Studies on phytochemical and In-Vitro antioxidant potential of *Justicia beddomei (Clarke) Bennett*

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Submission Date: 12-5-2012; Review Completed: 10-7-2012; Accepted Date: 4-11-2012

ABSTRACT

Introduction: The current study was carried out to investigate the phytochemical constituents and *in vitro* antioxidant potential of the aerial parts of *Justicia beddomei* (Clarke) Bennett belonging to the family Acanthaceae. The aerial part of *J. bebbomei* is traditionally used as a hepatoprotective, antitussive and antispasmodic. **Methods:** The dried powdered aerial parts of *J. beddomei* were extracted using petroleum ether, chloroform, ethyl acetate and methanol using a soxhlet extractor and preliminary phytochemical screening was performed using standard protocols. All the extracts were evaluated for their potential antioxidant activities using tests such as DPPH, hydroxyl radical, superoxide anion radical scavenging abilities, β-carotene-linoleic acid model, reducing power ability and total phenolic and flavonoid contents. **Results:** Preliminary screening revealed the presence of bioactive components especially phenolics and flavonoids in all the extracts. The phenolic and flavonoid content was found to be highest in methanolic extract and lowest in petroleum ether extract. All extracts showed strong antioxidant activity when compared with the standard. **Conclusion:** The results of the present study indicate that the aerial part extracts of *J. beddomei* is a good source of antioxidant constituents.

Keywords: Free radicals, Antioxidant, *Justicia beddomei*, *in vitro* study, phenolics.

INTRODUCTION

Free radicals and other reactive oxygen species (ROS) are generated continuously via normal physiological process, more so in pathological conditions. These free radicals are associated directly or indirectly with most of the pathologies known to date. Oxidative damage caused by free radicals and ROS plays a significant role in the etiology of cancer, emphysema, cirrhosis, arthritis etc.[1] The free radical theory of ageing predicts that the rate of aging is dependent on the level of oxidative stress, i.e the balance between pro-oxidants and antioxidants and the consecutive oxidative damage.[2] Due to this reason synthetic antioxidants are widely used but their safety and toxicity have been an important concern.[3] Much attention has been focused on medications which are known to contain a variety of antioxidants to inhibit lipid peroxidation to protect the human body from oxidative damage by free radicals. There has been an increasing demand to evaluate the antioxidant properties of direct plant extracts or isolated products rather than looking for synthetic ones.[4] Epidemiological studies showed that phytonutrients are beneficial in protecting human body against damage caused by ROS and nitrogen species.[5] Therefore the development and utilization of more effective antioxidants of natural origin are desired.[6]

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DOI: 10.5530/ax.2012.4.5

The genus Justicia comprises about 300 species world over and nearly 50 species occur in lndia.[7,8] About 20 species have been chemically investigated and reported to contain lignans, triterpenoidal glycosides and amide. The plants of *Justicia* genus are rich sources of bioactive lignans particularly arylnaphthalides.[8,9] The plant has been found to be having anti diabetic, anti-inflammatory and anthelmintic properties.[10,11] The plant is distributed in the hilly regions of Kerala. Various parts are widely used in folk medicine as an expectorant, diuretic, antispasmodic, depurative and styptic. Leaves are used in diarrhoreal bleeding and irritative cough. Flowers are used in ophthalmic preparations and the roots along with the leaf juice are used in pthisis, cough, and asthma.[12] Along with turmeric it is used in an anti ageing cream to prevent wrinkles in the skin. However no reports on phytochemical or antioxidant studies on aerial part of *Justicia beddomei* are reported till date. Thus in the light of knowledge that *Justicia* species contains lignans[13] having wide folklore uses we are aiming to evaluate the antioxidant properties of various extracts of aerial parts of *Justicia beddomei* using *in vitro* models.

MATERIALS AND METHODS

Plant material

The plant material was collected from hilly regions of Kerala, India. The plant was botanically identified by Dr. V. Chelladurai, Research Officer Botany, (Rtd) CCRAS, Government of India. A voucher Specimen has been kept in the department of chemistry (NCP/CH/PS/JB01), National College of Pharmacy, Calicut.

Extraction

The aerial parts of *J. beddomei* were dried, grounded into fine powder and sieved through No. 20 mesh sieve. About 100 g of powdered aerial part was subjected to soxhlet extraction with 500 ml of petroleum ether, followed by chloroform, ethyl acetate and methanol. After extraction, the extracts were concentrated and dried at room temperature. The percentage yield of petroleum ether, chloroform, ethyl acetate and methanol were 3.2, 4.4, 7.6 and 8.8% w/w respectively.

Chemicals and instruments

Linoleic acid, β-carotene, ferrozine and l, l-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). Butylated hydroxytoluene (BHT), ascorbic acid (AA), Potassium ferricyanide, nitroblue tetrazolium (NET), trichloroacetic acid (TCA), and ferric chloride were purchased from SD Fine Chemicals (Mumbai, India). All other drugs and chemicals used for the work were purchased commercially and were of analytical grade. U-V spectrophotometer (Shimadzu) was used to measure the absorbance.

Phytochemical screening

All the extracts were subjected to phytochemical tests for various chemical constituents like flavonoids, lignans, alkaloids, glycosides, phenols, terpenoids, saponins and steroids.[14,15,16]

Total phenolic assay

The total phenolic content of extracts of *Justicia beddomei* were determined by using the Folin-Ciocalteu assay.[17] About 1 ml of extracts or standard solution of gallic acid (20, 40, 60, 80 and 100 mg/L) was added to 25 ml volumetric flask, containing 9 ml of distilled deionised water (dd H_2O). A reagent blank using dd H_2O was prepared. 1 ml of Folin-Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to the mixture and total volume made up to 25 ml with dd H_2O and mixed. After incubation for 90 min at room temperature, the absorbance against prepared reagent blank was determined at 750 nm with UV-Vis Spectrophotometer. Total phenolic content of the extracts were expressed as mg Gallic acid equivalents (GAE)/100 g dry weight.

Total flavonoid assay

Total flavonoid content was measured by the aluminum chloride colorimetric assay.[18] An aliquot (1 ml) of extracts or standard solution of catechin (20, 40, 60, 80 and 100 mg/L) was added to 10 ml volumetric flask containing 4 ml of dd H_2O. To the flask 0.3 ml 5 % $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $A1C1_3$ was added. At 6th min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with dd H_2O. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content was expressed as mg catechin equivalents (CE)/100 g dry weight.

Reducing power assay

The total reducing power of all the extracts was determined following the method of Oyaizu.[19] Different amount of extracts (25–400 μg/ml) were mixed with equal volume of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture followed by centrifugation at 3000 rpm for 10 min. A 2.5 ml

portion of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeC1_3$. The absorbance was measured at 700 nm and ascorbic acid was used as the standard compound.

Antioxidant assay using β-carotene-linoleate model system

The antioxidant activity of all the extracts was evaluated by the β carotene-linoleate model system suggested by Miller *et al.*[20] A solution of carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. This solution (2 ml) was pipetted into a 100 ml round-bottom flask. After chloroform was removed under vacuum, 40 mg of purified linoleic acid, 400 mg of tween-40 emulsifier and 100 ml distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into test tubes containing different concentrations of the extracts (0.2 ml). As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The tubes were placed at 50°C in a water bath, and measurement of absorbance was recorded after 2 hours; a blank, devoid of β-carotene, was prepared for back-ground subtraction. The same procedure was repeated with the butylated hydroxyl toluene (BHT), as a positive control. Percentage of antioxidant activity was calculated using the following equation:

$$\text{Antioxidant activity} = \frac{\beta\text{-carotene content after 2 h of assay}}{\text{Initial } \beta\text{-carotene content}} \times 100$$

Determination of DPPH radical scavenging activity

The antioxidant activity was assessed quantitative using 2, 2-diphenyl-1 picrylhydrazyl (DPPH) method.[21] The solution of DPPH (0.1 mM) was prepared with methanol. About 0.5 ml of extract solution, prepared at different concentration in the range of 25–400 μg/ml were added to 5 ml of 0.l mM methanolic solution of DPPH. After 30 minutes of incubation in dark at room temperature (30 ± 2°C), the absorbance was read against a sample blank at 517 nm. BHT was used as a reference compound. The percentage inhibition of the extracts and standard was calculated.

Superoxide anion scavenging activity [non-enzymatic]

Measurement of superoxide anion scavenging activity of *Justicia beddomei* extracts was based on the method described by Liu *et al*[22] with slight modification.[23] 1 ml of Nitro blue tetrazlium (NBT) solution (156 μM/L NBT in l00 mM/L Phosphate buffer, pH 7.4), 1 ml NADH solution (468 μM/L in l00 mM/L Phosphate buffer, pH-7.4) and 0.1 ml of sample solution of extracts (25–400 μg/ml) in water were mixed. Reaction started by adding l00 μL of Phenazine methosulphate [PMS] solution (60 μM/L in l00 mM/L Phosphate buffer, pH-7.4) to the mixture incubated at 25°C for 5 minutes. Catechin was used as a reference compound. Absorbance was measured against control samples at 560 nm and percentage inhibition calculated.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of extracts (25–400 μg/ml) were assayed.[24] The reaction mixture contained 500 ml of 2-deoxyribose (2.8 mM) in phosphate buffer (50 mM, pH 7.4), 200 ml of premixed ferric chloride (100 mM) and EDTA (100 mM) solution (1:1; v/v), 100 ml of H_2O_2 (200 mM) without or with the extract solution (100 ml). The reaction was triggered by adding 100 ml of 300 mM ascorbic acid and incubated for 1 h at 37°C. A solution of TBA in 1 ml (1%; w/v) of 50 mM NaOH and 1 ml of 2.8% (w/v; aqueous solution) TCA was added. The mixture was heated for 15 min on a boiling water bath and then cooled. The absorbance was measured at 532 nm with ascorbic acid as the reference compound. Percentage inhibition of superoxide anion radicals was calculated.

Calculation of 50% inhibitory concentration (IC_{50})

The concentration (μg/ml) of the extract required to scavenge 50% of the radicals was calculated by using the percentage scavenging activities at five different concentrations of the extracts. Percentage inhibition (I%) was calculated using the formula:

$$I\% = A_c - A_t / A_c \times 100$$

Where A_c is the absorbance of the control and A_t is the absorbance of the sample.

Statistical analysis

All the experiments were carried out in triplicate and results expressed as mean ± SEM. Significant differences among means were evaluated by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Phytochemical screening

Various bioactive components such as flavonoids, phenolics and lignans were prominently revealed during the

preliminary phytochemical screening. Phenolics and flavonoids were present in all the extracts whereas saponins were absent in all the extracts. Terpenoids, steroids and lignans were absent in ethyl acetate and methanol extracts were as glycosides and alkaloids were present in petroleum ether and chloroform extracts.

Total phenolic content

The total phenolic content of different extracts was measured using colorimetric Folin–Ciocalteu (F-C) method. The F-C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 760 nm.[25] The content of phenolic compounds in methanolic, ethyl acetate, chloroform and petroleum extracts were found to be 16.19 ± 0.083, 13.21 ± 0.052, 9.69 ± 0.034, 4.66 ± 0.23 mg GAE/g respectively. These results suggest that the greater levels of antioxidant activity were due to higher phenolic contents and their activity against free radicals can be related to their scavenging ability owing to their hydroxyl groups.

Total flavanoid content

Flavanoids are a class of secondary plant phenolics with powerful antioxidant properties.The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the ortho dihydroxyl groups in the A or B ring of flavonoids.[26] The amount of flavanoids found in methanolic, ethyl acetate, chloroform and petroleum ether extracts was 8.08 ± 0.12, 4.14 ± 0.13, 1.02 ± 0.23 and 0.78 ± 0.36 mg CE/g. Several studies have shown that many flavonoids are effective in blocking oxidant induced neuronal injury.[27]

Reducing power ability

In reducing power, the presence of antioxidants reduce Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} formation can be monitored spectrophotometrically.[28] The reducing power of all the extracts and the standard increased with an increase in their concentration. It has been observed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts.[29] At concentration 400 µg/ml, the methanolic extract (0.7013 ± 0.194) showed greater absorbance than ascorbic acid (0.6016 ± 0.338). Reducing power of the *Justicia beddomei* extracts are shown in Table 1.

Antioxidant assay using β-carotene linoleate model

The antioxidant activity of the extracts were measured by the bleaching of β-carotene and is shown in the Table 2. The addition of BHT and various *Justicia beddomei* extracts prevented the bleaching of β-carotene to different degrees. In the absence of antioxidants, β-carotene undergoes a rapid discoloration due to the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. These free radicals attack highly unsaturated β-carotene molecules. As a result β-carotene will be oxidized, subsequently losing its chromophore and characteristic orange colour, which can be monitored spectrophotometrically. The presence of different antioxidants can hinder the extend of β-carotene bleaching by neutralizing the linoleate free radicals and other free radicals formed in the system.[30] In the present study *Justicia beddomei* extracts were found to hinder the extend of β-carotene bleaching by neutralizing free radicals. The methanolic extract of *Justicia beddomei* showed a significant ($P < 0.05$) antioxidant activity (IC_{50} = 86.11 ± 0.236 µg/ml) when compared with that of the standard BHT (IC_{50}= 70.58 ± 0.530 µg/ml) (Table 2).

DPPH radical scavenging activity

The Scavenging activity of a stable radical is considered a valid and easy assay to evaluate scavenging activity of natural compounds.[31] The method is based on the reduction of DPPH solution in the presence of a hydrogen donating antioxidant. Thus the purple colour of DPPH will get reduced to yellow colored diphenyl-picryl hydrazine.[32]

Table 1 Reducing power ability of *J. beddomei*

CONC µg/ml	Absorbance (nm)				
	PEJ	CEJ	EAEJ	MEJ	ASCORBIC ACID
25	0.0126 ± 0.026	0.0346 ± 0.325	0.5262 ± 0.302	0.0628 ± 0.046	0.6628 ± 0.462
50	0.0822 ± 0.032	0.0926 ± 0.185	0.1064 ± 0.144	0.2332 ± 0.144	0.7082 ± 0.262
100	0.1122 ± 0.012	0.2456 ± 0.192	0.2692 ± 0.565	0.3652 ± 0.057	0.8922 ± 0.322
200	0.1372 ± 0.133	0.2962 ± 0.120	0.4685 ± 0.046	0.5722 ± 0.243	0.9502 ± 0.126
400	0.1864 ± 0.275	0.3256 ± 0.205	0.6016 ± 0.338	0.7013 ± 0.194	0.9816 ± 0.156

PEJ: Pet Ether Extract of Justicia beddomei; CEJ: Chloroform Extract of Justicia beddomei; EAEJ: Ethyl Acetate Extract of Justicia beddomei; MEJ: Metanolic Exract of Justicia beddomei; All values determined were mean ± SEM; n = 3.

Table 2 *In vitro* antioxidant activity of aerial part of *J. beddomei*

Extract/standard	IC_{50} (µg/ml)			
	Hydroxyl radical	Superoxide radical	DPPH radical	β-carotene-linoleate model
PEJ	350.60 ± 0.162*	364.13 ± 0.182*	369.05 ± 0.159*	342.85 ± 0.204*
CEJ	208.30 ± 0.174*	186.71 ± 0.032*	214.68 ± 0.330*	191.66 ± 0.314*
EAEJ	103.04 ± 0.129*	158.25 ± 0.029*	173.25 ± 0.185*	93.75 ± 0.217*
MEJ	97.89 ± 0.362*	112.97 ± 0.322*	89.68 ± 0.107*	86.11 ± 0.236*
ASCORBIC ACID	–	68.32 ± 0.522	–	–
BHT	–	–	48.04 ± 0.152	70.58 ± 0.530
CATECHIN	35.32 ± 0.236	–	–	–

*PEJ: Petroleum ether extract of J. beddomei; CEJ:- Chloroform extract of J. beddomei EAEJ: Ethyl acetate extract of J. beddomei; MEJ: Methanolic exract of J. beddomei. All values determined were mean ± SEM; n = 3. *P < 0.05 when compared with standard.*

All extracts showed a concentration-dependent anti-DPPH radical scavenging ability, of which methanolic extract (89.68 ± 0.107 µg/ml) exhibited prominent activity. It thus appears that the extracts possess hydrogen donating ability and act as antioxidant. However, the scavenging ability of BHT (48.04 ± 0.152 µg/ml) a known antioxidant used as positive control was greater than all the extracts (Table 2).

Superoxide anion scavenging activity

The Superoxide anion radical scavenging activity of *Justicia beddomei* extracts are shown in Table 2. Superoxides are produced from molecular oxygen due to the oxidative enzymes of body as well as via non enzymatic reaction such as auto oxidation by catecholamines. In the present study superoxide radical reduces NBT to a blue coloured formazan which is measured at 560 nm.[33] All the extracts prevented formation of blue coloured formazan and the percentage inhibition was concentration dependent. The methanolic extract (112.97 ± 0.322 µg/ml) of the aerial part of *J. beddomei* exhibited notable superoxide scavenging ability when compared with ascorbic acid (68.32 ± 0.522 µg/ml), which was used as a positive control.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity.[34] The ability of extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and they seem to be good scavengers of active oxygen species, thus reducing the rate of reaction. All extracts scavenged the generated hydroxyl radicals and prevented degradation of 2-deoxy-2-ribose in a dose dependent manner. However the antioxidant activity exhibited by catechin, a positive control was more pronounced than all the extracts (Table 2).

CONCLUSION

The present study clearly reveals that *Justicia beddomei* possess remarkable antioxidant property. Among the extracts, methanolic extract showed strong free radical scavenging activity. A co-relation was also observed between methanol and ethyl acetate extracts for their total phenolic and flavonoid content. These results conclude the use of *Justicia beddomei* in the traditional medicine useful and reasonable. Further studies are ongoing for the isolation and identification of individual phytoconstituents responsible for the biological activity of *Justicia beddomei* and also *in vivo* studies are needed for better understanding of their mechanism of action.

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