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Phytochemical Investigation, Isolation and Characterization of Betulin from Bark of *Betula Utilis*

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Betula utilis is a hardy perennial plant of moderate size up to 20 M in height, forming the upper limit of forest vegetation. It inhabitates along the Himalayan range from Bhutan westwards, ascending to an altitude of 4200 M. The bark of *Betula utilis* contains sitosterol, betulin, betulic acid, oleanolic acid, acetyloleanolic acid, lupeol, lupenone, methyl betulonate, methyl betulate and a new triterpenoid karachic acid. The ethanolic extract of powdered drug of *Betula utilis* was prepared. Most of the constituents were found to be present in the ethanolic extract. Thus it was concluded that constituents of *Betula utilis* bark are more soluble in polar solvents. The ethanolic extract showed the presence of alkaloids, carbohydrates, flavonoids, saponins and triterpenes. After identification of crude extract, the main work was to isolate the desired compound (betulin). So, fractionation of ethanolic extract was done by suspending it in water and then extracted it with n-hexane and dichloromethane. Betulin was isolated from dichloromethane fraction using column chromatography. Ethyl acetate and n-hexane in various ratios (1:10; 1:5; 1:3; 1:2) was used as eluent for separation of desired compound from the dichloromethane fraction. After isolation of desired compound, it was subjected to characterization. For characterization studies; melting range, TLC and spectroscopic techniques (UV, IR, Mass and NMR) were utilized.

Keyword: Altitude, Betulin, Ethanolic extract, Column chromatography, TLC, Isolation

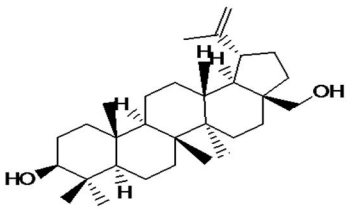
1. Introduction

Betula utilis is a hardy perennial plant of moderate size up to 20 M in height, forming the upper limit of forest vegetation. It inhabitates along the Himalayan ranges from Bhutan westwards, ascending to an altitude of 4200 M.

Scientific Classification

Kingdom	: Plantae
Phylum	: Tracheophyta
Class	: Magnoliopsida
Order	: Corylales
Family	: Betulaceae
Genus	: *Betula*
Species	: *utilis*

The bark of *Betula utilis* contains sitosterol, betulin, betulic acid, oleanolic acid, acetyloleanolic acid, lupeol, lupenone, methyl betulonate, methyl betulate and a new triterpenoid karachic acid[1,2]. Leucocyanidin and polymeric leucoanthocyanidins are also present in this plant.



BETULIN

1.2 Description:

In its native habitat, *B. utilis* tends to form forests, growing as a shrub or tree reaching up to 20 m (66 ft) tall. It frequently grows among scattered conifers, with an undergrowth of shrubs that typically includes evergreen *Rhododendron*. The tree depends on moisture from snowmelt, rather than from the monsoon rains. They often have very bent growth due to the pressure of the deep winter snow in the Himalaya. Leaves are ovate, 5 to 10 cm (2.0 to 3.9 in) long, with serrated margins, and slightly hairy. Flowering occurs from May–July, with only a few male catkins, and short, single (sometimes paired) female catkins. The perianth has four parts in male flowers, and is absent in the female flowers[3]. Fruits ripen in September–October. The thin, papery bark is very shiny, reddish brown, reddish white, or white, with horizontal lenticels. The bark peels off in broad, horizontal belts, making it very usable for creating even large pages for texts. A fungal growth, locally called *bhurja-granthi*, forms black lumps on the tree weighing up to 1 kg. The wood is very hard and heavy, and quite brittle. The heartwood is pink or light reddish brown[4].

White, paper-like bark

Image-1

Close-up showing lenticels

Image-2

1.3 Conservation:

Deforestation due to overuse of the tree has caused loss of habitat for many native groves of *B. utilis* (locally called *bhojpatra* in the Indian Himalaya). The first high-altitude *bhojpatra* nursery was established in 1993 at Chirbasa, just above Gangotri, where many Hindus go on pilgrimage to the source of the sacred Ganges river. Dr. Harshvanti Bisht, a Himalayan mountaineer, established the first nursery and continues to expand the reforestation of *bhojpatra* in the Gangotri area and inside Gangotri National Park[5]. About 12,500 bhojpatra saplings had been planted in the area by the year 2000. In recent years, attempts have been made to ban the collection of *bhojpatra* trees in the Gangotri area[6].

1.4 Varieties and Cultivars:

Many named varieties and cultivars are used in landscaping throughout the world. In the eastern end of the tree's native distribution, several forms have orange- or copper-colored bark. *Betula utilis* var. *jacquemontii*, from the western end of the native habitat, is widely used because several cultivars have especially white bark. These include:

- 'Doorenbos'
- 'Grayswood Ghost'
- 'Jermyns'
- 'Silver Shadow'
- 'Snow Queen'

B. utilis 'Fascination' has an orange- to mahogany-colored bark, which peels off to reveal a polished-looking layer underneath. The bark of 'Wakehurst Place Chocolate', as the name implies, is dark brown to nearly black [7].

2. Material and Methods:

2.1 Phytochemical Investigation:

- Collection of plant material: - The bark of *Betula utilis* (Bhojpatra) was collected in the month of December from Munsiary village (near Sarmoli village) of Pithoragarh district of Uttarakhand state.
- Identification and authentication of plant:- The bark of *Betula utilis* (Bhojpatra) was identified and authenticated from National Botanical Research Institute, Lucknow under the Ref. No.: NBRI/CIF/132/2009 dated 11-01-2010.
- Drying of plant material: The bark of *Betula utilis* (Bhojpatra) was dried in shade.
- Coarse powder of the plant: The dried bark of *Betula utilis (*Bhojpatra) was cut into small pieces and then powdered with the help of mixer grinder.

2.2 Preparation of Crude Extract

The powdered bark of *Betula utilis* (45 gm) was defatted with petroleum ether (1.25 L) and then the defatted powder of bark of *Betula utilis* was extracted with 95% ethanol (1.25 L) in the soxhlet apparatus at 40ºc for about 72 hours. After the completion of extraction, the extract was concentrated at vacuum rotary evaporator to get a yellowish brown residue which was placed in vacuum desiccator for 4-5 days for drying and then used for subsequent experiments. The process is repeated with rest of drug again[8,9].

Table 1: Extractive value of different extracts of *Betula utilis*

Plant name	Parts used	Method of Extraction	% Yield of Petroleum ether extract	% Yield of 95% Ethanolic extract
Betula utilis	Bark	Continuous Hot Percolation (Soxhlet Extraction)	6.88%	20.75%

2.3 Isolation of Compound:

2.3.1 Fractionation of Ethanol Extract:

15 gm of ethanolic extract was suspended in water (300 ml) and then extracted with n-hexane (300 ml × 3) and dichloromethane (300 ml × 3) using the principle of liquid/liquid extraction. Then both the fractions are concentrated in vacuum rotary evaporator and concentrated fractions are put for drying in desiccators for 4 - 5 days. Then both the fractions were characterized by TLC, and found that the R_f of the aimed compound (Betulin) match with the TLC of dichloromethane fraction. So, the dichloromethane fraction was used for further isolation by column chromatography.

Percentage yield of n-hexane fraction: The % yield of n-hexane fraction was found to be 22% w/w.

Percentage yield of dichloromethane fraction: The % yield of dichloromethane fraction was found to be 70.20% w/w.

2.3.2 Isolation of Active Component from Dichloromethane Fraction

The column (3.5 × 60 cm) was prepared with silica gel (60-120 mesh) in n-hexane by wet method and column is put for overnight. Then the dichloromethane fraction (8 gm) was poured to silica gel open column chromatography and step gradient technique was used to run the column. Various ratios of ethyl acetate and n-hexane (1:10, 500 ml; 1:5, 500 ml; 1:3, 500 ml; 1:2, 350 ml) were used for the isolation of active component. The fractions of 50 ml were collected; characterized by TLC and same type of fractions are mixed; then again characterized by TLC and found that the aimed compound (betulin) is present as a single spot in the fraction collected from the 1:2 (ethyl acetate and n-hexane) solvent system and the R_f value for this fraction was found to be 0.4705 which is similar to the R_f value of betulin in this solvent system.

2.4 Characterization of Isolated Compound: Physical Properties

Colour: yellowish white

State: solid

Solubility: soluble in chloroform and ethyl acetate

Melting Point: 242-246 °C (determined by open capillary method)

R_f Value: 0.4705 in ethyl acetate and n-hexane (1:2)

3. Result and Discussion

After identification of crude extract, the main work was to isolate the desired compound (betulin). So, fractionation of ethanolic extract was done by suspending it in water and then extracted it with n-hexane and dichloromethane. Betulin was isolated from dichloromethane fraction using column chromatography. Ethyl acetate and n-hexane in various ratios (1:10; 1:5; 1:3; 1:2) was used as eluent for separation of desired compound from the dichloromethane fraction. After isolation of desired compound, it was subjected to characterization. For characterization studies; melting range, TLC and spectroscopic techniques (UV, IR, Mass and NMR) were utilized.

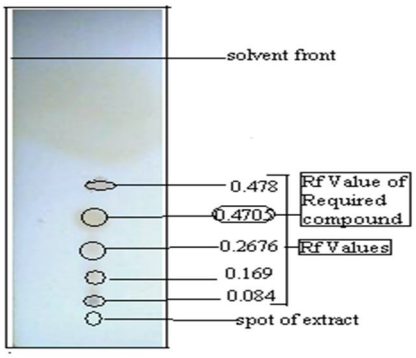


Fig. 1: TLC of isolated compound [Betulin] in iodine chamber

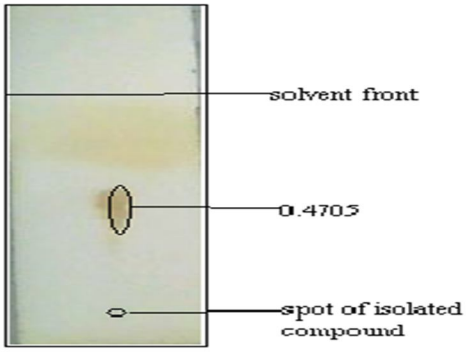


Fig. 2: TLC of ethanolic extract in iodine chamber

Table 2: Partition coefficient data for isolated compound betulin:

S. No.	Concentration of solution containing isolated compound (Betulin)	Absorbance at 318 nm wavelength (λ_{max})
1.	5 µg/ml solution in Concentrated H_2SO_4	0.087
2.	10 µg/ml solution in Concentrated H_2SO_4	0.242
3.	15 µg/ml solution in Concentrated H_2SO_4	0.324
4.	20 µg/ml solution in Concentrated H_2SO_4	0.402
5.	25 µg/ml solution in Concentrated H_2SO_4	0.540
6.	30 µg/ml solution in Concentrated H_2SO_4	0.631
7.	Octanol phase (Unknown concentration)	0.270
8.	Water Phase (Unknown concentration)	0.110

So, partition coefficient (P) of isolated compound (Betulin) = 2.35.

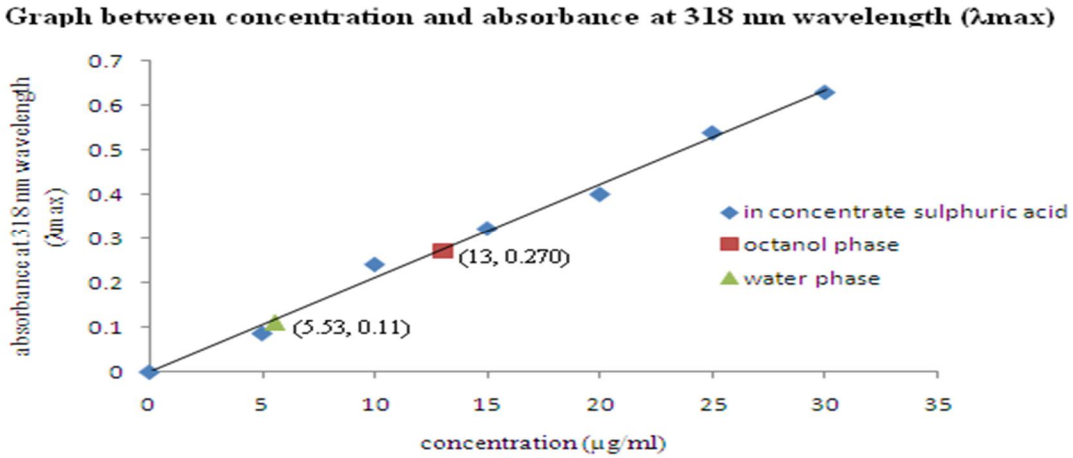


Fig. 3: Graph shows partition coefficient of data in water phase, octanol phase and in concentrate H_2SO_4

2.5 Spectral Techniques:

UV Spectroscopy:

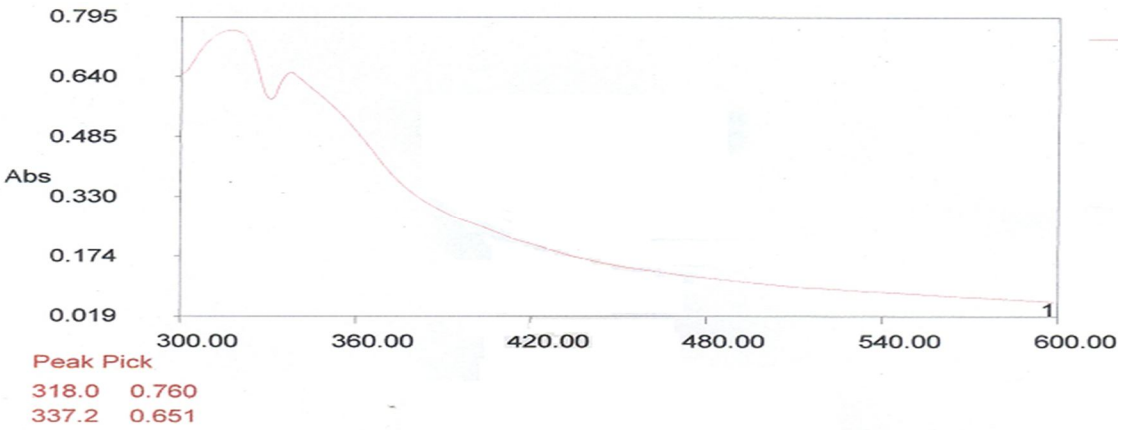


Fig. 4: UV spectra of isolated compound Betulin

IR Spectroscopy:

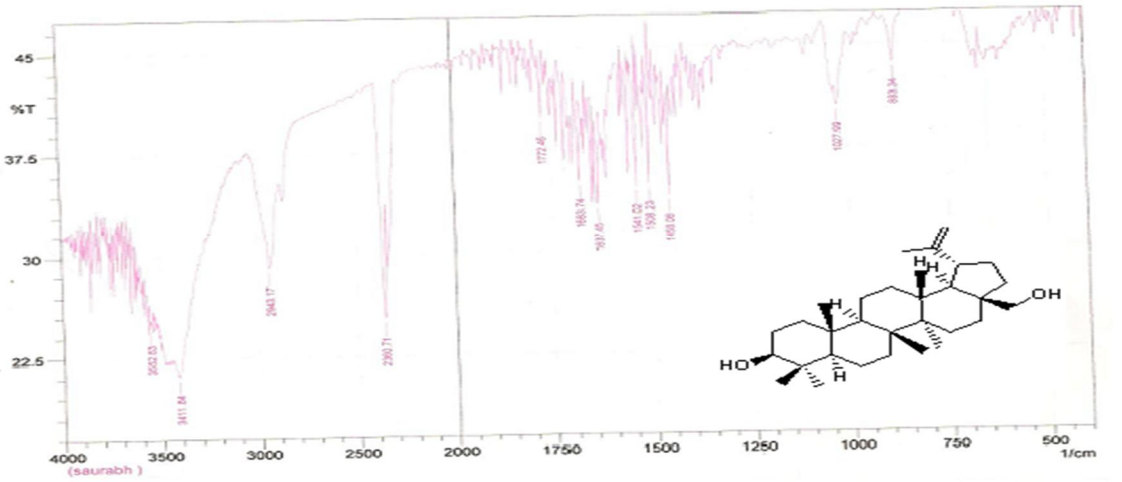


Fig. 5 IR characterization of isolated compound Betulin

Mass Spectroscopy:

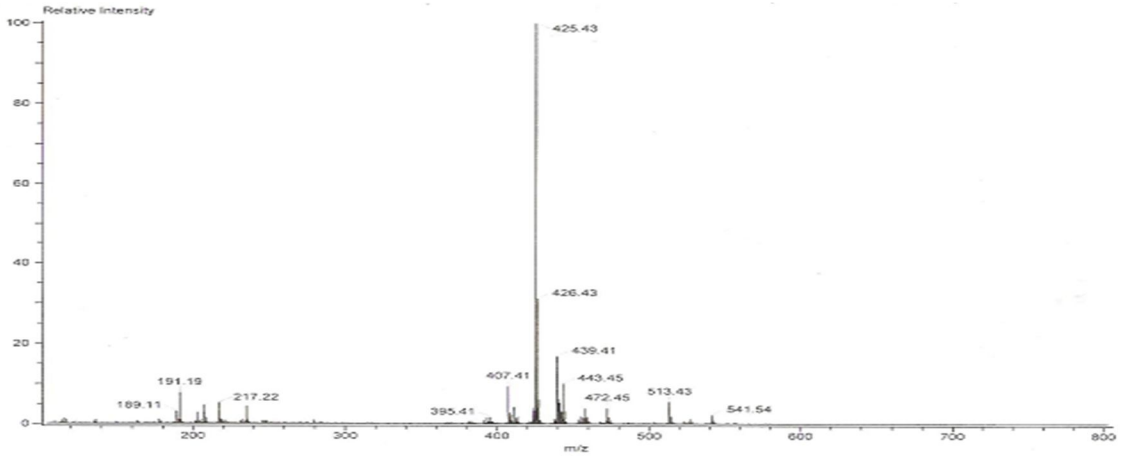


Fig. 6 Mass spectra of isolated compound Betulin

NMR Spectroscopy:

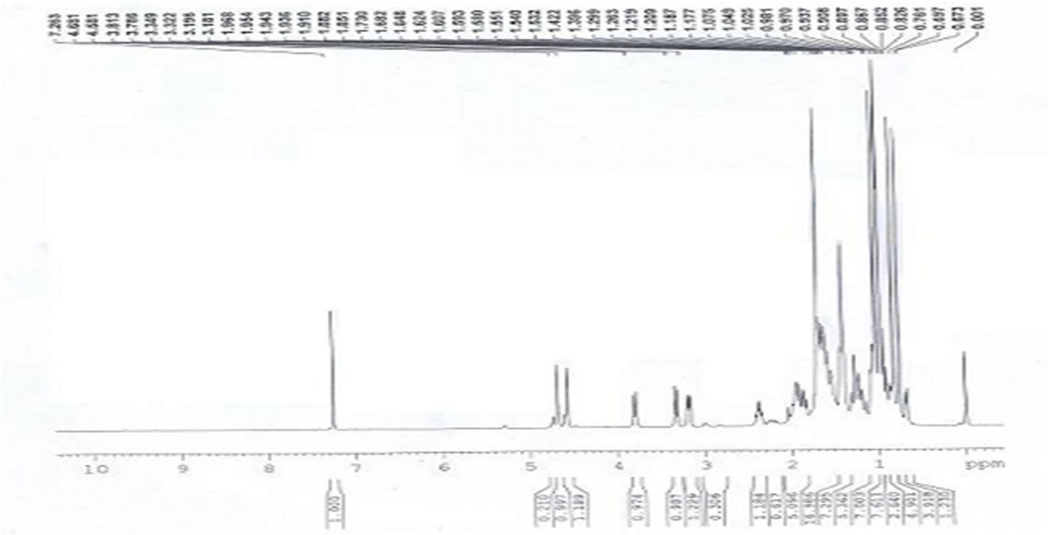


Fig. 7: ^{1}H-NMR Spectra of isolated compound Betulin

Melting range of the isolated compound, determined by open capillary method, was found to be 242-246°C, which is uncorrected and comparable with that of standard betulin which is 248-251°C.

Partition coefficient of isolated compound was found to be 2.35 in octanol and water.

Wavelength of maximum absorbance (λ_{max}) of isolated compound was found to be 318 nm in concentrated sulphuric acid which is very close to 316 nm which is of standard betulin in concentrated sulphuric acid.

- FT-IR spectrum of isolated compound gave characteristic peaks of groups present in the isolated compound like C-H (alkane), -CH_3 (bending), C=C, C-O and -OH group.
- Mass spectroscopy of isolated compound was performed, by DART technique, showing a molecular ion peak at M^++1 *i.e.* at 443.43, while calculated molecular weight of betulin (molecular formula-$C_{30}H_{50}O_2$) is 442.
- Proton NMR spectroscopy was used for the confirmation of structure of isolated compound; in which various peaks in $CDCl_3$ were found at δ value 1.0-1.1 (t, 7H); 1.11-1.3 (m, 5H); 1.4 (d, 7H); 1.5-1.7 (m, 17H); 1.8-2.1 (m, 5H); 2.14-2.3 (m, 1H); 2.31-2.46 (m, 1H); 3.0 (d, 1H); 3.2 (q, 1H); 3.34 (d, 1H); 3.8 (d, 1H); 4.6 (d, 1H); 4.68 (s, 1H); 4.75 (s, 1H) and it shows the presence of 50 hydrogens in the compound.

On the basis of characterization studies of isolated compound, it was concluded that the isolated compound may be betulin and its structure is determined as follows.

4. Conclusion

On the basis of characterization studies, the isolated compound has physical properties (colour, state, solubility, melting range and R_f value) which are identically resemble with the standard betulin. Spectral data shows that the isolated compound has λ_{max} which is mostly similar to standard betulin; IR peaks of various functional groups of betulin are found in this isolated compound; M^++1 peak was also obtained in the mass spectra of the isolated compound and above all, 50 protons are found in the ^{1}H-NMR spectra of isolated compound. So, it can be concluded that the isolated compound is similar to the molecular formula $C_{30}H_{50}O_2$, which corresponds to the molecular formula of betulin. So, the isolated compound was found to be betulin.

5. Acknowledgement

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