

Original Research Article

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A Simple Method for the Characterization of Antioxidant Property of Different Extracts of Bark of Gethi (*Boehmeria rugulosa*)

**Anubhuti Sharma*, Rajesh Khulbe, Salej Sood, Pawan Kumar Agrawal,
Jagdish Chandra Bhatt and Arunav Pattanayak**

ICAR-Vivekananda Institute of Hill Agriculture Almora, Uttarakhand–263 601, India

*Corresponding author

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Boehmeria rugulosa is a multipurpose tree of Urticaceae family and is widely distributed in Indian Himalayas. The tree is used mainly for fodder and for making traditional bowls and vessels. The bark is also reported to be used for making bread of non-glutenous crops soft and tasty. The bark of the tree is known to possess medicinal properties as well. The available information on its potential bioactivity and antioxidant property, however, is meagre. The study on the antioxidant property of the *B. rugulosa* bark was, therefore, undertaken. The antioxidant property of methanol, acetone and acidified methanol extracts of the bark with three different concentrations (0.5mg/ml, 5 mg/ml and 10mg/ml) was determined by phytochemical screening, estimation of total phenolics, total antioxidant activity, total flavonoids and in vitro antioxidant screening models viz., DPPH scavenging activity, ferric reducing antioxidant power (FRAP) and ABTS scavenging activity. The methanolic extracts for 5 mg/ml concentration showed marked quantity of phenolics and flavonoids and promising levels of antioxidant activity followed by acidified methanol and acetone extracts, indicating potency of the methanolic extracts for therapeutic applications.

Introduction

Free radicals are toxic by-products formed naturally as a result of aerobic metabolism in our body. Free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Naskar *et al.*, 2011). Antioxidants are radical scavengers which protect the human body against free radicals stress that may cause severe diseases (Kokczka *et al.*, 2015). Plants are potent biochemical factories and have been components of phytomedicine since time immemorial. These plant-based active components can be derived from any part of plant like bark, leaves, flowers, roots, fruits, seeds, etc. (Venkateswara Rao *et al.*, 2013;

Anubhuti *et al.*, 2016). The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolites present in the plant. Secondary metabolites from plants have important biological and pharmacological activities, such as anti-oxidative, anti-allergic, antibiotic, hypoglycemic and anti-carcinogenic (Borneo *et al.*, 2008; Katalinic *et al.*, 2004; Mulabagal and Tsay, 2004; Sharma *et al.*, 2016). Subsequently antioxidant-based drugs or formulations have gained appreciation for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer (Adamu *et*

al., 2014; Devasagayam *et al.*, 2004). Major active antioxidant compounds include flavonoids, phenolics, lignans, and catechins etc. It has been reported that flavonoids and phenolic acids are widely distributed as secondary metabolites with antioxidant properties (Sharma *et al.*, 2013; Margaret *et al.*, 2015). The bark of *Boehmeria rugulosa* possesses high antioxidant activity and contains appreciable amounts of iron and zinc (Khulbe *et al.*, 2014). However, the extent of antioxidant content in the bark powder and the appropriate concentration with polar extract for suitable pharmaceutical application is not available. The present work, therefore, was undertaken to determine the optimum concentration at which total phenolic content and flavonoids in various extracts of the Gethi (*Boehmeria rugulosa*) are highest, as well as to examine antioxidant activity of the plant extracts using *in vitro* model system.

Materials and Methods

Plant material

Bark samples of the *Boehmeria rugulosa* were collected from north western hills of Uttarakhand, India and thoroughly dried under sunlight. Dried samples were ground into a uniform powder using a blender and stored in polythene bags at room temperature.

Total phenols, flavonoid and antioxidant properties in powdered bark samples were determined. Antioxidant activity was further evaluated by measuring DPPH activity, FRAP value and ABTS activity.

Sample preparation

Samples were extracted by a minor modification of the method of Rehman (2006) and Demiray *et al.*, (2009) using three solvent systems (methanol, acetone and acidified methanol) with three different concentrations

(0.5mg/ml, 5 mg/ml and 10mg/ml). The samples were homogenized in pestle and mortar at room temperature with the three different solvents. Extracts were centrifuged at 4000 rpm for 30 min and the residues were re-extracted under the same conditions. Supernatants were pooled and mixed. Extracts were stored at 4°C for biochemical studies.

Total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu method as described by Singleton *et al.*, (1999). Appropriate volume (0.1 ml) (1mg ml⁻¹) of the extracts was briefly oxidized with Folin–Ciocalteu reagent (0.75ml) and the reaction was neutralized with sodium carbonate. Absorbance was measured at 725nm. The results were expressed as gallic acid equivalents (g/100 g as GAE).

Total antioxidant activity

Total antioxidant activity was estimated using the method of Prieto *et al.*, (1999) with slight modification. 1.23 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added to 20 µl of the extract and the contents were incubated at 90°C for 90 min, cooled to ambient temperature and the absorbance was measured at 695 nm. The antioxidant capacity was expressed as trolox (g/100 g of extract) equivalent.

DPPH scavenging assay

DPPH assay was used for determination of free radical scavenging of extracts following the method of Chang *et al.*, (2001). The scavenging effects on DPPH radicals were determined by measuring the decrease in absorbance at 517 nm due to the DPPH radical reduction, indicating the antioxidant activity of the compounds in a short time. 10

μl of sample (5 mg/ml) was mixed with 90 μl of 50 mM Tris-HCl buffer (pH 7.4) and 200 μl of 0.1 mM DPPH-ethanol solution. When DPPH reacts with an antioxidant, it donates hydrogen and gets reduced. The resulting decrease in absorbance at 517 nm was recorded using a UV-Vis spectrophotometer (Thermo-Scientific, UV-Vis spectrophotometer). Trolox was used as a positive control. The results were expressed as trolox equivalents (g/100 g).

Ferric reducing activity power (FRAP) assay

The FRAP assay was carried out according to Stratil *et al.*, (2006) with slight modification using freshly prepared FRAP reagent. The 200 μl of methanolic extract of each sample was mixed into 1.3ml of the FRAP reagent. The tubes were vortexed and left at 37 °C for 40 min, and the absorbance was measured at 595 nm. The absorbance changes in the test mixture were compared to those obtained from standard mixture of trolox equivalent (0.1μm/l – 1.0μm/l). FRAP values were expressed as μM trolox equivalents per gm.

ABTS radical scavenging assay

The ability of the test sample to scavenge ABTS⁺ radical cation was compared to trolox standard (Re *et al.*, 1999). A stock solution of ABTS radicals was prepared by mixing 5.0 ml of 7 mM ABTS solution with 88 μl of 140 mM potassium persulfate, and kept in the dark at room temperature for 12-14 hrs. An aliquot of stock solution was diluted with phosphate buffer (5 mM, pH 7.4) containing 0.15 M NaCl in order to prepare the working solution of ABTS radicals to an absorbance of 0.70±0.02 at 734 nm. A 65 μl aliquot of sample solution was mixed with 910 μl of ABTS radical working solution, incubated for 10 min at room temperature in the dark, and then absorbance was measured at 734 nm.

The percent reduction of ABTS⁺ to ABTS was calculated according to the following equation (Amadou *et al.*, 2011):

$$\text{ABTS } (\%) = 1 - (\text{absorbance of sample}/\text{absorbance of control}) \times 100$$

Results and Discussion

The yield of extract obtained from 10 g of dry plant material using the three different solvents was highest for methanol (Table 1).

The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (Table 2). The total phenolic contents in the examined extracts ranged from 1.25 to 3.98 gallic acid/100g. The bark extract showed highest concentration of phenolic content at 5mg/ml in methanol followed by acetone and then in acidified methanol. The concentrations used to prepare extracts also showed variations. Earlier workers have also reported that high solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction (Stankovic *et al.*, 2011; Mohesh and Ammar, 2008; Zhou and Yu, 2004). Thus it can be stated that the total phenolic contents in plant extracts of the species *B. rugulosa* depends on the type of extract, i.e. the polarity of solvent used in extraction.

The concentration of flavonoids in bark extracts of *B. rugulosa* was determined using spectrophotometric method with aluminum chloride and was expressed in terms of catechin equivalent. The total flavonoid content in extracts of different polarities from *B. rugulosa* showed different results in the range of 14.97 to 38.79 gm catechin equivalents/100g. Methanolic extract of *B. rugulosa* had the highest total flavonoid content (38.79 g CE/100 g) and lowest (14.97

g CE/100 g) for the bark extract. Of the three extracts used, methanolic extract contained the highest flavonoid activity (38.79 g/100g) at 5 mg/ml concentration. However, the concentration of flavonoids in methanol extract was closer to the concentration of acetone extract concentration. The lowest flavonoid activity was measured in acidified methanol extract (14.97g/100g) at 10mg/ml. Acetone extract also contained the highest activity at 5 mg/ml, whereas acidified methanolic extract showed maximum activity at 0.5 mg/ml. These results showed that the concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation (Min and Chun-Zhao, 2005).

In order to determine the relationship between the level of total phenols and free radical scavenging activity, the total antioxidant activity from these extracts was estimated. Among the different extracts used with different concentrations, the highest total antioxidant activity of the methanolic extract was observed in 5mg/ml (35.16 gm trolox equivalent) (Table 4). These results are in agreement with Banerjee *et al.*, (2012) where they reported that the polar solvent had the higher antioxidant activities. However, different concentrations of the extracts showed remarkable variation in the activity. Acetone extract with 0.5 mg/ml concentration showed maximum activity (24.48 gm trolox equivalent) whereas acidified methanolic extract showed minimum activity (15.36 mg/ml) at 10 mg/ml concentration. All the three extract showed higher activity at 5 mg/ml with slight variation in acetone and acidified methanol extracts.

The antioxidant activity of bark extracts using the three solvent systems with three different concentrations was determined by DPPH reagent. A freshly prepared DPPH solution exhibits a deep purple colour with absorption

maxima at 517 nm. The intensity of purple colour generally decreases when antioxidant molecules quench DPPH free radicals by providing hydrogen atoms and thereby converting them into a bleached product 2,2-diphenyl-1-hydrazine. This conversion into a substituted analogous hydrazine results in a decrease in absorbance at 517 nm (Amarowicz *et al.*, 2003). The obtained values for DPPH radical scavenging varied from 1.40 to 3.27 trolox equivalent for different extracts with the methanolic extract being the most effective among the three extracts. The DPPH radical scavenging activity of the extract was higher at 5.00 mg/ml concentration i.e. 3.00 mg/g DPPH radical scavenging. These results are in agreement with Stankovic (2011) where he observed that methanolic extract from *M. peregrinum* manifested the strongest capacity for neutralization of DPPH radicals.

The antioxidant capacities using FRAP, ABTS and DPPH assays of different polar extracts from bark extracts are shown in Table 5.

In FRAP method, antioxidant capacities are in the range of 7.3–33.8 μ M trolox equivalents for different extracts. Methanolic extract exhibited highest FRAP capacity (33.8 μ M trolox equivalents), while the lowest capacity (7.3 μ M trolox equivalents) was shown by acidified extract. FRAP assay measures the ability of the plant extracts to reduce ferric to ferrous at low pH causing the formation of ferrous-tripyridyltriazine complex (Fidrianny *et al.*, 2014). The methanol extract at 5 mg/ml exhibited better ferric reducing property than acetone and acidified methanol extracts.

For ABTS scavenging activity also methanolic extract showed highest scavenging capacity (99.59 % inhibition) towards quenching of ABTS, whereas acidified methanolic acid had lowest %

inhibition (62.01%). A moderate activity was found for acetone extracts. The extraction of antioxidant substances of different chemical structure was achieved using solvents of

different polarity. The results are based on the ability of an antioxidant to decolorize the ABTS⁺ cation radical.

Table.1 The yields of solid residue after extraction and evaporation from 10 g dried plant parts

Extract yields (g)	Concentration (mg/ml)		
Solvents	0.5	5	10
Methanol	0.84 ± 0.06	0.88 ± 0.07	0.86 ± 0.02
Acetone	0.71 ± 0.01	0.72 ± 0.02	0.70 ± 0.02
Acidified methanol	0.46 ± 0.03	0.49 ± 0.02	0.46 ± 0.01

Each value is the average of three measurements ± standard deviation

Table.2 Total phenolic contents in the plant extracts expressed in terms of gallic acid equivalent (gm of GA/100g of extract)

	Concentration (mg/ml)		
Solvents	0.5	5	10
Methanol	1.68±0.09	3.98±0.22	1.39±0.05
Acetone	1.94±0.07	1.80±0.09	1.25±0.10
Acidified methanol	1.86±0.11	2.52±0.10	3.17±0.17

Each value is the average of three analyses ± standard deviation.

Table.3 Concentrations of flavonoids in the plant extracts expressed in terms of equivalent (gm of CE/g of extract)

	Concentration (mg/ml)		
Solvents	0.5	5	10
Methanol	32.53±1.85	38.79±1.26	21.22±2.13
Acetone	22.11±1.69	25.44±0.98	16.27±0.66
Acidified methanol	21.82±2.15	15.42±0.89	14.97±0.84

Each value is the average of three analyses ± standard deviation.

Table.4 Concentrations of total antioxidant activity in the plant extracts expressed in terms of trolox equivalent (gm of trolox/g of extract)

	Concentration (mg/ml)		
Solvents	0.5	5	10
Methanol	31.13±0.61	35.16±0.95	21.99±0.82
Acetone	24.48±0.55	23.14±2.97	17.23±1.96
Acidified methanol	21.28±1.58	19.18±1.05	15.36±1.23

Table.5 Concentrations of different antioxidant activity (DPPH, FRAP, ABTS) in the bark extracts

	DPPH			FRAP			ABTS		
	0.5 (mg/ml)	5 (mg/ml)	10 (mg/ml)	0.5 (mg/ml)	5 (mg/ml)	10 (mg/ml)	0.5 (mg/ml)	5 (mg/ml)	10 (mg/ml)
Solvents									
Methanol	2.71±0.00	2.7±0.00	1.4±0.00	17.16±.205	33.8±.0005	13.7±.006	69.742±1.605	88.145±1.383	99.599±.308
Acetone	3.27±.004	3.0±.001	1.5±.000	20.51±.221	26.8±.017	7.3±.004	65.575±7.524	76.339±0.394	97.554±.000
Acidified methanol	2.93±.010	2.5±.000	1.4±.000	30.7±.050	24.8±.007	14.2±.006	62.007±5.872	80.964±0.345	98.283±0.00

Figure.1a Colinear relationships between nutritional parameters studied at 0.5 mg/ml concentration

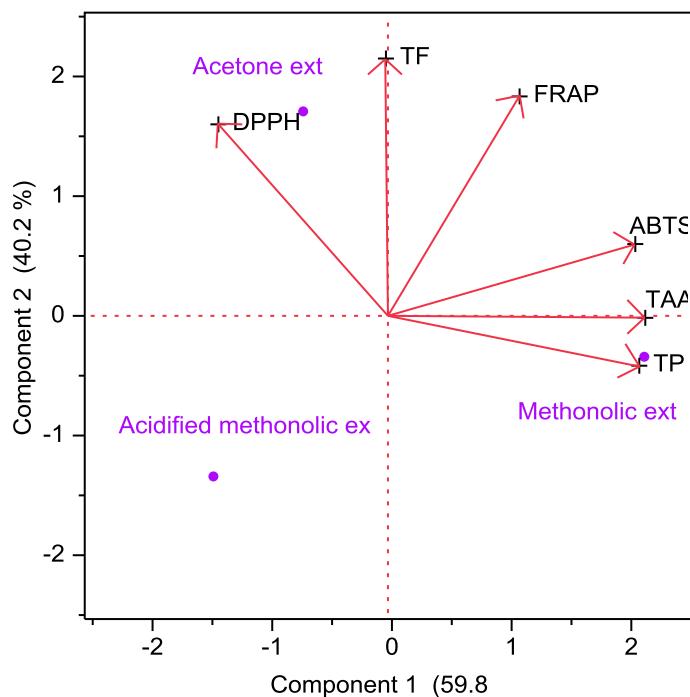


Figure.1b Colinear relationships between nutritional parameters studied at 5 mg/ml concentration

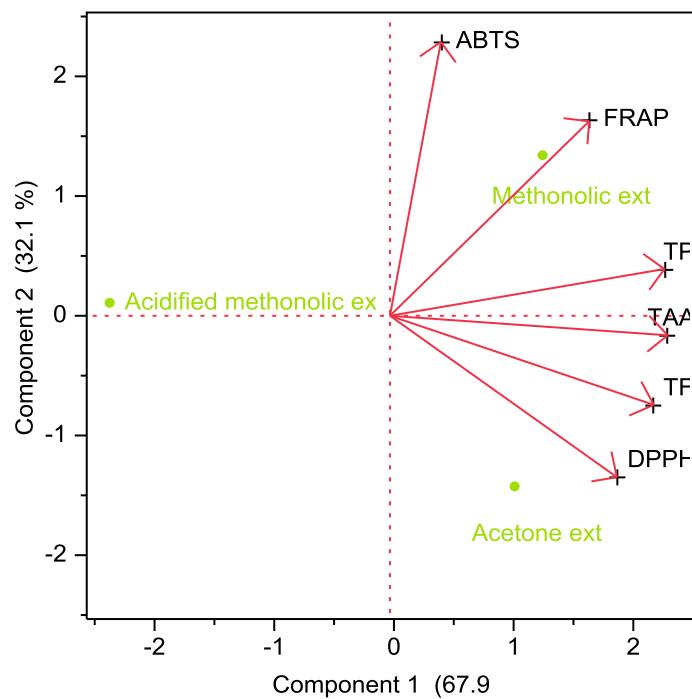
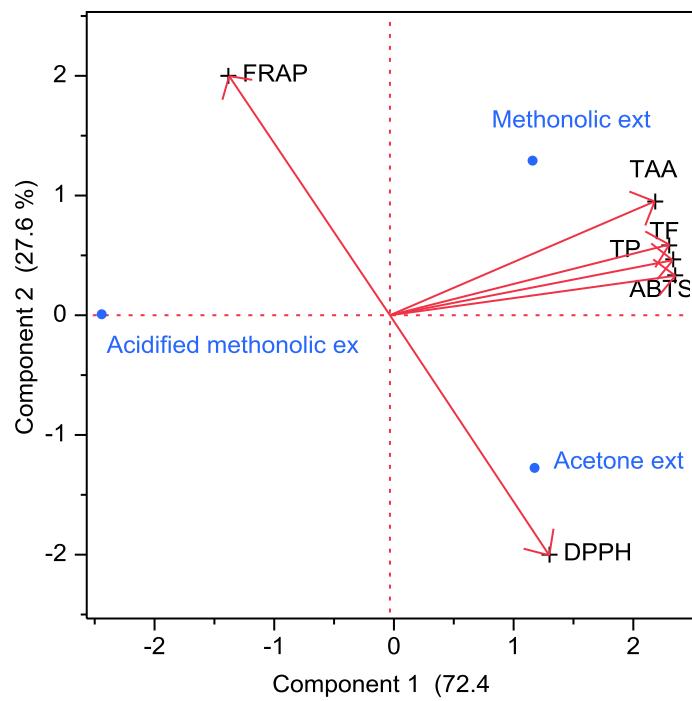


Figure.1c Colinear relationships between nutritional parameters studied at 10mg/l concentration



Phenolic compounds have antioxidant properties and can protect against degenerative diseases, such as heart diseases and cancer, in which reactive oxygen species like superoxide anion, hydroxyl radicals and peroxy radicals are involved (Rhodes and Price, 1997; Tosun *et al.*, 2009). A significant linear correlation was found between the values for the concentration of phenolic compounds and the antioxidant activity of extracts from *B. rugulosa* (Figure 1a,b,c). Similar results with high linear correlation between the values of phenol concentration and antioxidant activity were observed by Canadanovic-Brunet *et al.*, (2008), Borneo *et al.*, (2008), Katalinic *et al.*, (2004) and Yeasmen and Islam (2015).

Plant phenolics have been recognized to be a therapeutic target for cancer treatment and cardiovascular disease in the next decades (Fidriyani *et al.*, 2014) and also have the ability to prevent oxidant potentials of free radicals as natural source of antioxidants. Stankovic (2011) reported the presence of high concentrations of phenols in the extracts obtained using polar solvents. They also reported that extracts with highest antioxidant activity have the highest concentration of phenolic acids also. Numerous other studies also mention that phenolic content of plants may contribute directly to their antioxidant action (Koczka *et al.*, 2015; Tosun *et al.*, 2009; Stankovic, 2011). Thus, phenolic compounds are potentially health promoting due to their antioxidant property.

Flavonoids are a group of polyphenolic compound with known free radical scavenging activity (Frankel *et al.*, 1995). A positive correlation between total phenolic content and free radical scavenging activity has been reported earlier also (Koczka *et al.*, 2015). Thus, it can be inferred that the polyphenolics present in Gethi (*Boehmeria rugulosa*) contribute to the antioxidant

property. Methanolic extract of gethi bark showed high value of total phenolics and flavonoids indicating high antioxidant activity in the subsequent assays.

The methanol extracts of leaves of *Acorus calamus* have also shown striking DPPH scavenging activity (at 20 μ g/mL), ferric ion chelating potential (at 18.8 μ g/ml) and reductive ability (concentration dependant) whereas, methanol extract of rhizome exhibited better superoxide radical scavenging potential (at 30.5 μ g/mL) (Devi and Ganjewala, 2011). Further, it has been reported earlier also that total phenolic content and total flavonoid content of Gethi bark powder is substantially higher than finger millet and sorghum (Khulbe *et al.*, 2014).

In this study we report that all the three extracts under study exhibited concentration dependent free radical scavenging activity, and also that at 5mg/ml concentration of methanol extract of Gethi bark powder exhibited higher phenolic acid content flavonoid content, total antioxidant activity with slight variations. The higher antioxidant activity and higher content of health-promoting phenolics and flavonoids in Gethi bark powder make it additionally beneficial as a food ingredient.

In conclusion the results of the present study reveal the potential value of Gethi (*Boehmeria rugulosa*) bark in pharmacy and therapeutics. Methanolic extract exhibited greater potency for extraction of phenolic compounds from Gethi (*Boehmeria rugulosa*) than other solvents showing that solvents of high polarity yield highest concentration of phenolic compounds, flavonoid content and total antioxidant activity in the extracts. The high content of phenolic compounds and significant linear correlation between the concentration of phenolic compounds,

flavonoid content and antioxidant activity indicated that these compounds contribute to the high antioxidant activity of the bark. Based on this information it may be concluded that *Boehmeria rugulosa* is a good source of antioxidant substances of high importance. Further studies on this plant species need to be directed on the formulation of natural pharmaceutical products of high value using its natural active components.

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