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STROBILUS EXTRACTIVES OF *THUJA ORIENTALIS* AS NOVEL ANTIBACTERIAL AGENT AGAINST SOME PATHOGENIC BACTERIA

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ABSTRACT

Thuja orientalis is a well known red cedar commonly used as an externally applied tincture or ointment for the treatment of ringworm, warts and thrush. Antibacterial potentiality of the crude extract and different solvent extracts of the strobilus of *Thuja orientalis* was evaluated on microbial strains like gram negative *Pseudomonas fluorescens*, *Pseudomonas putida* and gram positive *Bacillus mycoides*, *Bacillus licheniformis* using agar well diffusion method during the present study. Chloroform: methanol (1:1 v/v) extracts showed the best result against the test bacteria. M.I.C. values of the aforesaid extract were found to be 0.36 mg/ml and 0.96 mg/ml against *Pseudomonas fluorescens* and *Pseudomonas putida* respectively while 1.09 mg/ml and 1.12 mg/ml for *Bacillus mycoides* and *Bacillus licheniformis* respectively. The chloroform: methanol (1:1 v/v) extract of strobilus of *Thuja orientalis* may act as a novel source to obtain an effective antibacterial agent.

KEY WORDS: ***Thuja orientalis*, Strobilus, Solvent Extracts, Antibacterial activity, Minimum inhibitory concentration.**

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INTRODUCTION

The microbial diversity is numerous in any particular ecosystem and biosphere[1] causing various human health hazards. Plants with immense medicinal application are widely used in the folklore medicines in India since antiquity. A number of scientists have focused their interest to investigate plant derived bioactive substances for the welfare of human health[2]. Several medicinal plant species have been known to produce chemical compounds and metabolites with preventive and curative properties against some fungal and bacterial diseases [3, 4, 5, 6]. Moreover phytochemicals are also being used as potential vector control agents[7, 8, 9, 10] for prevention of hazardous diseases. On the other hand, resistance against anti-biotics become an unceasingly therapeutic problem due to arbitrary consumption of medicines. Moreover, artificially derived chemical drugs are not only high-priced but are also with huge side effects. Therefore, the demand on the plant based therapeutics is rising due to their eco-friendliness, biodegradability, non narcotic nature, having minimal side effects and easily available at reasonable prices. Therefore to treat numerous diseases and to encourage the usage of herbal products, it is important to isolate the bioactive phytochemical compounds. Our present study concerns about the antibacterial potentiality of strobilus of *Thuja orientalis* against a few fish pathogenic bacteria namely *Pseudomonas putida*, *Pseudomonas fluorescens*, *Bacillus licheniformis* and *Bacillus mycoides* under laboratory conditions. *P. fluorescens* affects the physiology of nerve cells [11] and also acts as an expedient pathogen in immune compromised fish like Koi[12]. *P. putida* was found to be allied with rainbow trout disease in fishes[13]. *B. licheniformis* is commonly associated with food poisoning causing "ropy bread"[14]. The abortions of livestock are the most serious environmental hazard caused by this bacterium[15]. *B. mycoides* serves as a persuasive bacterial pathogen of channel catfish [16]. *Thuja orientalis* (L.) known as *Platycladus orientalis* of family-Cupressaceae is an evergreen and monoecious ornamental shrub which has been used in different activities such as antipyretic, antitussives, astringent, diuretic, refrigerant and stomachic[17]. The secondary phytochemicals of *T. orientalis* such as flavonoids and terpenoids[18], sesquiterpenoids in essential oils of different parts of the plant[19] showed different biological activities. The essential oil from seed coats of *T. orientalis* possesses antimicrobial activity[20] as well as antioxidant activity[21]. Based on the above fact, this is the first ever attempt to control some fish pathogenic bacteria with the extracts of strobilus of *T. orientalis* as per our literature review was concerned.

MATERIALS AND METHODS

Plant material

The strobilus of *T. orientalis* were precisely collected from Burdwan district (23°16'N, 87°54'E), WB, India during spring (mid-March to mid-April 2014). They were taxonomically legitimated by Professor Ambarish Mukherjee, Department of Botany, The University of Burdwan, Burdwan. The herbarium of the specimen has been deposited in the Department of Zoology, The University of Burdwan, having the Voucher specimen no. GCZD- 13. Initially the strobili were cleaned with distilled water followed by drying on paper towel in the laboratory at (37 ± 2 ^{o}C).

Test microorganism

Four bacterial strains viz. *P. fluorescens* (MTCC 103), *P. putida* (MTCC 1654), *B. mycoides* (MTCC 7343) and *B. licheniformis* (MTCC 530) were collected from Mosquito, Microbiology and Nanotechnology Research Units, Parasitology Laboratory. The bacteria were cultured in nutrient broth Hi-Media, M002 (Hi-Media Laboratories Limited Mumbai, India) at 37 ± ^{o}C all the above bacteria except *P. fluorescens* which is kept at 30 ± ^{o}C for optimum growth. The test micro organisms were maintained on nutrient agar slants at 4^{0}C with regular periods of subculture.

Antibiotics

Antibiotic discs (Span Diagnostics Limited, India) of different concentrations such as Amoxicillin (30µg), Kanamycin (30 µg), Nalidixic acid (30 µg) Chloramphenicol (30 µg), Tetracycline (30 µg), Norfloxacin (10 µg), Ampicillin (10 µg), Penicillin (10 µg), Ofloxacin (5 µg) and Ciprofloxacin (5 µg) were tested against those bacteria during the present assay and used as positive control.

Procurements of plant extracts

Crude extraction

The strobili of *T. orientalis* were minced in a mechanical grinder and the fluid was filtered by Whatman's no-1 filter paper. The filtrate was esteemed as a stock solution (100% concentration) for later bioassay experiments. The required concentrations 0.5%, 1.0%, 1.5%, 2.0% and 2.5% were set up by adding distilled water to the stock solution.

Differential solvent extraction

For solvent extraction, fresh and clean strobili of *T. orientalis* were air dried for 3 months approximately in shed. 100 g dried pods were put into the column of the Soxhlet apparatus and in the ratio of 1:10, 1 lit solvent was loaded into the solvent chamber. Three different solvents viz. chloroform: methanol (1:1, v/v), ethyl acetate and water were passed in a non-polar to polar fashion through the same column one after another respectively. The extraction period was 72 h with maximum 8 h a day for each solvent. Elutes were collected from chamber and made concentrated through air dried at room temperature for solvent elimination. The extractive of each solvent was preserved at 4°C for further bioassay.

Sensitivity Test

Following the disc diffusion method [22] antibiogram was carried out with commonly used antibiotics. Aseptically the test bacteria were isolated from the slant with inoculating loops and transferred to 5.0 mL of sterile distilled water containing test tubes. Until the turbidity equalled to 0.5 McFarland (10^{8}CFU/mL), sufficient inoculums were added. For each bacterium, one millilitre of the test tube suspension was added to the 15–20 mL of nutrient agar and transferred to the agar

plate (9 cm in diameter).The inoculated agars were set aside at room temperature for 25 min and after that antibiotic sensitivity test discs were placed on the surface of solid agar. The plates were incubated for 24 h at 37° C in the incubator. Antibiotic sensitivity was assayed by measuring the clear zones of inhibition formed around the discs. Diameters of the inhibition zone were interpreted as responsive, intermediate and resistant as per manufacturer's instructions.

Antibacterial Bioassay

The antibacterial assay was conducted by agar well diffusion method [23]. The bacterial strains grown on nutrient agar at 37°C for 18 h were suspended in saline solution (0.85% NaCl) and attuned to a turbidity of 0.5 Mac Farland standards (10^8 CFU/ml). The suspension was inoculated in Petri plates (90 mm diameter) and wells of 5 mm diameter were punched off after solidification of the agar, and filled with 30 ml each of 1000 mg/ml extracts. DMSO was taken as control for solvent extracts and sterile distilled water as control for crude extract. The plates were incubated for 24h at 37°C and antibacterial activities were evaluated by measuring the diameters of inhibition. The experiments were repeated thrice.

Determination of Minimum Inhibitory Concentration (MIC)

The value of MIC was determined by the dilution method as described by the National Committee for Clinical Laboratory Standards 1993 [22]. The cultures were watered down in Mueller-Hinton broth at a density adjusted to turbidity of 0.5 Mac Farland standards. Equal volume of 0.5 ml of each extract (by serial dilutions from the suspension of chloroform: methanol (1:1 v/v) and ethyl acetate solvent extracts stock solution) and nutrient broth were mixed in test tubes. 0.1 ml of standardized inoculums (5 x 10^5 CFU/ml) was added to each tube and incubated at 37°C for 24 h. Two control tubes were maintained for each test batch, one the antibiotic control (tube containing extract and the growth medium without inoculums) and another bacterium control (the tube containing growth medium, physiological saline and the inoculums). The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tube was regarded as MIC.

Statistical analysis

The results are presented as mean ±SD, analysed by using Excel and Easy plot software.

RESULTS

In Table-1 antibiogram assay of the test Gram positive and Gram negative bacterial strains were illustrated against some broadly used antibiotics and the given data are calculated by taking the mean value of three sets of observations. *P. fluorescens*, *P. putida*, *B. licheniformis* and *B. mycoides* strains were found to be resistant to antibiotics Ampicillin (10 µg), and Penicillin-G (10 µg). Susceptibility of all bacteria to Chloramphenicol (30 µg) was higher than Kanamycin (30 µg), Ofloxacin (5 µg) and Ciprofloxacin (5 µg). Among all the antibiotics assessed against the test microorganisms, *B. licheniformis* exhibited maximum sensitivity to Tetracycline (30 µg). The crude extracts exhibited the highest antibacterial potentiality against *P. fluorescens* (19 mm diameter of inhibitory zone). Among the solvents used the chloroform: methanol (1:1 v/v) extracts exhibited greatest antibacterial activity against *P. fluorescens* and *B. licheniformis,* followed by *P. putida and B. mycoides* (Table -2). The minimum inhibitory concentration (MIC) value was calculated for determining effectiveness of various extracts used but only chloroform: methanol (1:1 v/v) extracts inhibited the bacteria at lowest MIC value (Table-3).

Table 1

Susceptibility of four reference bacterial strains to some antibiotics in nutrient agar

Antibiotics (µg/ml)	Diameter of the inhibitory zones (mm)			
	P. fluorescence	*P. putida*	*B. mycoides*	*B. licheniformis*
Amoxicillin (30)	15	0	0	9
Kanamycin (30)	27	15	18	22
Chloramphenicol (30)	30	25	20	22
Nalidixic acid (30)	0	11	0	20
Tetracycline (30)	12	0	0	25
Norfloxacin (10)	24	16	8	0
Ampicillin (10)	0	7	0	0
Penicillin G (10)	0	0	0	0
Ofloxacin (5)	19	20	21	14
Ciprofloxacin (5)	19	22	17	11

Table 2
Antimicrobial sensitivity assay of crude and chloroform: methanol (1:1 v/v) extracts of strobilus of Thuja orientalis

Extracts of strobilus	Diameter of the inhibitory zones (mm)			
	P. fluorescence	*P. putida*	*B. mycoides*	*B. licheniformis*
Crude extract (1.0%)	19	10	12	17
Solvent extract (chloroform: methanol) (1:1v/v)	30	22	19	26
Solvent extract (Ethyl acetate)	2	1	0	1
Sterile distilled water	0	0	0	0
Dimethylsulphoxide	0	0	0	0

Table 3
Minimum inhibitory concentration of bioactive fraction of chloroform: methanol (1:1v/v) from strobilus of Thuja orientalis

Bacteria	M.I.C. Value (mg/ml)
Pseudomonas fluorescens	0.36
Pseudomonas putida	0.96
Bacillus mycoides	1.09
Bacillus licheniformis	1.12

DISCUSSION

The antibacterial drug preparations from botanicals were documented as an alternative medicine for synthetic drugs. The plant extracts are cost-effective and efficient herbal drugs may be prepared to combat microbial infections. The finding of this study indicates the effective *in vitro* activity of the crude and solvent extracts of *T. orientalis* against selective bacterial species. Mukherjee et al (2015)[24] showed the antibacterial property of *M. oleifera* against *P. fluorescens, B. licheniformis, B. mycoides* and *P. putida* at quiet low concentration of petroleum ether pods extracts with inhibition zone of 28.6 µg/ml and 32.0 µg/ml respectively for gram negative bacteria *P. fluorescens* and *P. putida* while 39 µg/ml and 42 µg/ml values were obtained for gram positive bacteria *B. mycoides* and *B. licheniformis* respectively. However, in the present study, antibacterial activity of crude extract of strobilus of *T. orientalis* against *P. fluorescens, B. licheniformis, B. mycoides* followed by *P. putida* was remarkable with an inhibition zone of 19, 17, 12 and 10 mm respectively. Duhan et al (2013)[25] established the antibacterial activity of various extracts (viz. methanol, acetone and ethyl acetate) of *T. orientalis* against human pathogenic microbes *Staphylococcus aureus, Bacillus subtilis*, *Pseudomonas aeruginosa*, *Acaligens faecalis* and *Klebsiella pneumonia.* Methanol and acetone extracts of leaf exhibited maximum activity against *B. subtilis* with zones of inhibition of 20.33 mm and 17.83 mm, respectively. All the extracts of stem of *T. orientalis* were found to be highly effective against *P. aeruginosa. S. aureus* was found to be sensitive to leaf extracts prepared in methanol, acetone and ethyl acetate with 13.66, 14.03 and 15.00 mm zone of inhibition, respectively. Likewise, in the present piece of work, among all the active fractions, the chloroform: methanol (1:1 v/v) extract of *T. orientalis* showed highest inhibition zone at 30 ppm doses against the test bacteria with an inhibition zone of 30, 22, 19 and 26 mm accordingly. Jasuja et al (2013)[26] also established the antibacterial properties of leaves of *T. orientalis* of methanol: distilled water (70:30) extract against *S. aureus, B. subtilis, Escherichia coli* and *Agrobacterium tumefaiens.* The minimum inhibitory concentrations (MICs) of the extract ranged from 0.55 to 1.15 mg/ml. The antimicrobial property of the present study showed the MIC value at relatively low concentration of active fraction ranging from 0.36 to 1.12 mg/ml. Compared with the commercially available antibiotics, the inhibition zone exhibited by chloroform: methanol (1:1 v/v) extract of *T. orientalis* was quiet significant (Table- 1 and Table- 2). In brief, the findings of the current investigation reveal that the strobilus extract of *T. orientalis* exhibits remarkable antimicrobial activity against the test fish pathogenic bacteria. Further laboratory investigations are required for illuminating the actual chemical compound responsible for antibacterial activity and also to identify the chemical personality of the active ingredient.

CONCLUSION

The strobilus extract of *T. orientalis* were proved to be a bioactive antibacterial agent which could be used for treatment of the fish diseases in future. Conversely, isolation of individual compounds and their biological activities required to be exposed further to develop its importance and open new opportunity in the field of investigation.

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