Assessment of killing kinetics assay and bactericidal mechanism of crude methanolic bark extract of *Casuarina equisetifolia*

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Abstract: *Casuarina equisetifolia L.* is an important medicinal plant widely used to treat various diseases particularly ulcers, diabetes, cough, diarrhea and many infectious and skin diseases. The aim of this research study was to examine the killing mechanism and killing kinetics assay of methanolic bark extract of *C. equisetifolia* against some highly resistant human pathogens. The comparison on antibacterial activity of extract was firstly done with six different well reputed antibiotics using disk diffusion method. The broth dilution method was used to measure the MIC and MBC values. The mechanism of killing was identified by scanning electron microscopy (SEM) technique. Results showed that higher inhibitory zones were produced by methanolic plant extract than that of some tested antibiotics. The lower MIC and MBC values indicated the antibacterial potency of plant extract. The extract of *C. equisetifolia* produced a more drop in optical density of *S. aureus, MRSA B. subtilis and S. epidermidis* up to 12 hrs. The complete destruction of the cell membrane of *MRSA* was observed after 12 h treatment with plant extract. It is concluded that crude bark extract of *C. equisetifolia* is potent antimicrobial agent and produced both bacteriostatic and bactericidal effects. Its killing time was extremely faster especially against *MRSA*. The cell membrane rapturing is a suggested killing mechanism of plant extract.

Keywords: Antimicrobial activity, *Casuarina equisetifolia*, killing mechanism, killing kinetic assay, Methicillin resistant *Staphylococcus aureus*.

INTRODUCTION

Antimicrobial resistance is renowned as one of the greatest burden to human health worldwide (Walker *et al.*, 2009). One of the widespread factors linked with the antibiotic resistance is consumption behavior of the global population. Improper or injudicious exercise of these agents in a variety of disciplines like medicine, dentistry, veterinary medicine, food industry and agriculture may be considered to play a task in coming out of multi-resistant bacterial strains (Epstein *et al.*, 2000). Therefore global alarm of antibiotic resistance is a big dare for current medicine, and it seems to return public health to the "Pre-antibiotic era" where no successful or appropriate treatment against bacterial infections was accessible (Cars *et al.*, 2008). High expenditure of manufacturing of synthetic compounds, increasing resistance towards presently used antimicrobials along with many side effects, call for an action to reduce this problem. Plants are well known for antimicrobial activities. In the battle of microbial infections, plants are considered as strong tool due to presence of anti-infective agents such as emetine, quinine, berberine, tannins, terpenoids, alkaloids and flavonoids (Cowan 1999).

Casuarina equisetifolia L. (*Casuarinaceae*) is a beautiful tree, with needle shaped branch lets and with dropping branches. Different parts of this plant are used such as bark reported as anti- acne as well as to treat diarrhea, dysentery, lotion for swelling headache and fever, antimicrobial and anticancer (Essien *et al.*, 2016, Gumgumjee and Hajar 2012, Shafiq *et al.*, 2014). The knowledge regarding its bacterial killing kinetics and killing mechanism against different pathogenic strains is insufficient. In order to promote *C. equisetifolia*as a bacteriostatic and bactericidal plant, pathogenic strains of different microorganisms were kept for observation to determine its antimicrobial activity. Therefore, this study was planned to investigate the antibacterial actions of bark extract of *C. equisetifolia* against some highly

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resistant microorganisms. The bacterial killing kinetics assay and mechanism of bacterial killing by bark extract of *C. equisetifolia* was also examined.

MATERIALS AND METHODS

Collection of plant material

Barks of *C. Equisetifolia* were collected from different areas of Karachi Pakistan, after the identification and confirmation of plant taxonomy by Meritorious Prof. Dr. Ghazala H. Rizwani, belong to the Dept. of Pharmacognosy, Faculty of Pharmacy, University of Karachi. The voucher specimen of bark (0091) was submitted in the Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Pakistan. The collected bark was shade dried and pulverized by electric grinder in order to prevent enzymatic degradation and fungal growth in bark.

Chemicals

Methanol was procured from Merck (Darmstadt, Germany). DMSO was acquired from Sigma- Aldrich (St. Louis, USA), Distill water (always prepared freshly by distillation), Muller Hinton agar (MHA), Muller Hinton broth (MHB), Nutrient agar and Antibiotic discs namely amoxicillin, erythromycin, gentamycin, levofloxacin, ofloxacin and tetracycline were obtained from Oxoid LTD Basingstoke (Hemisphere England). All collected antibiotic discs are of 10µg.

Collection of different ATCC standard and clinical isolates

The different pathogenic strains were collected from Dr. Essa and Darul Sehat Hospital Karachi Pathological Laboratories in Karachi Pakistan. ATCC control cultures used in this study were *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella pneumonia* (ATCC 43816), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19165),

Salmonella enterica (ATCC 14028) and *Escherichia coli* (ATCC 8939). Moreover, the eleven pathogenic clinical bacterial isolates were used including *Bacillus subtilis* (MT 0208), *Staphylococcus aureus* (MT 0420), *Streptococcus pyogenes* (MT 0151), *Streptococcus fecalis* (MT 0891), *Staphylococcus epidermidis* (MT 0712), *Salmonella. enterica* (MT 1205), Methicillin-resistant *S. aureus (MRSA) (MT 0511), Pseudomonas aeruginosa* (MT 0073), *Klebsiella pneumonia*, (MT 1631), *Salmonella typhi* (MT 0136) and *Escherichia coli* (MT 1823). All collected isolates from the hospitals laboratories were identified by pathologists on the basis of bacterial morphology, cultural and biochemical reactions. All pathogenic strains were placed on sterile nutrient slants at 4^{o}C before used.

Plant extraction

The 2 kg dried material of bark was soaked in methanol (90%) using Soxhlet extractor. The solution was filtered and evaporated by rotary vacuum evaporator (Buchi, Switzerland) under reduced pressure at 40^{o}C. This process was performed in multiple times to obtain a crude extract of bark of *C. equisetifolia*.

Antibacterial susceptibility testing

Firstly, culture was inoculated in sterile Muller Hinton broth and incubated at 37^{o}C for 24 h and its turbidity was matched with 0.5 Mc Far land standard to achieve 10^6 cell/mL. A comparative antimicrobial susceptibility of *C. equisetifolia* bark was performed using Kirby-Bauer disc diffusion method. This method was approved by Clinical Laboratory and Standard Institute (CLSI) for antimicrobial testing (Balouiri *et al.*, 2016). Six different antimicrobial discs and the plant disc of methanolic bark extract (5 mm in diameter) were used. These discs placed on the pre inoculated MHA plates with respective cultures and were incubated (Lotus Co Model 215 incubator) at 37°C for overnight. Zones of inhibitions (including the diameter of disc) were measured in millimeter.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The broth dilution method was used to estimate the MIC and MBC of methanolic bark extract of *C. Equisetifolia* (Yu *et al.*, 2004). First, 100µL crude bark extract of plant was used to prepare the concentration of 50mg/mL then made several diluted concentrations up to 0.5mg/mL using serial dilution method. All concentrations were prepared in nutrient broth and tween 20 was used to solubilize the plant extract. The concentration of each collected tested strain was adjusted to 1×10^8cfu/mL. The inoculated microbial plates were incubated overnight at 37 °C. The MIC refers as the minimum concentration of the antimicrobial agent used to inhibit the visible growth of microorganisms. Turbidity indicates the growth of tested isolates in the test tubes. The culture broth was incubated in Tyramide Signal Amplification (TSA) system at 37°C for 24 hrs. The MBC value defined as the minimum concentration of the antimicrobial agent at which pathogenic strain was completely killed. The experiment was performed in triplicates.

Study of killing kinetics assay

The killing kinetics study of methanolic bark extract of *C. equisetifolia* was carried out according to the modified method of Ibrahim et al. (Ibrahim and Lim, 2015). A total of 500 mL conical flask containing 100 mL bark extract of plant and nutrient broth were used with concentration of MIC of each tested isolate. The isolates were grown in logarithmic phase for 2 h prior to the exposure of the methanolic bark extract of *C. equisetifolia*. The concentration of 1×10^8 cells/mL for each strain was used. Four milli liters bacterial culturewas withdrawn to determine the optical density at 540 nm after every 4h interval. The killing kinetic plot was constructed between optical density of culture and time in hrs.

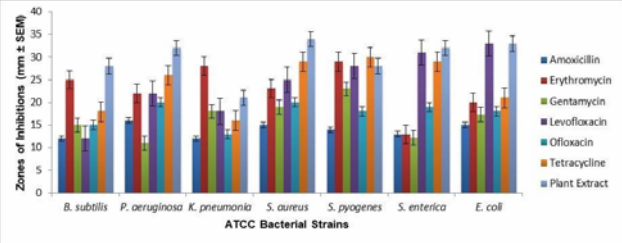

Fig. 1: Antibiotic and plant extract susceptibility of ATCC bacterial strains.

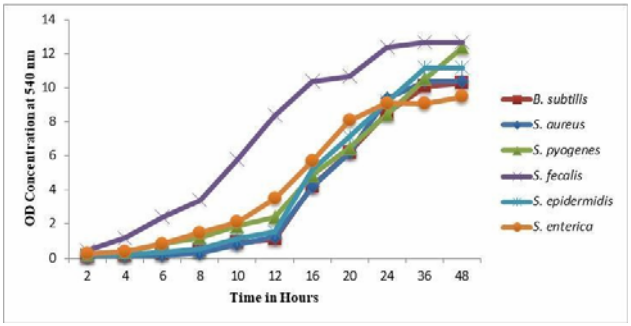

Fig. 2a: Growth Profile of different microorganisms after exposure to methanolic extract of *C. equisetifoliaat* MIC of each isolate.

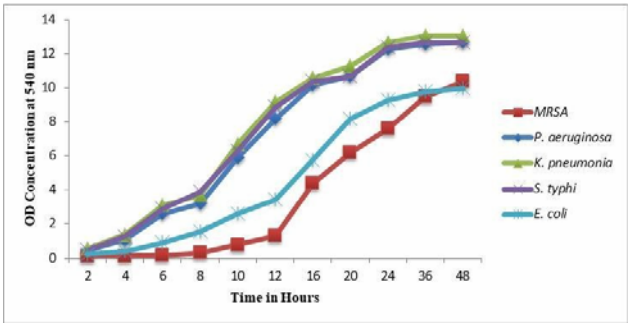

Fig. 2b: Growth Profile of different microorganisms after exposure to methanolic extract of *C. equisetifolia* at MIC of each isolate.

Morphological study of MRSA cells

The morphological study of *MRSA* cells at initial stage and after exposed to methanolic bark extract of *C. equisetifolia* was performed using scanning electron microscopy (SEM) technique (Okonogi *et al.,* 2011). The suspension of tested isolates before or after exposure to the plant extract was dropped into a membrane filter and dried. Then the bacterial culture was fixed with glutaraldehyde solution prepared in phosphate buffered. Subsequently, the fixed isolates were stained for 2 h with already prepared solution of OsO_4 in phosphate buffered and dehydrated using water-methanol mixture. The filter membrane was examined with SEM before coated with gold particles.

STATISTICAL ANALYSIS

All the experimental studies were performed in triplicates. All results achieved in this study were presented as the means ± standard deviation. The data were subjected to analyzed using SPSS (version 22) software and analysis was carried out using the One-way ANOVA at $p < 0.05$. Furthermore, the differences in antibacterial activity between the extract and collected antibiotics were evaluated using Post Hoc Tukey's test.

RESULTS

Antibacterial activity

The antimicrobial activities of different antimicrobial agent and crude methanolic bark extract of *C.equisetifolia* agains thighly resistant pathogens are presented in table1 and 2 respectively. The tested antibiotics and plant extracts susceptibility against some ATCC cultures are shown in

Fig. 1. Among the tested isolates *S. aureus, B. subtilis, S. epidermidis* and *MRSA* were highly susceptible to the extract. However, the zones of inhibition produced by the plant extract were larger than that of some well-known commercial antibiotics. The MIC and MBC values of methanolic bark extract of *C. equisetifolia* showed positive results in screening test of different microbes (table 3). The results of MIC and MBC tests showed that extract of *C. equisetifolia*had strong inhibitory and bactericidal activity against *S. aureus, S. epidermidis, MRSA,* and *E. coli* observed the same value of both MIC and MBC were 6.5, 6.5, 7.0 and 7.0mg/mL respectively. The results of statistical analysis are given in table 4.

Antibacterial killing kinetics

The growth profiles of different test microorganisms after exposed with the methanolic bark extract of *C. equisetifolia* at MIC of each microorganism (fig. 2a and 2b). This killing kinetics study against highly resistant pathogens was performed over a period of 48 hours. The methanolic bark extract of *C. equisetifolia* produced a more drop in optical density of *S. aureus, MRSA B. subtilis and S. epidermidis* up to 12 hrs. Furthermore, exposure to methanolic bark extract of *C. equisetifolia* all tested microorganisms showed stationary growth phase after 36 hrs.

MRSA morphology study

The scanning electron microscope (SEM) study was performed on *MRSA* since it is known as a most common pathogen for human and cause serious infections including wounds infections, urinary tract infections and different systemic infections. Under the SEM study of *MRSA* cells after exposed to extract of *C. equisetifolia* at different time interval, it was observed that the *MRSA* cells was shrunk within few hrs. The normal cells of *MRSA* at initial stage of exposure to extract were completely destroyed after treatment to 12h with plant extract.

Table 1: Antimicrobial agent susceptibility of highly resistant clinical isolates

Clinical isolates	Zone of inhibitions in mm					
	Amoxicillin	Erythromycin	Gentamycin	Levofloxacin	Ofloxacin	Tetracycline
B. subtilis	7.5 ± 0.424	22.1 ± 0.123	8.3 ± 1.329	10.0 ± 1.543	10.4 ± 1.240	16.1 ± 0.412
S. aureus	9.5 ± 0.874	20.2 ± 0.415	10.6 ± 0.211	22.2 ± 0.121	7.4 ± 1.742	23.2 ± 0.171
S. pyogenes	9.0 ± 0.987	24.0 ± 1.204	16.2 ± 0.326	22.7 ± 1.438	11.4 ± 1.240	22.5 ± 0.024
S. fecalis	8.7 ± 0.474	23.6 ± 0.603	22.7 ± 0.721	23.0 ± 0.556	14.6 ± 0.721	22.2 ± 0.457
S. epidermidis	9.5 ± 0.185	27.0 ± 1.924	18.2 ± 0.173	23.5 ± 1.564	12.8 ± 1.380	16.6 ± 1.202
S. enterica	7.2 ± 0.187	13.8 ± 2.474	9.2 ± 0.250	27.1 ± 2.532	7.8 ± 0.610	11.0 ± 0.627
MRSA	6.7 ± 1.044	12.4 ± 0.875	7.2 ± 0.271	14.5 ± 0.814	9.5 ± 0.472	13.8 ± 1.072
P. aeruginosa	8.0 ± 1.987	16.4 ± 1.178	11.2 ± 0.326	15.0 ± 1.653	10.5 ± 0.900	16.7 ± 0.119
K. pneumonia	7.6 ± 0.491	17.8 ± 1.698	14.7 ± 0.610	13.4 ± 0.651	9.2 ± 0.764	12.0 ± 1.946
S. typhi	6.2 ± 0.269	13.4 ± 1.824	16.2 ± 1.482	27.4 ± 2.113	10.9 ± 1.626	10.5 ± 1.622
E. coli	15.4 ± 0.418	20.2 ± 0.710	17.3 ± 0.135	16.6 ± 0.542	8.2 ± 1.462	16.3 ± 0.187

Results are mean ± S.D of triplicate experiments, (n = 10). All antibiotics are of 10 µg/discs

Table 2: Antimicrobial activity of crude MeOH bark extract against clinical isolates at different concentrations

Clinical isolates		Zone of inhibitions in mm		
	10 mg/mL	15 mg/mL	20 mg/mL	30 mg/mL
B. subtilis	20.3 ± 0.674	22.5 ± 1.080	24.1 ± 0.875	30.1 ± 1.105
S. aureus	22.1 ± 0.994	24.2 ± 0.816	28.6 ± 2.452	34.8 ± 2.292
S. pyogenes	15.1 ± 0.732	19.2 ± 2.442	21.0 ± 1.763	24.9 ± 1.790
S. fecalis	7.6 ± 1.171	14.9 ± 0.992	16.3 ± 0.947	18.2 ± 1.229
S. epidermidis	16.3 ± 0.674	24.5 ± 1.584	28.4 ± 1.574	33.1 ± 1.444
S. enterica	17.1 ± 2.232	21.0 ± 2.053	26.0 ± 1.825	30.7 ± 1.636
MRSA	17.4 ± 0.541	25.7 ± 1.024	28.7 ± 1.374	32.8 ± 1.074
P. aeruginosa	15.1 ± 0.737	17.1 ± 1.191	19.4 ± 1.570	21.6 ± 1.745
K. pneumonia	13.0 ± 0.816	14.4 ± 0.845	17.2 ± 1.031	19.6 ± 1.170
S. typhi	11.2 ± 0.816	11.9 ± 1.375	16.1 ± 1.792	18.1 ± 1.911
E. coli	16.0 ± 0.812	21.2 ± 0.918	26.5 ± 2.011	29.2 ± 1.544

Table 3: MIC and MBC of crude methanolic bark extract of *C. Equisetifolia*

Clinical Isolates	MIC (mg/mL)	MBC (mg/mL)
B. subtilis	7.5 ± 0.325	8.0 ± 0.721
S. aureus	6.5 ± 1.275	6.5 ± 1.752
S. pyogenes	9.5 ± 0.512	9.5 ± 1.970
S. fecalis	9.5 ± 0.780	12.5 ± 2.240
S. epidermidis	6.5 ± 0.874	6.5 ± 0.987
S. enterica	7.5 ± 1.740	10.0 ± 1.027
MRSA	7.0 ± 1.971	7.0 ± 1.770
P. aeruginosa	10.0 ± 1.017	12.5 ± 2.870
K. pneumonia	10.0 ± 1.247	12.0 ± 2.404
S. typhi	10.0 ± 2.470	12.5 ± 3.017
E. coli	7.0 ± 1.791	7.0 ± 2.190

Results are mean ± S.D of triplicate experiments, (n = 10)

Table 4: Statistical analysis of One-way of variance (Anova) and Post Hoc Tukey's Test

Microorganisms	One-way Anova (Level of Significance)	Multiple Comparisons with Plant extract (Level of Significance)			
		Erythromycin	Tetracycline	Amoxicillin	Ofloxacin
B. subtilis	0.021	0.015	0.027	0.011	0.010
S. aureus	0.013	0.023	0.014	0.021	0.025
S. pyogenes	0.031	0.017	0.018	0.010	0.021
S. enterica	0.010	0.011	0.008	0.020	0.011
P. aeruginosa	0.041	0.013	0.021	0.021	0.019
MRSA	0.011	0.041	0.042	0.004	0.013
E. coli	0.003	0.029	0.023	0.019	0.027

*P<0.05

DISCUSSION

In this research, ATCC cultures (both Gram positive and Gram negative) were used as standard and comparative account a plant extract at different concentration and clinical isolates was investigated. Tested ATCC organisms found to be sensitive against marketed antibiotics although clinical isolates showed resistant pattern against such collected antibiotics disc. Resistance was seen more in *P. aeruginosa* towards marketed discs followed by *K. pneumonia*. Among Gram positive microorganisms *MRSA* was highly resistant against all tested antibiotics.

S. aureus and *B. subtilis* also found resistant organisms particularly towards amoxicillin, gentamycin and ofloxacin. The more resistant found in clinical isolates towards amoxicillin among all tested antibiotics. Antimicrobial potential of crude bark methanolic extract of *C. equisetifolia* was examined at different concentration against standard ATCC cultures and clinical isolates. Extract showed significant results against *S. aureus* and *S. epidermidis* which usually involved in skin lesions and wound infections. Several studies are there reporting the resistance of this organisms against different well reputed antibiotics (Levy, 1998, Raafat *et al*., 2017). The MeOH extract showed significant antimicrobial activity even at lowest concentration i.e. 10mg/mL reflects that results are comparable with zones produced by commercial antibiotics. These significant zones of inhibitions produced by plant extract demonstrated that compounds having antibacterial activity were present in extract when methanol used in solvent extraction process. Manivannan et al., also indicated the prime importance of solvent extraction process to get significant antimicrobial activity (Manivannan *et al.,* 2011). Furthermore, the results of statistical (Anova and Tukey's Post Hoc) analysis indicated that the significant differences found between the antibacterial activity of plant extract and collected antibiotics.

More sensitive results produced by plant extract towards Gram positive organism. Current findings are much similar with (Ahsan *et al.,* 2009). Slight variations in results found may be due to the multiple factors including geographical locations, plant age, methodological variation and storage conditions. The MIC values of extract (6.5 to 10 mg/mL) showed inhibitory effects of plant even at lowest concentration justified the traditional use of plant in management of infectious diseases. According to Sahgal et al, MIC difference exists because of morphological structure and composition of bacterial cells (Sahgal *et al.,* 2011).

The bark extract of *C. equisetifolia* showed significant bactericidal and bacteriostatic effects after short exposure with the tested isolates (figs. 2a and 2b). It was observed that the methanolic bark extract inhibit the growth of most of the tested isolates up to 12 hrs. The findings of antibacterial killed kinetics assay showed that the plant extract produced their antibacterial activity more rapidly against Gram-positive organisms. The extract of *C. equisetifolia* killed and inhibits the growth of *MRSA* up to 8 hrs.

The number of studies involved in finding mechanisms involved in killing of bacteria (Dholvitayakhun *et al.,* 2017, Limsuwan *et al.,* 2017). The most shining feature of this plant is a dynamic result against *MRSA* which is in current scenario a big problem to handle. Therefore SEM study was performed on *MRSA* cells after exposure to the methanolic extract at different time interval in order to identify the possible bactericidal mechanism. SEM findings reflects morphological and cytological alterations in *MRSA* cells which leads to collapsed and lysed with losing their virulence after treated with plant extract for 12 hrs. In short, *MRSA* cells collapsed after treatment with methanolic bark extract of plant due to presence of active compounds in extract.

CONCLUSION

This research finding evident that crude bark extract of *C. equisetifolia* is potent antimicrobial agent and produced both bacteriostatic and bactericidal effects especially against *MRSA*. The lower MIC and MBC values reflected the antibacterial potency of plant. According to the results of morphological study on *MRSA* cells, it is suggested that extract killed bacteria through alteration in structure of cell membrane. It is recommended that, focused and systematic research is required to get new biological, chemical and pharmacological findings. These findings are helpful in clinical setting involving *MRSA* as an infectious cause.

REFERENCES

Ahsan M, Islam K, Haque M and Mossaddik M (2009). In vitro antibacterial screening and toxicity study of some different medicinal plants. *World Journal of Agricultural. Sciences*, **5**(5): 617-621.

Balouiri M, Sadiki M and Ibnsouda SK (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *J. Pharm. Anal.*, **6**: 71-79.

Cars O, Högberg LD, Murray M, Nordberg O, Sivaraman S, Lundborg CS, So AD and Tomson G (2008). Meeting the challenge of antibiotic resistance. *BMJ*., (Online), 337.

Cowan MM (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev.*, **12**: 564-582.

Dholvitayakhun A, Trachoo N, Narkkong NA and Cushnie TT (2017). Using scanning and transmission electron microscopy to investigate the antibacterial mechanism of action of the medicinal plant Annona squamosa Linn. *J. Herb. Med.*, **7**: 31-36.

Epstein JB, Chong S and Le ND (2000). A survey of antibiotic use in dentistry. *J. Am. Dent.Assoc.*, **131**: 1600-1609.

Essien EE, Newby JM, Walker TM, Ogunwande IA, Setzer WN and Ekundayo O (2016). Essential oil constituents, anticancer and antimicrobial activity of Ficusmucoso and Casuarina equisetifolia leaves. *Am. J. Essent. Oils Nat. Prod.,* **4**: 01-06.

Gumgumjee NM and Hajar AS (2012). Antimicrobial efficacy of Casuarina equisetifolia extracts against some pathogenic microorganisms. *J. Med. Plants Res.*, **6**: 5819-5825.

Ibrahim D and Lim SH (2015). *In vitro* antimicrobial activities of methanolic extract from marine alga Enteromorpha intestinalis. *Asian Pac. J. Trop. Biomed.*, **5**: 785-788.

Levy SB (1998). The challenge of antibiotic resistance. *Sci. Am.*, **278**: 46-53.

Limsuwan S, Moosigapong K, Jarukitsakul S, Joycharat N, Chusri S, Jaisamut P and Voravuthikunchai SP (2017). Lupinifolin from Albiziamyriophylla wood: A study on its antibacterial mechanisms against cariogenic Streptococcus mutans. *Arch. Oral Biol.*, pii: S0003-9969(17)30342-4.

Manivannan K, Anantharaman P and Balasubramanian T (2011). Antimicrobial potential of selected brown seaweeds from Vedalai coastal waters, Gulf of Mannar. *Asian Pac. J. Trop. Biomed.*, **1**: 114-120.

Okonogi S, Prakatthagomol W, Ampasavate C and Klayraung S (2011). Killing kinetics and bactericidal mechanism of action of Alpiniagalanga on food borne bacteria. *Afr. J. Microbiol. Res.,* **5**: 2847-2854.

Raafat D, Leib N, Wilmes M, François P, Schrenzel J and Sahl HG (2017). Development of *in vitro* resistance to chitosan is related to changes in cell envelope structure of Staphylococcus aureus. *Carbohydr. Polym.,* **157**: 146-155.

Sahgal G, Ramanathan S, Sasidharan S, Mordi, M., Ismail S and Mansor S (2011). *In vitro* and *in vivo* anticandidal activity of *Swietenia mahogani* methanolic seed extract. *Trop. Biomed.*, **28**: 132-137.

Shafiq Y, Naqvi BS, Rizwani GH, Usman M, Shah BA, Aslam M and Hina B (2014). Anti-acne activity of *Casuarina equisetifolia* bark extract: A randomized clinical trial. *Bangladesh J. Pharmacol.*, **9**: 337-341.

Walker B, Barrett S, Polasky S, Galaz V, Folke C, Engstrom G, Ackerman F, Arrow K, Carpenter S and Chopra K (2009). Looming global-scale failures and missing institutions. *Sci.,* **325**:1345-1346.

Yu J, Lei J, Yu H, Cai X and Zou G (2004). Chemical composition and antimicrobial activity of the essential oil of *Scutellaria barbata*. *Phytochem.,* **65**: 881-884.