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ANTIBACTERIAL EFFICACY OF STEROIDS FROM AEGLE MARMELOS (LEAF, STEM AND FRUIT); AN IN VITRO APPROACH

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ABSTRACT The development of resistance of microbes against the commonly used antibiotics threatens the effective treatment and prevention of infections caused by bacteria, viruses, fungi and parasites. This drives the screening of medicinal plants for development of novel bioactive compounds advocating good efficacy against pathogenic microorganism having fewer side effects. The present study aims to investigate the *in vitro* antibacterial efficacy of steroids of different plant parts (viz leaves, stem and fruit) of *Aegle marmelos*. Among all the extracts steroids of leaves were most active because most of the bacteria found susceptible. However highest zone of inhibition was found against *R.planticola* (IZ 21.16±0.503 mm, AI 0.672, MIC 0.039mg/ml, MBC 0.078 mg/ml and TA 166.66 ml/g) shown by fruit extract followed by *B. subtilis* (IZ 19.26±0.230mm, AI 0.583, MIC 0.039 mg/ml, MBC 0.078mg/ml and TA 153.84 ml/g) against which stem extract was observed to be most active, Moreover *A. tumifaciens* was most resistant, none of the extracts showed activity against it. The low value of MIC indicates the strong bio efficacy of the plant extracts. The finding of the present investigation offer scientific evidence to support that the phytocompounds are promising in the development of alternative medicines.

KEYWORDS : Antibiotics, Antibacterial, Phytocompounds, MIC, MBC

INTRODUCTION:

The natural product formed inside plants are non-nutritive even non-essential for their survival and their normal physiology, but these metabolites can be of great importance to the mankind as they may be used as stimulant, hallucinogen, antimicrobial agents and medicine (Cowan, 1999). A number of interesting outcomes have been found with use of mixture of natural products to treat diseases, most notably the synergistic effects and poly pharmacological application of plant extracts (Gibbons, 2003). The growing incidence of infectious diseases due to development of increasing antibiotic resistant pathogens justify attempt to search for new antimicrobial agents as well as compounds that are capable of inhibiting resistance bacterial mechanism to classical drugs. The continuous use of same class and type of antimicrobial compounds seems to contribute to a significant increase in resistant bacteria (Dias *et al.*, 2014). Infection with antibiotic resistant bacteria negatively impact on public health, due to increased incidence of treatment failure and severity of diseases (Samy *et al.*, 2013). Development of resistant bacteria due to chromosomal mutation is more commonly associated with the horizontal transfer of resistant determinants borne on mobile genetic elements (Walsh and Fanning, 2008) against indiscriminate use common synthetic drugs. This 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).So the use of and search for drugs and dietary supplements has been accelerated. Medicinal plants thus offer significant potential for the development of novel antimicrobial therapies and adjunct treatments (Mahady, 2005).

A known set of six bacteria was selected to examine antibacterial activity of steroids from different parts of *Aegle marmelos*. *S. aureus* is a commensal found on skin as well as in the nose and throat of human. It causes a range of infections, from minor skin infections to abscesses, endocarditis and sepsis, cause of food poising induced by heat resistant enterotoxin A and it is one of the leading causes of nosocomial infections (Lowy, 1998). *Bacillus subtilis* is a rod shaped bacteria that are Gram-positive (Perez, 2000). *Bacillus subtilis* contains catalase, and enzyme that is responsible in the decomposition of hydrogen peroxide to water and oxygen, and superoxide dismutase, an enzyme that catalyzes the breakdown of superoxide into oxygen and hydrogen peroxide (Bandow, 2002). The *Raoultella planticola* is an environmental organism which causes infection such as bacteraemia (Hu AY *et al.*, 2012), soft tissue infection (O'Connell *et al* 2010), pancreatitis (Alves *et al* 2007)] and urinary tract infection (Olson *et al.*, 2013). *Klebsiella pneumoniae* is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium. Although found in the normal flora of the mouth, skin and intestines (Ryan and Ray 2004). It can cause destructive changes to human lungs if aspirated. It is clinically the most significant member of the *Klebsiella* genus of Enterobacteriaceae. *Agrobacterium* is a serious pathogen of walnuts, grape vines, and stone fruit. *Pseudomonas aeruginosa* is an opportunistic bacterium migrates from its natural environment, colonizing and infecting a wide range of organisms (Pirnay *et al.*, 2011, Silby *et al.*, 2009) and it is hard to treat due to its ability of acquiring resistance against multiple classes of antibiotics

MATERIALS AND METHODS

Plant material of different parts (leaf, stem and fruit) of *Aegle marmelos* were collected from Jaipur, (India) voucher specimens of the plants have been deposited at the Herbarium, Department of Botany, University of Rajasthan.

Extraction of Steroids

Each of the dried materials was finely powered/chopped and defatted in a soxhlet for 24 hrs on a water bath. Each of the mixture was filtered; the residual mass was refluxed with 15% ethanolic HCL for 4 hr and the extract filtered. The filtrates were then separated with ethyl acetate fractions was brought to neutrality by repeated washings with distilled water and passed over sodium sulphate so as to remove traces of moisture. Neutral ethyl acetate fraction of all the samples was then dried in vacuo separately, reconstituted in chloroform, filtered, dried and weighted before subjecting it to analysis (Tomita *et al.*, 1970).

Test Pathogens

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

Antibacterial screening of extracts:

For antibacterial screening of extracts Disc diffusion assay (DDA) (Andrews 2001 and Gould and Bowie 1952) was performed. MH agar (for bacteria) base plates were seeded

with the standard size of bacterial suspension (1×108 CFU/ml). Sterile filter paper discs (6mm in diameter) were impregnated with 100 l of each of the extract (10mg/ml concentration) to give a final concentration of 1mg/disc, left to dry in vaccuo to remove residual solvent, which might interfere with the determination. Extract discs were then placed on the seeded agar plates. Each extract was tested in triplicate along with standard streptomycin (1mg/disc) for bacteria. The plates were kept at 4°C for 1h for diffusion of extract, thereafter were incubated at 37±2°C for 24 hours. Zone of inhibition (IZ) or depressed growth of microorganisms was measured and the 'Activity Index' (AI) for each extract was calculated. The experiment was performed three times to minimize the error and the mean values were recorded for final estimation of the resultant values.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal (MBC)

Minimum inhibitory concentration (MIC) was determined for plant extract showing antimicrobial activity against test pathogens in disc diffusion assay (Basri and Fan 2005). Broth micro dilution method was followed for determination of MIC values. Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make 10mg/ml final concentration and then was added to broth media of 96-wells of microtiter plates using two fold serial dilution. Thereafter 100μl inoculum of standard size was added to each well. Bacterial suspensions were used as negative control, while broth containing standard drug was used as positive control. The microtiter plates were incubated at 37±2°C for 24h for bacteria, 27±2°C for 48h for yeast and 27±2°C for 5-7 days for fungi. Each extract was assayed in duplicate and each time two sets of microtiter plates were prepared, one was kept for incubation while another set was kept at 4°C for comparing the turbidity in the wells of microtiter plate. The MIC values were taken as the lowest concentration of the extract in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms. The minimum bacterial concentration (MBC) was determined by sub culturing 50μl from each well showing no apparent growth. Least concentration of extract showing no visible growth on sub culturing was taken as MBC.

Total activity (TA)

Total activity is the volume at 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 (Eloff, 2000).

Statistical analysis:

Statistical analysis, in form of, mean value and standard deviation were calculated for the test bacteria. Data were analyzed by one way ANNOVA and values were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The present study carried an exhaustive analysis to identify metabolite from medicinal plants and its broad applications towards pathogenic microbes. The finding of new bioactive compounds from medicinal plants is a never ending process to meet the perpetual demand for novel bio-molecules with antimicrobial properties to fight against human and plant pathogens. Here steroids from different parts of plants were examine for their antibacterial efficacy (Table 1-3) The results of the present study revealed that the antimicrobial activity of extracts of *Aegle marmelos* species, varied greatly depending upon the different parts of the plants. Among all the extracts steroids of leaves were most active because most of bacteria found susceptible. Highest zone of inhibition was found against *R.planticola* (IZ 21.16±0.503 mm, AI 0.672, MIC 0.039mg/ml, MBC 0.078 mg/ml and TA 166.66 ml/g) shown by fruit extract followed by *B. subtilis* (IZ 19.26±0.230mm, AI 0.583, MIC 0.039 mg/ml, MBC 0.078mg/ml and TA 153.84 ml/g) against which stem extract was observed to be most active, *P. aeruginosa* (IZ 12.7±0.692 mm, AI 0.508, MIC 0.156mg/ml, MBC 0.312 mg/ml and TA 41.66 ml/g). *A. tumifaciens* was most resistant, none of the extracts showed activity against it. Of the Results confirmed that the antibacterial activity was found both extracts from different parts and organism dependent. As far as our literature survey could as certainly, we could reach there are few reports on the antibacterial activity were found but mostly the crude extracts without MIC, MBC and TA determination. The crude extracts in different solvents (Water, Methanol and Pet ether) of *Aegle marmelos* showing antimicrobial activity were reported by Jaishree Sharma and Padma Kumar (2017) and Devi *et al* (2020) Such investigation indicate only there antimicrobial potential but are not helpful in establishing them as antibiotics hence can't replace the existing one. In the present investigation IZ, AI, MIC, MBC and TA of each extract were determined. The low value of MIC indicates the strong bio efficacy of the plant extracts. The finding of the present investigation offer a scientific evidence to support that the phytocompounds are promising in the development of alternative medicines.

CONCLUSION

A.marmelos is an important medicinal plants and rich source of bioactive compounds. Little work has been done on their biological activity. Hence extensive investigation is needed to exploit bioactive compound from the plant for medicinal purpose. From the above observations it was concluded that results of the present study reveals that steroids from different parts (leaves, stem and fruit) of *A. marmelos* shows antibacterial properties which might be helpful in preventing the development of various disease and can be used as alternative system of medicine.

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Table I: Antibacterial activity of steroids from different parts

Plant part/ Pathogens	leaf		Stem		Fruit	
	IZ	AI	IZ	AI	IZ	AI
B.subtillis	12.06±0.115	0.402	19.26±0.230	0.583	-	-
S.aureus	6.9±0.1	0.276	-	-	7.96±0.115	0.306
A.tumifaciens	-	-	-	-	-	-
R.planticola	-	-	11.16±0.057	0.429	21.16±0.503	0.672
K.pneumonae	7.03±0.0577	0.242	7.4±0.360	0.264	-	-
P.aureginosa	11.1±0.265	0.444	-	-	12.7±0.692	0.508

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.

Table II: MIC and MBC of active extracts

Plant part/ Pathogens	leaf		Stem		Fruit	
	MIC	MBC	MIC	MBC	MIC	MBC
B.subtillis	0.312	0.625	0.039	0.078		
S.aureus	0.625	1.25			0.625	1.25
A.tumifacians	-	-			-	-
R.planticola	-	-	0.312	0.625	0.039	0.078
K.pneumonae	0.625	1.25	0.625	1.25	-	-
P.aureginosa	0.312	0.625	-	-	0.156	0.312

MIC- Minimum inhibitory concentration, MBC- Minimum bactericidal concentration, MFC- Minimum fungicidal concentration

Table III: Total activity of active extracts

Extracts	Quantity of extract g/dry weight mg/ml	Total activity mg/g					
		Bs	Sa	At	Rp	Kp	Pa
Leaf	2.5	8.01	4	-	-	4	8.01
Stem	6	153.84	-	-	19.23	9.6	-
Fruit	6.5	-	10.04	-	166.66	-	41.66

B.s.- Bacillus subtilis, S.a.- Staphylococcus aureus, A.t.- Agrobacterium tumifacian, K.p.- Klebsiella pneumonae, R.p.- Roultella planticolla, P.a.-Pseudomonas aeruginosa, A.f.- Aspergillus flavus, T.m.- Trichophyton mentegrophyte
Total activity = Extract per gram dried plant part/MIC of extract

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