ASIAN JOURNAL OF BIOMEDICAL & PHARMACEUTICAL SCIENCES

e-ISSN: 2249-622X

RESEARCH ARTICLE

Free radical scavenging activity of bark extracts of *Bauhinia variegata* L.

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Received:
30th May 2013
Received in revised form:
15th July 2013
Accepted:
30th July 2013
Available online:
10th Aug 2013

OPEN ACCESS

Online ISSN 2249–622X
http://www.ibiopharm.com

ABSTRACT

Bauhinia variegata L. is traditionally used in treating a variety of ailments in India. Phytochemical screening and *in vitro* free radical scavenging activity of aqueous and ethanolic bark extracts of *Bauhinia variegata* was assessed by studying its ability to scavenge DPPH, Nitric oxide, hydroxyl radical and reducing power. Phytochemical analysis revealed the presence of steroid, phenol/ tannin, glycoside/ sugar, carbohydrate and terpenoids. Ethanolic extract showed significant nitric oxide scavenging activity, whereas aqueous extract was comparatively more potential against both ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species) generation systems. The results support its traditional use in curing various diseases` and as a source of natural antioxidants which protect cells against oxidative stress.

Keywords: *Bauhinia variegata*, Phytochemicals, DPPH, Nitric oxide, hydroxyl radical, reducing power.

1. INTRODUCTION:

Antioxidant activity of herbs is one of the reason, plants are extensively used in traditional medicine. Oxidation results in the production of Reactive Oxygen species (ROS) the byproducts of biological reactions which include superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide which are known to induce oxidation of lipids, damage cell membranes and cause DNA mutation [1]. At high concentrations free radicals generate oxidative stress which is reported to be causative in most degenerative diseases such as cancer[2], cardiovascular disease[3], neural degeneration[4], diabetes, obesity[5] and aging. Several mechanisms in the body neutralize the oxidative stress by antioxidant action which involves suppressing the formation of free radicals and scavenging them, act as reducing agents and quenchers of singlet oxygen formation [6].

Bauhinia variegata L. belongs to the family fabaceae and is widely distributed throughout India. Different parts of the plant are used traditionally for curing a variety of diseases. The stem bark is used as an astringent, antileprotic, antigoitrogenic, antitumour and in the treatment of fever, skin diseases and wound healing [7-10]. The stem bark is reported to contain 5, 7– dimethoxy and 5, 7- dihydroxy flavanone-4-O-α-L rhamnopyrosyl-β-D-glycopyranosides, kaempferol-3-glucoside, lupeol and betasitosterol possessing anti-inflammatory potential [11, 12]. Exploring active pharmacological compounds in plants traditionally used as medicine is gaining interest. Natural antioxidants such as plant polyphenols play an important role in inhibiting and scavenging free radicals [13]. From this viewpoint the present study was carried out to evaluate the *in vitro* free radical scavenging activity of aqueous and ethanolic bark extracts of *B. variegata*.

2. MATERIALS AND METHODS

2.1. Collection and Identification of plant materials

The bark of *B. variegata* L. was collected in the month of June 2012, from Yenepoya University campus, Mangalore, Karnataka, India. The plant material was taxonomically identified by a botanist. The fresh bark collected were washed with distilled water to remove dust and was shade dried, pulverized by a mechanical grinder and stored in airtight containers for further use.

2.2 Preparation of extracts

Aqueous and ethanolic extracts of *B. variegata* bark were prepared as per the guidelines of Raaman [14]. The extracts were prepared by maceration technique. The bark powder (10g) was extracted with ethanol (200ml) and the solution was concentrated in a water bath at 60°C to obtain a

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brownish black ethanolic extract (EBV – ethanolic extract *Bauhinia variegata*). About 50g of the bark powder was dissolved in 300 ml of distilled water. The solution was concentrated under reduced pressure by lyophilizer (OPERON-FDB-5003) to yield the aqueous extract (ABV– aqueous extract *Bauhinia variegata*). The yield of the extracts was noted and was kept in a refrigerator until further use.

2.3. Phytochemical analysis

Preliminary phytochemical tests are used to detect the presence of various organic functional groups, which is the indicative of type of phytochemicals present in the plant. These tests indicate the presence of different class of constituents present in the extract. Tests were performed as per the methodology mentioned by Harborne [15].

Tests for Alkaloids

a. *Dragendroff's test:* To a few mg of extract dissolved in alcohol, a few drops of acetic acid and Dragendroff's reagent were added and shaken well. An orange red precipitate formed indicates the presence of alkaloids.
b. *Wagners's tests:* To a few mg of extract dissolved in acetic acid, a few drops of Wagner's reagent was added. A reddish brown precipitate formed indicates the presence of alkaloids.
c. *Mayer's test:* To a few mg of extract dissolved in acetic acid, a few drops of Mayer's reagent was added. A dull white precipitate formed indicates the presence of alkaloids.
d. *Hager's test:* To a few mg of extract dissolved in acetic acid, 3 ml of Hager's reagent was added, the formation of yellow precipitate indicates the presence of alkaloids.

Test for Carbohydrates

a. *Molisch's test:* To the extract, 1 ml of α-naphthol solution and conc. sulphuric acid were added along the sides of the test tube. Violet colour formed at the junction of the two liquids indicates the presence of carbohydrates.
b. *Fehling's test*: A few mg of extract was mixed with equal quantities of Fehling's solution A and B. The mixture was warmed on a water bath. The formation of a brick precipitate indicates the presence of carbohydrates.
c. *Anthrone-sulphuric acid test:* A few mg of the extract was mixed with equal quantity of anthrone and treated with two drops of conc. sulphuric acid. It was then heated gently on a water bath. Dark green colour formed indicates the presence of sugar/glycoside.

Test for Steroids

a. *Libermann-Burchard test:* To the extract was dissolved in chloroform, 1ml of acetic acid and 1 ml of acetic anhydride were added, then heated on a water bath and cooled. Few drops of conc. sulphuric acid was added along the sides of the test tube. Appearance of bluish green colour indicates the presence of steroids.
b. *Salkowski test:* The extract was dissolved in chloroform and equal volume of conc. sulphuric acid was added. Formation of bluish red to cherry red colour in chloroform layer and green fluorescence in the acid layer indicates the presence of steroids.

Test for Saponins

a. To a few mg of extract, distilled water was added and shaken. Stable froth formation indicates the presence of saponins.

Test for Tannins

a. To the extract, a few drops of dilute solution of ferric chloride was added, formation of dark blue colour shows the presence of tannins.

Test for flavonoids

a. Shinoda's test: To the extract in alcohol, a few magnesium turnings and few drops of conc. Hydrochloric acid were added and heated on a water bath. Formation of red to pink colour indicates the presence of flavonoids.

Test for Phenols

a. To the extract in alcohol, added two drops of alcoholic ferric chloride. Formation of blue to blue black indicates the presence of phenol.

Test for Coumarins

a. To the extract in alcohol, a few drops of 2 N sodium hydroxide solution was added. Dark yellow colour formation indicates the presence of coumarins.

Test for Triterpenoids

a. The extract was warmed with tin bits and few drops of thionyl chloride. Formation of pink colour indicates the presence of triterpenoids.

Test for Carboxylic acid

a. Extract dissolved in water is treated with sodium bicarbonate. Brisk effervescence indicates the presence of carboxylic acid.

2.4. Antioxidant Activity:

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. All other chemicals used were of analytical grade.

DPPH-radical scavenging activity

DPPH-radical scavenging activity was determined according to the technique outlined by George [16]. A 0.002% of DPPH in methanol was prepared and 1 ml of this solution was added to 1 ml of different concentrations of extracts (10µg/ml to 100µg/ml and standard (Ascorbic acid), allowed to stand for 30 min at room temperature.

All tests were performed in triplicate. The change in color from purple to yellow was measured at 517nm in a spectrophotometer (SYSTRONICS 2201). Methanol with extract served as the blank and DPPH in methanol without the extracts served as the positive control. The percentage of radical scavenging activity was calculated using the following formula:

% Antioxidant scavenging activity = $[(A_0 – A_1) / A_0]$ x 100

Where, A_0 = Absorbance of control. A_1 = Absorbance of sample

Nitric oxide radical scavenging assay

Free radical scavenging activity by nitric oxide scavenging test was assessed by the Griess-liosvay reaction proposed by Garret [17]. 2 ml of Sodium nitroprusside (10µM) in 0.5 ml of standard phosphate buffer solution was incubated with 0.5 ml of different concentration of the test extracts (10 µg/ml - 100 µg/ml) dissolved in standard phosphate buffer (0.025M, pH 7.4) and the tubes were incubated at 25 °C for 2½ hr. To 0.5 ml of incubated mixture 1 ml of 0.33% sulfanilic acid was added and allowed to stand at room temperature for 5 min. After incubation 1 ml of 0.1% of naphthyl ethylenediamide dichloride was added, mixed the content and incubated at room temperature for 30 minutes. All tests were performed in triplicate. The absorbance of the mixture at 540 nm was measured with a Double beam UV-visible Spectrophotometer (SYSTRONICS 2201). Distilled water served as the blank. Nitric oxide radical scavenging activity was calculated according to the following formula:

% inhibition = $[(A_0 – A_1) / A_0]$ x 100

Where, A_0 = Absorbance of control. A_1 = Absorbance of sample

The activity was compared with ascorbic acid, which was used as a standard antioxidant.

Reducing power assay

The reducing power of the extracts was assessed by the method proposed by Oyaizu [18]. A 0.75 ml of various concentrations of the extracts (10 µg/ml - 100µg/ml) was mixed with 0.75 ml of phosphate buffer (0.2 M pH 6.6) and 0.75 ml of potassium ferricyanide (1% v/v). Incubated at 50 °C for 20 min. The reaction was stopped by adding 0.75 ml of 10% trichloroacetic acid, centrifuged at 800 rpm for 10 minutes. 1.5 ml of supernatant was mixed with 1.5 ml distilled water and 0.1 ml ferric chloride (0.1%). Incubated at room temperature for 10 minutes and the absorbance at 700 nm were measured with Double beam UV-visible Spectrophotometer (SYSTRONICS 2201). All tests were performed in triplicate. Higher absorbance of reaction mixture indicates the greater reducing power as compared to ascorbic acid (Standard). The percentage of radical scavenging activity was calculated using the following formula:

% Antioxidant scavenging activity = $[(A_0 – A_1) / A_0]$ x 100

Where, A_0 = Absorbance of control. A_1 = Absorbance of sample

Hydroxyl radical scavenging activity

The scavenging activity for hydroxyl activity was assayed according to the method of Yu [19]. About 60 µl of ferrous chloride (1mM), was added to 90 µl of 1, 10 phenanthroline (1mM). About 2.4ml of phosphate buffer saline (0.2 M, pH 7.4) was added to the mixture, followed by the addition of 150 µl of hydrogen peroxide (0.17 M) and 1.5 ml of different concentrations of the extracts (10 µg/ml - 100µg/ml). The mixture was incubated for 5 min at room temperature. All tests were performed in triplicate. The absorbance of the mixture was read at 560 nm in a Double beam UV-visible Spectrophotometer (SYSTRONICS 2201) against blank (distilled water). The hydroxyl radical scavenging activity was calculated according to the following formula.

% inhibition = $[(A_0 – A_1) / A_0]$ x 100

Where, A_0 = Absorbance of control. A_1 = Absorbance of sample

2.5 Statistical Analysis

The data obtained have been presented as Mean ± SEM. The difference between the control group and test extracts treated group was analyzed by employing one way ANOVA (Analysis of Variance) followed by Dunnett's multiple 't' test as post hoc test . A $p<0.05$ was considered as statistically significant.

3. RESULTS

Aqueous extract of *B. racemosa* yielded 17% and ethanolic extract was 0.8% respectively. These extracts were subjected for preliminary phytochemical screening and antioxidant analysis.

Phytochemicals

The results of preliminary phytochemical study are tabulated in Table I. The results revealed the presence of pharmacologically active chemical compounds such as steroid, phenol/ tannin, glycoside/ sugar, carbohydrate and terpenoids in both the extracts.

TEST	EBV	ABV
Alkaloid	-	-
Coumarin	-	-
Flavone	-	-
Carbohydrate	+	+
Steroid	+	+
Phenol	+	+
Tannin	+	+
Glycoside/sugar	+	+
Terpenoid	+	+
Carboxylic acid	-	-
Saponins	-	-

Table I- Preliminary phytochemical tests of different extracts of Bauhinia variegata

EBV – ethonalic extract Bauhinia variegata; ABV – aqueous extract Bauhinia variegata

DPPH scavenging activity

The percentage of DPPH radical scavenging activity of *Bauhinia* extracts is presented in Figure 1.

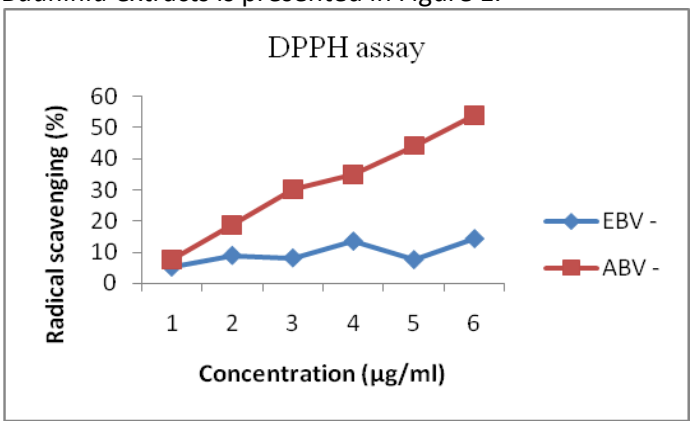

Fig 1: DPPH radical scavenging activity of aqueous and ethanolic extracts of *B. racemosa*

EBV produced only a weak radical scavenging activity in this assay. The observed inhibition in test extract given groups was found to be statistically non-significant in comparison to control tubes. ABV shows dose dependent marked anti-oxidant activity at the higher dose level. The free radical scavenging effect observed with 40, 60, 80 and 100 µg/ml dose level was found to be statistically highly significant. The weak to moderate activity observed at lower dose level of 10 and 20 µg/ml dose level was found to be statistically non-significant.

Nitric oxide scavenging activity

Figure 2 shows the measure of nitric oxide scavenging activity of *Bauhinia* aqueous and ethanolic extracts. EBV produced moderate to good nitric oxide scavenging activity. The activity was found to be significant with respect to the inhibition observed at 40 and 80 µg/ ml concentration ($p<0.05$) while with remaining concentration it was found to be highly significant with a $p<0.01$. However, the observed effect is not dose dependent; at higher concentration there was a tendency towards decrease in the scavenging activity. ABV do not possess significant free radical scavenging activity with respect to nitric oxide formation. Though moderate inhibition was observed at 10µg/ml dose it was found to be statistically non-significant.

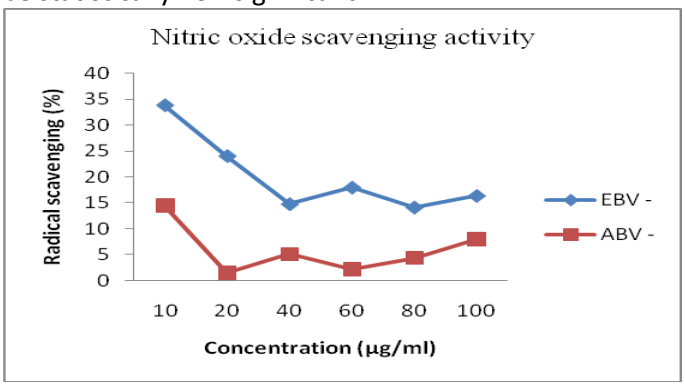

Fig 2: Nitric oxide scavenging activity of aqueous and ethanolic extracts of *B. racemosa*

Hydroxyl radical scavenging activity

Antioxidant activity of aqueous and ethanolic *Bauhinia* bark extracts by hydroxyl radical scavenging activity is presented in Figure 3. EBV drug produced only a weak and statistically non-significant hydroxyl radical scavenging activity. Though moderate activity was observed at lower dose level it was found to be statistically non-significant. ABV shows a moderate but statistically non-significant scavenging of hydroxyl radical scavenging in the dose range between 10 to 60µg/ ml. At higher doses the effect is only marginal.

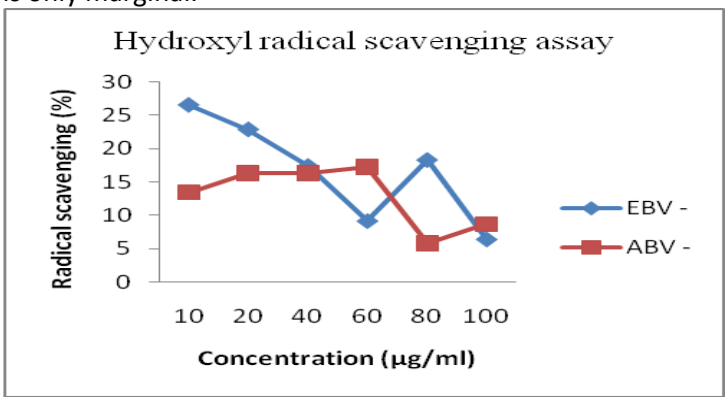

Fig 3: Hydroxyl radical scavenging activity of aqueous and ethanolic extracts of *B. racemosa*

Reducing power assay

The capacity of *Bauhinia* extracts to reduce Fe^{3+} to Fe^{2+} is shown in Figure 4. Though an apparent moderate reducing power decrease was observed in the EBV extract given the effect was found to be statistically non-significant in comparison to control group tubes. The observed effect was also not dose dependent. ABV at the dose level of 10, 20, 40 µg/ ml produced significant reducing effect. However, the observed effect was not dose dependent. At 60 and 100 µg/ ml a moderate but statistically significant reduction was observed in comparison to the control values. At 80µg/ ml dose level only a marginal reduction was observed.

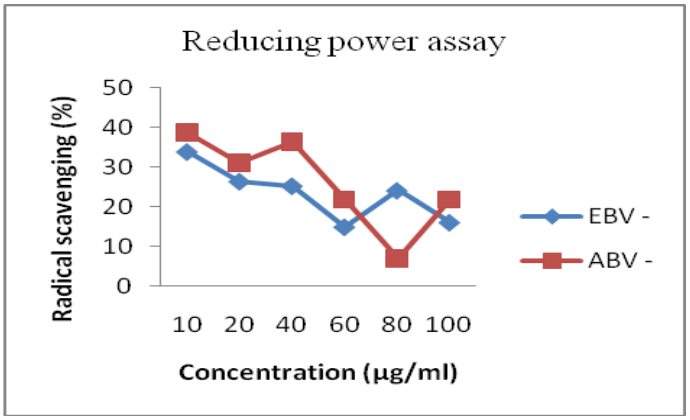

Fig 4: Reducing power assay of aqueous and ethanolic extracts of *B. racemosa*

4. DISCUSSION

Antioxidant capacities of the plants are correlated with their polyphenolic contents as reported in many studies. Phenolics are important antioxidants because of their high

redox potentials, which act as reducing agents, hydrogen donors, and singlet oxygen quenchers [20]. In this study the free radical scavenging potential of *B. variegata* bark extracts may be linked to its phenolic content. The aqueous extract (ABV) produced significant scavenging effect in DPPH test in a dose dependent manner indicating its efficacy in scavenging electron transfer based systems. It also produced good, though dose independent reducing power which further supports the scavenging capacity. However, the extract was found to be devoid of nitric oxide scavenging activity. Since nitric oxide is involved in generation of Reactive Nitrogen Species (RNS) - it indicates that the extract do not possess RNS scavenging activity. The extract possesses only weak hydroxyl scavenging potential. The ethanolic extract (EBV) produced significant scavenging effect only in one system that is nitric oxide scavenging test. In other tests it exhibited only a non-significant weak to moderate inhibition. This suggests that the EBV is efficacious mainly in scavenging nitric oxide based free radicals. From the above results it can be inferred that ABV has got better free radical scavenging potential in comparison to EBV. The present study supports the *in vitro* antioxidant potential of *B. racemosa* but further studies of their activity in biological systems is necessary to confirm their usefulness as natural source of antioxidants.

5. ACKNOWLEDGEMENT:

The author is grateful to Yenepoya University for permission to carry out this study.

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Conflict of Interest: None Declared

Cite this article as:

Bhagya Bhaskar. Free radical scavenging activity of bark extracts of *Bauhinia variegata* L.Asian Journal of Biomedical and Pharmaceutical Sciences, 2013, 3: (22), 9-13.

