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GC-MS analysis, evaluation of phytochemicals, antioxidant, thrombolytic and anti-inflammatory activities of *Exacum bicolor*

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Abstract

The aim of the present study was to investigate the GC-MS analysis, phytochemical screening, antioxidant, thrombolytic and anti-inflammatory activities of methanol extract of leaves of *Exacum bicolor*. FTIR analysis confirmed the presence of alcohol, phenols, alkanes, aromatic compounds, aldehyde and ethers. GC-MS analysis revealed the presence of eight phyto-constituents. The total phenol, flavonoid and alkaloid contents were 18.0 ± 0.2 mg/GAE/g, 13.1 ± 0.4 mg QE/g and 108.0 ± 1.2 mg AE/g respectively. The DPPH assay exhibited potent antioxidant abilities with IC_{50} 8.8 µg/mL. Significant thrombolytic activity was demonstrated by clot lysis method (45.1 ± 0.8%). The methanol extract showed significant membrane stabilization on human red blood cell with IC_{50} value of 37.4 µg/mL. There was a significant correlation (R^2>0.98) with total phenolic content versus antioxidant and anti-inflammatory activity. The above results confirmed that *E. bicolor* could be a promising antioxidant, thrombolytic and anti-inflammatory agent.

Introduction

Oxidative stress plays a pivotal role in the development of human diseases (Rajendran et al., 2014). Anti-oxidants that can scavenge or neutralise the reactive oxygen species are beneficial in reducing the oxidative stress (Bandyopadhyay et al., 1999). Plants rich in anti-oxidants have ability to protect against oxidative cell damage that can lead in the treatment of many human diseases including diabetes, cancer, Alzheimer's, cardio-vascular diseases, chronic inflammation, thrombus formation and several degenerative diseases in humans (Danino et al., 2008; Deore et al., 2008; Dinstel et al., 2013). The free radical scavenging molecules such as flavonoids, phenols, tannins, alkaloids, amines, vita-mins and other metabolites possess anti-inflammatory, thrombolytic, anti-carcinogenic, antibacterial and anti-viral activities (Filomena et al., 2008). Inflammation is a key factor in all aspects of coronary disease including the initiation and progression of atherosclerotic plaque, plaque rupture, and thrombosis (atherothrombosis) where the oxidative stress is known to play a significant role (Freedman, 2008). Oxidative stress and inflamma-tion are intimately linked with both the evolution of cardiovascular disease and acute coronary syndromes (Pashkow, 2011). Due to short comings present in the synthetic drugs, research has been directed towards the development of herbal medicine which are considered safer due to their natural activity.

Exacum bicolor Roxb., a member of family *Gentianaceae*, is a herbaceous plant possessing antioxidant and anthel-mintic activities (Ashwini and Majumdar, 2014; Ashwini and Majumdar, 2015). Ethanopharmacologically *E. bicolor* is used for curing human ailments like diabetes, malaria, skin disorders, fungal diseases and inflamma-tion (Marles and Farnsworth, 1995; Reddi et al., 2005; Pullaiah, 2006; Khare, 2007). No scientific report is

available till date to validate these folkloric uses. *E. bicolor* is endemic to peninsular India and presently considered as an endangered species (Sreelatha et al., 2007; Brilliant et al., 2012). Chemically *E. bicolor* consists of protocatechuic, apigenin, luteolin, vanillic, ρ-coumaric acids, secoiridoids and ρ-hydroxybenzoic (Das et al., 1985; Khare, 2007). Hence, the present study is focussed to evaluate the anti-inflammatory and thrombolytic activities for the first time.

Materials and Methods

Plant collection and extraction

Plant material was collected from Kumar Parvatha, Western Ghats, Karnataka and authenticated by Regional Research Institute Bangalore, India (Accession No.: 557). The leaves were dried at room temperature in the shade. Twenty grams of the dried powdered leaves sample was soaked in 150 mL of methanol and was shaken intermediately. After 7 days the solution was filtered and was evaporated to dryness.

Infrared spectral analysis

The methanol leaf extract of *E. bicolor* was subjected to IR spectrum which was determined using Fourier transform infrared spectrophotometer (FTIR-Perkin-Elmer). The extract was ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4,000–400 cm^{-1} (Bunghez et al., 2011).

Gas chromatography-mass spectrometry (GC-MS) analysis

The methanol leaf extract was subjected for GC-MS analysis equipped with Thermo GC-Trace ultra Ver: 5.0, Thermo MS DSQ II and equipped with column DB - 5MS capillary standard non-polar (length 30 m x inner diameter 0.25 mm film thickness 0.25 µm) was used for analysis. Helium gas was used as the carrier gas at constant flow rate 1 mL/min and an injection volume of 1 µL. The oven injector temperature 70°C and raised to 260°C at 6°C/min. Overall runtime was 40.5 min.

Identification of components

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with relative retention time and mass spectra of the known components stored in the NIST library.

Preliminary phytochemical screening

The preliminary qualitative phytochemical study of the methanol leaves extract were screened for alkaloids (Meyer's test), flavonoids (Shinoda test), saponin, steroids, terpenoids, glycosides (Salkowski's test), phenols, tannins, amino acids, proteins (ninhydrin test), carbohydrates (Fehling's test), coumarins, quinones, oxalates and phlobotannins and acids (Harborne, 1973).

Total phenols

The total phenolics content in methanol extract was determined by FC method with minor modifications (Singleton and Rossi 1965). To 1 mL of extract (25, 50, 75, 100 µg/mL) 0.5 mL FCR (diluted 1:10 v/v) was added and allowed to stand for 5 min. To the above solution 20% sodium carbonate (1 mL) was added and allowed to stand for half hour in dark. The sample was read against the blank at 765 nm using UV-spectrophotometer. Results were expressed as in mg/g of dry weight of gallic acid equivalent (GAE) which was used as standard. All experiments were carried out in triplicates and represented as mean ± SE.

Estimation of flavonoids

Total flavonoid content was determined by aluminium chloride method using quercetin as a standard (Chang et al., 2002). 1 mL of plant extract (25, 50, 75, 100 µg/mL) and 0.5 mL of 5% sodium nitrite was added to the above solution 0.5 mL of 10% aluminium chloride was added. Incubated at room temperature for 6 min and 2 mL of 1 M sodium hydroxide was added to the reaction mixture. Volume was made up with distilled water and the absorbance was measured at 420 nm spectrophotometrically. Results were expressed as quercetin equivalents (mg QE/g dry weight). All the tests were performed in triplicates and represented as mean ± SE.

Estimation of alkaloids

Alkaloids were estimated according to Shamsa et al. (2008). *E. bicolor* methanolic extract (100 mg) was dissolved in 2 N HCL. 1 mL of the filtrate was washed with chloroform. The pH of the solution was adjusted to 7 with 0.1 N NaOH. Atropine was used as standard to which BCG solution and phosphate buffer was added. The mixture was shaken well and using chloroform and made up to 10 mL in a volumetric flask. The absorbance was measured at 470 nm against the blank. The values were expressed as atropine equivalent (AE) mg/g dry weight. All experiments were carried out in triplicates and represented as mean ± SE.

Antioxidant assay

DPPH(1,1-Diphenyl-2-picryllhydrazyl) radical scavenging activity

Free radical scavenging activity of *E. bicolor* methanol extract was determined according to Braca et al. (2003). The reaction mixture consist of extract (25, 50, 75, 100 µg/mL), 2 mL of 0.002% methanol solution of DPPH. This solution was incubated for 30 min in dark. The absorbance was read at 517 nm using spectrophotometer. Control was prepared by omitting the extracts,

ascorbic acid was used as standard and the percentage inhibition activity was calculated using the equation:

$$\%Inhibition = [(A_{Control}-A_{extract})/A_{control}] \times 100$$

Where $A_{Control}$ is the absorbance of the control and $A_{extract}$ the absorbance of the extract. All the tests were performed in triplicates represented as mean ± SE.

ABTS·+[2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)+] radical cation scavenging assay

ABTS radical cation decolorization assay was determined with minor modifications (Katalinic et al., 2006). ABTS·+ was produced by reacting 2 mM ABTS with 17 mM potassium persulfate and allowed to stand in dark at room temperature for 12-16 hours before use. To 1 mL of extract (25, 50, 75, 100 µg/mL) 2 mL of ABTS reagent was added. The absorbance was read at 734 nm and percentage inhibition was calculated using formula. Ascorbic acid was used as standard.

$$\% \text{ scavenging activity} = [(A_0-A_t)/A_0] \times 100.$$

Where A_0 is the absorbance of the control and A_t is the absorbance of the extract. All the tests were performed in triplicates and represented as mean ± SE.

In vitro thrombolytic activity

The thrombolytic activity was evaluated by the previously developed method (Prasad et al., 2007). Streptokinase (SK) vial was procured commercially of the brand name myokinase manufactured by Biocon (India) Ltd., Bangalore of 15,00,000 I.U. 5 mL of distilled water was used to dissolve. 100 µL (30,000 I.U) from the stock was used for further studies. Blood sample was drawn from human volunteers (n=50) who are healthy and do not show any history of acquiring oral contraceptive or undergoing anticoagulant therapy.

100 mg of powdered extract was dissolved in 10 mL distilled water. Blood was collected and distributed in pre weighed sterile eppendorf tube (0.5 mL/tube) and incubated at 37°C for 45 min. After clot formation, the serum was completely removed without disturbing the clot and each tube with the clot was again weighed to determine the clot weight (clot weight = weight of clot with tube – weight of empty tube). To each pre-weighed tube containing the clot, 100 µL of *E. bicolor* methanol leaf extract (test extract), SK (positive control) and distilled water (negative control) were separately added to the tubes. Tubes were incubated at 37°C for 90 min. The fluid released after the incubation period was removed and tubes were weighed again to observe the difference in weight after clot disruption. Percentage of clot lysis was calculated by calculating the weight that is taken before and after clot.

In vitro anti-inflammatory activity

Anti-inflammatory activity was assessed by human red blood cell (HRBC) membrane stabilization method (Gandhisan et al., 1991). The blood sample was collected from healthy human volunteer. Equal volume of alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) was mixed and centrifuged at 3,000 rpm. The packed cells were washed with isosaline for 3 times and 10% suspension was made with isosaline. Various concentration of extracts (25, 50, 75, 100 µg/mL), 1 mL of phosphate buffer, 2 mL hyposaline and 0.5 mL of HRBC suspension were added. It was incubated at 37°C for 30 min and later centrifuged at 3,000 rpm for 20 min. The haemoglobin content of the supernatant solution was estimated at 560 nm spectrophotometrically. Diclofenac (10, 25, 50, 75, 100 µg/mL) was used as reference standard and control was prepared by omitting the extracts. The percentage of hemolysis and protection of HRBC membrane was calculated as follows:

$$\%Hemolysis = (OD \text{ of test sample} / OD \text{ of control}) \times 100$$

$$\%Protection = 100 - \%Hemolysis$$

Statistical analysis

All the results are expressed as mean ± SEM. IC_{50}, percentage of clot lysis analysis was done using Graph pad prism 6 using ANOVA with post hoc analysis by DMRT. p values ≤0.05 were considered to be significant. The Correlation coefficient (R^2) values of all the different concentration of extracts between total phenols with DPPH assay and anti-inflammatory activities were evaluated.

Results

The FTIR spectrum (Figure 1) analysis revealed the identity of the functional groups of the active components present in the plant based on the peaks and the values in the IR region. *E. bicolor* exhibited characteristic absorption bands at 3436, 2919, 1689, 1604, 1402, 1370, 1239, 1101 and 1080 cm^{-1}. These results of FTIR analysis confirmed the presence of alcohols, phenols, alkanes, amines, aromatic compound, aldehyde and ethers.

The GC-MS analysis of phytoconstituents in methanol extract of leaves of *E. bicolor* revealed the presence of eight major phytoconstituents (Figure 2; Table I). The identification of the compounds was confirmed based on the retention time, peak area and the molecular formula. The major phytocomponents reported are 1-methyl 2-(3-oxocyclohexyenyl)imidazole (5.8%), erythrocentaurin (1.0%), neophytadiene (4.0%), hexadecanoic acid (5.4%), 6-octadeccenoic acid (12.0%), (+-)-inophylum D (2.7%), 4,6,8(14)-cholestatriene (6.0%) and methyl 3,4-diphenylpyrrolo[2,1,5-cd] indolizine-1-carboxylate (5.5%).

The preliminary qualitative phytochemical screening of the methanol extract revealed the presence of alkaloids, flavonoids, saponin, steroids, terpenoids, glycosides,

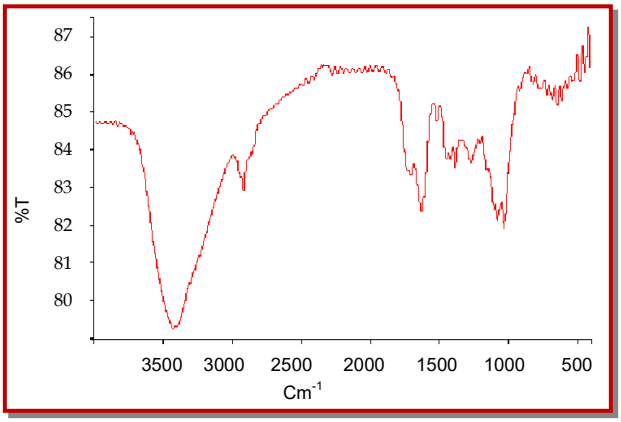

Figure 1: FTIR spectrum of *E. bicolor* (leaf) methanol extract

phenols, tannins, amino acids, proteins, carbohydrates and acids. The quantitative analysis of *E. bicolor* leaf methanol extract was based on the total phenols flavonoids and alkaloids. The total phenols, flavonoid and alkaloid content in methanol extract were found to be 18 ± 0.2 mg/ GAE/g, 13.1 ± 0.4 mg QE/g and 108 ± 1.2 mg AE/g respectively.

The results of DPPH assay showed that methanol extract of *E. bicolor* had a good inhibitory activity in dose-dependent manner. The percentage inhibition of methanol extract was 91.8% at 100 µg/mL concentration with an IC_{50} value of 8.8 µg/mL. In terms of ABTS cation radical scavenging assay, the percentage inhibition of *E. bicolor* leaf extract was 76.9% at 100 µg/mL concentration with an IC_{50} value of 43.7 µg/mL. The extract also exhibited dose dependent inhibitory activity. Ascorbic acid was used as standard for both DPPH and ABTS assay with the IC_{50} value of 6.0 µg/mL and 11.3 µg/mL respectively.

In the present study, thrombolytic activity was evaluated by clot disruption method. Streptokinase, positive control, showed 54.8 ± 0.7% clot lysis. Clots when treated with sterile distilled water (negative control) showed only 8.3 ± 0.5% of clot lysis. The mean difference in clot lysis percentage between positive and negative control was significant ($p<0.0001$). The tested methanolic extract of *E. bicolor* exhibited 45.1 ± 0.8% of clot lysis.

In vitro anti-inflammatory activity of *E. bicolor* extracts at different concentrations showed significant stabiliza-

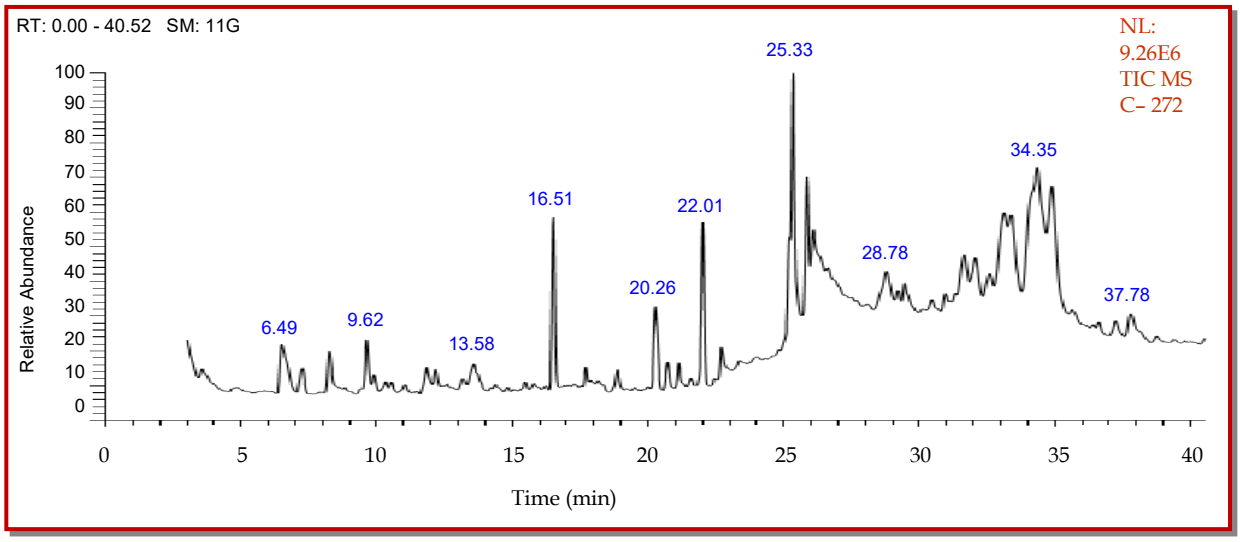

Figure 2: GC-MS analysis of *Exacum bicolor* methanol leaf extract

Table I

Chemical profile identified by GC-MS analysis of methanol leaf extract of *E. bicolor*

SL. No.	RT	Compounds	Molecular formula	MW	%Area
1	16.51	1-Methyl 2-(3-oxocyclohexyenyl)imidazole	$C_{10}H_{12}N_2O$	176	5.8
2	17.72	Erythrocentaurin	$C_{10}H_8O_3$	176	1.0
3	20.26	Neophytadiene	$C_{20}H_{38}$	278	4.0
4	22.01	Hexadecanoic acid	$C_{17}H_{34}O_2$	270	5.4
5	25.33	6-Octadeccenoic acid	$C_{19}H_{36}O_2$	296	12.0
6	28.78	(+-)-Inophylum D	$C_{25}H_{24}O_5$	404	2.7
7	33.13	4,6,8(14)-Cholestatriene	$C_{27}H_{42}$	366	6.0
8	34.35	Methyl 3,4-Diphenylpyrrolo[2,1,5-cd] indolizine-1-carboxylate	$C_{24}H_{17}NO_2$	351	5.5

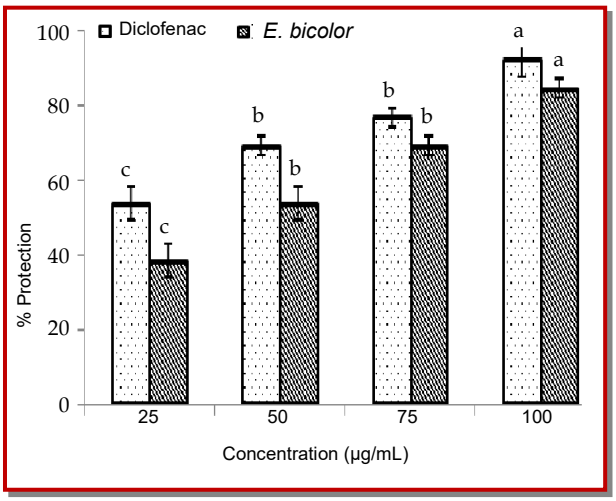

Figure 3: *In vitro* anti-inflammatory activity by HRBC membrane stabilization method of standard diclofenac and *E. bicolor*

tion towards HRBC membranes. The percentage protection (Figure 3) at concentration 100 µg/mL was more when compared to the other concentrations in *E. bicolor* (84.6 ± 2.6%) and diclofenac (92.3 ± 4.4%) as standard. The IC_{50} values of *E. bicolor* and diclofenac were 37.4 and 28.0 µg/mL respectively.

A positive, correlation of R^2=0.9867 was found between the total phenols and DPPH assay which was highly significant (p=0.007). Whereas the total phenols were also significantly (p=0.006) correlated with anti-inflammatory activity (R^2= 0.9916).

Discussion

FT-IR showed typical bands arising as a result of strong O-H stretching (3550-3200 cm^{-1}) intramolecular bonds. Other bands, occurred at 3000-2840 cm^{-1} (C-H stretching), 1710-1685 cm^{-1}, showed strong (C=O stretching) conjugated aldehyde peaks at 1650-1600 cm^{-1} resulted in medium (C=C stretching) conjugated alkene, signals at 1150-1085 cm^{-1} gave strong (C-O stretching) aliphatic ether, 1450-1375 cm^{-1} resulted medium C-H bending alkane with methyl group, at 1085- 1050 cm^{-1} strong (C-O stretching) primary alcohol. Based on the FT-IR spectrum, *E. bicolor* leaf extract contains various phytocompounds with functional groups such as phenols, amines, aldehyde, alkanes, carboxylic acids and alcohols.

The GC-MS analysis of the plant extracts are becoming a valuable tool for detection of phytochemicals which can be aimed before the process of large scale purification (Vinay et al., 2014). In the present study, the GC-MS analysis of methanol extract of *E. bicolor* majorly contained phenols, alkanes, terpenoids, alkaloids which might be responsible for various medicinal activities (Sellamuthu et al., 2009). According to the previously reported literature erythrocentaurin, a monoterpene alkaloid has also been identified from *Enicostemmahys sopifolium* and *Swertia lawii* (Ghosal et al., 1974). Neophytadienea (major component) and hexadecanoic acid were also present in *Centaurium erythraea* (Jovanovic et al., 2009) which belongs to family *Gentianaceae*. The present study was in accordance with the above literature.

Phytochemicals or secondary metabolites are chemical compounds which are formed during the plants normal metabolic processes and plants use them to protect themselves during stress related conditions (Ning et al., 2009). In the current study, the leaf extract of *E. bicolor* possesses 18 ± 0.2 mg/GAE/g of phenols, 13.1 ± 0.4 mg QE/g of flavonoids, 108 ± 0.0 mg AE/g of alkaloids when determined spectrophotometrically. According to Baba and Malik (2014), *Gentiana kurroo* methanol leaf extract showed 34 ± 1.8 mg GAE/g of phenols and 20 ± 1.5 mg RE/g flavonoids respectively. The total phenols in methanol extract of *S. chirata* were about 38.4 ± 0.4 mg GAE/g (Tupe et al., 2013).

Phenolic compounds are widely distributed in various plant species which have received considerable attention (Li et al., 2006). Phenolic antioxidants provided tremendous potential benefits because of their ability to scavenge reactive oxygen species (Bakirel et al., 2008). The total antioxidant power evaluates health beneficial effects because of the co-operative action of antioxidants. Therefore, it is desirable to measure the radical scavenging capacity level by more than one method (Fu et al., 2014).

DPPH antioxidant scavenging assay is based on the ability of DPPH, to decolorize in the presence of antioxidants. The visible deep purple color is produced due to the DPPH free radical which contains an odd electron. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured by absorbance (Nuengchamnong et al., 2009). The methanolic extract of *E. bicolor* in the present study exhibited potent antioxidant activity at 100 µg/mL (91.2 ± 0.7%) with lower IC_{50} value (8.8 µg/mL). According to Vaijanathappa et al. (2008) the IC_{50} value of methanol extract of *Enicostemma axillare* was 325.5 ± 5.9 µg/mL. In case of *S. chirata*, IC_{50} value was 87.6 ± 0.4 (Tupe et al., 2013). According to Baba and Malik (2014), *Gentiana kurroo* showed 91% of inhibition at 600 µg/mL. In comparison to earlier findings, *E. bicolor* exhibited relatively strong radical scavenging activities and might serve as effective radical scavenger.

ABTS method has chromophores which are soluble in both aqueous and organic solvents, and may therefore serve the need to simultaneously measure hydrophilic and lipophilic antioxidants (Cekic et al., 2009). In this assay, ABTS radical cation was generated directly in

stable form using potassium persulfate and the anti-oxidant activity is measured in terms of decolorization (Sanchez-Moreno 2002). The free radical scavenging ability of *E. bicolor* was determined using ABTS radical cation which exhibited 76.9 ± 0.9% inhibition and the IC_{50} value was 43.7 µg/mL. Tupe et al., (2013) reported that in *S. chirata* the IC_{50} value was 71.7 ± 1.7 µg/mL. But the present study with *E. bicolor* showed better antioxidant capacities when compared to *S. chirata.*

Atherothrombotic diseases occur as serious impacts of the thrombus formed in the blood vessels. Thrombo-lytics dissolve blood clots and are used in treating myocardial infarction, cerebral stroke, high cholesterol hypertension, hypotension, arrhythmias, congestive heart failure, coronary artery disease, unwanted blood clots and arteriosclerosis (American Pharmacist Asso-ciation, 2010). The synthetic drugs have limitations and can lead to serious and sometimes fatal consequences. Studies have been conducted to find plants having anti-thrombotic compound which might lead to lower or no adverse effects (Prasad et al., 2007). In case of *E. bicolor,* the methanol leaf extract was assessed by clot disruption method which revealed 45.1 ± 0.8% of clot lysis while the streptokinase (positive control) and water (negative control) demonstrated 56.2 ± 0.6% and 8.3 ± 0.5% lysis of clot respectively. The results were also statistically significant ($p<0.0001$). According to the previous study, *S. chirata* showed clot lysis of 46.1% and 31.9% in ethanolic and chloroform extract respectively (Hossain et al., 2012). According to Dhande et al., (2014) the crude extracts of *S. chirata* were found to have significant ($p<0.01$) thrombolytic activity at 1 mg/mL. Whereas, the *E. bicolor* methanolic leaf extract showed better thrombolytic activity with lower concen-tration (100 µg/mL) when compared to the above study.

Plants with antioxidants properties are used for minimizing the inflammation related diseases (Wong et al., 2006). Erythrocyte membrane is an analogue of lysosomal membrane. The plant extract plays an impor-tant role in stabilizing lysosomal membrane which limits the inflammatory response by preventing the release of activated neutrophils, which might further cause tissue damage and inflammation (Murugasan et al., 1981). The extract may inhibit this process and acts as an anti-inflammatory compound. Investigation on *in vitro* anti-inflammatory activity by human red blood cell membrane stabilization method in *E. bicolor* exhibited 87.2 ± 2.6% of protection at 100 µg/mL with IC_{50} value of 37.4 µg/mL, whereas Leelaprakash and Dass (2011) reported 30 ± 0.0% inhibition of hemolysis in *Enicostemma axillare* methanol extract of which the present study exhibited potent anti-inflammatory activity.

In order to understand the relationship between the antioxidant activity with total phenolic content, the correlation between the DPPH scavenging activity and the total phenolics were measured. Our results revealed that there is a strong and significant correlation (R^2=0.9867) between total phenolic content and DPPH free radical scavenging activity. There was also a positive correlation between total phenol contents and anti-inflammatory activity (R^2 = 0.9916).

Conclusion

Methanolic leaf extract of *E. bicolor* possesses phyto-chemicals which might be playing a major role in anti-oxidant, anti-inflammatory and thrombolytic activities. The positive correlation between total phenolic content with DPPH scavenging capacities and anti-inflamma-tory activity showed that total phenols might be the major contributor for these biological activities. *E. bicolor* extracts may be exploited as a source of bene-ficial compounds for oxidative stress related diseases in humans.

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Jain University

Ethical Issue

The collection of blood sample from the human volunteers was done with the approval by Institutional Ethics Committee of Kempegowda Institute of Medical Sciences (KIMS), Bangalore, Karnataka with registration No. ECR/216/Inst/Kar/2013. The volunteer donors were supplied with a consent form which informed detail of investigations.

Conflict of Interest

Authors declare no conflict of interest

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