Anti-biofilm activity and time kill kinetic effects of *Salacia oblanga* wall leaf and root extracts against clinical multi-drug resistance bacteria

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ABSTRACT

Introduction and Aim: Antibiotic resistance and biohazard nature of synthetic drugs is contemporary threatening issues in the health sector. The alternative research is focused on plants which attribute for various compounds that exhibit therapeutic properties. Therefore, the present aim of the work is to evaluate the anti-biofilm activity and time-kill kinetics of *Salacia oblonga* Wall.

Materials and Methods: The extraction procedure was carried out in soxhlet apparatus using low polar to high polar solvents. Anti-biofilm activity and time kill kinetics was carried out to evaluate antibacterial effect of *S. oblonga* leaf and root extracts on selected bacterial strains. The results were expressed as mean ± standard deviation (SD) of three replicates. The data from the experiments was subjected to one-way analysis of variance (ANOVA) wherever applicable.

Results: The effectiveness of ethyl acetate leaf and root extracts at (1 × MIC) on the attachment and inhibition of biofilm formation is found significant at 2.8mg/ml and 2.3mg/ml (50%) and 3.4mg/ml and 3.0mg/ml (90%) against *M. luteus* and 3.7mg/ml and 3.4mg/ml (50%) and 4.4mg/ml and 4.1mg/ml (90%) for *M. tuberculosis* respectively. Results of time killing kinetics indicated that leaf and root extracts were significant against *M. luteus* and *M. tuberculosis* killed 50 and 100% at 1 × MIC after 3 and 5 h respectively.

Conclusion: Based on the results, we conclude that leaf and root extracts showed significant activity on biofilm formation and time kill assay.

Keywords: *Salacia oblonga;* anti-biofilm; time kill kinetics; ethyl acetate extract.

INTRODUCTION

The extensive use of antibiotics led to the emergence of antibiotic-resistant bacteria and caused the increase in mortality rates in humans and animals (1). The ability of contemporary antimicrobial to treat common infectious diseases has been declined. The continuous rise of pathogenic organisms with multidrug resistance and the ineffectiveness of current antimicrobials is a serious threat to humans and as well the whole nation (2). In 2017, World Health Organization (WHO) and The Center for Disease Control and Prevention released the list of pathogenic organisms such as *Staphyloccocus aureus, Klebsiella spp, Streptococcus pneumoniae, Escherichia coli* etc., with increased ability to form a biofilm and promote high levels of resistance towards existing and third-generation antibiotics (3). As a result, innovative methods to find for novel drugs must be developed. The use of chemically derived drugs is halted because of its attributed abrupt symptoms on health and environment. To combat antimicrobial resistance and existing hazardous effects of synthetic drugs, medicinal plants are considered as an alternative source of drug discovery and complementary treatment for multidrug-resistant pathogens including bacteria (4). In ancient days, plant based medicaments are prescribed in the form of crude drugs such as poultices, powders, teas, tinctures, and other herbal formulations (5). Now a days the use of plants as medicines includes the identification and isolation of active molecules like morphine isolated from opium (6), others include codeine, cocaine, quinine, digitoxin isolated from plants are still in use (7, 8).

Biofilm is a network of proteins, carbohydrates, and other organic compounds that help the pathogen to bind and form a strong attachment to the surfaces (9). This biofilm formation is a key factor of adherent pathogen and considered as one of the indirect modes of resistance by bacteria towards various existing antibiotics (10, 11). The time-kill activity of bactericidal activity of plant extracts can be assessed *in vitro* by serial sampling and counting viable bacteria. Time-kill studies monitor bacterial cell viability and death over a different range of antimicrobial concentrations has been frequently used to determine the efficacy of antimicrobials over time.

We framed out our study to evaluate anti-biofilm activity and time killing kinetics of *S. oblonga* Wall

because species of *Salacia* genera have been reported prominent antimicrobial properties (12, 13).

MATERIALS AND METHODS

Collection of plant material

S. oblonga plants were collected from the natural habitat, Yeleswaram forest, East Godavari, Andhra Pradesh, situated at 17.2833˚N 82.1000˚E (Fig. 1a) and are authenticated by taxonomist Dr. M. Venkataratnam (Retired Professor of Botany, Andhra University, Visakhapatnam). The plants are grown in the GITAM greenhouse. Leaf and root samples were collected from the plants maintained in the GITAM greenhouse, Visakhapatnam.

Extraction

Ethyl acetate was selected as the solvent of choice to extract the powdered leaf and root. The ethyl acetate can extract compounds of different range of polarities, high yielding, easy to remove from the extract and it is nontoxic to bioassay systems. 500 grams of ground dry leaf and root powder was extracted with approximately 3.5 L ethyl acetate using Soxhlet apparatus and subjected for dryness under reduced pressure by Rota-vapor at 40-50˚ C for 3 h to collect the crude extract.

Anti-biofilm Assay

Bacterial and fungal Strains

Bacillus subtilis (ATCC 6633), *Bacillus cereus*, (ATCC 14579) *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 43816), *Escherichia coli* (ATCC 8739), *Proteus vulgaris* (ATCC 13315), Methcillin- resistant *Staphylococcus aureus* (MRSA, NCTC 13616) were obtained from the Kakatiya Medical College, Warangal Urban. *Micrococcus luteus, Mycobacterium tuberculosis* were requisitely obtained from local TB hospital, Bheemaram, Warangal Urban. MRSA was cultured and maintained on Mannitol Salt Agar medium augmented with 7.5% sodium chloride. *Mycobacterium Sps (M. tuberculosis* and *M. luteus)* were maintained on Middle Brook 7H10 selective media. The other bacterial strains were maintained on Luria-Bertani (LB) medium (purchased from Hi-media Laboratories, Mumbai, India). All the bacterial cultures were incubated at 37°C for 24 h. All strains were subcultured on to nutrient agar medium for bioassays examination. The cultures were grown and the turbidity was adjusted with sterile broth to obtain a half of MC Farland standard (1x108 - 5x108 cfu/ml). This was used as starting inoculum for the assay.

Growing a biofilim

The ability of the extracts to prevent further biofilm development or destruction of preformed biofilm was investigated by the method previously described (14). A 100µl aliquot of standardized concentration of cultures with OD560 =0.02(1.0×10 6CFU/ml) was added into individual flat-bottomed 96-well micro titre plates containing LB medium. The micro titre plate was incubated to develop a multilayer biofilm for about 24h (irreversible attachment phase) and 48h (mature biofilm) at 37°C. Following, 100µl aliquots of leaf and root extracts 1 × MIC, 2 × MIC, 4 × MIC were added into the wells of a 96-well micro titre plate and the plates were incubated further at 37°C for 24h. Chloramphenicol at the same concentration as the extracts served as positive control while both DMSO and sterile distilled water served as negative controls. The biofilm biomass was assayed using the crystal violet (CV) staining assay (15).

Crystal violet staining assay

Staining the Biofilm

The assay was carried out according to the method previously described [15]. Briefly, the cells were dumped by shaking and turning the plate. Gently, wash the micro titre plates repeatedly for 3-4 times with sterile distilled water, air dry, and then oven-dried at 60°C for 35-45min. This step removes unattached cells and media components that can be stained in the following step and mainly minimises the background staining of well. Transfer 125µl of 0.1% solution of crystal violet to each well and incubate at room temperature for 10-15min. Rinse the plate 4-5 times with sterilised distilled water to rid the plate of all excess cells and dye. At this stage, biofilm was observed as purple rings at the sidewall of the well. The plate is dried overnight and followed for quantitative assessment.

Quantitative assessment of biofilm

A quantitative assessment of biofilm formation was done by adding 125µl of 33% of acetic acid to each well. The micro titre plate is incubated at room temperature for 10-15 min. A 125µl aliquot of the solubilised solution was transferred to a fresh and sterile micro titre plate and the absorbance was measured at 590nm using a micro plate reader. The mean absorbance of the samples was determined, and the percentage inhibition of biofilm was determined using the equation below [14]:

Percentage (%) of inhibition = OD Negative control-OD Experimental/OD of Negative control x 100

Time-Kill Kinetics Assay

Time-kill kinetics of leaf and root ethyl acetate extracts was evaluated by the previously described method (16). Aliquots of leaf and root extracts 1 × MIC, 2 × MIC, 4 × MIC were prepared. An inoculum size of 1.0 × 106 CFU/ml was transferred to tubes containing nutrient broth and incubated 37oC for 24 h. A control test was performed for the organisms without the extracts or reference antibiotics. Aliquots of 1.0 ml of the medium were taken at time intervals of 0, 1, 2, 3, 4, 5, 6, 12, and 24 h and inoculated

aseptically into freshly prepared 20 ml nutrient agar plates and incubated at 37oC for 24 h. The CFU of the organisms was determined and the experiments were performed in triplicate. A graph was plotted between log CFU/ml versus time.

RESULTS

Anti-biofilm activity

The effectiveness of ethyl acetate leaf and root extracts on the attachment and inhibition of biofilm formation is given in Table 1 and Fig. 1, 2. The 50% and 90% of biofilm formation activity was inhibited by leaf and root extracts (1 × MIC) found significant at 2.8 mg/ml and 2.3mg/ml (50%) and 3.4mg/ml and 3.0mg/ml (90%) against *M. luteus* and 3.7mg/ml and 3.4mg/ml (50%) and 4.4mg/ml and 4.1mg/ml (90%) for *M. tuberculosis* respectively. Following, Methicillin-resistant *S. aureus* and *E.coli* biofilm formation activity inhibition of leaf and root extracts (2 × MIC) found at 5.9mg/ml and 5.2mg/ml (50%), 7.0mg/ml and 6.8mg/ml (90%), 7.0mg/ml and 6.6mg/ml (50%), 7.4mg/ml and 7.2mg/ml (90%) respectively. On the other hand, biofilm inhibition of *Bacillus cereus* and *B. subtilis* (ATCC 6633) and *K. pneumoniae* (ATCC 43816) by leaf and root extracts (2 × MIC) was found at 7.6mg/ml and 7.2mg/ml (50%), 8.4mg/ml and 8.0mg/ml (90%), 7.8mg/ml and 7.4mg/ml (50%), 8.8 mg/ml and 8.2mg/ml (90%), 8.3mg/ml and 8.0 mg/ml (50%), 9.6mg/ml and 9.0mg/ml (90%) respectively. Whereas, *S. typhimurium*, *P. aeruginosa* and *P. vulgaris* anti-biofilm formation was also investigated by leaf and root extracts and found good at (2 × MIC) with 12.5mg/ml and 12.0 mg/ml (50%), 15.8mg/ml and 14.5 mg/ml (90%), 13.8mg/ml and 13.3mg/ml (50%), 16.7mg/ml and 16.0 mg/ml (90%), 12.6mg/ml and 12.1mg/ml (50%), 15.3mg/ml and 14.8mg/ml (90%) respectively. According to published reports [17], percentage anti-biofilm formation values by extracts between 0 to 100% indicate inhibition of biofilm, while development of growth is considered by values below 0%. Inhibition of biofilm above the 50% is regarded as good activity, while between 0 and 49% it is poor.

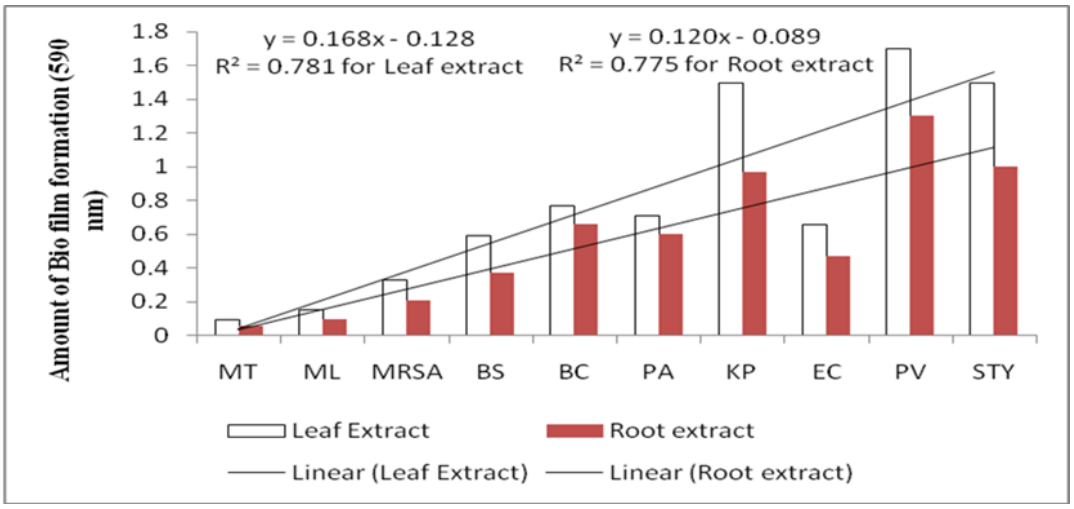

Fig. 1: Inhibitory effect of *S. oblonga* leaf and root ethyl acetate extract on biofilm formation of MT-*M. tuberculosis*, ML-*M. leteus*, MRSA-Methcillin-resistant *S. aureus*, BS-*B. subtilis*, BC-*B. cereus*, PA-*P. aeruginosa*, KP- *K. pneumoniae*, EC-*E. coli*, PV- *P. vulgaris*, STY- *S. typhimurium*

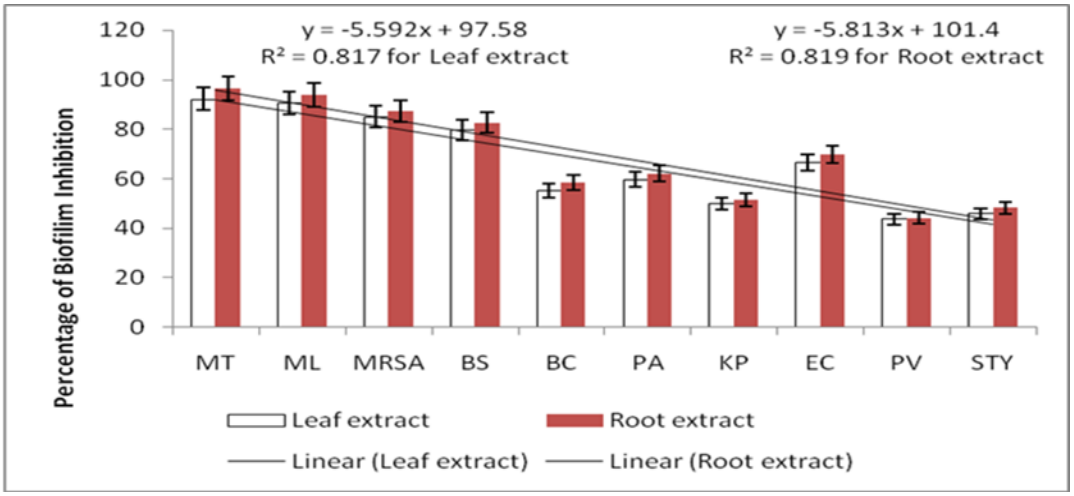

Fig 2: Inhibitory effect of *S.oblonga* leaf and root ethyl acetate extract on percentage of biofilm formation of MT-*M. tuberculosis*, ML-*M. leteus*, MRSA-Methcillin-resistant *S. aureus*, BS-*B. subtilis*, BC-*B. cereus*, PA-*P. aeruginosa*, KP- *K. pneumoniae*, EC- *E. coli*, PV- *P. vulgaris*, STY- *S. typhimurium*

Table 1: Biofilm inhibition activity by ethyl acetate extracts of Leaf and Root extracts of *Salacia oblonga*

Inhibition of biofilm development (mg/ml)				
	Leaf		Root	
Bacterial strains	BIC 50%	BIC 90%	BIC 50%	BIC 90%
Mycobacterium tuberculosis	3.7	4.4	3.4	4.1
Micrococcus leteus	2.8	3.4	2.3	3.0
Methcillin-resistant Staphylococcus aureus	5.9	7.0	5.2	6.8
Bacillus subtilis	7.8	8.8	7.4	8.2
Bacillus cereus	7.6	8.4	7.2	8.0
Pseudomonas aeruginosa	13.8	16.7	13.3	16.0
Klebsiella pneumonia	8.3	9.6	8.0	9.0
Escherichia coli	7.0	7.4	6.6	7.2
Proteus vulgaris	12.6	15.3	12.1	14.5
Salmonella typhimurium	12.5	15.8	12.0	14.5

Minimum biofilm concentration of extracts that showed 50% and 90% inhibition of biofilim formation.

Time kill kinetics of *S. oblonga* extracts

The organisms selected for the killing rate test against the leaf and root ethyl acetate extracts. As shown in Fig 3, the results indicated that leaf and root extracts killed 50% of *M. luteus* CFU at 1 × MIC after 3h of contact time interval with each of the extracts was 53.1% and 56.3% respectively. Following *M. luteus*, the killing rate reaction noted for *M. tuberculosis* 50% of CFU at 1 × MIC after 3h of contact time interval by leaf and root extracts with 50.1% and 53.9% respectively. By the end of 5 h 100% *M. luteus* and *M. tuberculosis* CFU were killed by leaf and root extract with 90.8%, 98.6%, and 87.3%, 96.9% respectively. Methicillin- resistant *S. aureus* and *E. coli* cells after 4h contact were killed 50% CFU at 2 × MIC with 58.3% and 62.1%, 60.4% and 66.8% and 100% CFU at 4 × MIC with 81.8% and 95.2%, 91.4% and 95.2% after 6h interval by leaf and root extracts respectively. While, *B. cereus* and *B. subtilis* and *K. pneumonia* were killed 100% CFU after 12h of incubation by leaf and root extracts at 4 × MIC with 92.7% and 94.6%, 91.9% and 95.8%, 82.1%, and 92.8% respectively. Whereas, *S. typhimurium*, *P. aeruginosa* and *P. vulgaris*, cells were killed 100% CFU after at the end of the incubation period (24 h) by leaf and root extracts at 4 × MIC with 79.2% and 81.9%, 76.4% and 80.5%, 70.1%, and 73.6% respectively.

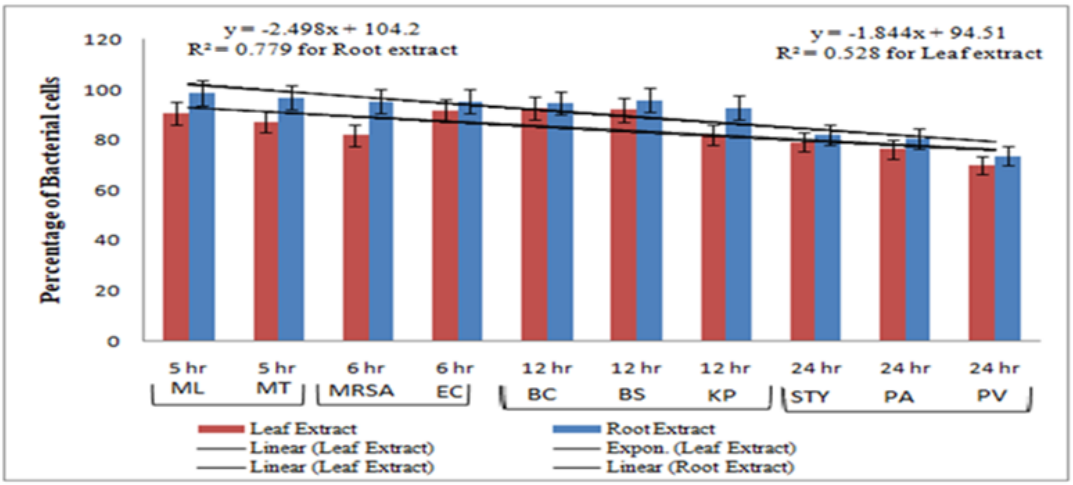

Fig. 3: Time kill kinetics of Leaf and Root ethyl acetate extracts against resistant pathogenic organisms. MT-*M. tuberculosis*, ML-*M. leteus*, MRSA-Methcillin-resistant *S. aureus*, BS-*B. subtilis*, BC-*B. cereus*, PA-*P. aeruginosa*, KP-*K. pneumoniae*, EC- *E. coli*, PV-*P. vulgaris*, STY- *S. typhimurium*

DISCUSSION

In this study, the ability of the plant extracts was evaluated against the biofilm of the selected bacteria species. This seems to be the first report to determine the anti-biofilm activity of the *S. oblonga* plant extracts. The ability of plant extracts and antibacterial compounds to inhibit biofilm formation and or destruction of biofilm holds great importance in the reduction of colonisation of surfaces and epithelial mucosa by bacteria (17). As expected, the leaf and root extract exceptionally inhibited biofilm formation of the bacteria tested. However, *S. typhimurium*, *K. pneumonia*, and *P. vulgaris* prevented the activity of extracts and showed biofilm production. As shown in the results, the 50% and 90% of biofilm inhibition of

M. luteus and *M. tuberculosis*. The result can be attributed due to the presence of anti-Mycobacterium agents in the crude extract of *S. oblonga* which were probably stable even above 37ºC. Generally Mycobacterium sp. replicating period varies from 23hr to 69hr (17), but the constant growth of Mycobacterium sp. is reported at 24hr at 1 × MIC at least concentrations indicate remarkable anti-attachment activity of the extracts. On the other hand, the 50% and 90% of biofilm inhibition of Methicillin- resistant *S. aureus* (MRSA) and *E. coli* is noted at 2 × MIC considerable concentrations. While, *B. subtilis, B. cereus, P. aeruginosa, K. pneumonia, P. vulgaris, S. typhimurium* 50% and 90% biofilm inhibition by extracts was slightly found at high concentration of 2 × MIC. The outstanding ability of the leaf and root extracts to interfere and abrupt the initial stage of biofilm formation of the selected bacterial isolates perhaps attributed mechanism of interference with forces like electrostatic interaction forces (electric forces between positive and negative charges mainly between nuclei and electrons), Lifshitz–Van der Waals (intermolecular attraction forces) Brownian movement of cells which leads to the collision, sedimentation that helps the aggregation and adherence of bacteria to surfaces (18). Moreover, plant extracts may also inhibit the requirement of nutrients which are essential for bacteria growth and also for adhesion (19). Thus, the leaf and root extracts may hold importance for a decrease in colonisation and thereby preventing infectious diseases.

Based on time-kill kinetic assay graph, leaf and root extracts of *S. oblonga* at 1 × MIC showed bactericidal activity against all the bacterial strains tested. The rate of killing of bacteria relies on the concentration of extract and duration of exposure (20). Interestingly, both extracts had a greater effect on *M. luteus* and *M. tuberculosis* which showed bactericidal activity 50% and 90% directly at 1 × MIC. Whereas, the death rate of Methicillin- resistant *S. aureus* (MRSA) and *E. coli* varied with concentration and time of exposure. These bacterial cells were killed 50% after 4 h of incubation at 2 × MIC and 90% after 5hr of incubation at 4 × MIC. Even though, the strong resistance ability of MRSA (21) and *E. coli* (22) towards major antibiotics available, they were effectively killed by the leaf and root extracts. This indicates that *S. oblonga* leaf and root extracts are the potential sources for isolating bactericidal agents against Methicillin- resistant *S. aureus* (MRSA) and *E. coli*. The bactericidal effect of both extracts against *B. subtilis, B. cereus, K. pneumonia* found good at 4 × MIC where 90% of bacterial cells were killed after 12 h of incubation. Whereas, *P. aeruginosa, P. vulgaris, S. typhimurium* killed 90% after 24 h of incubation.

The literature survey revealed that there are only a few reports are published on antibacterial activity of *S. oblonga* wall leaf and root and found no reports on anti-biofilm assay and time killing kinetics. Therefore, our experiment data of anti-biofilm activity and time-kill studies might be the first report. Among the leaf and root extracts, results were significant with root extract. However, the continuation of an experiment with root will be difficult because the plant is being overexploited. Works had been done for conserving the plant and maintaining the genetic diversity through micro and vegetative propagation (23, 24). In the view of results with leaf extracts are competed with root, therefore, leaf extract could become the source of active compounds against various bacteria and help the plant from overexploitation.

CONCLUSION

According to our results the inhibition of biofilm formation and time kill study of bacteria was found significant with leaf and root extracts. In the view of these results, the investigation will be continued for isolation of active principle for activity.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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