



The Sensitivity of Pathogenic Bacteria Isolated from Fish Labeo, Catla and Channa Worked with to *Alangium salvifolium*

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

The study was conducted to investigate the mixture of *Alangium salvifolium* extracts with distinguished organic solvents which were generally used to screen for antibacterial activity against *Macroccoccus brunensis*, *Bordetella hinzii*, *Morganella morganii*, *Salmonella bongori*, *Klebsiella oxytoca*, *Alcaligenes faecalis*, *Achromobacter xylosoxidans*, *Comamonas testosteroni*, *Pseudomonas aeruginosa*, and *Pseudomonas plecoglossicida* that obtained from seven fish pathogens. Three pathogens were elucidated in the study which is conducted by using SDS-PAGE to its localisation. The extracted solvents (Acetone) gave good and enhanced antimicrobial activity in their way. The present investigation is an essential step for developing plant-based drugs which are eco-friendly for the management of the pathogenic bacteria of a fish pathogen. Development of commercial formulation of botanicals used to enhance the probability of managing such pathogenic bacteria. Further investigations are necessary for developing commercial formulations based on fields, animal trails, and toxicological experiments. This research may be footprint towards managing the newer antimicrobial entities against the fish pathogen.

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1. INTRODUCTION

Communicable diseases are severe concerns regarding Aquaculture worldwide. Most of the bacteria pose significant threats to aquaculture farming with potentiality in concern with damage to mankind and other animals [1]. The conventional use of synthetic antimicrobial agents is in concern with the result to more resistant bacterial strains of the aquatic environment [2,3]. Considering this, there is an urgent need to search for alternative methods by which management of antibiotic-resistant bacteria without any toxicity can be easily reachable to the consumers, which are eco-friendly and effective to them. The global scenario is also supporting the development of modern drugs to from less toxic plant products with proven medicinal properties [4,5]. In India, medicinal plants are widely used by people for treatment and remedies [6]. The plant *Alangium salvifolium* is a small tree or shrub, native to South India and Ceylon. It belongs to the family Alangiaceae commonly known as *Ankolah*, a well known medicinal plant in Ayurveda [7]. The roots and the fruits of this plant are used in the treatment of rheumatism and haemorrhoids. Externally it is used to treat bites of dogs, rabbits, and rats [8]. The plant is also popular in anti-inflammatory, anti-fertility and cardiotoxic activities. Its dried seeds have traditionally been used to treat various ailments [9,10]. Therefore, the objective of the present study is to evaluate the antibacterial activity and elucidation evaluation of various organic solvent extracts that are obtained from *Alangium*, in most frequently isolated bacteria in the aquaculture industry.

2. MATERIALS AND METHODS

2.1 Plant Material and Extraction

Seeds of *Alangium salvifolium* (fig-1) were collected from the rural area of Bhopal (M.P), India in April 2017. These herbs were authenticated by Botanical Survey of India, Centre Circle (BSI), Allahabad, India and preserved in the herbarium of the department. All collected plant seeds were oven dried at 40°C and extracted with ethanol (EtOH), methanol (MeOH) and Acetone. For organic extractions, twenty grams of seeds (fine, coarse powder) sample was Soxhlet extracted with 350 ml each solvent and liquid portion was evaporated under

vacuum. For the antibacterial assay, each extract was re-dissolved in Tween-20 to obtain a final concentration of 100 mg/ml. Three strengths of extracts and standard drug concentration used in the antimicrobial assay. The Phytochemicals qualitative was an evaluation done by the use of a standard procedure phytochemical screening. All extraction parameters and Phytochemicals assay were summarised in Table 2 [11].



Fig. 1. Image of *Alangium salvifolium* (www.cimap.org)

2.2 Antibacterial Assay

The disc diffusion assay (Kirby-Bauer Method) was used to screen the herbal extracts for antibiotic activity [12]. Different gram-negative microorganism used for the study of their names and MCC No., listed in Table 1. The microorganism isolated from fish (Labeo, Catla, and Channa) body and identified their possible MTCC number and culture from the Department of Biotechnology, Barkatullah University, Bhopal, (M.P.) India. The pure culture of each bacterial strain was grown on Tryptone Soya Agar (TSA) plates and incubated for 2 days at 37°C. All extracts were sterilised by filtering through a 0.22 µm filter (Millipore), and sterile filter paper discs (Glass Micro-fiber filters, Whatman®; 6 mm in diameter) were impregnated with 15 µl of extract. There were four replicates on each plate and two plates for each extract tested for each bacterium. Positive controls consisted of different antimicrobial susceptibility test discs (Bioanalyzer). The fresh, pure 100% extracts obtained from the plant used to suitably dilute up to the concentrations of 20,

Table 1. Name of pathogenic bacterial/ microorganism

S.N	Codes	Bacterial strains	MCC no.
1.	Bact-1	<i>Morganella morganii</i>	1111
2.	Bact-2	<i>Salmonella bongori</i>	2025
3.	Bact-3	<i>Klebsiella oxytoca</i>	48267
4.	Bact-4	<i>Achromobacter xylosoxidans</i>	67785
5.	Bact-5	<i>Comamonas testosteroni</i>	827E
6.	Bact-6	<i>Pseudomonas aeruginosa</i>	E-120
7.	Bact-7	<i>Pseudomonas plecoglossicida</i>	F-20289

50, and 100 mg/ ml and applied onto the test organism using Kirby Bauer filter paper disc diffusion method.

2.3 Elucidation of Bacteria

2.3.1 Protein Extraction

108 CFU/ml, bacterium grew on TSB medium containing MIC concentrations of extracts. A control experiment was conducted in the absence of extracts. After the cultures were incubated at 37°C with shaking at 150 RPM for 12 h, the samples were centrifuged for 10 min at 6,000 g. The volume of suspension was then corrected with distilled water to have the powder to water ratio as 1:10(w/v). The suspension was stirred for 1h, followed by centrifugation at 4500 rpm for 30min to recover protein extract. The alkaline supernatant was separated and the pH was adjusted to 3.8 (by 0.1M HCl) to collect protein content as a precipitate by centrifugation (20 min, 4500 rpm) considering the corresponding approximate pI (16). The collected protein was once rinsed with distilled water to remove trace acid and stored in the freezer (-70°C).

2.3.2 SDS-PAGE assay

Detecting Purification and Determining Quantity of Sample Proteins Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on the crude protein extract as well as those sample fractions collected from the two column chromatography experiments using Laemmli and Favere' procedure [13,14]. Molecular mass marker proteins were employed to estimate molecular weights of protein samples. Electrophoresis was performed at 70 V through the stacking gel (5%), and at 120 V through the separation gel (12%).

2.4 Statistical Analysis

The obtained data were expressed as the average between the replicates [(mean value ±

standard error (n = 3)] and the coefficient of variation (C.V) among the replication were analyzed using SAS software for accuracy and precision. All tests were used in order to evaluate the differences in the inhibition zones among the plant extracts.



Fig. 2. Infected fish with bacterial pathogen

3. RESULTS AND DISCUSSION

In this present paper, I have evaluated Indian medicinal plant *Alangium salviifolium* for its antimicrobial potential. Preliminary Phytochemicals study of this plant collected from Madhya Pradesh, which reveals that there is a presence of alkaloids, glycosides, tannins, saponins, flavonoids, phenols, and steroids [11] separately for all extracts. Different extracts of AS is in the concentration range of (20, 50 and, 100mg/ml) were evaluated for antimicrobial activity, as shown in Table 3. During the study, a total of seven (07) bacteria were isolated from the selected herbal medicines. The results obtained from the antibacterial activity of various extracts of *A. Salviifolium* as discussed clearly indicated that only extract-4 (Acetone) have the inhibitory action on the test organism under *in vitro* conditions. The extract-4 gave the best activity index almost upon all forming an inhibitory zone of 11 mm to 34 mm and activity index 0.197 to 2.429 respectively. In the present study, the ethanolic, methanolic and Acetone extracts of *A. Salviifolium* seeds were used on all gram-negative bacteria used in the work. *Morganella morganii* has positively inhibited the ethanolic seed extracts at a minimum concentration of 20 mg/ml for a zone of 16 mm

diameter, while methanolic seed extracts are not found to be encouraging the antimicrobial property even at 100 mg/ml concentration against the same in the present study. Three strains selected for elucidation activity with the help of SDS-PAGE at the molecular level. We found a number of differences in the band pattern if, compared with the control strain protein pattern (molecular size) in PAGE. The *M. morganii*, from the experiments of antimicrobial studies was known to be sensitive for the two extracts; the extract-1 and extract-3. The bacterial whole cell lysates obtained as lysate-1, lysate2 and lysate-3 as lysate from the drug untreated bacteria as control, lysate from bacteria treated with extract-4 respectively. The banding pattern was depicted in the Fig. 2. The first lane of the gel designated as L₁ reveals the banding pattern of bacterial cell lysate without any treatment with extracts/drug, with a total

number of 8 bands; the second lane L₂ indicates the banding pattern of whole cell bacterial lysate treated with extract-1 with 3 clearly visible bands and a very feeble fade mark beyond the 14.4 KD molecular weights. From these results, it can be clearly pointed out that the extract-1 and extract-3 has a definite impact on cellular and protein functionality that may be responsible for inhibition and or killing of the microorganism *M. morganii*. Bands resulting from the direct colony *C. testosterone* the same intensity as those of the standard SDS-PAGE studies was showed to be sensitive for all three extracts; the extract-1, extract-2, extract-3. The lysates obtained as lysate-1, lysate-2, and lysate-4 as lysate from the drug untreated bacteria as control, The lanes L₂, L₃, L₄, and L₅ are the whole cell lysates of *C. testosterone* treated with the drug extract-1, extract-2, and extract-3 respectively. From the observation obtained from the SDS-PAGE gel describes the complete denaturation of the

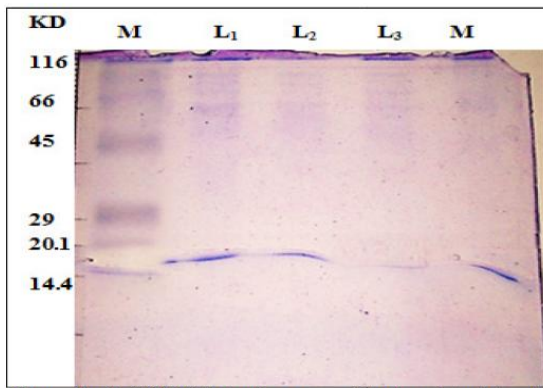


Figure No. 3: SDS-PAGE banding pattern of the bacterial whole cell lysates of all the 3 bacterial strains used in present study. (L₁ = *Morganella morganii*, L₂ = *Comamonas testosterone*, L₃ = *Pseudomonas plecoglossicida*; where M= molecular weight marker).

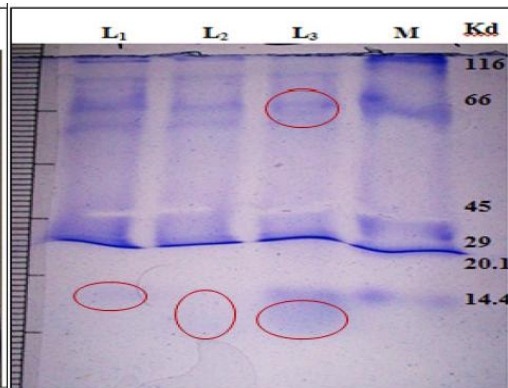


Figure No.4: SDS-PAGE banding pattern of the bacterial whole cell lysates of *Morganella morganii* after treated with phytochemical extracts. (L₁ = Control, L₂ = extract-4 treated and L₃ = extract-7 treated; M= molecular weight marker).

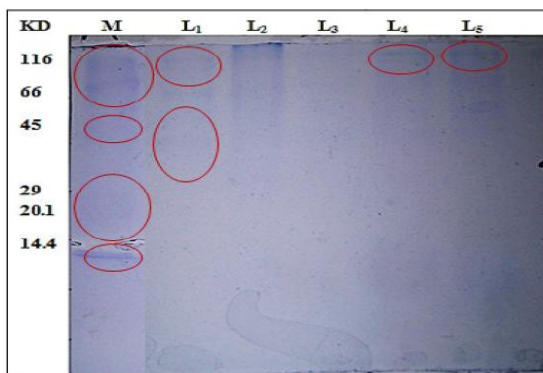


Figure No.5: SDS-PAGE banding pattern of the bacterial whole cell lysates of *Comamonas testosterone* after treated with phytochemical extracts. (L₁ = Control, L₂ = extract-3 treated, L₃ = extract-5 treated, L₄ = extract-7 treated and L₅ = extract-8 treated; where M= molecular weight marker).

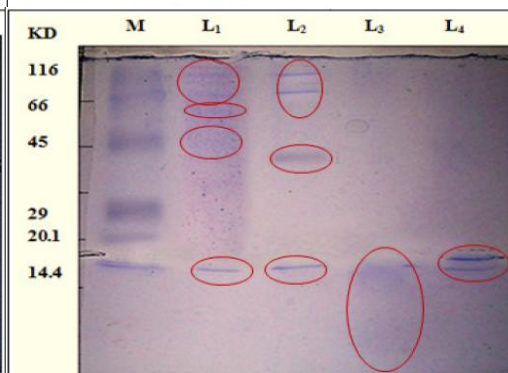


Figure No.6: SDS-PAGE banding pattern of the bacterial whole cell lysates of *Pseudomonas plecoglossicida* after treated with phytochemical extracts. (L₁ = Control, L₂ = extract-2 treated, L₃ = extract-4 treated, L₄ = extract-7 treated and L₅ = extract-8 treated; where M= molecular weight marker).

Table 2. Plant extraction and qualitative analysis of extracts

S. no	solvent	Ratio	Temp (hrs)	Time (.c)	% Yield	Phytochemical analysis								
						Alkaloids	Glycosides	Flavonoids	Steroids	Phenolics	Amino acids	Carbohydrate	Proteins	Saponin
1	EtOH	1:7	48	31	3.6	+	-	-	+	-	-	-	-	-
2	MeOH	1:8	42	31	4.8	+	+	-	+	+	+	+	+	+
3	Acetone	1:8	50	16	4.0	-	-	-	+	+	+	-	-	-

*+ (present) – (absent)

Table 3. The antimicrobial activity of different solvent extracts of *A. salvifolium* and antimicrobial activity index against some fish pathogenic bacteria

S. no.	Microorganisms	DRUG CONC. (ug/ml)	EtOH	MeOH	Acetone	stad	AI1)	AI(STD (02)	AI (03)	
1	<i>Morganellamorganii</i>	20	--	--	16±0.22	21	--	--	0.762	
		50	11.1±0.25	--	22±0.34	23	0.492	--	0.956	
		100	14.2±0.11	--	34±0.44	14	1.015	--	2.429	
2	<i>Salmonella bongori</i>	20	6.5±0.14	--	7±0.27	9	0.722	--	0.777	
		50	11.6±0.45	10±0.36	16.3±0.55	21	0.553	0.477	0.776	
		100	14±0.33	--	22±0.16	29	0.483	--	0.759	
3	<i>Klebsiella oxytoca</i>	20	--	4±0.23	11±0.57	9	--	0.445	1.223	
		50	--	--	5.7±0.53	21	--	--	0.197	
		100	10±0.39	11±0.49	17±0.38	29	0.345	0.379	0.587	
4	<i>Alcaligenesfaecalis</i>	20	10.4±0.29	5±0.53	15±0.15	21.4	0.486	0.232	0.700	
		50	--	--	18.1±0.32	26	--	--	0.696	
		100	14±0.35	--	22.3±0.19	33.1	0.422	--	0.674	
5	<i>Comamonastestosteroni</i>	20	--	9.4±0.14	11±0.11	19	--	0.495	0.578	
		50	--	--	11±0.16	22 ±0.16	27	--	0.408	0.814
		100	11±0.27	13.2±0.13	18±0.39	32	0.344	0.413	0.562	
6	<i>Pseudomonas aeruginosa</i>	20	2±0.59	10±0.45	11±0.11	17	0.118	0.589	0.648	
		50	7±0.21	9±0.11	13±0.16	24	0.292	0.375	0.541	
		100	10±0.36	120.15	17±0.26	29	0.588	0.413	0.586	
7	<i>Pseudomonas plecoglossicida</i>	20	8±0.16	9±0.26	10±0.21	20	0.400	0.450	0.500	
		50	10±0.21	11±0.11	13±0.11	26	0.385	0.424	0.500	
		100	14±0.31	15±0.41	29.7±0.17	32	0.438	0.468	0.928	

IZ= Inhibition zone (in mm) includes the diameter of disc (6 mm); Standards: streptomycin (1.0 mg/disc), Flucanazole (1.0 mg/disc); AI- activity index = IZ of test sample / IZ of standard. Values are mean of triplicate readings (mean ± S.D)

bacterial functional and structural protein as there is spreading to protein from the lysate throughout the length of gel without band formation. The lysates in lane L₄ and L₅ has one clear stable band in the range of 116 KD common to control lysate (Fig. 3). The *in vitro* treatment of bacteria *P. plecoglossicida* with extracts 1 & 4 describes the dissociative effect on bacterial cell protein when analyzed in Fig. 4 on the gel as higher molecular weight protein bands were completely disappeared from gel when compared to the banding pattern of the control lysate; moreover it spreads and accumulated towards the low molecular weight region as faded spots. It requires further studies to better understand the more defined target of the phytochemical drug extracts in the inhibitory action of bacterial strains. The comprehensive investigation of cellular proteins is called proteomics, which is an important area of research that in the expansion of the post-genome era [15]. Recently, the investigation of complex biochemical processes, the discovery of new proteins and investigation of protein-protein interactions are being done using proteomics and as a powerful tool. Moreover, the evaluation of protein profiles in response to multiple stress mechanisms, such as sensitivity to antibiotics or modifications related to antibiotic resistance, could represent a valid and integrating approach for the development of new therapeutic strategies. In complement, Mass Spectrometry (MS)-based proteomics and Bioinformatics were shown to be suitable for evaluation of the effect of protein extracts, whole cell versus outer membrane proteome (OMP), on the identification of Gram-negative organisms. The effect of phytochemical extracts can be easily evaluated by SDS-PAGE analysis if it was compared with control bacterial stain in Fig. 3. The present study represents the first practical application for the localization of microbial killing drugs of aquaculture diseases, a field lacking in terms of technological advancement. As the number of isolates from each region was dissimilar and low, it would not be advisable to determine a frequency profile of pathogens by location, neither the prevalence of bacterial genera by fish species, but we emphasize the importance of drawing a regional profile in aquaculture health monitoring programs and preventive management, therefore, in case of disease outbreak, treatment measures are different in each region, since factors such as light, water quality and soil contamination, quantity of parasites, management, etc are also peculiar to each locality.

4. CONCLUSION

This particular study enforces to conclude that, various extracts of seeds of *A. salvifolium* (W.) shows substantial antimicrobial activities against the gram-negative strain viz., *Macrococcus brunensis*, *Bordetella hinzii*, *Morganella morganii*, *Salmonella bongori*, *Klebsiella oxytoca*, *Alcaligenes faecalis*, *Achromobacter xylosoxidans*, *Comamonas testosteroni*, *Pseudomonas aeruginosa*, and *Pseudomonas plecoglossicida* especially with the Acetone, and Ethanol extracts. Furthermore, modern techniques (wet lab) and *in-silico* (dry-lab) study required to get concrete conclusion which needed to be explored for future research.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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