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PHYTOCHEMICAL SCREENING, TOTAL PHENOLICS, FLAVONOID CONTENT AND ANTIOXIDANT POTENTIAL OF DIFFERENT PARTS OF *BETULA UTILIS* D. DON FROM KASHMIR HIMALAYA

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Keywords:

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ABSTRACT: The aim of this study was to compare the phytochemical composition, total phenolic content (TPC), total flavonoids content (TFC) and antioxidant activity of aerial and underground parts of *Betula utilis*. Qualitative phytochemical screening of the three parts indicates the presence of alkaloids, flavonoids, phytosterols, tannins, terpenoids, phenols, proteins, amino acids, anthraquinone glycosides and steroids. Total phenolic content determined by Folin-Ciocalteu method ranged from 0.03 ± 0.003 to 33.6 ± 2.1, 0.34 ± 0.02 to 23.6 ± 0.67 and 0.08 ± 0.005 to 16.9 ± 1.9 mg/g of dry weight of roots, leaf and bark extracts, respectively, expressed as gallic acid equivalents. The total flavonoid contents as measured by aluminium chloride method ranges from 0.2 ± 0.003 to 7.3 ± 0.31, 0.3 ± 0.08 to 4.3 ± 0.08 and 0.3 ± 0.008 to 1.3 ± 0.03 mg/g of dry weight of leaves, bark and root extracts respectively, expressed as rutin equivalents. The results showed that TPC and TFC values were higher in methanol extract of root (33.6 ± 2.1) and leaves (7.3 ± 0.31). The antioxidant capacities in the forms of DPPH (2, 2-diphenyl-1-picrylhydrazyl) were evaluated by spectrophotometric methods. A direct correlation between total phenolic content of leaves and free radical scavenging activity was revealed (r = 0.72).

INTRODUCTION: Phenolic compounds are common plant secondary metabolites with many physiological functions in plants, due to antioxidant properties that has positive effects for human health [1, 2]. Antioxidants play important roles in counteracting pathogenic processes related to cancer, cardio-vascular disease, muscular degeneration, cataracts and asthma, and can enhance immune function. Antioxidant defences shield the body from the adverse effects of free radicals generated as by-products of normal metabolism [2].

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In addition to anti-oxidative roles, phenolic compounds from different plants had been reported to have antimicrobial activity against various pathogenic micro-organisms [3, 4]. There is an increasing interest in medicinal plants as an alternative to synthetic drugs, especially against microbial agents because of the growth of antibiotic resistance [5]. The search for new antimicrobial agents like phenolic compounds has subsequently turned out to be crucial.

A huge number of plants are well known in traditional medicine system for their medicinal and therapeutic potentials worldwide alike *Betula utilis* which is a deciduous tree belonging to the family Betulaceae. It is a moderate size tree that can grow up to 20 m in height in alpine Himalayas. The bark is shining, smooth, reddish white or white with

horizontal lenticels. The outer bark is having numerous thin papery layers, exfoliating in wide horizontal rolls. Its bark is used in the treatment of various diseases such as wound healing, skin disinfectant, bronchitis, convulsions, leprosy and diseases of the blood and the ear [6]. It is used as an antiseptic, carminatic and antibacterial agent [7]. The purpose of this study is to evaluate the total phenolics content, total flavonoids content and anti-oxidant activity of *B. utilis* bark, leaves and roots extracts by using various solvents.

MATERIALS AND METHODS:

Chemicals: HPLC grade methanol, hexane, petroleum ether, ethanol, dichloromethane and chloroform were procured from the Merck. Specialties Pvt. Ltd. gallic acid, rutin, aluminium trichloride, DPPH (1, 1–diphenyl - 2 - picryl - hydrazyl), and ferric chloride were purchased from Sigma-Aldrich.

Plant Material: The aerial parts of the plant (bark and leaves) and roots were collected from Gulmarg at an altitude in the months of August-September 2016. The voucher specimen was deposited to the herbarium, Department of Botany, Punjabi University Patiala, India (accession no. = 61231). The aerial and underground parts of the plant were washed thoroughly with distilled water to clean the dust and topical fauna. Samples were air dried, powdered, sieved, weighed and stored in air tight container and subsequently referred to as powdered drug.

Extraction Procedure: Ten grams (10 g) of powdered plant materials were successively extracted by maceration with water, methanol, hexane, petroleum ether, ethanol, dichloromethane and chloroform for 72 h stirring. The macerates were filtered and concentrated using a rotary evaporator at 40-50 °C. The obtained extracts were stored at 4 °C until further use.

Phytochemical Analysis: Phytochemical analysis of dichloromethane (DCM), hexane, petroleum ether, chloroform, ethanol, methanol and water extract was done for presence/absence of metabolites such as flavonoids, alkaloids, terpenoids, cardiac glycosides, saponins, phenolics, proteins, amino acids, anthraquinone glycosides, phytosterols, tannins and steroids [8] .

Determination of Total Phenolic content (TPC): The total phenolic content (TPC) was determined by the Folin-Ciocalteu method [9,] with some modifications. 0.5 ml of test assay was treated with 0.5 ml Folin-ciocalteu reagent (1N) and 20 % sodium carbonate solution sequentially. The sample is mixed over vortex. The reaction mixture was left for 30 minutes after mixing. The solution was maintained up to 12.5 ml with water. The absorbance was recorded at 765 nm wavelength. The content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Determination of Total flavanoid content (TFC): Total phenolic contents (TPC) in extracts were measured as described by Nadhiya and Vijaya-lakshmi [10]. To 1 ml of extract (100 µg mL-1), 3 ml of methanol, 0.2 ml of 1 M potassium acetate, 0.2 ml of 10 % aluminium chloride and 5.6 ml of distilled water was added and left at room temperature for 30 minutes. Absorbance of the mixture was read at 420 nm using UV spectro-photometer. The content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

***In-vitro* Antioxidant Potential by DPPH Radical-Scavenging Activity:** The ability of the plant extract to scavenge DPPH free radicals was assessed by the method of Shahat *et al.*, 2015 with slight alterations [11]. The stock solutions of extracts were prepared in methanol to accomplish the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 200, 150, 100, 50 and 10µg/ml. Diluted solutions (1 ml each) were mixed with 1 ml of methanolic solution of DPPH in concentration of 1 mg/ml.

After 30 min incubation in darkness at room temperature (23 ^{o}C), the absorbance was recorded at 517 nm. Control sample contained all the reagents except the extract. Percentage inhibition was calculated using equation 1, whilst IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard error (n = 3).

$$\% \text{ inhibition} = \frac{(\text{A of control} - \text{A of sample})}{\text{A of control}} \times 100 \quad (1)$$

RESULTS:

Preliminary Phytochemical Screening: Results obtained for qualitative screening of phyto-chemicals in stem bark and roots of *B. utilis* are presented in **Table 1**. Of the twelve phytochemicals screened for, all the twelve were found to be present in various solvent extracts. In all, more phytochemicals were found present in the stem bark than in the leaves and roots. Remarkably, cardiac glycosides, saponins, proteins, amino acids, anthraquinone glycosides, phytosterols, tannins and steroids were not present in roots but were present in stem bark. This suggests that the stem bark offers a wider array of phytochemicals than the root and leaves. From this analysis, methanolic extract of stem bark was found to have more constituents compared to leaves and root extracts.

TABLE 1: QUALITATIVE ANALYSIS OF THE PHYTOCHEMICALS IN BARK, LEAVES AND ROOTS

	Bark							Leaves							Roots						
Phytoconstituents	A	M	E	C	PE	H	DCM	A	M	E	C	PE	H	DCM	A	M	E	C	PE	H	DCM
Flavonoids	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	+	-	-	-	-	-
Alkaloids	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-
Terpenoids	+	+	+	+	+	+	-	-	+	+	-	-	-	+	-	+	+	-	-	-	-
Cardiac Glycosides	+	+	-	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
Saponins	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenolics	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
Proteins	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Amino acids	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinone glycosides	_	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Phytosterols	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tannins	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Steroids	-	-	-	+	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-
Total no. of phytoconstituents	10	11	3	6	6	2	3	1	7	7	3	2	1	2	nil	4	2	1	1	nil	nil

A*: Aqueous; M: Methanol: E: Ethanol; C: Chloroform; PE: Petroleum ether; H: Hexane; DCM: Dichloromethane

Total Phenolic and TFCs: Bark, leaves and root extracts using water and six different organic solvents (methanol, ethanol, dichloromethane, chloroform, petroleum ether and hexane) have been prepared to examine the free radical scavenging activity and contents of total phenols and flavonoid concentrations. Total phenolic content was estimated by using Folin-Ciocalteu reagent. Total phenolic content of the different extracts of *B. utilis* was solvent dependent and expressed as milligrams of gallic acid equivalents (GAE). **Table 2** summarizes that total phenolic compounds in extracts varied widely, ranging from 0.07±0.002 and 33.6 ± 2.1 mg GA/g. In methanolic, extracts from roots, very high values of total phenolic contents are measured (33.6 ± 2.1 mg GA/g). In the analysis of results for the concentrations of total phenolic compounds in all root extracts, it is noticed that the highest concentration of phenolic compounds is in the extracts obtained using solvents of high and moderate polarity. The concentrations of phenols in the bark extract range from 0.08 ± 0.005 to 16.9 ± 1.9 mg GA/g. In seven different leaves extracts, the highest content of phenols (23.6 ± 0.67 mg GA/g) is measured in the aqueous extract. In other extracts from leaves, the contents of phenolic compounds vary from 0.34 ± 0.02 to 23.6 ± 0.67 mgGA/g.

Flavonoids are the major components of the phenolic compounds. The concentration of flavonoids in plant extracts from leaves, bark and roots of *B. utilis* is determined using spectro-photometric method with $AlCl_3$. The summary of quantities of flavonoids identified in the tested extracts is shown in **Table 3**. Values designating concentration of flavonoids in the extracts of aerial and underground parts of plants are very uneven. The concentrations of flavonoids in plant extract range from 0.15 ± 0.003 to 7.23 ± 0.31 mg RU/g. The highest concentrations of flavonoids in leaves are measured in methanol, ethanol and water extracts, while DCM, chloroform, petroleum ether and hexane extracts are with small amounts of flavonoids. Concentration of flavonoids in the extracts from roots is smaller in the comparison to

extracts from bark, but there is not much variation in the values. In extracts from bark the highest concentration of flavonoids is in methanolic and ethanolic extract.

TABLE 2: EFFECT OF DIFFERENT SOLVENTS ON EXTRACTION OF TOTAL PHENOLS FROM DIFFERENT PARTS OF *B. UTILIS*

Total phenols mg GA/g DW							
Parts	**Water**	**Methanol**	**Ethanol**	**Dichloromethane**	**Chloroform**	**Petroleum ether**	**Hexane**
Bark	3.5 ± 0.4	16.9 ± 1.9	6.5 ± 0.2	1.2 ± 0.02	0.5 ± 0.02	0.08 ± 0.005	0.2 ± 0.003
Root	11.1 ± 0.27	33.6 ± 2.1	29.1 ± 0.67	0.3 ± 0.02	0.4 ± 0.03	0.03 ± 0.003	0.07 ± 0.002
Leaf	23.6 ± 0.67	21.8 ± 0.5	12.6 ± 0.38	0.7 ± 0.02	0.9 ± 0.19	0.34 ± 0.02	0.5 ± 0.02

*All analyses are the mean of triplicates ± standard error. Values are expressed as mg gallic acid/g of dry plant material

TABLE 3: EFFECT OF DIFFERENT SOLVENTS ON EXTRACTION OF TOTAL FLAVONOIDS FROM DIFFERENT PARTS OF *B. UTILIS*

Total flavonoids mg Ru/g DW							
Parts	**Water**	**Methanol**	**Ethanol**	**Dichloromethane**	**Chloroform**	**Petroleum ether**	**Hexane**
Bark	0.4 ± 0.038	4.3 ± 0.08	3.2 ± 0.012	0.8 ± 0.05	0.5 ± 0.05	0.3 ± 0.08	0.3 ± 0.11
Root	0.34 ± 0.02	1.3 ± 0.03	1.1 ± 0.02	0.8 ± 0.07	0.5 ± 0.009	0.3 ± 0.008	0.6 ± 0.04
Leaf	1.3 ± 0.018	7.3 ± 0.31	5.3 ± 0.03	0.2 ± 0.003	0.8 ± 0.6	0.2 ± 0.007	0.3 ± 0.018

All analyses are the mean of triplicates ± standard error. Values are expressed as mg rutin/g of dry plant material

DPPH Radical Scavenging Assay: It is notable that the antioxidant activity of plant extracts containing polyphenol components is because of the ability to be donors of hydrogen atoms or electrons and to catch the free radicals. The antioxidant proprieties of extracts were calculated in terms of their efficient IC_{50} concentration corresponding to the sample concentration that reduced the initial DPPH• absorbance of 50 %. These IC_{50} values are given in **Table 4**.

The obtained values for antioxidant activity range from 513.3 ± 6.9 to 43.9 ± 2.8mg/ml. The results revealed that methanolic extract of root has highest antioxidant action among all the extracts tested as denoted by the low IC_{50} value of 43.9 ± 2.8mg/ml.

Correlation Analysis between TPC, TFC and Antioxidant Activity (DPPH): To establish the suitability, reliability and relationship amongst TPC, TFC and free radical scavenging activity, correlation analysis was performed. The correlation coefficients (R) for aerial and underground plant parts are given in **Table 5**. The total phenolic content showed strong positive correlation with leaves (R=0.7198). This suggests that phenols are the main compounds responsible for the antioxidant activity in *B. utilis*.

TABLE 4: ANTIOXIDANT (DPPH SCAVENGING) ACTIVITY OF INVESTIGATED PLANT EXTRACTS PRESENTED AS IC_{50} VALUES (µg/ml)

Type of extract	Bark	Roots	Leaves
Water	112.7 ± 2.6	133.4 ± 2.9	126.3 ± 2.2
Methanol	88.0 ± 2.4	43.9 ± 2.8	85.2 ± 3.1
Ethanol	84.8 ± 2.5	46.6 ± 3.5	200.9 ± 5.2
Chloroform	101.5 ± 3.2	144.3 ± 4.7	252.9 ± 3.3
Petroleum ether	426.4 ± 5.5	504.2 ± 7.5	513.3 ± 6.9
Hexane	233.1 ± 3.5	303.5 ± 4.9	480.1 ± 5.3
Dichloromethane	86.8 ± 3.2	160.8 ± 4.6	329.7 ± 1.7

TABLE 5: CORRELATIONS BETWEEN THE IC_{50} VALUES OF ANTIOXIDANT ACTIVITIES AND PHENOLIC AND FLAVONOIDS CONTENT OF *B. UTILIS*

IC_{50} of DPPH radical scavenging activity	Correlation R2	
	Phenol	**Flavonoids**
Bark	0.18	0.21
Roots	0.46	0.51
Leaves	0.72*	0.48

Each value in the table is represented as mean ± S.E (n = 3). *indicates significance at $P < 0.05$.

DISCUSSION: The preliminary phytochemical screening tests might be valuable in the detection of the bioactive principles and consequently may lead to the drug discovery and development. The

phytochemical compounds detected are known to have therapeutic significance. For instance, alkaloids have been reported as powerful poison and many alkaloids derived from medicinal plants show biological activities like, anti-inflammatory [12], antimalarial [13], antimicrobial [14], cytotoxicity, antispasmodic and pharmacological effects [15].

Similarly, steroids derived from plants are known to have cardio tonic effect and furthermore have antibacterial and insecticidal properties [16]. These are very often used in medicines because of their their well-known biological activities. Tannins are known to have antibacterial [17], antitumor and antiviral activities [18]. These work by precipitating microbial protein thus making nutritional protein inaccessible for them. Other phytochemicals called cardiac glycosides have been utilized to treat congestive heart failure and cardiac arrhythmia [19].

In the present study total phenolic content (TPC), total flavonoid contents (TFC) and free radical scavenging activity of *B. utilis* varied among the different plant parts extracted in various solvents. Phenolics or polyphenols are secondary plant metabolites that are most usually present in plants of high medicinal value. Phenolic compounds contribute to the antioxidant potential of plants by neutralizing free radicals and preventing de-composition of hydroperoxides into free radicals referred to have therapeutic uses such as anti-oxidant, anti-carcinogenic, antimutagenic activities [20].

These are additionally known to decrease cardio-vascular risks [21]. Besides, these have a metal chelation potential [22]. Results of the assays for phenolics described in the present report, indicated a wide variation in the total phenolic content in the different extracts, showing the higher phenolic content in the methanol, which is in concurrence with Shukla *et al.,* 2017 [23]. In the present study, we found the presence of higher amounts of phenolics in root part of the plant.

Also TPC in the bark was more as reported by Pandey *et al.,* which varied from 0.41 mg/g to 14.47 mg/g gallic acid equivalent (GAE) [24]. The difference in the concentration of phenolics in the respective solvents is due to the polarity of the solvents used for extraction. Higher solubility of the phenols in polar solvents keeps higher concentration of these compounds in the extracts obtained by using the particular solvent during extraction [25]. In our present study the TPC in the bark was more as reported by Pandey *et al.,* varied from 0.41 mg/g to 14.47 mg/g gallic acid equivalent (GAE) [24].

Flavonoids are one class of secondary plant metabolites that are otherwise called Vitamin P. These metabolites are generally used in plants to produce yellow and other pigments which play an important role in the colors of plants. Flavonoids are easily ingested by humans and they seem to display important anti-inflammatory, anti-allergic and anti-cancer activities [26]. These are likewise strong antioxidants as these are capable of effectively scavenging the ROS due to their phenolic hydroxyl groups [27]. Higher amounts of flavonoids were recorded in the methanolic extract of leaf as compared to other plant parts which was also reported by Cao *et al*., in *B. pendula* [28].

Concentration of flavonoids in the extracts from bark and roots is smaller in the comparison to extracts from leaves. These results are in accordance with the literature. Several diaryl-heptanoids, phenylbutanoids, lignans and phenol glycosides have been determined in wood, bark and stem of various birch species extracts [29, 30], whereas flavonol derivatives were identified as the most abundant phenolics in birch leaves according to the results of previous studies on leaves from *Betula* sp. [31]. Polar extracts (methanol, ethanol, and water extracts) showed more flavonoids than apolar extracts.

Various techniques are utilized to determine the radical scavenging effects of antioxidants. The DPPH method is a preferred technique since it is quick, simple and reliable and does not require a special reaction and device. DPPH is a stable, synthetic radical that does not deteriorate in water, methanol, or ethanol. The free radical scavenging activities of extracts depend on the capacity of antioxidant compounds to lose hydrogen and the structural conformation of these components [32]. The DPPH free radical, which is at its maximum wavelength at 517 nm, can easily get an electron or hydrogen from antioxidant molecules to become a stable diamagnetic molecule [33]. Owing to the DPPH radical's ability to bind H, it is considered to have a radical scavenging property.

A solution of DPPH radicals prepared in methanol is converted into DPPH-H (diphenylhydrazine) molecules in the presence of an antioxidant agent, as shown in the following equation. Discoloration occurs due to the decreasing quantity of DPPH radicals in the environment. The discoloration of the DPPH therefore reflects the radical scavenging activity of the analysed extract [34].

In our present investigation, methanolic extracts exhibited a potent antioxidant activity, which is in accordance with Shukla *et al.*, 2017 [23]. The hydroxyl radical scavenging activity of methanolic root extract was found to be higher than all the extracts which can be inferred from their IC_{50} values in *B. utilis*. It was observed that the radical scavenging effect of the leaf extracts was positively correlated with their total amount of phenolic compounds (r = 0.7198). Poor correlations determined between the total phenols and radical scavenging capacity of the bark and root extracts, indicate to the synergistic or antagonist effects of molecules such as polyphenols and triterpenes present in the bark and roots of *B. utilis* [35].

CONCLUSION: The results of the preliminary phytochemical screening of the plant extracts showed presence of alkaloids, flavonoids, phyto-sterols, tannins, terpenoids, phenols, flavonoids, proteins, amino acids, anthraquinone glycosides and steroids. Results of our study suggest the great value of the species *B. utilis* for use in pharmacy and phytotherapy. In light of this data, it could be concluded that this plant is natural sources of antioxidant substances of high significance. It is noticed that the highest concentration of phenolic compounds in the extracts were obtained with solvents of high polarity; the methanolic extract showed greater power of extraction for phenolic compounds from *B. utilis*. The high contents of phenolic compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity indicated that these compounds contribute to the strong antioxidant activity.

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CONFLICT OF INTEREST: We declare that we have no conflict of interest.

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