British Journal of Pharmaceutical Research
14(6): 1-9, 2016; Article no.BJPR.31817
ISSN: 2231-2919, NLM ID: 101631759

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Antioxidant Activities and HPLC-DAD Based Phenolic Content Determination of *Bauhinia scandens*

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Authors' contributions

This research work was carried in alliance of all authors. Author MSH outlined the study, wrote the protocol and checked the manuscript. Authors SAN, AH, MAUI, ZI and SD executed the experimental works. Authors MAH and MSR assisted to complete the experimental works. Author AFMMI performed the statistical analysis. Author MSH handled the literature searches and analyzed of the data. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2016/31817
Editor(s):
(1) R. Deveswaran, M.S. Ramaiah College of Pharmacy, Bangalore, India.
Reviewers:
(1) Hasan Hüseyin Doğan, Selçuk University, Turkey.
(2) Eliton da Silva Vasconcelos, Federal University of Sao Carlos, UFSCar, Brazil.
(3) Ayona Jayadev, All Saints College, Thiruvananthapuram, Kerala, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/18008

Original Research Article

Received 25th January 2017
Accepted 21st February 2017
Published 2nd March 2017

ABSTRACT

Aim of Study: *Bauhinia scandens* L. (Leguminosae) is a valuable plant used for various ailments. This study investigated the in vitro antioxidant potential and quantification of selected bioactive plant polyphenol compounds of the ethanolic extracts of *Bauhinias scandens* (EEBS).
Study Design: Assessment of antioxidant activities and HPLC-DAD based phenolic contents analysis.
Place and Duration of Study: Department of Pharmacy, Gono Bishwabidyalay, Savar, Dhaka, Bangladesh, between March 2015 and February 2016.
Methodology: DPPH free radical scavenging, total phenolic and total flavonoid content assay

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were used to evaluate in vitro antioxidant capacity. Moreover, phenolic compounds were identified and quantified by HPLC-DAD.

Results: EEBS showed good free radical scavenging effects with IC_{50} 13.5 µg/ml whereas for standard ascorbic acid, IC_{50} value was 8.25 µg/ml. For EEBS, total phenolic content was 47.33 ±0.01 mg GAE/gram of dry extract while total flavonoid content was 6.59 ±0.04 mg QE/g dry extract. The experimental results indicated that EEBS contained a high concentration of gallic acid, ellagic acid and rosmarinic acid (215.39, 72.24 & 50.96 mg/100 g of dry extract, respectively). Vanillin was also detected at moderate concentration in EEBS (39.85 mg/100 g of dry extract). Vanillic acid and syringic acid were also detected at lower concentration (12.34 & 17.15 mg/100 g of dry extract, respectively).

Conclusion: Experimental results suggest that ethanolic extracts of *Bauhinias scandens* contain high amount of phenolic constituents which could be responsible for the biological activities of this plant extract.

Keywords: Bauhinia scandens; DPPH; HPLC; phenolic content; flavonoid content.

1. INTRODUCTION

Vegetables, spices, and herbs contain important natural substances such as antioxidants. Several studies have dealt with antioxidant activity to find new sources of natural antioxidants to be used in foods, cosmetics, medicine, and other purposes [1,2]. Antioxidants play an important role in health care to prevent and scavenge free radicals; alleviate chronic diseases and degenerative ailments such as cancer, autoimmune disorders, hypertension, atherosclerosis; and delay the ageing process [3,4,5], The use of herbal medicine has become increasingly popular worldwide and medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects [6]. New drugs of plant origin and new methods of producing them will continue to be an important parts of the service and thus Plants are considered as are of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates [7].

Bauhinia scandens is a genus of more than 500 species of flowering plants in the subfamily Caesalpinioideae of the large flowering plant family Fabaceae, with a pantropical distribution. The genus was named after the Bauhin brothers, Swiss-French botanists. Many species are widely planted in the tropics as orchid trees, particularly in India, Sri Lanka, Vietnam and southeastern China. *Bauhinia*, along with a northern temperate genus *Cercis* L. and several tropical genera, bear bilobate, bifoliolate, or sometimes unifoliolate leaves, which constitute the tribe CercideaeBronn as sister to the remaining legumes in the molecular phylogenetic trees [8-11].

Other common names include mountain ebony and kachnar (India and Pakistan). In the United States of America, the trees grow in Hawaii, coastal California, Texas, Louisiana, and Florida [12]. Several ethnomedicinal studies and local practitioner suggested that *Bauhinia scandens* is a flowering plant having different medicinal properties used locally by local practitioner for the use of different ailments like Alzheimer, cardiac disease, tumor etc. The antioxidative effect is mainly due to phenolic components, such as flavonoids [13], phenolic acids, and phenolic diterpenes [14], which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [15]. Many of these phytochemicals possess significant antioxidant capacity that may be associated with lower incidence and lower mortality rates of cancer in several human populations [16].

Although numerous biological activities of *Bauhinia scandens* was studied but its antioxidant activities and phenolic contents analysis yet not reported. As a part of our routine investigation on medicinal plants to evaluate antioxidant as well as biological potentiality, in this research article, we assessed the antioxidant activities of the ethanolic extract of *Bauhinia scandens* and determined phenolic constituents present in it using HPLC-DAD method.

2. MATERIALS AND METHODS

2.1 Plant Material

The whole plants of *Bauhinia scandens* were collected from Sitakunda, Chittagong, Bangladesh. The plants were identified by the experts of Bangladesh National Herbarium,

Mirpur, Dhaka. The accession no is DACB 40636 for *Bauhinia scandens*.

2.2 Chemicals and Reagents

Analytical grade chemicals and reagents were used for this study. Methanol, ethanol and folin-ciocalteu reagent were acquired from Merck (Germany). Gallic acid, quercetin and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were acquired from Sigma Chemicals (USA). Sodium carbonate and potassium acetate were acquired from Merck (Germany) Limited. Aluminium chloride was acquired from Fine Chemicals (India).

2.3 Preparation of Extract

After drying and grinding, 250 g powder of *Bauhinia scandens* was weighed and taken in a conical flask containing about 1800 ml ethanol. This was left for 3 days with periodical stirring to allow for the extraction of active ingredients. Then the soaked samples were filtered. Final filtrate was then collected. The obtained filtrates were evaporated by Rotary evaporator until constant weight was obtained. The crude extracts were collected after complete evaporation. Then the dried crude extract was preserved at +4ºC. Dark green viscous concentrate was found after evaporation and this concentrate was designed as ethanol extract of *Bauhinia scandens* (EEBS).

2.4 *In Vitro* Antioxidant Methods

2.4.1 Estimation of total phenolic content

The content of total phenolic compounds in plant ethanolic extracts was determined by using the Folin-Ciocalteu Reagent (FCR) [16]. 1.0 ml of plant extract (200 µg/ml) or standard of different concentration solution was taken in a test tube.5 ml of Folin-Ciocalteu (diluted 10 fold) reagent solution was added to the test tube.7.5% Sodium carbonate solution (4 ml) was added to the same test tube and mixed well. Test tubes containing standard solutions were incubated for 30 minutes at room temperature to complete the reaction but the test tubes containing extract solution were incubated for 1 hour at room temperature to complete the reaction. Then the absorbance of the solution was measured at 765 nm using a spectrophotometer against blank. A typical blank solution containing the solvent used to dissolve the plant extract. The Total content of phenolic compounds plant extracts in Gallic acid equivalents (GAE) was calculated using the following equation:

$$C = (c \times V)/m$$

Where; C = total content of phenolic compounds, mg/g plant extract, in GAE, c = the concentration of Gallic acid established from the calibration curve (mg/ml), V = the volume of extract in ml, m = the weight of crude plant extract in gm.

2.4.2 Estimation of total flavonoid content

Total flavonoid was determined using the Aluminum chloride colorimetric method described by Wang and Jiao [17]. 1 ml of plant extract (200 µg/ml) or standard of different concentration solution was taken in a test tube. 3 ml of ethanol was added to the test tube. Then, 200 µl of 10% aluminium chloride solution was added into the same test tube followed by the addition of 200 µl of 1 M potassium acetate solution into the test tube. Finally, 5.6 ml of distilled water was mixed with the reaction mixture. The reaction mixture then incubated for 30 minutes at room temperature to complete the reaction. Then the absorbance of the solution was measured at 415 nm using a spectra photometer against blank. Ethanol served as blank. The Total content of flavonoid compounds in plant ethanol extracts in quercetin equivalents was calculated by the following formula equation.

$$C = (c \times V)/m,$$

Where; C = total content of flavonoid compounds, mg/gm plant extract, in quercetin equivalent, c = the concentration of quercetin established from the calibration curve in mg/ml, V = the volume of extract in ml, m = the weight of crude plant extract in gm.

2.4.3 DPPH free radical scavenging assay

DPPH is a reactive free radical that acts as an electron acceptor (oxidant/ oxidizing agent) and causes oxidation other substances. On the other hand, antioxidants act as electron donors (reductant/ reducing agent). Antioxidants neutralize DPPH by being oxidized themselves. DPPH is found as dark-colored crystalline powder composed of stable free-radical molecules and forms deep violet color in solution. The scavenging of DPPH free radical (neutralization) is indicated by the deep violet color being turned into pale yellow or colorless [18]. The stock solution was serially diluted to

achieve the concentrations of 800 µg/ml, 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml. Each test tube containing 1ml was properly marked. 2 ml of 0.004% DPPH solution in the solvent was added to each test tube to make the final volume 3 ml (caution: DPPH is light sensitive, so making the solution and adding it to the test tubes should be done in minimum light exposure). The mixture was incubated in room temperature for 30 minutes in a dark place. Then the absorbance was measured at 517 nm. DPPH was calculated by the following formula equation

$$\% \text{ Inhibition} = \left(1 - \frac{Absorbance of sample}{Absorbance of Control}\right) \times 100$$

2.5 Determination of Polyphenolic Compounds by HPLC System

2.5.1 Chemicals used

Arbutin (AR), gallic acid (GA), hydroquinone (HQ), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), Syringic acid (SA), (-)-epicatechin (EC), vanillin (VL), *p*-coumaric acid (PCA), *trans*-ferulic acid (FA), rutin hydrate (RH), ellagic acid (EA), benzoic acid (BA), rosmarinic acid (RA), myricetin (MC), quercetin (QU), *trans*-cinnamic acid (TCA), and kaempferol (KF) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was obtained from Merck (Darmstadt, Germany).

2.5.2 HPLC detection and quantification of polyphenolic compounds

Detection and quantification of selected phenolic compounds in the ethanol extract were determined by HPLC-DAD analysis as described by Ismet et al. [19] with some modifications. It was carried out on a Dionex UltiMate 3000 system equipped with quaternary rapid separation pump (LPG-3400RS) and photodiode array detector (DAD-3000RS). Separation was performed using Acclaim® C_{18} (5µm) Dionex column (4.6 x 250 mm) at 30 ºC with a flow rate of 1 ml/min and an injection volume of 20 µl. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C) with the gradient elution program of 5%A/95%B (0-5 min), 10%A/90%B (6-9), 15%A/75%B/10%C (11-15), 20%A/65%B/15%C (16-19 min), 30%A/50%B/20%C (20-29 min), 40%A/30%B/30%C (30-35) and 100%A (36-40 min). The UV detector was set to 280 nm for 22.0 min, changed to 320 nm for 28.0 min, again change to 280 nm for 35 min and finally to 380 nm for 36 min and held for the rest of the analysis period while the diode array detector was set at an acquisition range from 200 nm to 700 nm. For the preparation of calibration curve, a standard stock solution was prepared in methanol containing arbutin (AR), (-)-epicatechin (ECA) (5 µg/ml each), gallic acid (GA), hydroquinone (HQ), vanillic acid (VA), rosmarinic acid (RA), myricetin (MC) (4 µg/ml each), caffeic acid (CA), Syringic acid (SA), vanillin (VL), *trans*-ferulic acid (FA) (3 µg/ml each), *p*-coumaric acid (PCA), quercetin (QU), kaempferol (KF) (2 µg/ml each), (+)-catechin hydrate (CH), ellagic acid (EA) (10 µg/ml each), *trans*-cinnamic acid (TCA) (1 µg/ml), rutin hydrate (RH) (6 µg/ml) and benzoic acid (BA) (8 µg/ml). A solution of the extract was prepared in ethanol having the concentration of 10 mg/ml. Prior to HPLC analysis, all the solutions (mixed standards, sample, and spiked solutions) were filtered through 0.20 µm syringe filter (Sartorius, Germany) and then degassed in an ultrasonic bath (Hwashin, Korea) for 15 min. Data acquisition, peak integration, and calibrations were calculated with Dionex Chromeleon software (Version 6.80 RS 10).

3. RESULTS AND DISCUSSION

3.1 Percentage Yield of Plant Extracts by Maceration Process

The percentage yield of the crude extract of *Bauhinia scandens* is shown in Table 1. Remarkable yield (9.61%) was found from the extraction of *Bauhinia scandens* with ethanol.

3.2 Antioxidant Study

3.2.1 DPPH free radical scavenging assay

When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. The IC_{50} value was found 13.5 µg/ml for EEBS and 8.25 µg/ml for ascorbic acid.

3.2.2 Total phenolic content analysis

The total phenolic contents of the EEBS were calculated using the standard curve of gallic acid ($y = 0.003x + 0.204$; $R^2 = 0.956$). Total phenolic content was found 47.33 ±0.01 (mg/g, gallic acid equivalents) for EEBS.

Table 1. Percentage yield values of the crude extracts

Plants used	Solvent volume (ml)	Powder weight (gm)	Extract obtained (gm)	Yield (%)
Bauhinia scandens	1800	250	24.023	9.61

Table 2.Total phenolic and flavonoid contents of the ethanol extract of *Bauhinia scandens*

Sample	Total phenolic content (mg of gallic acid equivalent per g of dry extract)	Total flavonoid content (mg of quercetin equivalent per g of dry extract)
Bauhinia scandens	47.33 ±0.01	6.59 ±0.04

Data are represented as the mean ± SD, (n=2)

3.2.3 Determination of total flavonoid content

The total flavonoid contents were calculated using the standard curve of quercetin (y = 0.003x + 0.109; R^2 = 0.915) and were expressed as quercetin equivalents (QE) per gram of the plant extract. Total flavonoid content was found 6.59 ±0.04 (mg QUE/gm) for EEBS.

3.3 Polyphenolic Compounds Analysis of EEBS

The chromatographic separations of polyphenols in standard and ethanol extract are shown in Fig. 3.5 and 3.6 respectively. The content of each phenolic compound was calculated from the corresponding calibration curve and presented as the mean of five determinations as shown in Table 3.

The experimental results indicated that ethanol extract of *Bauhinia scandens* contained a high concentration of gallic acid, ellagic acid and rosmarinic acid (215.39, 72.24 & 50.96 mg/100 g of dry extract, respectively). Vanillin was also detected at moderate concentration in the ethanol extract of *Bauhinia scandens* (39.85 mg/100 g of dry extract). Vanillic acid, and syringic acid were also detected at lower concentration (12.34 & 17.15 mg/100 g of dry extract, respectively).

DPPH radical scavenging is a widely used method to evaluate the free radical scavenging activity of compounds or antioxidant capacity of plant extracts [20]. It is considered as a basic and rapid screening method which demonstrated the antioxidant effect by decrease in the absorbance at 517 nm [21]. DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation [22]. The greater the decolorizing action, the higher the antioxidant activity, and is reflected by lower IC_{50} value. Substances which are able to perform this reaction can be considered as antioxidants and, therefore, radical scavengers. In the present study, all the plant extracts showed dose dependent scavenging of DPPH free radicals in a way similar to that of the standard antioxidant ascorbic acid. However, the EEBS exhibited good antioxidant potency (IC50= 13.5 µg/ml) which was close to antioxidant effect of ascorbic acid (IC50= 8.25 µg/ml). Presence of total phenol content and flavonoid in the plant extracts may be a reason for this DPPH-scavenging activity. So, present study showed that the extracts have the proton-donating ability due to presence of phenolics and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants [21].

Table 3. Contents of polyphenolic compounds in the EEBS (n=5)

Polyphenolic compound	EEBS	
	Content (mg/100 g of dry extract)	% RSD
GA	215.39	1.97
VA	12.34	0.42
SA	17.15	0.33
VL	39.85	0.03
EA	72.24	0.08
RA	50.96	0.05

Presence of polyphenols has been reported to be responsible for the antioxidant activity in plant extracts [23]. Phenolic constituents react with active oxygen radicals such as hydroxyl radical, superoxide anion radical and lipid peroxyl radical [24]. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors which can stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions

(termination of the Fenton reaction) [25]. Oxidation reactions have been reported to play a central role in atherogenesis and epidemiological studies have shown an association between cardiovascular diseases and low plasma concentrations of ascorbate, tocopherol and β-carotenes [26]. Polyphenols have been shown to inhibit the Cytochrome P450 superfamily of enzymes that metabolizes many pro-carcinogens to reactive compounds before they react with DNA and induce malignant transformation, thus reducing the formation of reactive intermediates [27]. Glutathione reductace activity in rats has also been shown to be induced by certain polyphenols. The results of the present study strongly suggest that phenolics are important components of the tested plant extracts. EEBS contain considerable amount of phenolic compounds which indicate the antioxidant activity of extracts.

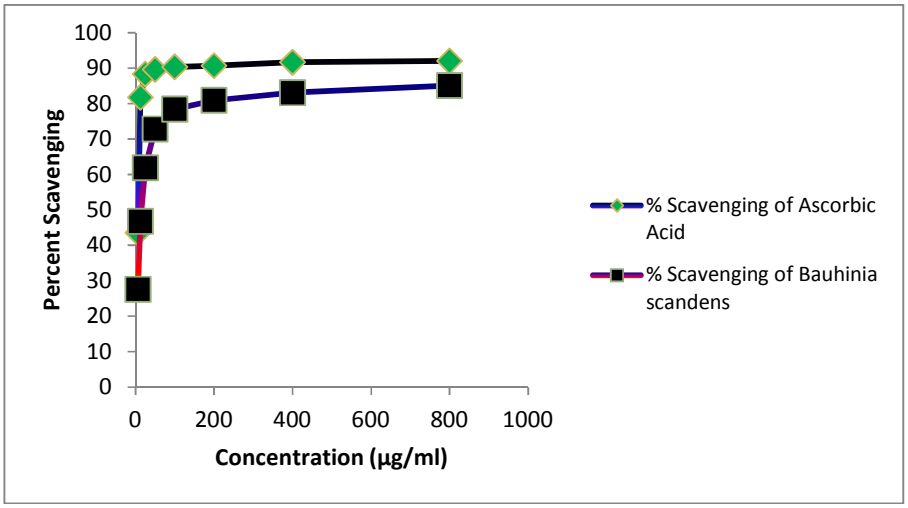

Fig. 1. DPPH Free radical scavenging assay of ascorbic acid and EEBS

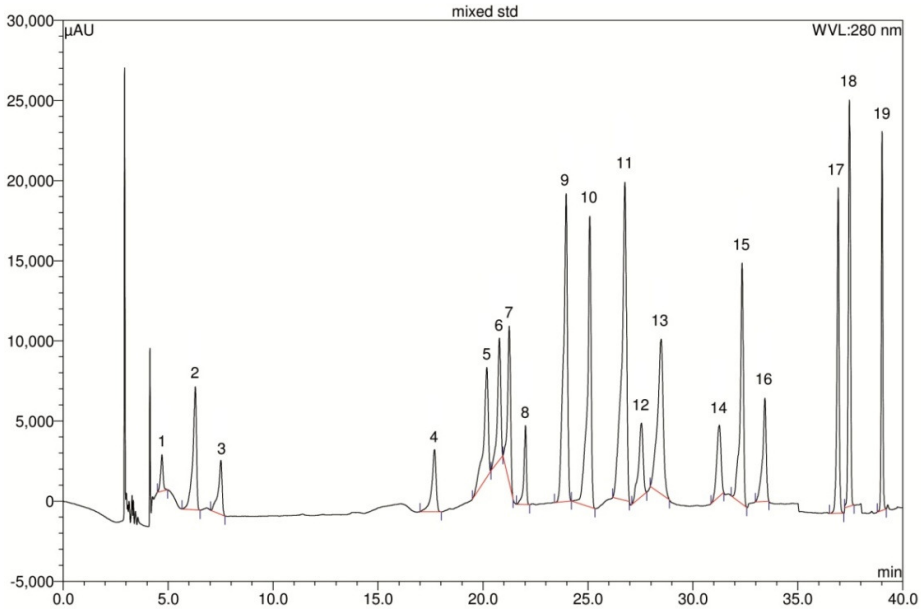

Fig. 2. HPLC chromatogram of a standard mixture of polyphenolic compounds

Peaks: 1, arbutin; 2, gallic acid; 3, hydroquinone; 4, (+)-catechin; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, (–)-epicatechin; 9, vanillin; 10, p-coumaric acid; 11, trans-ferulic acid; 12, rutin hydrate; 13, ellagic acid; 14, benzoic acid; 15, rosmarinic acid; 16, myricetin; 17, quercetin; 18, trans-cinnamic acid; 19, kaempferol

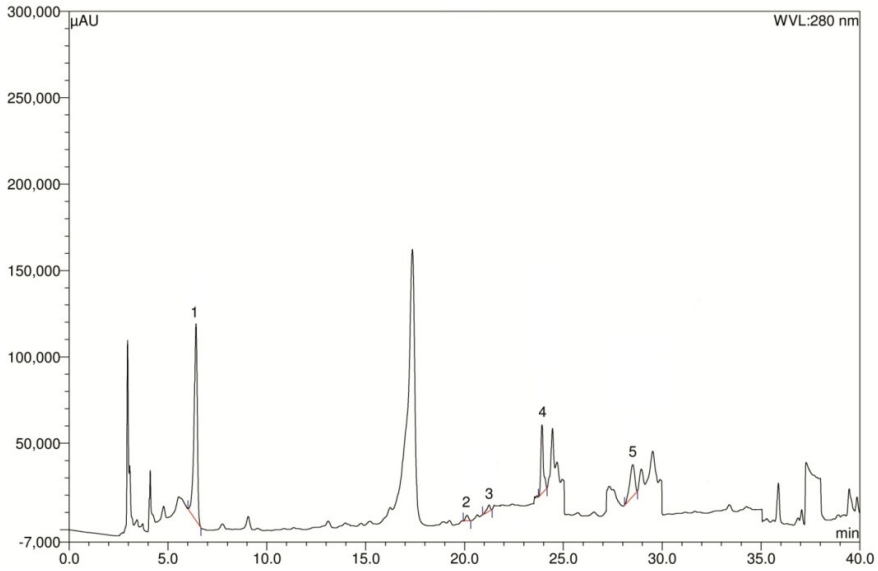

Fig. 3. HPLC chromatogram of ethanol extract of *Bauhinia scandens*
Peaks: 1, gallic acid; 2, vanillic acid; 3, syringic acid; 4, vanillin; 5, ellagic acid; 6, rosmarinic acid

Flavonoids are known for their antioxidant properties, and the flavonoid content of the investigated plant is of interest when evaluating its antioxidant properties. A positive correlation between the content of flavonoids and the antioxidant capacity in plant extracts has been found. Principally, the procedure involves the formation of complex between flavonoid and $AlCl_3$ that produces a yellow colored solution. The absorbance is then measured spectrophotometrically to determine the presence of flavonoid compounds [28]. The extracts of the present study were found to have notable amounts of flavonoids.

Presence of the different types of phenolic compounds in the plant extract by HPLC method also ensures the antioxidant capacity of the ethanolic extract of *Bauhinia Scandens.*

4. CONCLUSION

In present study, the total phenolic content and anti-oxidant activity of EEBS were evaluated. The observed antioxidant activities are due to the presence of considerable amount of flavonoids and phenolics in the EEBS. Results of the study provided a scientific claim to the antioxidant activity of EEBS.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby announce that all experiments have been examined and approved by the appropriate ethics committee and have been performed following ethical standard.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/18008