In Vitro Antibacterial and Antioxidant Studies of *Croton roxburghii* L., from Similipal Biosphere Reserve

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Abstract In vitro antibacterial activities of acetone, ethanol, methanol and water extracts of leaves and bark of *Croton roxburghii* L. studied against ten human pathogenic bacterial strains showed significantly higher activity in acetone extract and least activity in case of aqueous. Minimal inhibitory concentration (MIC) values of all extracts ranged between 0.62 and 10 mg/ml, while minimal bactericidal concentration (MBC) values ranged from 1.25 to values greater than 10 mg/ml. The antioxidant assays viz. DPPH, hydrogen peroxide scavenging, iron reducing and iron chelating assays along with total phenol and ascorbic acid content were carried out with aqueous extracts of leaves and bark. While the total phenol contents in leaves and bark extracts were 0.766 ± 0.014 and $0.735 \pm 0.028\%$ respectively their ascorbic acid contents were found to be 0.252 ± 0.019 and $0.431 \pm 0.013\%$ respectively. DPPH activities in both (leaves and bark) extracts increased with the increase in concentrations. Iron chelating capacity of leaves extract is significantly higher than that of the bark. Leaves extract showed an increase in percentage of scavenging property with the increase in concentrations. Plant extracts showed low amount of iron reducing property at all concentrations. Hydrogen peroxide scavenging properties of bark was low than that of the leaves.

Keywords Antimicrobial activity · Medicinal plant · Reactive oxygen species · Crude plant extracts

The discovery of antibiotics during the nineteenth century has substantially decreased the number of deaths caused due to bacterial infections. However, the injudicious use of these substances has led to the emergence of multidrug resistant pathogens. Besides the antibiotics often induced hypersensitivity, immune-suppression and allergic reactions in host [1]. Since plants possess bioactive antimicrobial substances, which are safer to use without any side effects, there is need to screen out medicinal plants with potent antimicrobial activities. Medicinal plants are also known to possess antioxidant properties and their consumption reduces the risk of developing chronic diseases.

Reactive oxygen species (ROS) are formed as a result of normal metabolic activity and from exogenous sources. ROS formed often results in causing cellular and subcellular damage by peroxidation of membrane lipids, denaturation of DNA strands and cellular proteins [2]. In multicellular organisms, cell protects itself from the damage caused by the ROS system by various enzymatic pathways which includes catalase, glutathione peroxidase, glutathione reductase, superoxide dismutase, lipid peroxidase and non enzymatically by beta carotene, vitamin A, C, E. Recently, there is growing urge to discover natural antioxidants from medicinal plants and the phytochemicals are generally safer than the chemical and synthetic formulations.

Croton roxburghii L., is a potential medicinal plant from Similipal Biosphere Reserve (SBR) in the state of Orissa (India), has been used in the treatment of various disorders like infertility, fever and wounds, etc. by the local folks and in many parts of India [3] including SBR, which is one of the grand repository of medicinal plants from eastern part of the country. Looking at the limited studies on bioactivity medicinal plants from SBR [4], an attempt has been made

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to evaluate the antibacterial and antioxidant properties viz. DPPH, hydrogen peroxide scavenging, iron reducing and iron chelating assays along with total phenol and ascorbic acid contents of leaves and bark extracts of *Croton roxburghii*.

Materials and Methods

Plant Material

Fresh leaves and bark of *Croton roxburghii* L were collected from forests of Similpal Biosphere Reserve (20° 17'–22° 10' N latitude and 85° 57'–85° 47' E longitude), Orissa, during March to April 2008. The plant was identified by Dr. A. K. Biswal, Department of Botany, North Orissa University and a voucher specimen (No. NOU 1091) was deposited in the same department. The samples were initially rinsed with 1% mercuric chloride, dried, homogenized to powder and subjected to extraction with different solvents.

Preparation of Plant Extracts

Crude extracts of acetone, ethanol, methanol and aqueous were obtained by extracting 25 g of fine powder from dried bark and leaves of *C. roxburghii* with respective solvents in a soxhlet apparatus. Extracts were evaporated to dryness by vacuum distillation and stored at 4°C for further analysis. Percentage yield of crude extracts for both leaves (acetone 2.44%, ethanol 3.04%, methanol 0.66%, aqueous 1.71%) and bark (acetone 0.58%, ethanol 2.68%, methanol 0.38%, aqueous 1.69%) were calculated.

Test Microorganisms

Ten pathogenic bacteria, *Staphylococcus aureus* (MTCC 1144), *Shigella flexneri* (MTCC 441), *Escherichia coli* (MTCC 1089), *Bacillus subtilis* (MTCC 7164), *Bacillus licheniformis* (MTCC 7425), *Bacillus brevis* (MTCC 7404), *Vibrio cholerae* (MTCC 3904), *Pseudomonas aeruginosa* (MTCC 1034), *Streptococcus aureus* (lab isolate) and *Staphylococcus epidermidis* (MTCC 3615) used in the study, were obtained from Institute of Microbial Technology (IMTECH), Chandigarh. 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 loopful of cells from stock cultures to test tubes of Muller-Hinton broth (MHB) that were incubated without agitation for 24 h at 37°C.

Determination of Antibacterial Activity

In vitro antibacterial activity of crude extract of each solvent was screened by agar well diffusion method [5]. Wells were prepared in seeded Muller–Hinton Agar (MHA) plates. Prior to introduction of test compounds, 0.1% inoculum suspension was swabbed uniformly and were allowed to dry for 5 min. 200 µl test compound (from a concentration of 50 mg/ml with 100% dimethylsulphoxide) was introduced in the wells. Plates were incubated overnight at 37°C. Antibacterial spectrum of extract was determined in terms of zone sizes around each well. Diameters of zone of inhibition produced by extracts were compared with those of antibiotic Gentamycin (10 µg/disc). Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were seen on those bacterial strains which showed zones of inhibition against the plant extracts [6]. Experiments performed in triplicates.

Antioxidant activity

Total phenolic compounds were determined with Folin–Ciocalteu's Phenol reagent (FCP) according to the method of Slinkard and Singleton [7] with minor modifications. Absorbance was measured at 700 nm using spectrophotometer (SYSTRONICS-114). Phenolic content was expressed as guaiacol equivalents % dry weight. The ascorbic acid content was estimated following the methods of Swain and Tripathy [8] with slight modifications. Absorbance was measured at 660 nm using spectrophotometer. Results were expressed in % dry weight. The DPPH radical scavenging effect was determined by following standard methods of Hatano et al. [9]. Absorbance was measured at 517 nm. Percentage scavenging effect was calculated using the following equation.

$$\% \text{ scavenging} = (A_0 - A_1)/A_0 \times 100$$

where A_0 = Absorbance of control and A_1 = Absorbance of test sample.

Reducing power of aqueous extract of leaf and bark was determined by the method of Oyaizu et al. [10]. Absorbance was read at 700 nm. Hydrogen peroxide (H_2O_2) scavenging assay was carried adapting the method of Gulcin et al. [11] with minor changes. Absorbance was measured at 240 nm. Percentage scavenging was calculated from control value. Ferrous ion chelating activity was assessed as described by Zhao et al. [12]. Absorbance of the solution was measured spectrophotometrically at 562 nm. Percentage of inhibition of ferrozine- Fe^{2+} complex formation was calculated using the formula given below:

$$\text{Metal chelating effect (\%)} = (A_0 - A_1)/A_0 \times 100$$

where A_0 = Absorbance of control and A_1 = Absorbance of test sample. BHT was used as a reference standard.

Statistical Analysis

Experiments were carried out in three different batches of plant materials. The means of all the parameters were examined for significance by analysis of variance (ANOVA) and in case of significance mean separation were accomplished by Bonferroni post hoc test using SPSS (version 10) statistical software package. Differences were considered significant at a probability level of $P < 0.05$.

Results

Antibacterial Activity

The results of antibacterial activity of different crude extracts of *Croton roxburghii* leaves and bark against ten human pathogenic bacterial strains are presented in Table 1. All the extracts showed antibacterial activity against almost all the bacterial strains which however differ in their degree of inhibition. The results of antibacterial activity depended largely upon the plant part, solvent used for extraction and the organism tested. MIC and MBC of extracts were carried out for those which showed in vitro antibacterial activity and are given in Table 2. MIC values ranged from 0.62 to >10 mg/ml, while MBC values varied from 1.25 mg/ml to values greater than 10 mg/ml for different extracts. Two of the extracts such as acetone and ethanol had promising MIC value of 0.62 mg/ml for *S. flexneri*, *B. brevis* and *P. aeruginosa*.

Antioxidant Activity

The total amount of phenolic content present in the aqueous extract of leaves and bark samples were found to be 0.766 and 0.735% respectively. Total amount of ascorbic acid content present in the aqueous extract of both leaves and bark samples were found to be 0.252 and 0.431% respectively. Aqueous extract of both leaves and bark showed significantly higher DPPH activity in comparison to the standard BHT at all three (50, 75 and 100 μ g/ml) concentrations (Fig. 1a). Bark showed significantly higher activity than leaves in all three concentrations ($P < 0.05$). Linear regression analysis was carried out to evaluate the dose related DPPH activity. The r^2 values of standard, leaves and bark were found to be 0.935, 0.841 and 0.979, respectively indicating the existence of a strong, linear and positive relation between concentration and DPPH activity in both leaves and bark extract (Fig. 1a). The iron chelating

Table 1 Inhibition zone (in mm) of *Croton roxburghii* leaf and bark extracts against pathogenic bacteria

Strains	Leaf				Bark				Gentamycin (10 μ g/disc)
	Acetone	Ethanol	Methanol	Aqueous	Acetone	Ethanol	Methanol	Aqueous	
<i>Staphylococcus aureus</i>	32.66 $\pm$ 1.52 ^a	24.33 $\pm$ 1.154 ^a	18 $\pm$ 0 ^a	–	28.33 $\pm$ 0.577 ^a	18.33 $\pm$ 1.52 ^{ac}	12.33 $\pm$ 1.154 ^{ac}	13.33 $\pm$ 0.577 ^{bc}	26 $\pm$ 0
<i>S. flexneri</i>	30.66 $\pm$ 1.52 ^a	18.66 $\pm$ 1.154 ^a	24.33 $\pm$ 0.577 ^a	20.33 $\pm$ 0.577 ^b	14 $\pm$ 0 ^a	12.33 $\pm$ 0.577 ^{ac}	18.66 $\pm$ 1.52 ^{ac}	16.33 $\pm$ 0.577 ^{bc}	23 $\pm$ 0
<i>B. licheniformis</i>	28.33 $\pm$ 1.154 ^a	30.66 $\pm$ 1.52 ^a	23 $\pm$ 0 ^a	–	22.66 $\pm$ 1.52 ^a	20.33 $\pm$ 0.577 ^{ac}	15.33 $\pm$ 0.577 ^{ac}	15.33 $\pm$ 0.577 ^{bc}	20 $\pm$ 0
<i>B. brevis</i>	32.33 $\pm$ 0.577 ^a	34.66 $\pm$ 1.52 ^a	30.33 $\pm$ 1.52 ^a	21 $\pm$ 0 ^b	34.33 $\pm$ 1.154 ^a	30.33 $\pm$ 0.577 ^{ac}	22.33 $\pm$ 1.154 ^{ac}	14.33 $\pm$ 0.577 ^{bc}	22 $\pm$ 1
<i>V. cholerae</i>	29.33 $\pm$ 1.52 ^a	22.66 $\pm$ 0.577 ^a	25.33 $\pm$ 0.577 ^a	–	23.66 $\pm$ 1.154 ^a	13 $\pm$ 0 ^{ac}	19.66 $\pm$ 0.577 ^{ac}	16.33 $\pm$ 0.577 ^{bc}	17 $\pm$ 0
<i>P. aeruginosa</i>	27.33 $\pm$ 0.577 ^a	24.33 $\pm$ 0.577 ^a	14 $\pm$ 0 ^a	18 $\pm$ 0 ^b	20.33 $\pm$ 0.577 ^a	20.66 $\pm$ 0.577 ^{ac}	20.33 $\pm$ 0.577 ^{ac}	14 $\pm$ 0 ^{bc}	18 $\pm$ 0
<i>Streptococcus aureus</i>	26.66 $\pm$ 1.154 ^a	17 $\pm$ 0 ^a	20.33 $\pm$ 0.577 ^a	–	27.66 $\pm$ 1.154 ^a	24.33 $\pm$ 0.577 ^{ac}	16.33 $\pm$ 0.577 ^{ac}	13 $\pm$ 0 ^{bc}	–
<i>S. epidermidis</i>	20.33 $\pm$ 0.577 ^a	19.66 $\pm$ 1.154 ^a	22.33 $\pm$ 0.577 ^a	12.33 $\pm$ 0.577 ^b	17 $\pm$ 0 ^a	16 $\pm$ 0 ^{ac}	18.66 $\pm$ 1.52 ^{ac}	22.66 $\pm$ 0.577 ^{bc}	20 $\pm$ 0
<i>B. subtilis</i>	18.66 $\pm$ 0.577 ^a	15 $\pm$ 0 ^a	22.66 $\pm$ 1.154 ^a	16 $\pm$ 0 ^b	20.33 $\pm$ 0.577 ^a	18 $\pm$ 0 ^{ac}	16 $\pm$ 0 ^{ac}	14 $\pm$ 0 ^{bc}	19 $\pm$ 0
<i>E. coli</i>	14.33 $\pm$ 0.577 ^a	18.33 $\pm$ 0.577 ^a	12.33 $\pm$ 0.577 ^a	10.33 $\pm$ 0.577 ^b	14.66 $\pm$ 0.577 ^a	14 $\pm$ 0 ^{ac}	13 $\pm$ 0 ^{ac}	12.33 $\pm$ 0.577 ^{bc}	18 $\pm$ 1

Concentration 50 mg/ml, Same superscripts indicate no significant difference within the same plant part

Table 2 MIC and MBC of *Croton roxburghii* leaves and bark extracts on bacterial strains

Strains	Leaf				Bark			
	Acetone MIC MBC	Ethanol MIC MBC	Methanol MIC MBC	Aqueous MIC MBC	Acetone MIC MBC	Ethanol MIC MBC	Methanol MIC MBC	Aqueous MIC MBC
<i>Staphylococcus aureus</i>	5	5	5	–	>10	>10	>10	5
	10	10	10	–	>10	>10	>10	10
<i>S. flexneri</i>	0.62	5	10	>10	10	5	>10	>10
	1.25	5	>10	>10	>10	10	>10	>10
<i>B. licheniformis</i>	>10	>10	>10	–	2.5	5	10	2.5
	>10	>10	>10	–	5	10	>10	5
<i>B. brevis</i>	0.62	10	5	5	1.25	5	2.5	2.5
	1.25	>10	10	10	2.5	5	5	2.5
<i>V. cholerae</i>	>10	10	10	–	10	1.25	2.5	10
	>10	>10	>10	–	>10	2.5	5	>10
<i>P. aeruginosa</i>	5	0.62	5	2.5	5	5	5	5
	10	1.25	10	5	10	10	5	10
<i>Streptococcus aureus</i>	5	10	5	–	5	10	>10	10
	10	>10	5	–	10	>10	>10	>10
<i>S. epidermidis</i>	2.5	1.25	5	10	2.5	5	>10	5
	5	2.5	10	10	5	10	>10	10
<i>B. subtilis</i>	2.5	5	5	10	2.5	10	10	5
	5	10	10	>10	5	>10	>10	10
<i>E. coli</i>	5	2.5	>10	5	2.5	5	>10	10
	10	5	>10	10	5	10	>10	>10

capacity of leaves extract is significantly higher than that of the bark extracts in all three concentrations. The leaves extract showed an increase in percentage of scavenging property with the increase in concentration. The r^2 value of the leaves extract found to be 0.979, indicative of the presence of a strong, linear and positive concentration—percentage inhibition response (Fig. 1b). All the extracts show low amount of iron reducing property at all concentrations (Fig. 1c). There were no significant difference in the reducing power of the leaves and bark extract at any of the three concentrations. The r^2 values of BHT, leaves and bark were found to be 0.841, 0.999 and 0.999 respectively suggesting of a linear and positive response between concentrations and reducing capacity of the samples taken. Scavenging activity of hydrogen peroxide in both leaves and bark extracts of *C. roxburghii* from the reference compound (BHT) were significantly lower but both leaves and bark extracts were able to show certain amount of inhibitory action on free radical generations. At 1.2 and 4 mg/ml the leaves were more efficient in scavenging in comparison to that of the bark samples. However, both leaves and bark sample showed a positive and linear response between the concentration and percentage inhibition

(Fig. 1d). The r^2 values of leaves and bark were found to be 0.77 and 0.823, respectively.

Discussions

Significantly higher antibacterial activity was observed in acetone extract, whereas least activity observed in case of aqueous extract with intermediate values for ethanol and methanol extracts. However, no significant differences were found between acetone and ethanol and in between acetone and methanol. Both gram positive and gram negative bacteria were susceptible to plant extracts which stands in contrast to earlier reports that plant extracts were most active against gram positive than gram negative organisms [13]. Human body is always vulnerable to attack of many kinds of pathogens. In such a scenario remediation includes adaptation of antibiotics and other synthetic formulated medicines. Both antibiotics and formulated drugs often offer side effects and antibiotic resistance. So it is now a challenge to discover new alternatives. Plant extracts being natural in origin are ideal to use.

Among the different phytochemicals, *Croton roxburghii* possess phenolic compounds to the extent of 0.766 which

Fig. 1 Free radical scavenging activities of aqueous extract of *Croton roxyburgii* leaves and bark **a** DPPH **b** Ferrozine **c** Reducing **d** Hydrogen Peroxide. (Absorbance values are mean of triplicates). Same superscripts indicate no significant difference

may have several biological activity including antioxidant activity [14]. Results of total ascorbic acid content indicate the presence of ascorbic acid both in bark and leaves. Natural ascorbic acid, being an antioxidant, is vital for the body performance.

The results of the present study suggest that the DPPH scavenging activities are shown both by the leaves and bark extracts. However, the bark in all the concentrations has significant effects in comparison to the leaves. In both leaves and bark, the activity gets elevated with the elevation in the extract concentration. DPPH assay is widely used as a model system in the assessment of scavenging activities of several natural compounds. DPPH radical is scavenged by antioxidants through the donation of protons forming the reduced DPPH [15]. As metal chelation is one of the important properties of antioxidants so, the chelating of ferrous ions by the plant extract was studied. Transition metal, mostly iron, plays a key role in oxygen radical reactions and subsequently results in bringing about oxidative damage in the biomolecules like proteins and DNA [16]. Results show that leaves are significantly more efficient than the bark extracts in iron chelating phenomenon. Bark extracts are able to show the chelating activity at higher concentrations. Chelating activity in leaves also increase with the increase in the concentration of the extract.

Result of reducing assay suggested that the extract have reducing power and it increases with the increase in concentration. But, the aqueous extracts of leaves and bark showed no significant amount of difference in their activity at any of the three concentrations. Reducing capacity of a compound may serve as indicator of its potential antioxidant capacity [17]. Hydrogen peroxide (H_2O_2) is considered to be a weak oxidizing agent; however, its potential to produce highly reactive oxygen species like hydroxyl radical through Fenton reaction is very high [18]. Thus, removal of H_2O_2 is very important for medicinal value and protection of food systems. The results of the study reveals that both the leaves and bark extract have got some amount of hydrogen peroxide scavenging activities but, the amounts are very low. Aqueous extracts of both leaves and bark could able to show an increase in the free radical inhibition with the increase in concentrations. The antioxidant scavenging capacity of compounds has been attributed to various mechanisms, like, chelating metal ions, reductive capacity and free radical scavenging [19]. Cellular damage or oxidative injury arising from free radicals or ROS now appear to be one of the fundamental mechanism for a number of human diseases, such as ischemic injury, inflammation, cancer, atherosclerosis, neurodegenerative diseases (Parkinson's and Alzheimer's) and ageing. Free radicals are generated from both endogenous and

exogenous sources. A number of evidence suggests that ROS can be scavenged through chemoprevention utilizing natural antioxidant compounds are present in foods and medicinal plants.

Present study concludes that *Croton roxburghii* possess antibacterial and antioxidant potential. Further, in vivo studies are also necessary to evaluate these activities and to establish the medicinal value of the plant.

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