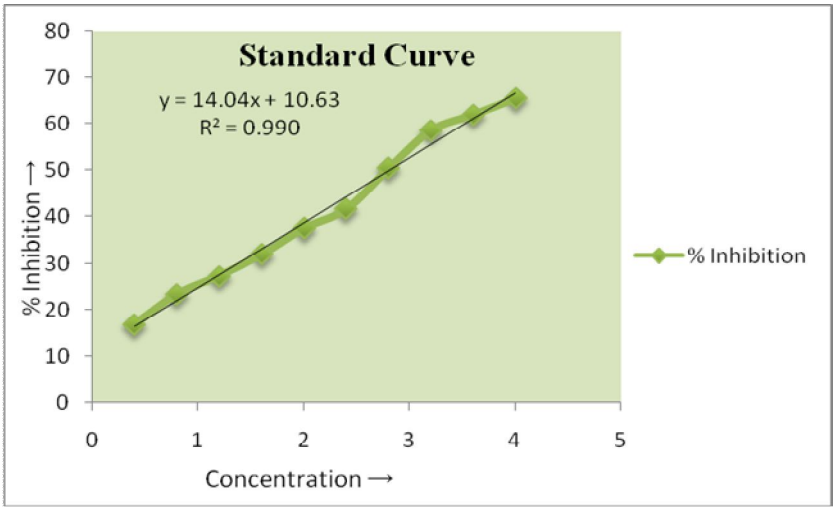

Fig. 5: Graphical representation of concentration (µg) Vs percentage inhibition of methanolic solution of ascorbic acid (standard antioxidant)

X Axis = Concentration in µg/ml

Y Axis = % Inhibition

From the equation, When Y=50, X= 2.80

Table 2: Values of absorbance and percentage inhibition with increase in concentration of methanolic extract of *A. marmelos.*

Concentration (µg/ml)	Absorbance	Percentage Inhibition
8	1.825 ± 0.061	22.99
16	1.723 ± 0.102	27.29
24	1.622 ± 0.102	31.56
32	1.499 ± 0.199	36.75
40	1.422 ± 0.127	40.00
48	1.329 ± 0.169	43.92
56	1.275 ± 0.165	46.20
64	1.154 ± 0.194	51.30
72	1.106 ± 0.169	53.33
80	0.99 ± 0.193	58.22

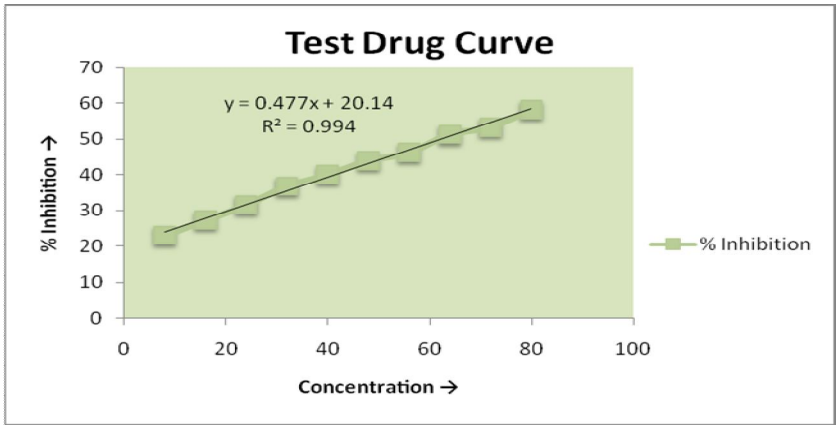

Fig. 6: Free radical (DPPH) scavenging activity of methanolic extract of *A. marmelos* in *in-vitro* system.

Graphical representation of the concentration required inhibiting 50 percent of free radicals (Each point represents the mean percentage inhibition of triplicate experiments).

X Axis = Concentration in µg/ml

Y Axis = percentage Inhibition

From the equation, When Y=50, X= 62.59.

DISCUSSION

It may be concluded from this study that the methanolic extract of *Aegle marmelos* is very active against the free radicals. The free radical scavenging and antioxidant activity found in *Aegle marmelos* may be associated with their main phytochemical compounds like flavonoids, phenols and tannins. This gives the support that the fruit can be used as the medicinal plant to treat free radical damages in cells.

Antimicrobial

Table 3: Inhibition zones for *E. coli* of various concentrations along with their standard mean S_3

Plates	Zone of Inhibition							
	S_1	S_3	S_2	S_3	S_4	S_3	S_5	S_3
1	0.660	0.766	0.760	0.900	1.400	1.000	1.030	0.960
2	0.600	0.833	1.010	1.000	1.100	1.130	1.100	0.960
3	0.733	0.900	0.780	0.900	0.730	0.760	1.060	1.060
Mean	0.664	0.833	0.850	0.988	1.076	0.960	1.120	0.990

Correction Factor = S_T(Average inhibition of S3 in all concentrations [0.833+0.988+0.960+0.990 = 0.954]) – S_3 (Average inhibition of S_3 in individual concentration)

Final Inhibition = Total inhibition of individual concentration +/- Correction Factor

Table 4: Preparation of standard curve of tetracycline against *E. coli*

Concentrations	Total Inhibitions	$S_T - S_3$ (of Individual Concentration)	Correction Factor	Final Inhibitions
S_1	0.664	0.833	+ 0.121	0.785
S_2	0.850	0.988	- 0.034	0.816
S_3	0.954	0.954	0.000	0.954
S_4	1.076	0.960	- 0.052	1.027
S_5	1.120	0.99	- 0.036	1.084

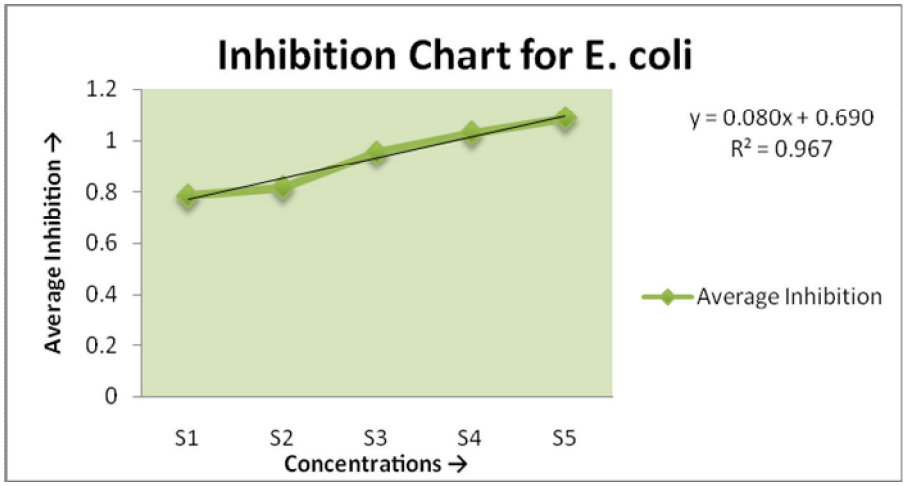

Fig. 7: Standard Curve of tetracycline against E. coli

Table 5: Inhibition zones for *S. aureus* of various concentrations along with their standard mean S_3

Plates	Zone of Inhibition							
	S_1	S_3	S_2	S_3	S_4	S_3	S_5	S_3
1	0.630	0.830	0.700	0.900	1.060	1.030	1.160	1.000
2	0.630	0.900	0.960	1.030	1.060	1.200	1.230	1.030
3	0.760	0.930	0.730	0.900	0.830	0.930	1.160	0.930
Mean	0.670	0.880	0.790	0.940	1.160	1.060	1.180	0.980

Correction Factor = S_T(Average inhibition of S3 in all concentrations [0.880+0.940+1.060+0.980 = 0.965) – S_3 (Average inhibition of S_3 in individual concentration)

Final Inhibition = Total inhibitions of individual concentration +/- Correction Factor

Table 6: Preparation of standard curve of tetracycline against *S. Aureus*

Concentrations	Total Inhibitions	$S_T - S_3$ (of Individual Concentration)	Correction Factor	Final Inhibitions
S_1	0.670	0.880	+ 0.085	0.755
S_2	0.790	0.940	+ 0.025	0.815
S_3	0.965	0.965	0.000	0.965
S_4	1.160	1.060	- 0.095	1.065
S_5	1.180	0.980	- 0.015	1.165

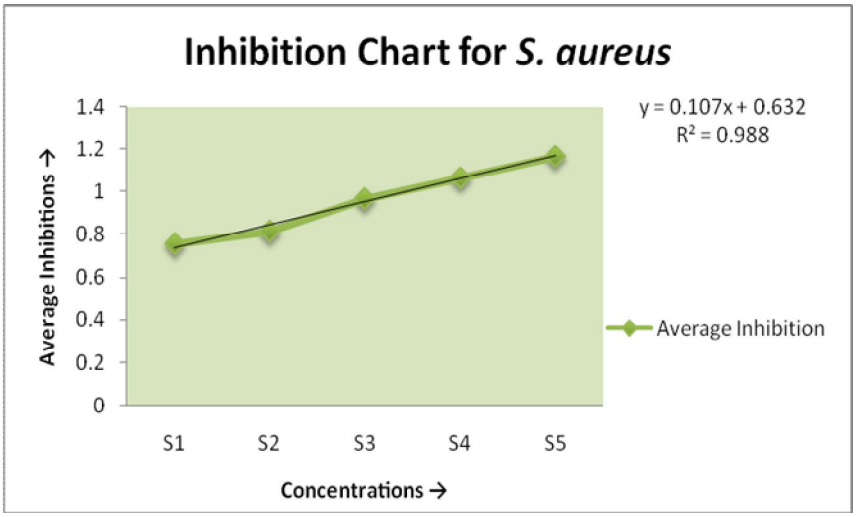

Fig. 8: Standard Curve of tetracycline against *S. aureus*

The MIC of test drug for E. *coli* was tested to be 2350 µg/ml and this concentration of test drug was used to test inhibition against both E. coli and S. aureus. These readings were compared with Standard Drug of concentration 2.5 µg/ml. The comparison chart is as below,

Table 7: Test drug (*A. marmelos*) inhibition against *E. Coli*

Plates	Standard Mean (S_3)	Test Drug Inhibition	Correction Factor	Final Inhibition
1	0.930	0.430	- 0.020	0.410
2	0.870	0.470	+ 0.040	0.510
3	0.930	0.400	- 0.020	0.380
Mean	0.910	0.433	-	0.430

Table 8: Test drug (*A. marmelos*) inhibition against *S. Aureus*

Plates	Standard Mean (S_3)	Test Drug Inhibition	Correction Factor	Final Inhibition
1	0.930	0.530	- 0.050	0.480
2	0.870	0.530	+ 0.010	0.540
3	0.860	0.430	+ 0.020	0.450
Mean	0.880	0.496	-	0.490

DISCUSSION

In the present study standard curves of standard drug tetracycline was prepared individually against two bacterial strains viz. *E. coli* and *S. aureus*. These standards curves were used for determination of median inhibition (inhibition of median concentration S_3) against the two strains. Again using this median concentration of standard tetracycline, activity of test drug (*A. marmelos*) was evaluated for the two strains and was compared with the media inhibition of the standard drug. The minimum inhibitory concentration of methanolic fruit extract was found to be 2350 µg/ml for both *E. coli* and *S. aureus*.

The result clearly showed that the *A. marmelos* methanolic fruit extract has significant activity against *E. coli* and *S. aureus*, the average inhibition of the test drug against *E. coli* is 0.43 which is comparable with the inhibition of tetracycline, 0.954. Again the average inhibition of test drug was found to be 0.49 which was again comparable with the standard, which showed its median inhibition of 0.965 against *S. aureus*. From the study it was also concluded that *Aegle marmelos* is more active against *S. aureus* in comparison to *E. coli*.

Thus, the drug *A. marmelos* could be used as medicinal supplement as it showed sufficient antibacterial activity at high concentration as compared to standard drug tetracycline. The activity could be due the presence of tannins (9 %), phenols and coumarins in the fruit [17]. Further in the near future the drug could we useful for isolation of antibacterial compound which could we used with other drugs to give synergistic effect.

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