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Full Length Research Paper

Antimicrobial activity of *Diospyros melanoxylon* bark from Similipal Biosphere Reserve, Orissa, India

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The antimicrobial activity of five extracts of *Diospyros melanoxylon* Roxb. bark collected from Similipal Biosphere Reserve, Orissa was evaluated against human pathogenic bacteria and fungi. The extracts including both polar and non polar solvents; petroleum ether, chloroform, ethanol, methanol and aqueous were evaluated for their antimicrobial activity against three gram positive and five gram negative bacteria as well as three fungal strains. Although, all the five extracts exhibited promising antibacterial activities, yet maximum activity was observed in ethanol extract. In case of antifungal activity, except petroleum ether extract none of the extracts were found to be active against the fungal strains. MIC values for most of the extracts ranged from 1.5 to 6 mg/ml, while MBC values varied from 3 mg/ml to values greater than 12 mg/ml. Phytochemical analysis exhibited the presence of steroids, alkaloids, glycoside, proteins, tannins, phenolic compounds, carbohydrates, gums and mucilage in acetone, methanol and ethanol extracts with maximum phytochemicals in ethanol extract. Least phytochemicals was observed in case of petroleum ether. These results, so obtained, demonstrate the broad spectrum activity of *D. melanoxylon* bark extracts which may be useful in treatment of various microbial infections. However, the active components responsible for antimicrobial activity need to be evaluated.

Key words: Phytochemicals, *Diospyros melanoxylon*, ebenaceae, antibacterial.

INTRODUCTION

Phytomedicines have been an integral part of traditional health care system in most parts of the world for thousands of years. According to World Heath Organisation, greater than 80% of world population depends on traditional medicine for their primary healthcare needs (Duraipandiyan et al., 2006). Studying medicinal plants with ethno-botanical importance and folklore reputation has become the more important need in recent times in order to promote the use of herbal medicines and to determine their potential as source of new drugs (Parekh and Chanda, 2007). In recent years, use of antimicrobial drugs in treatment of infectious diseases have developed multiple drug resistance (Service, 1995) and with increase in production of new antibiotics, by pharmaceutical industry, resistance to these drugs have also increased (Nascimento et al., 2000). Hence, scientists are shifting their attention to folk medicine in order to find new leads for better drugs against microbial infections. Plant materials are known as source of new antimicrobial agents, as a result search has been to discover new antibacterial drugs of plant origin. A number of compounds like vincristine, quinine, salicyclic acid, eligitalis, morphine, and codeine have been derived from plants which are having enormous therapeutic potential (Parekh and Chanda, 2007). Still medicinal properties of many plants are yet to be investigated for phytochemistry and pharmacognosy and there is an urgent need to identify lead substances that are active towards resistant pathogens (Recio, 1989).

Similipal Biosphere Reserve (20° 17'-22° 10'N latitude and 85° 57'- 85° 47' E longitude), located in Mayurbhanj district of Orissa (India), is a unique habitat of mixed tropical forest which harbor varied flora and fauna. The ecosystem is enriched with variety of medicinal plants. Survey made by Saxena and Brahmam (1989) has re-

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ported occurrence of more than 500 medicinal plants of Similipal Biosphere Reserve. However, very limited studies on medicinal plants in general and antimicrobial activity of phytochemicals from Similipal Biosphere Reserve have been done (Thatoi et al., 2008; Mohanta et al., 2007). Hence in the present paper an attempt has been made to study the antimicrobial activity of Indian medicinal plant, *Diospyros melanoxylon* Roxb. (Ebenaceae) bark against some selected human bacterial and fungal pathogens. Selection of the medicinal plant for the present study was based on its ethnomedicinal usages and preliminary screening of antimicrobial activity made by the authors (Thatoi et al., 2008). This plant, commonly known as kendu, has been used for treatment of various ailments like diarrhoea and dyspepsia (Ambastha, 1986). The bark is also diuretic, carminative, laxative, styptic and used as an astringent lotion for eyes (Mallavadhani et al., 1998). *D. melanoxylon* Roxb. is normally found in dry deciduous forest and is widely distributed in forests of Similipal Biosphere Reserve. Wood of the tree, besides being a source of Indian ebony, is used for making boxes, combs, ploughs and beams. The fruits are eaten and sold commercially. E sTeeds are prescribed as cure for mental disorders, palpitation of heart and nervous breakdown (Rathore, 1972) Te objective of this study was to evaluate the potential of the plant bark extracts on standard human pathogenic microorganism and the phytochemicals present in the extracts.

MATERIALS AND METHODS

Plant material

D. melanoxylon Roxb. (Ebenaceae) barks were collected from the forests of Similipal Biosphere Reserve, Orissa, India in the month of March to April 2007. The plant was identified by Dr. A. K. Biswal, Department of Botany, North Orissa University and a voucher specimen (No.140) was deposited in the Department of Botany, North Orissa University. The dried bark was homogenized to powder and subjected to extraction with different solvents

Crude extraction

Successive petroleum ether, chloroform, ethanol, methanol and aqueous extraction (yield 1.07, 0.8, 5.6, 4.8 and 2.06% respectively) from dried *D. melanoxylon* bark were done using Soxhlet apparatus. The extracts were evaporated to complete dryness by vacuum distillation and stored at 4°C in airtight bottles for further use.

Microorganisms tested

The bacterial strains used to asses the antibacterial property of the crude extracts of *D. melanoxylon* bark included three gram positive and five gram negative bacteria. Three fungal strains were also included in the study (Table 1). All bacterial cultures and fungus *A. niger* were obtained from Institute of Microbial Technology, Chandigarh. Rest fungi were clinical isolates. The organisms were maintained on nutrient agar (Hi Media, India) slopes at 4°C and subcultured before use. Active cultures for experiments were prepared by transferring a loop-full of cells from stock cultures to test tubes of Muller-Hinton broth (MHB) for bacteria and Sabourand dextrose broth for fungus that were incubated without agitation for 24 h at 37 and 25°C, respectively. Cultures were diluted with fresh Muller-Hinton and Sabourand dextrose broth to attain an inoculum size of 2.0×10^6 cfu/ml for bacteria and 2.0×10^5 spore/ml for fungal strains.

Determination of antimicrobial assay

In vitro antimicrobial activity of crude extract against both bacterial and fungal strains was screened by agar well diffusion method (Khalid et. al., 2007). Mueller-Hinton agar (MHA) (Hi-Media, India) was used as bacteriological medium. MHA plates were prepared by pouring molten media into sterile Petri plates. The plates were allowed to solidify for 5 min. Wells were prepared in seeded agar plates. 0.1% inoculums suspension was swabbed uniformly and the inoculums were allowed to dry for 5 min. The extracts were diluted in 100% DMSO. Antimicrobial activity was evaluated at a concentration of 50 mg/ml. The test compound (200 µl) was introduced in the wells. Plates were incubated overnight at 37°C. The antimicrobial spectrum of the extract was determined in terms of zone sizes around each well. The diameters of zone of inhibition produced by extracts were compared with those of standard antibiotics like ciprofloxacin for bacteria and clotrimazole for fungus. Minimal inhibitory concentration (MIC) was determined by microdillution method (Eloff, 1998). Minimal inhibitory concentration and minimal bactericidal concentration (MBC) (Dewanjee et al., 2007) was seen on those bacterial strains which showed zones of inhibition against the plant extracts.

Phytochemical screening

All the five solvent extracts (petroleum ether, chloroform, ethanol, methanol and aqueous) of bark were evaluated for the presence of different phytochemicals using procedures of Mukherjee (2002) and Parekh and Chanda (2007).

RESULTS AND DISCUSSION

The antimicrobial activity of different crude extracts of *D. melanoxylon* bark was determined against eight human pathogenic bacterial strains and three fungal strains which are reported in Table 1. All the five extracts of *D. melanoxylon* bark viz., petroleum ether, chloroform, ethanol, methanol and aqueous showed promising antibacterial activities against almost all bacterial strains. Among the extracts, maximum activity was observed in ethanol extract whereas aqueous extract showed the least activity against all the bacterial strains. Both gram positive and gram negative bacteria were susceptible to the plant extracts which is in contradiction to earlier reports by some workers (Rabe and Van Staden, 1997; Vlietinck et al., 1995) that plant extracts were most active against gram positive microorganisms than gram negative ones. In the present work, among gram positive bacteria *Staphylococcus aureus* was the most susceptible with inhibition zones of 20.33, 26.33 and 27.33 mm in petroleum ether, ethanol and methanol extracts, respectively, whereas in case of gram negative bacteria, *Escherichia coli* was most susceptible with inhibition zones of 18.33, 26.66 and 22.33 mm, respectively, in

Table 1. Antibacterial activity of *D. melanoxylon* bark extracts against bacterial strains.

Strains	Inhibition zones in diameter mm					
	Petroleum ether (50 mg/ml)	Chloroform (50 mg/ml)	Ethanol (50 mg/ml)	Methanol (50 mg/ml)	Aqueous (50 mg/ml)	Ciprofloxacin (100 µg/ml)
Bacteria						
Staphylococcus aureus MTCC 1144 (G+)	20.33±0.577	12.66±1.52	26.33±0.577	27.33± 0.577	16.00±1	20.00±0
Staphylococcus epidermidis MTCC 3615 (G+)	21.66±1.52	14.33±0.577	22.66±1.52	22.66±1.52	-	10.00±0
Bacillus licheniformis MTCC 7425 (G+)	20.33±0.577	14.00±0	21.66±1.52	18.33±0.577	-	14.00±0
Escherichia coli MTCC 1089 (G-)	18.33±0.577	16.33±0.577	26.66±0.577	22.33±0.577	14.00±0	28.00±0
Pseudomonas aeruginosa MTCC 1034 (G-)	16.33±0.577	-	29.66±1.52	22.33±0.577	19.33±0.577	26.00±0
Pseudomonas fluorescens MTCC 1748 (G-)	18.33±0.577	14.00±0.33	26.66±0.577	14.00± 0	14.00±1	16.00±0
Salmonella typhii MTCC 3216 (G-)	21.66±1.52	12.33±0.577	24.66±1.52	16.33±0.577	-	20.00±0
Vibrio cholerae MTCC 3904 (G-)	15.33±0.577	12.00±0	22.66±0.577	16.33±0.577	20.66±1.52	14.00±0
Fungus						**Clotrimazole (100 µg/ml)**
Aspergillus niger MTCC 871	-	-	-	-	-	20.00±0
Trichosporon rubrum	13.33±0.577	-	-	-	-	27.00±0
Aspergillus fumigatus	-	-	-	-	-	20.00±0

Values are mean ± S.E.M. of 3 replications, - = No inhibition, G+ = Gram positive, G- = Gram negative.

Table 2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *D. melanoxylon* bark extracts on bacterial strains.

Extracts	*S. aureus*[a]		*S. epidermidis*[a]		*B. licheniformis*[a]		*E. coli*[a]		*P. aeruginosa*[a]		P. *fluorescens*[a]		*S. typhii*[a]		*V. cholerae*[a]	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Petroleum ether	3	3	6	>12	6	6	3	6	6	6	6	>12	6	>12	3	>12
Chloroform	6	>12	6	>12	6	6	6	6	-	-	6	>12	6	>12	6	>12
Ethanol	6	6	6	>12	6	12	3	>12	1.5	3	3	>12	3	>12	3	>12
Methanol	3	>12	6	>12	6	12	3	>12	1.5	3	6	>12	3	>12	3	>12
Aqueous	3	>12	-	-	-	-	3	>12	3	3	6	>12	-	-	3	>12

a = Values in mg/ml; - = no inhibition.

petroleum ether, ethanol and methanol extracts. In case of antifungal activity of different extracts on three pathogenic fungi it was found that except petroleum ether none of the extracts were active against the fungal strains. Out of three fungi tested, only *Trichosporon rubrum* showed an inhibition zone less than 15 mm in petroleum ether extract whereas other two fungi did not show any inhibition zone. Results were compared with standard antibiotics Ciprofloxacin and Clotrimazole. Minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) of extracts with positive responses were tested for different strains which are given in Table 2. In the present study, MIC was determined by micro-dilution method (Eloff, 1998) where DMSO was used as a solvent. MIC values ranged from 1.5 to 6 mg/ml, while MBC values varied from 3 mg/ml

Table 3. Phytochemical analysis of different extracts of *Diospyros melanoxylon* bark.

Phytochemical	Chloroform	Acetone	Ethanol	Methanol	Aqueous
Steroid	-	+	+	+	-
Alkaloid	-	+	+	+	-
Cardiac glycoside	-	+	+	-	+
Protein and amino acid	+	+	+	+	+
Tannin and phenolic compounds	-	+	+	+	+
Ascorbic acid	+	+	+	-	+
Fixed oil and fats	+	+	+	-	+
Anthraquinone	-	-	-	-	-
Triterpenoid	-	-	-	-	-
Carbohydrate	+	+	+	+	+
Saponin	+	-	+	+	+
Gums and mucilage	-	+	+	+	-

to values greater than 12 mg/ml for different extracts. Two of the extracts such as ethanol and methanol, had the lowest MIC of 1.5 mg/ml for *P. aeruginosa.*

There have been reports of antimicrobial activity of root, leaf and bark of *D. anisandra* (Borges-Argaez et al., 2007) and *D. peregrina* fruits (Dewanjee et al., 2007).

Similarly there are also reports of antipyretic properties of *D. mespiliformis* (bark) and *D. variegata* (stem) (Adzu et al., 2002; Trongsakul et al., 2003). Antimicrobial activity of *D. melanoxylon* has been reported on its leaves, because leaves of coppiced plants are used as wrappers in the bidi (cigarette) industry (Mallavadhani et al., 2004). Even inhibitory activity of the leaves of this plant against *Plasmodium falciparum* (Simonsen et al., 2001) has also been reported. The present paper reports antimicrobial activity of the bark of *D. melanoxylon* from Similipal Biosphere Reserve. Further, preliminary phytochemical screening of the bark extracts studied, showed that ethanol extract contains most of the phytochemicals like alkaloids, steroids, tannins, saponins, ascorbic acid etc. (Table 3). Aminoacids and carbohydrates were universally present in all the extracts. The presence of such phytochemicals may be correlated with the fact that ethanol extract showed maximum activity against the bacterial strains. The active constituents of plants usually interfere with growth and metabolism of microorganisms in a negative manner (Aboaba et al., 2006). Phenolic compounds like tannins found in plant cells are potent inhibitors of hydrolytic enzymes used by plant pathogens. Other compounds like saponins also have antifungal properties. Many plants release phenolic compounds that are toxic to microbial pathogens (Aboaba and Efuwape, 2001). Hence, these phytochemicals present in the crude extracts may be responsible for antimicrobial activity of plant extracts which need further investigation.

In the present study a variety of gram positive and gram negative bacteria and three fungal strains were selected for screening of antimicrobial effect of five extracts (polar and non-polar) of a single plant part to perceive the antimicrobial spectrum as well as authenticate the ethnomedicinal uses. *D. melanoxylon* bark has been used against diarrhoea, dyspepsia by ethnomedicial practiotioners (Ambastha, 1986). Thus, present findings of antimicrobial activity of *D. melanoxylon* have fairly good degree of correlation with ethnomedicinal uses of the plant. Preliminary results of this investigation appear to indicate that bark of *D. melanoxylon* have high potential antimicrobial activity. Novel bioactive compounds from the bark need to be isolated and screened for their pharmaceutical and biotechnological applications in order to cure chronic and infectious diseases.

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