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Phytochemical screening and antibacterial activity of alkaloids and flavonoids of different parts of *aegle marmelos* linn. Against pathogenic bacteria

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Abstract:
Objective: *Present study aims to investigate the antibacterial efficacy of flavonoids and alkaloids from different parts of Aegle marmelos Linn. against selected pathogens.*
Method: *Different parts (Leaf, Stem and Fruit) of Aegle marmelos were collected and air dried and soxhlet extracted by using standard methods for flavonoid and alkaloid extraction. Antibacterial activity of selected extracts was tested by Disc Diffusion Assay and MIC, MBC were calculated by Broth Dilution Assay respectively.*
Result: *MIC and MBC values of the extracts were also in desired range i.e. from 1.25mg/ml to 0.0195mg/ml which ensure their use as an alternative to existing antibiotics against selected pathogens*
Conclusion: *the study reveals that flavonoids and alkaloids of different parts (leaf, stem and fruit) of Aegle marmelos has considerable antibacterial activity.*
Keywords: *Aegle marmelos; antibacterial; minimum inhibitory concentration; minimum bactericidal concentration; alkaloid and flavonoid.*

I. Introduction

World health organization has also pointed out that more than 80% of the world population depends on plants to meet their primary health care needs [1].In recent years, multiple drug resistance in both human and plant pathogenic microorganisms has been developed due to indiscriminate use of synthetic drugs. This drives the need to screen medicinal plants for noval bioactive compounds as they are biodegradable, safe and have fewer side effects (2) *Aegle marmelos* Linn. of family Rutaceae is commonly called as 'Beal' is the well known Indian medicinal plant of therapeutic importance. Beal tree is commonly found in Hindu sacred grooves. It is considered sacrilegious and enormous quantities of the leaves are collected to be used during ritual ceremonies.(3). Various phytoconstituents like alkaloids, coumarins and steroids have been isolated and identified from different parts of the tree such as leaves, fruits, wood, root and bark. Root and fruit contain alkaloids, coumarins and furocumarins such as aegelenine, aegeline (4), skimmianine, marmelosin, aurapten, epoxyaurapten, marmin, marmesin, xanthotoxin, scopoletin (5), decursinol and haplopine (6), umbelliferone (7), 6-methyl-4-chromanone (8), skimmin, psoralen, 6,7-dimethoxycoumarin, tembamide (9). Fruit in addition, contain xanthotaxol, alloimperatorin and alkaloid like marmeline identified as N-2-hydroxy-2-[4 - (3′, 3′-dimethyl allyloxy) phenyl] ethyl cinnamide (10). β- sitosterol and its glycoside are also present in the fruit (11)

Present study was carried out to investigate the antibacterial activity against Gram positive bacteria (*S.aureus, Bacillus subtilis and Roultella planticola*) and Gram negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumonia,* and *Agrobacterium tumefaciens*).

II. Material And Method

Different parts of *A.marmelos* (Leaves, stem and fruit) were collected from campus of University of Rajasthan Jaipur. Plant was identified by the senior taxonomist at department of Botany University of Rajasthan and (voucher specimen no.RUBL211335) was submitted in 'Herbarium', Botany Department University of Rajasthan.

Preparation of extracts

a) Flavonoids

Selected parts of the plant were collected separately washed with sterile water, shade dried, and finely powdered using grinder. Each sample was subjected to extraction, following the method of Subramannian and Nagarjan (12). One hundred gram of each sample was soxhlet extracted in 80% hot methanol (500 ml) on water bath for 24 hours and filtered. Filtrate was re-extracted successively with petroleum ether (Fraction I), ethyl ether (fraction II) and ethyl acetate (Fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances whereas ethyl ether and ethyl acetate fractions were analyzed for free

and bound flavonoids respectively. Ethyl acetate fraction of each sample was hydrolyzed by refluxing with 7% H_2SO_4 for 2 hours and the filtrate was then extracted with ethyl acetate in separating funnel. Ethyl acetate extract thus obtained was washed with distilled water to neutrality. Ethyl ether (free flavonoids) and ethyl acetate fraction (bound flavonoids) were dried in vaccuo, weighed and were stored at 4^0C.

b) Alkaloids

Alkaloids were extracted from different parts of the plant by well established methods after preliminary detection of alkaloids. Finely powdered samples (100 g) of plant parts were extracted with 10% acetic acid in ethanol for four hours, Extracts were then concentrated and were made alkaline by NH_4OH.The precipitate thus obtained was collected by centrifugation, washed with 1%NH_4OH, filtered, dried in vaccuo and weighed. Extracts thus obtained were stored at 4^0C in air tight glass vials for further use (13).

Test Pathogens

Six pathogens were selected in total which include *Staphyllococcus aureus* (3610), *Pseudomonas aeruginosa*(1934), *Bacillus subtilis*(121), *Klebsiella pneumonae* (4030), *Agrobacterium tumifacian*(431), *Roultella planticola.*(530).The pathogens were procured from IMTECH Chandigarh, (India).Bacterial strains were grown and maintained on Muller-Hinton agar medium.

Antimicrobial Assay

'Disc Diffusion Assay' (14) was performed for screening. Muller-Hinton agar plates were seeded with bacterial inoculum (1.10^7 CFU/ml).Sterile paper discs of Whatmann no.1(6 mm in diameter)were impregnated each with 100 microlitre of the extract of concentration (10mg/ml) to give final concentration of 1 mg/disc. Discs were left to dry in vaccuo to remove residual solvent, which might interfere with the determination. Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested with references to streptomycin (1mg/disc) as standard for bacteria. The plates were kept at 4^0 C for diffusion of extracts, thereafter were incubated at 37^0 C. Activity index of each extract was calculated by the standard formula viz.
Activity index = IZ produced by extract / IZ produced by standard
Where, IZ =Zone of inhibition

Table-1a-Antimicrobial activity of extracts of A.marmelos against B.subtillis ,S. aureus, A.tumifacian

Microorganism		***B.subtillis***		***S. aureus***		***A.tumifacian***	
Plant parts	**Extracts**	**IZ**	**AI**	**IZ**	**AI**	**IZ**	**AI**
Leaf	E1	21.93±0.404	0.645	10.56±0.602	0.422	15.16±0.251	0.689
	E2	16.66±0.416	0.49	10.23±0.236	0.409	10.56±0.416	0.48
	A	21.73±0.315	0.658	13.46±0.503	0.489	-	-
Stem	E1	-	-	10.56±0.513	0.391	-	-
	E2	-	-	7.06±0.208	0.282	-	-
	A	15.13±0.321	0.581	-	-	13.1±0.458	0.485
Fruit	E1	10.96±0.776	0.365	15.3±0.360	0.566	-	-
	E2	10.1 ±0.458	0.336	7.3±0.360	0.270	9.96±0.152	0.332
	A	27.53±0.450	0.786	13.03±0.152	0.592	24.2±0.346	0.756

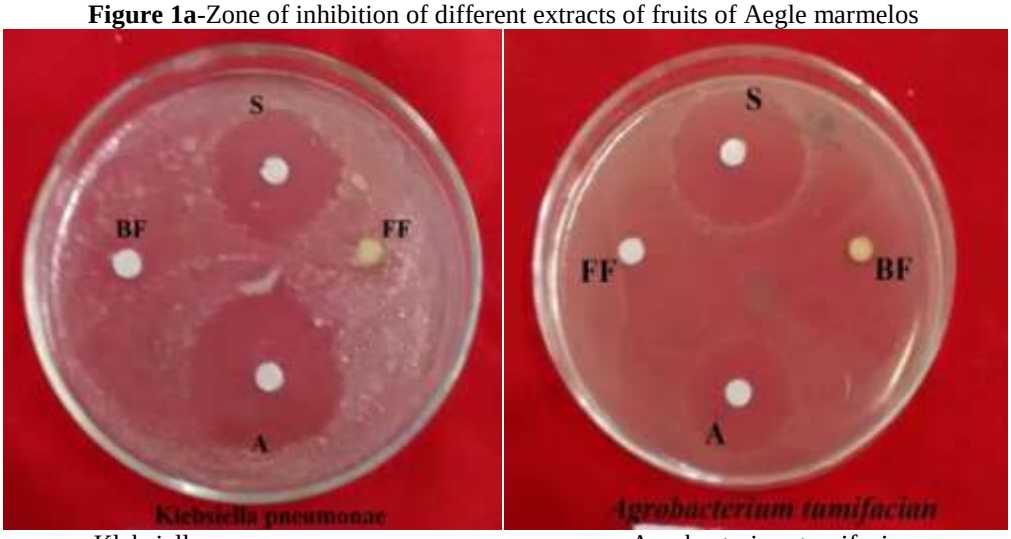
All values are mean± SD; n=3; IZ Inhibition zone in mm (including 6mm diameter). AI-Activity index (IZ developed by extract/IZ developed by standard) (-) –No activity, E1-Free flavonoids;E2-Bound flavonoids IZ of standard drug streptomycin against *B.subtillis* (35mm), *S. aureus* (26mm), *A.tumifacian* (32mm)

Table-1b-Antimicrobial activity of extracts of A.marmelos against K.pneumonae, R.planticola ,P.aeruginosa

Microorganism		***K.pneumonae***		***R.planticola***		***P.aeruginosa***	
Plant parts	**Extracts**	**IZ**	**AI**	**IZ**	**AI**	**IZ**	**AI**
Leaf	E1	7.2±0.300	0.3	13.26±0.305	0.247	15.5±0.5	0.574
	E2	9.1±0.173	0.379	20.03±0.251	0.646	11.43±0.404	0.423
	A	8.6±0.0.529	0.296	8.93±0.208	0.288	-	-
Stem	E1	-	-	7.86±0.416	0.262	12.53±0.503	0.481
	E2	-	-	-	-	7.2±0.264	0.276
	A	20.03±0.351	0.572	15.63±0.602	0.521	7.03±0.251	0.281
Fruit	E1	-	-	19.96±0.152	0.570	17.5±0.503	0.583
	E2	-	-	9.23±0.305	0.263	8.6±0.529	0.286
	A	31.16±0.623	1.15	10.23±0.251	0.409	-	-

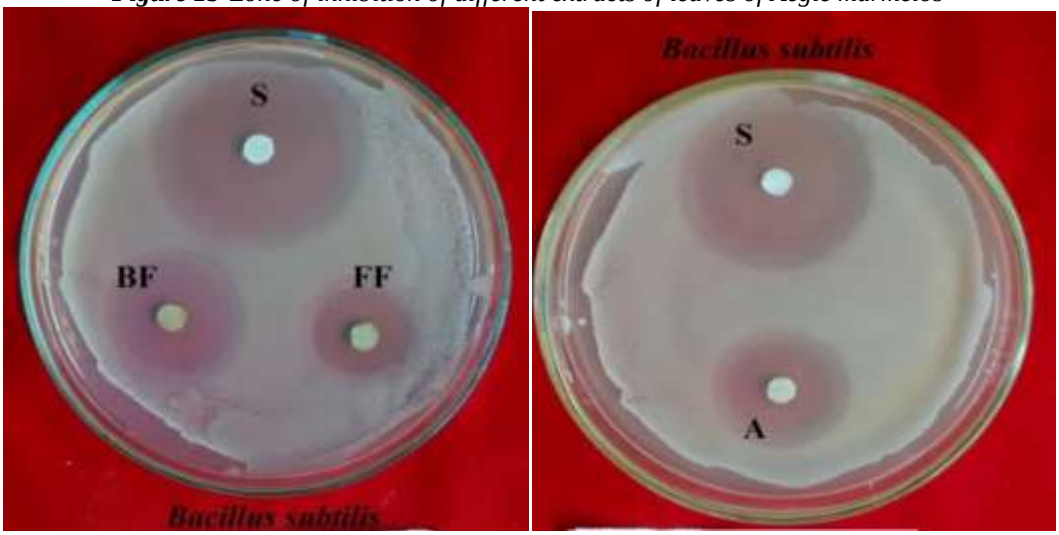
IZ of standard drug streptomycin against *K.pneumonae* (27mm) *R.planticola* (31mm) *P.aeruginosa* (30mm).

Figure 1a-Zone of inhibition of different extracts of fruits of Aegle marmelos



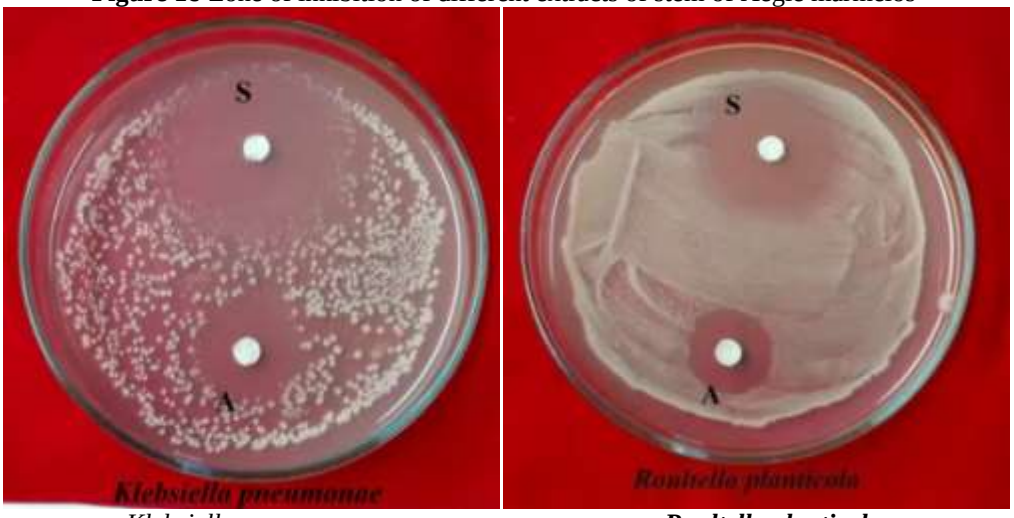
Klebsiella pneumonae Agrobacterium tumifacian

***Figure 1b**-Zone of inhibition of different extracts of leaves of Aegle marmelos*



Bacillus subtilis ***Bacillus subtilis***

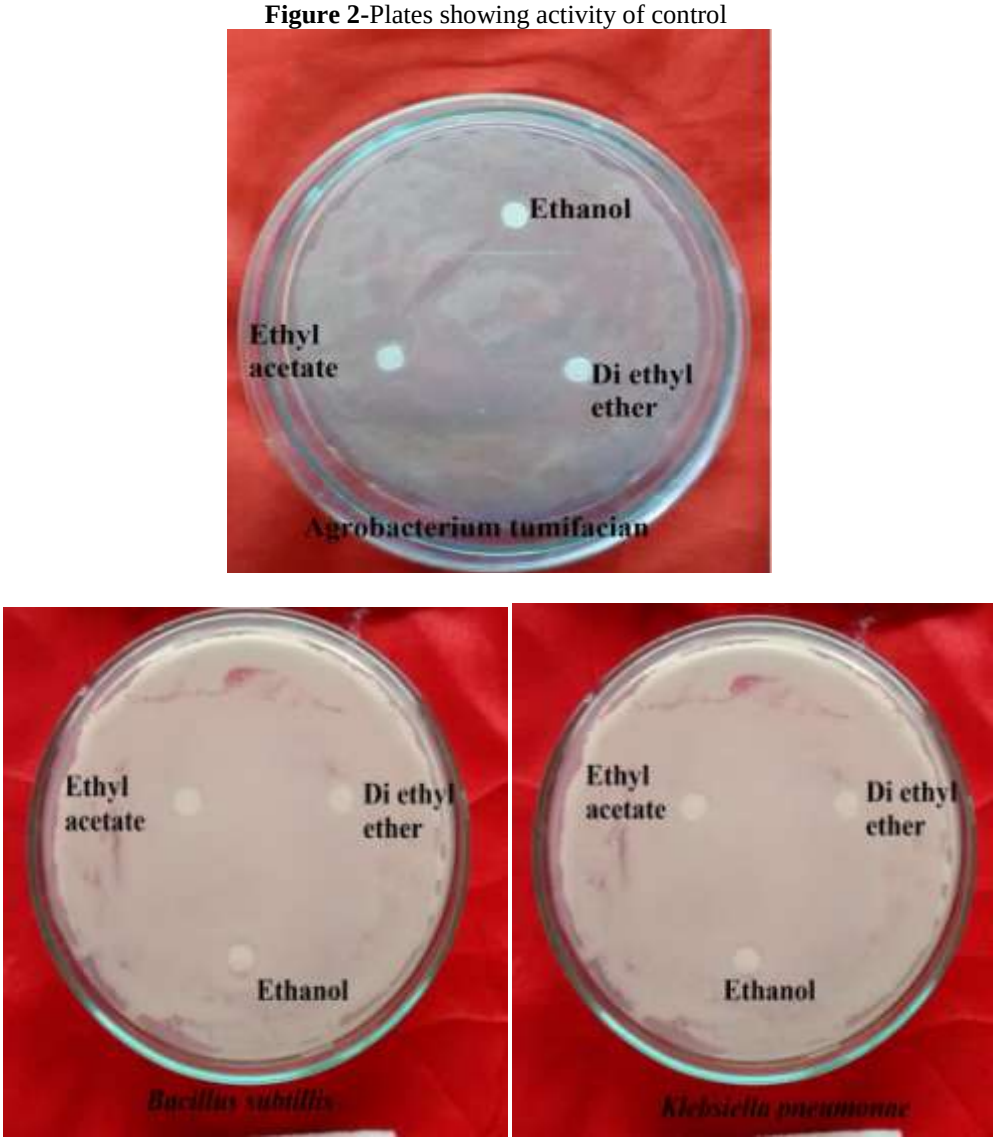
Figure 1c-Zone of inhibition of different extracts of stem of Aegle marmelos



Klebsiella pneumonae ***Roultella planticola***

BF-Bound flavonoid, FF-Free flavonoid, A-Alkaloid, S-Standard

Figure 2-Plates showing activity of control



Determination of minimum inhibitory concentration and minimum bactericidal concentration

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against test pathogens. Broth micro dilution method (15) was followed for determination of MIC values. Plant extracts were re-suspended in acetone (acetone has no activity against test microorganisms) to make 10 mg/ml final concentration. Two fold serially diluted extracts were added to broth media of 96 wells of micro titre plates. Thereafter 100µl inoculum of bacteria was added to each well. Bacterial suspensions were used as negative control, while broth containing standard drug was used as positive control. Micro titre plates were then incubated at 37^0 C for 24 hours. Each extract was assayed in triplicate and each time two sets of micro titre plates were prepared, one was kept for incubation and another was kept at 4^0 C for comparing the turbidity in the well of micro titre plate. The MIC values were taken as the lowest concentration of the extracts in the well of micro titer plate that show no turbidity after incubation. The turbidity of the well in the micro titer plate was interpreted as the visible growth of micro organism. The minimum bactericidal concentration was determined by sub culturing 50µl from each well. Least concentration of extract showing no visible growth on sub culturing was taken as MBC. (Table 2a and 2b).

Table 2a MIC and MBC of active flavonoid extracts of A.marmelos against different pathogens

Plant parts	***Leaf***				***Stem***				***Fruit***			
Micro	E1		E2		E1		E2		E1		E2	
organism	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B.subtillis	0.078	0.039	0.156	0.078	-	-	-	-	0.625	0.312	0.625	0.312
S. aureus	0.625	0.312	0.625	0.312	0.625	0.312	1.25	0.625	0.156	0.78	1.25	0.625

A.tumifacian	0.156	0.78	0.625	0.312	-	-	0.312	0.156	-	-	-	-
K.pneumonae	1.25	0.625	0.625	0.312	-	-	-	-	-	-	-	-
R.planticola	0.312	0.156	0.078	0.039	1.25	0.625	-	-	0.078	0.039	0.625	0.312
P.aeruginosa	0.312	0.156	0.625	0.312	0.312	0.156	1.25	0.625	0.156	0.078	0.625	0.312

E1- Bound flavonoids,E2 – Free flavonoids, MBC- Minimum inhibitory concentration, MBC- Minimum bactericidal concentration

Table 2b MIC and MBC of active alkaloid extracts of A.marmelos against different pathogens

Plant parts / Microorganism	***Leaf***		***Stem***		***Fruit***	
	A		A		A	
	MIC	MBC	MIC	MBC	MIC	MBC
B.subtillis	0.078	0.039	0.312	0.156	0.039	.0195
S. aureus	0.312	0.156	-	-	0.312	0.156
A.tumifacian	-	-	-	-	0.039	.0195
K.pneumonae	0.625	0.312	0.078	0.039	0.039	.0195
R.planticola	0.625	0.312	0.312	0.156	0.625	0.312
P.aeruginosa	-	-	1.25	0.625	-	-

A-Alkaloids, MIC- Minimum inhibitory concentration, MBC- Minimum bactericidal concentration

Total activity determination

Total activity is the volume up to which test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g (16)(Table 3).

Table-3 Total activity of active extracts of Aegle marmelos.

Plant parts	Quantatiy of extract g/dry weight mg/ml		Microorganisms					
			B. s.	S. a.	A. t.	K. p.	R. p	P. a.
	Extracts		Total activity ml/g					
Leaf	E1	2.5	32.05	4	16.02	2	8.01	8.01
	E2	2	12.82	3.2	3.2	3.2	25.64	3.2
	A	136	1743.5	435.8	-	217.6	217.6	-
Stem	E1	4		6.4	-	-	3.2	12.82
	E2	1.5	-	1.2	48.02	-	-	1.2
	A	53	169.8	-	-	679.4	169.8	42.5
Fruit	E1	1	0.125	0.156	-	-	0.0156	0.125
	E2	5	0.625	1.25	-	-	0.625	0.625
	A	188	482	0.001	482	482	0.003	-

E1- Bound flavonoids,E2 – Free flavonoids, A- Alkaloids of respective parts. B.s.-*Bacillus subtilis*, S.a.-*Staphylococcus aureus*, A.t.- *Agrobacterium tumifacian*, K.p.- *Klebsiella pneumonae*, R.p.- *Roultella pla*nticolla,P.a.-*Pseudomonas aeruginosa*.

Total activity = $\frac{\text{Extract per gram dried plant part}}{\text{MIC of extract}}$

III. Result

Antimicrobial potency of flavonoids (free and bound), alkaloids was assessed using various parameters and quantity of extracts per gram of plant material was also determined. In the present study six extracts were screened and all the extracts were found active against one or the other test pathogen. Most of the extracts showed broad spectrum antibacterial activity but the best activity was shown by alkaloids of fruits against *Klebsiella pneumonae* (IZ-31.16mm, MIC-0.039mg/ml,MBC-.0195mg/ml,TA=482ml/g),which is more than the standard streptomycin. Alkaloids of fruit also showed considerable activity against *Bacillus subtillis* (IZ-27.53mm, MIC-0.039 mg/ml, MBC-.0195 mg/ml, TA=482ml/g) and *Agrobacterium tumefaciens* (IZ-24.2mm, MIC -0.039, MBC-.0195 mg/ml ,TA=482ml/g) which was almost near to standard. Bound flavonoid extracts of fruits was most active against *Roultella planticola* (IZ-19.96mm, MIC-0.078 mg/ml, MBC-0.039 mg/ml,TA-0.0156 ml/g) and free flavonoid extract of fruit showed satisfactory activity against all of the tested pathogens except *Klebsiella pneumonae* but was very active against *Bacillus subtillis* (IZ-10.1mm,MIC= 0.625 mg/ml, MBC-0.312 mg/ml, TA=0.625 ml/g).Both leaf bound flavonoids (IZ-21.93mm,MIC-0.078 mg/ml, MBC-0.039mg/ml,TA=32.05ml/g).and alkaloids (IZ-21.73mm,MIC-0.078 mg/ml,MBC-0.039 mg/ml, TA=1743.5 ml/g) were most active against *Bacillus subtillis* whereas the free flavonoid extract of leaves was most active against *Roultella planticola* (IZ-20.03mm, MIC-0.078mg/ml, MBC-0.039 mg/ml, TA=25.64ml/g). Stem alkaloids showed considerable activity against all the tested pathogens but was most active against *Klebsiella pneumonae* (IZ-20.03, MIC-0.078 mg/ml, MBC-0.039 mg/ml,TA=679.4 ml/g). Bound flavonoid extracts of stem also showed satisfactory activity against *Pseudomonas aeruginosa* (IZ-12.53mm, MIC-0.312 mg/ml,

MBC-0.156 mg/ml, TA=12.82ml/g), *Staphyllococcus aureus* (IZ-10.56mm, MIC-0.625 mg/ml, MBC-0.312 mg/ml TA=6.4ml/g). Free flavonoids of stem showed marginal antibacterial activity against *Staphyllococcus aureus* and *Pseudomonas aeruginosa*. Among all the extracts, leaves bound flavonoids were found to be the most active as had shown activity against all of the test pathogens.Fruit alkaloid shown activity against *Klebsiella pneumonae, Bacillus subtillis, Agrobacterium tumefaciens* indicating their antibacterial efficacy but more investigation is required to establish it as antibiotic. MIC and MBC values were calculated for the extracts that had shown activity in Diffusion Assay and the range of MIC and MBC of the extracts recorded were 1.25-0.039mg/ml and 0.625-0.0195mg/ml respectively. In this investigation lowest MIC was 0.039 for *B. subtillis, K. pneumonae, Agrobacterium tumefaciens,* indicating the significant potency of the test extracts.MIC and MBC values were found similar for various extracts indicating their bactericidal potential. Quantity of extract obtained per gram of the plant parts and total activity (TA) was calculated and recorded (Table 3). Most of the extracts showed high value of (TA) against *Pseudomonas aeruginosa, Staphyllococcus aureus, Roultella planticola*

IV. Discussion

Continuous and urgent need to discover new plant based antimicrobial compounds due to increase in the number of new and reemerging infectious diseases (17).

Aegle marmelos has been previously studied for its antibacterial activities. Methanolic, Pet ether and chloroform extract of leaves had already been screened against *S. aureus, P. aeruginosa, K. pneumoniae, B. subtilis* and *E. coli,*(18).Mostly crude extracts were screened for whole plants parts without MIC/MBC and TA determination. Such studies could only indicate their antimicrobial potential but thesis not sufficient to establish them as antibiotic. The present screening was carried for flavonoids and alkaloids with determination of MIC, MBC and TA. Low values of MIC and MBC indicate the strong antimicrobial efficacy of the plant. Further phytochemical studies and deep investigation may establish them as novel antibiotics with less or no side effects.

V. Conclusion

From the result of screening of antibacterial activity of flavonoids and alkaloids of the fruit stem and leaves of *Aegle marmelos* used for study, alkaloids were exhibited best antibacterial activity. Among all the plant parts leaves were considered most effective because almost all the test extracts of leaves shows antibacterial activity compared to other extracts. Controls are used to cross check the activity of the extracts in which they dissolved for the antibacterial activity free flavonoids are dissolved in diethyl ether, bound flavonoids are dissolved in ethyl acetate and alkaloids are dissolved in ethanol. All the controls are inactive so it is concluded that the activity of extracts are purely due to extracted compounds and are not affected by the solvent in which they dissolved Traditional herbal medicine is preferred to grant the benefits of modern science and technology to serve further global needs. The drugs derived from herbs may have the possibility of using in medicine because of its good antibacterial activity. Further research in the focusing on the isolation of the individual compounds and finally subjecting to the clinical trials promises to open new path in the use of plants for therapeutic system.

VI. Abbreviations

g – Gram
ml – Milliliter
mm – Millimeter
C – Celcius
IZ – Inhibition zone
MIC- Minimum inhibitory concentration
ul – Microliter
MBC- Minimum bactericidal concentration

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