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Research Article

EVALUATION OF ANTIBACTERIAL POTENTIAL OF LEAF AND LEAF DERIVED CALLUS EXTRACTS OF *ORTHOSIPHON ARISTATUS* (BLUME) MIQ.

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ABSTRACT

Objective: To evaluate the antibacterial efficacy of chloroform, petroleum ether, ethyl acetate, methanol, ethanol and aqueous extracts of leaf and leaf derived callus of *Orthosiphon aristatus* against *Bacillus cereus, Bacillus subtiltis, Staphylococcus aureus, Streptococcus pyogenes, Enterobater aerogenes, Escherichia coli, Proteus mirabilis,* and *Klebsiella pneumoniae.*

Materials and Methods: The leaf segments were cut into small pieces of size 1-2 sq cm and were cultured on Murashige and Skoog solid medium supplemented with different auxins alone and in combination. Antibacterial efficacy was performed by disc diffusion method followed by minimal inhibitory concentration (MIC) determination by two-fold serial dilution method. Leaf and leaf callus extracts were subjected to the qualitative phytochemical analysis.

Results: Maximum callus formation percentage was obtained from the leaf segments cultured on MS medium supplemented with 2, 4-D (2 mg/l). Ethanolic leaf extract showed maximum inhibition activity with 28 mm zone of inhibition against *P. mirabilis* with MIC value of 0.32 mg/ml. Out of the callus extracts, ethanolic extract showed the maximum bio-efficacy against *S. aureus* with 26 mm zone of inhibition and MIC value of 0.64 mg/ml. Results revealed that both leaf and leaf derived callus extracts are effective against Gram-positive and Gram-negative test bacteria.

Conclusion: The bioefficacy study confirms the strong antibacterial potential of leaf and leaf derived callus extracts of *O. aristatus*.

Keywords: Leaf, Leaf callus, Antibacterial efficacy, *Orthosiphon aristatus.*

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INTRODUCTION

Plants as medicine have been used since ages and form the backbone of the primary health care system. It is necessary to establish a relation between traditional therapeutic value, biological activity, and chemical composition of medicinal plants. Popular observation and scientific validation of medicinal plants will significantly contribute to the development of natural and novel drug system. In India, medicinal plants constitute 50% of the higher flowering plant species [1]. Till date, approximately 20% of the medicinal plants have been subjected to the different biological assays [2]. Pharmaceutical industries invest considerable time and money in the development of the natural product-based drugs. Number of attempts has been made by far to develop an antibiotic of plant origin and still the efforts are on for this lead discovery. Although hundreds of medicinal plant species have been evaluated for their antibacterial properties, a vast majority has not been adequately evaluated [3].

Despite the advancement in science and technology infectious diseases claim millions of lives annually, especially in developing countries [4]. The treatment of infectious diseases faces a serious of issue of development of resistance by microbes against available antibiotics, which has necessitated a continuous search for the new antimicrobials of plant origin [5]. Today, multi-drug resistant bacteria such as methicillin resistant *Staphylococcus aureus*, vancomysin-resistant *Enterococci* Sp, *Klebsiella pneumoniae, Escherichia coli,* and *Pseudomonas aeruginosa* pose a serious threat to the human health. There is also an emergence of bacterial pathogens with intrinsic resistance to the available antibacterial agents, thus making them obsolete [6].

A proper approach needs to be adopted to counter this menace of antibiotic resistance. The search for new antimicrobials of plant origin could possibly counter the infectious disease threat and may lead to the discovery of natural and novel drug. Plants are rich source of antimicrobials and play an important role in the process of drug discovery and development [7]. In contrast to the synthetic antimicrobials, plant origin antibiotics are devoid of any ill effects and possess high therapeutic potential to counter infectious diseases. Screening of callus extracts for biological activities paves a way for the mass production of biologically viable secondary metabolites and thus provides an alternative to the *in vivo* plant material thereby minimizing any possible future threat to the natural habitat of medicinal plants.

Orthosiphon aristatus (Blume) Miq. also called as cat's whiskers, is an ethnomedicinal perennial herb and belongs to the family Lamiaceae, a native of Indonesia. It is 10-25 cm tall with quadrangular stem, leaves opposite, ovate to rhomboid with terminal inflorescence [8]. The plant is one of the most popular traditional folk medicines extensively used in Southeast Asia for the treatment of wide range of ailments. In Indonesia, leaves are used to treat rheumatism, diabetes mellitus, hypertension, tonsillitis, epilepsy, menstrual disorder, gonorrhoa, syphilis, renal calculi, gallstone, acute and chronic nephritis, gout arthritis, urinary tract, renal diseases, and fever [9-12]. It is also traditionally used to treat edema, eruptive fever, influenza, hepatitis, jaundice, and biliary lithiasis [13,14]. The medicinal property of the leaves of *O. aristatus* is attributed to the presence of bitter glycoside orthosiphon [15]. *O. aristatus* is patchily distributed and very rare in the field, hence, the conservation of this species is needed to ensure its sustainable utilization [16]. Bioefficacy potential of callus cultures sends a ray of hope for such highly medicinal plants, which if not protected may become extinct in near future.

In the present study, an attempt was made to screen the leaf and leaf callus extracts of *O. aristatus* for their antibacterial efficacy.

MATERIALS AND METHODS

Collection of plant material and callus induction

O. aristatus plants were collected from the Nilgiri Hills, Western Ghats of Tamil Nadu (November 2012), and was authenticated by Dr. Md. Umer Sharief, Scientist C, at Botanical Survey of India, Yercaud-Salem, Tamil Nadu, India, and are being maintained in the medicinal plant garden of Department of Studies in Botany, University of Mysore, Mysuru. Healthy leaf explants were collected from the mother plant and were washed under running tap water for 10 minutes to remove the soil and dust particles, followed by treatment with fungicide bavistin (1% w/v) for 5 minutes. Explants were then washed thrice with sterile double distilled water and were treated with mercuric chloride (0.01% w/v) for 5 minutes followed by washing thrice with sterile double distilled water. Inside laminar airflow, leaf explants were cut into small pieces (1 sq cm) and were inoculated onto the MS medium containing 3% sucrose, 0.08% agar and fortified with different concentrations of auxins. Before autoclaving at 121°C for 15 minutes, pH of the medium was adjusted to 5.8 using 1 N NaOH and 1 N HCl. The culture flasks were maintained in the incubation chamber under 16 hr photo period at light intensity of 25 $\mu mol/s^2/m^2$ for 4 weeks. Each experiment was performed with five replicate and repeated thrice. The callus cultures were maintained for the period of 5 months and were periodically subcultured with 2-3 weeks of interval onto the fresh MS medium for callus proliferation. Subsequently, the callus was harvested at the transfer age of 3 weeks, kept in hot oven at 60°C for 24 hrs till a constant dry weight was obtained, and the callus was then further exploited for extraction and antibacterial evaluation.

Dried leaves and the *in vitro* leaf derived callus were coarse powdered using electric homogenizer. Aqueous extraction was carried out by mixing leaf and leaf callus coarse powder separately with deionized water in the ratio of 1:5 (w/v) in conical flask and allowed to settle in an oven at 50°C for 72 hrs with occasional shaking. After 72 hrs, the extracts were filtered using whatman filter paper [17]**.** The filtrate was lyophilized to dryness and stored in vials at 5°C for further use. Solvent extraction was carried out by taking 25 g of dry leaf and callus coarse powder and filled in a thimble separately and extracted sequentially with 200 ml of petroleum ether, chloroform, ethyl acetate, ethanol, and methanol in soxhlet extractor for 48 hrs. The solvent extracts were concentrated under reduced pressure and were stored at 5°C in vials for further use.

The test bacteria; Gram-positive bacteria: *Bacillus cereus* (MTCC 430), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 1144), *Streptococcus pyogenes* (MTCC 422). Gram-negative bacteria: *Enterobacter aerogenes* (MTCC 111), *Escherichia coli* (MTCC 1687), *Proteus mirabilis* (ATCC 7002), *Klebsiella pneumoniae* (MTCC 7407) were procured from MTCC Chandigarh, India. All the test bacteria were freshly subcultured once in a week during work on nutrient agar slants (Himedia) and were incubated at 37°C for 24 hrs. From 24 hr old slant culture, a loop of inoculum was transferred to the 8 ml nutrient broth (Himedia) and incubated for 24 hrs at 37°C which was used as fresh suspension culture. The turbidity of bacterial suspension cultures was adjusted by adopting barium sulfate ($BaSO_4$) turbidity standard which is equivalent to 0.5 McFarland standard [18], which gives 10^6 CFU/ml bacteria (1×10^6 cell/ml). Antibacterial activity of leaf and leaf callus extracts was screened by adopting disc diffusion assay [19,20]. Sterilized 20 ml nutrient agar medium was transferred to the pre-autoclaved petriplates (90 mm) and allowed to solidify. 50 µl of broth suspension bacterial culture (24 hr old) containing 10^6 CFU/ml (0.5 McFarland standard) was put on to the solidified nutrient agar and was spread evenly using autoclaved glass spreader. Four impregnated discs (6 mm) were placed on inoculated nutrient agar in each petriplate, (i) leaf extract impregnated disc (10 mg/disc), (ii) leaf callus extract impregnated disc (10 mg/disc), (iii) standard streptomycin/gentamycin antibiotic disc (10 mg/disc) as reference drug, and (iv) solvent impregnated disc (100 µl) as solvent negative control. The plates were kept in refrigerator at 5°C for 20 minutes to let extracts and controls diffuse into the agar. The plates were then incubated for 24 hrs at 37°C. Antibacterial activity was evaluated by measuring the diameter of zone of inhibition after 24 hrs around each disc against the test bacteria. The experiment was repeated thrice, and statistical analysis was carried out using SPSS, DMRT with $p \leq 0.5$.

The microplate (96 well) method was used to determine the minimal inhibitory concentration (MIC) of leaf and leaf callus extracts [21]. Initially, 100 µl sterilized nutrient broth was put in each well of microplate. All the extracts (leaf and callus) were tested at 5 mg/ml and were serially diluted two-fold to 1 µg/ml after which 100 µl of bacterial cultures (10^6 CFU/ml) were added to each well. The final volume in each well was 200 µl. In each assay, streptomycin was used as reference drug. Control wells were prepared with one containing culture medium only and the other with normal bacterial suspension culture. The contents of each well were mixed on a microplate shaker at 900 rpm for 1 minute before incubation for 24 hrs [22]. After 24 hr, 20 µl TTC solution (2,3,5-triphenyl tetrazolium chloride) prepared in deionized water (20 mg/ml) was added to each well and incubated for 1 hr in dark [1,21]. The microbial growth in each well was determined by reading the respective absorbance at 600 nm using Universal Micro Plate Reader. The qualitative phytochemical analysis of leaf and leaf callus extracts was carried out by the method described by Zhang *et al*., (2016) [23].

RESULTS

Callus induction was observed along the cut edges of leaf segments after 2 weeks of inoculation on MS medium supplemented with auxins. Callus induction percentage varied with respect to the concentration of the growth regulator used and the age of the explant. Young explants cultured on MS medium augmented with 2,4-D (1 mg/l-3 mg/l) showed high percentage of callus induction and proliferation. IBA and NAA supplemented medium induced hard and compact callus with low percentage of proliferation (Table 1).

Antibacterial activity was carried out by disc diffusion assay, and the results revealed the antibacterial property of both *in vivo* leaf extracts and *in vitro* leaf callus extracts. Both leaf and leaf callus extracts inhibited the growth of test Gram-positive and Gram-negative bacteria. Out of the six leaf extracts (petroleum ether, chloroform, ethyl acetate,

Table 1: Influence of auxins on callus induction from the leaf segments of *O. aristatus*

MS medium+plant growth regulator mg/l	Mean percentage of callus induction	MS medium+plant growth regulator mg/l	Mean percentage of callus induction
2,4-D		IAA	
0.5	68	0.5	43
1	86	1	47
1.5	90	1.5	56
2	90	2	62
2.5	92	2.5	64
3	98	3	70
3.5	72	3.5	76
4	70	4	79
4.5	74	4.5	82
5	68	5	78
IBA		NAA	
0.5	32	0.5	30
1	42	1	36
1.5	58	1.5	42
2	58	2	46
2.5	62	2.5	53
3	68	3	58
3.5	72	3.5	60
4	65	4	63
4.5	60	4.5	68
5	62	5	68

O. aristatus: Orthosiphon aristatus

ethanol, methanol, and aqueous extracts), maximum growth inhibition was reported in ethanolic leaf extract against *P. mirabilis* with 28 mm zone of inhibition. Chloroform and petroleum ether extracts showed no or very less growth inhibition against test bacteria. Antibacterial activity and zone of inhibition of leaf extracts against test bacteria are given in Table 2.

Leaf callus extracts also showed promising growth inhibition against both Gram-positive and Gram-negative test bacteria. Nonpolar solvent callus extracts, i.e., chloroform and petroleum ether did not show any bacterial growth inhibition against any test bacteria. The maximum zone of inhibition was reported in ethanolic callus extract against *S. aureus* with 26 mm of inhibition zone. The zone of inhibition of different leaf callus extracts against different test bacteria is given in Table 3.

The extracts (both leaf and leaf callus) which inhibited the growth of test pathogens as revealed by disc diffusion method were subjected to MIC determination which provides quantitative data of minimum concentration of active extract which inhibits the bacterial growth. The MIC values of active leaf and leaf callus extracts were determined by two-fold serial dilution microplate method. The MIC values of leaf and leaf callus extracts are presented in Tables 4 and 5, respectively.

DISCUSSION

The study was aimed to evaluate the antibacterial property of leaf and leaf callus extracts of *O. aristatus.* Effect of auxins on callus induction has been reported earlier by many tissue culturists [24-26]. In the present study, 2,4-D supplemented MS medium was found to induce maximum callusing in leaf segments. Our results are in concurrence with the earlier findings carried out on *Plumbago zeylanica* [24] and *Withania somnifera* [27].

In the present study, leaf and leaf callus extracts of *O. aristatus* inhibited the growth of both Gram-negative and Gram-positive bacteria. Out of the extracts tested, non-polar solvent extracts (chloroform and petroleum ether) both from leaf and leaf callus showed little or no zone of inhibition against test bacteria. Moreno *et al.* (2006) [28] explained in their studies that less polar compounds diffuse slowly into the medium as they are hydrophobic in nature which prevents their uniform diffusion through agar medium. Out of all the extracts tested polar solvent (ethanol, methanol and aqueous) extracts of both leaf and leaf callus showed promising results.

Successful prediction of natural compounds from the plant or callus extract depends largely on the type of solvent used in the extraction

Table 2: Antibacterial activity (zone of inhibition in mm) of leaf extracts of *O. aristatus*

Leaf extracts							
Pathogen	**Aqueous**	**Ethanol**	**Methanol**	**Ethyl acetate**	**Petroleum ether**	**Chloroform**	**Streptomycin**
B. cereus	2±0.43	3±0.65	15±0.45	4±0.65	5±0.54	-	40±0.55
B. subtilis	4±0.32	7±0.65	9±0.34	18±0.19	-	-	35±0.65
S. pyogenes	-	18±0.32	-	-	-	-	38±0.43
S. aureus	12±0.65	14±0.78	17±0.45	10±0.65	3±0.54	-	35±0.23
E. coli	-	12±0.67	20±0.34	-	-	-	40±0.53
P. mirabilis	-	28±0.65	15±0.36	-	-	-	38±0.54
K. pneumoniae	-	7±0.76	26±0.56	-	2±0.23	-	35±0.56
E. aerogenes	-	13±0.54	-	-	-	-	35±0.76

O. aristatus: Orthosiphon aristatus, B. cereus: Bacillus cereus, B. subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, P. mirabilis: Proteus mirabilis, K. pneumonia: Klebsiella pneumonia, E. aerogenes: Enterobacter aerogenes

Table 3: Antibacterial activity (zone of inhibition in mm) of leaf callus extracts of *O. aristatus*

Leaf callus extracts							
Pathogen	**Aqueous**	**Ethanol**	**Methanol**	**Ethyl acetate**	**Petroleum ether**	**Chloroform**	**Streptomycin**
B. cereus	-	3±0.27	10±0.45	-	-	-	40±0.55
B. subtilis	-	8±0.25	2±0.54		-	-	35±0.65
S. pyogens	-	10±0.15	-	-	-	-	38±0.43
S. aureus	3±0.42	22±0.62	3±0.45	12±0.54	-	-	35±0.23
E. coli	-	-	-	-	-	-	40±0.53
P. mirabilis	-	2±0.48	14±0.67	-	-	-	38±0.54
K. pneumonia	-	20±0.41	14±0.52	-	-	-	35±0.56
E. aerogenes	-	-	-	-	-	-	35±0.76

O. aristatus: Orthosiphon aristatus, B. cereus: Bacillus cereus, B. subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, P. mirabilis: Proteus mirabilis, K. pneumonia: Klebsiella pneumonia, E. aerogenes: Enterobacter aerogenes

Table 4: Minimal inhibitory concentration (mg/ml) of leaf extracts of *O. aristatus* against test bacteria

Test bacteria								
Extracts	***B. cereus***	***B. subtilis***	***S. pyogenes***	***S. aureus***	***E. coli***	***P. mirabilis***	***K. pneumoniae***	***E. aerogenes***
Aqueous	-	-	-	2.50	-	-	-	-
Ethanol	-	2.50	1.25	2.50	1.25	0.32	2.50	1.25
Methanol	0.62	1.25	-	0.62	0.62	1.25	0.62	
Ethyl acetate	2.50	1.25	-	1.25	-	-	-	-
Petroleum ether	5	-	-	2.50	-	-	2.50	-
Chloroform	-	-	-	-	-	-	-	
Streptomycin	0.062	0.062	0.062	0.12	0.25	0.25	0.50	0.25

O. aristatus: Orthosiphon aristatus, B. cereus: Bacillus cereus, B. subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, P. mirabilis: Proteus mirabilis, K. pneumonia: Klebsiella pneumonia, E. aerogenes: Enterobacter aerogenes

Table 5: Minimal inhibitory concentration (mg/ml) of leaf callus extracts of *O. aristatus* against test bacteria

Test bacteria								
Extracts	***B. cereus***	***B. subtilis***	***S. pyogenes***	***S. aureus***	***E. coli***	***P. mirabilis***	***K. pneumoniae***	***E. aerogenes***
Aqueous	-	-	-	1.25	-	-	-	-
Ethanol	5	2.50	1.25	0.64	-	2.50	1.25	-
Methanol	2.5	1.25	-	5	-	0.62	0.62	-
Ethyl acetate	-	-	-	1.25	-	-	-	-
Petroleum ether	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-
Streptomycin	0.062	0.062	0.062	0.12	0.25	0.25	0.50	0.25

O. aristatus: Orthosiphon aristatus, B. cereus: Bacillus cereus, B. subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, P. mirabilis: Proteus mirabilis, K. pneumonia: Klebsiella pneumonia, E. aerogenes: Enterobacter aerogenes

process. Traditional practioners widely use water as a solvent, but in our study, we found that organic solvents such as ethanol and methanol extracts exhibited more antibacterial activity against test bacteria than aqueous extract. Our studies are in concurrence with the earlier studies carried out by Martin, (1995) [29], Sanches *et al.,* (2005) [30], Aboaba *et al.,* (2006) [31], and Durmaz *et al.,* (2006) [32], who reported that generally water extracts of plants do not have much activity against bacteria. Eloff, (1998) [21] reported in his study that ethanol and methanol extracts are most effective than water and other nonpolar solvent extracts, and the same is in concurrence with our study.

In the present study, leaf extracts were more effective against the Gram-negative bacteria which are more complex than Gram-positive ones; their cell walls are more complex and make them less susceptible to antibacterial agents [33,34]. According to Gupta and Saxena, (1984) [35] who reported that the judgment of strong antibacterial activity is based on MIC values between 0.05 and 0.50 mg/ml, moderate activity on values between 0.6 and 1.50 mg/ml, and week activity on values above 1.50 mg/ml. In the present investigation, results revealed that the MIC values of the ethanolic leaf and leaf callus extract are 0.32 mg/ml and 0.64 mg/ml, respectively, which demonstrates their strong antibacterial activity against *P. mirabilis* and *S. aureus,* respectively. Antibacterial activity of leaf callus extracts has been reported earlier in several medicinal plants earlier like *Mentha arvensis* [1] and *Barleria lupulina* [36]. Callus cultures are considered to be very useful in the production of antimicrobial principles and are considered a *de novo* production of the needed metabolites through cell culture [37,38]. Tejavathi and Rao, (1996) [39] reported in their study that callus and regenerated plants sometimes show enhancement of secondary metabolites when compared to *in vivo* plants. Hence, an attempt was made to carry out the antibacterial study of *in vivo* plant material and *in vitro* derived leaf callus of *O. aristatus.*

CONCLUSION

The study shows a strong correlation with the reported traditional medicinal use for the treatment of infectious diseases. Both leaf and leaf callus extracts exhibited antibacterial activity. The extracts were more effective against gram negative bacteria. Callus mediated cell lines can be explored for the mass production of viable antibacterial secondary metabolites. Further studies need to carry out to isolate and characterize the active principles from leaf and callus cultures.

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