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In vitro antibacterial activity of various tissue types of *Dumortiera hirsuta* (Sw) Nees from different altitudes of eastern Himalaya

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ABSTRACT

Objective: To study the antibacterial activity of methanol and ethanol extracts of vegetative, male sexual and female sexual reproductive thalli of the bryophyte *Dumortiera hirsuta* collected from five different altitudes of eastern Himalaya against some human pathogenic bacteria. **Methods:** Well diffusion method, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were carried out against strains of human pathogenic bacteria. **Results:** Different tissue types of the bryophyte collected from various altitudes have shown to inhibit the human pathogenic bacterial strains in vitro. Maximum antibacterial activity was noted against *S. marcescens* (ATCC 13880) for both the 80% methanolic and ethanolic extracts of both the vegetative and the reproductive thalli. The extracts of the reproductive thallus showed less antibacterial activity than that of the vegetative thallus. Specimens collected from higher altitude showed slightly lesser antibacterial activity than their lower altitude counterparts. **Conclusions:** Antibiotic activity was noted to be dependent on solvent system used for extraction, altitude and tissue type. Analyses of extracts may yield some bioactive molecule responsible for antibacterial efficacy of the bryophyte.

1. Introduction

Bryophytes contain a number of secondary metabolites with diverse pharmacological activities. This group of plants were reported to possess antibacterial[1], antifungal[2], wound healing[3], antioxidant[4] and cytotoxic[5] efficacy. Their plant body is rich in several bioactive compounds such as terpenoids, phenols, glycosides, and fatty acids[6]. Due to their continuous effort to survive against a number of biotic and abiotic stresses, they are able to biosynthesize diverse types of secondary compounds[7]. Reports on traditional use of bryophytes as medicine are available[8] but very few attempts have been taken to exploit the pharmacological, clinical and medicinal potential of bryophytes. Ethnic use of bryophytes[9], when scientifically verified, might serve an exciting aspect to explore the immense bioactive potential of this less used plant group. The present study aims to explore the antibiotic potential of the said bryophyte as a non-conventional and unexploited source of antibiotics against some human pathogenic bacteria. Secondly, the altitudinal and vegetative and reproductive tissue specific variation of antibacterial activity is also observed.

Dumortiera hirsuta (Sw) Nees is distributed over a very wide range of geographical range. It is a thalloid liverwort growing on moist shady places by the streams and waterfalls. The thallus is dark green or yellowish green in colour. The male receptacles contain bristles and are borne on a very short stalk while the female receptacles are long stalked. Lack of air pores distinguishes the species from other hepatics such as *Marchantia polymorpha* and *Conocephalum conicum*.

2. Materials and methods

2.1 Collection of plant materials

The vegetative and male and female receptacles bearing

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thalloid plant body of *Dumortiera hirsuta* were collected at different seasons during the years 2010–2011 from five different altitudes of eastern Himalaya, India viz. Darjeeling town, Lava, Lolegaon, Rishap and Kalimpong. Altitudes and latitudes of the five locations are mentioned in Table 1. The voucher specimen was identified from the key to the specimen[10] and deposited at the department of Zoology and Molecular Biology, Presidency University, Kolkata.

2.2 Preparation of methanol and ethanol extracts

Fresh vegetative and male and female reproductive thalli were collected and washed in running tap water to remove soil particles from the ventral surface. The thalli were repeatedly washed in the detergent Teepol® followed by Bavistin to remove microbial contamination. Lastly, these were thoroughly washed by autoclaved distilled water. The plant material was air dried and powdered (40 mesh size) using an electrical mixer grinder. The fine powder (100g) was extracted in a Soxhlet apparatus for 4 days with 80% methanol and ethanol separately (3 changes of 100ml each). The extracts were pooled and a rotary evaporator was applied to evaporate the solvent under reduced pressure. The crude extracts were refrigerated at −4℃ for future use.

2.3 Antibacterial assay

2.3.1 Bacteria tested

Antibacterial activity of *Dumortiera hirsuta* was tested against ATCC strains of some common human pathogenic bacteria viz, *Shigella dysenteriae* (ATCC 9361), *Enterobacter cloacae* (ATCC 13047), *Staphylococcus aureus* (ATCC 25923) and *Serratia marcescens* (ATCC 13880). These strains were cultured on nutrient agar (Himedia, India) plates. For the purpose of storage, grown bacteria were then picked from the medium, and maintained in nutrient agar stab at room temperature and in nutrient broth containing 10% glycerol (Merck, Germany) at −20℃ until testing.

2.3.2 Evaluation of antibacterial activity

Antibacterial activity of the extract was determined by agar–diffusion assay, with the following modifications. Bacterial strains were first grown in Mueller– Hinton Broth (MHB) (Himedia, India) under shaking condition (120 rpm) for 4 h at 37 ℃ and after the incubation period, 1ml of culture were spread on MH Agar plates. In the inoculated MHA plate, wells were made by using sterile 6 mm cork borer. The wells were filled with 200 μl of the plants extracts (re–suspended in 80% methanol and ethanol). The concentrations of extract employed were 100, 150, and 200 mg/ml. The same assay was performed using the solvents only (80% methanol and ethanol, without the plant extract) to check the antibacterial activities of the solvents (negative control) and Tetracycline (150 μg/ml, 200 μl) was used as positive control. Zone diameter was measured after 24 h incubation at 37 ℃.

2.3.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC of the extract was assessed using the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards[11,12]. An inoculum of the microorganism was prepared from 24 h MHB cultures and suspensions were adjusted with turbidity equivalent to that of a 0.5 McFarland standard. Suspensions were further diluted 1:10 in sterile MHB to obtain a final inoculum of 5×10^5 CFU/ml. The 96– well round bottom sterile plates were prepared by dispensing 180 μl of the inoculated broth into each well. A 20 μl aliquot of the plant extract was added. The concentrations of plant extract tested were 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.25, 2.5 and 2.75 mg/ml. Dilutions of tetracycline served as positive control, while broth with 20 μl of 80% methanol/ ethanol were used as negative controls. Plates were covered and incubated for 24 h at 37 ℃. After incubation, minimum inhibitory concentrations (MIC) were read visually.

MBC of the extracts was determined by spreading 100 μl of samples from the 96– well plate used for MIC determination (after 24 h incubation) onto sterile MH Agar plates. After incubation for 24 h at 37 ℃, MBC values were determined as the lowest concentration of extracts, at which, no visible colonies of bacteria were seen after the incubation period.

Values of MIC and MBC were determined after performing the experiments in triplicate.

2.4 Statistical analyses

Calculations were carried out in triplicate with their mean values and standard error[13]. Values are expressed as mean ± S.D. Statistical significance was determined using Student's t–test. Values with $P < 0.05$ were considered significance. Preparation of tables was done by Microsoft Office Excel

Table 1
Place of collection of the plant materials.

SL. No.	Place of collection	Altitude (ft)	Location
(A1)	Darjeeling town, West Bengal, India	6710	27°3′13″N 88°15′59″E
(A2)	Lava, West Bengal, India	7200	27°5′11″N 88°39′47″E
(A3)	Lolegaon, West Bengal, India	5500	27°0′0″N 88°31′59″E
(A4)	Rishap , West Bengal, India	8500	27°6′33″N 88°38′57″E
(A5)	Kalimpong, West Bengal, India	4100	27°4′11″N 88°28′44″E

Table 2
Antibacterial activity of vegetative and reproductive thalli extracted in 80% methanol. (Zone diameter values are expressed in mm.)

Populations at different altitudes	Micro-organisms	Vegetative thallus			Reproductive thallus					
					Male sexual			Female sexual		
		100 μg/ml	150 μg/ml	200 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml
A1	*S. aureus*	6.5±0.891	7.7±0.437	8.0± 0.572	6.5±0.788	7.5±0.848	7.8±0.789	6.5±0.520	7.2±0.823	7.5±0.320
	S. dysenteriae	7.5±0.653	8.7±0.452	9.5±0.817	7.6±0.912	8.5±0.556	9.2±0.752	7.2±0.616	8.1±0.286	9.0±0.092
	E. cloacae	6.2±0.24	6.7±0.981	7.2±0.485	6.3±0.547	6.8±0.448	6.9±0547	6.1±0.785	6.4±0.925	6.8±0.411
	S. marcescens	9.5±0.526	9.7 ± 0.531	10.2 ± 0.985	9.5±0.478	9.6±0.805	9.9±0.639	9.3±0.328	9.4±0.207	9.5±0.829
A2	*S. aureus*	6.7±0.211	7.8±0.897	8.3±0.283	6.5±0.175	7.4±0.780	7.9±0.483	6.5±0.193	7.1±0.132	7.5±0.857
	S. dysenteriae	7.5±0.371	8.9±0.855	9.4±0.845	7.4±0.051	8.4±0.543	9.2±0.575	7.1±0.332	8.0±0.357	9.1±0.747
	E. cloacae	6.2±0.489	6.4±0.654	6.6±0.541	6.1±0.485	6.4±0.484	6.7±0.321	6.1±0.128	6.5±.0785	6.6±0.415
	S. marcescens	9.4±0.222	9.5±0.250	9.8±0.496	9.1±0.701	9.5±0.486	9.7±0.479	9.0±0.915	9.2±0.117	9.3±0.544
A3	*S aureus*	6.2±0.470	7.3±0.550	7.9±0.219	6.2±0.724	7.2±0.344	7.5±0.866	6.7±0.150	7.0±0.530	7.4±0.782
	S. dysenteriae	7.6±0.585	8.8±0.575	9.5±0.462	7.4±0.820	8.3±0.817	9.1±0.635	7.2±0.531	8.0±0.391	9.3±0.277
	E. cloacae	6.1±0.156	6.4±0.841	6.4±0.523	6.1±0.347	6.2±0.549	6.4±0.649	6.2±0.581	6.3±0.781	6.3±0.984
	S. marcescens	9.5±0.496	10.8±0.479	12.1±0.909	9.1±0.173	9.5±0.852	9.8±0.817	9.1±0.584	9.3±0.145	9.6±0.743
A4	*S. aureus*	6.5±0.489	7.5±0.631	8.9±0.554	6.8±0.913	7.6±0.930	7.7±0.150	6.5±0.424	7.4±0.882	7.6±0.857
	S. dysenteriae	7.5±0.586	8.6±0.751	9.5±0.454	7.5±0.757	8.4±0.080	9.3±0.477	7.1±0.530	8.3±0.835	9.2±0.327
	E. cloacae	6.3±0.894	6.5±0.541	6.8±0.052	6.1±0.045	6.3±0.458	6.4±0.842	6.2±0.482	6.3±0.291	6.5±0.651
	S. marcescens	9.4±0.351	9.2±0.503	9.7±0.056	9.2±0.845	9.1±0.141	8.9±0.744	9.0±0.729	9.1±0.811	9.1±0.572
A5	*S. aureus*	6.8±0.479	7.6±0.292	8.1±0.682	6.5±0.714	7.6±0.355	7.5±0.702	6.6±0.391	7.1±0.352	7.4±0.402
	S. dysenteriae	7.6±0.280	8.7±0.531	9.6±0.518	7.5±0.430	8.4±0.785	9.4±0.228	7.4±0.145	8.2±0.743	9.1±0.829
	E. cloacae	6.3±0.521	6.4±0.359	6.5±0.641	6.1±0.656	6.2±0.961	6.4±0.522	6.3±0.871	6.4±0.651	6.6±0.548
	S. marcescens	9.5±0.539	9.8±0.210	10.0±0.272	9.4±0.191	9.6±0.301	9.8±0.552	9.0±0.882	9.3±0.822	9.5±0.842

worksheet (2007).

3. Results

3.1 Antibacterial activity

In the present investigation, antibacterial activities of the 80% methanol and ethanol extracts of the vegetative and male and female reproductive thalli of *D. hirsuta* were studied against four human pathogenic bacterial strains, *S. dysenteriae* (ATCC 9361), *E. cloacae* (ATCC 13047), *S. aureus* (ATCC 25923) and *S. marcescens* (ATCC 13880). In all the experiments, both 80% methanolic and ethanolic extracts of the vegetative thallus showed more potent antibacterial activity than that of the reproductive thallus against the bacteria tested. Among the extracts, the 80% methanolic extract of both the vegetative and the reproductive thalli showed highest antibacterial activity against *S. marcescens* (ATCC 13880). At the highest of the concentrations tested, the maximum mean zone of inhibition of the 80% methanolic extract of the vegetative thallus and reproductive structures, against *S. marcescens* (ATCC 13880) were (12.1±0.909) mm and (9.9±0.639) mm respectively. In case of ethanolic extract, the highest mean zone of activity was observed also against *S. marcescens* (ATCC 13880), showing maximum mean zone diameter of (10.5±0.466) mm and (9.3 ±0.697) mm for the vegetative and the reproductive thallus respectively. Minimum antibacterial activity of the 80% methanol extract was observed against *E. cloacae* (ATCC 13047), maximum mean zone being (7.2±0.485) mm, and that of the ethanolic extract was observed against *S. aureus* (ATCC 25923), maximum mean zone of inhibition (6.7±0.645) mm. In general, vegetative thallus has shown higher antibacterial activity than their reproductive counterparts. Male and female reproductive structures bearing thalli were found to show more or less similar level of activity whereas the plants collected from lower altitude have exhibited higher antibacterial efficacy than that of the plants collected from higher altitude (Tables 2 and 3). Tetracycline (30 μg/ml) was used as the positive control and yielded mean zone of inhibition ranging from (14.5±0.652) mm to (11.1±0.636) mm (Table 4), and the negative controls (80% methanol and ethanol) showed no activity against the bacteria tested.

3.2 MIC and MBC

The MIC values varied from (0.5 to 1.25) mg/ml and from (0. 5 to 1) mg/ml for 80% methanolic and ethanolic extracts respectively. The range of the MBC values were from (1 to 2.5) mg/ml and from (1 to 2) mg/ml for 80% methanolic and ethanolic extracts respectively. Lowest MIC value of the 80% methanolic extract was observed against both *S. dysenteriae* (ATCC 9361) and *S. marcescens* (ATCC 13880) (0.5 mg/ml) and lowest MBC values were observed against *S. marcescens* (ATCC 13880) (1 mg/ml). Lowest MIC value of the ethanolic extract was observed against *S. marcescens* (ATCC 13880) (0.5 mg/ml) and lowest MBC values were observed also against *S. marcescens* (ATCC 13880) (1 mg/ml). (Table 5)

Table 3

Antibacterial activity of vegetative and reproductive thalli extracted in ethanol. (Zone diameter values are expressed in mm.)

Populations at different altitudes	Micro-organisms	Vegetative thallus			Reproductive thallus					
					Male sexual			Female sexual		
		100 μg/ml	150 μg/ml	200 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml
A1	*S. aureus*	6.4±0.472	6.6±0.154	6.6±0.682	6.2±0.543	6.3±0.965	6.4±0.585	6.1±0.241	6.3±0.578	6.5±0.595
	S. dysenteriae	6.5± 0.612	6.9±0.651	7.9±0.457	6.5±0.308	6.8±0.409	7.0±0.636	6.6±0.392	6.8±0.473	7.0±0.528
	E. cloacae	6.9 ±0.321	7.6±0.347	8.3±0.615	6.6±0.642	7.4±0.659	8.1±0.433	6.5±0.687	7.5±0.321	8.2±0.667
	S. marcescens	8.1±0.712	8.8±0.453	9.2±0.541	8.2±0.317	8.3±0.563	8.9±0.856	8.1±0.840	8.4±0.439	8.8±0.985
A2	*S. aureus*	6.1±0.545	6.2±0.585	6.4±0.288	6.2±0.548	6.2±0.689	6.4±0.867	6.2±0.891	6.3±0.476	6.4±0.453
	S. dysenteriae	6.6±0.972	6.9±0.812	7.5±0.331	6.5±0.468	6.7±0.639	6.9±0.199	6.5±0.763	6.7±0.434	6.9±0.532
	E. cloacae	6.7±0.743	7.2±0.224	7.9±0.241	6.5±0.582	7.2±0.803	8.1±0.594	6.7±0.697	7.9±0.623	8.2±0.675
	S. marcescens	8.2±0.694	8.9±0.252	10.5±0.466	8.1±0.486	8.4±0.586	8.7±0.143	8.2±0.365	8.5±0.523	8.9±0.682
A3	*S. aureus*	6.2±0.165	6.3±0.156	6.4±0.896	6.1±0.486	6.3±0.984	6.4±0.626	6.2±0.948	6.3±0.326	6.4±0.329
	S. dysenteriae	6.5±0.812	6.8±0.434	7.2±0.527	6.5±0.910	6.8±0.564	7.0±0.496	6.6±0.624	6.8±0.241	6.9±0.675
	E. cloacae	6.9±0.577	7.5±0.623	8.1±0.812	6.8±0.725	7.4±0.592	7.9±0.964	7.0±0.113	7.3±0.466	7.8±0.430
	S. marcescens	8.0±0.929	8.6±0.523	9.1±0.678	8.1±0.547	8.7±0.586	9.3±0.697	8.0±0.443	8.5±0.480	8.9±0.528
A4	*S. aureus*	6.1±0.476	6.4±0.983	6.7±0.645	6.2±0.984	6.2±0.321	6.2±0.651	6.2±0.489	6.4±0.653	6.5±0.836
	S. dysenteriae	6.6±0.872	6.9±0.480	7.5±0.953	6.6±0.391	6.9±0.475	7.0±0.365	6.7±0.924	6.8±0.217	7.1±0.667
	E. cloacae	7.0±0.309	7.5±0.217	8.2±0.596	7.1±0.751	7.3±0.827	8.1±0.624	7.2±0.216	7.5±0.741	7.9±0.426
	S. marcescens	8.1±0.730	8.5±0.741	9.2±0.863	8.0±0.508	8.4±0.504	8.9±0.592	8.1±0.564	8.5±0.775	9.0±0.468
A5	*S. aureus*	6.2±0.398	6.3±0.665	6.5±0.658	6.2±0.566	6.3±0.659	6.5±0.984	6.3±0.546	6.4±0.656	6.6±0.0312
	S. dysenteriae	6.7±0.241	7.0±0.171	7.3±0.195	6.6±0.399	6.9±0.942	7.1±0 475	6.5±0.331	6.7±0.872	7.0±0.255
	E. cloacae	6.9±0.466	7.4±0.412	8.2±0.137	7.0±0.196	7.6±0.628	8.0±0.827	7.2±0.395	7.6±0.309	8.1±0.223
	S. marcescens	8.2±0.527	8.6±0.687	9.3±0.135	8.5±0.252	8.9±0.835	9.2±0.504	8.4±0.352	8.6±0.203	8.9±0.244

Table 4

Mean zone of inhibition shown by positive control tetracycline. (Zone diameter values are expressed in mm.)

Micro-organisms	Zone diameter of tetracycline (30 μg) (Positive control)
S. aureus	11.1±0.636
S. dysenteriae	12.5±0.365
E. cloacae	14.5±0.652
S. marcescens	14±0.558

Table 5

MIC and MBC of two extracts of *Dumortiera hirsuta* (mg/mL).

Microorganisms	80% Methanol (mg/ml)		Ethanol (mg/ml)	
	MIC	MBC	MIC	MBC
S. aureus	1.25	2.5	1	2
S. dysenteriae	0.5	1.5	0.625	1.25
E. cloacae	1	2.25	0.75	1.5
S. marcescens	0.5	1	0.50	1

4. Discussion

Plant extracts and products have been reported as antimicrobial agents[14]. Ethanol and methanol extracts were found to be useful in several antimicrobial experiments[15,16]. The polar extracts (ethanol and methanol) have revealed the presence of several bioactive phyto-constituents such as alkaloids, flavonoids, saponins, triterpenoids etc.[17]. Human pathogenic bacterial strains were reported to be inhibited by plant extracts in vitro[18,19,20]. Plants extracted in different solvent systems have been proved as antibiotic against multidrug resistant bacteria[21,22]. Bryophytes have been reported to possess antibiotic activity[23]. Antibacterial[24] and antifungal[25] activities of bryophytes have been reported in many recent investigations. Among the several compounds bibenzyls[26], flavonoids[27], sphagnum acid [p-hydroxy-beta-(carboxymethyl)-cinnamic acid][28], sesquiterpenes[29] etc. isolated from different bryophytes have been reported to possess antibiotic property.

D. hirsuta has been investigated for various pharmacological activities. In vitro and in vivo experiments have proved the cytotoxic efficacy of riccardin D, a macrocyclic bisbibenzyl compound isolated from the species[30,31]. Crude extracts of the species has been tested for radical scavenging and antimicrobial activities and different sesqui- and diterpenoids and aromatic compounds have been isolated[32].

Lunularin, isolated from the same has shown cytotoxicity and antimicrobial effects against *Pseudomonas aeruginosa*[33]. Earlier sesquiterpenoids and some other bioactive constituents were isolated from the species[34,35]. Dumortane derivatives[36] and carboxylated α-pyrone derivatives[37] have been reported from the species.

Secondary metabolites of botanical origin have been reported to possess different pharmacological activities. Content of such metabolites may vary depending on several factors such as altitude, latitude, tissue type, season etc. Altitude is considered as one of the prime factors responsible for such variation[38,39]. Phenol, considered as one of the primary bioactive molecule present in plant kingdom, was also reported to vary in plants growing at different altitudes[40]. Temperature has been considered to be responsible for this phenomenon[41]. The variation of secondary metabolites has been recorded due to different exposure to ultraviolet (UV) radiation[42] at different altitudes. Bryophytes are said to be affected by different levels of UV radiation[43]. Secondary metabolites in vegetative and reproductive tissues of plants have been investigated[44]. Tissue age specific variation of secondary metabolites was observed in plant species[45]. Variation in antibiotic activity in the present investigation may be ascribed to the differential effect of temperature and UV radiation on the secondary metabolite production in different tissue types of the bryophyte collected from different altitudes. Possible variation in secondary metabolites in vegetative and reproductive thalli could be the reason behind the different antibiosis phenomenon shown by the two types of thalli.

The present study indicates antibacterial efficacy of the hepatic which is comparable to the standard antibiotic. It also shows the variability of activity of vegetative and reproductive plant bodies of the same species collected from various altitudes which was tentatively attributed to the quantitative and qualitative variation of certain bioactive secondary metabolites. Bioactivity guided fractionation and isolation of novel compounds can be utilized against bacteria and bacteria related diseases as an alternative to conventional synthetic drugs, prolonged use of which has resulted into development of drug resistance in certain microbes. Furthermore, the possible toxicity of herbal formulations must be considered and rigorous clinical trials should be performed to use such alternative sources of medicine.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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