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Antibacterial Activity of Pteridophytes and *Nigella sativa* Against Antibiotic Resistant Bacteria Isolated from Wastewater Environment

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Abstract: Bacterial resistance to antibiotics has become a global problem impacted partly by use of antibiotics in various environments. New antimicrobial agents are therefore urgently needed to overcome this problem. This study was carried out to investigate the tetracycline (TC) and ampicillin (AMP) resistant bacteria in wastewater environments of Rajshahi Metropolitan City, Bangladesh. In this investigation ethanol and acetone extracts of Dryopteris sp. (leaves), *Pteris vittata* L. (leaves) and *Nigella sativa* L. (seeds) were examined for their antibacterial activity. A total of 34 TC and AMP resistant bacteria were isolated from wastewater sources. Among them, a random selection of eight strains (three gram negative and 5 gram positive) was used for this study. The zone of inhibition for *Dryopteris* sp. leaves ranged from 6-11 mm and 6-12 mm and for *Pteris vittata* L. leaf extract, from 6-12 mm and 6-13 mm within ethanol and acetone extracts, respectively. Acetone extract of *Nigella sativa* L. seeds at the concentration of 2500 µg/ml had a marked sensitivity (7-18 mm) towards three tested gram positive bacteria in this study. The results of the present work provide essential baseline information for the use of the studied plants in the fight against drug resistant phenotypes.

Keywords: Antibacterial Activity, Pteridophytic Plants, *Nigella sativa*, Leaves Extracts, Antibiotic Resistant Bacteria

1. Introduction

Antibiotics are poorly absorbed in human and animal gastrointestinal tracts with a majority of them being excreted unchanged in feces and urine which eventually find their way into the environment through sewage, hospital wastewater and household wastewater. These bacteria are capable of spreading their genes into water-indigenous microbes, which contains resistance genes also [1]. As a result antibiotic resistant bacteria enter into water environments from various sources [2]. Antibiotic resistant bacteria and drug resistance genes now a days are an important environmental contamination issue which has received increased attention not only abroad [3-5] but also in Bangladesh [6]. Resistance against antimicrobials may cause severe problems in human and animal health care [4, 5]. Waste water environment occurs largely in distributing of antibiotic resistant bacteria that concern to human health. The resistance can spread through food crops irrigated with affected water [7].

Due to the increase of resistance to antibiotics by a variety of organisms is a major concern the modern medicines face [8].There is a pressing need to develop new and innovative antimicrobial agents [9]. Among the potential sources of new agents, the medicinal value of certain plants has been an ancient concept. The antimicrobial properties of plants have been reported through numerous studies from around the world and many have been used as the therapeutic alternatives because of their antimicrobial properties [10,11].Potential antibacterial activity of medicinal plants, such as *Lawsonia inermis*, L., *Mimosa pudica* L., and *Achyranthesaspera* L. were found against a number of gram-positive and gram-negative bacteria which were isolated from diarrheal patients [12,13]. Among the plants, seeds of *Nigella sativa* L. have been used for centuries for the treatment of various infectious diseases [14].More recently however, additional alternative plants have been attracting

more attention in the search for novel and potential molecules. For several years the medicinal value of the pteridophytes has been known [15].Hence, the present study was undertaken specifically to investigate resistance patterns in bacterial strains isolated from wastewater and the role of ethanol and acetone extracts of *Pteris vittata* L.(leaves), *Dryopteris* sp. (leaves) and *Nigella sativa* L.(seeds) as a potential antimicrobial agent against these isolated bacteria.

2. Materials and Methods

2.1. Sampling Sites and Sample Collection

This cross-sectional study was conducted from different sites of Rajshahi Metropolitan City, Bangladesh. Specifically, the study sites were assigned to different types of wastewater flowing drainage systems. These sites receive a high amount of wastewater on a daily basis, consisting of three types of wastewater: hospital effluent, municipal and household drainWastewater samples were collected using sterile bottles and performed in the early hours of the day. All samples were placed in an ice box and transported to the Plant Pathology, Mycology and Microbiology Laboratory, Rajshahi University, Bangladesh and processed within two hours of collection.

2.2. Isolation of Antibiotic Resistant Bacteria

To screen for resistant bacteria in wastewater samples, from 10ml of previously prepared 10-fold diluted sample in PBS, 100 µl was spread at 25 ºC for 24–48 hours in a nutrient agar (NA) medium supplemented with ≥32 µg/ml of tetracycline (TC) and ampicillin (AMP), respectively(original concentration was 2560 µg/ml). Strains that were resistant to ≥32 µg/ml of antibiotics were defined as "resistant strains" [3]; this criterion (≥32 µg/ml) has been commonly used [16]. The plates without the antibiotic treatment were used as control. After incubation, representative colonies were picked up and sub-cultured based on colony morphology. After obtaining pure colonies isolated organisms were identified by gram staining method following standard methods [17].

2.3. Plant Materials and Preparation of Extracts

Disease free leaves of *Dryopteris* sp. and *Pteris vittata* L. were collected from the Botanical Garden of Rajshahi University, Rajshahi, and the seeds of *Nigella sativa* L. were collected from Katakhali bazar, Rajshahi. These plants were substantiated by Taxonomist Dr. A.H.M. Mahbubur Rahman, Associate Professor, Department of Botany, Rajshahi University, Rajshahi, Bangladesh. A voucher specimen of those plants was deposited in the herbarium of Department of Botany, Rajshahi University, Rajshahi, Bangladesh.

The extracts of plant materials were prepared according to the method described by [12]. Fresh plant materials were first cleaned, oven dried at 40 °C and subsequently pulverized to a fine powder. Five grams of powdered plant sample was then taken and continuously shaken with 20 ml each of ethanol and acetone within a water bath at 60 °C for 10 h. The extracts were filtered through a Whatmann No. 1 filter paper and evaporation of solvents were made using a rotary evaporator. The evaporated extracts in respective solvents were dissolved (10 mg/ml). For MIC determination of studied plant extracts a two-fold serial dilution method was followed. Ten milligrams of the studied plant extracts were dissolved in 2 ml of the respective solvent to obtain a concentration of 5 mg/ml (5000 µg/ml) which after serial dilution yield2500, 1250, 625, 312.5 and 156.25 µg/ ml, respectively[18]. Discs measuring 6 mm in diameter were soaked with each extract and air dried under aseptic condition inside laminar flow.

2.4. In Vitro Antibacterial Activity

Antibacterial tests were performed by preparing a bacterial suspension followed by a disc diffusion test [19].For preparation of bacterial suspension, one single colony of bacteria was picked and inoculated onto a 5 ml nutrient broth (Merck, Germany) and incubated at 25 °C for overnight. The concentration of the bacteria for each disc diffusion assay was standardized to 108 cfu/ml based on the Mcfarland standard No.1. One hundred microliters (100µl) of inoculums (108 cfu/ml) were spread by a spreader on the NA media. Following, the previously prepared four different concentrations on2500, 1250, 625, 312.5 and 156.25 µg/ ml discs were placed on the surface of the medium seeded with the respective bacterial sample by sterile forceps. As a negative control only respective solvents (without extract) containing sterile blank discs were used and as a positive control TC/AMP (32 µg/ml) was used. After incubation, the antibacterial property of leaves of *Dryopteris* sp. and *Pteris vittata* L. leaves and the seeds of *Nigella sativa* L. were determined by measuring the zone of inhibition against individual studied bacteria. Each assay was conducted three times.

3. Results and Discussions

A total of 34 TC and AMP resistant bacteria were isolated from wastewater sources. Among these, eight strains were randomly selected and used for this study (Table 1). The antibacterial activities of the extracts obtained from the plants under study by the disc diffusion method are shown in Table 2. An acetone extracts had grater activity than ethanolic extracts against antibiotic resistant environmental bacteria. The acetone and ethanol extracts of *Nigella sativa* revealed significant antibacterial activity relative to pteridophytic plant extracts (Fig. 1).

Table 1. *List of antibiotic resistant bacteria used in this study.*

Strain ID	Resistant to	Gram staining reaction
$081013HS_2$	TC*	- ve
$081013HS_4$	TC	- ve
$081013HS_5$	TC	- ve
$081013HS_1$	TC	+ ve
$081013HS_6$	AMP	+ ve
$081013HS_7$	AMP	+ ve
$081013HS_{12}$	AMP	+ ve
$081013HS_{14}$	AMP	+ ve

TC, tetracycline; AMP, ampicilin; * resistant to 32 µg/ml of antibiotics.

Table 2. *Inhibitory properties of plant extracts on antibiotic resistant bacteria.*

		Strain ID		Concentration of plants extracts									
				312.5µg/ml		625 µg/ml		1250µg/ml		2500 µg/ml		NC	
				ET	AC	ET	AC	ET	AC	ET	AC	ET	AC
Pteriodophytes	*Dryopteris* sp.	081013HS$_1$	Zone of inhibition (mm)	7±0.20	7±0.33	8±0.26	8±0.26	9±0.13	9±0.13	10±0.20	10±0.26	+	+
		081013HS$_2$		6±0.13	6±0.13	8±0.33	7±0.06	9±0.13	9±0.40	10±0.33	11±0.20	+	+
		081013HS$_4$		6±0.13	7±0.13	7±0.26	8±0.06	8±0.13	9±0.33	9±0.20	11±0.26	+	+
		081013HS$_5$		6±0.20	6±0.06	7±0.26	7±0.20	9±0.33	8±0.13	9±0.26	11±0.26	+	+
		081013HS$_6$		6±0.31	7±0.20	7±0.26	7±0.33	8±0.06	9±0.20	11±0.13	11±0.26	+	+
		081013HS$_7$		6±0.33	6±0.40	7±0.40	8±0.06	8±0.26	10±0.20	10±0.33	10±0.13	+	+
		081013HS$_{12}$		7±0.20	6±0.26	7±0.26	8±0.13	8±0.26	9±0.40	11±0.20	11±0.13	+	+
		081013HS$_{14}$		7±0.26	8±0.20	8±0.33	9±0.20	9±0.13	10±0.17	10±0.33	12±0.66	+	+
	Pteris vittata	081013HS$_1$		6±0.13	7±0.46	8±0.26	9±0.13	10±0.20	10±0.26	10±0.26	11±0.26	+	+
		081013HS$_2$		7±0.26	7±0.26	8±0.26	9±0.13	9±0.26	11±0.26	11±0.33	11±0.13	+	+
		081013HS$_4$		6±0.40	7±0.26	7±0.13	8±0.26	8±0.33	9±0.33	9±0.26	10±0.20	+	+
		081013HS$_5$		7±0.20	6±0.40	8±0.33	8±0.26	9±0.26	9±0.26	10±0.13	10±0.40	+	+
		081013HS$_6$		7±0.26	8±0.13	9±0.26	9±0.20	11±0.26	10±0.13	11±0.26	12±0.26	+	+
		081013HS$_7$		7±0.26	8±0.13	10±0.33	9±0.33	12±0.26	11±0.26	12±0.13	13±0.13	+	+
		081013HS$_{12}$		6±0.13	7±0.26	11±0.33	10±0.33	12±0.26	11±0.26	12±0.20	12±0.20	+	+
		081013HS$_{14}$		7±0.26	8±0.13	8±0.26	10±0.17	10±0.20	11±0.26	11±0.26	13±0.13	+	+
Oil seeds	*Nigella sativa*	081013HS$_1$		9±0.26	9±0.33	10±0.17	10±0.13	12±0.26	12±0.66	13±0.13	14±0.40	+	+
		081013HS$_2$		8±0.33	9±0.13	10±0.17	10±0.20	11±0.26	12±0.13	13±0.66	14±0.33	+	+
		081013HS$_4$		9±0.13	10±0.3	11±0.20	12±0.20	13±0.13	12±0.20	13±0.13	15±0.17	+	+
		081013HS$_5$		8±0.26	9±0.13	9±0.40	11±0.13	10±0.40	12±0.17	12±0.06	13±0.13	+	+
		081013HS$_6$		7±0.26	13±0.2	13±0.33	15±0.13	15±0.17	16±0.33	15±0.20	17±0.06	+	+
		081013HS$_7$		9±0.33	10±0.3	12±0.33	12±0.33	14±0.40	13±0.33	15±0.26	17±0.13	+	+
		081013HS$_{12}$		9±0.13	11±0.2	11±0.26	12±0.20	14±0.40	14±0.06	16±0.06	15±0.20	+	+
		081013HS$_{14}$		8±0.13	8±0.33	11±0.26	14±0.40	15±0.33	15±0.17	16±0.26	18±0.26	+	+

Values are represented as mean ± SD of triplet experiments; ET, ethanol; AC, acetone; NC, negative control; + bacterial growth (without plant extracts).

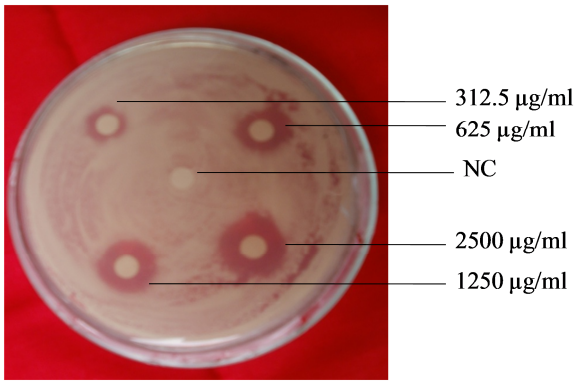

Fig. 1. *Representative profile of antibacterial activity of acetone extract of N. sativa against antibiotic resistant bacteria.*

Very little work has been done on the antimicrobial activity of pteridophytes, yet the ethanobotanical importance of these plants has been investigated and studied[20]. These plants are of great medicinal importance and are used by tribal and local people for remedies against various ailments [21,22]. In the present investigation both solvent (ethanol and acetone) extracts of *Dryopteris* sp. leaves were active against randomly selected isolated bacterial strains. The highest zone of inhibition of12 mm was observed at a concentration of 2500 µg/ml of ethanolic extract of *Dryopteris* sp. against AMP resistant gram positive bacteria. However, the antibacterial activity of *Dryopteris* sp. failed to show any significant differences between gram positive and gram negative bacteria. The effect of *Pteris vittata* leaf extract showed sensitivities to both of two extracts. Acetone extract showed the maximum significant inhibitory effect (13mm) compared to the ethanol extract against AMP resistant gram positive081013HS7 and081013HS14 strains. *The effect of leaf extract on pteridophytes has been studied against Agrobacterium tumifacience, E. coli, Salmonella arizonae, Salmonella typhi* and *Streptococcus aureus* in India[20]. Their results suggested that antimicrobial agents could be further enhanced through in vivo studies and isolation and characterization of active constituents for human health. Rani et al [15] reported on the antibacterial and antifungal properties of non-aqueous frond extracts of three pteridophytes and found most of the extracts in their study were effective against both Gram negative and Gram positive bacteria. The present study confirms the earlier findings that pteridophytes should be further screened for generating biologically active compounds.

The acetone extract of *Nigella sativa* exhibited the highest antibacterial activity at 2500 µg/ml concentration relative to the pteridophytes. The highest zone of inhibition was found at 18 mm followed by 17 mm against AMP resistant gram positive strains. An important observation was with regard to the concentration of both extracts of *N. sativa* causing a high level of inhibitory activity. The successive isolation of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Sharma and Patel [23] reported that acetone and aqueous extracts of medicinal plant material showed grater activity compared to ethanol extract against human

pathogenic bacteria.

The results from our present study indicated that the extracts of all plant materials studied showed antibacterial activity towards antibiotic resistant gram positive bacteria. These results are consistent with previous reports on related plants regarding gram positive bacteria [24, 25]. The resistance of gram negative bacteria to plant extracts was not unexpected as in general, this class of bacteria is more resistant than gram positive bacteria. Such resistance could be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism [25, 26].

4. Conclusion

It is concluded that antibacterial activity of *Nigella sativa* seed and pteridophytic plant extracts would be helpful in treating antibiotic resistant gram positive bacteria isolated from wastewater sources. The efficacy of plant extracts need to explore the exact mechanism of the interaction among the active phytoconstituents.

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