Evaluation of the DPPH radical scavenging activity, total phenols and antioxidant activities in Indian wild *Bambusa vulgaris* "Vittata" methanolic leaf extract

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ABSTRACT

Background: Antioxidants have the ability to protect organisms from damage caused by free radical-induced oxidative stress. A lot of research is being carried out worldwide directed toward finding natural antioxidants of plant origin. The antioxidant activity of the methanolic extract of *Bambusa vulgaris* "Vittata" (BVV) leaves is reported along with screening for photochemical constituents of the Indian, wild BVV methanolic leaf extract. **Materials and Methods:** The antioxidant activity was tested spectrophotometrically, measuring the ability of the plant extract to scavenge a stable DPPH• free radical and the total phenolic and flavonoid contents. **Results:** Preliminary studies show the presence of carbohydrates, reducing sugars, flavonoids, steroids, saponins, alkaloids, tannins, anthraquinones and glycosides. The antioxidant activity of the investigated extract has a scavenging ability of hydroxyl peroxide radicals (421.74 ± 25.61 mg/ml) and DPPH• radical scavenging activity (around 95%). The high contents of total phenolic compounds (22.69 ± 0.084 mg GAE/g of dry extract) and total flavonoids (159.80 ± 0.047 mg Quercetin/g of dry extract) indicated that these compounds contribute to the antioxidative activity. **Conclusions:** Our findings provide evidence that the crude methanolic extract of *BVV* is a potential source of natural antioxidants, and this justified its uses in folkloric medicines.

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Key words: *Bambusa vulgaris* "Vittata," DPPH, flavonoids, FRP, H_2O_2 scavenging, phenolics

Access this article online
Website: www.jnatpharm.org
DOI: 10.4103/2229-5119.73586
Quick Response Code:

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INTRODUCTION

Antioxidants have the ability of protecting organisms from damage caused by free radical-induced oxidative stress.[1]

Presently, the probable toxicity of synthetic antioxidants has been condemned. It is strongly believed that regular consumption of plant-derived phytochemicals may drift the balance toward an adequate antioxidant status.[2] Thus, in recent years, interest on natural antioxidants, especially of plant origin, has increased manifolds.[3]

Bambusa vulgaris "Vittata" (BVV), variously known as "painted bamboo," "tiger bamboo" or "yellow bamboo," belongs to the true grass subfamily Bambusoideae of Poaceae, with culms that are yellow in color with green stripes of varying width. The clums are thick walled and densely tufted, having a diameter of about 5–8 cm and reach a height of about 4–8 m.[4] It is cultivated as an ornamental plant in gardens because of its culms. The newly emerged shoots are edible.[5] It flowers rarely, having a flowering cycle of about 80 ± 8 years. It also fails to produce seeds. The leaves of BVV have been reported to have an antimicrobial activity.[6] The culms of this species have been used in China for the treatment of edema in older men.[7]

Bamboo species finds its importance as a novel food additive due to the high natural antioxidant content.[8] Worldwide, bamboo has made a mark in the field of functional and medical health food with its rich antioxidant resource and specific functional factors.[9]

A review of the literature did not throw any light on the antioxidant study of this species. The aim of this study was to assess the *in vitro* antioxidant activity of the methanolic extract of BVV leaves. For this purpose, the factors responsible for the potent antioxidant and radioprotecting ability of BVV, have been evaluated for the total free radical

scavenging activity, preliminary phytochemical assay, hydrogen peroxide ability and hydroxyl inhibitory activity. Attempts have also been made to quantitatively identify important phytochemicals (phenolics and flavonoids) and correlate its phenolic content with the free radical scavenging reactions.

MATERIALS AND METHODS

Chemicals and Reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), quercetin, sodium nitrite ($NaNO_2$), trichloroacetic acid (TCA), ascorbic acid, ferric chloride ($FeCl_3$), 2-deoxy-2-ribose, butylated hydroxytoluene (BHT), gallic acid, magnesium ribbon, acetic anhydride and ammonium hydroxide (NH_4OH) were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemical, Mumbai, India. Ethanol, chloroform, methanol, glacial acetic acid, benzene, hydrogen peroxide, ethylenediamine tetraacetic acid (EDTA), potassium di-hydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4), potassium hydroxide (KOH), sodium hydroxide (NaOH), potassium ferricyanide ($K_3Fe(CN)_6$), Fehling's solution, Mayer's reagents, sodium carbonate (Na_2CO_3), conc. HCl and conc. H_2SO_4 were procured from Merck, Mumbai, India. Folin-Ciocalteu reagent was from Sisco Research Laboratory, Mumbai, India. Aluminum chloride ($AlCl_3$) was obtained from Sd Fine Chemicals Limited, Mumbai, India. All chemicals and solvents are analytical grade.

Plant Material and Extraction

BVV leaves were collected from Siliguri in June 2009 and identified by P. P. Paudyal (Consultant, Bamboo Mission Sikkim, India). A voucher specimen (SUK/KRR/BVV001) has been submitted in Bambusetum, Kurseong Research Range, Sukna, Darjeeling.

Dried, ground leaves of BVV (10 g) were extracted in a Soxhlet apparatus using 80% aqueous methanol (the ratio of plant material to solvent was 1:15 m/v).[10] The extraction was carried out at boiling temperature for 6 h. The extracts obtained were evaporated under pressure at 50°C to a constant weight. The extract was stored at 4°C until required. Before use, the BVV leaves extract was dissolved in double-distilled water (DDW) in desired concentrations.

Determination of Plant Extract Yield

The yield of evaporated dried leaves extracts of BVV based on dry weight basis was calculated from the following equation:

Yield (g/100 g of dry plant material) = (W1 × 100) / W2

Where, W1 and W2 were the weight of the extract after the solvent evaporation and the weight of the dry plant material, respectively.

Preliminary Phytochemical Screening

The presence or absence of the phytochemical constituents of powdered plant material was analyzed using the following standard methods:

Carbohydrates: Leaves (500 mg) of BVV were boiled in 30 ml DDW and filtered, 1 ml filtrate + 1 ml of Molisch's reagent + 1 ml conc. H_2SO_4. The presence of carbohydrate is inferred by a reddish ring.[11]

Reducing sugars: One milliliter of the above filtrate + 2 ml of Fehling's solution were boiled for 5 min. A brick red precipitate indicates the presence of reducing sugar.[11]

Tannins: Two milliliters of the filtrate + 1 ml $FeCl_3$. A blue-black or greenish-black precipitate confirms tannins.[11]

Saponnins: Frothing test: 0.5 ml filtrate + 5 ml DDW, shaken for 30 s, persistent frothing indicates saponnins.[11]

Flavonoids: Shinoda's Test: 200 mg plant material was extracted with 5 ml ethanol and filtered; 1 ml filtrate + magnesium ribbon + conc. HCl were added to this. A pink or red color indicates the presence of flavonoids.[11]

Steroids: Liebermann–Burchard's test: 200 mg of the plant material in 10 ml chloroform, filtered. 2 ml filtrate + 2 ml acetic anhydride + 1 ml conc. H_2SO_4 are added to this. A blue-green ring shows the presence of steroids.[12]

Alkaloids: Plant material (200 mg) was boiled in 20 ml of 1% H_2SO_4 in 50% ethanol and filtered; filtrate + 5 drops conc. NH_4OH + 20 ml chloroform were added and the two layers were separated. The chloroform layer was extracted with 20 ml dilute H_2SO_4. On addition of extract + 5 drops of Mayer's reagent, a creamy/brownish-red/orange-red precipitate indicates the presence of alkaloids.[11]

Anthraquinones: Borntrager's test: 100 mg of powdered plant in 5 ml of chloroform, filtered. 2 ml filtrate + 2 ml 10% NH_4OH were added to this. A bright pink color confirms the presence of anthraquinones.[11]

Glycosides: Keller–Kiliani test: 2 ml filtrate + 1 ml glacial acetic acid + 1 ml $FeCl_3$ + 1 ml conc. H_2SO_4. A green-blue color indicates the presence of glycosides.[12]

Hydrogen Peroxide Scavenging

This activity was determined according to a previously described method[13] with minor changes. An aliquot of H_2O_2 (2 mM) and various concentrations (100–1000 µg/ml) of samples were mixed (1:0.6 v/v) and incubated for 10 min

at room temperature. After incubation, the absorbance of hydrogen peroxide at 230 nm was determined against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction. The percentage scavenging activity of hydrogen peroxide by the BVV leaves extract was calculated as follows:

% scavenging activity [H_2O_2] = [Abs (control) – Abs (standard) / Abs (control)] × 100

where, Abs (control): absorbance of the H_2O_2 (2 mM) as control

Abs (standard): absorbance of the extract/standard

Determination of the Total Flavonoid Content

The total flavonoid content was determined with the aluminum chloride ($AlCl_3$) method[14] using quercetin as a standard. The plant extract (0.25 ml) was added to 1.25 ml DDW followed by 75 µl of 5% $NaNO_2$. After 5 min at room temperature (RT), $AlCl_3$ (0.15 ml, 10%) was added. After a further incubation for 6 min at RT, the reaction mixture was treated with 0.5 ml of 1 mM NaOH. Finally, the reaction mixture was diluted with 275 µl of DDW. Further incubation for 20 minutes at RT was performed and the absorbance was measured at 510 nm. All tests were performed in triplet. The flavonoid content was calculated from a quercetin standard curve.

Determination of the Total Phenolic Content

Total phenolic content was determined using the Folin-Ciocalteu (FC) reagent method[15] with slight modification. Briefly, the leaf extract (0.5 ml) was mixed with 0.5 ml of FC reagent (previously diluted 1:1 with distilled water) and incubated for 5 min at RT, then 1 ml of 2% Na_2CO_3 solution was added. After incubation at RT for 10 min, the absorbance was measured at 730 nm. All tests were performed in triplicates. Gallic acid monohydrate was used as the standard. The total phenolic content was expressed as gram of gallic acid equivalents (GAE) per 100 g extract.

DPPH Radical Scavenging Activity

The free radical scavenging capacity of the extracts was determined using DPPH.[16] The DPPH solution (0.006% w/v) was prepared in 95% methanol. The methanol extract of the BVV leaves was mixed with 95% methanol to prepare the stock solution (1 mg/ml). Freshly prepared DPPH solution was taken in test tubes and extracts were added followed by serial dilutions (100–1000 µg) to every test tube such that the final volume was 2 ml, and discoloration was measured at 517 nm after incubation for 30 min in the dark (Themo UV1 spectrophotometer, Thermo Electron Corporation, England, UK). Measurements were performed at least in triplicate. Ascorbic acid was used as a reference standard and dissolved in DDW to make the stock solution with the same concentration (1 mg/ml). The control sample was prepared, which contained the same volume without any extract and 95% methanol was used as the blank. Percent scavenging of the DPPH free radical was measured using the following equation:

DPPH scavenging effect (%) = (Ao – A1) / Ao X 100

where, Ao was the absorbance of the control and A_1 was the absorbance in the presence of the sample (methanolic leaf extract of BVV)

The actual decrease in absorption induced by the test compounds was compared with the positive controls. The IC_{50} value was calculated using the dose inhibition curve.

Reducing Power

The reducing power of the BVV leaf extract was determined according to the method previously described.[17] Different concentrations of the extract (250–2,500 µg) in 1 ml of DDW were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of TCA (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with DDW (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as a blank solution. The absorbance of the final reaction mixture of the two parallel experiments was taken and expressed as mean ± standard deviation.

Statistical Analysis

Results are expressed as mean ± S.E.M. of triplets. The groups were compared by two-way ANOVA using Graph Pad Prism, Version 5.0 (Graph Pad Software, San Diego, CA, USA). *P*-values <0.001 were considered significant.

RESULTS

Plant Yield

The yield of the BVV leaf methanolic extract was 3.33%.

Preliminary Phytochemical Screening

Preliminary phytochemical screening of the extract revealed the presence of various bioactive components, of which flavonoids and tannins were the most prominent, and the result of the phytochemical test has been summarized in Table 1. Reports are also available where the phenolic compounds and flavonoids are found to be associated with the antioxidative activity in biological systems.[18,19]

Determination of the Total Phenolic Content

Phenolic compounds may contribute directly to the

antioxidative action. The total phenolic content was 22.69 ± 0.084 mg/ml, gallic acid equivalent per 100 mg plant extract.

Determination of the Total Flavonoid Content

The total flavonoid content of the 70% methanolic extract of the BVV leaves was 159.80 ± 0.047 mg/ml quercetin equivalent per 100 mg plant extract.

DPPH Scavenging Activity of the BVV Methanolic Leaf Extract Compared with Standard Ascorbic Acid

In this present study, the antioxidant activity of the methanol extracts of the BVV leaves was investigated using the DPPH scavenging assay, reducing power of the extract and by determining the total antioxidant capacity of the extract. All these have proven the effectiveness of the methanol leaf extract of BVV compared with the reference standard antioxidant ascorbic acid. The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron that is responsible for the absorbance at 540 nm and also for the visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Comparison of the antioxidant activity of the extract and ascorbic acid is shown in Figure 1. The methanol extract of BVV exhibited a significant dose-dependent inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of 532 µg/ml as compared with the standard ascorbic acid (269.53 µg/ml).

Hydrogen Peroxide Scavenging

Figure 2 shows that the leaf extract is a good scavenger of H_2O_2 (IC_{50} = 521.74 ± 25.61 mg/ml) compared with standard ascorbic acid (IC_{50} = 700 ± 0.3 mg/ml). The IC_{50} value [Figure 2] of the extract was lesser than that of the standard.

Reducing Power Assay

The reductive capabilities of the plant extract compared with ascorbic acid have been depicted in Figure 3. The reducing power of the extract of BVV leaves was found to be remarkable, which increased gradually with a rise in the concentration. As illustrated in Figure 3, Fe^{3+} was transformed to Fe^{2+} in the presence of the extract and the reference compound ascorbic acid to measure the reductive capability. At 0.25 mg/ml, the absorbance of the plant extract and ascorbic acid was 0.026 and 0.016, respectively, while at 2.0 mg/ml, the absorbance of both the extract and ascorbic acid were almost the same. From the figure, it can be inferred that a low dose of the extract shows the maximum reducing capability when compared with the standard.

DISCUSSION

During oxidative stress and exposure to radiation, excessive free radicals are produced that are known to cause damage to the biomolecules.[20,21] Antioxidant and radioprotection studies indicate that BVV should possess

Table 1: Preliminary phytochemical screening of the *Bambusa vulgaris* "Vittata" methanolic leaf extract

Chemical compounds	Result
Saponins	+
Steroids	+
Alkaloids	+
Tannins	+
Carbohydrates	+
Flavonoid	+
Anthraquinone	+
Glycosides	+
Reducing sugars	+

- = Compound not detected; + = compound detected

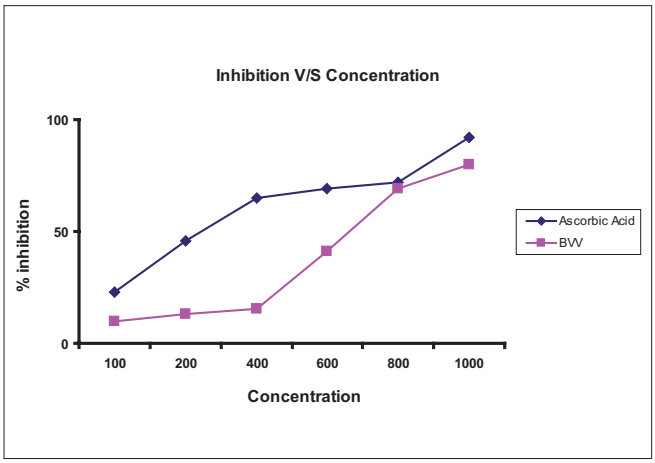

Figure 1: Dose inhibition curve and IC_{50} values of the methanolic leaf extract of *Bambusa vulgaris* "Vittata"

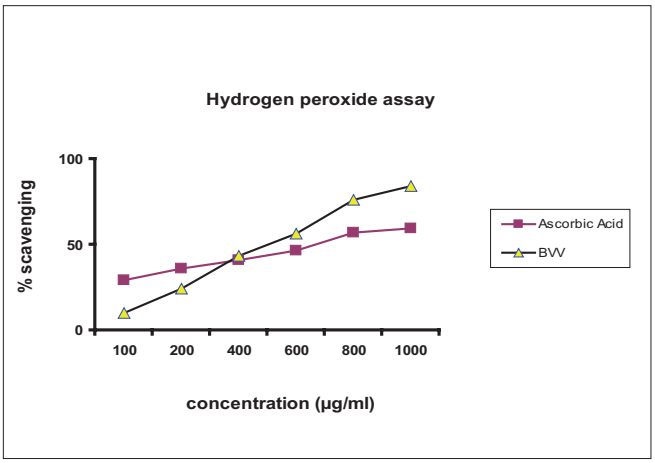

Figure 2: Hydrogen peroxide scavenging of the methanolic extract of *Bambusa vulgaris* "Vittata" in comparison with a standard (ascorbic acid) at λ = 230 nm

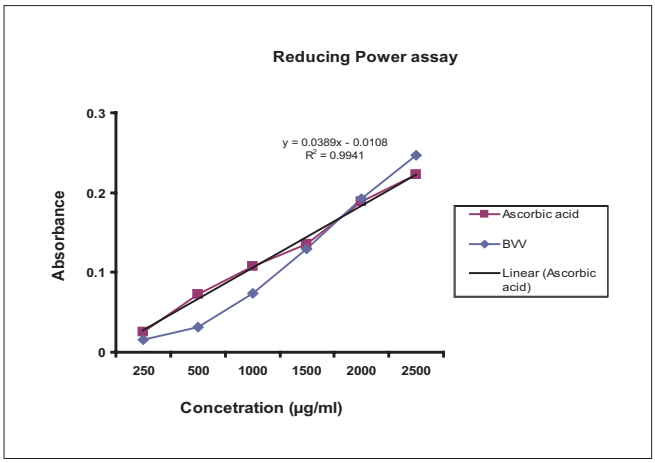

Figure 3: Reducing power activities of the methanolic extract of *Bambusa vulgaris* "Vittata" in comparison with a standard (ascorbic acid) at λ = 700 nm

the ability of either inhibiting free radical formation or itself be a free radical scavenger. Our methanolic extract of BVV leaves showed similar results as previously reported for Bambusa species.[22] The dose inhibition curve and IC_{50} values of the leaf extract are shown in Figure 1. In the dose-response experiment, it could be observed that total inhibition of the enzymes was never achieved. The maximum inhibition was in the range of 75–85% in the presence of the 25 mg/ml extract. With the addition of a larger amount of extract to the DPPH assay mixture, the degree of inhibition decreased, indicating a pro-oxidant effect. The explanation for the higher IC_{50} (IC_{50} = 269.53 µg/ml) value found in the experiment was because the sample used was a crude extract, with the compound(s) reacting as antioxidants.

Our result shows that the methanolic extract of BVV leaves had high effects on scavenging OH-, as reported by previous authors.[23,24] The antioxidative effect of BVV is mainly due to phenolic components, such as phenolic acids, and flavone C-glucosides.[25]

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides.[26] In this respect, polyphenolic compounds like flavonoids and phenolic acids commonly found in plants have been reported to have multiple biological effects, including an antioxidant activity.[27,28]

For measurements of the reductive ability, it has been found that the Fe^{3+}–Fe^{2+} transformation occurred in the presence of plant extract, as shown by Oyaizu.[17] Similar trends have been observed with the BVV leaf extract. Earlier authors[29] have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The presence of reductones is thought to be associated with the reducing properties,[30] which in turn have been shown to exert an antioxidant action by donating a hydrogen atom that breaks the free radical chain.[31] Reductones are also reported to react with certain precursors of peroxide thus preventing peroxide formation.

CONCLUSION

The BVV leaf methanolic extract showed a strong antioxidant activity by inhibiting DPPH, hydrogen peroxide and reducing power activities when compared with the standard L-ascorbic acid, quercetin and gallic acid. In addition, the BVV was found to contain a noticeable amount of total phenols and flavonoids, which play a major role in controlling oxidation. The results of this study show that the BVV leaves can be used as an easily accessible source of natural antioxidant. However, the phytoconstituents responsible for the antioxidant activity of BVV are not much clear. Therefore, a further study is needed to determine the mechanism behind the antioxidant activity of this plant.

ACKNOWLEDGMENT

The authors are thankful to the Department of Food Processing Industries and Horticulture, Government of West Bengal, for providing grants. We thank Dr. T. L. Shantha, Director, and Dr. MB Nagaveni, Head, Maharani Lakshmi Ammanni College for Women, for supporting this research. We are also grateful to the Directorate of Forests, Government of West Bengal, Silviculture (Hills) Division, Darjeeling, for providing the necessary help, support and information. We would also like to thank Mr. Ajay Kr. Dubey, Divisional Forest Officer, Silviculture (Hills) Division, Darjeeling, and Mr. Prasanta Kr. Ghosh, Range Officer, Kurseong Research Range, Sukna, Darjeeling. The authors are also obliged to the bamboo taxonomist, Mr. P. P. Paudyal, Consultant, Bamboo Mission, Sikkim, for helping in identifying the species of bamboo.

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Source of Support: Nil, **Conflict of Interest:** None declared.