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# Effective Method of Extraction of Betulin Diacetate from Birch Bark

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ABSTRACT

A new method for extraction of betulin diacetate from birch bark was developed using a mixture of anhydride acetic acid and acetic acid with percentages of 64% and 36% consecutively. The temperature of extraction was 130 °C which gave a good yield, the extraction time didn't exceed 48 hours. The structure and the purity of betulin diacetate was confirmed by the following measurements and techniques: melting point, element analysis, HPLC, $^{1}$H NMR, $^{13}$C NMR, DEPT and FTIR spectroscopy.

## 1. Introduction

Natural products have been used to treat human disease for thousands of years and play an increasingly important role in drug discovery and development. In fact, the majority of anticancer and anti-infectious agents are of natural origin [1, 2].

Triterpenoids are one of the most important classes of natural products occurring widely in the plant kingdom. The derivatives of triterpenoids have been one of most interesting areas of research in the past few years vested to their broad range of biological and medicinal properties [3-8].

The birch tree (*Betula sp., Betulaceae*) is one of the substantial source of pentacyclic triterpenoids. Extracts of the outer bark of different types of birch predominantly contain pentacyclic triterpenoids of the lupan family. The main component of all extracts is betulin (lup-20(29)-ene-3b, 28-diol), which imparts a white color to birch bark. The betulin content in the outer bark varies from 10 to 40% depending on the birch type, growing place and conditions, age of the tree, season, etc.

It is well known that betulin (Fig. 1) and its chemical derivatives exhibit a wide range of important biological effects on animal and human health [9]. Anti-inflammatory [10, 11], antiviral (including anti-HIV) [7, 12, 13], hepatoprotective [14, 15], gastroprotective [16, 17], anti-proliferative and anti-cancer [18, 19] properties have previously been demonstrated. Betulin also moderates the biosynthesis of cholesterol and fatty acids, and so ameliorates diet-induced obesity and reduces the size and improves the stability of atherosclerotic plaques (evidenced by reduced accumulation of macrophages) [20]. It can be also used in the treatment of type II diabetes via promotion of insulin sensitivity of cells.

Besides this, cosmetic applications have also been reported, and betulin and birch bark extracts are used as additives in cosmetology and food products [21]. Thus, betulin of high purity can be found widely used in the pharmaceutical and cosmetic industries.

The betulin esters are mainly prepared by acetylation of betulin and require the necessary step of the betulin isolation from the upper bark of the *Betula pendula* Roth birch [22, 23].

Among these esters, this present work highlighted the synthesis of betulin diacetate because of its interesting biological activities hepatoprotective, hypolipidemic, cholegogic, and antioxidant properties and is a promising pharmaceutical [24-28]. Furthermore the protection of C-3 and C-28 using acylation method can serves as the raw material for many organic syntheses such as the synthesis of betulic acid, sulphur-containing betulin derivatives, amino derivatives of betulin diacetate, and especially conversions involving the isopropenyl group which are relatively unstudied [29, 30].

The main aim of the present study was to develop a novel method for isolating betulin diacetate (Fig. 1) directly from outer birch bark (raw material) in sufficiently high yield and purity in order for it to be further used for the synthesis of its derivatives using as simple and efficient as possible process, that could be scaled up to a large-scale industrial production.

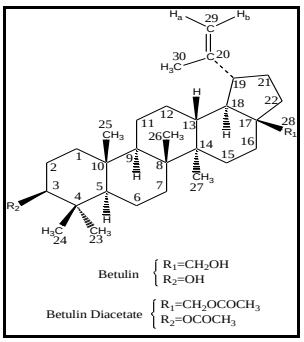


**Fig. 1** Chemical structure of betulin derivatives

## 2. Experimental Methods

### *2.1 Plant Material*

Collection of plant material: the bark of *Betula pendula* Roth birch was collected on June 2015 from the forests of tomsk region.

Drying of plant material: the bark of *Betula pendula* Roth birch was dried in shade.

Coarse powder of the plant: the dried bark of *Betula pendula* Roth birch was cut into small pieces and then powdered with the help of mixer grinder.

### *2.2 Instrumentation*

The $^{1}$H NMR spectra were measured on a Varian Mercury-VX 300 MHz or a Chemagnetics CMX 400 MHz spectrometer with chemical shifts reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 7.26 ppm as an internal standard ).

The $^{13}$C NMR spectra were obtained on a Varian Mercury-VX 75 MHz or a Chemagnetics CMX 100 MHz S8 spectrometer with chemical shifts

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reported as parts per million (in $CDCl_3$ at 23 °C, solvent peak at 77.0 ppm as an internal standard).

Fourier-transform infrared (FTIR) spectra were obtained directly from the products using the high-attenuated total reflectance technique in a Bruker Tensor 27 FT-IR Spectrometer.

The spectra were recorded in the range of 400 to 4000 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ over 16 scans.

High performance liquid chromatography (HPLC) was performed in the isocratic mode. A C18 symmetry analytical column from waters with the size of 3.9 x 150 mm, 5 mm particle size was used. The mobile phase consisted of a mixture of acetonitrile, water solution (65:35, v/v). The flow rate was set to 0.75 mL/min, and the oven temperature 25 °C. The injection volume was 5 µL, and the detection wavelength was set at 220 nm.

Melting points were obtained with Buchi apparatus without correction.

The elemental analysis of recrystallized products was carried out with the help of element analyzer FLASHTM.

The upper bark of the *Betula pendula* Roth birch was used as a starting material. The upper bark was chopped into fractions of 10–20 mm.

### *2.3 Extraction and Isolation*

In a round bottom flask equipped with a magnetic stirrer and condenser, 100 g of dried birch bark was placed, and 1 L of mixture of anhydride acetic acid 64% and acetic acid 36% was added at a temperature of 130 °C, the reaction mixture was refluxed at a temperature of 130 °C for 48 h. After the completion of extraction, the reaction mixture was filtered from residual and the extract was concentrated at vacuum rotary evaporator to get 400 mL of a black brown solution which was poured into 400 mL of cold water forming a white precipitate that was separated by filtering, washed with distilled water and dried in the air. The product was recrystallized from ethanol to give 25 g.

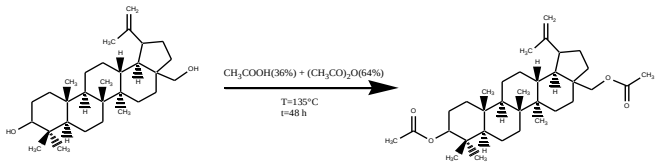


**Fig. 2** Acetylation reaction of betulin

### *2.4 Characterization of Isolated Compound*

#### *2.4.1 Physical Properties*

Colour: yellowish white; State: solid; Solubility: soluble in chloroform and ethyl acetate; Melting Point: 219-220 °C (determined by open capillary method); $R_f$ Value: 0.6 in ethyl acetate and n-hexane (1:4).

#### *2.4.2 Elemental Analysis*

According to the data of elemental analysis of betulin diacetate, found (%): C 77.9, H 10.2, O 11.9. Calculated (%): C 77.6, H 10.3, O 12.1

## 3. Results and Discussion

In the previous reported synthesis of diacetate betulin from outer birch bark which was conducted by Kyznetsuva et al [31]. They obtained diacetate betulin with three byproducts cited as following betulin, lupeol acetate and lupeol. Moreover their method of extraction needed two steps, starting by the activation of birch bark using explosive autohydrolysis then the synthesis of betulin diacetate using acetic acid.

However in the present work the extraction was optimized by the addition of anhydride acetic acid which led to the total conversion of betulin to its acetate (Fig. 2) without formation of other byproducts.

After isolation of desired compound, it was subjected to characterization. For identification studies; melting range, HPLC and spectroscopic techniques (IR and NMR) were utilized.

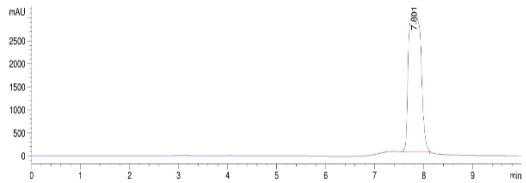


**Fig. 3** HPLC chromatogram of Isolated compound

Betulin diacetate was identified as only product by HPLC (Fig. 3) with purity of 99%, as was evident by infrared spectrum (Fig. 9), which indicated the absence O-H stretching band and the presence of C-H (alkane), $-CH_3$ (bending), C=C, C-O and C=O group.

Proton NMR, $^{13}C$ NMR and DEPT spectroscopy were used for the confirmation of structure of isolated compound; in which various peaks in $CDCl_3$ were found at δ value.

### *3.1 The $^1H$-NMR and $^{13}C$ NMR Spectra of the Product*

#### *3.1.1 The C-3 Proton*

The $^1H$ NMR spectrum of betulin (Fig. 4) showed the signal for the C-3 methine proton, approximating to a quartet, centered at δ 3.16 ppm. On formation of the diacetate this multiplet underwent an acetylation shift and moved downfield to δ 4.50 ppm (Δδ = 1.34 approx.).

The formation of the diacetate was evidenced both by the appearance of two proton singlet at δ 2.06 and δ 2.08 ppm in its $^1H$ NMR spectrum (Fig. 5), and the presence of resonances for two methyl groups at δ 21.04 and δ 21.30 ppm with the signals for the corresponding carbonyls at δ 171.02 and δ 171.63 ppm in its $^{13}C$ NMR.

#### *3.1.2 The C-28 Methylene Protons*

The $^1H$ NMR spectrum of betulin diacetate showed a two singlet at δ 2.06 and 2.08 ppm, assignable to the acetate methyl and two doublet, attributable to two methylene protons, centered at δ 3.87 and 4.29 ppm consecutively , which was shifted downfield from its position at δ 3.32 and 3.72 ppm in the spectrum of the original alcohol (Fig. 4). The change in the chemical shift of the doublet (Δδ = 0.55 ppm) was attributable to acetylation of this primary OH group and the quoted values are consistent with a $-CH_2$-OH group at the 28 position of the molecule. The concept of diastereotopicity was introduced in this case where the two protons of $CH_2$ group are considered diastereotopic due to adjacent chiral center. The methylene protons did not give a simple singlet as might be expected, it was attributable to the fact that they were nonequivalent protons, and replacement of one of them with a different substituent would result in a pair of diastereoisomers.

#### *3.1.3 The Olefinic Methylene Protons*

The $^1H$ NMR spectrum for the betulin diacetate showed a double singlet at δ 4.62 and 4.72 ppm consecutively and was attributable to the protons of the terminal methylene of an olefinic group, these two protons are diastereotopic. The situation in this case is simple with gem-alkene protons, it is easy to see how they are different if we add two protons to the double bond. However, it is more complicated for $sp^3$ carbons such as the methylene protons of $C_{28}$.

The $^{13}C$ NMR spectrum of the diacetate showing signals at δ 109.88 and δ 150.12 ppm were attributable to a terminal methylene carbon and a quaternary carbon atom.

#### *3.1.4 The Methyl Group Protons*

A singlet, attributable to a vinylic methyl group, showing some broadening due to an NOE, appeared at δ 1.70 in the $^1H$ NMR spectrum of the original compound, and its derivatives. The $^1H$ NMR also showed a set of singlets representing six methyl groups at δ 0.87, 0.90, 0.99, 1.06, 1.45 and 1.70 ppm, characteristic of the lupane series triterpenoids. A comparison of the $^{13}C$ NMR chemical shift values for the diacetate, with those recorded in the literature, enabled the six methyl groups to be identified as occurring at the C-23, C-24, C-25, C-26, C-27 and C-30 positions in the molecule.

**Table 1** $^1H$-NMR spectral data of betulin diacetate

| Chemical shift (ppm) | Multiplicity | J (Hz) |
|---|---|---|
| 0.87 | m(3H, 5-H) | - |
| 0.90 | s(3H, $CH_3$) | - |
| 0.99 | s(6H, $CH_3$) | |
| 1.06 | s(3H, $CH_3$) | J= (7.9,15.3 Hz) |
| 1.45 | s(3H,$CH_3$) | J= (8.8,15.3 Hz) |
| 1.03-1.95 | m(10H,$CH_2$) | |
| 1.30 | m(1H,CH) | |
| 1.60 | m(1H,CH) | |
| 1.65 | m(1H,CH) | |
| 1.70 | s(3H,$C_{30}H_3$) | |
| 2.06 | s(3H,$CH_3CO$) | |
| 2.08 | s(3H,$CH_3CO$) | |
| 2.47 | td(1H, 19-H) | J = 11.0, 5.8 Hz |
| 3.87 and 4.29 | d (2H each, 28-H) | 2J = 11.0 Hz |
| 4.50 | q (1H, 3-H) | |
| 4.62 and 4.72 | s (2H each, 29-H) | |

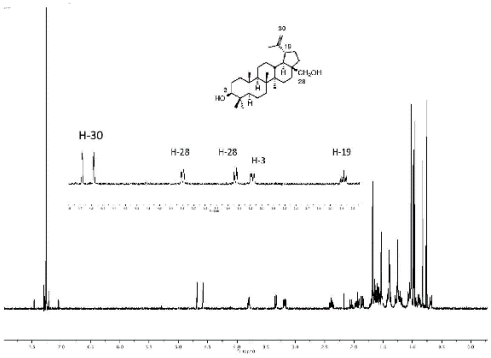


**Fig. 4** $^{1}$H NMR spectrum of standard betulin

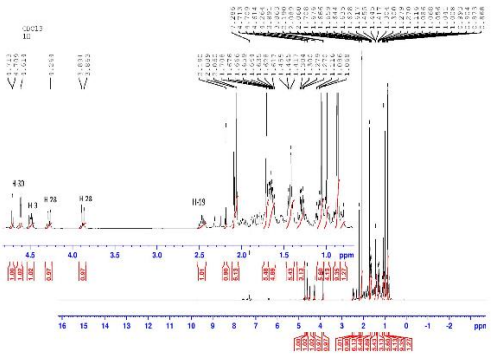

**Fig. 5** $^{1}$H NMR spectrum of betulin diacetate

### *3.2 DEPT and $^{13}$C NMR Spectra of the Product*

On the other hand the $^{13}$C NMR (Fig. 6) and DEPT spectra (Fig. 7) revealed the presence of 34 signals including 11 characteristic downfield methene carbons peaks at δC 38.33(C-1), 23.66(C-2), 18.13(C-6), 34.52(C-7), 20.76(C-11), 25.10(C-12), 27.01(C-15), 29.53(C-16), 29.69(C-21), 34.09(C-22), 62.78(C-28), 109.88(C-29), and olefinic peaks at δC 150.12 (C-20), δC 109.88 (C-29).

Furthermore six quaternary carbons were revealed at δC 37.76(C-4), 40.85(C-8), 37.02(C10), 42.64(C14), 46.26(C17), 150.12(C-20), as well as the carbon peaks at 171.02 and 171.63 of both carbonyl group.

Additionally, methylene carbon signals were showed at δC 80.88(C-3), 55.33(C-5), 50.25(C-9), 37.51(C-13), 48.73(C-18), 47.68(C-19), 19.06(C-30). Finally characteristic methyl carbon peaks showed at δC 14.69 (C-27), δC 15.99 (C-26), δC16.12(C-25), δC 16.46 (C-24), δC 27.90 (C-23), δC 21.04 and 21.30 of the both methyl carbons of acetyl group.

The chemical shift assignments for these carbons and all others in the compound are given in Table 2.

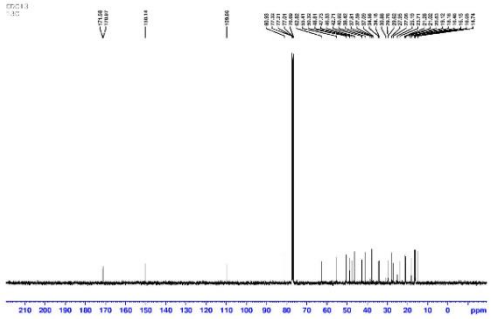

**Fig. 6** $^{13}$C NMR spectra of betulin diacetate

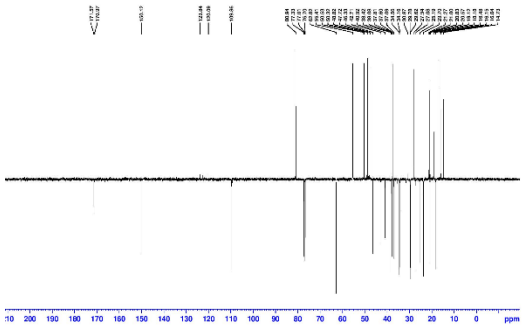

**Fig. 7** DEPT spectrum of betulin diacetate

**Table 2** $^{13}$C NMR spectral data of betulin diacetate

| Carbon | Chemical Shift (ppm) | Carbon | Chemical Shift (ppm) | Carbon | Chemical Shift (ppm) |
|---|---|---|---|---|---|
| 1 | 38.33($CH_2$) | 11 | 20.76($CH_2$) | 21 | 29.69($CH_2$) |
| 2 | 23.66($CH_2$) | 12 | 25.10($CH_2$) | 22 | 34.09($CH_2$) |
| 3 | 80.88(CH) | 13 | 37.51(CH) | 23 | 27.90($CH_3$) |
| 4 | 37.76(C) | 14 | 42.64(C) | 24 | 16.46($CH_3$) |
| 5 | 55.33(CH) | 15 | 27.01($CH_2$) | 25 | 16.12($CH_3$) |
| 6 | 18.13($CH_2$) | 16 | 29.53($CH_2$) | 26 | 15.99($CH_3$) |
| 7 | 34.52($CH_2$) | 17 | 46.26(C) | 27 | 14.69($CH_3$) |
| 8 | 40.85(C) | 18 | 48.73(CH) | 28 | 62.78($CH_2$) |
| 9 | 50.25(CH) | 19 | 47.68(CH) | 29 | 109.88($CH_2$) |
| 10 | 37.02(C) | 20 | 150.12(C) | 30 | 19.06(CH) |
| | | | | $OCOCH_3$ | 21.04($CH_3$) |
| | | | | $OCOCH_3$ | 21.30($CH_3$) |
| | | | | $OCOCH_3$ | 171.02(C) |
| | | | | $OCOCH_3$ | 171.63(C) |

### *3.3 IR Spectrum of the Product*

IR spectrum of betulin diacetate (Fig. 8) has the absorption band corresponding to the stretching vibrations of the C–H bonds in the $CH_2$ groups occur at ν(C–H) = 2916.65 cm$^{-1}$, ν(C–H) = 2850.02 cm$^{-1}$, in the $CH_3$ groups, at ν (C–H) = 2887.13 cm$^{-1}$. Deformation vibrations of the C–H bonds in the $CH_2$ groups occurs at δ(C–H) =1470.82 cm$^{-1}$ (planar scissoring vibration); characteristic absorption for the $CH_3$ group is at δ(C–H) =1374.99 cm$^{-1}$. Frequency of the stretching vibrations of the carbonyl group is ν(C=O) =1739.93 cm$^{-1}$.

**Table 3** Basic IR Absorptions of Betulin Diacetate

| Wave number (cm $^{-1}$) | Type of vibration | Nature of functional group |
|---|---|---|
| 2916.65 | $CH_2$ stretching | alkane |
| 2887.13 | $CH_3$ stretching | alkane |
| 3070.90 | C=C-H stretching | alkene |
| 1470.82 | $CH_2$ deformation | alkane |
| 1374.99 | $CH_3$ deformation | alkane |
| 1739.93 | C=O stretching | carbonyl |
| 1644.49 | C=C stretching | alkene |
| 879.79 | C=C-H deformation | alkene |
| 1086.83 | C-O stretching | ether |
| 1246.14 | C-O stretching | ether |

Characteristic frequency of the stretching vibrations of the double bond $R_1R_2C=CH_2$ are ν(C=C-H) = 3070.90 cm$^{-1}$, ν(C=C) = 1644.49 cm$^{-1}$. The bands at ν(C–H) = 1644.49 cm$^{-1}$, γ(C–H) = 879.79 cm$^{-1}$ correspond to the stretching vibrations and non-planar deformation vibrations, respectively of the C–H bonds at carbon with the double bond. The stretching vibration of the C–O bond is observed at ν(C–O) =1086.83 cm$^{-1}$, 1246.14 cm$^{-1}$. The basic absorptions of betulin diacetate is recorded in the Table 3.

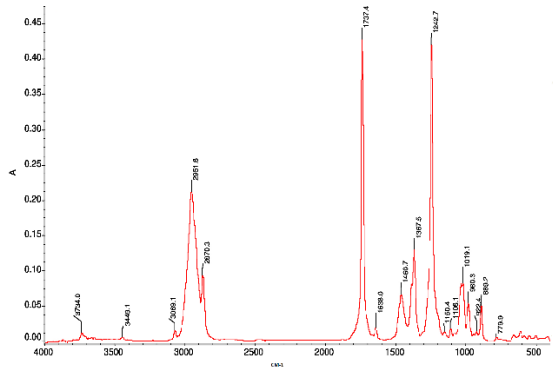


**Fig. 8** IR spectrum of betulin diacetate

## 4. Conclusion

Based on 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 diacetate. Spectral data shows that mostly IR peaks of various functional groups of betulin diacetate are found in this isolated compound, 54 protons are found in the

$^1$H-NMR spectra of isolated compound. Therefore, 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 diacetate. So, the isolated compound was found to be betulin diacetate.

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