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Evaluation of *Bauhinia variegata* L. bark fractions for *in vitro* antioxidant potential and protective effect against H₂O₂-induced oxidative damage to pBR322 DNA

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Bauhinia variegata L. (Fabaceae) is widely used in Indian traditional medicine. The present study was undertaken to evaluate the protective effect of different fractions from the crude methanolic extract of *B. variegata* L. bark against free radicals and H₂O₂-induced oxidative damage to pBR322 DNA. 80% methanol extract (MEB) of the bark was fractionated into non-polar fractions, including hexane (HEB), chloroform (CFB) fractions and Polar fractions, including n-butanol (NBB), ethyl acetate (EAB) and remaining extract (REB). The extract/fractions were analysed for total phenolic and flavonoid content and antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power assays while the genoprotective activity was assessed using DNA nicking assay. The polar fractions, MEB, EAB, NBB and REB showed greater antioxidant activity (EC₅₀ 44.07, 58.09, 69.68 and 51.81 µg/ml, respectively) in comparison to non-polar fractions. However, all the fractions effectively protected pBR322 plasmid DNA from the H₂O₂ induced damage. The phytochemical analysis of the polar fractions revealed them to be rich in the phenolic/flavonoid content and that significant correlation existed between phenolic/flavonoid content and antioxidant activity. Our studies showed that the phytoconstituents of *B. variegata* harbour the potential to combat various free-radicals and need further investigation.

Key words: Antioxidant, flavonoid, free radical, phenolic compound, scavenging activity, reactive oxygen species.

INTRODUCTION

Reactive oxygen species (ROS) cause the oxidation of biomolecules leading to cellular damage. The tissue injury caused by ROS may include DNA and protein damages, oxidation of important enzymes and these events could consequently result in the occurrence of various free radical-related diseases (Ammar et al., 2008). Natural antioxidants, present in medicinal and dietary plants that might help attenuate oxidative damage, are gaining much interest (Silva et al., 2005). Plant polyphenols act as strong antioxidants and they protect cell constituents against oxidative damage, thus averting the deleterious effects on nucleic acids, proteins

and lipids in cells (Rice-Evans, 2001). There are reports regarding their effects against the development of degenerative diseases, such as cancer (Hertog et al., 1996), cardiovascular diseases (Vita, 2005), neural degeneration (Youdim et al., 2002), diabetes and obesity (Tsuda et al., 2003).

Mechanisms of antioxidant action can include suppressing reactive oxygen species formation, scavenging reactive oxygen species or upregulating or protecting antioxidant defenses. Sources of natural antioxidants include plant phenolics which occur in all parts of plants like fruits, vegetables, seeds, leaves, roots and bark. In the field of nutrition, health and medicine, crude extracts of these parts rich in phenolics are under extensive research, because they retard oxidative degradation of lipids and thereby improve the quality and nutritive value of foods (Rice-Evance et al., 1995).

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Bauhinia variegata L. (Fabaceae) is a medium-sized deciduous tree, commonly known as Kachnar. Its traditional uses are against bronchitis, leprosy, tumors and ulcers (Kirtikar and Basu, 1993). Raj Kapoor et al. (2003) have reported its antiulcer activities. A flavonol glycoside, 5,7,3',4'-tetrahydroxy-3-methoxy-7-O- α -L-rhamnopyranosyl(1 $\rightarrow$ 3)-O-beta-galactopyranoside, isolated from *B. variegata* showed anti-inflammatory potential (Yadava and Reddy, 2003). The present study was aimed to evaluate the antioxidant and DNA protective potential of different extract/fractions of the bark of *B. variegata*. The antioxidant activity was assessed in terms of the DPPH radical scavenging activity and the reducing potential of the extract/fractions. To determine the DNA protective activity, DNA nicking assay was carried out. Further, the extracts/fractions were analyzed for the total phenolic and flavonoid content.

MATERIALS AND METHODS

Chemicals

Ethidium bromide and Rutin were purchased from Sigma-Aldrich, USA. 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, L-ascorbic acid, gallic acid and potassium ferricyanide were obtained from HiMedia Pvt. Ltd., Mumbai, India. pBR322 (CsCl₂ purified) was procured from Genei, Bangalore, India. All other chemicals and solvents were of analytical grade. Double distilled water was used for preparation of stock solutions.

Plant material and extraction

The stem bark of *B. variegata* L. was collected in the month of August, from Botanical Garden of Guru Nanak Dev University, Amritsar (Punjab, India). The plant material was taxonomically identified from the Division of Botany, Forest Research Institute (F.R.I.), Dehradun, India and a Voucher specimen (0391/Herb) was deposited in the herbarium of Guru Nanak Dev University, Amritsar, Punjab, India.

The bark was washed with distilled water to remove dust and was oven-dried at $\pm 40^\circ\text{C}$. Dried and powdered bark was percolated with 80% methanol and was vacuum dried with Buchi Rotavapor to obtain the dried methanol extract (MEB). MEB was dissolved in distilled water and the aqueous extract was fractionated with different solvents, including hexane, chloroform, n-butanol and ethyl acetate in the order of their increasing polarity to obtain respective fractions which includes hexane fraction (HEB), chloroform fraction (CFB), n-butanol fraction (NBB), ethyl acetate fraction (EAB) and the remaining fraction (REB) (Figure 1).

Phytochemical analysis

Determination of total phenolic content

Total phenolics content (TPC) was assayed using the Folin–Ciocalteu reagent, following the method of Singleton and Rossi (1965). To 100 μl of extract/fraction, 0.5 ml of Folin–Ciocalteu reagent was added, and the mixture was shaken and allowed to stand for 6 min, before adding 1.5 ml of 20% Na₂CO₃ solution. The solution was then adjusted with deionized water to a final volume of 5 ml and mixed thoroughly. After incubation for 2 h, the absorbance

was read at 765 nm. Total phenolic content (three replicates per treatment) were expressed as mg gallic acid (standard phenolic compound) equivalents per gram (mg GAE/g) through the calibration curve with gallic acid. The calibration curve range was 20 to 200 $\mu\text{g/ml}$ ($R^2 = 0.99$).

Determination of total flavonoid content

The total flavonoid content (TFC) was assayed by following the method of Zhisen et al. (1999). In 1 ml of extract/fraction, de-ionized water, 5% NaNO₂ and 10% AlCl₃ were added. After incubation for 5 min, 1 M NaOH was added, followed by the addition of 2.4 ml de-ionized water to get the final volume of 10 ml. The absorbance was read at 510 nm. Rutin was taken as standard flavonoid compound and total flavonoid contents (three replicates per treatment) were expressed as mg rutin equivalents per gram (mg RE/g) through the calibration curve with rutin. The calibration curve range was 20 to 200 $\mu\text{g/ml}$ ($R^2 = 0.988$).

Antioxidant activity

DPPH-radical scavenging activity

Radical scavenging capacity was determined according to the technique reported by Ohinishi et al. (1994). A 0.1 mM solution of DPPH in methanol was prepared and 2 ml of this solution was added to 300 μl of various concentrations of extracts/fractions (20 to 200 $\mu\text{g/ml}$) and the reference compound. After 30 min, absorbance was measured at 515 nm (Systronics 2202 UV-Vis spectrophotometer). Ascorbic acid (AA) was used as standard reference compound. All tests were performed in triplicate. An aliquot without test sample was taken as control. Percent inhibition was calculated by comparing the absorbance values of control and samples using the following equation:

$$\% \text{ Antioxidant activity} = \left[\frac{(\text{absorbance of control} - \text{absorbance of test sample})}{\text{absorbance of control}} \right] \times 100.$$

The EC₅₀ values (effective concentration at which the extract/fraction is able to scavenge 50% of the radicals generated) for all the extract/fractions were also calculated using regression analysis.

Reducing power assay

The reducing power of the extract/fractions of *B. variegata* bark was assessed by the method proposed by Oyaizu (1986). 1 ml of various concentrations (40 to 200 $\mu\text{g/ml}$) of different extracts/fractions was mixed with 200 μM potassium phosphate buffer, pH 6.6, and 1% potassium ferricyanide [K₃Fe (CN)₆]. After incubation at 50°C for 20 min, an aliquot of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 *g* for 10 min. The upper layer of the solution was mixed with 2.5 ml distilled water and 0.5 ml FeCl₃ (0.1%) and absorbance was measured at 700 nm. The increase in absorbance of the reaction mixture was interpreted as increase in reducing activity of the extract/fractions and the results were compared with ascorbic acid (standard reference compound).

DNA nicking assay

The ability of *B. variegata* bark extract/fractions to protect DNA damage was evaluated using the DNA nicking assay as described by Lee et al. (2002). 100 $\mu\text{g/ml}$ concentrations of the different

Figure 1. Scheme of preparation of different extract/fractions of *B. variegata* L. bark.

extract/fractions and Fenton's reagent (30 mM H_2O_2 , 50 mM ascorbic acid, 80 mM FeCl_3) was added to pBR322 plasmid DNA (1 μl) and the final volume of the mixture was brought up to 20 μl . The mixture was then incubated for 30 min at 37°C and the DNA was analyzed on a 1% agarose gel followed by ethidium bromide staining. The DNA strand breaks were analysed with Gel Doc XR system (Bio-Rad, USA).

Statistical analysis

All the experiments were performed in triplicates and the data has been presented as Mean $\pm$ Standard deviation. The significance of the data was assessed using one way ANOVA with significance level set as $p \leq 0.05$. The Pearson correlation analysis was also carried out between the various parameters.

RESULTS AND DISCUSSION

High antioxidant capacities are observed in plants with

high level of phenolic compounds (Moyo et al., 2010). The redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers account for the antioxidant potential of medicinal plants (Hakkim et al., 2007). The phenolic content is considered as an important plant constituent as its hydroxyl groups have considerable scavenging ability (Diplock, 1997). The flavonoids, which are the largest and most studied polyphenols, are gaining interest as antioxidants because of their high capacity to scavenge free radicals (Souza et al., 2008). There are reports regarding the anti-inflammatory, anti-allergic, anti-viral and anti-carcinogenic properties of flavonoids (Nijveldt et al., 2001). Flavonoids prevent hydroxy radical-induced damage (Husain et al., 1987) by donating an electron to neutralize the species (Morel et al., 1994). Biological properties of different *Bauhinia* species have been investigated in numerous experimental *in vivo* and

Table 1. Total phenolic content (TPC) and total flavonoid content (TFC) of different extract/fractions of *Bauhinia variegata* L. bark.

Extract/fraction (g)	Total phenolic content (mg GAE/g of extract)	Total flavonoid content (mg RE/g of extract)
MEB (108.197)	440.0 ± 3.02	937.5 ± 5.37
HEB (3.127)	200.0 ± 2.67	204.1 ± 4.63
CFB (4.76)	372.5 ± 5.97	262.5 ± 2.88
NBB (10.946)	492.5 ± 3.86	737.5 ± 4.76
EAB (12.628)	585.5 ± 3.45	812.5 ± 5.93
REB (14.764)	429.5 ± 4.77	684.3 ± 3.85

MEB, methanol extract; HEB, hexane fraction; CFB, chloroform fraction; NBB, n-butanol fraction; EAB, ethyl acetate fraction; REB, remaining extract.

in vitro models and the results indicate that the reported therapeutic properties are mainly due to the presence of flavonoids (Rao et al., 2008). Results obtained in the present study revealed that there is present a considerable level of total phenolic and flavonoid content in the polar extract/fractions viz., MEB, EAB, NBB and REB of *B. variegata* bark (Table 1).

Scavenging effect on DPPH

The DPPH assay is extensively used for the evaluation of free-radical scavenging potential of antioxidative compounds. DPPH is a free radical generating compound and antioxidants scavenge these radicals either by donating hydrogen (Wanga and Zanga, 2003) or through electron transfer reaction (Foti et al., 2004). In its radical form, DPPH[•] absorbs at 515 nm, but upon reduction by an antioxidant (AH) or a radical species (Re), the absorption decreases.

The methanol extract, the n-hexane, ethyl acetate and n-butanol fractions of *Opuntia monacantha* Haw. (Cactaceae) were assessed for their antioxidant activity by measuring their ability to scavenge DPPH radicals and the samples were able to inhibit the activity of DPPH radicals in a dose-dependent manner (Valente et al., 2010). Similarly, Hemalatha et al. (2010) utilized the DPPH assay to assess the free radical scavenging activity of aqueous extract of *Melothria maderaspatana* Linn. (Cucurbitaceae) and reported it to scavenge the free radicals considerably. The radical scavenging activities of *B. variegata* bark extracts/fractions showed that the polar fractions scavenged the DPPH radicals more significantly. The scavenging of radicals increased with increasing concentrations of the extract/fractions which showed similar amount of percent radical scavenging afterwards. The non-polar fractions, including, HEB and CFB showed insignificant scavenging activity (Figure 2).

The EC₅₀ values showed that MEB extract (EC₅₀ 44.07 ± 1.87 µg/ml) was most effective in scavenging free radicals followed by EAB (EC₅₀ 49.68 ± 0.42 µg/ml), REB (EC₅₀ 51.81 ± 2.01 µg/ml) and NBB (EC₅₀ 58.09 ± 1.85 µg/ml) fractions (Table 2).

Reducing power assay

The capacity of a compound to reduce Fe³⁺ to Fe²⁺ serves as an indication of its potential antioxidant activity (Meir et al., 1995). Depending on the reducing power of the test compound, the colour of the FeCl₃/K₃Fe(CN)₆ system changes to different shades of green. At a wavelength of 700 nm, greater absorbance indicates greater reducing power. Among the different extract/fractions of *B. variegata* bark, the concentration dependent reducing power followed the order of MEB>REB>EAB>NBB>CFB>HEB (Figure 3). The higher reducing power of MEB may be attributed to its higher flavonoid content as compared to other fractions. Similar increase in reducing power with increase in concentration was observed in a study conducted on aqueous extracts of leaves, flowers and stem of *Moltkia petraea* (Tratt.) Griseb. (Koncic et al., 2010). The methanol extracts of the aerial parts of *Leonurus cardiaca* L. subsp. *Persicus* (Labiatae), *Grammosciadium platycarpum* (Apiaceae), *Onosma demawendicum* (Boraginaceae) were investigated by Ebrahimzadeh et al. (2010) and were found to reduce Fe³⁺ ions significantly. An increase in absorbance with increasing concentration of the extracts has also been reported.

Genoprotective activity

The DNA nicking assay assesses the conversion of supercoiled DNA (Form I) to nicked circular form (Form II) as a result of exposure to OH[•] radicals generated by the Fenton's reagent. The cleavage of one of the phosphodiester chains of the supercoiled DNA results in the formation of nicked circular form (Singh et al., 2009).

Figure 2. DPPH radical scavenging activity of *B. variegata* L. bark extract/fractions. AA, L-Ascorbic acid; MEB, methanol extract; HEB, hexane fraction; CFB, chloroform fraction NBB, nbutanol fraction; EAB, ethyl acetate fraction; REB, remaining extract.

Table 2. EC₅₀ values (µg/ml) of different extracts/fractions of *B. variegata* L. bark in DPPH radical scavenging assay.

AA (Standard)	MEB	HEB	CFB	EAB	NBB	REB
15.07	44.07	ND	ND	58.09	49.68	51.81

ND, Not determined.

Figure 3. Reducing power of *B. variegata* L. bark extract/fractions.

Figure 4. The protective effect of different extract/fractions of the bark of *B. variegata* L. against H_2O_2 -induced oxidative damage studied through DNA nicking assay. Lane 1: pBR322 DNA+ Double distilled water; Lane 2: pBR322 DNA + Fenton's reagent; Lane 3: pBR322 DNA + MEB+ Fenton's reagent; Lane 4: pBR322 DNA + HEB+ Fenton's reagent; Lane 5: pBR322 DNA + CFB+ Fenton's reagent; Lane 6: pBR322 DNA + EAB+ Fenton's reagent; Lane 7: pBR322 DNA + NBB+ Fenton's reagent; Lane 8: pBR322 DNA + REB+ Fenton's reagent.

If multiple nicks or double strand breaks occur, nicked circular form gets converted to linear form (Form III). In Figure 4, it is clearly observable that co-incubation of plasmid DNA with *B. variegata* bark extract/fractions have significantly inhibited the conversion of Form I DNA to Form II and III. Similar results were obtained in a study conducted by Prakash et al. (2007), where it was observed that red onion peel, rich in polyphenols, was able to protect pBR322 plasmid DNA against the damage caused by Fenton's reagent. *Moringa oleifera* Lamarack (Moringaceae) extracts have also been reported to inhibit the $OH^\cdot$ dependent damage of puc18 plasmid DNA (Singh et al., 2009).

Correlation of phytochemical contents with antioxidant activities

To determine the relationship between TPC/TFC levels, DPPH scavenging activity and reducing power of extract/fractions of *B. variegata* bark, Pearson correlation analysis was carried out. The results revealed that the total phenolic content correlated significantly ($r = 0.8303$; $p \leq 0.05$) with DPPH radical scavenging activity whereas its correlation with reducing power was relatively low ($r = 0.7679$; $p \leq 0.10$). A very significant correlation ($p \leq 0.01$) was observed among the amount of total flavonoids present and the ability of extract fractions to scavenge DPPH radicals ($r = 0.9726$) as well to reduce Fe^{3+} ions ($r = 0.9581$). Further, the DPPH scavenging activity correlated extremely highly ($r = 0.9741$; $p \leq 0.001$) with the reducing power of extract/fractions (Table 3).

Table 3. Correlation between total phenolic/flavonoid content and antioxidant activity.

Parameter	Correlation value (r) [#]
TPC/DPPH	0.8303 ^a
TPC/Reducing power	0.7679 ^b
TFC/DPPH	0.9726 ^c
TFC/Reducing power	0.9581 ^c
DPPH/Reducing power	0.9741 ^d

#Correlation was assessed using Pearson correlation analysis (Microsoft excel). ^aSignificant correlation at $p \leq 0.05$; ^bsignificant correlation at $p \leq 0.10$; ^csignificant correlation at $p \leq 0.01$; ^dsignificant correlation at $p \leq 0.001$.

Conclusion

In the present study, methanol extract of *B. variegata* bark and its fractions were evaluated for antioxidant and DNA protective activity. The results of the present research work leads to the conclusion that MEB and its polar subfractions (EAB, NBB and REB) have significant antioxidant activity and potential to prevent H_2O_2 -induced oxidative damage to pBR322 DNA. The potent antioxidative activity and DNA protection ability of *B. variegata* bark extract/fractions may be attributed to their richness in phenolic/flavonoid compounds. Moreover, there was a significant correlation between antioxidant activity and the total phenolic/flavonoid content. Further isolation and characterizations of these extract/fractions is in progress so as to identify the specific antioxidative/genoprotective compounds.

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