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Evaluation of Antioxidant and Antidiabetic capacity of plant *Boehmeria rugulosa* Bark

Anchal[1]*, Abha shukla[1], R.K. Shukla[2] and Ritu tyagi[1]

[1]Department of chemistry, Kanya gurukula campus, Gurukula Kangri Vishwavidyalaya, Haridwar, Uttarakhand, India
[2]Department of Chemistry, Gurukula Kangri Vishwavidyalaya, Haridwar, Uttarakhand-249407, India

Abstract: In the present study, phytochemical screening, antioxidant and antidiabetic activities of different solvent extracts of Boehmeria rugulosa bark investigated. Successive extraction was carried out with different solvents (petroleum ether, acetone, ethyl acetate and ethanol), using Soxhlet extractor. These extracts were screened for the presence of chemically active compounds by standard methods. The results revealed the presence of saponins, tannins, flavonoids, sugars, terpenoids etc. Among the various extracts acetone has highest Total phenolic content (TPC) (709.931±0.174mg GAE/g dw). Results indicates that the acetone extract of bark exhibit the efficient 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (6.832±0.677µg ml^{-1}) and highest Ferric reducing antioxidant power (441.21±1.499 µM/ml, FRAP value =2.157). Acetone extract of boehmeria rugulosa bark also possessed the significant inhibition activities on α-amylase (668.547±0.02 µg ml^{-1}) and α-glucosidase (691.546±1.466 µg ml^{-1}) enzyme. All extracts exhibited outstanding antidiabetic as well as antioxidant activity. Therefore, the results indicates that the Boehmeria rugulosa bark can served as potential antioxidant as well as antidiabetic agent in food and pharmaceutical industries.

Keywords : Urticaceae, Boehmeria rugulosa, phytochemical, phenolic content, DPPH, FRAP, alpha glycosidase, Alpha amylase etc.

Introduction

From the ancient time plants are the major source of many pharmacological activities due to presence of biological active constituents, known as phytoconstituents such as alkaloids, tannins, flavonoids, terpenoids, steriods, carbohydrates, protein and phenolic compounds etc. The mixture of these phytoconstituents may provide better protection than single phytochemical due to their united effects (1).Diabetes mellitus is a chronic disorder caused by partial or complete deficiency of insulin, which resulting the glucose accumulates in the blood, leading to various chronic complications (2). Many studies have been focused on the inhibition of α-Amylase and α-glucosidase enzymes, which are responsible for hydrolysis of starch to oligosaccharides and monosaccharides, thus avoiding a sharp increase in the blood glucose level and eliminate the symptoms of diabetes(3). Furthermore, Interest in finding the antioxidant ability of phenolic compounds in plants could be attributed to their properties such as reducing agents, hydrogen donors, singlet hydrogen quenchers and/or metal ion-chelators (4). Therefore, natural antioxidants can also prevent the key enzymes α-amylase, α-glucosidase and restraint the post-prandial hyperglycemic conditions which are a probably approach to rehabilite the type 2

diabetes mellitus (5). Phenolic compounds are commonly found in both edible and nonedible plants, and they have been reported to have various biological activities, including antioxidant activity and anti-diabetic. The plant constituents play an important role in the maintenance of health and protection from coronary heart disease and cancer (6). Boehmeria rugulosa is an important medicinal plant belonging to the Urticaceae family. The aqueous extract of leaves is used for diabetes mellitus and paste obtained from the stem bark has been used as a remedy bone for bone fractured (7). Bark also used as soap and detergent (8), dying cloths (9) cut and wounds (10) by the local communities. So till date the present study reveals that no more scientific data was reported on the bark of Boehmeria rugulosa.

Thus in the present study, we analysed the biological activities of the crude extract from the bark of B. rugulosa. To better understand the biological activities of B. rugulosa bark, we determined the relationship between the amounts of phenolic compounds and antioxidant activities. To determine the potential of B. rugulosa bark extracts as antidiabetic agents, we investigated the effect of extracts on the activity of α-glucosidase and α-amylase enzymes.

Experimental Section

Chemical and reagents

2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) (Sigma Aldrich), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich), Folin's ciocalteau phenol reagent (Merck), Gallic acid (Loba chemie), Ascorbic acid (Rankem India), Tris buffer (Merck), p-Nitrophenyl-α-D-glucopyranoside [p-NPG](SRL Pvt. Ltd.), α-amylase (SRL Pvt.Ltd), Dimethyl sulphoxide [DMSO](Merck), 3,5-Dinitrosalicylic acid [DNSA] (SRL Pvt. Ltd.); α-glucosidase (SRL Pvt Ltd); Acarbose (Bayer India Limited),Sodium carbonate (CDH), Petroleum ether (Merck), Ethanol (Merck), Ethyl acetate (Merck), Acetone (Merck) were purchased. All other solvents and chemical used were analytical grade.

Collection of plant material

The bark of plant Boehmeria rugulosa was collected from the rishikesh region. Voucher specimens have been deposited at the Herbarium of the Botanical Survey of India Dehradun in November 2015 with accession No. 115901.A voucher specimen has been deposited in medicinal plants Herbarium Department of Chemistry, Kanya Gurukula campus, Gurukula Kangri Vishwavidyalaya with registry no. 1/4.The plant materials were washed, dried in shade and grinded to powder and stored in polythene begs for further use.

Preparation of Extracts

Soxhlet extractor was used for the preparation of the extracts. Successive solvent system was used for the extraction. Briefly 100 gm of powdered of bark were loaded in thimble of soxhlet extractor and extracted with petroleum ether, ethyl acetate, acetone, ethanol with increasing order of their polarity. A minimum of 60 cycles of siphoning was done for successive solvent, and the process was continued for 60 hrs until the solvent in the extractor siphon tube became colourless. Extracts were concentrated at reduced pressure in a rotary vacuum evaporator and refrigerated until further use.

Phytochemical screening

Phytochemical analysis for various phytoconstituents obtained from the Bark extracts of B. rugulosa was undertaken using standard qualitative methods [11-12]. The obtained extracts were screened for the presence of biologically active compounds like alkaloids, carbohydrates, glycosides, protein, amino acid, steroids and triterpenoids, fats and oil, tannins and phenolic group, flavonoids etc.

Total phenolic content

The total phenolic content was determined using spectrophotometric method (13-14) with some little modification. Extracts were diluted with methanol to form a concentration of 1000 µg ml^{-1}. The reaction mixture was prepared by mixing of 1 ml of extract, 10 ml of 10% Folin-Ciocalteu's reagent dissolved in water, and 8 ml of 7.5 % sodium carbonate was added after 8 minutes. Further, total volume is made up to mark by adding distilled water in volumetric flask (20 ml).The complete reaction mixtures were incubated for about 45 min in the dark and at room temperature of about 25°C±2. The same procedure was followed with gallic acid

standard dilutions range of (25-700 µg ml^{-1})and also with blank where methanol is used in place of extract. After incubation, the absorbance was measured at 765 nm with UV-VIS spectrophotometer. Calibration curve of gallic acid was use for calculations. The total phenolic content of extracts was expressed as mg gallic acid equivalents (GAE) / gram of dry mass by following equation.

$T = C \times V/M$
Where,
T= Total phenolic content mg/gm of plant extract in GAE,
C= Concentration of Gallic acid from the calibration curve,
V = Volume of the extract in ml,
M =Weight of the plant extract in gm.

Antioxidant activity

DPPH free radical scavenging assay

The extracts were examined for antioxidant activity. The antioxidant activity of the plant extracts were investigated using DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay [16-18]. A working solution of 0.004% was freshly prepared by dissolving 10 mg of DPPH in 250 ml of methanol. The various concentrations of extracts (1-1000 µg ml^{-1}) were prepared in methanol. 3ml of DPPH solution was added to 1ml of each sample. This reaction mixture put in dark for 30 minutes. After 30 minutes, absorbance was measured at 517 nm. Ascorbic acid was used as a standard. The scavenging activity was calculated as follows:

[DPPH radical scavenging activity (%) = [(Abs. of control – Abs. of sample)/ (Abs. of control) × 100]

IC_{50} of each extract and standard ascorbic acid was calculated by graphical method by plotting % inhibition *vs* concentration.

Ferric reducing antioxidant potential assay (FRAP)

Ferric reducing antioxidant power (FRAP) value was calculated using the method of Benzie and Strain (19). The FRAP solutions was prepared by mixing of 300 mM acetate buffer (3.1g NaOAc .$3H_2O$ and 16 ml Glacial HOAc), pH 3.6, 10mM TPTZ (2, 4, 6- tri-(2-pyridyl)-1, 3, 5-triazine) solution in 40 mM HCL, and 20 mM $FeCl_3.6H_2O$ solution. The FRAP reagent was prepared mixing 300 mM acetate buffer (pH=3.6), 10 mM TPTZ and 20 mM $FeCl_3$.6H2O in a ratio of 10:1:1 at 37°C. The reaction mixture contained 1 ml of extract (100µg ml^{-1}) and 10 ml of working FRAP reagent. The mixture was incubated at 37°C for 30 min. The antioxidant potential of samples was determined from standard curve plotted using ascorbic acid in the concentration range between (100 - 600 µM). Working FRAP reagent serves as blank and 1ml of methanol with 10ml of working FRAP reagent act as control. Calculations were made by calibration curve. Results were expressed as µM mL^{-1}.

Antidiabetic Activity

Anti-diabetic activity of each extract was assessed by alpha amylase and alpha glycosidase inhibitory method.

Alpha-Amylase Inhibition Activity

The α-amylase inhibitory activity of extracts was performed using DNSA method with a little modification (20).

Briefly, 1 ml of each solution of different concentration (1-5000 µg ml^{-1}) of extract and standard acarbose in DMSO was incubated with 1 ml of α-amylase (concentration 3 mg ml^{-1} in 20 mM phosphate buffer containing (6.7 mM NaCl, pH 6.9) for 30 min at 37ºC. After pre incubation, 1 ml of 1% starch solution in 20 mM phosphate buffer, pH 6.9, was added. The reaction mixtures were then incubated for 15 minutes at 37ºC. The reaction was stopped by adding 1 ml of DNSA color reagent (96 mM 3,5-dinitrosalicylic acid and 5.315 M sodium potassium tartrate in 2 M (NaOH). The tubes containing resultant mixture were then incubated in a boiling water bath for 5 min and then cooled to room temperature. The absorbance was taken at 540 nm with a UV-Vis spectrophotometer (Agilent Technologies cary-60 spectrophotometer) after diluting each tube with 9 ml of deionised water. For correcting background absorbance (absorbance due to extracts or standard) the

enzyme was replaced by 1 ml buffer solution with similar test procedure. The α-amylase inhibitory activity was calculated by equation.

$$\text{α-amylase inhibitory activity (\%Inhibition)} = \frac{[(AC^{+} - AC^{-}) - (AS - AB)]}{(AC^{+} - AC^{-})} X100$$

Where AC^{+} represents absorbance of pure control having 100% enzyme activity (DMSO and Enzyme), AC^{-} symbolize absorbance of blank for pure control having 0% enzyme activity (DMSO and Buffer), AS represent absorbance of sample or standard (sample/standard and Enzyme) and AB symbolize for background absorbance due to sample and standard (sample/standard and Buffer). IC_{50} of each extracts and standard acarbose was calculated by graphical method by plotting % inhibition *vs.* concentration.

Alpha-Glucosidase Inhibition Activity

The α-glucosidase inhibitory activity of extracts was determined according to cetto *et al*. with a little modification (20). Briefly, 1 ml of each solution of different concentration (1-5000 µg ml^{-1}) of extracts or standard acarbose in DMSO was incubated with 1 ml of α-glucosidase (1U ml^{-1} in 100 mM phosphate buffer pH 6.8) for 30 min at 37ºC. After pre incubation, 1 ml of, p-NPG in 100 mM phosphate buffer, pH 6.8, was added. The reaction mixtures were then incubated for 15 minutes at 37ºC. The reaction was stopped by adding 4 ml 0.5 M tris buffer. The absorbance was taken by UV-VIS spectrophotometer (Agilent Technologies cary-60) at 410 nm. For correcting background absorbance the enzyme was replaced by 1 ml buffer solution with similar test procedure. The % inhibition and IC50 was calculated in similar way as mentioned in α-amylase activity. Earlier 0.1 M NaOH was used to stop the reaction but it brought auto degradation of p-NPG which causes a steady increase in absorbance at 410 nm.

Statistical analysis

All determinations were carried out in triplicates and data were analyzed by ANOVA followed by Tukey's multiple comparisons test for significant differences. Values were considered significant at $p<0.0001$.

Result And Discussion

Extractive yield

Percent extractive values of various extracts are depicted. This shows that Ethanol has the maximum yield and acetone has the lowest yield. The extractive yield with their consistency after complete removal of respective solvent is shown in table 1 below. The extractive yields in (% w/w) are illustrated in Table 1.

Table 1: Extractive yield of different extracts of B. rugulosa Bark

Extracts	% yield	Consistency
Petroleum ether	2.634	Oily, viscous
Ethyl acetate	3.710	Semisolid
Acetone	2.308	Sticky
Ethanol	3.752	Sticky

Table 2:Phytoconstituents present in different extracts of B. rugulosa Bark

Phytoconstituents and Test performed		**Extracts**			
		PE	**EA**	**AC**	**ETH**
Alkaloid	Mayer's test	-	+	+	+
	Wagner's Test	-	+	+	+
	Hager's Test	-	+	+	+
	Dragendroff's test	-	+	+	+
Carbohydrate	Molisch's Test	+	+	+	+
	Fehling's Test	+	-	+	+
	Benedict's Test	-	+	+	+

	Barfoed's test	-	-	+	+
Flavonoids	Alkaline test	-	+	+	+
	Lead acetate test	-	+	+	+
	Shinoda Test	-	+	+	+
	Sulphuric acid test	-	+	+	+
Tannins	Ferric chloride test	-	+	+	+
Glycosides	Keller-Killiani Test	+	+	+	+
	Legal's Test	+	+	+	+
	Borntrager's test	+	+	+	+
Terpenoid	Liebermann burchard test	+	+	+	+
	Salwoski test	+	+	+	+
	Salwoski test (Triterpenes)	+	+	+	+
Steroids	Liebermann burchard test	-	+	+	+
Fat and Oil	Saponification test	+	-	-	-
	Filterpaper test	+	-	-	-
Saponin	Foam test	-	+	+	+
Protein and amino acid	Ninhydrin	-	-	-	-
	Biuret	-	-	-	-
Phytosterol	Salwoski test	-	+	+	+
	Liebermann burchard tes	-	+	+	+

+ Present,-Absent

Phytochemical screening

Phytochemicals present in bark of B. rugulosa is shown in Table 2. The bark of the B. rugulosa was contains the alkaloids, carbohydrates, flavonoids, tannins, saponins, steriods, fat and oils, amino acids and protein etc. Some of the major constituents like alkaloids responsible for analgesic and antimicrobial activity (21) Flavonoids and tannins act as antioxidant and tannins are also reported for antibacterial activity (22). Saponins are likely to demonstrate anti inflammatory, anticancer and antidiabetic and antibacterial activities. Terpenoids are also responsible for antibacterial activity (23). The presence of these phytochemicals is inductive for therapeutic value of B. rugulosa and also for the use of this plant traditionally in a number of diseases.

Table 3: Total phenolic content of Boehmeria rugulosa Bark extracts.

Extracts	TPC (mg GAE/g dw)
Petroleum ether	8.572±0.211
Ethyl acetate	73.750±0.231
Acetone	709.931±0.174
Ethanol	632.173±0.28

*****mg GAE/g means milligram of extract equivalent to gallic acid per gram of dry weight of extract. Values are expressed as mean±SD of the three replicates. P<0.0001

Total phenolic content (TPC)

TPC gives a measure of to what extent phenolic compounds are present in the extract. In plants, some of the phytoconstituents such as flavonoids, tannins, phenolic acids etc are responsible for the TPC (14). Total phenolic content of extracts were studied using Folin-ciocalteu method. Absorbance of the samples was measured at 760 nm and the amount of the total phenolic in mg GAE/gm extracts were then analyzed and interpreted. By the regression equation of gallic acid calibration curve ($y = 0.0026x+0.1015$, $R^2 = 0.9988$), the total phenolic content of each extracts was calculated and expressed as gallic acid equivalent (GAE) to facilitate the comparison. As shown in Table 3 the acetone extract of *Boehmeria rugulosa* had the highest phenolic content followed by Ethanol extract. Result shows the significant differences in total phenolic content among the four samples.

Antioxidant activity

DPPH free radical scavenging assay

Phenolic group shows the potent antioxidant activity due to its redox potential, which plays an important role in scavenging activity of four extracts (Petroleum ether, ethyl acetate, acetone and ethanol) of bark of B. rugulosa were tested using DPPH is a stable and nitrogen cantered free radical and gives the characteristic absorption at 517 nm. The purple colour of DPPH changes to yellow colour. So plants secondary metabolites which have tendency to donate radical exhibit good antioxidant power. DPPH radical scavenging activity of B. rugulosa bark extracts and their comparison with standard (ascorbic acid) is illustrated in table 4. Acetone extract exhibit (IC_{50} = 6.832± 0.677 µg/ml) good antioxidant power followed by the Ethanol (IC_{50} = 25.956±1.071 µg/ml).

Table 4:DPPH radical scavenging activity of Boehmeria rugulosa bark extracts compared with ascorbic acid

Extracts/standards	IC_{50} value ((mg/ml)
Ascorbic acid	24.001±0.009
Petroleum ether	1990.678±0.294
Ethyl acetate	68.995±0.549
Acetone	6.832±0.677
Ethanol	25.956±1.071

*Values are Means ± SEM for groups of 3 observations with $p < 0.0001$

Table 5: Ferric reducing antioxidant potential (FRAP) Assay) of Boehmeria rugulosa Bark

Extracts/standards	**Ferric reducing Antioxidant power (µM/ml)**	**FRAP value**
Ascorbic acid	249.68±5.758	2.000
Petroleum ether	74.92±0.117	0.680
Ethyl acetate	149.69±3.928	1.748
Acetone	441.21±1.499	2.157
Ethanol	294.72±0.900	2.008

*Values are Means ± SEM for groups of 3 observations with $p < 0.0001$

Ferric reducing antioxidant potential (FRAP Assay)

Antioxidant activity determined by FRAP method is shown in Table 5. FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{+3} TPTZ] complex and producing a coloured ferrous tripyridyltriazine [Fe^{+2} TPTZ]. Generally, the reducing properties are associated with the presence of compounds which exert the action by breaking thr free radical chain by donating a hydrogen atom or an electron to the metal atom. FRAP assay treats the antioxidants in the sample as a reducant in a redox-linked colorimetric reaction.

In the present study, FRAP assay is used because it is comparatively more accurate, quick and reaction is reproducible and linearly related to the molar concentration of the standard antioxidants. The results were expressed as µM/ml using the standard curve equation (y=0.0016+0.0066, R^2 =0.9886) where y is the absorbance at 593 nm and X is the ferric reducing antioxidant ability in 50 µg/ml of extracts. The unit µM/ml means the quantity of Fe^{+3} in the µM that can be reduced to Fe^{+2} by per ml of extract or ascorbic acid. The higher the FRAP value he greater is the antioxidant activity (20).

The acetone extract of Boehmeria rugulosa bark possessed a good reducing power and FRAP activity followed by the ethanol extract. Acetone extract has the higher FRAP value than the standard.

Antidiabetic activity

Alpha-Amylase Inhibition Activity

The *in vitro* α- amylase inhibitory activity of *Boehmeria rugulosa* bark extracts compared with acarbose is illustrated in Table 6. IC_{50} value is the concentration of extract or standard drug which is required to inhibit 50 percent of the enzyme in reaction mixture**.** All the extracts signifies a dose dependent α- amylase inhibitory activity. Acarbose showed percentage alpha amylase inhibition of 3.756% - 89.239% on varying concentration from (1-5000 μg ml^{-1}) with an IC_{50} value 617.23 μg ml^{-1}. Lower IC_{50} value corresponds to greater potency and better therapeutic efficacy. Acetone extract reflects the highest alpha amylase inhibitory activity (IC_{50} = 668.547±0.02 μg ml^{-1}). The IC_{50} value of acetone extract is nearly comparable with acarbose and thus can be regarded as an excellent inhibitory activity.

Table 6: Alpha amylase inhibitory activity of Boehmeria rugulosa bark extracts compared with acarbose

Extracts/standards	IC_{50} value (μg ml^{-1})
Acarbose	617.23±0.15
Petroleum ether	-
Ethyl acetate	1473.246±0.11
Acetone	668.546±0.02
Ethanol	1374.021±0.059

*Values are Means ± SEM for groups of 3 observations with $p < 0.0001$

Table 7: Alpha glucosidase inhibitory activity of Boehmeria rugulosa bark extracts compared with acarbose

Extract/Standard	IC50 value (μg ml-1)
Acarbose	358.42 ± 1.52
Petroleum eyher	-
Ethyl acetate	807.221±2.791
Acetone	691.546±1.466
Ethanol	871.022±1.115

* Results are expressed as mean of 3 values ± standard deviation

Alpha-Glucosidase Inhibition Activity

The *in vitro* α- glucosidase inhibitory activity of *boehmeria rugulosa* bark extracts compared with acarbose is illustrated in Table 7. Theα-glucosidase inhibition on changing the concentration of each extract and helps in estimation of IC_{50} value of each extract as well as standard acarbose. Again the highest alpha glucosidase inhibitory activity was demonstrated by acetone extract (IC_{50} = 691.546±1.466 μg ml^{-1}) followed by ethyl acetate extract (IC_{50} = 807.221±2.791μg ml^{-1}). The IC_{50} value of acetone extract is better indicates its extremely potent nature.

Previous literature evidenced alkaloids, tannins and flavonoids as potent antidiabetic activity (24) and these phytoconstituents are cited in all extracts except petroleum ether extract. Further naturally occurring extracts could be well tolerated as antidiabetic agent in comparison to synthetic inhibitors with a number of side effects like abdominal discomfort, diarrhoea and flatulence etc.

Conclusion

The present study was aimed to perform phytochemical evaluation, total phenolic content, antioxidant activity and antidiabetic activity of each extract of *Boehmeria rugulosa bark*. Since this is the first report regarding phytochemical screening and antidiabetic activity, antioxidant activity along with total phenolic content on bark of this plant and provide referential information for correct identification. These two activities of bark established a scientific proof for its traditional claim.

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