

Article - Biological and Applied Sciences

Investigation of Mode of Action of Anti Bacterial Activity of *Salacia Oblonga* Extract Against Drug Resistant Pathogen

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HIGHLIGHTS

- *S. Oblonga* ethyl acetate extract has shown good growth inhibition against drug resistant pathogens
- Extract acts on membrane of the drug resistant pathogen *S. aureus*.
- Phytochemicals were identified from the crude extracts.

Abstract: *Salacia oblonga* Wall belonging to family Celastraceae contains vital phytoconstituents and has been used since long for the treatment of diabetes, inflammation and burn wounds. *S. oblonga* ethyl acetate root extract was evaluated for antibacterial activity towards drug resistant pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Further 260 nm absorbing material was estimated in the control and treated cells. Interestingly 260 nm absorbance material is higher in the *Staphylococcus aureus*. Further the effect of the plant extract on drug resistant pathogen *S. aureus* was examined by scanning electron microscopy (SEM). SEM results have shown that treated bacterial cells have changed in morphology, size and reduced in number. Based on these results it can be concluded that *S. oblonga* extract acts on membrane of the drug resistant pathogen *S. aureus*.

Keywords: *Salacia oblonga*; Antibacterial activity; Drug resistant pathogen; and SEM.

INTRODUCTION

Antimicrobials are very important medical treatment since the first identification of antibacterial dyes by Ehrlich in the beginning of twentieth century. However, bacteria resistant to antibiotics were recognized as a serious problem in clinical environments such as hospital and healthcare facilities [1]. The health problems related to microbial infection are seriously affected by the widespread antibiotic resistance [2]. Antibiotic resistance is a profound and growing global problem to public health, when bacteria mutates, antibiotics no longer work in people who require them to treat infections. Bacterial resistance is closely associated with the usage of antimicrobial compounds in clinical practice. Long duration usage of antibiotics for therapy can lead to the development of resistance in microorganism that initially is sensitive to antibiotics [3]. They adapt gradually and develop resistance to antibiotics, the emergence of phenotype resistant to antibacterial agents depend on various factors.

Medicinal plants are rich source of modern drugs, pharmaceutical intermediates and chemical entities for synthetic drugs [4]. Plant derived substances have recently gained great interest due to many advantages [5]. Medicinal plants are considered to be a source of potential antimicrobial crude drugs as well as novel compounds with antimicrobial activity, with possibly new modes of action. This leads to an assumption that some naturally occurring plant compounds can kill antibiotic resistant strains of bacteria [6]. Many of the diverse compounds derived from plants have been proven as antimicrobial agents against various human pathogens [4, 7].

Salacia oblonga wall is an important medicinal plant from genus *Salacia* which is found in the Western Ghats of India and Srilanka. Roots of *S.oblonga* have been used as anti diabetic since ancient times in Indian medicinal system. *S. oblonga* possesses significant medicinal properties viz antibacterial, antioxidant, and anti-inflammatory [8-12]. The present study aimed at understanding the mode of action of *S. oblonga* root extract for growth inhibition of drug resistant *S. aureus*.

MATERIAL AND METHODS

CULTURE COLLECTION

Strains of *Staphylococcus aureus* (MTCC-7443), *Pseudomonas aeruginosa* (MTCC-2295) and *Klebsiella pneumoniae* (MTCC-3384), were procured from microbial type culture collection (MTCC), IMTECH, Chandigarh, India. The bacteria were cultured on Mueller Hinton Agar (MHA) and activated in MH broth at 37°C for 24 h before experimentation. The bacterial cultures were maintained on MH agar and were subcultured every fortnight.

PLANT MATERIAL AND EXTRACT PREPERATION

The plants were collected from Western Ghats Karnataka, with the help of plant taxonomist. The plant was separated into aerial and root parts, shade dried and ground to fine powder with the help of blender. Homogenized powder was dissolved in different solvents and 100 g dried powder was used for extraction with the help of soxhelt apparatus

and concentrated using rotary evaporator (AKA RV 10). The weight of concentrated crude extract was calculated and prepared to 1 mg/ml stock in specific solvent. The extracts were stored at 4° C for further use.

ANTIBACTERIAL ASSAY

The antimicrobial activity of plant extract was investigated by agar well diffusion method [13-14]. The Mueller Hinton agar (MHA) was transferred onto the petriplates with an inoculum size of 10^6 colony forming units (c.f.u)/ml. The wells were prepared in the MHA plates with the help of a borer (8mm). The extracts were used at a concentration of 1 mg/ml for analyzing the antibacterial activity. A broad spectrum standard antibiotic amikacin (concentration 50 µg/ml) was used as a positive control, whereas the solvent served as negative control. The MHA plates were incubated overnight at 37°C for allowing bacterial growth. After incubation, the zones of inhibition observed around the wells were measured and tabulated for the extracts. All the experiments were performed in triplicate.

ESTIMATION OF 260 nm ABSORBING MATERIAL

A modified method from Carson et al (2002) [15] was used in this assay. Suspensions of bacteria were prepared from the culture on MHA medium. The bacterial cells were treated with the *S. oblonga* extract with the MIC concentration, and then incubated for 24 h. Samples were centrifuged and filtered through 0.2µm pore size filter and subjected to OD 260 nm. Absorbance was estimated for control and treated cells by spectrophotometer (UV-2450 UV-Visible spectrophotometer SHIMADZU).

SEM ANALYSIS

Samples were treated with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PH 7.2) for 24 hrs at 4°C and post fixed in 2% aqueous osmium tetroxide for 4 h. Dehydrated in series of graded alcohols and subjected to critical point drying with CPD unit. The analyzed samples were mounted over the stud with double-sided carbon conductivity tape, and a thin layer of gold coat over the samples was done by using an automated sputter coater (Model – JEOL JFC- 1600) for 3 minutes and analyzed under scanning electron microscope (SEM-Model: JOEL-JSM 5600) at required magnifications as per the standard procedure of Bozzola et al. 1998 [16] at RUSKA Labs, college of Veterinary science, SVVU, Rajendranagar, Hyderabad, India.

GC-MS ANALYSIS

GC - MS analysis was done by injecting the samples into a HP - 5MS in a capillary column (30 m length x 250 µm dia. x 0.25 µm film thickness), Agilent Technologies, USA GC-MS model, consisting of 6890 N Gas Chromatograph coupled with 5,973 insert MSD (Mass Selective Detector). The injector was set at 250°C and the detector at 280°C. The stepped temperature program-was as follows: held at 50°C for 2 min and from 50 to 280°C at the rate of 10°C/min, held for 5 min. The total running time was of 30 min. The GC-MS interface temperature was at 280°C. The injection volume was 1 µl. The solvent delay was 3 min and was injected in a splitless mode. The MS scan range was from 35 – 6,000 Da.

Compound identification was done by comparing the retention time of the compounds with that of the available spectral data with the NIST library of the corresponding compounds.

RESULTS

ANTIBACTERIAL ASSAY

In the present study antimicrobial activity of *Salacia oblonga* wall extracts were evaluated by agar well diffusion method against drug resistant pathogens viz. *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Different solvent extracts of *S. oblonga* were evaluated for antibacterial activity, among them ethyl acetate extracts have shown superior activity compared to the other solvents. The aerial and root parts were separately analyzed for the activity. ethyl acetate extracts displayed good antimicrobial activity against drug resistant pathogens (Figure 1). Root and aerial extracts displayed the inhibition zones of $19.2\pm$ mm and $15.12\pm$ mm against *S. aureus*, $19.77\pm$ mm and $20.27\pm$ against *Klebsiella pneumoniae* and $17.8\pm$ mm and $18.17\pm$ mm against *Pseudomonas aeruginosa* respectively.

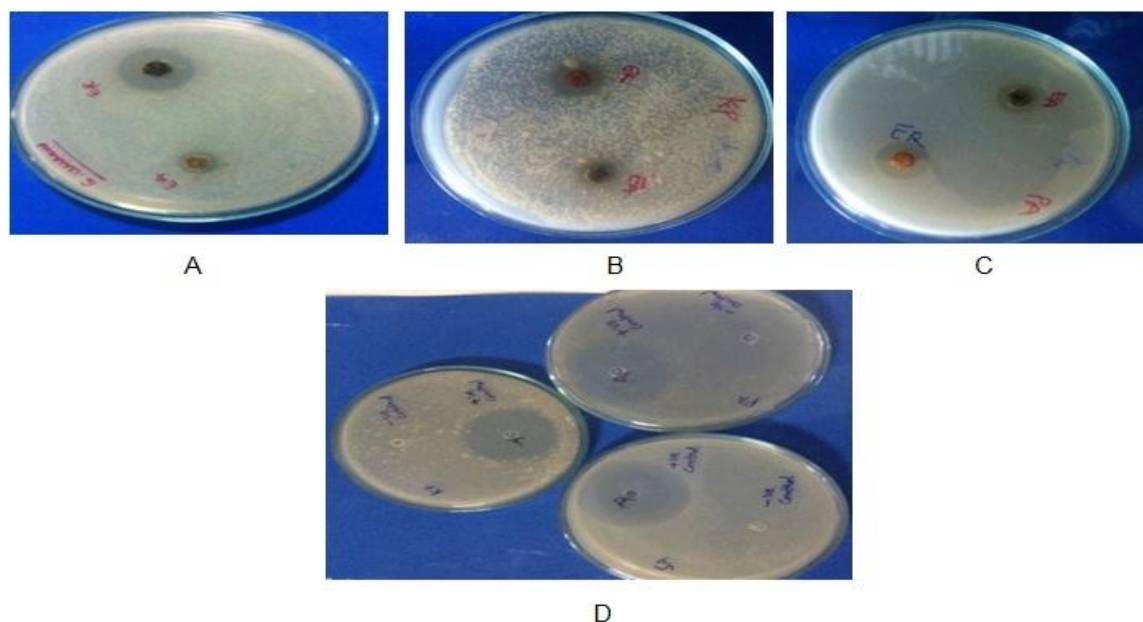


Figure 1. Effect of *S. oblonga* extracts against drug resistant pathogens. Zones of inhibition exhibited by aerial and root extracts of *S. oblonga* towards pathogen A: *S. aureus* B: *K. pneumoniae* C: *P. aeruginosa* D: Amikacin (positive control) and solvent (negative control).

ESTIMATION OF 260 nm ABSORBING MATERIAL

Leakage through bacterial cytoplasmic membrane was analyzed by determining the absorbance at 260 nm after treating the organisms with MIC concentration of the extracts. The results demonstrated that *S. aureus* have shown significant increase in OD at 260 over the control after 24 h and *P. aeruginosa* and *K. pneumoniae* exhibited increased OD values over the control cells. However, *P. aeruginosa* and *K. pneumoniae* have shown lesser OD values compared to *S. aureus* (Figure 2).

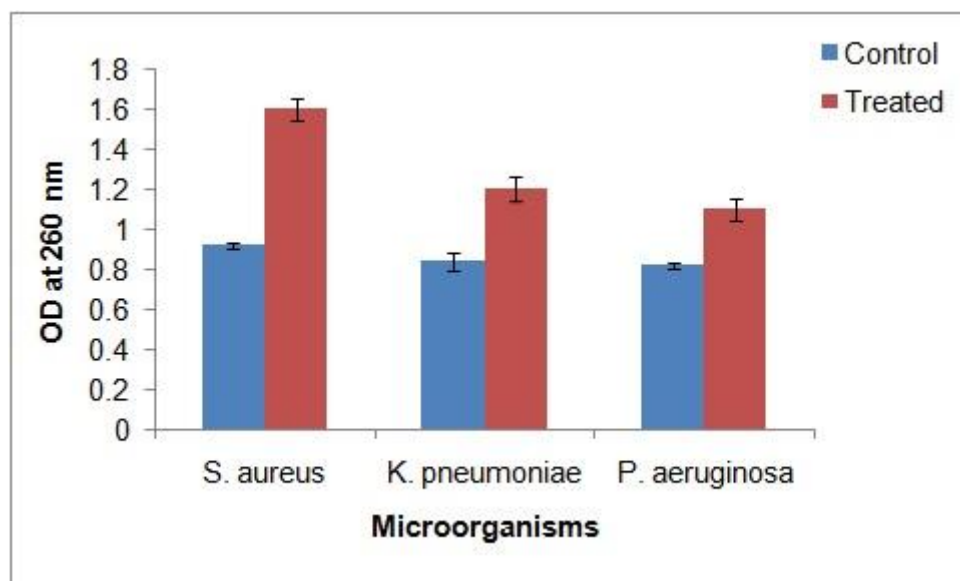


Figure 2. Absorbance of the cell material content of bacteria at 260 nm after treating with MIC concentration of the extract for 24 h. Values are mean \pm SD (n=3).

SEM ANALYSIS

Scanning electron microscopy studies were carried out to determine the morphology of the bacteria after treatment with *S. oblonga* extract for 24 h. The cells without treatment exhibited normal morphology of *S. aureus*. In contrast, the cells exposed to MIC concentration of the extract showed change in number, morphology and reduced size of the individual cells (Figure 3). This indicates that *S. oblonga* extract might act on membrane of *S. aureus*, thereby losing the membrane integrity and releasing the cytoplasmic content, that further leads to cell death.

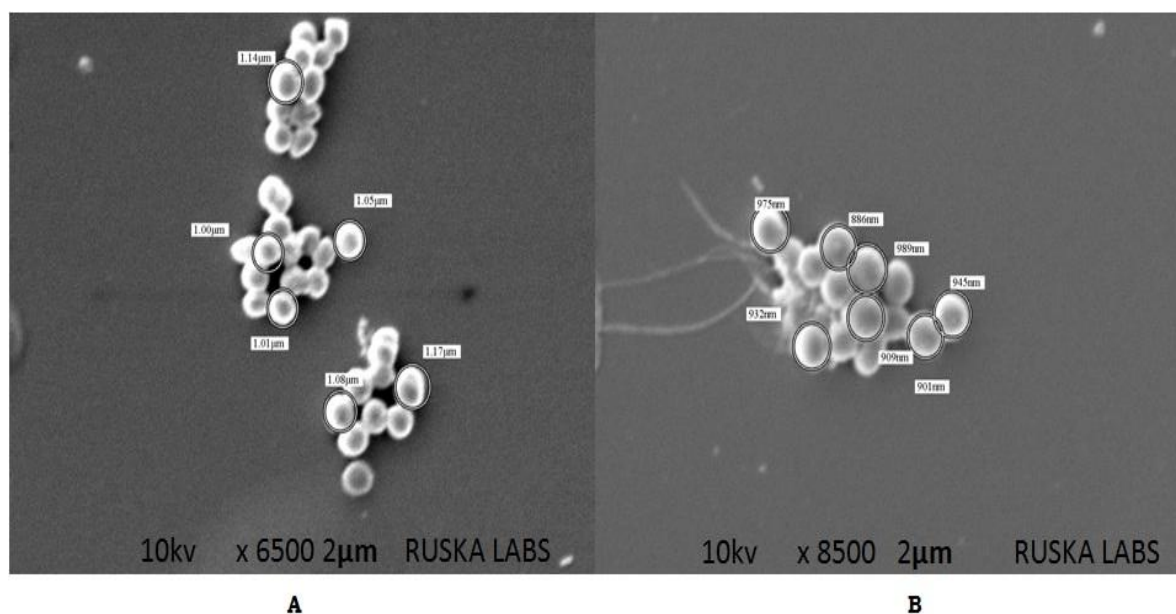


Figure 3. SEM photographs displaying the effect of *S. oblonga* extract on *S. aureus* A) control cells B) treated with plant extract (80 μ g/ml).

GC-MS ANALYSIS

In order to identify the phytochemicals from the crude extracts GC-MS analysis was carried out (Table 1).

Table 1. Content and composition of compounds in the ethyl acetate root extract of *S. oblonga* analyzed by GC – MS.

RT ^a	Library Compound	<i>S. Oblonga</i> root ^b extract
7.754	1,2,3-Propanetriol, monoacetate	2.31
10.700	Hexadecanoic acid, 3-hydroxy-, methyl ester	8.25
12.601	1,2,3-Propanetriol, diacetate	0.96
13.339	4-Tetradecene, (E)	0.85
13.791	1,3,5,6,7-Pentamethylbicyclo[3.2.0] hepta-2,6-diene	0.65
14.850	Benzonitrile	1.02
15.554	Phenol, 2,4-bis(1,1-dimethylethyl)	1.27
16.830	2-Tetradecene, (E)	1.22
17.116	1,E-11, Z-13-Hexadecatriene	2.00
18.786	Bicyclo[4.3.0] nona-3,7-diene, 8-(N-pyrrolidiny)-,cis	0.93
18.941	9,10-Dibromo-(+)-camphor	1.50
19.977	1-Nonadecene	1.08
21.253	Vitamin E	2.47
22.534	Tetradecanoic acid	11.99
22.838	5-Eicosene, (E)	1.64
24.840	9-Octadecenoic acid, (E)	14.56
25.132	Octadecanoic acid	5.25
25.453	Cyclopentadecane	0.73
	3H-Indazol-3-one,	
27.580	1,2-dihydro-5-methyl-2-(4-methylphenyl) ester	3.78
	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)	
29.640	ester	5.41
30.562	Olean-12-en-11-one	0.60
31.169	Eicosane	1.60
31.730	Hop-22(29)-en-3.beta.-ol	2.47
32.233	γ-sitosterol	6.66
32.548	Squalene	5.57
32.868	β-amyrin	3.2
	Cycloheptane,	
	4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-v	
33.549	inyl	6.39
	9,19-Cycloergost-24(28)-en-3-ol,4,14-dimethyl-,acet	
33.830	ate,(3.beta.4.alpha.5.alpha.)	4.13
	2,2,4,4a,6a,8a,9,12b,14a-Octamethyl-1,2,3,4,4a,5,6,	
	6a,6b,7,8,8a,9,12,12a,12b,13,14,14a,14b-eicosahyd	
33.984	ropicene	4.73

^a Retention time in minutes; ^b Relative area percentage (peak area relative to the total peak area percentage)

DISCUSSION

S. oblonga extracts displayed growth inhibition against drug resistant pathogens. *S. aureus* pathogenesis is due to a combination of nasal carriage and bacterial immune evasive strategies which is responsible for many infections [17]. Presence of Mec A gene in *S. aureus* confers resistance to many commercial antibiotics [18]. *P. aeruginosa* is a gram-negative opportunistic bacterium, one of the most important characteristic of this bacteria is antibiotic resistance which is attributed by the action of multidrug efflux pump with chromosomally encoded antibiotic resistant genes. In addition to this *P. aeruginosa* develops intrinsic resistance by mutation in the genome or horizontal gene transfer of antibiotic resistance determinants [17]. *K. pneumoniae* is a gram negative, non motile encapsulated bacteria and exhibits resistance to many commercial antibiotics [19, 20]. These three drug resistant pathogens displayed effective growth inhibition by *S. oblonga* extracts. Our previous results explained that extracts have low minimum inhibitory concentration 80µg/ml (MIC) and minimum bactericidal concentration 160µg/ml (MBC) values against drug resistant pathogens [21].

Plant secondary metabolites affect the microbial cells in different ways. These include disruption of membrane function and structure, interruption of DNA and RNA synthesis and function, interference with intermediate metabolism, induction of coagulation constituents and interruption of normal cell communication (Quorum sensing) [7, 22-23]. The antibacterial action generally includes the following sequence of events. Plant phytochemicals interact with the cell membrane and diffuse through the membrane (phytochemicals from the plant penetrate into the interior of the cell) and interact with intracellular constituents. Antimicrobial properties of secondary metabolites and their mode of action is dependent on several factors [3].

Leakage in the cytoplasmic membrane was analyzed by determination of the absorbance at 260 nm to detect the release of cell materials including nucleic acids, metabolites and ions [24]. Nucleic acid and its related compounds, such as pyrimidines and purines, absorb UV light at a wavelength of 260nm. The presence of these materials in a suspension may be used as an indicator of damage to the cell membrane causing leakage of the materials into the surrounding [25]. Diterpene compounds isolated from the medicinal plant *Salvia miltiorrhiza* acted on membrane resulting in increased 260 nm absorbance material [26]. Similar results were also reported from the diterpene compounds of the *Alpinia nigra* [27]. The UV absorption of *S. aureus* culture supernatant was shown in Figure 2, the release of 260 nm absorbing material from the *S. aureus* increased when treated with the MIC concentration of the extract, significantly higher than that of control values. 260 nm absorbance material and proteins were used as an indicator of irreversible damage to the membrane integrity [28]. Our results have shown leakage of 260 nm absorbing material suggesting damage of cytoplasmic membrane. The lipophilic antibacterial compounds present in the crude extract might interact directly with the membrane proteins resulting in alteration of membrane permeability and leakage of intracellular content [29]. The leakage of nucleic acids and proteins could cause the disorder

of function in the synthesis of proteins and DNA materials and the inhibition of bacterial growth.

To examine the antibacterial mode of action, it is important to estimate changes in bacterial membrane permeability, integrity, morphology and surface structure [30]. SEM analysis was done to understand the effect of *S. oblonga* extracts against *S. aureus*. Our findings have been supported by research groups with other medicinal plant [25]. The morphological changes in *E coli* and *Salmonella* were observed by SEM analysis after treatment with *Acacia* extracts [3]. Our results were also in corroboration with their findings. Further identification of phytochemicals from the crude extract was done by GC-MS analysis. Hexadecanoic acid 3-hydroxy methyl ester, Tetradecanoic acid, 9-Octadecenoic acid, (E), and γ -sitosterol were present in higher quantity, these compounds might be responsible for the antibacterial activity of the extract against drug resistant pathogens.

CONCLUSION

Our study clearly demonstrated that *S. oblonga* extracts exhibited good anti bacterial activity against drug resistant pathogens. Estimation of 260 nm absorbance material and SEM results prove that the extract might be working on cell membrane which further leads to cell death. Bioactive phytochemicals from the crude *S. Oblonga* extract were identified.

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Conflict of interest: Authors have no conflict of interest

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ERRATUM

In the Article “Investigation of Mode of Action of Anti Bacterial Activity of Salacia Oblonga Extract Against Drug Resistant Pathogen” with Running title: “Effect of S. Oblonga extract on drug resistant pathogen”, DOI number: <http://dx.doi.org/10.1590/1678-4324-2019180051>, published in the journal Brazilian Archives of Biology and Technology, vol. 62, page 1.

That read:

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Read:

“¹Jawaharlal Nehru Technological University Hyderabad, Institute of Science and Technology, Centre for Biotechnology, Hyderabad, Telangana, India;”