

IN VITRO AND IN VIVO ANTIMICROBIAL EFFECTS OF WRIGHTIA TINCTORIA (ROXB.) R. BR. AGAINST EPIZOOTIC ULCERATIVE SYNDROME IN CHANNA STRIATUS

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ABSTRACT

Murrels are often affected by the dreadful disease Epizootic Ulcerative Syndrome (EUS) and the primary causative agent of EUS is the fungus *Aphanomyces invadans* and the opportunistic bacterial pathogen is *Aeromonas hydrophila*. The antibacterial activity and antifungal activity of *W. tinctoria* were studied against selected pathogens and *in vivo* antibiotic effect in EUS affected striped murrel *Channa striatus* was also examined. The leaves of a medicinal herb namely *Wrightia tinctoria* (Roxb.) R. Br. was evaluated against five fish pathogens. Phytochemical screening of the leaves revealed the presence of steroids, reducing sugars, alkaloids, phenolic compounds, flavonoids, saponins and tannins. The TLC chromatogram of the extracts revealed several coloured bands. Antibacterial activities of the extracts were determined by the agar disc diffusion method. Chlortetracycline (5mcg/disc) was used as positive control for comparison of the inhibition zones. The extract showed efficient antibacterial activity against *Aeromonas hydrophila* with maximum zone of inhibition. The Minimum Inhibitory Concentration of the extract against the pathogens was determined. Growth of *Aphanomyces invadans* was inhibited by 85-90%. The herbal paste applied topically on the lesions showed a positive effect by controlling and curing the lesions. Thus, the active extract of *W. tinctoria* could be taken to the next step of bioassay guided purification to characterize the novel antimicrobial agents.

Keywords: Murrel, *Wrightia tinctoria*, EUS, Antibacterial activity, Antifungal activity.

INTRODUCTION

Murrels commonly called snakeheads, of the family Channidae (Ophiocephalidae) are the most common and dominant group of air-breathing freshwater fish. They are highly regarded as food fish in south-east Asian countries due to their taste, flavor, fewer intramuscular spines, medicinal and recuperative properties [1]. They are often affected by the dreadful disease Epizootic Ulcerative Syndrome (EUS) and encounter heavy loss in capture and culture fisheries. The primary causative agent of EUS is the fungus *Aphanomyces invadans* [2] and the opportunistic bacterial pathogen is *Aeromonas hydrophila* characterized as virulent [2, 3] or cytotoxic [4] which invades the blood stream and causes lethal septicemia resulting heavy mortality [5]. Moreover, *Vibrio sp.*, *Pseudomonas sp.* and *Salmonella sp.* [5-8] and virus [9] have also been detected in EUS affected murrels. The development of drug resistance as well as appearance of undesirable side effects of certain antibiotics [10] in EUS affected murrels have led to the search of new antibacterial agents, particularly medicinal plants as potential source of new antimicrobial agents [11], since they are safe without side-effects [12] and also eco-friendly.

Wrightia tinctoria (Roxb.) R. Br. belonging to the family Apocynaceae, is a small deciduous tree, distributed all over India [13]. Five flavonoid compounds, viz: indigotin, indirubin, tryptanthrin, isatin and rutin were isolated and identified from their leaves [14], for antimicrobial, hepatoprotective activity and to treat psoriasis, wound healing [15-17] and abdominal pain. With this background, antibacterial activity and antifungal activity of *W. tinctoria* were studied against selected pathogens and *in vivo* antibiotic effect in EUS affected striped murrel *Channa striatus* was also examined.

MATERIALS AND METHODS

The leaves of *W. tinctoria* (Roxb.) R. Br. were collected from the herbal garden of St. Xavier's College, Palayamkottai. Common fish pathogens *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Staphylococcus epidermidis*, *Aeromonas salmonicida* and *Vibrio alginolyticus* were purchased from Microbial Type Culture Collection (MTCC), Chandigarh. The bacterial strain were cultured in nutrient broth at 37°C and maintained on nutrient agar (HiMedia) slant at 4°C [18]. The fungus, *Aphanomyces invadans*, causative agent of EUS was isolated from diseased *C. striatus*, identified and the culture was stored on Czapek Dox Agar slants at 4°C until use.

The dried leaves were then powdered and macerated with 95% methanol (100 g dried powder sample/500 ml of 95% methanol) for 7 days at room temperature. The filtrated solvent was removed under vacuum at 40°C by using a rotary evaporator. The obtained crude extract was stored at 4°C until use. The condensed extracts were used for preliminary screening of phytochemicals such as alkaloids, steroids, reducing sugars, catechins, anthroquinones, flavonoids, terpenoids, sugars, phenols, saponins, tannins and amino acids. The presence of phytochemicals from methanol extract was qualitatively determined [19].

Thin layer chromatography

10 ml methanol extract of each sample was taken and evaporated; the paste of the evaporated extracts was used for TLC analysis. A combination of Petroleum ether, Methanol, Ethyl acetate, Benzene and Hexane in the ratio 2:1:1:1:1.5 was used as solvent mixture. The pre-coated silica aluminum plates were used for TLC studies of secondary metabolites. The extracts to be analyzed were spotted on the plates and they were placed in TLC chamber and the chromatogram was developed using the solvent mixture. The TLC plates were taken out and visualized in visible light, UV light (254 nm & 366 nm) and iodine chamber and spots were marked. The migration pattern was recorded and the R_f value of each spot was calculated using the formula, R_f value = Distance traveled by the solute/Distance traveled by the solvent and the values were tabulated.

Invitro studies

Antibacterial assay was carried out by disc diffusion method [20] using microbial cell suspension whose concentration was equilibrated to 0.5 McFarland standards (3 x 10⁸ cfu /ml). For this, 0.1ml (10⁻⁵ cfu /ml) of 24 h old bacterial culture was placed on Muller Hinton agar medium and spread throughout the plate by spread plate technique. The sterile filter paper disc of 6mm diameter was loaded with 5, 10, 15, 20 and 25µl of plant extracts dissolved in DMSO, placed on the surface of the medium and incubated at 37°C for 24 h. Antibacterial activity was recorded by measuring the diameter of zone of inhibition. Chlortetracycline (5 mcg / disc) was used as positive control and negative controls were maintained using paper disc loaded with 25 µl of DMSO. The entire test was performed in triplicate. The Minimum Inhibitory Concentration (MIC) of methanol extract was determined as the lowest

concentration of the plant extract inhibiting the visible growth of organism.

To the plant extract amended Muller Hinton Agar plate, 5 ml of the standardized inoculum was spread. After incubating the plates at room temperature (27° C) for 4 days or until the measurable growth, the diameter of the fungal mycelium was measured in test (plant extract amended) and also in the positive control. The mycelial growth inhibition in percentage was calculated as: The percentage of mycelial growth inhibition = (Control - Test)/Control X 100 [21].

The antibacterial activity was calculated by applying the expression: % RIZD = (IZD sample - IZD negative control) / IZD antibiotic standard × 100, where RIZD is the relative inhibition zone diameter and IZD is the inhibition zone diameter in mm [22]. All values were expressed as mean ± standard deviation. Data estimated for the Inhibition Zone Diameter of each concentration were analyzed using one way analysis of variance (ANOVA). P value < 0.05 was considered as significant. Using Originpro software the values of the inhibition zone was statistically analyzed through one way analysis of variance (ANOVA) followed by Tukey's test. Values of P<0.05 were considered statistically significant.

In vivo studies

Diseased *Channa striatus* (32.7± 1.75 cm and 247 ±18 g) with distinct dermal lesions including ulcers were purchased from Melapalayam fish market and were reared in cement tanks (15mX3mX2m) and fed with chicken intestine. Leaves of *W. tinctoria* (15 g) were weighed and were made into a paste was prepared using a mortar and pestle by adding 10ml of coconut oil. Fifteen diseased striped murels with moderate lesions were

taken from the cement tanks and divided into three groups each with five individuals. The herbal paste was applied topically on the lesions of the fish and were kept undisturbed in plastic troughs (Capacity: 50 litres) for 10 minutes and were again introduced into the cement tanks (3m×1m×1m). The treatment was given once in a day and continued for 3 days. Everyday fishes were taken from the rearing tanks and observed for wound healing and after that, the treatment was repeated. Meanwhile, the rearing tanks were cleaned and supplied with well oxygenated water from a nearby bore well. Water quality parameters were measured and recorded.

RESULTS AND DISCUSSION

Water quality parameters were recorded as pH - 7.0, Chloride - 200 ppm, Total Hardness - 525 ppm, Fluoride - 0.5 mg/l, Iron - 0.5 mg/l, Residual Chlorine - nil and Nitrate - 0.45 mg/l. Medicinal plants are the vital source of innumerable number of antimicrobial compounds. Several phytoconstituents like flavonoids [23], phenolics and polyphenols [24], tannins [25], terpenoids [26], sesquiterpenes [27], etc., are effective antimicrobial substances against a wide range of microorganisms. Phytochemical evaluation of the methanol extract of *W. tinctoria* showed the presence of steroids, reducing sugar, alkaloids, phenolic compounds, flavonoids, saponins and tannins, whereas triterpenoids, sugars, catechin, anthraquinones and amino acids were absent. The TLC chromatogram of the methanol leaf extract of *W. tinctoria* revealed 11 bands. The chromatogram viewed under various lights showed variously coloured bands and their Rf values ranged between 0.10 and 0.94 (Table 1). Gabriela [28] suggested that the colours of the separated spots in TLC and their position relative to standard substances are important characteristics for the plant extract identification.

Table 1: TLC fingerprint profile of *W. tinctoria* under various lights

S. No.	Colour of the bands				Rf value
	Visible light	UV light (366 nm)	UV light (254 nm)	Iodine vapour	
1.	Yellow	Orange fluorescence	Yellow	Light brown	0.94
2.	Grey	Black	Brown	Grey	0.86
3.	Brownish green	Orange fluorescence	Dark green	Green	0.82
4.	Bluish green	Black	Brown	Dark green	0.72
5.	Dark green	Fluorescence	Black	Light green	0.64
6.	Brownish green	Black	Yellowish green	Dark green	0.56
7.	Yellow	Orange fluorescence	Black	Yellow	0.46
8.	Green	Black	Yellowish brown	Brown	0.36
9.	Dark grey	Fluorescence	Black	Greyish green	0.26
10.	Light grey	Black	Brown	Grey	0.16
11.	Grey	Black	Black	Grey	0.10

The methanol leaf extract of *W. tinctoria* showed efficient activity against all the bacteria used in this study. The zone of inhibition against the five pathogens ranged from 7.0 to 15.6 mm. The highest inhibition zone 15.6 mm was formed against *Aeromonas hydrophila* at the highest concentration with 67.1% of RIZD, followed by 13.6 mm of inhibition zone against *Pseudomonas fluorescens* with 51.3% of RIZD. The least inhibition zone (10.3 mm) was found for *Staphylococcus*

epidermidis at the highest concentration with 25 µl extract. 10µl of the methanol extract was observed as the minimum inhibitory concentration against *A. hydrophila*, *A. salmonicida* and *V. alginolyticus* with 7.3, 7.6 and 7.0 mm of inhibition zones respectively (Table 2). The antibacterial activity possessed by the leaf extract was almost significant with the positive control used against all the bacteria with a certain concentration and the data are given in Table 3.

Table 2: Antibacterial activity of the methanolic leaf extract of *W. tinctoria*

S. No	Bacteria	Inhibition zone diameter (mm)/RIZD (%)					Positive control
		Methanol extract					
		5µl	10µl	15µl	20µl	25µl	
B ₁	<i>Pseudomonas fluorescens</i>	-	-	8.3±0.3/15.5	12.6±0.3/44.5	13.6±1.4/51.3	14.8
B ₂	<i>Aeromonas hydrophila</i>	-	7.3±0.3/9.1	9.6±0.3/25.1	13.0±0.5/48.9	15.6±0.3/67.1	14.3
B ₃	<i>Staphylococcus epidermidis</i>	-	-	6.6±0.3/5.1	8.0±0.5/17.2	10.3±0.3/37.0	11.6
B ₄	<i>Aeromonas salmonicida</i>	-	7.6±0.6/9.8	8.2±0.3/13.4	10.5±0.5/27.6	13.3±0.3/44.7	16.3
B ₅	<i>Vibrio alginolyticus</i>	-	7.0±0.5/7.1	7.6±0.3/11.4	9.6±0.8/25.7	12.3±0.3/45.0	14.0

Table 3: Statistical analysis of antimicrobial activity of *W. tinctoria* against fish pathogens

Bacteria	Concentration	MS value	F value	P value	Significance
<i>Pseudomonas fluorescens</i>	5µl	-	-	-	-
	10µl	-	-	-	-
	15µl	54.0000	64.8000	0.0012	Significant
	20µl	4.1666	5.0000	0.0890	Not Significant
	25µl	0.6666	0.1739	0.6980	Not Significant
<i>Aeromonas hydrophila</i>	5µl	-	-	-	-
	10µl	73.5000	55.1250	0.0017	Significant
	15µl	32.6666	24.5000	0.0077	Significant
	20µl	2.6666	1.6000	0.2745	Not Significant
	25µl	2.6666	2.0000	0.2302	Not Significant
<i>Staphylococcus epidermidis</i>	5µl	-	-	-	-
	10µl	-	-	-	-
	15µl	37.5000	112.5000	0.0004	Significant
	20µl	20.1666	30.2500	0.0053	Significant
	25µl	2.6666	8.0000	0.0474	Significant
<i>Aeromonas salmonicida</i>	5µl	-	-	-	-
	10µl	112.6666	135.2000	0.0003	Significant
	15µl	112.6666	338.0000	0.00005	Significant
	20µl	60.1666	90.2500	0.0006	Significant
	25µl	13.5000	40.5000	0.0031	Significant
<i>Vibrio alginolyticus</i>	5µl	-	-	-	-
	10µl	73.5000	73.5000	0.0010	Significant
	15µl	60.1666	90.2500	0.0006	Significant
	20µl	28.1666	16.9000	0.0147	Significant
	25µl	4.1666	6.2500	0.0667	Not Significant

Similar studies have been carried out by Khyade and Vaikos [29] with various solvent extracts of bark of *W. tinctoria* and *W. arborea* against gram positive and gram negative organisms. The chloroform extracts of *W. arborea* showed broader spectrum of antibacterial activity when compared with *W. tinctoria*. Kannan et al. [30] found that the methanolic and ethanolic leaf extract of *W. tinctoria* were active against bacteria and the hexane extract was active against dermatophytic fungi. They also suggested that the active principles may be useful in the tropical treatment of superficial skin infections. In the present study, methanolic extract of *W. tinctoria* was found to be effective in treating the fungus *A. invadans*, which is the primary causative agent of EUS in fishes. Methanolic extract of *W. tinctoria* exhibited good inhibitory efficacy against both *Staphylococcus* and *Salmonella* sp. [31].

Sridhar et al. [32] reported that hexane extract of *W. tinctoria* was active against dermatophytic fungi, suggesting that the active principles may be useful in the topical treatment of superficial skin infections. They also reported that the methanol extract was active against *Aspergillus flavus* and *Mucor indicus*. Similarly, *W. tinctoria* showed varying degree of inhibition against growth of *Curvularia luenata*, *Botrytis cinerea*, *Aspergillus niger*, *Aspergillus flavus*, *Trichophyton rubrum* and *Epidermophyton floccosum*. A similar report of 51–100% inhibition was observed in the leaf extracts against different dermatophytes [30,33], *Rauvolfia serpentina* [34], *Holarrhena antidysentrica* [35] and *Vinca rosea* [36].

An important characteristic of medicinal plant extracts and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will ultimately lead to death [37]. It has been suggested that, phytochemical extracts from plants hold promise to be used in allopathic medicine as potential sources of antimicrobial agents [38]. The active secondary metabolites synthesized with established potent antimicrobial activities have promised the application of medicinal herbs in pharmaceuticals, alternative medicines and natural therapies [18]. Hence, the antimicrobial activity of *W. tinctoria* against the fish pathogens might be due to the presence of steroids, reducing sugar, alkaloids, phenolic compounds, flavonoids, saponins and tannins. Harikrishnan et al. [39], illustrated the effect of intramuscular injection of azadirachtin (a tetra-nortriterpenoid), camphor (a terpenoid) and curcumin (a polyphenol) on the hematological parameters post injection of *A. hydrophila*. Similar

effect was found when treating the infected fish with neem paste [40] and turmeric paste [41].

In the present study, *W. tinctoria* was found to reduce the growth of *A. invadans* by 85-90% *invitro*. Herbal paste of *W. tinctoria* when applied externally on diseased *C. striatus* was found to heal the dermal lesions within 5 days *invivo*. After 2nd and 3rd day post application of herbal paste, healing symptoms were noticed and signs of dermal lesions and ulcers vanished and the wound was completely healed by the 5th day. No mortality was observed during the course of the study. The use of heavy antibiotics in aquaculture should be reduced and replaced with alternative medicines for prevention from fish diseases to avoid the emergence of antibiotic resistance in pathogenic and environmental bacteria. The herbal plants may be used as potential and promising source of pharmaceutical agents against fish pathogens in the organic aquaculture [42]. Sathianarayanan and Rajasekaran [43] reported that oral administration of ethanolic Leaf extract of *W. tinctoria* showed significant *invivo*, immunomodulatory activity and exerting its effect through diverse mechanisms that may involve cellular pathways. It is therefore suggested that the active extract of *W. tinctoria* could be taken to the next step of bioassay guided purification to characterize the novel antimicrobial agents.

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