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Original Article

Comparative Analysis of Fatty Acids and Antioxidant Activity of *Betula utilis* Bark Collected from Different Geographical Region of India

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ABSTRACT

Objective: The present study investigated the comparative analysis of fatty acid and antioxidant activity from the bark of *Betula utilis*, collected from Kashmir and Sikkim coded as BUK and BUS respectively. **Methods:** Fatty acid constituents were analyzed by GC-MS (gas chromatography mass spectroscopy) in BUK and BUS bark. *B. utilis* bark extracts of both the locations were phytochemically investigated and radical scavenging activity was evaluated by DPPH in all solvent fractions. **Results:** Phenolic content were found to be higher in methanolic extracts of BUK bark, 5.8 ± 0.1 mg/gm and flavonoid content were higher in the water extract of BUK bark, 6.16 ± 0.2 mg/gm. The radical scavenging activity was found to be higher methanolic, alcoholic and water extracts of BUK bark. The lowest IC_{50} value for radical scavenging activity of methanolic and water extracts of *B. utilis* bark collected from Kashmir were found 18.7 ± 1.1 and 18.2 ± 0.3 µg/mL, respectively. Thirteen fatty acids were identified in the sample BUK as well as BUS in which the major fatty acids were found-Palmitic acid, linoleic acid and oleic acid. BUK possess the highest amount of Palmitic acid (18.07%) in oily portion of the bark. **Conclusion:** The present study concluded that BUK and BUS extracts have shown significant antioxidant activity in comparison to standard but BUK possess potent radical scavenging activity over BUS, and higher amounts palmitic acid, linoleic acid and oleic acid was present in the extract, and may a play an important role in nutritional and pharmaceutical applications.

Key words: *Betula utilis*, Free radicals, Antioxidant, Total phenol, Flavonoid.

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INTRODUCTION

Betula utilis D. Don or Himalayan Silver Birch (*Betulaceae*) commonly known as Bhojpatra (Hindi). *B. utilis is* a moderate-sized tree that grows up to 20 m in height. The bark is smooth, shining, reddish white or white, with white horizontal lenticels, the inner layers pink. It is widely distributed in northern India and is generally known for its therapeutic applictions.[1] Traditionally, bark was used for the treatment of astringent, acrid, antibacterial, anticonvulsant, constipation, expectorant and as a tonic. The bark contains betulin, lupeol, oleanolic acid, acetyloleanolic acid, betulinic acid, lupenone, sitosterol, methyle betulonate, methyl betulate and a new triterpenoid, karachic acid. It is aromatic and has the antiseptic properties.[2,3]

The Fatty acids are widely occurring in natural fats and dietary oils and they play an important role as nutritious substances and metabolites in living organism. Recent studies have also clearly shown the important impact of fatty acids in particularly unsaturated fatty acids on human health in the prevention of, Human diseases.[5]

In the present study, the fatty acid analysis and antioxidant activity from the bark of *B. utilis* collected from two different geographical locations (Kashmir and Sikkim) in India was studied. Earlier the fatty acid composition from the bark of *B. utilis* has been reported which was collected from Uttarakhand, but comparative analysis of fatty acids and antioxidant activity not yet reported previously.

MATERIAL & METHODS

Plant Material

B. utilis bark collected from Kashmir and Sikkim codes as BUK and BUS respectively. The plant materials were authenticated at the Herbarium of the CSIR-National Botanical Research Institute, Lucknow, where voucher specimens BUK (253494) and BUS (255812) are deposited. The bark was collected and washed thoroughly, dried in shade and then powdered in a mixer-grinder and used for the further study.

Extraction and fractionation

Shade-dried bark of *B. utilis* were milled into powder and then extracted with methanol and alcohol in an extractor for 36 h. The extract was evaporated in a rotatory evaporator and dried by vacuum pump. The methanol extract was suspended in water and extracted successively with hexane, ethyl acetate, chloroform, and water for five times at room temperature. The resulting four extracts were evaporated under vacuum to dryness the hexane, ethyl acetate, chloroform and water fractions. They were quantitatively re-dissolved in methanol. The stock solution was kept at 4^0C in the dark until further analysis.

Determination of Total Phenolic Content

Total Phenolic content was determined by the folin-ciocalteu method[6] and calculated using gallic acid as a standard. 20 µl, 40 µl, 60 µl, 80 µl, 100 µl (1 mg/ml) of methanol, alcohol, hexane, ethyl acetate, chloroform and water extract of BUK and BUS (bark), 0.5 ml of the folin-ciocalteu (50%) reagent was added while mixing gently. After 2 min, 1 ml of sodium carbonate (20% solution) and 12.5 ml of distilled water was added. The content were mixed and allowed to stand for 2 h. The optical density of the samples was measured at 720 nm and total phenolic content were expressed as equivalent to Gallic acid.

Table1: Total Phenoilc and Flavonoid content present in BUK and BUS bark

Extracts	Total Phenolic Content (mg/gm)	
	BUK	BUS
Methanol	5.8 ± 0.1	4.88 ± 0.5
Alcohol	5.2 ± 0.09	3.05 ± 0.13
Hexane	2.2 ± 0.2	2.21 ± 0.02
Ethyl acetate	2.6 ± 0.1	2.90 ± 0.4
Chloroform	2.63 ± 0.2	2.48 ± 0.4
Water	5.46 ± 0.2	4.5 ± 0.5
	Total Flavonoid Content (mg/gm)	
	BUK	BUS
Methanol	5.4 ± 0.4	4 ± 0.03
Alcohol	5.5 ± 0.5	5.38 ± 0.3
Hexane	2.3 ± 0.2	3 ± 0.18
Ethyl acetate	2.2 ± 0.09	2.3 ± 0.14
Chloroform	4.1 ± 1.0	3.0 ± 0.04
Water	6.16 ± 0.2	4.0 ± 0.08

All results are presented as mean ± SEM of three assays.

Table 2: IC_{50}, total antioxidant values of different fractions of BUK and BUS bark

Extracts	DPPH radical scavenging activity (IC_{50}: µg/mL)	
	BUK	BUS
Methanol	18.7 ± 1.1	23.0 ± 2.8
Alcohol	21.1 ± 0.7	28.34 ± 1.2
Hexane	361.1 ± 13.3	131.5 ± 0.9
Chloroform	53.5 ± 0.3	109.4 ± 1.1
Ethyl acetate	29.1 ± 0.2	27.0 ± 0.3
Water	18.2 ± 0.3	21.2 ± 1.4
Quercetin[a]	13.8 ± 0.9	
Blank	-	

All results except blank control are presented as mean ± SEM of three assays; [a]: Standard antioxidant.

Table 3: Fatty acid composition from the bark of BUK and BUS

Fatty acid constituents	Area%	
	BUK	BUS
Myristic acid	1.16	0.41
Pentadecanoic acid	1.6	0.63
Palmitic acid	18.07	12.30
Margaric acid	1.23	1.15
Oleic acid	11.71	12.39
Linoleic acid	17.09	12.4
Linolenic acid	1.82	3.37
Stearic acid	1.06	0.13
Arachidic acid	3.71	2.31
Heneicosanoic acid	1.31	0.38
Behenic acid	4.63	7.04
Lignoceric acid	1.18	13.74
Cerotic acid	-	3.5
Total		
Others (Unidentified)	35.43	30.25
Identified	64.57	69.75

Table 4: Fatty acids categories in BUK and BUS

Fatty acid Category %	Extracts	
	BUK	BUS
Saturated fatty acid	33.95	41.59
Monounsaturated fatty acid	11.71	12.39
Polyunsaturated fatty acid	18.91	15.77

Determination of Total Flavonoid Content

Total flavonoid content was determined by and calculated using rutin as a standard. 20 µl, 40 µl, 60 µl, 80 µl, 100 µl (1 mg/ml) of methanol, alcohol, hexane, ethyl acetate, chloroform and water extract of BUK and BUS (bark), 1.5 ml of the sodium nitrite (5%) reagent was added while mixing gently. After that 0.15 ml aluminium chloride (10%), 1 ml of sodium hydroxide (1 N solution) was added. The content were mixed and allowed to stand. The optical density of the samples was measured at 415 nm and total flavonoid content was expressed as (1 mg/ml) rutin.

Determination of DPPH Radical–Scavenging Activity

DPPH radical-scavenging activity was determined by the method of Shimada, Fujikawa, Yahara, and Nakamura.[7] Briefly, a 2 ml of DPPH methanol solution was added to sample solution (1 mg/ml) at different volume- 20 µl, 40 µl, 60 µl, 80 µl, 100 µl. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free-radical scavenging activity. The scavenging activity of sample was expressed as 50% effective concentration (EC_{50}), which represented the concentrated of sample having 50% of DPPH radical-scavenging effect. The inhibition of the DPPH radical by the sample was calculated based on the formula below.

$$\text{Inhibition}\% = \frac{\text{Ac} - \text{As} \times 100}{\text{Ac}}$$

*Ac=Absorbance of the control

*As=Absorbance of the sample

Fatty acid analysis

Formation of fatty acid methyl ester (FAME)

The hexane extract (500 mg) in concentrated sulphuric acid (2 mL) and methanol (20 mL) was heated under reflux on a water bath for 3 h. It was cooled to room temperature and extracted with petroleum ether (3×20 mL) and water in a separating funnel. The petroleum ether extract was dried over Na_2SO_4. The extract was dried under reduced pressure at 40°C. Prepared fatty acid methyl ester (FAME) was stored for further analysis.

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was performed with a Thermo Fisher TRACE GC ULTRA coupled with DSQ II Mass Spectrometer instrument using a TR 50MS column (30 m x 0.25 mm ID x 0.25 µm, film thickness); carrier gas, helium; temperature programming, 5 min. delay for solvent, at 50°C temperature, hold time 5.0 min, rising at 4°C/min to 250°C and finally held isothermally for 5 min. The injector temperature was 230°C and carrier flow was constant flow 1 ml/min, in split mode (1:50) with injector volume 1 µl. The ion source temperature was set at 220°C, transfer line temperature was 300°C, and the ionization of the sample components was performed in EI mode at an ionization voltage of 70 eV. Mass range was used from m/z 50 to 650 amu.

Compound identification

Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library (NIST/Wiley) or with authentic compounds.[8]

Statistical analysis

All the measurements were done in triplicate for each test and statistical analysis was performed and all the data were expressed as mean ± SEM. For statistical comparison among several means, The IC_{50} value was calculated using linear regression analysis of the percent inhibition obtained using different concentrations. The regression equation was obtained and the concentration required to produce 50% effect (IC_{50}) was calculated. All the data on scavenging percentage were subjected to a one-way analysis of variance (ANOVA) (at P≤0.05) with comparison to standard.

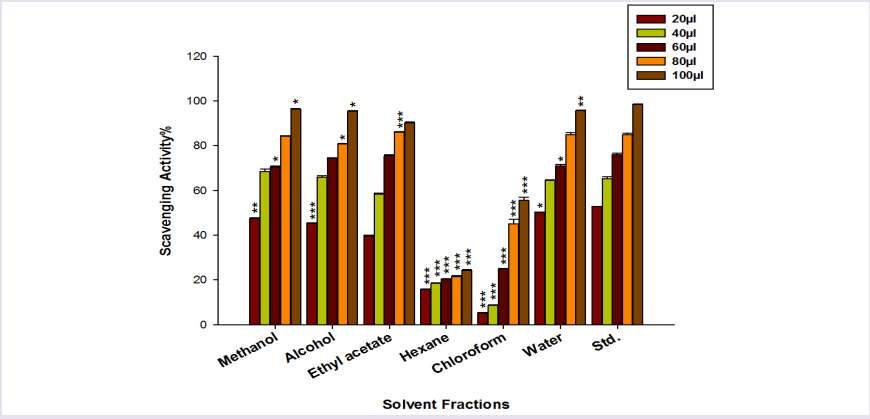

Figure 1: Scavenging activity of BUK bark in different solvent fractions. All results are presented as mean ± SEM of three assays; *P<0.05, **P<0.001, ***P<0.0001when compared with Standard antioxidant. Bars attributed by no astric (*) are not significantly different (P≤0.05).

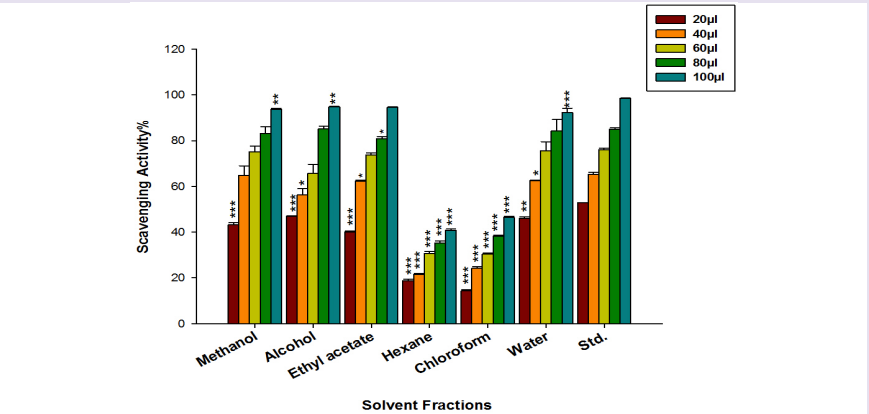

Figure 2: Scavenging activity of BUS bark in different solvent fractions. All results are presented as mean ± SEM of three assays; *P<0.05, **P<0.001, ***P<0.0001 when compared with Standard antioxidant. Bars attributed by no astric (*) are not significantly different (P≤0.05).

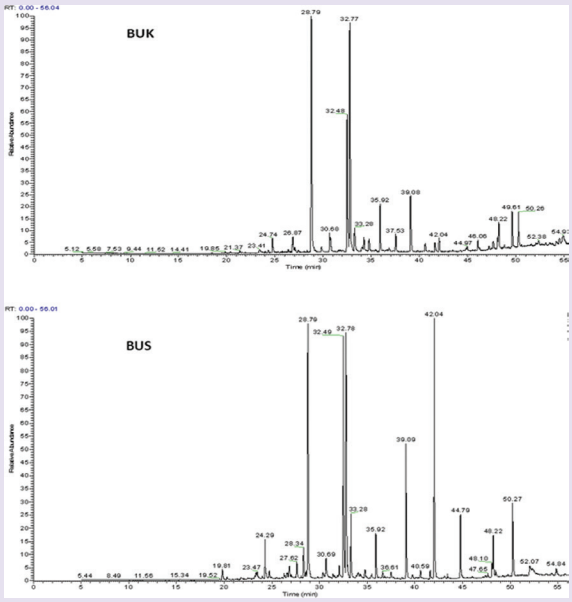

Figure 3: GCMS chromatogram of fatty acids from *Betula utilis* bark collected from Kashmir (BUK) and Sikkim (BUS).

RESULTS

Total Phenolic Content

Plant phenolics are commonly found in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. In addition, they have a metal chelation potential.[9] The standard curve was constructed using Gallic acid. The results showed the highest phenolic content ispresent in methanolic extract of BUK bark representing. 8 ± 0.1 mg/gm (Table 1).

Total Flavonoid Content

It has been recognized that flavonoid show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process.[10,11] The results showed the highest flavonoid content is present in water fraction of BUK bark representing 6.16 ± 0.2 mg/gm. The standard curve was constructed using Rutin (Table 1).

Antioxidant assay

Plants are potential sources of natural antioxidants. It produces various antioxidative compounds to counteract reactive oxygen species (ROS) in order to survive.[12] In present study the maximum antioxidant activity was showed by methanol, alcohol and water extracts of the BUK bark as shown by using DPPH assay, 96.3 ± 0.2, 95.4 ± 0.3 and 95.6 ± 0.1% respectively at volume 100 µg/ml (Figure 1). BUS bark showed highest scavenging activity by alcohol, ethyl acetate and methanol extracts, 94.8 ± 0.2, 94.5 ± 0.2 and 93.7 ± 0.4 respectively. (Figure 2). For the each fraction, the IC_{50} values were calculated from the curves plotted as shown in Table 2.

The methanolic and water extract of BUK indicates the lowest IC_{50} value 18.7 ± 1.1 and 18.2 ± 0.3 µg/ml respectively. It was not significantly different from the standard. All other extracts were found to have moderate scavenging activity with compare to standard as they are significantly different with Quercetin (P≤0.0001).

Fatty acid analysis

The fatty acid composition of BUK and BUS bark was analyzed and shown in Table 3. Thirteen fatty acids were identified in BUK and BUS representing 64.57% and 69.75% respectively. The major fatty acids found were Palmitic acid, Linoleic acid, Linolenic acid, oleic acid. The most abundant fatty acid was recorded Palmitic acid in BUK bark (18.07%) and Lignoceric acid in BUS bark (13.74%). All the identified fatty acids were categorized according to the presence of unsaturation (Table 4). The saturated and monounsaturated fatty acid content was higher in BUS bark (41.59%), (12.39%) respectively (Figure 3). Total polyunsaturated fatty acid content was highest in BUK bark accounting (18.91%). The present study revealed the comparative analysis of Fatty acids in *B. utilis* bark collected from Kashmir and Sikkim, which play a significant role in various pharmaceutical applications.

DISCUSSION

The fatty acid composition in *B. utilis* bark, collected from Uttarakhand region was studied and found that nineteen fatty acids were identified in the oily portion of the bark, representing 79.12% of the total oil, the major fatty acids was found Linoleic, Oleic and Palmitic acid. The most abundant fatty acid identified was Linoleic acid (17.66%).[13] In addition, radical scavenging activity was also evaluated from the leaves of *B. utilis*, collected from Bangalore. Results from this study indiated that the methanolic extract of *B. utilis* leaves showed a potent DPPH scavenging activity (8.4 µg/ml IC_{50}).[14]

In the present study, we found the presence of higher amounts of Palmitic acid (18,07%) in oily portion of the bark. Total phenolic and flavonoid content was analyzed in all solvent extracts and found highest amount of phenols and flavonoids in methanol, alcohol and water fraction of BUK bark. The antioxidant activity has been also been evaluated in BUK and BUS, whereas BUK has showed highest scavenging activity in methanolic, alcoholic and water extracts. This may be due to the presence of higher amounts of phenol and flavonoids in these extracts. e. Moreover, additional health benefits may be achieved from *B. utilis* due to the presence of flavonoids and phenols, which demonstrated anti-inflammatory, antifungal, and antioxidant properties[15-18] and the essential fatty acids palmitic, linolenic and linoleic acids implicated in human health promotion.[19-22]

CONCLUSION

The present study show the presence of significant amounts of Palmitic, Linoleic and Oleic acid, total phenolic and flavanoid content in *B. utilis* bark extract which may increases the nutritional value and adds to the overall health benefits.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ABBREVIATION USED

BUK: *Betula utilis* Kashmir; **BUS:** *Betula utilis* Sikkim; **GC-MS:** Gas chromatography-Mass spectroscopy; **µl:** Micro liter; **FAME:** Fatty acid methyl ester.

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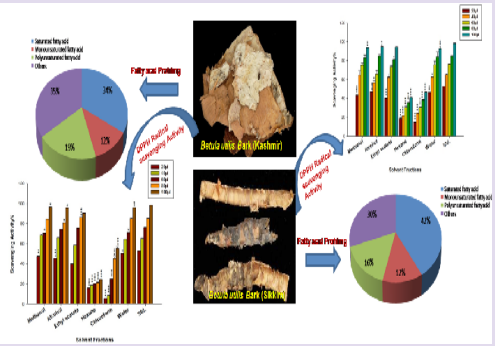
PICTORIAL ABSTRACT



SUMMARY

- *Betula utilis* bark collected from Kashmir (BUK) and Sikkim (BUS), extracted with methanol and alcohol then fractionated with four solvents-Hexane, ethyl acetate, chloroform and water successively.
- Fatty acid methyl esters were analyzed by GC-MS. The saturated and monounsaturated fatty acid content was higher in *Betula utilis* bark of Sikkim where as total polyunsaturated fatty acid content was higher in *Betula utilis* bark of Kashmir.
- Total phenolic, Total Flavonoid Content and Antioxidant activity were evaluated for all the solvent fractions, *Betula utilis* bark of Kashmir was found to have significant Antioxidant activity.

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