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RESEARCH ARTICLE

ANTI BACTERIAL AND BIOFILM INHIBITORY ACTIVITIES OF AEGLE *MARMELOS* METHANOL LEAF EXTRACT

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Abstract

The Aegle *marmelos* commonly known as BAEL belongs to family Rutaceae plays a role in traditional culture and medication from ancient periods. This plant lacks sufficient evidences regarding the values and components it has. Therefore, we framed out our studies to evaluate the phytochemical analysis, antibacterial activity, antibiofilm activity. These studies are evaluated using different solvents like methanol, acetone, chloroform, toluene leaf extracts of Aegle *marmelos*. We evaluated the potency of different solvents leaf extracts using Agar well diffusion method. Antibacterial activity was also evaluated using ELISA plate assay. The potency of different solvents extracts to inhibit biofilm of selected microbial strains. In accordance to results, the leaf extracts revealed the presence of several biologically active phytochemicals with highest quantities of carbohydrates, phenols, alkaloids, flavonoids, tannins, saponins, steroids, aminoacids etc. The antibacterial activity was found significant against microbial strains of both gram positive and gram negative bacteria. These strains showed susceptibility nature towards the different solvents extracts with zone of inhibitions (mm). On the other hand, the inhibition of biofilm was also significant at all tested concentrations. The biofilm inhibition of microbial strains was found significant at 1 XMIC, 2 XMIC, 3 XMIC. Based on our studies here we conclude that the different solvents leaf extracts possessed inhibitory activity against selected human pathogenic organisms.

Introduction:-

Aegle marmelos belonging to the family Rutaceae is well known Indian medicinal plant commonly called as Bael tree [1-2]. It has been reported that the fruit of this plant is used as astringent and also in the treatment of digestive problems, diarrhoea and dysentery [3]. The leaves are extensively used in the treatment of asthma, inflammation, febrifuge, hypoglycaemia [3]. It has been also reported that this plant is widely used in the treatment of diabetes, antioxidant, anticancer etc [4-6].

The emergence of multidrug resistance pathogens to the contemporary antibiotics made difficulties in the treatment against bacterial infections. In addition, the cost effective and hazardous effects of synthetic drugs, a continuous effort put forward to discover the new and drugs with cost affordable and safe. In this concern, among the various

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sources of drug discovery, medicinal plants have been given immense importance because of their diverse chemical structures with wide range of therapeutic action. The ability of producing Biofilm is a greater resistance strategy shown by the bacteria towards the several antibiotics action. Biofilm is a complex matrix composed by extra cellular polymeric substances (EPS) [7]. The EPS form a outer layering of the bacterial cells, hence the bacteria can escape from environmental stress and host defense mechanism and as well as different types of antibiotic treatment [8].

In context to medicinal value of *Aegle marmelos*, the current study is aimed to evaluate the antibacterial activity and biofilm inhibition ability of leaf extracts of *Aegle marmelos.*

Material and Methods:-

Collection and identification of plant material

A survey was conducted in the nearest villages of Warangal rural and the parts of *Aegle marmelos* plant was collected from Oglapur village. The plant was authenticated by Rtd. Professor, V. Thirupathaiah, Department of Botany, Kakatiya University, Warangal Urban, Telangana.

Extract preparation

The fresh leaves of *Aegle marmelos* are properly washed under running water for 1 hour. The rinsed leaves were shade dried and made fine powder. 500 g of powdered is extracted in soxhlet using various solvents like Toluene, Chloroform, Acetone and Methanol. The solvent was evaporated by evaporator to collect crude extracts.

Phytochemical Analysis

Phytochemical analysis of the extracts was carried out using standard procedure described by Trease and Evans (1996) [9].

Molisch's assay for carbohydrates

To 2ml of *Aegle marmelos* leaf crude extracts, 2-3 drops of alpha naphthalene solution are added and shaken for 2-3min. 1ml of con. H_2SO_4 Sulphuric acid was gently added from the sides of the test tube. The samples were observed for deep violet color at the junction of two different layers, indicating the presence of carbohydrates in the tested samples.

Fehling's assay for reducing sugars

1ml of Fehling's A and B solutions are mixed with 2 ml of *Aegle marmelos* leaf crude extracts and incubated in the water bath for 10 min. The samples were observed for yellow color formation. The brick-red precipitate confirms the presence of reducing sugars in tested crude extracts.

Screening for phenols

1ml of *Aegle marmelos* leaf crude extracts are mixed with few drops of $FeCl_3$. The samples are observed for intense color to confirm the presence of phenols.

Ninhydrin assay for amino acids

To 3 ml of *Aegle marmelos* leaf crude extracts, add 3 drops of 5% v/w lead acetate solution and incubate in the water bath for about 10 min. The samples for change in color to purple or blue indicate the amino acid present in the crude extracts.

Biuret assay for proteins

3ml of *Aegle marmelos* leaf crude extracts are mixed with 2ml of NaOH and then add few drops of 1% $CuSO_4$ solution along the sides of the test tube. The samples were observed for violet or pink color, which confirms the crude extracts' presence.

Baljet's assay for glycosides

2 ml of *Aegle marmelos* leaf crude extracts are mixed with 2 ml of sodium picrate solution and observed for orange color, indicating the presence of glycosides in the tested extracts.

Screening for tannins
The equal amounts of *Aegle marmelos* leaf crude extracts and 1% of potassium dichromate solution are incubated at room temperature for 2-3min. The samples were observed for voluminous precipitate, which confirms the presence of tannins.

Nacl test
1ml of *Aegle marmelos* leaf crude extracts is mixed with a small amount of distilled water and followed by the addition of a pinch of NaCl. The samples were maintained at room temperature for 3-5min for observation precipitation, indicating tannins within tested extracts.

Screening for Flavonoids
Alkaline Reagent assay
To 2ml of *Aegle marmelos* leaf crude extracts, add a few drops of 1 N NaOH. The samples are observed for yellow color and turn colorless. The addition of dilute acid indicates the presence of flavonoids in the extracts.

Lead acetate assay
To 2ml of *Aegle marmelos* leaf crude extracts, add a few drops of lead acetate solution. The samples are observed for yellow color precipitation, which indicates the presence of flavonoids in the crude extracts.

Screening for Alkaloids
Mayer's assay
1ml of *Aegle marmelos* leaf crude extracts was mixed with 1ml of Mayer's reagent. The samples are maintained at room temperature and observed for reddish-brown precipitate, which confirms the alkaloids in the crude extracts.

Wagner's assay
1ml of *Aegle marmelos* leaf crude extracts are mixed with 1ml of Wagner's reagent. The samples are maintained at room temperature and observed for reddish-brown precipitate, which confirms the alkaloids in the crude extracts.

Dragendroff's assay
1ml of *Aegle marmelos* leaf crude extracts was mixed with 1ml of Dragendroff's reagent. The samples are maintained at room temperature and observed for reddish-brown precipitate, which confirms the alkaloids in the crude extracts.

Hager's assay
1ml of *Aegle marmelos* leaf crude extracts was mixed with 1ml of Hager's reagent. The samples were maintained at room temperature and observed for yellow-colored precipitate, which confirms the alkaloids in the crude extracts.

Screening of Saponins
Froth assay
Aegle marmelos leaf crude extracts are mixed with 20ml of distilled water and vigorously shaken for 15 minutes. The appearance of a 1 cm layer of froth confirms the presence of saponin in the crude extracts.

Foam Test
Approximately 0.5gm of *Aegle marmelos* leaf crude extracts is vigorously shaken with 2 ml of distilled water. If foam produced persists for more than 10 minutes, it indicates saponin presence in the tested crude extracts.

Antibacterial evaluation
Procuring bacterial strains
Microbial strains for antibacterial evaluation were obtained from Department of Microbiology, Chaitanya Deemed to be University Hanamkonda, Warangal, Telangana, India. Three Gram positive strains, *Bacillus cereus* (ATCC 14579), *Bacillus subtilis* (ATCC 6633), Methicillin-resistant *Staphylococcus aureus* (MRSA, NCTC 13616) and three Gram negative strains, *Klebsiella pneumoniae* (ATCC 43816), *Escherichia coli* (ATCC 8739), *Proteus vulgaris* (ATCC 13315) are used in the evaluation. The test organisms are stored on LURIA-BERTANI agar plates.

Antibacterial assay
The antibacterial of *Aegle marmelos* leaf extracts was evaluated by agar well diffusion method [10]. Briefly, 24 h old bacterial culture was transferred on to the media plates. Using 6mm sterile cork borer, wells are created on each media plate. The wells are filled with two different concentrations (75, 100 µg/well) of *Aegle marmelos* leaf extracts. The results compared with known standard Chloramphenicol.

Minimum inhibitory concentration (MIC) assay using plant extracts
The MIC off *Aegle marmelos* leaf extracts was determined by broth dilution technique [11]. Briefly, Fresh culture of experimental strains was diluted with 100 fold nutrient broth medium. *Aegle marmelos* leaf extract stock solution was prepared so that the concentrations is increased (1.25, 2.5, 5, 10, 20, 40 ml that contain 6.25, 12.5, 25, 50, 100, 200 µg of the extract). The experimental strains are inoculated into the *Aegle marmelos* leaf extracts as mentioned above concentrations and maintained at room temperature. The tubes with lowest or negligible visible growth of the experimental strains at a particular plant concentration are recorded as MIC.

Anti-biofilm Assay
Based on the results of antibacterial activity, the methanol and acetone extracts are further evaluated for biofilm assay.

Growing a Biofilm
The *Aegle marmelos* leaf extracts were determined for their attributed inhibitory or destruction property of the biofilm development by the experimental strains [12]. A 100µl culture of each test bacterial strains (information presented in 4.2.2) was transferred 96-well plate containing nutrient broth. The plates are maintained at room temperature for 24 and 48h for the development of biofilm. Following that, 100µl aliquots of *Aegle marmelos* leaf extracts at 1 × MIC, 2 × MIC, 4 × MIC are added to the wells and maintained at 37°C for 24h. Chloramphenicol and DMSO are used as positive and negative control respectively. The crystal violet (CV) staining assay described by Djordjevic *et al*, (2002) [13] was used to determine the percentage of biofilm inhibition by the tested extracts.

Crystal violet staining assay
Staining the Biofilm
The assay was carried out according to the method previously described [13]. Briefly, the cells were dumped by shaking and turning the plate. Gently, wash the microtitre plates repeatedly 4-5 times with double distilled and sterilized water. The plates are dried at 60°C for 35-45min using hot air oven. During this step, the unattached cells and utilized media will wash away and prevents the background staining of the well in further steps. The wells are filled with 125µl of 0.1% crystal violet and maintained at 37 ^{0}C for 15-20 min. The wells are then washed with sterile double distilled water to remove the excess dye. Following, the plates are observed for biofilm on the side walls of the wells. The plates are dried at room temperature overnight and then subjected to quantitative assessment.

Quantitative assessment of biofilm
Quantitative determination of biofilm present in the each well is initiated by the addition of 125µl of 33% of acetic acid. The plate is maintained at 37 ^{0}C for 15-20 min. following, that 125µl of the solubilized solution is transferred from each well on to a fresh sterile plate. Using microplate reader the absorbance is measured at 590nm. The percentage of biofilm inhibition is determined using the following equation [12].

$$\text{Percentage (\%) of inhibition} = \frac{\text{OD Negative control-OD Experimental X 100}}{\text{OD of Negative control}}$$

Statistical analysis
The results of antibacterial activity and anti-biofilm were shown as Mean±SD. The student '*t*' was performed to calculate the probability value. The $P<0.05$ is considered as significant.

Results:-

Phytochemical Analysis
The tests were conducted to evaluate the different types of phytochemicals were given positive. All the extracts were showed positive to Phenols, Carbohydrates, Reducing sugars, Proteins, Amino acids, Glycosides, Steroids, Flavonoids, Alkaloids, Tannins, Gums, Saponins, Volatile oils. The test were conducted to evaluate Coumarins was given negative. The results are shown in table 1.0.

Table 1.0:- Phytochemical analysis of *Aegle marmelos* leaf extracts.

Phytochemicals	ACT*	CHL*	TOL*	MET*
Phenols	+	+	+	+
Coumarins	-	-	-	-
Carbohydrates	+	+	+	+
Amino acids	+	+	+	+
Glycosides	+	+	+	+
Steroids	+	+	+	+
Flavonoids	+	+	+	+
Alkaloids	+	+	+	+
Tannins	+	+	+	+
Saponins	+	+	+	+
Gums	+	+	+	+
Oils	+	+	+	+
Reducing sugars	+	+	+	+
Proteins	+	+	+	+
***ACT-Acetone, CHL-Chloroform, TOL-Toluene, MET-Methanol				

Antibacterial evaluation

The present study was carried out to evaluate the antibacterial activity of Aegle *marmelos* leaf extract. The results are found in concentration dependent manner. Among the tested extracts, methanol extract pronounced paramount antibacterial activity against selected multidrug resistant pathogenic bacterial strains. The results are represented in Mean ±_SD. The methanol extract is highly active against E. *coli* with 27.3mm. Following to E. *coli*, bacterial strains P. *vulgaris*, B. *subtilis*, B. *cereus* were also found highly susceptible towards the methanol extract with the zone of inhibition 25.4, 23.9, 22.1 mm respectively. Next to methanol extract, acetone extract exhibited significant activity against tested bacterial strains. E. *coli* was found more susceptible towards acetone extract with zone of inhibition 21.2 mm. The chloroform and toluene extracts were exhibited moderate activity against the bacterial strains tested (Table 1.1). The Minimum Inhibitory Concentration (MIC) of methanol against E. *coli*, P. *vulgaris*, B. *subtilis*, B. *cereus* were found significant at 3.2, 4.5, 5.8, 6.0 µg/ml respectively. Whereas, the MIC values of acetone extract recorded against E. *coli*, P. *vulgaris*, B. *subtilis*, B. *cereus* are 4.4,6.3,5.5,6.0 µg/ml respectively. On the other hand toluene and chloroform extracts are showed average activity against K. *pneumonia*, B. *subtilis*, B. *cereus* with MIC>100 µg/ml. The results were compared with reference drug Ciprofloxacin (Table 1.2).

Table 1.1:- Antibacterial activity of *Aegle marmelos* leaf extracts.

PE/Std	Conc (µg/mL)	Zone of Inhibitions (mm)					
		EC	PV	KP	BS	BC	MRSA
Toluene	25	3.6±0.5	1.5±0.1	5.4±0.5	5.3±0.01	4.6±0.07	2.2±0.5
	50	4.2±1.8	2.3±2.5	7.1±1.2	8.8±1.3	7.5±0.1	2.9±0.2
	100	5.8±0.07	3.3±1.5	9.8±1.0	13.9±1.0	12.2±0.5	3.5±1.1
Chloroform	25	2.0±0.5	1.5±0.1	3.2±1.5	3.2±0.03	3.0±1.5	3.0±0.5
	50	3.3±1.8	2.2±2.5	5.8±0.1	5.4±1.1	5.1±2.2	4.9±2.0
	100	6.1±0.07	3.9±1.5	7.7±0.5	7.7±0.01	6.9±0.01	5.8±1.1
Acetone	25	7.1±0.5	5.5±0.1	6.1±0.03	6.6±1.1	5.2±1.2	6.1±0.5
	50	13.8±1.8	8.9±2.5	9.5±1.2	10.1±1.5	9.6±0.1	11.3±2.0
	100	21.2±0.07*	15.3±1.5	14.7±0.5	15.5±0.05	17.3±0.5*	20.7±1.1*
Methanol	25	9.5±0.5	8.1±0.1	7.3±1.7	8.2±0.3	7.3±0.5	8.8±0.5
	50	16.4±1.8	14.8±2.5	12.2±0.09	13.6±0.01	11.5±1.1	15.2±2.0
	100	27.3±0.07*	25.4±1.5*	19.6±1.5	23.9±1.2*	22.1±0.01*	26.8±1.8*
Ciproflaxacin	10	35.3±0.07	32.6±1.5	29.5±1.5	38.3±0.1	37.1±0.5	31.9±1.1
EC-E.*coli*, PV-P. *vulgaris*, KP- K. *pneumonia*, BS-B.*subtilis*, BC-B.*cereus*, MRSA-Methicillin *Staphylococcus aureus*, Values are expressed as Mean ± SD. n=4, *P<0.05							

Table 2.2:- Minimum Inhibitory Concentrations of *Aegle marmelos* leaf extracts.

BACTERIA	Plant Extracts				
	AC	CHL	TOL	MET	CIP
E.*coli*	4.4	44.3	38.4	3.2	2.9
P.*vulgaris*	6.3	69.1	59.7	4.5	3.9
K. *pneumonia*	>10	>100	>100	>10	3.2
B. *subtilis*	5.5	>100	>100	5.8	2.5
B. *cereus*	7.2	78.3	105.9	>100	3.1
MRSA	>100	>150	>150	>100	4.6

Antibiofilm activity

Based on the significant antibacterial activity of methanol and acetone extracts against tested bacterial strains, there extracts were selected for further evaluation of antibiofilm formation activity against the tested bacterial strains. The biofilm studies were carried out using three different concentrations 1 X MIC, 2 X MIC, 3 X MIC. According to our results, the methanol extract showed 48%, 62% and 84% of biofilm inhibition of E. *coli* at 1 X MIC, 2 X MIC, 3 X MIC respectively (Fig. 1). The methanol extract is also highly active against P. *vulgaris*, B. *subtilis* and B. *cereus* with biofilm inhibition percentage 42%, 55%, 70% recorded at 3 X MIC respectively. On the other hand acetone extract also exhibited good biofilm inhibition property against tested bacterial strains. The highest biofilm inhibition percentages 55% and 69% recorded against E.*coli* at 2 X MIC and 3 X MIC respectively (Fig.2). The biofilm inhibition at 50 and 90% by the methanol and acetone extracts was shown in table 1.3.

Table1.3:- Biofilm inhibition activity of *Aegle marmelos* leaf extracts.

Aegle marmelos **Leaf**				
Bacterial strains	Methanol extract		Acetone extract	
	BIC 50%	BIC 90%	BIC 50%	BIC 90%
MRSA	7.9	13.3	11.3	20.7
B. *subtilis*	11.5	19.2	15.9	30.5
B. *cereus*	12.4	21.1	14.4	27.6
K. *pneumonia*	15.7	27.6	18.8	33.9
E. *coli*	6.0	12.1	7.7	15.9
P.*vulgaris*	10.1	18.3	13.9	25.1

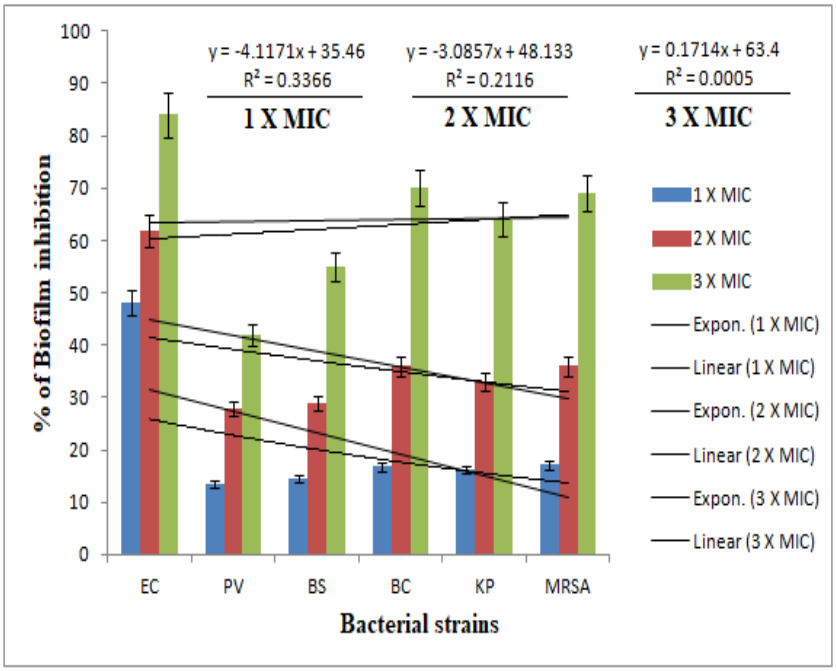

Figure 1:- Percentage of Biofilm inhibition by the Aegle marmelos leaf Methanol extract.

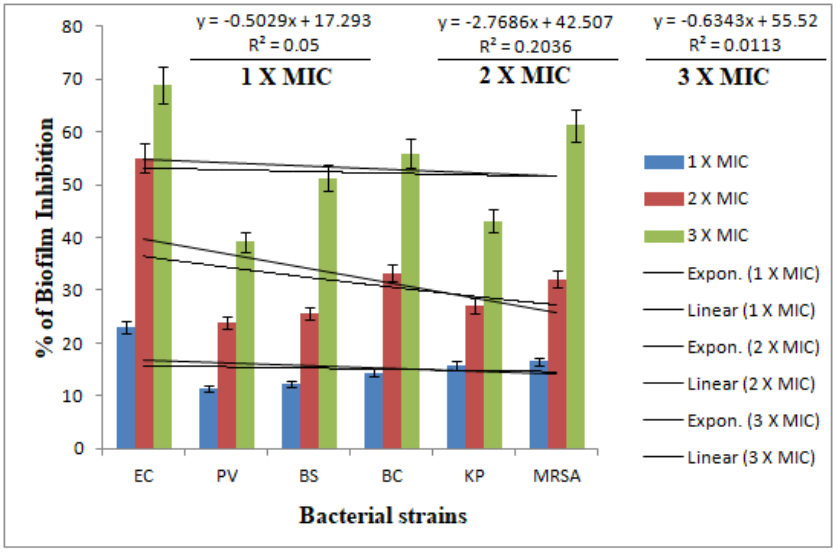

Figure 2:- Percentage of Biofilm inhibition by the *Aegle marmelos* leaf Methanol extract.

Discussion:-

The preliminary qualitative tests conducted to different solvent extracts of *Aegle marmelos* revealed to possess tannins, saponins, alkaloids, gums, steroids, carbohydrates, amino acids, proteins, glycosides. The test conducted for coumarins was given negative. The antibacterial activity of *Aegle marmelos* solvent leaf extracts was found concentration dependent. The present study supports the previously published reports that the antibacterial activity is directly proportional to the concentration of plant extracts [14]. Among, methanol, toluene, acetone, chloroform extracts, the methanol and acetone extracts were pronounced significant activity ($P<0.05$) against all bacterial strains tested. Whereas, toluene and chloroform extracts exhibited moderate activity. Comparing to methanol and acetone extracts, the methanol extract equally inhibited the tested Gram positive and negative bacteria used in the work. Whereas, the acetone extract showed its activity slightly high against Gram positive bacteria comparing to Gram negative bacteria. With reference to our results the susceptibility nature of E. *coli* was found high towards methanol and acetone extracts ($P<0.05$) with zone of inhibition 27.3 and 21.2 mm respectively found significant. It is evident that E. *coli* is highly sensitive to methanol leaf extract [15-17]. Following, MRSA-Methicillin resistant Staphylococcus *aureus* with zone of inhibitions 26.8 and 20.7 mm also found more susceptible towards methanol and acetone extracts respectively ($P<0.05$). In addition, the methanol extract was significant ($P<0.05$) against P. *vulgaris*, B. *subtilis*, B. *cereus* with zone 25.4, 23.9, 22.1 mm respectively. Whereas, the acetone extract exhibited average activity against P. *vulgaris*, B. *subtilis*, B. *cereus* with zone of inhibitions 15.3, 15.5, 17.3 mm respectively. The methanol extract may adopt one of the underlying antibacterial mechanisms is decreasing the pH of cytoplasm and as well as bacterial membrane stability especially against E.*coli*, MRSA and Bacillus *subtilis* and Bacillus *cereus* [18]. On the other hand, the toluene and chloroform extracts showed poor activity. The grater attributed activity of methanol and acetone might probably due to secondary metabolites such as tannins, alkaloids, flavonoids, saponins, steroids present in the *Aegle marmelos* leaf extract that involve in different antibacterial mechanisms *viz.*, restricting the pathway of cell wall formation, dissolution of the cell wall by which the leakage of enzymes and proteins, weakening the cell wall tissue and subsequent increase of permeability of antimicrobial components such as flavonoids and alkaloids etc. Due to Covid-19 lock down and the time concern we did not conduct the quantitative analysis of the above mentioned phytochemicals, which could act as major discussion point of anti bacterial activity variations of the toluene, chloroform, acetone and methanol extracts. Even though, the antibacterial activity of the parts of this plant is well known, the emergence of drug resistant bacterial strains and as well side effects of synthetic drugs made us to focus once again in to well known medicinal plant research for finding the new principles to overcome the various resistant strategies shown by the bacterial species.

To the best of our knowledge, there are no any literature on anti biofim formation activity of *Aegle marmelos* plant extracts or isolated compounds have been was evaluated so far. Therefore our work could become the first report. The inhibition of biofilm formation by methanol and acetone extracts was determined three different concentrations with reference to Minimum Inhibitory Concentration (Mentioned in the method). Among the bacterial resistant strategies, the biofilm formation is considered as a counter mechanism shown by bacteria towards antibiotics [19].

This biofilm formation helps the bacteria strongly to attach and survive in biotic and as well abiotic circumstances [20]. The percentage of biofilm inhibition was found concentration dependent. Mainly methanol extract exhibited significant biofilm inhibition activity against all the bacterial strains. The biofilm inhibition concentration 50% and 90% of the methanol extract against E. *coli* is recorded at 6.0 and 12.1 µg/mL respectively. Following, the BIC 50 and 90% of MRSA was noted at 7.9 and 13.3 µg/mL respectively. Whereas, the inhibition of biofilm formation by K. *pneumonia*, Bacillus sps, was found slightly lower. On the other hand, the biofilm inhibition activity by the acetone extract was found more difficult comparing to methanol extract. In exception to E.*coli* the other bacterial strains were prevented the mechanism of biofilm inhibition. The BIC 50 and 90% of acetone extract against E.*coli* were noted are 7.7 and 15.9 µg/mL respectively. The anti-biofilim property exhibited by the both extracts is might be due to the inhibition of Quorum sensing molecules, especially acyl homoserine lactones (AHLs) by the poly phenolic contents such as flavonoids, alkaloids etc [21].

The antibacterial activity and anti biofilm activity of plant extracts are not enough to discover a drug against any pathogenic organism. Such studies require a proper documentation of further screening process such as, isolation of actual principle agent that responsible for activity, further conformational study with isolated compounds, bioavailability studies, dose fixation studies etc., are necessarily need to be evaluated for its use as medicine. In future prospects, we suppose to extend our research to fulfill the above mentioned areas of work.

Conclusion:-

Based on the data of antibacterial and antibiofilm activity, the methanol and acetone extracts exhibited significant activity comparing to toluene and chloroform extracts. E. *coli* and Methicillin resistant Staphylococcus *aureus* are found more susceptible comparing to other bacterial strains.

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