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In Vitro antioxidant activities of *Breynia Vitis-Idaea* extracts

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ABSTRACT

The present study was under taken to study the antioxidant potential of hexane, ethyl acetate and methanol extracts of Breynia vitis-idaea (Burm.f.) Fischer. (Euphorbiaceae) aerial parts. Antioxidant activities of these extracts were studied using in vitro assays and compared with the standard antioxidant compounds like ascorbic acid and rutin. All the three extracts showed significant radical scavenging activities, total antioxidant activity and reducing power. These activities showed the dose dependency. Radical scavenging activities and antioxidant nature of the extracts may be attributed to the presence of phenolic compounds such as flavonoids in these plant extracts. These studies prove the potential antioxidant nature of the extracts of Breynia vitis-idaea aerial parts.

Keywords: *Breynia vitis-idaea,* antioxidant, methanol extract, ethyl acetate extract.

INTRODUCTION

Free radicals of oxygen, nitrogen and sulfur molecules are highly reactive in the biological systems due to their unpaired electrons. These reactive oxygen /nitrogen species (ROS/RNS) are produced during the cellular metabolism play a significant role in cell signaling, apoptosis, gene expression and ion transportation. However, excessive accumulation of ROS can cause oxidative stress, which results in the damage of DNA, RNA, proteins and lipids inhibiting their normal functions. The abnormal functioning of these biomolecules can lead to an increased risk for cardiovascular disease, cancer, autism and other diseases [1, 2]. Therefore, minimizing oxidative stress will promote our physical condition and prevent some degenerative diseases in which free radicals are involved [3].

The agents which can scavenge the free radicals and inhibit the harmful effects caused by the oxidants are called antioxidants. They can lessen the severity of destruction caused by the free radicals by neutralizing them ahead of the damages caused to lipids, proteins, enzymes, carbohydrates and DNA [4]. Antioxidants can be classified into two major classes i.e., enzymatic and non-enzymatic. The enzymatic antioxidants are produced endogenously and include superoxide dismutase, catalase, and glutathione peroxidase. The non-enzymatic antioxidants include tocopherols, carotenoids, ascorbic acid, flavonoids and tannins which are obtained from natural plant sources [5, 6].

A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases [7]. There are some synthetic antioxidant compounds such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylhydroquinone which are commonly used in processed foods. However, there is a widespread agreement that synthetic antioxidants need to be replaced with natural antioxidants because some synthetic antioxidants have shown potential health risks and toxicity, most notably possible carcinogenic effects .Therefore, it is of great importance to find new sources of safe and inexpensive antioxidants of natural origin in order to use them in foods and pharmaceutical preparations to replace synthetic antioxidants [2, 5, 8].

Breynia vitis-idaea (Burm.f.) Fischer.(Euphorbiaceae)is an evergreen, glabrous tree or large shrub. Found in the Gangetic plain, western peninsula, china, Malay peninsula and Sri Lanka. These plants are planted as ornamental hedge in garden. Bark is yellowish grey, leaves are alternate dark brown or black when dry, flowers are small, greenish yellow or pink, and dull red, purple or white berries. Root, leaves and bark are medicinal. Roots decoction is used as mouthwash. A new sulfur-containing spiroketal glycoside, breynin I (1), and a new terpenic glycoside, breyniaionoside E (2), together with 10 known compounds, were isolated from the aerial parts of *B. vitis-idaea* [9].

Prior to this study, there is no report on the antioxidant activity of *B. vitis-idaea* in the available literatures. The aim of this study was to investigate the antioxidant activity of the different fractions of the methanolic extract of the leaves of *B. vitis-idaea* using *in vitro* models. Total phenolic content of the fractions were also determined in order to evaluate a relationship between the antioxidant activity and the phytochemical constituents.

EXPERIMENTAL SECTION

Plant material: Aerial parts of *B. vitis-idaea* were collected in and around Madikeri, Coorg in the month of January and shade dried .The plant was authenticated by comparing the herbarium with the previously deposited herbariums in Department and the specimen was deposited in the Department of Botany, FMKMC College, Madikeri. Karnataka, India.

Preparation of the extract: 100 g of the dried powder of *B. vitis-idaea* was extracted in a soxhlet extractor with hexane (500ml), ethyl acetate (500ml) and (500ml) methanol. The mark was pressed and the expressed solvent was mixed with the main extract. Each extract was concentrated to constant weight in a rotary shaker evaporator and labeled hexane extract (HE),

ethyl acetate extract (EAE) and methanol extract (ME) respectively. The extracts were stored at -20ºC until use.

Drugs and chemicals:1,1-Diphenyl-2-picryl hydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzo-thiozoline-6-sulphonic acid) diammonium salt(ABTS), were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dimethylsulphoxide (DMSO) and riboflavin were from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Sodium carbonate (Na_2CO_3), Rutin and Folin-Ciocalteu were from Merck (India) Ltd, India. All other chemicals used in the study were obtained commercially and were of analytical grade.

Phytochemical screening: Preliminary phytochemical screening of the crude extracts of the aerial parts was carried out with the methods with little modifications [10].

DPPH radical scavenging assay: To 2ml of DPPH (100 µM) solution, 100 µl of various concentrations of the extracts or the standard (ascorbic acid) solution were added separately. The reaction mixtures were incubated at 37 ºC for 30 min. Absorbance of each solution was measured at 517 nm [11]. The radical scavenging activity of extracts calculated as the percent DPPH radical scavenging effect.

Scavenging of ABTS radical cation: ABTS radical cat ion ($ABTS^{\cdot+}$) scavenging activity was measured according to the method of Re et al. (1999) [12]. ABTS was dissolved in water to a concentration of 7mM and $ABTS^{\cdot+}$ was produced by mixing with 2.45mM potassium persulfate. The mixture was allowed to stand in dark at room temperature for 12-16h. The ABTS radical cat ion solution was diluted with ethanol to adjust its absorbance to 0.70 ± 0.02 at 734nm. To determine the scavenging activity, 0.2ml of various concentration of the extracts or the standard were mixed with 2ml distilled DMSO and 0.32ml $ABTS^{\cdot+}$ solution. The absorbance was measured after 20 min at 734nm. The percentage of inhibition was calculated by the equation:

$$\text{Inhibition Percentage (\% IP)} = \frac{Ac - At}{Ac} \times 100$$

Where Ac and At are the absorbance of the control and the test respectively. From the plot of concentration against IP (%) a linear regression analysis was performed to determine the IC_{50} value.

Hydroxyl radical scavenging assay: Various concentrations of the extracts or standard (500µl) mixed with 500µl of reaction mixture containing 200 µl of 2-deoxy2-ribose (28 mM in 20 mM KH_2PO_4 buffer, pH 7.4), 200 µl of 1.04 mM EDTA and 200 µM $FeCl_3$ (1:1v/v), 100 µl of 1.0 mM hydrogen peroxide (H_2O_2) and100 µl of 1.0 mM ascorbic acid. Test samples were kept at 37ºC for 1 h. The free radical damage imposed on the substrate, deoxyribose was measured using the thiobarbituric acid. One ml of 1% thiobarbituric acid (TBA) and 1.0 ml 2.8% trichloroacetic acid (TCA) were added to the test tubes and were incubated at 100ºC for 20 min. After cooling, the absorbance was measured at 532 nm against a blank. Rutin was used as a positive contro[13,14].

Hydrogen peroxide scavenging activity: Scavenging activity of hydrogen peroxide by the extract was determined by the method of Ruch *et al*. (1989)[15].Extracts (4 mL) prepared in DMSO at various concentration were mixed with 0.6 mL of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the extract withoutH_2O_2.

Total antioxidant activity: A spectrophotometric method has been developed for the quantitative determination of antioxidant capacity. The assay is based in the reduction of MO (V1) to MO (V) by the sample analyze and the subsequent formation of a green phosphate / MO (V) complex at acidic pH. The method has been optimized and characterized with respect to linearity interval, repetitivity and reproducibility and molar absorption coefficients for quantitation of several antioxidants, including vitamin E [16].

To 0.1ml of extract, 0.9ml 99% DMSO and 2ml of total antioxidant(mixture of 0.6M H_2SO_4, 28mM sodium monophosphate and 4mM ammonium molybdadte) reagent were added and incubated in a thermal block at 95^0C for 90 min. Optical density was measured at 695nm after cooling the reaction mixture. Ascorbic acid was used as standard.

Total Reducing power: Various fractions (0.1mL) were mixed with 2.5mL of 0.2M phosphate buffer (pH 6.6) and 2.5mL of1% potassium ferricyanide. After the mixture was incubated at 50ºC for 20 minutes, 2.5mL of 10% trichloroacetic acid, 2.5mL distilled water and 0.5mL of 0.1% ferric chloride solution were added and then the absorbance was measured at 700nm against a blank. The blank consists of all the reagents without the sample. Increasing absorbance of the reaction mixture indicates increasing reducing power [17].

Total Phenolic Contents: The total phenolic compounds in the extract were determined according to the method of Singleton and Rossi as cited in Çoruh et al. (2007) [18] with some modifications. To 0.1m of EAE, 2ml of 2% (w/v) sodium carbonate solution was added and vortexed vigorously. After 3min, 0.1 ml of 1:1 diluted Folin Ciocalteu's phenol reagent was added and vortexed again. Same procedure was followed for the standard solution of tannic acid (0.05-0.3mg/ml). Each mixture was incubated at room temperature for 30min and the absorbance was measured at 750nm. The total phenolic content in the extracts were expressed as tannic acid equivalent in mg/g (TA mg/g).

Statistical analysis: All experiments were performed in triplicate (n=3) and results were expressed as mean ± SEM. Statistical analysis was carried out with (Prism package version 3.0) using ANOVA followed by Turkey's test ($P<0.05$).

RESULTS AND DISCUSSION

In the present study, *B. vitis-idaea* aerial parts were extracted with hexane, ethyl acetate and methanol were analyzed for the presence of phytochemicals and subjected to *in vitro* antioxidant studies. Phytochemical analysis of the extracts of *B.vitis-idaea* showed the presence of steroids, terpenoids and a moderate amount of carotenoids in the HE. The EAE and ME were containing flavonoids. ME even contained a slight amount of terpenoids. None of the fractions were positive for alkaloids and saponins(Table 1).

Table.1: Yield and phytochemical analysis of the extracts of *B. vitis-idaea.*

Extract	Yield (%)	Steroids	Terpenoids	Alkoloid	Flavonoids	Saponins	Carotenoids
HE	1.25 ± 2.31	+ + +	+ + +	-	+	-	+ +
EAE	8.34 ± 2.91	-	-	-	+ + +	-	-
ME	12.56 ± 11	-	+	-	+ + +	-	-

Results are expressed as mean± SEM of three parallel measurements for yield of extracts. phytochemical analysis was done in triplicates for each test. +++ = appreciable amount; ++ = moderate amount; + = trace amount; - = completely absent.

The antioxidant capacity of the plant extract largely depends on both the composition of the extract and the test system. It can be influenced by a large number of factors, and can not be fully evaluated by one single method. It is necessary to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action [19]. All the three extracts of *B.vitis-idaea* showed significant antioxidant activity in the different methods of antioxidant assays tested (Table 2). Highest DPPH scavenging activity was observed in EE (IC_{50} 284 ± 6.34µg/ml) followed by ME (284 ± 6.34µg/ml) and HE(744 ± 6.98µg/ml). Similar trend was observed in scavenging activity of ABTS+, radical scavenging of the hydroxyl radicals and H_2O_2 scavenging activity as well. The activities of these extracts were concentration dependant.

Table 2: Antioxidant activities of the fractions of *B. vitis-idaea.* IC50 (µg/ml)

Standard /Extract	DPPH	ABTS	OH	H_2O_2
HE	744 ± 6.98	653.23 ± 6.40	854.43 ± 5.23	950.12 ±7.98
EAE	153 ± 3.07	87.45 ± 5.78	246.98 ± 2.88	245.32 ± 4.32
ME	284 ± 6.34	156.32± 3.21	352.67 ± 1.89	422.41 ± 1.22
Ascorbic acid	6.13 ± 2.02	11.25 ± 0.49	-	-
Rutin	43.23 ± 4.14	-	35.65 ± 9.65	56.12 ± 4.67

Results are expressed as mean± SEM of three parallel measurements.

Free radicals generated during the course of metabolic process of an organism are known to play role in several disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases due to weak natural antioxidant defense mechanism. Antioxidant principles present in the plants have been shown to possess free radical scavenging activity. DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts [21]. DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow colored product, diphenylpicryl hydrazine, with the addition of the fractions in a concentration-dependent manner. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups. The significant DPPH scavenging potential of *B. vitis-idaea* extracts may be due to hydroxyl groups present in the phytochemicals.

The ability of extract/s to scavenge DPPH prompted us to look for the ability these extracts to inhibit the formation of ABTS+. ABTS•+ radicals is widely used to screen antioxidant activity of fruits, vegetables, foods and plants, and is applicable to both lipophilic and hydrophilic

antioxidants [22]. In particular, it is recommended to be used for plant extracts because the long wavelength absorption maximum at 734 nm eliminates color interference in plant extracts [3].

Hydroxyl radical is the most deleterious and reactive among the ROS and it bears the shortest half-life compared with other free radicals. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe2+) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid [23].

Hydrogen peroxide itself is not particularly reactive with most biologically important molecules, but is an intracellular precursor of hydroxyl radicals which is very toxic to the cell.Thus, scavenging of H_2O_2 is a measure of the antioxidant activity of the plant extracts. All the fractions of *B.vitis-idaea* scavenged hydrogen peroxide which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralizing it into water. Plants have been reported to exhibit antioxidant activity due to the presence of antioxidant compoundssuch as phenolics, proanthocyanidins and flavonoids [23,24].

The antioxidant capacities of the *B. vitis-idaea* extracts were much lesser than the standard ascorbic acid. The results obtained in this study were in parallel to findings of previous studies by other research groups. This implies that the plant extracts may be useful for treating radical-related pathological damage especially at higher concentration [4].

Total antioxidant activity and Total phenolic contents of HE, EAE and ME was studied using Ascorbic acid and Tannic acid as standards respectively (Table 3). All the fractions showed significant total antioxidant activity. Total antioxidant activities of the extracts were expressed µg equivaletnts of ascorbic acid/mg. EAE >HE>ME in terms of total antioxidant activity. Phenolic content of the extract was expressed as mg equivalent of TA/g. Phenolic content was highest in EE with 67.22 ± 0.31 mg equivalent of TA/g. Phenolic content was lowest in HE; while in ME, intermediate between EAE and HE with a value of 53.62 ± 0.02 mg equivalent of TA/g.

Table3: Total antioxidant activity (phosphomolybdate method) and total phenolic content of different extracts of *B. vitis-idaea*

Extracts	Total anti oxidant activity (µg equivalent of Ascorbic acid/mg)	Total phenolic content (mg equivalent of Tannic acid /g)
HE	30.12 ± 0.65	3.70 ± 0.01
EAE	45.43 ± 0.32	67.22 ± 0.31
ME	4.93 ± 0.21	53.62 ± 0.02

Values are expressed as mean± SEM of three parallel measurements.

The phosphomolybdate method has been routinely used to evaluate the total antioxidant capacity of the extracts [14].In the presence of the fractions, the Mo(VI) is reduced to Mo(V) and forms a green coloured phosphomolybdenum V complex which shows maximum absorbance at 695 nm. All the fractions showed significantly higher inhibition percentage (stronger hydrogen –donating ability) and positively correlated with total phenolic content.

Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities and

also decrease cardiovascular complications [25]. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. Total phenolic assay by using Folin-Ciocalteu reagent is a simple, convenient and reproducible method. It is employed routinely in studying phenolic antioxidants [26].

In the reducing power assay, the presence of antioxidants in the sample results in the reduction ofFe^{3+} to Fe^{2+} by donating an electron. The amount of Fe^{2+} can then be monitored by measuring the formation of Perl's blue at 700nm. Increasing absorbance indicates an increase in reductive ability [20]. Table.4 shows the total reducing power of the HE, EAE and ME in comparison with ascorbic acid standard at 700nm.Extracts showed an increase in reducing power of the extract with an increment in the extract concentration. The reducing power of the plant extracts were although not better than standard ascorbic acid yet showed considerable activity.

Table 4: Reducing power ability of different extracts of *B. vitis-idaea.*

Extracts	Absorbance at 700 nm				
	12.5 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
HE	0.023 ± 0.001	0.062 ± 0.002	0.094 ± 0.001	0.125 ± 0.003	0.211 ± 0.001
EAE	0.071± 0.002	0.144 ± 0.003	0.315 ± 0.002	0.507 ± 0.001	0.979 ± 0.001
ME	0.028 ± 0.001	0.086 ± 0.003	0.194 ± 0.001	0.283 ± 0.002	0.511 ± 0.003
Ascorbic acid	0.221 ± 0.001	0.368 ± 0.002	0.654 ± 0.001	0.853 ± 0.003	1.233 ± 0.004

Values are expressed as mean± SEM of three parallel measurements.

The reducing capacity of a compound may serve as a significant indicator of antioxidant activity potential of plant extracts. However, the activity of antioxidants has been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging [26]. Further studies are required to establish the mechanism by which the *B. vitis-idaea* extracts bring about antioxidant activity.

The antioxidant capacities and total phenolic contents of *B. vitis-idaea* were evaluated for the first time and the study showed a correlation between the phenol content of the extracts with their antioxidant potential. Several studies exhibited a close relationship between antioxidant activities and total phenolic content [27-32]. In this study, the antioxidant activity of *B. vitis-idaea* were also in agreement with the total amount of phenolics, which exhibited high associations with reducing power, superoxide scavenging activity and total antioxidant capacity Thus, the content of phenolic compounds could be used as an important indicator of antioxidant capacity. Strong antioxidant activities and medicinal functions of *B. vitis-idaea* makes it a promising source of natural antioxidants in food and pharmaceutical industries.

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