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Original Article

QUANTIFICATION OF PHYTOCHEMICAL CONTENTS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *EXACUM BICOLOR* (ROXB.), AN ENDEMIC MEDICINAL PLANT

APPAJI M ASHWINI, MALA MAJUMDAR*

Department of Biotechnology, Centre for Post Graduate Studies, Jain University, # 18/3, 9th Main, Jayanagar, 3rdBlock, Bangalore, Karnataka, 560011, India
Email: malamajumdar51@gmail.com

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ABSTRACT

Objective: To investigate different phytochemical contents and screening of antioxidant activity of sequentially extracted *Exacum bicolor* leaf with different solvents.

Methods: Leaf samples of *E. bicolor* were dried and subjected to sequential (hexane>chloroform>ethyl acetate>methanol>water) extraction using soxhlet apparatus. Total phenol contents (TPC) were estimated using Folin-ciocalteau method, flavonoids by aluminium chloride method, tannins by Folin-Denis reagent and alkaloids using bromocresol blue. *In vitro* antioxidant activities were estimated by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid $ABTS^{\cdot+}$and Nitric oxide (NO) scavenging assay.

Results: The total phenol, tannin and alkaloid contents in methanol extract were found to be 23.4±0.09 mg GAE/g, 23±0.57 mg TAE/g and 47.33±1.20 mg AE/g respectively. Hexane extract exhibited high flavonoid content (27.6±0.88 mg QE/g). Methanolic extracts exhibited dose dependent antioxidant activity against DPPH, $ABTS^{\cdot+}$and NO Scavenging methods; low IC_{50} value (14.84 µg/ml) was observed in DPPH scavenging activity. Findings showed positive and significant ($P \leq 0.05$) correlation between TPC/DPPH (R^2= 0.98).

Conclusions: The methanol extract showed higher antioxidative activity compared to other extracts. Positive correlation was obtained between TPC and DPPH activity signifying that the phenolic content may be responsible for the antioxidant activity of the plant. Further bioassay-guided purification of the bioactive compound is necessary. To the best of our knowledge, antioxidant activity and its correlation with TPC, is reported for the first time in *E. bicolor*.

Keywords: *Exacum bicolor*, DPPH, $ABTS^{\cdot+}$, NO scavenging assay, Correlation.

INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of the world's population as they are having little or no side effects and also they are economical [1]. Plants are the important source for free radical scavenging molecules. Free radicals are highly reactive particles with an unpaired electron and are produced by radiation or as by-products of metabolic processes that lead to disintegration of cell membranes and cell compounds [2].

A serious imbalance between the production of free radicals and antioxidant defence system is responsible for oxidative stress. Oxidative stress is related to ageing process and some chronic diseases such as diabetes, cancer, cardiovascular diseases and inflammatory diseases [3]. Synthetic antioxidants, such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA), have been widely used as antioxidants and may be responsible for side effects such as liver damage and carcinogenesis [4]. Therefore, the search for alternative source of natural antioxidant compound has gained importance nowadays.

During extraction, the solvent polarity plays a major role determining both quality and quantity of the extracted antioxidant compounds [5]. Higher the polarity, better the extraction of phenolic compounds which might be a major contributor for antioxidant activity [6]. Therefore, the investigation on phytochemicals and antioxidant activity from different non-polar and polar solvents will help to understand their importance and give information on rationale design or search for the new type of more potent bioactive compound helping in the treatment of oxidative stress related diseases.

E. bicolor Roxb. (Gentianaceae) is endemic to peninsular India [7] and now considered as endangered species [8]. They are extensively used locally in curing human ailments such as diabetes [9], antifungal [10], urinary, skin disorders [11], inflammation, purifies blood in treatment of malaria [12], paste is used against sores of leech bites [13], used as tonic for stomachic [14], febrifuge [15] also possess anthelmintic activity [16]. The plants possess secoiridoids, gentiopicroside and methylgrandifloroside [17]. As per literature survey, there are no reports of antioxidant activity of *E. bicolor.* Hence, *E. bicolor* has been selected in the present study for evaluating its antioxidant activity.

MATERIALS AND METHODS

Chemicals and reagents

Organic solvents (hexane, chloroform, ethyl acetate, methanol) SRL, Folin-Ciocalteu reagent (FC), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH); 2,2'-azi-no-bis (3-ethylbenzthiazoline-6-sulphonic acid $ABTS^{\cdot+}$, Gallic acid, Ascorbic acid, Quercetin from Sigma–Aldrich (Sydney, Australia). Shimadzu 1800 UV Visible spectrophotometer was used for the photometric study.

Plant collection

Whole plant of *E. bicolor* was collected from Kumar Parvatha located near the famous shrine of Lord Subramanya at Kukke Subramanya in Sullia taluk, at 12 ° 33' 53'' N 75 ° 23' 11'' E of South Canara district, Karnataka, during the month of September, 2012. The plant specimen was identified and authenticated by Regional Research Institute Bangalore, India [Accession no: SMPU/MADRI/BNG/2010-11/557]. The voucher specimen was deposited in Biotechnology Department of Jain University, Bangalore.

Preparation of plant extract

Twenty grams of the dried and powdered leaf samples were subjected to sequential [Hexane (H)>Chloroform (C)>Ethyl acetate (EA)>Methanol (M)>Water (W)] extraction using the Soxhlet apparatus. The resulting extracts were then evaporated to dryness using the rotary evaporator and stored at 4 °C for further use.

Estimation of total phenolic content

The total phenolic content in different solvent extracts was estimated using Folin-Ciocalteu's (FC) method with minor modifications [18]. Different concentrations of extracts were mixed with 0.5 ml FC reagent (diluted 1:10 v/v) and 5 min later 1 ml of sodium carbonate (20%) solution was added and allowed to stand in dark for 30 min at room temperature. Absorbance of sample was measured against the blank at 765 nm using UV-VIS spectrophotometer. Quantification was done on the basis of the standard curve of gallic acid. Total phenolic content was expressed as mg gallic acid equivalent (GAE) per gram of extract.

Estimation of flavonoids

Flavonoids were determined using aluminium chloride colorimetric method according to Chang *et al.* [19] with some modifications. An aliquot of 1 ml of leaf extracts were mixed with 0.5 ml of 5% sodium nitrite, after 5 min 0.5 ml of 10% aluminium chloride was added and the absorbance was measured at 420 nm. Flavonoid contents were determined from quercetin standard curve and were expressed as mg Quercetin equivalent (QE) per gram of extract.

Estimation of tannins

Amount of tannins in the leaf extracts were determined by Folin Denis method [20] extracts of various concentrations 1 ml mixed with 0.5 ml Folin-Denis reagent and after 5 min 1 ml of sodium carbonate solution was added. Absorbance of sample was measured against the blank at 700 nm. Tannic acid was used as standard the results were expressed as mg tannic acid equivalents (TAE) per gram of extract.

Estimation of alkaloids

Alkaloids were estimated [21] using 100 mg of extract dissolved in 2 N HCl and filtered. 1 ml of the filtered extract was taken and washed with chloroform in a separating funnel by adjusting the pH to 7.5 ml of BCG solution and 5 ml of phosphate buffer were also added. The mixture was shaken and complex extracted with chloroform by vigorous shaking, the extract was then collected in a 10 ml volumetric flask and diluted with chloroform. The absorbance of the complex in chloroform was measured at 470 nm in UV-Spectrophotometer against the blank. The results were expressed as mg Atropine equivalents (AE) per gram which was used as standard.

Antioxidant assay

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

Free radical scavenging activity of the different concentration of *E. bicolor* leaves extracts was determined by the method of Braca *et al.* [22] with little modifications. The reaction mixture (3 ml) consists of 1 ml of methanolic plant extract at different concentrations and 2 ml of 0.002% of methanolic solution of DPPH. Absorbance at 517 nm was determined after 30 min incubation in dark. Ascorbic acid was used as standard. The percentage inhibition activity was calculated by using the equation:

$$\% \text{ scavenging activity} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the extract.

$ABTS^{+}$ [2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging method

ABTS radical cation ($ABTS^{\cdot+}$) decolorization assay was determined with minor modifications [23]. $ABTS^{\cdot+}$was produced by reacting ABTS solution (2 mM) with 17 mM potassium persulfate and the mixture was allowed to stand in dark at room temperature for 12-16 h before use. 2 ml of ABTS reagent was added to different concentration of extracts. Ascorbic acid was used as standard. The absorbance was read at 734 nm and %Inhibition was calculated using following formula.

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

Where A_0 is the absorbance of the control and A_t is the absorbance of the extracts.

NO scavenging activity

NO scavenging activity was performed according to Sreejayan and Rao [24] with little modification where, sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of the *E. bicolor* extracts and incubated at 25 °C for 30 min. The incubated solutions were diluted with equal volume of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% *N*-(1-Naphthyl)ethylenediamine dihydrochloride). The absorbance of the chromophore formed were measured at 546 nm. The NO radical scavenging activity was expressed as the inhibition percentage (%I) and calculated as per the equation:

$$\%I = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound with all reagents. Ascorbic acid was used as standard.

IC_{50} value of the extracts

The half maximal inhibitory concentration (IC_{50}) value of each extract was determined, plotting the percentage of scavenging activity versus the concentration of extract using nonlinear regression analysis (curve fit). Lower the IC_{50} value, higher the radical scavenging effect.

Statistics

All analyses were performed in triplicate (n=3) using one way ANOVA and the data were presented as mean±SE. Graph Pad Prism for Windows version 7 (Graph Pad version 6 Software, San Diego, CA, USA) was used for all statistical analyses and IC_{50} determinations. P values ≤ 0.05 were considered to be significant.

RESULTS

Total Phenolic contents, Flavonoids, Tannins and Alkaloids

The amount of total phenols, flavonoids, tannins and alkaloids present in the sequential extracts of *E. bicolor* are shown in table 1. The methanolic extract recorded the highest amount of total phenols (23.4±0.09 mg GAE/g), tannins (23±0.57 mg TAE/g) and alkaloids (47.33±1.20 mg AE/g). Whereas the flavonoids were present in high amount in hexane (27.66±0.88 mg QE/g) extract.

Table 1: Total phenols, flavonoids and alkaloid content

Extracts	Total phenols (mg GAE/g)	Flavonoids (mg QE/g)	Tannins (mg TAE/g)	Alkaloids (mg AE/g)
H	3.6±0.04	27.66±0.88	2.33±1.52	8.66±0.88
C	7.5±0.12	21.33±0.88	12.33±1.52	15.0±0.57
EA	20.1±0.09	8.36±0.14	15.33±0.57	22.33±1.45
M	23.4±0.09	6.40±0.11	23.00±1.0	47.33±1.20
W	6.9±0.10	4.63±0.08	18.33±0.57	16.0±0.008

All values are expressed as mean±standard error (n=3). GAE=Gallic acid equivalents, QE=Quercetin equivalents, AE=Atropine equivalents H: Hexane, C: Chloroform, EA: Ethyl acetate, M: Methanol, W: Water extracts of *E. bicolor*.

DPPH radical scavenging activity

DPPH radical scavenging activity of hexane, chloroform, ethyl acetate, methanol and water extracts of *E. bicolor* are reported in fig. 1. All the extracts showed potent DPPH radical scavenging activity in concentration dependent manner. Among the solvent tested, the methanolic extract of *E. bicolor* leaves exhibited a maximum DPPH scavenging activity of (93.58±0.47%) at 100 µg/ml.

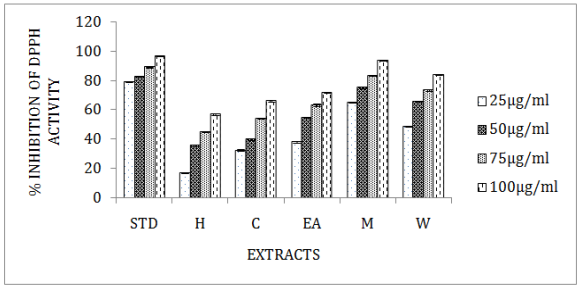


Fig. 1: DPPH radical scavenging assay
STD: Standard (Ascorbic acid), H: Hexane, C: Chloroform, EA: Ethyl acetate, M: Methanol, W: Water extracts of *E. bicolor*

ABTS $^{\cdot+}$assay

ABTS scavenging assay, the *E. bicolor* leaves extract showed dose dependent effect. The %inhibition of methanolic extract was 82.68±1.14 µg/ml and was highest compared to all other extracts at 100 µg/ml.

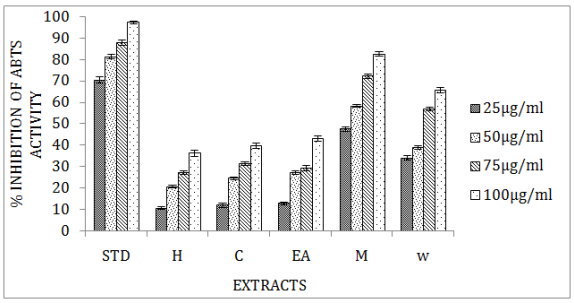


Fig. 2: ABTS assay
STD: Standard (Ascorbic acid), H: Hexane, C: Chloroform, EA: Ethyl acetate, M: Methanol, W: Water extracts of *E. bicolor*

NO scavenging assay

NO scavenging assay exhibited concentration dependent increase in %inhibition among all the extract, where the maximum %inhibition of the extracts in methanolic extract of about 77.19±2.32% at 100 µg/ml compared to the other extracts.

IC_{50} values

IC_{50} values of hexane, chloroform, ethyl acetate, methanol and water extracts of *E. bicolor* for DPPH radical scavenging activity were found to be 192.5 µg/ml, 73.54 µg/ml, 38.13 µg/ml, 14.84 µg/ml and 28.45 µg/ml respectively. In ABTS assay the IC_{50} values of hexane, chloroform, ethyl acetate, methanol and water extracts of *E. bicolor* were 258.2 µg/ml, 197.4 µg/ml, 107.1 µg/ml, 34.33 µg/ml, and 66.91 µg/ml respectively. NO scavenging assay exhibited IC_{50} values for hexane, chloroform, ethyl acetate, methanol and water extracts such as 363.1 µg/ml, 289.0 µg/ml, 228.3 µg/ml, 34.03 µg/ml and 72.48 µg/ml respectively (table 2).

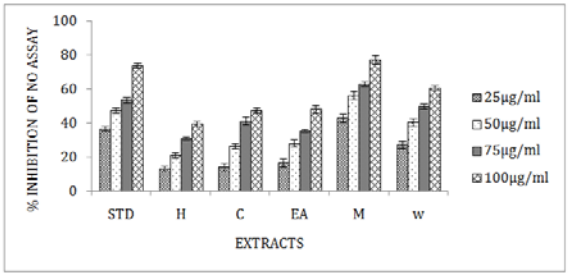


Fig. 3: Nitric oxide scavenging assay
STD: Standard (Ascorbic acid), H: Hexane, C: Chloroform, EA: Ethyl acetate, M: Methanol, W: Water extracts of *E. bicolor*

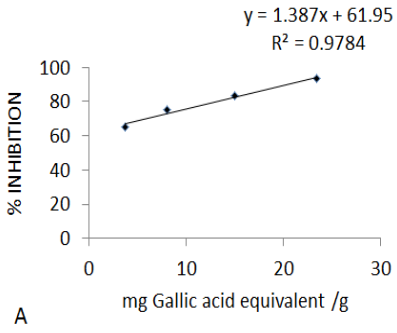
Table 2: IC_{50} values of different sequential extracts and standard (µg/ml)

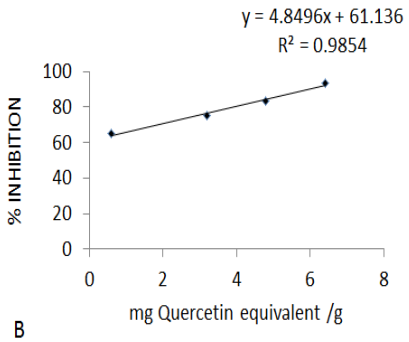
Extracts	DPPH	ABTS	NO
H	192.5	258.2	363.1
C	73.54	197.4	289.0
EA	38.13	107.1	228.3
M	**14.84**	**34.33**	**34.03**
W	28.45	66.91	72.48
STD	5.95	15.21	55.87

STD: Standard (Ascorbic acid), H: Hexane, C: Chloroform, EA: Ethyl acetate, M: Methanol, W: Water extracts of *E. bicolor*

Correlation of antioxidant activities with phenols, flavonoids and tannins contents

All the antioxidant activities showed positive and significant ($P \leq 0.05$) correlations with total phenol content, flavonoids and tannins of methanolic extracts (fig. 4).





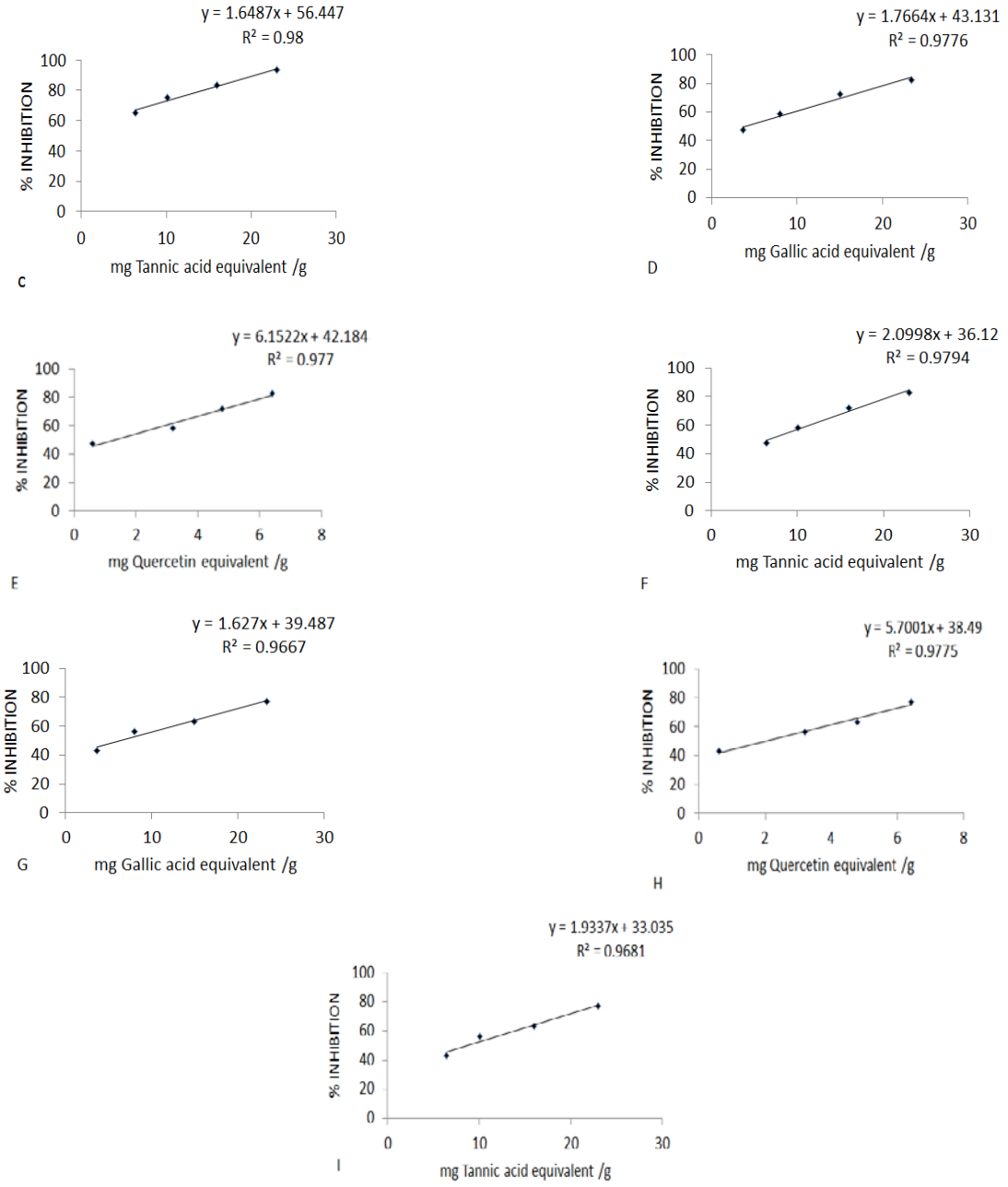


Fig. 4: The Correlation coefficient (R^2) values of all the different concentration of extracts between Total phenol contents (TPC) and antioxidant activities are tabulated

A: DPPH-Phenols; B: DPPH-Flavonoids; C: DPPH-Tannins; D: $ABTS^{+}$-Phenols; E: $ABTS^{+}$-Flavonoids; F: $ABTS^{+}$-Tannins; G: NO-Phenols; H: NO-Flavonoids; I: NO-Tannins

DISCUSSION

Phenolic compounds are widely distributed in plants which have gained much attention because of their possible beneficial antioxidative effects on human health to cure various diseases such as diabetes, cancer and other degenerative conditions [25]. In the present study, the methanolic extracts of *E. bicolor* leaves revealed high phenols, tannins and alkaloids (23.4±0.09 mg GAE/g, 23±0.57 mg TAE/g and 47.33±1.20 mg AE/g) respectively. Maximum amount of flavonoids (27.6±0.88 mg QE/g) were observed in the hexane extract. Earlier findings revealed that in *Swertia chirayita*, the leaf extract possess total phenols and flavonoid content as 67.53±2.08 mg/g GAE and 60.30±2.46 mg/g QE respectively. Misra *et al.* [26] has reported that the secondary metabolites are responsible for the antioxidant activity of the plants.

DPPH is a stable free radical containing an odd electron having a characteristic absorption at 517 nm. The deep purple colour usually gets decolorized when exposed to antioxidant in the solution. Lower the absorption, higher the radical scavenging activity of the extract [27]. Current study revealed that *E. bicolor* exhibited dose-dependent inhibition of DPPH radical scavenging activity. It was observed that the methanolic extract showed significantly higher activity compared to other extracts. However, ascorbic acid which was used as positive control showed highest inhibition at the same concentration (100 µg/ml). IC_{50} values of

hexane, chloroform, ethyl acetate, methanol and water extracts of *E. bicolor* for DPPH radical scavenging activity were found to be 192.5 µg/ml, 73.54 µg/ml, 38.13 µg/ml, 14.84 µg/ml and 28.45 µg/ml respectively (table 2).

The free radical scavenging ability of *E. bicolor* was also determined using $ABTS^{\bullet+}$radical cation. $ABTS^{\bullet+}$radical cation has been used for the evaluation of antioxidant activity of single compounds and complex mixtures. In this assay $ABTS^{\bullet+}$radical cation was generated directly in stable form using potassium per sulphate. Generation of radical before the antioxidants added prevents interference of compounds, which affect radical formation [28]. When stable absorbance is obtained the antioxidant sample is added to the reaction medium, and the antioxidant activity is measured in terms of decolourization. The IC_{50} values of hexane, chloroform, ethyl acetate, methanol and water extracts of *E. bicolor* were 258.2 µg/ml, 197.4 µg/ml, 107.1 µg/ml, 34.33 µg/ml and 66.91 µg/ml respectively.

NO is a reactive oxygen species which is associated with inflammation, cancer and other pathological conditions [29]. The ability of the extracts to scavenge NO may be considered advantageous for health as it can evade the ill effects of excessive NO generation. In *E. bicolor* the methanolic extract exhibited higher antioxidant activity than the other extracts with IC_{50} values for hexane, chloroform, ethyl acetate, methanol and water extracts were 363.1 µg/ml, 289.0 µg/ml, 228.3 µg/ml, 34.03 µg/ml and 72.48 µg/ml respectively.

The low EC_{50} value (27.70 µg/ml) of methanolic extract of *Swertia chirayita* exhibited effective antioxidant activity [30]. In *Swertia longifolia* the IC_{50} value of methanolic aerial part extracts showed 2.8 µg/ml [31]. In the present study, the methanolic leaf extract of *E. bicolor* showed low IC_{50} values for the three antioxidant assays exhibiting potent antioxidant activity compared to other extracts. Hence the methanolic extract of *E. bicolor* extract could be a good source of antioxidant.

The significant linear correlation (coefficient $R^2 \geq 0.98$and two-tailed p value ≤ 0.05) between the total phenolic content and DPPH activity of methanolic plant extracts concluded that the phenolic compounds might be responsible for the antioxidant activity [32].

CONCLUSION

In the present study the nonpolar to polar solvent systems showed difference in the total phenol content and antioxidant activity of *E. bicolor*. The polar solvents are more prominent than the nonpolar one in the present report, the former one can be a potential source of novel bioactive compounds for various stress related diseases. There was a significant positive correlation between total phenol content and DPPH activity, which signifies that the phenols may be the principal contributor of the antioxidant activities. To the best of our knowledge, antioxidant activity and its correlation with TPC, is reported for the first time in *E. bicolor*. Further investigations are required to purify the bioactive compounds and also to explore the pharmacologic and therapeutic potentials of the plant.

CONFLICT OF INTERESTS

We declare that we have no conflict of interest

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CONFLICT OF INTERESTS

Declared None

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