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In vitro antioxidant and *in vivo* anti-inflammatory activities of methanolic extracts from *Exacum bicolor* Roxb

Jeeshna MV
Department of Botany, Sree Narayana College, Thottada, Kannur, Kerala, India

Abstract
Exacum bicolor (Gentianaceae) is an endangered medicinal herb distributed in the northern part of Kerala, India. The leaves are used as traditional medicine to treat several inflammatory and antioxidant pathologies such as asthma, rheumatisms and burns. In this study, the antioxidant and anti-inflammatory activities of crude methanol extracts of leaves from *E. bicolor* were investigated. The leaf extracts showed significant *in vitro* antioxidant activity (total antioxidant activity, 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity and hydroxyl radical scavenging activity) and *in vivo* anti-inflammatory activity (carrageenan induced paw edema in mice and enzymic antioxidant status in serum). The extracts are reported to have considerable amount of phenolics (105.23 mg/g) and flavonoids (10.12 mg/g). In addition, the content and the composition of flavonoids were assessed by HPLC analysis. These result indicates that the endangered *Exacum bicolor* is a potential source of anti-oxidantal and antiinflammatory substances.

Keywords: *Exacum bicolor*, antioxidant, anti-inflammatory activities, luteolin, chlorogenic acid, HPLC analysis

1. Introduction
Exacum bicolor Roxb. (Gentianaceae) is an endangered medicinal herb widely distributed in the grasslands of northern Kerala India. Whole plant of this species is used as a tonic and febrifuge, and as antifungal agent in traditional health care (Rao, 1914; The Wealth of India, 1952; Pullaiah, 2006; Khare, 2007; Shiddamallayya *et al.*, 2010) [35, 50, 31, 21, 42] Being bitter in taste, local people take it as herbal remedy for diabetes and skin disorders (Reddi *et al.*, 2005) [36]. The traditional practitioners in Kerala have prescribed decoction of the leaf of this plant for the treatment of inflammatory conditions such as arthritis, and muscle and joint pain (Sreelatha *et al.*, 2007; Jeeshna, 2011) [48, 19].

Free radicals and reactive oxygen species. (ROS) are well known inducers of cellular and tissue pathogenesis leading to several human diseases such as cancer, inflammatory disorders, as well as in aging processes (Halliwell, 1994; Aviram, 2000; Finkel and Holbrook, 2000) [16, 3]. Antioxidants provide protection to living organisms from damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage and DNA strand breaking (Ghosal *et al.*, 1996) [11]. Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepato protective drugs have recently been shown to have antioxidant or radical scavenging mechanism as part of their activity (Lin and Huang, 2000; Repetto and Llesuy, 2002) [25, 37]. Moreover, several studies suggest that antioxidant and anti-inflammatory agents could be beneficial in the prevention and treatment of these pathologies (and Bezek, 1999; Rahman, 2002; Horton, 2003) [6, 34, 17].

In view of the several ethno botanical uses of *E. bicolor* described above, it has been worked out to isolate compound(s) from the extracts made by using high polar solvents, methanol for the *in vitro* antioxidant and *in vivo* anti-inflammatory activity using standard procedures.

2. Materials and methods
1. Plant Material
The leaves of *Exacum bicolor* were collected from the grasslands of Thirunelli forest Wayanad district, Kerala, India during the month of August, 2016.The plant specimen was identified and authenticated by Botanical Survey of India. The voucher specimen has been deposited for further reference at Sree Narayana College herbarium, Kannur, Kerala.

2. Chemicals and Reagents
Luteolin, chlorogenic acid, DPPH, 2,2-diphenyl-1-picrylhydrazyl, carrageenan, Folin-Ciocalteu reagent, quercetin, ethylene diamine tetraacetic acid (EDTA), ethylene diamine tetraacetic acid (DMSO), dimethyl sulfoxide (TCA), dithionitrobezoic acid (DTNB) and indomethacin were purchased from Sigma and Aldrich (St. Louis, MO). Formic acid and acetonitrite (HPLC grade) were purchased from Merck, Germany. Other chemicals and solvents were purchased from Merck chemicals Mumbai, India. All chemicals used were of analytical grade.

3. Plant material and extract preparation
The leaves were chopped to small pieces and dried in shade. The dried leaves (100g) were powdered and passed through sieve no. 20 and extracted with 600 ml of methanol in a soxhlet extractor for 18–20 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40–50^0 C). The methanol extracts yielded yellowish brown residue, weighing 6.37 g (6.37%). The extracts were preserved in a refrigerator till further use.

4. Animals

Healthy male albino rats of wistar strain (120-150 g) were obtained from the Small Animal Breeding Station, Mannuthy, Thrissur, Kerala, India and were maintained under standard environment conditions (22–28^0 C, 60–70% relative humidity, 12-h dark:12-h light cycle) and were fed with standard rat feed (M/S Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. Before performing the experiment, the ethical clearance was obtained from institutional animal ethics committee.

5. Determination of total phenolic content

Total phenolic content of the total extracts was determined using Folin-Ciocalteu reagent and chlorogenic acid as standard (Singleton and Rossi, 1965) [44]. Fifty milligrams of the extracts were weighed into plastic extraction tube and vortexed with 25 ml of the extraction solvent (40 ml acetone: 40 ml methanol: 20 ml water: 0.1 ml acetic acid). Then, the samples with the extraction solvent were heated at 60º C (water bath) for 1 h, allowed to cool to room temperature, and homogenized for 30 second with a sonicator. Two hundred microliters (three replicates) were introduced into screw cap test tubes; 1.0 ml of Folin- Ciocalteu's reagent and 1.0 ml of sodium carbonate (7.5%) were added. The tubes were vortexed and allowed to stand for 2 h. The absorption at 726 nm was measured UV/VIS spectrophotometer and the total phenolic content was expressed as milligram of chlorogenic acid equivalents per gram dry material.

6. Determination of total flavonoid content

Total flavonoids were estimated in the leaf extracts using a colorimetric method based on the formation of a complex flavonoid-aluminum, having the absorbivity maximum at 430 nm (Quettier-Deleu *et al.*, 2000) [33]. All determinations were made in triplicate and values were calculated from a calibration curve obtained with quercetin. Final results were expressed as milligram of quercetin equivalent per gram of dried weight.

7. *In vitro* antioxidant activity

The extracts and the isolated compounds were tested for their *in vitro* antioxidant activity using the standard methods. In all these methods, a particular concentration of the extract or standard solution was used, which gave a final concentration of 1000–0.45 g/ml after all the reagents were added. Absorbance was measured against a blank solution containing the extract or standard, but without the reagents. A control test was performed without the extracts or standards. Percentage scavenging and IC_{50} values±S.E.M. (IC_{50} value is the concentration of the sample required to inhibit 50% of radical) were calculated.

7.1. Total antioxidant activity

2 mg/ml of sample was taken and mixed with 1ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Then the reaction mixture was incubated at 95° C for 90 min (Prieto *et al.*, 1999) [30]. Absorbance of the sample was measured at 635 nm. Ascorbic acid was used as standard. The antioxidant activity was expressed as ascorbic acid equivalents (mg/g of sample).

7.2. DPPH radical scavenging activity

This experimental procedure was adapted from Blios (1958) [7]. Methanol solution of the sample extract at various concentrations (50, 100, 150, 200 and 250 µg/ml) was added separately to each 5 ml of 0.1 mM methanolic solution of DPPH and allowed to stand for 20 min at 27º C. After incubation, the absorbance of each solution was determined at 517 nm using spectrophotometer. Tannic acid was used as standard. The corresponding blank reading was also taken and the remaining DPPH was calculated by using the following formula:

$$\text{DPPH radical scavenging (\%)} = \frac{\text{sample absorbance}}{\text{Control absorbance}} \times 100$$

The DPPH solution without sample solution was used as control. All tests were run in triplicate and averaged. Ascorbic acid was used as positive control.

7.3. Hydroxyl radical scavenging activity

Various concentrations of the extract *viz.*, 20, 40, 60, 80, 100, 120 and 140 µg/ml were added separately with 1 ml of iron-EDTA solution (0.13 % ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018 %) and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH – 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80 to 90º C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1 ml of ice - cold TCA (17.5% w/v). Three millilitre of Nash reagent (75 g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at room temperature for 15 min (Klein *et al.*, 1991). The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrometrically at 412 nm against reagent blank. Tannic acid was used as standard. The hydroxyl radical scavenging activity (%) was calculated by the following formula:

$$\text{HRSA (\%)} = 1 - \frac{\text{Differences in absorbance of sample}}{\text{Differences in absorbance of blank}} \text{ X } 100$$

8. *In-vivo* anti-inflammatory activity

8.1. Carrageenan-induced paw edema

The anti – inflammatory activity of methanol extract of *E. bicolor* was investigated in carrageenan induced inflammatory model. For the experiment, the male wistar rats were divided into five groups such as Group I, Group II, Group III, Group IV and Group V (n = 6). The animals were fasted overnight prior to the start of the experiment. The first and second groups received distilled water, while the third group was treated with indomethacin (10 mg/Kg p.o.). The fourth and fifth groups were administered with the methanol extract of *E. bicolor* (200 and 400 mg//Kg per day p.o.). After one hour of the treatment, acute inflammation was produced to the Group II – V by the subplantar administration of 0.1 ml of 1 % carrageenan (CGN) in the right hind paw of the rats (Winter *et al.*, 1962). The thickness (mm) of the paw was measured immediately at 30 min interval for 4 hrs after the carrageenan injection by using vernier caliper.

At the end of experimental regimen, all the animals were subjected to mild diethyl ether anesthesia. Blood was then collected by cardiac puncture and allowed to clot for 20 – 30 min and centrifuged in a refrigerated centrifuge (4º C) at 3000 rpm for 10 min. Fresh serum samples were used to estimate various parameters *viz.,* total protein, superoxide dismutase, catalase, glutathione peroxidase, glutathione – S – transferase, nitric oxide and lipid peroxide.

8.2. Estimation of protein

For standard, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard solution, and for testing 0.1 ml of the sample were taken separately and the volume of all the test tubes were made upto 1.0 ml with distilled water. To this 5.0 ml of alkaline copper reagent was added to each test tube, mixed well and allowed to stand for 10 minutes. Then add 0.5 ml of Folin-Ciocalteau reagent to each test tube including the blank. Mixed well and incubated at room temperature for 30 min. After 30 min, the blue colour developed was read at 660 nm (Lowry *et al.,* 1951). Concentration of the enzyme specific activity was expressed as mg/dl.

8.3. Determination of enzymic antioxidant status

8.3.1. Estimation of superoxide dismutase activity (SOD) (EC 1.15.1.1)

The reaction mixture consisted of 100 µl of the serum, 0.05 ml of pyrogallol solution (0.2 mM pyrogallol in 10 mM HCl) was made upto the volume, 2.5 ml with tris-HCl buffer (50 mM tris- HCl buffer of pH, 7.0) and absorbance was read at 420 nm against reagent blank (Marklund and Marklund, 1974). A unit of enzyme was defined as the amount of the enzyme which inhibits the reaction by 50%. Specific activity was expressed as moles/ min/mg of protein.

8.3.2. Estimation of catalase activity (CAT) (EC NO.1.11.1.6)

The reaction mixture (1.5 ml) contained, 1 ml of 0.01 M phosphate buffer (pH – 7.0), 0.1 ml of enzyme preparation and 0.4 ml of 2 M hydrogen peroxide. The reaction was stopped by the addition of 2.0 ml dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). The tubes were kept in a boiling water bath for 10 min and cooled (Sinha, 1972). The absorbance was spectrophotometric ally measured at 620 nm. A system devoid of enzyme served as control. Activity of catalase was expressed as moles of hydrogen peroxide consumed/ min/mg protein.

8.3.3. Estimation of glutathione peroxidase (GPx) (EC 1.11.1.9)

To 0.4 ml of phosphate buffer (pH – 7), 0.2 ml of 4.0 mM of EDTA, 0.1 ml of 10 mM sodium azide, 0.2 ml of 4.0 mM of reduced glutathione and 0.1 ml of 2.5 mM of H_2O_2 were added separately to two test tubes labeled as test (T) and control (C). To the test tubes, added 0.2 ml of sample and to the control, added 0.2 ml of water. The contents were mixed well and incubated at 37 ºC for 10 min. The reaction was arrested with the addition of 0.5 ml of 10 % TCA. To determine the glutathione content, 1ml of supernatant was removed by centrifugation. To that, supernatant 3 ml of buffer and 0.5 ml of Ellman's reagent (19.8 mg DTNB in 1% sodium citrate) were added (Ellman, 1959). The colour developed was read at 412 nm. The activity was expressed in terms of µg of glutathione consumed /min/mg protein.

8.3.4. Determination of Glutathione – S- Transferase activity

To 1.0 ml of buffer, 0.1 ml of sample, 1.7 ml of water and 0.1 ml of CDNB (1 – chloro – 2, 4 dinitrobenzene) were added and incubated at 37 ºC for 5 min. After incubation, 0.1 ml of reduced glutathione was added (Habig *et al.,* 1974). The increase in optical density of the enzyme was measured against that of the blank at 340 nm. The enzyme activity was calculated in terms of µmoles of CDNB conjugate formed/min/mg protein.

8.3.5. Estimation of lipid peroxidation (LPO) levels

0.1 ml of the serum was treated with 2.0 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (0.375 % thiobarbituric acid (TBA), 15% trichloro acetic acid (TCA) and 0.025 N hydrochloric acid) and it was kept in boiling water bath for 15 min. After cooling, the tubes were centrifuged at 1000 rpm for 10 min. and the clear supernatant was taken for measurement (Niehaus and Samuelso, 1968) [28]. The absorbance of chromophore was read at 535 nm against a reagent blank that contained no serum. TBARS (thiobarbituric acid reactive substances) was expressed as mole of MDA (malondialdehyde)/mg protein.

8.3.6. Estimation of nitric oxide (NO) levels

Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulphate (300 mg/ml) to a final concentration of 15 mg/ml. After centrifugation at 10000 rpm for 5 min at room temperature, supernatant was collected (Green *et al.,* 1982) [12]. 0.1 ml of supernatant was mixed with 0.1 ml of Griess reagent (1% sulfanilamide and 0.1 % N –naphthylethy lenediamine hydrochloride in 2.5% phosphoric acid) at room temperature for 5 min and absorbance was measured at 540 nm.

9. HPLC analysis

9.1. Sample Preparation

5 ml of methanol was added to 2 g of powdered sample. The mixture was heated for 30 min at 40 ^{0}C. The residue was removed by centrifugation and the supernatant was diluted to 50 ml by adding methanol, then the solution was filtered through 0.45 µm filter for HPLC analysis.

9.2. Chromatography

Samples were analysed with Hitachi-6200 HPLC equipped with a L-1000 UV spectrometric detector and the separation was carried out on a Pursuit Column (250 x 4.6 mm, 5 - µm particle, symmetry C 18). The mobile phase was a gradient prepared from 100: 0.1 (v/v) water – formic acid (component A) and 100: 0.1 (v/v) acetonitrite – formic acid (component B); the gradient programme was from 84 – 60% for compound A in 30 min and then from 16 - 60 % for compound B in 30 min. The injection volume was 10 µl, the mobile phase flow rate was 0.7 ml/mi and the detection wave length was 255 nm.

10. Statistical analysis

Results are expressed as mean±S.E.M. Comparisons among the groups were tested by one-way ANOVA using Graph Pad Prism, Version 4.0 (Graph Pad Software, San Diego, CA, USA). When the *p*-value obtained from ANOVA was significant ($p < 0.05$), the Tukey test was applied to test for differences among groups.

3. Results

3.1. Total phenolic and flavonoid content

In *E. bicolor* the content of phenolics and flavonoids estimated was about 105.23 and 10.12 mg/g respectively. Many early studies report that flavonoid compounds and several polyphenolics in fruits and vegetables exhibit antioxidant activity (Luo *et al.,* 2002)

4. *In vitro* antioxidant activity

4.1. Total antioxidant activity

The total antioxidant activity was assessed by phosphomolybdenum assay and the activity of the extract is found to be 9.92 mg/g of ascorbic acid equivalents (Table 1).

4.2. DPPH radical scavenging activity

To evaluate the antioxidant efficiency of leaf extract, the radical scavenging capacity based on DPPH assay was determined and the results are shown in Table 1. The percentage of scavenging effect on the DPPH radical is increased with the increase in the concentration of extract from 50 -250 µg/ml. The percentage of inhibition of the DPPH radical was varying from 21.81% for 50 µg/ml of the extract to 53.74% for 250 µg/ml of extract. The IC_{50} value of the methanolic extract of *E. bicolor* is determined to be 235 µg/ml.

4.3. Hydroxyl radical scavenging activity

The data on antioxidant activity of the leaf extract based on hydroxyl radical scavenging activity results are shown in Table 1. The percentage of hydroxyl radical scavenging activity was determined to be increased with the increase in the concentration of extract from 20 to 140 µg/ml. The percentage of inhibition of the hydroxyl radical was varying from 2.68 % for 20 µg/ml of extract to 51.25% for 140 µg/ml extract. The IC_{50} value of the methanolic extract of *E. bicolor* was 130µg/ml.

Table 1: Total antioxidant, DPPH and hydroxyl radical scavenging activity of methanolic leaf extract of *Exacum bicolor*.

Total antioxidant activity		
Sample concentration (mg/ml)	**Activity (mg/g)**	
2	**9.92±0.008**	
DPPH radical scavenging activity		
Sample concentration (µg/ml)	Percentage activity	IC_{50} (µg/ml)
50	21.81 [a] ±0.09	235
100	30.07 [b] ±0.32	
150	38.47 [c] ±0.13	
200	46.39 [d] ±0.63	
250	53.74 [e] ±0.09	
Hydroxyl radical scavenging activity		
Sample concentration (µg/ml)	Percentage activity	IC_{50} (µg/ml)
20	2.68 [a] ±0.19	130
40	3.90 [a] ±0.28	
60	11.10 [b] ±1.00	
80	29.40 [a] ±1.98	
100	39.71 [d] ±2.35	
120	46.75 [e] ±2.55	
140	51.25 [e] ±2.94	

Values are expressed as mean±SD. (n=6).
Values within the same row not sharing common superscript letters (a-e) differ significantly at p<0.05 by DMRT.

5. *In vivo* anti-inflammatory activity

5.1. Carrageenan-induced paw edema

The results by using carrageenan induced inflammatory model for anti-inflammatory activity of methanol extract of *E. bicolor* are shown in Fig.1. The paw thickness of the normal rats was found to be 4.58 mm. The paw volume was increased from 4.58 to 6.62 mm at 60 minutes, then to 6.96 mm at 120 minutes, then to 6.49 mm at 180 minutes and then to 5.71 mm at 240 minutes in carrageenan induced rats. The increase in the paw thickness over a period of 4 hours indicates the release of first and second stage mediators of inflammation. Upon pretreatment of the animals with the extract at 200 mg/kg b.wt. one hour prior to induction, the paw thickness was found to be 4.40 mm and 6.36 mm at 60 minutes and 6.32 mm at 120 minutes and 5.96 mm at 180 minutes and 5.51 at 240 minutes. The group V rats were pretreated with the extract at 400 mg/kg b. wt. One hour prior to induction, the paw thickness was found to be 4.38 mm, 6.52 mm at 60 min, 6.10 mm at 120 minutes, 5.52 mm at 180 minutes and 4.93 mm at 240 minutes. In the standard drug pretreated groups, one hour prior to induction, the paw thickness was found to be 4.48 mm, 6.16 mm at 60 min and 4.64 mm at 240 minutes (Fig. 1). The increase in paw thickness upon carrageenan induction at 0.1 ml was found to be significantly reduced upon pretreatment of the animals with 400 µg/ml of the extract, the thickness was considerably minimized upto 0.55 mm when compared to group II rats that were CGN control group (Table 2) and it was comparable to that of the pretreatment of animal

with a standard drug, indomethacin. This considerable decrease in paw thickness upon treatment of extract indicates the presence of anti-inflammatory activity of the extract.

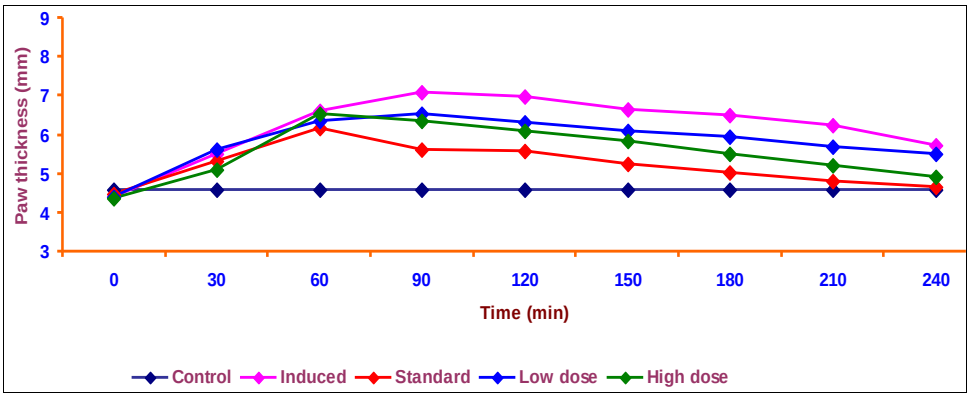

Fig 1: Paw thickness in the control and experimental rats at 30 minutes time intervals for 4 hours.

Table 2: Effect of methanolic leaf extract variant of *Exacum bicolor* on paw thickness in carrageenan induced rats.

Group	Initial paw thickness (mm)	Paw thickness after 4hr (mm)	Increase in paw thickness (mm)	Inhibition (%)
Group I (Control)	4.58 ±0	4.58 ±0	0	-
Group II (Induced)	4.41 ±0.28	5.71±0.34	1.3 ± 0.06	-
Group III (Standard)	4.48 ±0.16	4.64±0.24	0.16 ± 0.17	87.69
Group IV (Low dose)	4.40 ±0.21	5.51±0.17	1.11 ±0.32	14.61
Group V (High dose)	4.38 ±0.14	4.93±0.21	0.55 ±0.24	57.69

5.2. Determination of enzymic antioxidant status

5.2.1. Effect of *E. bicolor* extract on serum antioxidants

The results of activity of the enzymic antioxidants *viz.*, SOD, CAT, GPx and GST in serum of control and experimental rats revealed that the antioxidant enzymes were found to be significantly ($p<0.05$) reduced in the inflammation induced animals, while the same was elevated on treatment with methanolic leaf extract of *E. bicolor* (200 and 400 mg/Kg b. wt.) and standard, indomethacin (Table 3). The activity of the enzyme, SOD was found to be decreased from 3.03 µmoles/min/mg protein to 1.21 µmoles/min/mg protein in the carrageenan induced group of animals. Pretreatment of the animals with the extract at 200 and 400 mg/Kg b. wt., the activity rose upto 1.71 and 2.41 µmoles/min/mg protein respectively. Similar results were also obtained for the standard drug treatment. The activity of the CAT enzyme was also found to be decreased from 20.11 µmoles/min/mg protein to 11.95 µmoles/min/mg protein in the carrageenan induced group of animals. Pretreatment of the animals with the extract at 200 and 400 mg/Kg b. wt., the activity rose upto 14.09 and 17.85 µmoles/min/mg protein respectively. Similar results were also obtained for the standard drug treatment. In the induced group, the GPx activity was found to be 7.70 µmoles/min/mg protein. Pretreatment with extract at 200 mg/Kg b. wt. resulted in the increase of activity to 10.73 µmoles/min/mg protein and in 400 mg/Kg b. wt., it was increased to 14.68 µmoles/min/mg protein. Increase in activity for the standard drug, indomethacin treatment was also observed. The amount of GST was found to be 3.57 µmoles/min/mg protein in carrageenan induced rats, whereas in the rats which were pretreated with the extract at 200 and 400mg/ Kg b. wt., and the standard drug, indomethacin, the GST level was found to be 4.63, 6.19, 6.73 µmoles/min/mg protein of the experimental rats respectively.

5.2.2. Effect of *E. bicolor* extract on serum and lipid peroxide (LPO) and nitric oxide (NO) levels

The lipid peroxide of the induced rats was found to be significantly increased to 36.67 nmoles/min/mg protein. In the animals which were treated with the extract at two different doses *viz.*, 200 and 400 mg/Kg b. wt., the LPO level was found to have reduced significantly upto ($p<0.5$) 28.05 nmoles/min/mg protein and 23.22 nmoles/min/mg protein respectively (Table 4). Similar results were observed in the group of rats which were treated with the standard drug, indomethacin.

The level of serum nitric oxide (NO) was found to be 3.11 µmoles/dl protein in the normal rats. Nitric oxide was found to be increased to 8.89 µmoles/dl in the carageenan induced rats and in the rats of the groups III, IV and V which were treated respectively with the standard drug, indomethacin and 200 and 400 mg/Kg b.wt doses of extract, the levels were found to be 3.85, 6.88 and 5.53 µmoles/dl protein respectively (Table 4).

6. HPLC analysis

A chromatogram obtained from standards is shown in Fig.2 and 3. Acquisition of spectrum by using the UV spectrometric detector at 255 nm showed the presence of two flavonoids *viz.*, luteolin and chlorogenic acid. The contents of luteolin and chlorogenic acid were 815.86 µg/ml and 273.62 µg/ml respectively (Fig.4).

Table 3: Enzymic antioxidant status in the control and experimental rats.

Parameter (µmoles/min/mg protein)	Group I (Control)	Group II (Induced)	Group III (Standard)	Group IV (Low dose)	Group V (High dose)
SOD	3.03 ^{d} ±0.39	1.21 ^{a} ±0.22	2.66 ^{c} ±0.14	1.71 ^{b} ±0.23	2.41 ^{e} ± 0.19
CAT	20.11 ^{d} ±2.31	11.95 ^{a} ±0.48	20.12 ^{d} ±1.15	14.09 ^{b} ±0.33	17.85 ^{c} ±0.87
GPx	16.26 ^{d} ±0.96	7.70 ^{a} ±0.61	15.65 ^{d} ±0.45	10.73 ^{b} ±0.46	14.68 ^{c} ±0.42
GST	7.59 ^{e} ±0.31	3.57 ^{a} ±0.18	6.73 ^{d} ±0.49	4.63 ^{b} ±0.45	6.19 ^{c} ±0.16

Values are expressed as mean±SD. (n=6).
Values within the same row not sharing common superscript letters (a-e) differ significantly at p<0.05 by DMRT.

Table 4: Lipid peroxide and nitric oxide levels in the control and experimental rats.

Parameter	Group I (Control)	Group II (Induced)	Group III (Standard)	Group IV (Low dose)	Group V (High dose)
LPO (nmoles/mgprotein)	18.06 ^{a} ± 1.09	36.67 ^{e} ±1.67	21.02 ^{b} ±0.68	28.05 ^{d} ±0.62	23.22 ^{c} ±0.57`
NO (µmoles/dl)	3.11 ^{a} ±0.17	8.89 ^{e} ±0.47	3.85 ^{b} ±0.26	6.88 ^{d} ±0.26	5.53 ^{c} ±0.37

Values are expressed as mean±SD. n=6.
Values within the same row not sharing common superscript letters (a-e) differ significantly at p<0.05 by DMRT.

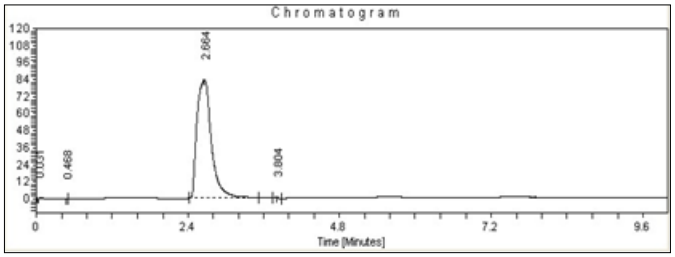

Fig 2: HPLC chromatogram obtained from a standard containing luteolin

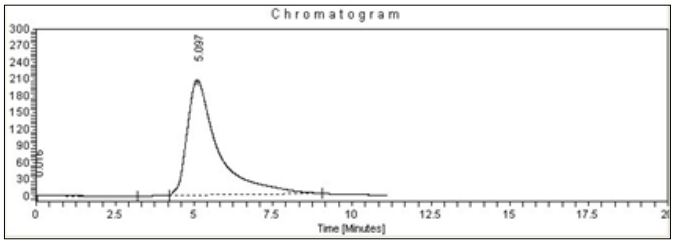

Fig 3: HPLC chromatogram obtained from a standard containing chlorogenic acid.

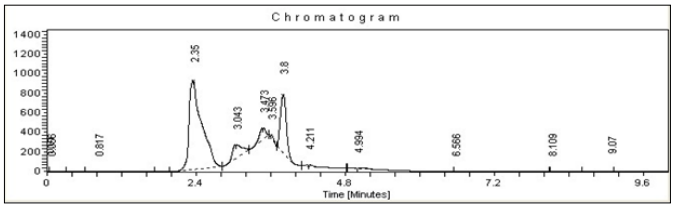

Fig 4: HPLC chromatogram obtained from the methanolic leaf extract of Exacum bicolor.

7. Discussion

E. bicolor leaves were analysed for phenolic and flavonoid contents. There are many study report on the positive correlation between phenolic flavonoid compounds and on the antioxidant activity. *In-vitro* antioxidant and *in vivo* anti-inflammatory activity. The results btained to provide evidence that the methanol extract of leaves of this species possesses high antioxidant and anti-inflammatory activities. The antioxidant activity probably due to the presence of respective phytochemicals which neutralize the free radicals that are routinely produced in the biological system (Baublis *et al.*, 1994; Vinson *et al.*, 1998) [5, 52]. Reactive oxygen species readily combine and oxidize biomolecules such as carbohydrates, proteins and lipids and thus making them inactive with subsequent damage to cells, tissues and organs (Punchard and Kelley, 1996; Krötz *et al.*, 2002) [32, 24]. Sangeetha *et al.* (2010) [38] observed similar type of results in the plant species, *Sphaeranthus indicus*.

In vitro antioxidant activity of the methanol extract of *E. bicolor* was investigated by DPPH and hydroxyl radical scavenging assay. Both methods have proven the effectiveness of the extract and comparable to that of the reference standard antioxidant, the tannic acid. Son and Lewis (2002) [46] explained that DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Antioxidant on interaction with DPPH, transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and convert it to 1–1 diphenyl –2– picryl hydrazine and the degree of discoloration indicates the scavenging activity of the drug. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is generally used as a substance to evaluate the antioxidant activity (Jun *et al.*, 2004; Shah *et al.*, 2010). In the present study, the leaf extract of *E. bicolor* had significant scavenging activity on the DPPH radical, which increased with the increase in the concentrations from 50 – 250 µg/ml (Table 1). The extract possess statistically high DPPH free radical scavenging activity (p<0.05). Similar trend of DPPH free radical scavenging activity was already observed in the species, *Piper nigram* (Singh *et al.*, 2008) [43], *Lippia alba* (Ara and Nur, 2009) [2], *Languas galanga* (Al-adhroey *et al.*, 2010) [1] and *Tephrosia purpurea* (Shah *et al.*, 2010) [40].

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins (Spencer *et al.*, 1984). The present study shows the ability of the extract to inhibit hydroxyl radical mediated deoxyribose degradation in concentration dependent way (Table 1). The extract had significant scavenging effects on the hydroxyl radical, which

increased with the increase in concentrations from 20 – 140 µg/ml. The methanol extract of the leaves of *E. bicolor* possess statistically high hydroxyl radical scavenging activity ($p<0.05$). Singh *et al.* (2008) [43] and Patil *et al.* (2009) [29] observed similar type of hydroxyl radical scavenging activity in the plants, *Piper nigram* and *Gmelina arborea* respectively.

The development of edema in the paw of the rat after the injection of carrageenan is due to the release of certain inflammatory mediators, istamine, serotonin and prostaglandin like substances (Vinegar *et al.,* 1969) [51]. Significantly high anti-inflammatory activity of the extract of *E. bicolor* (200 mg/Kg b.wt. and 400 mg/Kg b.wt.) determined may be due to the inhibition of such inflammatory mediators (Tables 2). Similar trend of results was also obtained for the plants like *Trigonella foeum-graecum* (Sharififar *et al.,* 2009) [41], *Rosa damascena* (Hajhashemi *et al.,* 2010) [14] and *Leucas cephalotes* (Baburao *et al.,* 2010). From the results it could be confirmed that the extract of *E. bicolor* at both the doses, 200 and 400 mg/Kg b. wt. posseses potent anti-inflammatory activity.

The antioxidant enzymes *viz.,* SOD, CAT, GPx and GST protect aerobic cells against oxygen toxicity and lipid peroxidation (Yamaguchy, 1991) [54]. Generally, SOD, CAT, GPx and GST are found to be decreased in carrageenan induced animals which may be due to enormous production of free radicals (Imadaya *et al.,* 1988) [18]. The decrease in SOD activity leads to declined production of hydrogen peroxide. As hydrogen peroxide is the substrate for the enzyme, catalase and GPx, they were also found to be decreased. On drug treatment, the activities of SOD, CAT, GPx and GST are brought to near normal levels, which may be attributed to the free radical scavenging activity of phytochemicals present in the drug (Sanz *et al.,* 1994) [39]. The results of the present study revealed that (Table 3) SOD, CAT, GPx and GST levels were decreased in carrageenan induced rats and while it was brought to near normal levels when the rats were pretreated with the extract of *E. bicolor* (Table 3). Chang *et al.* (2011) [8] also reported that the antioxidant level in the serum of carrageenan induced rat was brought to normal when it pretreated with ethanol extract of the plant species, *Phellinus linteus.*

Lipid peroxide (LPO) and nitric oxide (NO) are used as biomarker to show the index of oxidative stress, and causes cell membrane damage resulting in gradual loss of cell membrane fluidity, decreased the membrane potential and increased permeability to ions (Halliwell and Gutteridge, 1989; Kim *et al.,* 2005) [15, 22]. Significant decrease in LPO and NO levels in the carrageenan induced rats were observed when the animals were treated with the methanol extract of leaves of *E. bicolor* (Table 4). This results are also supported by Tabassum *et al.* (2010) [49] and Chitra *et al*. (2010) [9] using the extracts of *Ocimum sanctum* and *Camellia sinensis*, and *Strychnos nuxvomica* respectively.

8. Conclusion

On basis of the results obtained in the present study, it can be concluded that the methanol extract of leaves of *E. bicolor* had significant antioxidant and anti – inflammatory activities. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Plant polyphenolics and flavonoids in general are considered to be the effective free radical scavengers and so they serve as prominent antioxidants. The phytoconstituents identified in *E. bicolor* like polyphenols, and flavonoids *viz.*, luteolin and chlorogenic acid may be the responsible compounds for the inhibition of free radicals and inflammation. However, further clinical trials using human models are required to confirm the activities before going for commercialization of this species through obtaining appropriate and suitable drugs.

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