Natural Product Sciences
13(3) : 199-206 (2007)

Antioxidant and Free Radical Scavenging Potential of *Justicia gendarussa* Burm. Leaves *in vitro*.

K. Mruthunjaya[1] and V. I. Hukkeri[2]*

[1]*Department of Pharmacognosy and Phytochemistry, J.S.S. College of Pharmacy, Vidyanagar, S. S. Nagar, Mysore-570 015 Karnatakata, India*
[2]*Director, Herbals Research Division, Dr. Nargund Research Foundation, Dattatreya Nagar, BSK III Stage, 100 feet Ring Road, Bangalore-560 085, Karnataka, India.*

Abstract – Antioxidant activity of 70% aqueous ethanolic extract of leaves of *Justicia gendarussa* (EJ) was evaluated. EJ was prepared by cold maceration method. The antioxidant potency of EJ was investigated employing various established *in vitro* systems, such as DPPH radical scavenging, nitric oxide (NO) scavenging, β-carotene linoleic acid module system (β CLAMS), hydroxyl (OH) radical scavenging, anti lipid peroxidation. IC_{50} values were determined in each experiment. Also, ferric ion reduction capacity of extracts in presence and absence of chelating agent (EDTA) and total antioxidant capacity were determined. Preliminary phytochemical investigation was carried out to know the nature of constituents present in the leaves and correlate it with antioxidant activity. Further total phenolic content was determined in EJ. IC_{50} values of EJ were 123.09 ± 3.01, 643.0 ± 61.10, 132.3 ± 6.03, 68.5 ± 11.5 and 68.13 ± 1.38 µg/mL in DPPH radical scavenging, NO scavenging, β CLAMS, OH radical scavenging and anti lipid peroxidation activity respectively. In total antioxidant capacity assay, ascorbic acid equivalent value was found to be 205.56 ± 4.69 µg/mg of extract. Total phenolic content was found to be 43.76 ± 4.27 µg equivalent of gallic acid per mg of extract. Phytochemical investigation reveals the presence of flavonoids. The results indicate that EJ possess antioxidant activity and flavonoids are responsible for this activity.
Keywords – *Justicia gendarussa*, antioxidant, DPPH, free radical scavenging, hydroxyl radical scavenging, lignans

Introduction

Reactive oxygen species (ROS) can contribute to the etiology of disorders such as cancer, liver diseases, atherosclecrosis, respiratory diseases and inflammatory response syndrome. In recent years there is great deal of interest in developing agents to control damage induced by ROS in biological systems. As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

Justicia gendarussa belonging to family Acanthaceae is known for its medicinal properties in Ayurveda, an Indian system of medicine in inflammations, bronchitis, vaginal discharges, dyspepsia, eye diseases and fever (Kirthikar and Basu, 2001). Decoction of leaves is used to treat chronic rheumatism (Anonymus 1959). *Justicia* species found to contain lignans, naturally occurring phenolic dimers. Lignans reported to have various significant biological activities including antioxidant (Jyotishi and Bagavant, 1992a; 1992b). It is also reported to contain β-sitosterol, friedelin, lupeol and four simple *0*-substituted aromatic amines (Chakravarty *et al*., 1982). Its medicinal properties and presence of lignans inspired us to take up the particular study.

*Author for correspondence
Fax: +91-80-26421903; E-mail: hukkeri_vi@rediffmail.com

Experimental

Chemicals – α,α-diphenyl-β-picryl hydrazyl (DPPH), egg phospatidylcholine, β-carotene and γ-linoleic acid were obtained from Sigma Chemical Co.(St. Louis USA), butylated hydroxy toluene (BHT), ascorbic acid, ethylene-diaminetetraacetic acid (EDTA), Tween-40, deoxy-*d*-ribose, trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were obtained by Hi-Media Labs (Mumbai, India), 1,10-*O*-phenanthroline, ferric chloride ($FeCl_3$), hydrogen peroxide, ammonium molybdate, sodium dithionite were obtained from Ranbaxy Fine Chemicals (New Delhi, India), phenyl hydrazine, folin-ciocalteau phenol reagent were obtained from BDH Products (UK). Silymarin was kind gift from Dr. Chidambaramurthy K.N., CFTRI,

Mysore, India. All other chemicals used were of analytical grade. The solvents used for extraction were from Ranbaxy Fine Chemicals (New Delhi, India). The UV-visible spectrophotometric values were recorded in JASCO UV-500 spectrophotometer.

Plant material and extraction – The leaves of the plant were collected during September 2004 in Wynadu district of Kerala State, India, authenticated, by Dr. B.D.Huddar, Professor and Head, Department of Botany, HSK Science Institute, Hubli, India. The voucher specimen (KMJG01) of the plant is preserved in Department of Pharmacognosy, KLES' College of Pharmacy, Hubli, India. Leaves dried under shed, powdered and extracted with 70% aqueous ethanol by cold maceration. The extraction was done for 72 hours. After extraction the extract was separated from marc by filtration through filter paper. The marc was pressed in muslin cloth to remove the solvent which is left in the marc after filtration. Filtrate was preserved in a well closed container. Marc left after extraction was extracted by cold maceration for 3 more days with same amount of fresh solvent and the process was repeated for one more time. i.e. the drug was extracted 3 times with a gap of 3 days each. The 10^{th} day the filtrates were pooled and concentrated to syrupy liquid under reduced pressure using superfit rotary vacuum evaporator, dried and stored in dessicator. Same is used for below mentioned experiments.

Preliminary phytochemical investigations – The preliminary phytochemical screening of the extract was carried out to know the different constituents present in it as per the standard procedures. The extract was tested for alkaloids (Kokate *et al*., 1996), sterols and triterpenes (Peach *et al*., 1955a), saponins (Peach *et al*., 1955b), flavonoids (Geinssman, 1955; Trease *et al*., 1989), tannins (Kokate *et al*., 1996; Trease *et al*., 1989), carbohydrates (Hawks, 1971), cardiac glycosides, lactones and amino acids (Stevens, 1986).

HPLC fingerprinting of the extract – The high pressure liquid chromatography (HPLC) finger printing analysis was performed on a Shimadzu (Japan) chromatographic system, which included a binary pump model LC 10AT, a rheodyne injector with a 20 µl sample loop and a UV-Vis detector model SPD 10AVP. Spinchrom computer software was used to control the system and collect the data. The separation was performed on reverse phase packed column (RP C-18; Phenomenex, USA; 250 mm × 4.6 mm; particle size 5 µm). 3 mg/ml solution of EJ was prepared in methanol : water 7 : 3 and filtered through 0.45 µm membrane filter. The elution was performed using methanol (A) and water (B), employing a program from A : B-30 : 70, 50 : 50, 70 : 30, 90 : 10 and finally 100% A for 27 min. The flow rate was maintained at 1 mL/min. and the ratio changed at every 5 min. The chromatogram of the EJ was recorded at 275 nm.

DPPH radical scavenging activity (Singh *et al*., 2002) – Free radical scavenging potentials of the extracts was tested against a methanolic solution of DPPH. Different concentrations of EJ, BHT and ascorbic acid in 500 µL ethanol were taken and added with 5 mL of 100 µM DPPH in methanol to give final concentration of 9.09 to 181.82 µg/mL of EJ, 4.54 to 27.27 µg/mL of BHT and ascorbic acid. The mixtures were allowed to stand at room temperature for 20 minutes. The control was prepared as above without extract. The readings were read at 517 nm using methanol as blank. The absorbance of control was first noted at 517 nm. The changes in absorbance of the samples were measured. Scavenging activity was expressed as the inhibition percentage calculated using the formula **%** anti radical activity = [(Control Abs. – Sample Abs.) / Control Abs.] × 100. Each experiment was carried out in triplicate and results were expressed as mean % antiradical activity ± SD.

Nitric oxide scavenging activity (Govindarajan *et al*., 2003) – Scavenging of NO was determined using sodium nitroprusside (SNP) as NO donor. SNP (10 mM) in phosphate buffered saline was mixed with different concentrations of extract (100 to 1000 µg/ml) in ethanol, ascorbic acid as standard (25 µg/mL to 125 µg/mL) and incubated at 25 °C for 180 min, then Griess reagent (1% sulfanilamide, 0.1% naphylethylenediamine dihydrochloride and 3% phosphoric acid) volume equal to incubated solution was added. The absorbance was immediately measured at 546 nm. The NO scavenging activity was calculated from the formula, percentage NO scavenging activity = [(Abs of Control – Abs of Sample) / Abs of Control] × 100. Each experiment was carried out in triplicate and results were expressed as mean % NO scavenging activity ± SD.

Antioxidant assay using β-Carotene Linoleate Model System (β CLAMS) (Hidalgo *et al*., 1994; Singh *et al*., 2002) – The antioxidant activity of EJ was evaluated by slightly modified method of Hidalgo *et al.*, 1994. Briefly 5 mg β-carotene, 40 mg γ-linoleic acid and 400 mg of Tween-40 were mixed in 1 mL chloroform. Chloroform was removed under vacuum using the flash rotary evaporator at 40 °C. The resulting mixture was added with 20 mL water and emulsion was prepared. The emulsion was further diluted with 80 mL of oxygenated water. 100 to 600 µg of extract and BHT were added in separate test tubes and volume was made up to 0.4 mL

with ethanol. 0.6 mL of water and 3 mL of emulsion was added to each test tube. Absorbances of all samples were taken at 470 nm at zero time and test tubes were placed at 50 °C in water bath. Measurement of absorbance was continued at an interval of 30 minutes, till the color of β-carotene disappeared in the control reaction (t = 180 min). A mixture prepared as above without β-carotene emulsion served as blank and mixture without extract served as control. Dose response of antioxidant activity of EJ was determined at different concentrations. The antioxidant activity (%AA) of EJ was evaluated in terms of bleaching of β-carotene using the following formula. % AA = 100 $[1 - (A^o - A^t) / A^o\ 0 - A^t0)]$, where % AA = Antioxidant activity, A^o = absorbance of sample at zero time, A^00 = zero time absorbance of control, A^t = absorbance of sample after incubation for 180 min, A^t0 = absorbance of control after incubation for 180 min. Each experiment was carried out in triplicate and results were expressed as mean % antioxidant activity ± SD.

Hydroxyl radical scavenging activity (Chakrabarti *et al*., 1995) – The reaction volume contains different concentrations of EJ (from 5 to 80 µg) or standard gallic acid (0.5 to 4 µg), 2.8 mM deoxy-*d*-ribose and phenylhydrazine 0.2 mM incubated for 2 hours at 37 °C in incubator and hydroxyl radical scavenging was measured by thiobarbituric acid reactive substances (TBARS) method (Ohkawa *et al*., 1979). To each test tube was added 1 mL of 2.8% TCA containing 1% TBA and heated in boiling water bath for 20 min., cooled and absorbance was read at 532 nm. Percentage hydroxyl radical scavenging activity was calculated by the formula, percentage hydroxyl radical scavenging activity = $[(C - S) / C] \times 100$, where C is the absorbance of the control and S is the absorbance of the sample. Each experiment was carried out in triplicate and results were expressed as mean % OH radical scavenging activity ± SD.

Lipid peroxidation assay (Sudheerkumar *et al*., 2003) – Egg phospatidylcholine (20 mg) in chloroform (2 mL) was dried under vacuum in a rotary evaporator to give a thin homogenous film and further dispersed in normal saline (5 mL) with a vortex mixer. The mixture was sonicated to get a homogeneous suspension of liposomes. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1 mL), 150 mM potassium chloride, 0.2 mM ferric chloride, EJ (20 to 80 µg/mL) or standard Silymarin (1 to 10 µg) were added separately, in a total volume of 1 mL. The reaction mixture was incubated for 40 minutes at 37 °C. After incubation, the reaction was terminated by adding 1 ml of ice cold 0.25 M Sodium hydroxide containing 20% w/v TCA, 0.4% w/v of TBA and 0.05% w/v BHT. After keeping in boiling water bath for 20 min, the samples were cooled. The pink chromogen was extracted with a constant amount of *n*-butanol, and the absorbance of the upper organic layer was measured at 532 nm. % Anti lipid peroxidation activity was calculated by the formula, % Anti lipid peroxidation activity = $[(C - S) / C] \times 100$, where C is the absorbance of the control and S is the absorbance of the sample. Each experiment was carried out in triplicate and results were expressed as mean % Anti lipid peroxidation activity ± SD.

Reduction of Ferric Ions (Rajkumar and Rao, 1993) – The reaction mixture containing *O*-phenanthroline (0.5 mg), ferric chloride (0.2 mM) and extracts of different concentrations, 100 to 1000 µg or ascorbic acid 10 to 50 µg dissolved in ethanol, in a final volume of 5 mL and was incubated for 15 - 20 min. at ambient temperature. The absorbance at 510 nm was measured. In another set, sodium dithionite (0.3 mM) was added instead of the extract and the absorbance was taken as equivalent to 100% reduction of all the ferric ions present. Each experiment was carried out in triplicate and results were expressed as mean % reduction of ferric ions ± SD.

Reduction of Ferric Ions in presence of EDTA (Kunchandy and Rao, 1989) – The reaction mixture containing *O*-phenanthroline (0.5 mg), ferric chloride (0.2 mM), EDTA (0.2 mM) and extracts of different concentrations, 100 to 600 µg ascorbic acid 10 to 50 µg (dissolved in ethanol) in a final volume of 5 mL and was incubated for 15 - 20 min. at ambient temperature. The absorbance at 510 nm was measured. In another set, sodium dithionite (0.3 mM) was added instead of the extract and the absorbance was taken as equivalent to 100% reduction of all the ferric ions present.

Hydrogen peroxide scavenging activity (Ilhami, 2006) – The hydrogen peroxide scavenging ability of EJ was determined by simple UV spectroscopic method. Different concentrations of EJ and BHT from 5 µg to 50 µg were taken, volume adjusted to 3 mL with phosphate buffer and 1 mL of 30 mM H_2O_2 was added. After 10 min. the absorbance of the reaction mixture was recorded at 230 nm. Blank solution was containing the phosphate buffer without H_2O_2. Percentage scavenged $[H_2O_2]$ was calculated using the formula. Percentage scavenged $[H_2O_2] = [(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. Each experiment was carried out in triplicate and results were expressed as percentage scavenged $[H_2O_2]$ ± SD.

Total antioxidant capacity (Prieto *et al*., 1999; Govindarajan *et al*., 2003) – Total antioxidant capacity

was measured according to slightly modified method of Prieto *et al*., 1999. 100 μg of EJ, BHT and silymarin were taken in 0.1 mL of alcohol, combined separately in an eppendroff tube with 1.9 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 ºC for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1.9 mL of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid. The experiment was conducted in triplicate and values are expressed as ascorbic acid equivalents in μg per mg of extract (mean ± SD).

Total Phenolic Content (TPC) (Mathew and Abraham, 2006) – EJ was diluted with the alcohol 95% to a suitable concentration for analysis. TPC of EJ was assessed approximately by using the Folin-Ciocalteau phenol reagent method. To 100 μL of the extract (200 μg) was added 0.5 mL of Folin–Ciocalteau reagent and 1 mL of sodium carbonate (4% w/v) and the volume made upto to 2 mL, contents were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured in a UV-Vis. spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in micrograms per mg of sample, using a standard curve generated with gallic acid. The experiment was conducted in triplicate and values are expressed in mean ± SD.

Results and discussion

Preliminary phytochemical screening of EJ showed the presence of sterols, flavonoids, carbohydrates and glycosides.

The HPLC chromatogram of EJ measured at 275 nm is represented in Fig. 1. at a chromatogram of EJ at the concentration of 5 mg/mL showed that, it contains constituents eluting between 2 - 25 min with major peaks at 2.68, 2.833, 12.317, 23.37 and 23.46 min. The solvent system, concentration of sample and wavelength for detection (275 nm) was selected after trying different combinations. The method which is adopted was found the most suitable one with highest reproducibility.

As shown in Table 1, EJ strongly scavenged DPPH radical with the IC_{50} value of 123.09 ± 3.01 μg/mL. The scavenging was found to be dose dependent. Where as ascorbic acid and BHT used as standards were shown IC_{50} value of 4.91 ± 0.36 and 21.88 ± 2.12 μg/mL. DPPH radicals react with suitable reducing agents then losing colour stiochiometrically with the number of electrons consumed which is measured spectrophotometrically at 517 nm. Ascorbic acid is a potent free radical scavenger and BHT is known antioxidant and is used as preservative (Singh *et al*., 2002; Mathew and Abraham, 2006). So when compared to such pure components, IC_{50} value of 123.09 ± 3.01 μg/mL of EJ is quiet high, and shows that

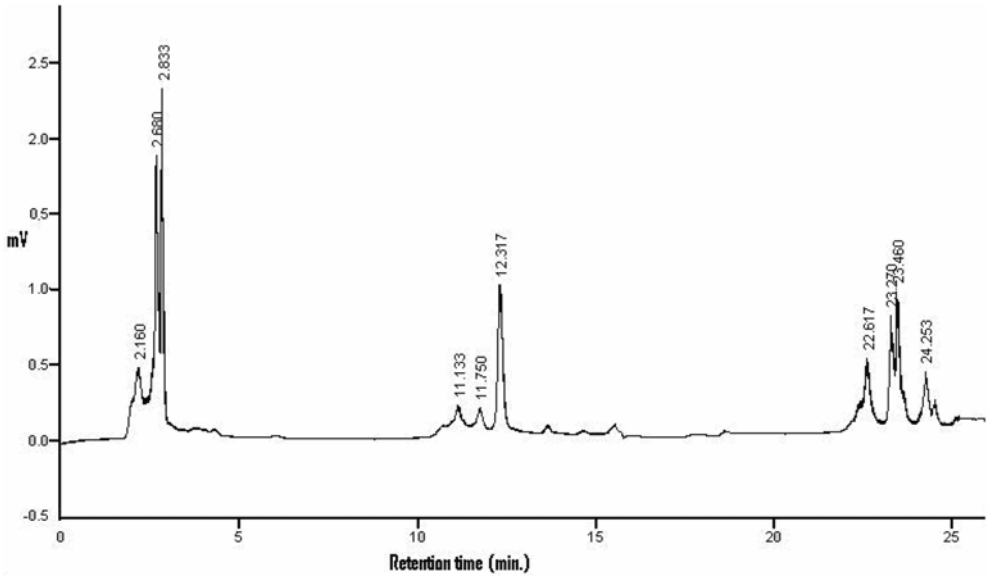

Fig. 1. HPLC finger printing. HPLC chromatogram of EJ detected at 275 nm separated on a RP-C18 column, Phenomenex, USA (250 × 4.6 mm; particle size 5 μm) using methanol: water in different ratio for 27 min.

Table 1. Percentage Free radical scavenging activity of EJ, BHT and Ascorbic acid in DPPH method

Conc. (µg/mL)	EJ	Conc. (µg/mL)	BHT	Ascorbic acid
09.09	06.34 ± 1.60	4.54	32.05 ± 2.62	49.19 ± 1.52
18.18	14.13 ± 1.57	9.09	36.96 ± 1.56	76.30 ± 2.65
36.36	22.97 ± 2.63	13.63	40.06 ± 0.95	80.01 ± 1.98
54.54	31.87 ± 1.03	18.18	47.03 ± 0.89	86.03 ± 1.21
72.73	38.29 ± 1.18	22.73	51.19 ± 3.21	93.90 ± 2.36
109.09	45.08 ± 1.30	27.27	59.98 ± 1.65	99.38 ± 1.87
145.45	51.85 ± 1.60		---	
181.82	63.49 ± 1.11		---	
IC_{50}	123.09 ± 3.01		21.88 ± 2.12	4.91 ± 0.36

Table 2. Nitric Oxide scavenging activity of EJ and Ascorbic acid in percentage

Conc. (µg/mL)	EJ	Conc. (µg/mL)	Ascorbic acid
100	17.09 ± 2.78	25	33.53 ± 2.17
200	26.65 ± 4.55	50	42.56 ± 2.58
300	34.21 ± 3.89	75	46.23 ± 2.10
400	41.98 ± 3.66	100	59.76 ± 4.16
600	48.79 ± 3.42	125	69.56 ± 0.53
800	53.61 ± 2.37		
1000	64.18 ± 0.98		
IC_{50}	643.0 ± 61.10		83.80 ± 3.75

EJ is potent DPPH free radical scavenger.

EJ also strongly inhibited NO in dose dependent manner (Table 2) with the IC_{50} being 643.0 ± 61.10 µg/mL. In the same manner ascorbic acid also scavenged NO and IC_{50} value being 83.80 ± 3.75 µg/mL. Nitric oxide is a potent pleiotropic mediator of physiological process such as smooth muscle relaxation, neuronal signaling, initiation of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodialtion and antimicrobial and antitumor activities. Studies in animal models have suggested a role for NO in the pathogenesis of inflammation and pain and NOS inhibitors have been shown to have beneficial effects on some aspects of the inflammation and tissue changes seen in models of inflammatory bowel disease (Govindarajan *et al*., 2003). Thus establishing the usage of the plant in the Indian indigenous system as an anti-inflammatory agent (Chopra *et al*., 1956 anonymus, 1959).

The antioxidant activity of EJ as measured by the bleaching of β-carotene is presented in Table 3. IC_{50} value

Table 3. Percentage Antioxidant property of EJ and BHT in β-CLAMS method

Conc. (µg/mL)	EJ	BHT
25	3.64 ± 1.58	20.71 ± 3.34
50	27.41 ± 0.61	36.17 ± 2.39
75	---	54.73 ± 0.39
100	43.91 ± 0.65	61.58 ± 1.43
150	55.79 ± 0.73	71.47 ± 0.33
IC_{50}	132.3 ± 6.03	68.51 ± 2.5

of EJ and BHT were found to be 132.3 ± 6.03 and 68.51 ± 2.5 µg/mL respectively. The mechanism of bleaching β-carotene is a free radical mediated phenomenon resulting from hydroperoxides formed from linoleic acid. β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radicals, upon the abstraction of hydrogen from one of its diallylic methylene group attacks the highly unsaturated β-carotene molecules. As β-carotene molecules loose their double bonds by oxidation, the compound looses its chromophore and characteristic orange color, which can be monitored spectrophotometrically at 470 nm. So in presence of antioxidants, β-carotene retains its color. Because antioxidants prevent abstract of hydrogen from linoleic acid from its diallylic methylene group by donating hydrogen from itself. Thus prevents the oxidation of β-carotene. Here the IC_{50} values of BHT which is used as standard and EJ are having near values. This shows that EJ can donate hydrogen to linoleic acid free radicals as much as BHT can. EJ can be used as natural antioxidant instead of BHT which is synthetic and use of BHT is said to unsafe and their toxicity is a problem of concern. (Kaur *et al*., 2006; Madhavi and Salunkhe., 1995).

Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using phenylhydrazine (Chakrabarti *et al*., 1995). Hydroxyl radical scavenging was measured by studying the competition between deoxy-*d*-ribose and sample extracts for hydroxyl radicals produced by phenyl hydrazine. The extent of deoxy-*d*-ribose degradation is measured as TBARS method of Ohkawa *et al*., 1979. EJ scavenged the hydroxyl radicals strongly with an IC_{50} value of 68.5 ± 11.5 µg/mL. IC_{50} value of GA is 3.3 ± 0.2 µg/mL (Table 4). The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cell. This radical has the capacity to join nucleotide in DNA

Table 4. Hydroxyl radical scavenging activity of EJ and Gallic acid (GA) in percentage

Conc. (μg/mL)	EJ	Conc. (μg/mL)	GA
5	8.167 ± 4.06	0.5	20.91 ± 6.51
10	29.34 ± 4.08	1	25.81 ± 5.65
20	40.20 ± 4.51	2	36.54 ± 3.97
50	44.14 ± 3.61	3	46.41 ± 2.81
80	53.68 ± 3.58	4	58.39 ± 2.62
IC_{50}	68.5 ± 11.5		3.3 ± 0.2

Table 5. Anti Lipid Peroxidation activity of EJ and silymarin in percentage

Conc. (μg/mL)	EJ	Conc. (μg/mL)	Silymarin
20	3.62 ± 1.54	1.0	3.13 ± 3.65
40	18.09 ± 0.44	2.0	29.66 ± 1.00
60	38.26 ± 1.54	5.0	43.34 ± 0.48
80	68.49 ± 1.86	7.5	59.44 ± 1.85
---	---	10.0	89.84 ± 1.48
IC_{50}	68.13 ± 1.38	IC_{50}	6.0 ± 0.2

and cause strand breakage which contributes to carcinogenesis, mutagenesis, and cytotoxicity. In addition, this species is considered to be one of the quick initiator of lipid peroxidation process, abstracting hydrogen atom form the unsaturated fatty acids (Singh *et al*., 2002; Lee *et al*., 2004). EJ has found to scavenge OH radicals but not as strongly as gallic acid. Because GA is very potent OH radical scavenger (Yen *et al*., 2002) and is a pure compound. Thus EJ may be useful as an antioxidant and may prevent damages that arise from OH radicals by scavenging them. This OH radical capacity of EJ may be due lignans and flavonoids as found present in EJ by qualitative chemical tests. It is found that flavonoids are potent OH radical scavenging agents (Amic *et al*., 2003; Dai *et al*., 2006).

EJ prevented lipid peroxidation strongly but less than the standard (silymarin). IC_{50} values of antilipid peroxidation of EJ and silymarin were 68.13 ± 1.38 and 6.0 ± 0.2 μg/mL respectively. The effect of EJ to prevent lipid peroxidation is shown in Table 5. In biological systems, malondialdehye (MDA) is very reactive species and takes part in the cross linking of DNA, with protein and also damaging the liver cells. The lipid peroxidation has been broadly defined as the oxidation deterioration of polyunsaturated lipids. The initiation of peroxidation sequence in membrane or polyunsaturated fatty acids is due to abstraction of a hydrogen atom from the double bond in the fatty acids. The free radicals tends to be stabilized by a molecular rearrangement to produce a conjugated dienes, which then easily react with an oxygen molecule to give a peroxy radical. Peroxy radical can abstract a hydrogen atom from another molecule or they can abstract hydrogen atom to give lipid hydroperoxide, R-OOH. A probable alternative fat of peroxy radicals is to form cyclic peroxidase; these cyclic peroxidase, lipid peroxidase and cyclic endoperoxidase fragment to aldehyde including MDA and polymerization product. MDA is the major product of lipid peroxidation and is used to study the lipid peroxidation process. (Jadhav *et al*., 1996). Determination of lipid peroxidase content was carried out indirectly by means of derivatizing MDA with TBA at high temperature and acidic condition (Halliwell and Guttridge, 1989). EJ has strongly inhibited the lipid peroxidation a shown in the Table 5, IC_{50} value of EJ is 68.13 ± 1.38 μg/mL and that of silymarin is 6.0 ± 0.2 μg/mL. this indicates that silymarin is more than ten times potent than EJ. Silymarin is a mixture of flavonolignan and is proven potent hepatoprotective and antioxidant agent (Varga *et al*., 2006; De *et al*., 1993). So when compared to such potent agent, the values obtained indicate that EJ is good antilipid peroxidation agent.

Fe^{2+} reacts rapidly with 1, 10-*O*-phenanthroline and forms red colored complex (λ_{max} 510) which is exceptionally stable. Extracts reacts with Fe^{3+} to reduce and convert it to Fe^{2+}. The degree of coloration measured at 510 nm indicates the reduction potential of the extracts (Rajkumar and Rao, 1993; Kunchady and Rao, 1989). As shown in the Table 6, in absence of EDTA, EJ has shown 41.82 ± 0.41% activity at 1000 μg concentration. IC_{50} of ascorbic acid is 39.15 ± 3.65 μg. But in presence of EDTA both EJ and ascorbic acid failed to show ferric ion reduction activity. Both EJ and ascorbic acid have reduced the Fe^{3+} to Fe^{2+} moderately. But in presence of EDTA (strong chelating agent), ferric ion reduction activity of EJ and ascorbic acid were nil. This is because of EDTA being strong chelating agent, complexed with ferric ions. EJ and ascorbic acid were not able to reverse this complexation. So there is no reduction of ferric ions. The reducing properties are generally associated with the presence of reductones. Antioxidant action of the reductones is based on the breaking of free radicals chain by the donation of a hydrogen atom. The reductones also react with certain precursors of peroxide, thus preventing formation of peroxide (Gordon, 1991).

H_2O_2 in phosphate buffer has the λ_{max} of 230 nm. In presence of extracts the reduction of absorbance at 230 nm indicates scavenging or breakdown of H_2O_2 (Ilhami, 2006). When breakdown of H_2O_2 occurs, there will be

Table 6. Ferric ion reduction activity of EJ and Ascorbic acid in percentage

EJ			Ascorbic acid		
Conc. (µgs)	In absence of EDTA	In presence of EDTA	Conc. (µgs)	In absence of EDTA	In presence of EDTA
100	22.14 ± 0.84	ND	10	18.87 ± 0.64	ND
200	27.24 ± 0.90	ND	20	26.63 ± 2.06	ND
300	30.03 ± 1.36	ND	30	38.72 ± 5.62	ND
400	31.01 ± 1.84	ND	40	48.60 ± 5.09	ND
600	33.85 ± 1.40	ND	50	60.95 ± 1.43	ND
800	38.34 ± 0.84	ND	--	--	--
1000	41.82 ± 0.41	ND	--	--	--
IC_{50}	ND			39.15 ± 3.65	

Concentration is the total extract present in reaction mixture in µgs.
ND - Not Detected at tested concentration.

Table 7. Percentage H_2O_2 scavenging activity of EJ and BHT

Conc. (µg/mL)	EJ	BHT
10	5.93 ± 0.29	15.34 ± 0.25
20	12.46 ± 0.58	22.16 ± 0.70
40	22.74 ± 0.25	30.66 ± 1.92
50	37.97 ± 0.43	33.86 ± 1.47

reduction of absorbance at 230 nm. As shown in Table 7, EJ and BHT scavenged the H_2O_2 to the extent of 37.97 ± 0.43% and 30.66 ± 1.92 at 50 µg/mL concentration. Both EJ and BHT both caused weak decomposition of H_2O_2 in a dose dependent manner as shown in Table 4. H_2O_2 scavenging capacity of EJ is found to be little higher than the BHT. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (–SH) groups. Hydrogen peroxide can cross cell membranes rapidly. Once enter inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. Two types of enzymes exist to remove hydrogen peroxide within cells. They are catalase and the peroxidase. The extract has shown to decompose H_2O_2 and thus may help in preventing the imbalance in oxidative stress and antioxidants including antioxidant enzymes like catalase and peroxidase in the biological system (Ilhami, 2006; Le *et al,* 2007; Kaur, 2006).

In total antioxidant capacity assay, it was found that 1 mg of EJ is equivalent to 205.56 ± 34.69 µg of ascorbic acid. Similarly 1 mg of slymarin and BHT were found equivalent to 197.22 ± 4.81 and 400.00 ± 22.05 µg of ascorbic acid respectively. In the same manner, in total phenolic content assay, it was found that 1 mg of EJ and 1 mg of silymarin were equivalent to 43.76 ± 4.27 and 42.49 ± 3.84 µg of gallic acid respectively. Total antioxidant capacity and total phenolic content were found to be more in EJ than the silymarin which is used as a standard. But total antioxidant capacity of EJ is less than the BHT. Silymarin was used as a standard because it is a mixture of flavonolignans and is potent well known hepatoprotective agent (Varga *et al*., 2006; De *et al*., 1993). The genus *Justicia* also found to contain lignans (Jyotishi and Bagavant, 1992a; 1992b) and also preliminary tests showed that EJ contains flavonoids. Hence total phenolic content of EJ is found to be more than the silymarin and because of this total antioxidant capacity of EJ is also more than the silymarin.

The data presented here indicate that the marked antioxidant activity of EJ extracts seems to be due to presence of flavonoids and lignans, which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction. Free radicals and reactive oxygen species are involved in a variety of pathological events such as aging, inflammation, cancer, atherosclerosis and diabetes (Lee *et al*., 2004). The plant would be useful for the treatment of various diseases mediated by free radicals. The flavonids and other components present in the extract found to suppress lipid peroxidation, hydroxyl radical formation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination. Overall, the plant would be useful as an antioxidant and free radical scavenging agent and thus help in treatment of many diseases mediated by ROS.

Acknowledgement

One of the authors, K.Mruthunjaya is highly thankful to JSS College of Pharmacy, and JSS Mahavidyapeetha, Mysore, India for providing financial assistance during the research work. And also thankful to Dr. K.N.Chidambara Murthy, CFTRI, Mysore, India, for providing pure chemicals that were required to carryout few experiments in the present study and Dr.L.V.G Nargund, Professor & Director, Drug Discovery Division, Dr. Nargund Research Foundation, Bangalore, India for his kind permission to utilize the research facilities available in their college.

References

Amic, D., Davidovic-Amic, D., Beslo, D., and Trinajstic, N., Structure-Radical Scavenging Activity Relationships of Flavonoids, *Croat. Chem. Acta* **76**, 55-61 (2003).

Anonymous, *The Wealth of India: Raw Materials*-Vol V, Publication & Information Directorate (CSIR), New Delhi, 1959.

Chakrabarti, S., Sonaye, B., Naik, A.A., and Nadkarni, P.P., Erythrocyte membrane protein damage by oxidation products of phenylhydrazine, *Biochem. Mol. Biol. Int.* **35**, 255-263 (1995).

Chakravarty, A.K., Dastidar, P.G., and Pakrash, S.C., Simple aromatic amines from *Justicia gendarussa*-^{13}C NMR spectra of the bases and their analogues. *Tetrahedron* **38**, 1797-1802 (1982).

Chopra, R.N., Nayar, S.L. and Chopra, I.C., *Glossary of Indian medicinal plants*, CSIR, New Delhi, 1956.

Dai, F., Miao, Q., Zhou, B., Yang, L, Liu, and Zhong-Li., Protective effects of flvonols and their glycosides against free radical-induced oxidative hemolysis of red blood cells, *Life Sci.* **78**, 2488-2493 (2006).

De, S., Ravishankar, B., and Bhavsar, G.C., Plants with hepatoprotective activity-A Review, *Indian Drugs* **38**, 363 (1993).

Geinssman, T.A., Flavonoids, in Peach, K., and Tracey, M.V., (eds.), *Modern Methods of Plant Analysis Vol. 3*, Springer Verlag, Berlin, 1955, pp. 467-474.

Gordon, M.F., The Mechanism of antioxidant action *in vitro*, in Hudson, B.J.F., (ed.), *Food antioxidants*, Elsevier Apllied Science, London, 1991, pp. 1-88.

Govindarajan, R., Rastogi, S., Vijayakumar, M., Shirwaikar, A., Rawat, A.K.S., Mehrotra, S., and Palpu, P., Studies on the Antioxidant Activities of *Desmodium gangeticum, Biol. Pharm. Bull.* **26**, 1424-1427 (2003).

Halliwell, B., and Guttridge, J.M.C., *Free radicals in Biology and Medicine*, Japan Scientific Press, Tokyo, 1989.

Hawks, P.B., *Hawk's Physiological chemistry*, Tata Macgraw Hill Publishing Company Ltd., New Delhi 1971.

Hidalgo, M.E., Fernandez, E., Quilhot, W., and Lissi, E., Antioxidant activity of depsides and desidones, *Phytochemistry* **37**, 1585-1587 (1994).

Ilhami, Gulcin., Antioxidant and antiradical activities of l-carnitine, *Life Sci.* **78**, 803-811 (2006).

Jadhav, S.J., Nimbalkar, Kulkarni, A.D., and Madhavi, D.L., Lipid oxidation in biological and food systems, in Madhavi, D.L., Deshpande S.S., Salunkhe, D.S., (eds.), *Food antioxidants,* Dekker, New york, 1996, pp. 5-63.

Jyotishi, S.G. and Bagavant, G., Lignans: their Pharmacological Activity and Occurrence in Justicia-I. *Indian J. Nat. Prod.* **8**, 3-9 (1992a).

Jyotishi, S.G. and Bagavant, G., Lignans: their Pharmacological Activity and Occurrence in Justicia-I. *Indian J. Nat. Prod.* **8**, 3-17 (1992b).

Kaur, G., Jabbar, Z., Athar, M., and Alam, M.S., *Punica granatum* (pomegranate) flower extract possesses potent antioxidant activity and abrogates Fe-NTA induced hepatotoxicity in mice, *Food Chem. Toxicol.* **44**, 984-993 (2006).

Kirthikar, K.R. and Basu, B.D., *Indian medicinal plants vol. 8*, Oriental enterprises, Dehradun, 2001.

Kokate, C.K., Purohit, A.P., and Gokhale, S.B., *Pharmacognosy*, Nirali Prakashan, Pune, 1996.

Kunchandy, E. and Rao, M.N.A., Effect of curcumin on hydroxyl radical generation through Fenton reaction, *Int. J. Pharm.* **57**, 173-176 (1989).

Lee, J., Koo, N., and Min, D.B., Reactive Oxygen Species, Aging, and Antioxidative Nutraceuticals, *Comp. Rev. Food Sci. Safety.* **3**, 21-33 (2004).

Li, Xin-Ming., Shi, Yong-Hui., Wang, Fang., Wang, Hai-Song., and Le, Guo-Wei., In vitro free radical scavenging activities and effect of synthetic oligosaccharides on antioxidant enzymes and lipid peroxidation in aged mice, *J. Pharm. Biomed. Anal.* **43**, 364-370 (2007).

Madhavi, D.L., and Salunkhe, D.K., Toxicological aspects of food antioxidants, in Madhavi, D.L., Deshpande, S.S., Salunkhe, D.K., (eds.), *Food Antioxidants*. Dekker, New York, 1995, pp. 267.

Mathew, S. and Abraham, T.E., *In vitro* antioxidant activity and scavenging effects of *Cinnamomum verum* leaf extract assayed by different methodologies, *Food Chem.l Toxicol.* **44**, 198-206 (2006).

Ohkawa, H., Ohishi, N., and Yagi, K., Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Anal. Biochem.* **95**, 351-358, (1979).

Peach, K. and Tracey, M.V., *Modern Methods of Plant Analysis Vol. 3*., Springer Verlag, Berlin, 1955a.

Peach, K. and Tracey, M.V., *Modern Methods of Plant Analysis* Vol. 4., Springer Verlag, Berlin, 1955b.

Prieto, P., Pineda, M., and Aguilar, M., Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phospho-molybdenum Complex: Specific Application to the Determination of Vitamin E1, *Anal. Biochem.* **269**, 337-341(1999).

Rajkumar, D.V. and Rao, M.N.A., Dehydrozingerone and isougenol as inhibitors of lipid peroxidation and as free radical scavengers, *Biochem. Pharmaco.* **46**, 2067-2072 (1993).

Singh, R.P., Murthy, K.N.C., and Jayaprakash, G.K., Studies on the Antioxidant activity of Pomegranate *(Punica granatum)* peel and seed extracts using *in vitro* models, *Journal of Agricult. Food Chem.* **50**, 81-86 (2002).

Stevens, H.M., Analytical techniques, in Moffat, A.C., Jackson, J.V., Moss, M.S., and Widdop, B., (eds.), *Clarke's isolation and Identification of drugs in Pharmaceuticals, Body fluids and post-mortem material*, The pharmaceutical Press, London, 1986, pp. 457-472.

Sudheerkumar, M., Jagadish, P.C., Sridhar, R.B., Kiran, B.S., and Unnikrishnan, M.K., *In-vitro* evaluation of antioxidant properties of *Cocos nucifera* Linn. Water, *Nahrung/Food.* **47**, 126-131 (2003).

Trease, G.E. and Evans, W.C., *Pharmacognosy,* ELBS, London, 1989.

Varga, Zs., Seres, I., Nagy, E., Ujhelyi, L., Balla, G., Balla, J., and Antus, S., Structure prerequisite for antioxidant activity of silybin in different biochemical systems *in vitro, Phytomedicine* **13**, 85-93 (2006).

Yen, Gow-Chin., Duh, Pin-Der., and Tsai, Hui-Ling., Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. *Food Chem.* **79**, 307-313 (2002).

(Accepted June 18, 2007)