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Original Article

ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF METHANOLIC EXTRACT OF *BAUHINIA RACEMOSA*

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ABSTRACT

Objective: To evaluate antimicrobial and *in vitro* antioxidant activities of methanolic extract of *Bauhinia racemosa* leaf.

Methods: The antimicrobial activity was determined by the agar-well diffusion method. The antioxidant potential was evaluated by using various *in vitro* methods such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), phospho molybdenum reduction assay and ferric (Fe 3+) reducing power assays.

Results: The maximum inhibition zone (26 mm) was found against *Proteus mirabilis* while lowest inhibition zone (20 mm) was observed against *Acinetobacter baumannii*.

The DPPH and ABTS were significantly inhibited by methanolic extract of *Bauhinia racemosa* leaf with IC_{50} of 9 µg/ml and 550µg/ml respectively, whereas methanolic extract of leaves showed good antioxidant potential using ferric reducing power assay (1.896 and phosphomolybdate assay (108.12 mg AAE/g) methods.

Conclusion: The results of present study showed that *Bauhinia racemosa* leaf possesses high potential antimicrobial and antioxidant activity and could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing oxidative stress-related degenerative diseases.

Keywords: *Bauhinia racemosa*, Antimicrobial, *In vitro* antioxidant, DPPH, ABTS, FRPA.

INTRODUCTION

The release of free radicals in biological systems causes various degenerative disorders, such as mutagenesis, carcinogenesis, cardiovascular disorders and ageing. Antioxidants are the compounds, which scavenge free radicals by inhibiting any one of the oxidative processes, like initiation, propagation and termination of free radicals [1].

Nowadays, the traditional medicines obtained from plant sources are being used worldwide for treatment of various life-threatening diseases. Currently, about 25% active components of plants are prescribed as medicines because of their less toxicity and economic viability [2]. Many plants have been claimed to have antibacterial and antioxidant activities. Development of multiple drug resistant microorganisms and oxidative stress induced diseases like cancer, cardiovascular, diabetes, atherogenesis, Alzheimer's and Parkinson's diseases are being the root cause to investigate potent antimicrobial and antioxidant agents from plant sources [3].

Since last few decades, a large number of synthetic antimicrobial and antioxidant agents used for the treatment of concerned diseases which are reported to have various toxic effects. Thus, there is a demand to investigate some antimicrobial and antioxidant agents from plant sources, which are devoid of such toxic effects.

Bauhinia racemosa Lam. (*B. racemosa*, family: Caesalpiniaceae) is a small deciduous tree with drooping branches, which grows in poor and very harsh climatic conditions. This plant is widely distributed throughout India, Ceylon, United States of America and China. The barks and leaves of *B. racemosa* are sweetish and astringent and are used in the treatment of a headache, fever, skin diseases, blood diseases, dysentery and diarrhea. *B. racemosa* is one of the most important herbal remedies, a decoction of its bark is recommended as a useful wash for ulcers. The bark is reported to have anti-oxidant and hepatoprotective activities. An extract of the leaves showed analgesic, antipyretic, anti-inflammatory, antispasmodic and anthelmintic activities. The tree has anti-tumor qualities and is widely used in Ayurveda to treat first stage cancer [4]. The root of *B. racemosa* contains a new tetracyclic lupeol, betulin, β-sitosterol, and tetracyclic 2, 2-dimethylchroman [5]. The seed contains flavonoids, crude protein and lipid [6]. The bark of the plant contains ß-sitosterol and ß-amyrin and the leaves contain flavonols (kaempferol, quercetin) and coumarins [7].

Till date, as no extensive antimicrobial and antioxidant activities of methanolic extract of leaves *B. racemosa* have been carried out, it was aimed to evaluate antimicrobial as well as antioxidant activity.

MATERIALS AND METHODS

Collection, identification and authentication of plant

Leaves of *B. racemosa* were collected from the herbal garden of Regional Plant Resource Centre, Bhubaneswar in the month of December 2014. The authentification of this plant was confirmed by Dr. P. C. Panda, senior scientist, Regional Plant Resource Centre, Bhubaneswar, Odisha, India. The voucher specimen was preserved in Department of Pharmacognosy, Siksha 'O' Anusandhan University, Odisha. Fresh leaves were washed under running tap water; air dried and then ground to coarse powder and stored for further use.

Preliminary phytochemical screening

Preliminary phytochemical screening of methanolic extract of *B. racemosa* was performed using standard method [8].

Antibacterial activity assay

Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative (*Acinetobacter baumannii, Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*) bacteria were used as a test organism and collected from Microbiology Department, Institute of Medical Sciences, Sum Hospital, Bhubaneswar, Odisha, India.

Antibacterial test of plant extract

One strain from each bacterial species having resistance to a maximum number of antibiotics was further used for monitoring antibacterial potentiality of methanolic extract, using the agar-well diffusion method as described previously by Nayak et. al [9]. Evaluation of antibacterial activities was done by measuring the diameter of zones of inhibition after use of methanol extract. The experiment was conducted thrice and results are presented. It was confirmed that 10 % DMSO had no inhibitory effect on any bacterium.

Determinations of MIC and MBC

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the methanolic extract of *B. racemosa* leaf were determined. The original stock solution was prepared with methanol at the concentration, 100 mg extract/ml in 10 % DMSO solution with distilled water. Each stock solution was diluted to obtain final concentrations of 1.562, 3.125, 6.25, 12.5, 25, 50 and 100 mg/ml with 10 % DMSO solution. An aliquot of 80 µl of each dilution of an extract was released to a well on a 96-welled (12 x 8) microtiter plate, along with an aliquot of 100 µl MH broth (Hi Media), an aliquot of 20 µl bacterial inocula (109 CFU/ml) and a 5 µl-aliquot of 0.5 % of 2,3,5-triphenyl tetrazolium chloride (TTC). After pouring all the cited to a well, the microtitre plate was incubated at 37 °C for 18 h. The pink coloration development due to TTC in a well indicated bacterial growth and the absence of the colour was taken as the inhibition of growth. The first well of the microtitre was the control without any extract. The MIC value was noted at the well, where no colour was manifested. Further, bacteria from each well of the microtitre plate were sub-cultured on a nutrient agar plate; the dilution level, which caused no bacterial growth on the nutrient agar plate, was observed and was taken as the MBC value [10].

DPPH radical scavenging activity

DPPH radical scavenging activity of the methanolic extract was determined according to the method described by Memarpoor-Yazdi *et al.* [11]. The extract was dissolved in distilled water at different concentrations. 200 µl of each sample was then mixed with 600 µl of methanol and 200 µl of DPPH (0.15 mmol in methanol). The mixture was shaken vigorously for 2 min and kept for 30 min in the dark at room temperature. The absorbance of the mixture was measured at 517 nm using a UV–Vis spectrophotometer. The control contains 800 µl of methanol and 200 µl of DPPH (0.15 mmol). All experiments were carried out in triplicate.

DPPH radical scavenging activity was calculated using the following equation:

$$\text{Inhibition } (\%) = [(\text{Ac} - \text{As})/\text{Ac}] \times 100$$

Where, As is the absorbance of methanolic extract at different concentration.

Ac is the absorbance of control

The 50% scavenging (IC_{50}) was then as the extract concentration required to scavenge then calculated by plotting the percentage of scavenging activity against the different concentrations (25–400 µg/ml).

ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay, as described by Pellegrini *et al.*, with minor modification [12]. The stock solutions contain 7 mmol ABTS solution and 2.45 m mol potassium per sulphate solutions. The working solution was prepared by mixing the above two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted to an absorbance of 0.700±0.05 at 734 nm for measurement. The photometric assay was conducted by mixing 2.7 ml of ABTS solution and 0.3 ml of methanolic extract for 45 s. The absorbance was measured at 734 nm after 15 min using a spectrophotometer. The blank was prepared in the same manner except the protein fraction. All experiments were carried out in triplicate.

ABTS radical scavenging activity was calculated using the following equation:

$$\text{Inhibition } (\%) = [(\text{Ac} - \text{As})/\text{Ac}] \times 100$$

Where A_S is the absorbance of methanolic extract at different concentration

Ac is the absorbance of control reaction

Total antioxidant capacity

The total antioxidant capacity of the methanolic extract of leaves of *B. racemosa* was evaluated by the phospho- molybdenum reduction assay method according to the procedure described by Oyaizu [13]. The assay was based on the reduction of Mo (VI) to Mo (V) by the methanolic extract of leaves of *B. racemosa* and subsequent formation of green phosphate/Mo (V) complex at acid pH. 1 ml of various concentrations (100-400 µg/ml) of extract was combined with1 ml of reagent solution (0.6M sulfuric acid, 28 mmol sodium phosphate and 4 mmol ammonium molybdate) and incubated at 95 °C for 90 min. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer. Antioxidant capacity was expressed as mg ascorbic acid equivalent per gram dry weight (mg AAE/g DW). All experiments were carried out in triplicate.

Ferric (Fe 3+) reducing power assay (FRPA)

Reducing capability of the methanolic extract was estimated by the method of Prieto *et al.* [14]. A volume of 2 ml of 0.2 mol phosphate buffer (pH 6.6) and 2 ml of potassium ferricyanide were mixed with 2 ml of methanolic extract (1 mg/ml) and incubated at 50 °C for 20 min. From the reaction mixture, 2 ml was taken after addition of 2 ml of 10 % TCA and was mixed with 0.4 ml of 0.1 % ferric chloride and 2 ml of distilled water. After 10 min of incubation, the optical density of the mixture was measured at 700 nm. High reducing power ability was associated with high absorbance value. All experiments were carried out in triplicate.

Statistical analysis

Analytical values were carried out using three independent determinations. Results were expressed as mean values and standard deviation of three independent determinations. Statistical analyses were determined using a statistical software program (SPSS for Windows version 11.0). The data were subjected to analysis of variance using the general linear model to determine significant differences between samples (p<0.05).

RESULTS

Preliminary phytochemicals analysis

Phytochemical screening suggests that methanolic extract contains various constituents which are given in the table 1. Preliminary phytochemicals analysis of a methanolic extract of *B. racemosa* leaf indicated the presence of alkaloids, saponin glycosides, steroids, tannins, flavonoids, triterpenoid and absence of carbohydrate, amino acids and anthracene glycosides.

Antibacterial activity of methanolic extract

The methanolic extract of *Bauhinia racemose* leaves was tested against antibacterial properties, by the agar-well diffusion method and values of the zone of inhibition were recorded in table 2. The methanolic extract was found most effective having a zone of inhibition 22 to 26 mm against *Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The methanolic extract of *B. racemosa* registered the highest value of diameter size of the zone of inhibition of 26 mm, against *Proteus mirabilis* and the lowest value of 20 mm against *Acinetobacter baumannii*.

MIC and MBC of methanolic extract

MIC and MBC values of methanolic extract of *B. racemosa* were recorded (table 3). MIC values of methanolic extract were found 1.51, 1.51, 1.51, 3.41, 4.27, 4.27, 3.41, 4.27 and 1.51 mg/ml against *Enterococcus faecalis, Staphylococcus aureus, Acinetobacter baumannii, Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* respectively. Similarly, the MBC values of rest methanolic extract were recorded in table 2 and found 3.41, 3.41, 3.41, 4.27,

9.63, 9.63, 4.27, 9.63 and 3.41 mg/ml against *Enterococcus faecalis, Staphylococcus aureus, Acinetobacter baumannii, Citrobacter freundii, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* respectively.

Table 1: Preliminary phytochemical analysis of methanolic extract of *Bauhinia racemosa*

S. No.	Phytochemical components	Results
1	Alkaloids	+
2	Anthracene glycosides	-
3	Saponins	+
4	Flavonoids	+
5	Tannins	+
6	Steroids	+
7	Triterpenoids	+
8	Carbohydrate	-
9	Amino acids	-

Table 2: Diameter inhibition of *Bauhinia racemosa* by the agar-well diffusion method

Bacteria	Diameter inhibition of methanolic extract(mm)	
	Methanolic extract	Gentamicin (30µg/ml)
Enterococcus faecalis	22±1.25	25±0.77
Staphylococcus aureus	21±0.58	28±0.64
Acinetobacter baumannii	20±1.19	22±0.83
Citrobacter freundii	22±0.48	20±1.52
Enterobacter aerogenes	25±0.61	27±0.67
Escherichia coli	24±1.29	28±1.49
Klebsiella pneumoniae	25±0.48	23±0.46
Proteus mirabilis	26±0.72	28±0.86
Pseudomonas aeruginosa	22±1.65	26±1.48

Table 3: MIC and MBC values of *Bauhinia racemosa* (mg/ml)

Bacteria`	Methanolic extract		Gentamicin (30µg/ml)	
	MIC(mg/ml)	MBC(mg/ml)	MIC(mg/ml)	MBC(mg/ml)
Enterococcus faecalis	9.63±0.56	21.67±0.42	1.51±0.28	3.41±0.37
Staphylococcus aureus	9.63±0.84	21.67±0.67	0.67±0.39	1.51±0.52
Acinetobacter baumannii	9.63±0.67	21.67±0.55	9.63±0.18	21.67±0.44
Citrobacter freundii	9.63±0.61	21.67±0.91	9.63±0.43	21.67±0.28
Enterobacter aerogenes	1.51±0.73	3.41±0.84	0.67±0.52	1.51±0.91
Escherichia coli	3.41±0.81	9.63±0.73	0.67±0.34	1.51±0.42
Klebsiella pneumoniae	1.51±0.19	3.41±0.57	3.41±0.37	9.63±0.55
Proteus mirabilis	1.51±0.87	3.41±0.86	0.67±0.67	1.51±0.51
Pseudomonas aeruginosa	9.63±0.26	21.67±0.35	1.51±0.43	3.41±0.73

DPPH radical scavenging activity

The DPPH radical scavenging capacity of leaf extract of *B. racemosa* and ascorbic acid were shown in fig. 1. The percentages of DPPH radical scavenging activity of the methanol extract were found 46.14, 52.87, 63.12, 70.98, 77.81 and 86.75 % at the concentrations of 5, 10, 20, 30, 40 and 50 µg/ml respectively. The methanolic extract exhibited highest percentage of inhibition (86.75 %) at the highest concentration, whereas ascorbic acid showed 92.7 % of inhibition.

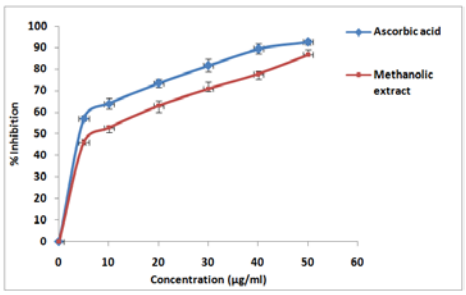

Fig. 1: DPPH radical scavenging activity of methanolic extract of *Bauhinia racemosa* and ascorbic acid
Values represent mean±SD of three replicates

The IC_{50} value of the extract was compared to IC_{50} ascorbic acid. The IC_{50}value of the extract was found to be 9 µg/ml and that of ascorbic acid was 2.5µg/ml from the regression equation.

ABTS radical scavenging activity

The capacity of methanolic extract for scavenging of ABTS radical was examined by ABTS radical decolorization method (fig. 2). ABTS is a nitrogen-centered free radical and is used to evaluate the antioxidant activity. The ABTS radical is generated by oxidation of ABTS with potassium persulfate, and when methanolic extract are added to the ABTS radical, it is converted into a stable non-radical form.

The results of the study indicated that methanolic extracts of *B. racemosa* at a concentration of 50,100, 200, 400 and 1000 µg/ml showed % inhibition of ABTS free radicals with the values of 17.62, 24.81, 30.42, 43.56 and 67.48 % respectively. The computational IC_{50} value of methanolic extract of *B. racemosa* leaves was 550 µg/ml whereas ascorbic acid was150 µg/ml.

Total antioxidant activity (Phospho molybdate assay)

Total antioxidant capacity of the methanol extract of *B. racemosa* expressed as the number of gram equivalents of ascorbic acid (Standard curve equation; y=0.007x+0.005; R^2= 0.98)) is shown in fig. 3. Total antioxidant activity of methanolic extract at highest concentration was found 108.12 (mg AAE/g).

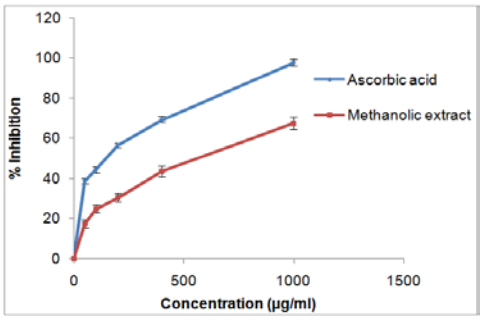

Fig. 2: ABTS radical scavenging activity of methanolic extracts of *Bauhinia racemosa* and ascorbic acid
Values represent mean±SD of three replicates

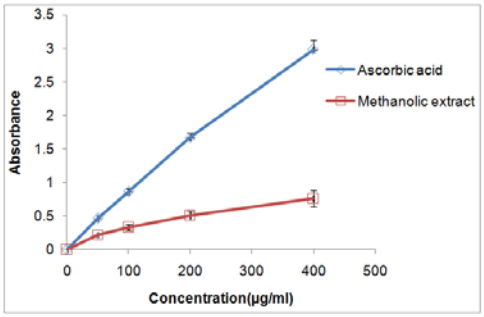

Fig. 3: Total antioxidant activity of methanolic extract of *Bauhinia racemosa* and ascorbic acid
Values represent mean±SD of three replicates

Ferric reducing power assay (FRPA)

The reducing power of methanolic extract of *B. racemosa* was shown in fig. 4. The reducing power increased with an increase in the concentration of the methanolic extract. The absorbance of extract at the highest concentration (400 µg/ml) was found 1.896. The above finding can be attributed to the presence of high amounts of reducing agent that can act as antioxidants which interact with free radicals to terminate radical chain reactions.

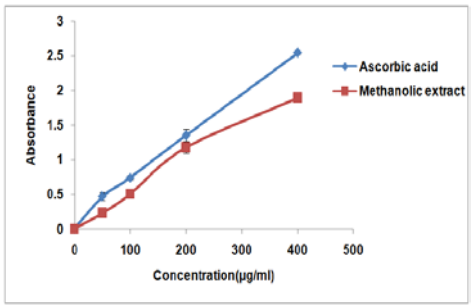

Fig. 4: Reducing power assay of methanolic extract of *Bauhinia racemosa* and ascorbic acid
Values represent mean±SD of three replicates

DISCUSSION

The importance of natural antibacterial and antioxidant agents are emerging into the limelight of modern research field at the alarming rate due to their less toxicity and easy availability. Every antibacterial drug is being resistant to conventional antibiotics after an active period and resistance is continuously emerging at an alarming rate [15].

Thus, the increase in resistance to antibacterial has created an urgent need for the search of new antibacterial agents from plant sources [16]. The Gram-positive bacterial strains are more susceptible to that of Gram-negative strains, which was previously reported by some researchers [17]. The methanolic extract of this plant contains alkaloids, saponin glycosides, steroids, tannins, flavonoids and triterpenoids, out of which, few metabolites exert antimicrobial activity through different mechanisms. The most common biological properties of alkaloids are their toxicity against cells of foreign organisms. The flavonoid exhibits antimicrobial activity by the formation of complex with cell wall and terpenoids also disrupt the cell membrane and shows antibacterial activity [18]. Hence, the presence of these compounds in this plant extract could explain the antimicrobial activities.

Free radicals are the molecules available with unpaired electrons and are involved in inflammation, reperfusion injury, cardiovascular disorders, rheumatoid arthritis, atherosclerosis, aging and neoplastic diseases [19]. Our results demonstrated that the methanolic extracts of leaves of *B. racemosa* possess free radical scavenging activity in DPPH, ABTS, reducing power activity and phospho molybdate assay.

The reactivity of methanol extract of *B. racemosa* extract was analyzed with DPPH, a stable free radical. As DPPH picks up one electron in the presence of a free radical scavenger, the absorption decreases, and the resulting discoloration is stoichiometrically related to the number of electrons gained [20]. Our findings correspond to the previous works i.e. Evaluation of *in vitro* antioxidant properties of methanol and aqueous extracts of *Parkinsonia aculeata* L. leaves by some researchers that the DPPH scavenging activity increased with increasing concentrations of the methanolic extract [21]. Thus, based on the results, the methanolic extract exhibited higher DPPH radical scavenging activity.

The ABTS radical scavenging assay involves a method that generates a green ABTS chromophore by the reaction of ABTS and potassium per sulphate. This radical action is generation by the oxidation of ABTS with potassium per sulphate. The methanolic extract showed ABTS scavenging activity and this observation is previously supported by Sahreen *et al.*, 2010 [22].

The phospho- molybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm [23].

A reducing power is associated with antioxidant potential having the ability to donate an electron and react with free radicals and then convert them into more stable metabolites and terminate the oxidative chain reaction [24].

The methanolic extract of *B. racemosa* might have sufficient amount of reductant, which may react with the free radicals to terminate free radical chain reactions. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing the power of methanol extract of leaves of *B. racemosa*. It causes the conversion of the Fe3+/ferricyanide complex to ferrous form. Fe+3 reductions are often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties [25].

The increase in absorbance of the reaction mixture indicated an increase in reducing the power of the extract was shown in fig. 4. The reducing power assay showed greater reduction capacity compared with the standard ascorbic acid. Hence, the methanolic extract of leaves of *B. racemosa* might be a potential source for the antioxidant phytoconstituents.

CONCLUSION

The spectra of antimicrobial activity displayed by the methanolic extract of *B. racemosa* could perhaps be explained by the presence of flavonoids, tannins, saponins and steroids.

The result of the present study signifies the potential of *B. racemosa* as a source of therapeutic agents, which may provide leads in the field of multidrug-resistant bacterial strains for searching

antimicrobial agents from plant sources. In this experiment, we conceded a systematic record on the relative free radical scavenging activity of the methanolic extract of *B. racemosa*. The finding of the study suggests that *B. racemosa* leaves could be a potential source of natural antioxidant that could have greater importance as therapeutic agents in preventing various oxidative stress related diseases.

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CONFLICT OF INTERESTS

Declared none

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