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Isolation, characterization of secondary metabolites from the *Diospyros malabarica* (Desr.) Kostel and to evaluate their *in-vitro* antidiabetic activity

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Abstract

Objectives: The present study was to isolate, characterize the secondary metabolites from the *Diospyros malabarica* (Desr.) Kostel stem bark and to evaluate their *in-vitro* antidiabetic activities.

Material and Methods: The *D. malabarica* stem bark powder was subjected to extraction, fractionation and column chromatography to isolate compounds. The identical eluents were collected and monitored on TLC and characterized them by IR, Mass and ¹HNMR and ¹³CNMR spectra. Later, they were subjected to *in-vitro* antidiabetic studies such as α -amylase, sucrase and α -glucosidase inhibitory activities.

Results: Four known compounds – betulin, betulinic acid, 16-hydroxy betulinic acid and lupeol were isolated from chloroform fraction of *D. malabarica* stem bark. The spectroscopic data confirmed their structures from reported compounds. The isolated compounds betulin and betulinic acid exhibited α -amylase and α -glucosidase inhibitory activities.

Conclusion: We isolated four known compounds from chloroform fraction of *D. malabarica* stem bark and betulin and betulinic acid exhibited significant *in-vitro* antidiabetic activities.

Keywords: *Diospyros malabarica*, *in-vitro* antidiabetic drugs

Introduction

Diabetes Mellitus is a group of metabolic disorder characterized by chronic hyperglycaemia resulting from defects in insulin secretion, insulin action or both. Type-2 diabetes (T2DM) is caused by combination of genetic factors related to impaired insulin secretion, insulin resistance and environmental factors (Obesity, overeating, lack of exercise, stress) and aging. Several clinical studies have demonstrated that chronic hyperglycaemia is associated with damage, dysfunction, and failure of vital organs and lead to retinopathy, nephropathy, neuropathy, cardiomyopathy, atherosclerosis and stroke. The impaired function of pancreatic β -cells may progress with prolonged T2DM, aging, obesity, insufficient energy consumption, alcohol, smoking, etc. are independent risk factors of pathogenesis of T2DM [1, 2]. The epidemic of diabetes mellitus and its complications poses a major global health threat. The International Diabetes Federation (IDF) estimated that 1 in 11 adults aged 20–79 years (415 million adults) had diabetes mellitus globally in 2015, and about 46.5% of them lived in three countries: China, India and the USA. The largest increase in DM will come from the regions experiencing economic transitions from low-income to middle-income levels [3]. The reasons for the escalating epidemic of diabetes mellitus are multiple, including population ageing, economic development, urbanization, unhealthy eating habits and sedentary lifestyles. Over 90% of diabetes mellitus cases are type 2 diabetes mellitus (T2DM) [4]. T2DM and its complications have contributed tremendously to the burden of mortality and disability worldwide. In 2015, IDF report estimated that DM and its complications caused 5.0 million deaths in adults aged 20–79 years, which is equivalent to one death every six seconds. The Global Burden of Diseases, Injuries, and Risk Factors Study 2015 estimated that a high fasting level of glucose was the third most common global risk factor for disability-adjusted life years (DALYs), accounting for 143 million DALYs in 2015 and a 22% increase in DALYs from 2005 to 2015 [5]. Globally, 45.8% (or 174.8 million cases) of all DM cases in adults were estimated to be undiagnosed; people with undiagnosed and untreated DM are at a greater risk of complications than those who are receiving treatment. Furthermore, medical expenditure for patients with diabetes mellitus is up to three times greater than for the general population without diabetes mellitus. The IDF conservatively estimated that in 2015, US\$673 billion.

(12% of global health expenditure) was spent on treating diabetes mellitus and its related complications [6]. Prolonged use of oral antidiabetic drugs will have drawbacks like resistance, hepatotoxicity, worsen heart disease, weight gain, bloating, flatulence, diarrhoea and abdominal discomfort and pain⁷. Many medicinal preparations contain one or more ingredients of plant origin. According to WHO, about 80% of the population in the developing countries use traditional medicine in the treatment of various diseases? Therefore, plant species used by different ethnic groups should be investigated in order to tap the incredible bio resources for sustainable harvesting of novel bioactive phytopharmaceuticals [8]. The interest in herbal drug research continues with an expectation to novel, safer and more effective compound with all the desired parameters of a drug that could replace the synthetic medicines. *D. malabaica* consists of 240 species, 59 of which are distributed throughout India, Thailand, Japan, Nigeria, South Africa and Philippines. In ayurvedic medicine and Indian medicinal plants: A compendium of 500 species mentioned that the root of *D. malabaica* is very effective in the polyurea, polydispisa and beneficial in the management of DM [9] Both the bark of the tree and unripe fruit has medicinal uses in Ayurveda. Plant pacifies vitiated pitta, kapha, burning sensation, inflammations, diarrhea, hemorrhoids, diabetes, skin diseases, fever, leucorrhea, urinary tract infections, splenomegali, anemia, burns and scalds [10]. Therefore we selected the plant *D. malabarica* stem bark for our research work. This plant contain a wide range of structurally diverse secondary metabolites have been identified including alkaloids, flavonoids, steroids, tannins, saponins and triterpenes. The reported biological activities are antioxidant [11], Antidiabetic [12, 13], Antiulcer [14], Antistress [15], Activity.

The literature survey revealed that the isolation work on *D. malabarica* stem bark was not carried out. The present study was conducted to isolate the secondary metabolites from bark of *D. malabarica* and they were screened for *in-vitro* antidiabetic activities.

Materials and Methods

Instrumental analyses: UV and IR were used to measure the absorbance and spectra UV-1800, Shimadzu, Japan and BRUKER ALPHA T – IR Spectrophotometer in our department. ¹H and ¹³C- NMR spectra were measured with a BRUKER AVANCE II 400 NMR Spectrophotometer and Mass spectra were recorded with WATERS Q-TOF MICROMASS (ESI-MS) from Panjab University, Chandigarh. Chemical shifts were given as δ (ppm) with tetramethylsilane (TMS) as an internal standard.

Plant materials: The *Diospyros malabarica* bark was collected nearby Dharwad and authenticated by Dr. S.S. Hebbar, Botanist, Preuniversity College, Dharwad -580 003.

Chemicals: Enzyme: (Type VIB: From porcine pancreas), Sigma, USA and 4-nitrophenyl- α -D-glucopyranoside (NPG), HiMedia were purchased. All other chemicals used were of analytical grade such as starch, sodium dihydrogen orthophosphate, disodium hydrogen phosphate, 3, 5-dinitrosalicylic acid, Potassium sodium tartarate, sodium hydroxide maltose monohydrate, dimethyl sulphoxide, sucrose. The pure drug meglitol was received as free sample from Dr. Veeresh Veerapur, Professor at Siddaganga College of Pharmacy, Tumkur, Karnataka.

Extraction and Isolation: The stem bark of *D. malabarica* (DM) was collected and washed, shade dried, cut into small pieces and crushed to moderately coarse powder. The root

powder was subjected to methanol extraction process by using soxhlet apparatus and obtained yield 34.25 g of methanol stem bark extract.

Fractionation: Methanol extract was subjected to liquid-liquid partition. 30 g of extract was dissolved in 300 ml of water and partitioned with hexane, chloroform and ethyl acetate using separating funnel. 12.3, 14.43 and 3.47 % w/v yield was obtained with hexane, chloroform and ethyl acetate respectively. Chloroform fraction was further subjected to column chromatography for separation of phytoconstituents.

Column chromatography: 12g of ethyl acetate fraction was packed in a column with silica (60-120) by wet packing method. The column was eluted with gradient mixture of chloroform: ethyl acetate and methanol. The flow rate was 2 ml/min. Fifty ml of eluents were monitored by pre-coated TLC plates. The first fraction eluted with chloroform: Ethyl acetate (90:10) and the R_f value of 0.66 denoted as SB2. The second fraction eluted with chloroform: Ethyl acetate (10:90) and R_f value was found to be 0.6, which was denoted as SB3. The third fraction eluted with ethyl acetate: methanol (90:10) and R_f value was 0.54, denoted as SB4. The fourth fraction eluted with ethyl acetate: methanol (70:30) and R_f value was found to be 0.8, denoted as SB5.

Later, the isolated compounds SB2, SB3, SB4 and SB5 structural elucidation was carried out with help of analytical instruments.

In-vitro antidiabetic studies

1. Alpha-amylase inhibition assay¹⁶: The samples (10 to 500 μg) were mixed with 200 μl of α -amylase soln, incubated for 10 min. Then 200 μl of 1% starch solution was added and incubated for 10 min. The enzyme reaction was stopped by adding 400 μl 3, 5 dinitro salicylic acid solution and transferred to a boiling water bath for 5 min. Then their absorbance was measured at 540 nm. Control represented 100% enzyme activity where, samples were replaced by buffer. The inhibition percentage of α -amylase was assessed by the following formula:

$$\text{Percentage inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

Isolation of enzyme¹⁷ Rats were sacrificed, intestine was removed and chilled with ice cold 80 mM phosphate buffer. The intestine was cut opened, the mucosa scraped off with a piece of glass rod and homogenized with cold phosphate buffer. Nuclei and large cell debris were removed by cold centrifugation at 2000 to 4000 rpm for 10 min and supernatant aliquote into 1.5 ml vials and stored at -20°C. The protein content of enzyme preparation was estimated by Lowry method.

2. Sucrase Inhibition assay: The samples (10 to 500 μg) or standard drug and 200 μl of enzyme solution were incubated for 10 min. Then 100 μl of sucrose solution (60 mM) was added and incubated for 10 min. The reaction was stopped by adding 200 μl of 3, 5-dinitrosalicylic acid reagent and transferred into a boiling water bath for 5min. The absorbance was measured at 540 nm by UV spectrophotometer. Control represented 100% enzyme activity where, samples were replaced by DMSO. The inhibition percentage of α -amylase was assessed by the following formula:

$$\text{Percentage inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

3. Alpha-glucosidase inhibition assay: Assay of α -glucosidase inhibition was performed according to Li *et al.* 2009. Samples (10 to 500 μ g) or standard and 200 μ l of enzyme solution were incubated for 10 min. Then 200 μ l of 2 mM p-nitrophenyl- α -D-glucopyranoside solution was added and incubated for 10 min. Then the reaction was stopped by adding 1 ml of 0.1M sodium carbonate. The absorbance was recorded at 405 nm by spectrophotometer. Control represented 100% enzyme activity where, samples were replaced by DMSO. The inhibition percentage of α -amylase was assessed by the following formula:

$$\text{Percentage inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

Results

In the present study, we isolated four compounds SB2, SB3, SB4 and SB5 were obtained by subjecting chloroform fraction to column chromatography.

Analysis of compound SB2 Physical state- White amorphous powder; Rf value- 0.66 [Chloroform: Ethyl acetate (90:10)]; Melting point- 225°C. The compound SB2 gave a positive response for Liebermann-Burchard test for triterpenoids.

Spectral characteristics Spectra of SB2 (Fig. -1-7)

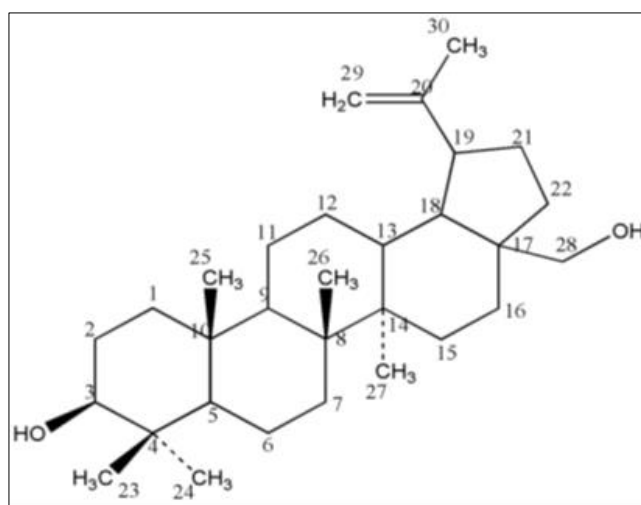
IR (KBr) - 3387.43 cm⁻¹ (br, OH), 2942.21 cm⁻¹ (C-H str in CH₃), 2868.35 cm⁻¹ (C-H str in CH₂), 1643.51 cm⁻¹ (C=C str), 1453.44 cm⁻¹ (C-H deformation in germinal dimethyl), 1033.40 cm⁻¹ (C-O str in 2° alcohol), 881.53 cm⁻¹ (=C-H deformation).

¹HNMR (DMSO) - δ 0.61 – δ 0.66 (t, 1H, C-5); δ 0.72 (s, 3H, C-24); δ 0.77 (s, 3H, C-25); δ 0.88 (s, 3H, C-23); δ 0.93 (s, 3H, C-27); δ 0.98 (s, 3H, C-26); δ 1.05 – δ 1.17 (m, 4H, C-11, 12); δ 1.23 – δ 1.27 (t, 3H, C-7, 9); δ 1.45 – δ 1.61 (m, 8H, C-1, 2, 6, 13, 18); δ 1.63 (s, 3H, C-30); δ 1.84 – δ 1.93 (m, 6H, C-16, 21, 22); δ 2.35 – δ 2.38 (t, 2H, C-15); δ 2.95 – δ 3.00 (m, 1H, C-19); δ 3.07 – δ 3.10 (m, 1H, C-3); δ 3.75 – δ

3.76 (d, 2H, C-28); δ 4.53 (s, 2H, C-29a); δ 4.64 (s, 2H, C-29b).

¹³CNMR (DMSO) - δ 39.32 (C-1); δ 18.72 (C-2); δ 79.15 (C-3); δ 39.52 (C-4); δ 54.83 (C-5); δ 17.92 (C-6); δ 36.63 (C-7); δ 40.40 (C-8); δ 49.81 (C-9); δ 38.26 (C-10); δ 24.73 (C-11); δ 6.60 (C-12); δ 38.44 (C-13); δ 42.14 (C-14); δ 27.99 (C-15); δ 29.29 (C-16); δ 47.34 (C-17); δ 7.30 (C-18); δ 48.14 (C-19); δ 150.17 (C-20); δ 28.95 (C-21); δ 33.78 (C-22); δ 27.09 (C-23); δ 5.62 (C-24); δ 15.83 (C-25); δ 15.64 (C-26); δ 14.43 (C-27); δ 57.97 (C-28); δ 109.43 (C-29); δ 20.32 (C-30);

Mass spectra: Molecular formula - C₃₀H₅₀O₂; Molecular weight – 442; (ESI-MS) (m/z) - 442.5 ([M+], C₃₀H₅₀O₂). The other peaks appeared at 411.5, 399.6, 385.5, 07.4, 203.6, 189.6 (100%), 175.4, 135.5 and 121.5. From the melting point, IR, ¹HNMR, ¹³CNMR and Mass spectra, Compound SB2 was designated as Betulin.



Betulin

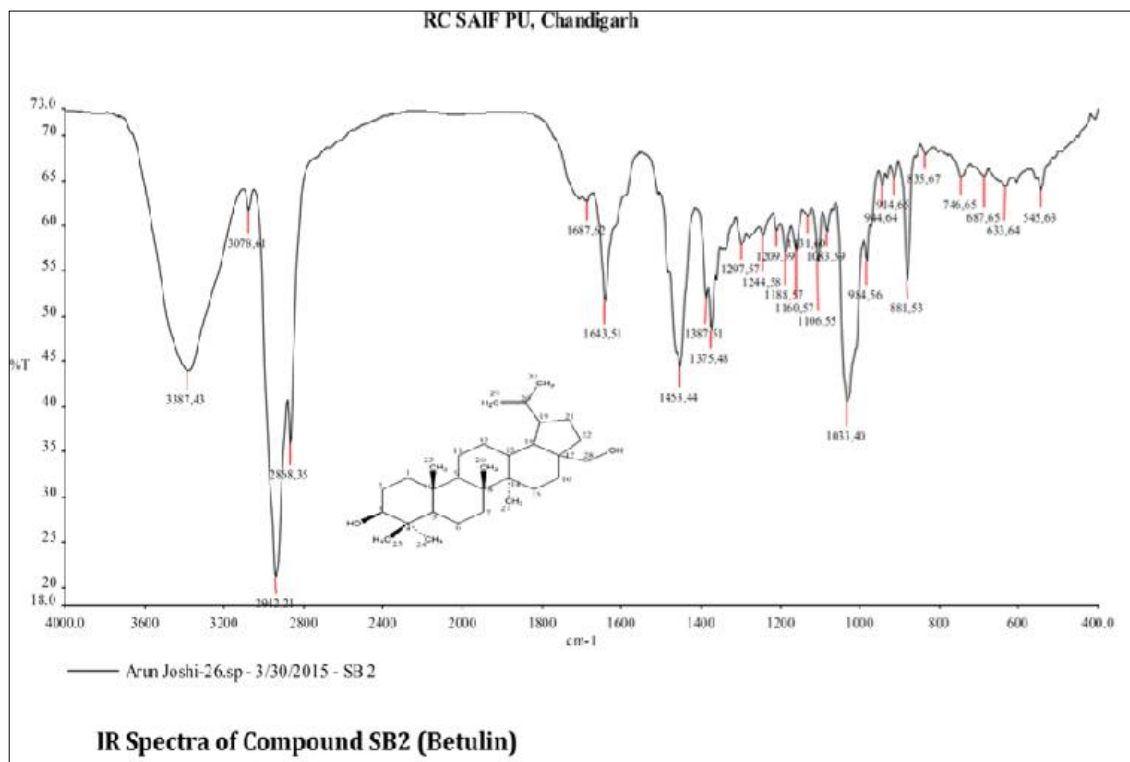


Fig 1: IR spectra of Betulin

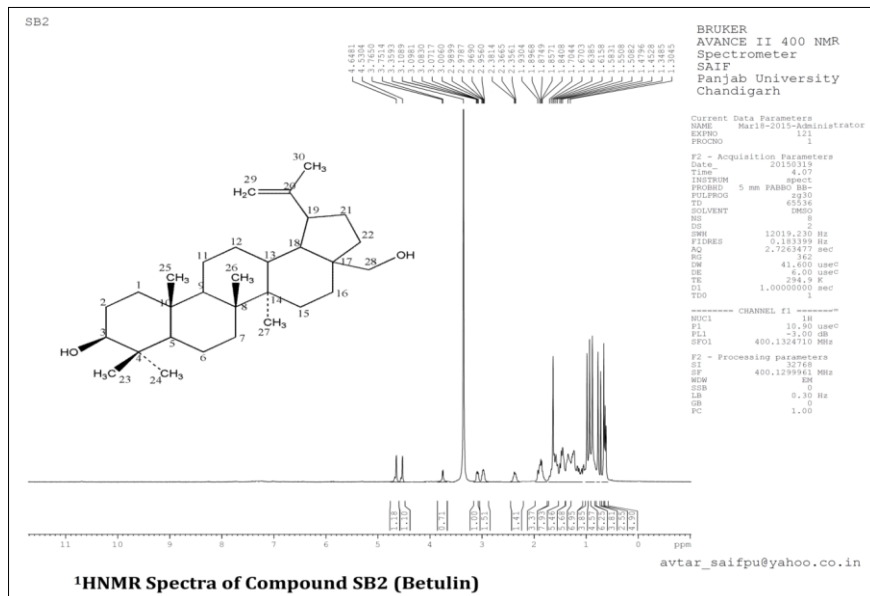


Fig 2: ¹H NMR Spectra of compound SB2 (Betulin)

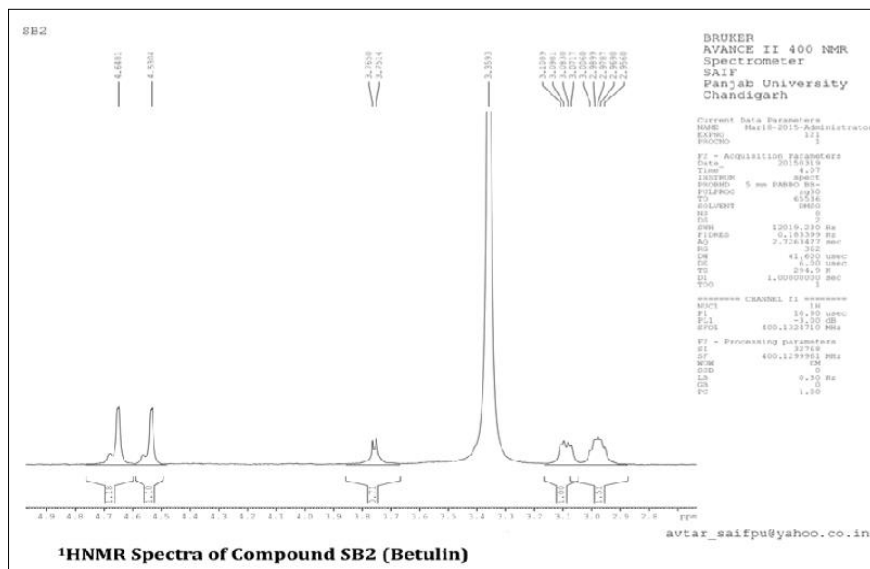


Fig 3: ¹H NMR Spectra of compound SB2 (Betulin)

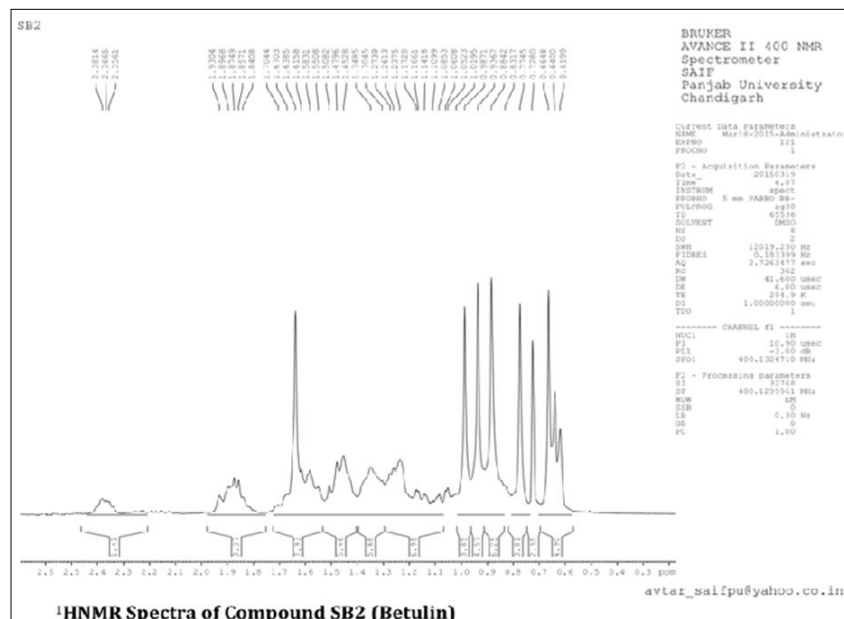


Fig 4: ¹H NMR Spectra of compound SB2 (Betulin)

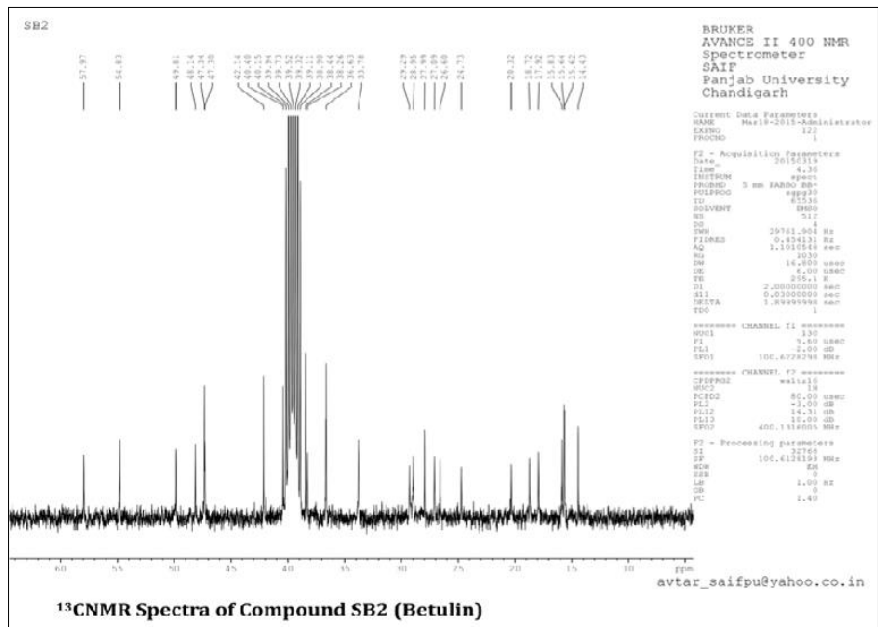


Fig 5: ¹H NMR Spectra of compound SB2 (Betulin)

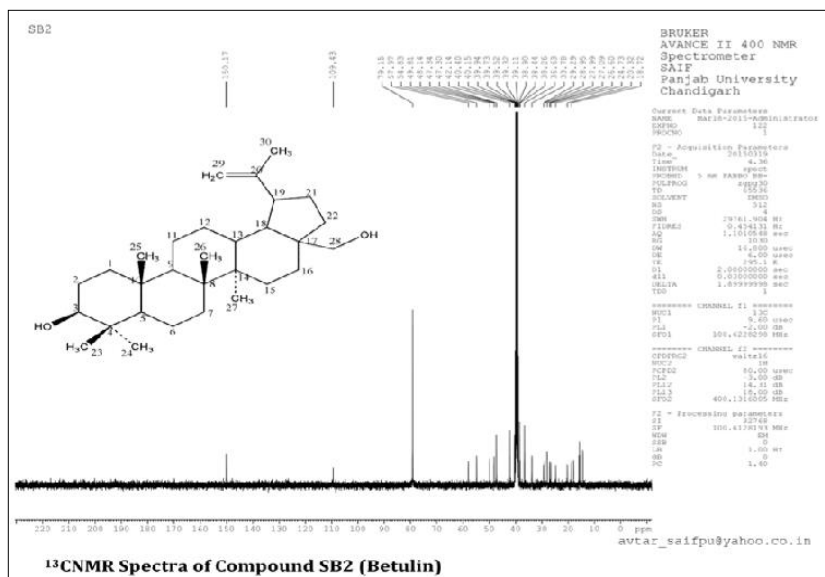


Fig 6: ¹H NMR Spectra of compound SB2 (Betulin)

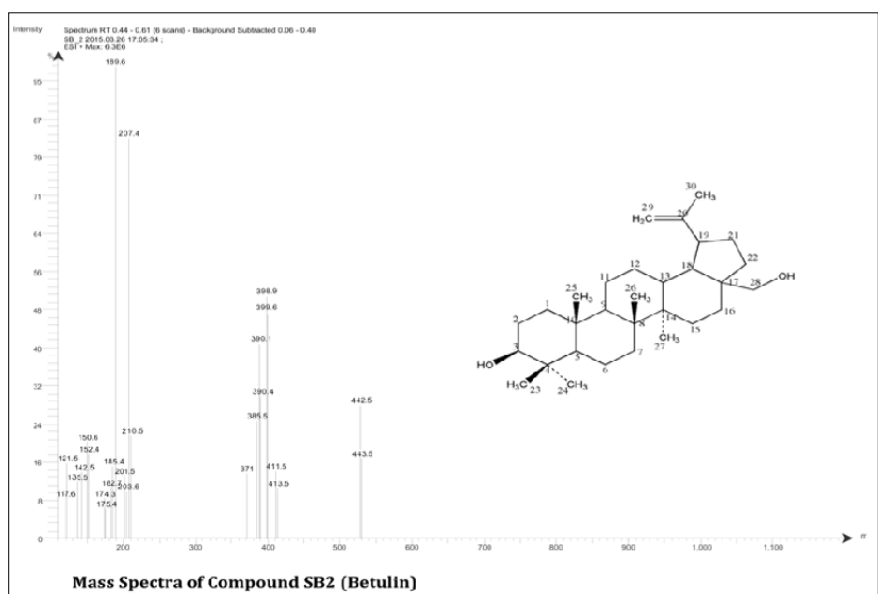


Fig 7: ¹H NMR Spectra of compound SB2 (Betulin)

Analysis of compound SB3: Physical state - White Amorphous Powder; Rf value - 0.6 (solvent system CHCl_3 : EtOAc 10:90); Melting Point - 317°C ; The compound SB3 gave a positive response for Liebermann- Burchard test for triterpenoids.

Spectral characteristics of compound SB3 - Spectra of SB3 (Fig. -8-14).

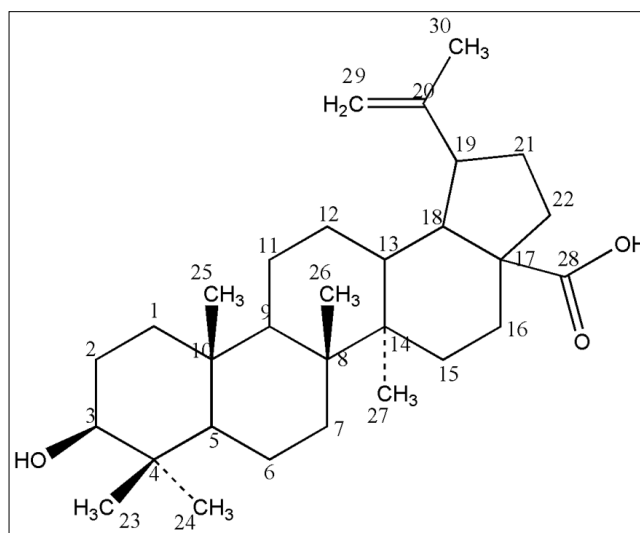
IR (KBr) - 3399.10 cm^{-1} (br, OH), 2950.70 cm^{-1} (C-H str in CH_3); 2847.12 cm^{-1} (C-H str in CH_2); 1714.21 cm^{-1} (C=O str in acid), 1634.36 cm^{-1} (C=C str); 1451.40 cm^{-1} (C-H deformation in geminal dimethyl); 1023.20 cm^{-1} (C-O str in 2° alcohol); 860.19 cm^{-1} (=C-H deformation).

$^1\text{H NMR}$ (DMSO) - δ 0.72 – δ 0.78 (m, 3H, C-1, 5); δ 0.97 (s, 3H, C-25); δ 1.02 (s, 3H, C-24); δ 1.05 (s, 3H, C-27); δ 1.09 (s, 3H, C-26); δ 1.11 (s, 3H, C-23); δ 1.15 – δ 1.26 (m, 5H, C-9, 11, 12); δ 1.34 – δ 1.45 (m, 6H, C-6, 7, 16); δ 1.48 – δ 1.57 (m, 7H, C-2, 18, 21, 22); δ 1.75 (s, 3H, C-30); δ 2.37 – δ 2.43

(m, 3H, C-13, 15); δ 3.50 – δ 3.56 (m, 2H, C-3, 19); δ 4.59 (s, 2H, C-29a); δ 4.66 (s, 2H, C-29b).

$^{13}\text{C NMR}$ (DMSO) - δ 39.04 (C-1); δ 27.39 (C-2); δ 78.49 (C-3); δ 39.11 (C-4); δ 55.57 (C-5); δ 18.55 (C-6); δ 33.78 (C-7); δ 40.44 (C-8); δ 50.10 (C-9); δ 38.44 (C-10); δ 21.07 (C-11); δ 24.73 (C-12); δ 38.78 (C-13); δ 42.17 (C-14); δ 29.92 (C-15); δ 32.32 (C-16); δ 47.37 (C-17); δ 48.14 (C-18); δ 49.47 (C-19); δ 150.15 (C-20); δ 30.28 (C-21); δ 38.26 (C-22); δ 27.95 (C-23); δ 16.32 (C-24); δ 16.51 (C-25); δ 16.77 (C-26); δ 15.75 (C-27); δ 180.87 (C-28); δ 108.48 (C-29); δ 19.12 (C-30).

Mass spectra- Molecular formula - $\text{C}_{30}\text{H}_{48}\text{O}_3$; Molecular weight - 456; (ESI-MS) (m/z) - 456.5 ($[\text{M}]^+$, $\text{C}_{30}\text{H}_{48}\text{O}_3$), the other peaks appeared at 440.5, 438.5, 426.4, 415.5, 262.4, 248.5, 208.7, 189.5, 163.5 (100%) and 135.6. From the melting point, IR, $^1\text{H NMR}$, $^{13}\text{C NMR}$ and Mass spectra, compound SB3 was designated as Betulinic Acid.



Betulinic acid

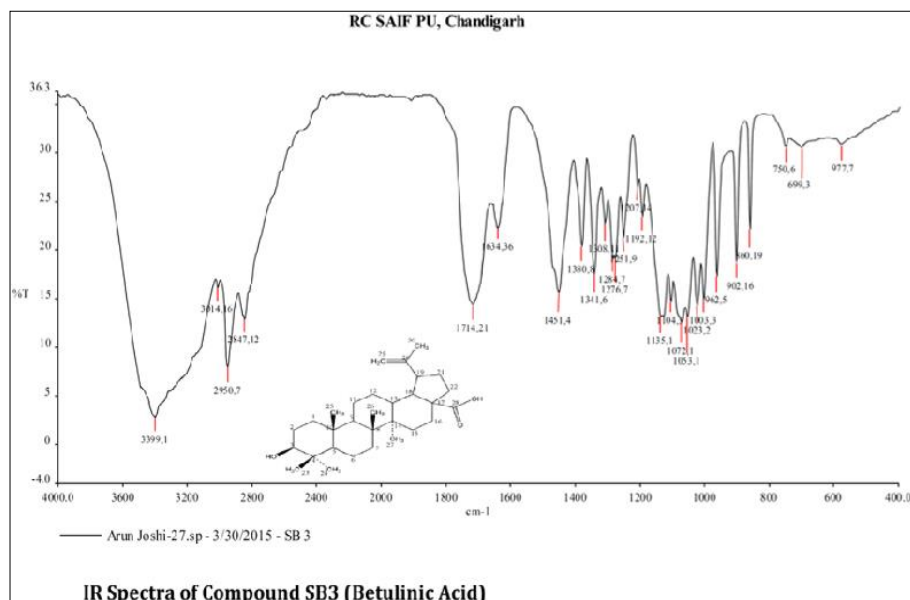


Fig 8: IR Spectra of Compound SB3 (Betulinic acid)

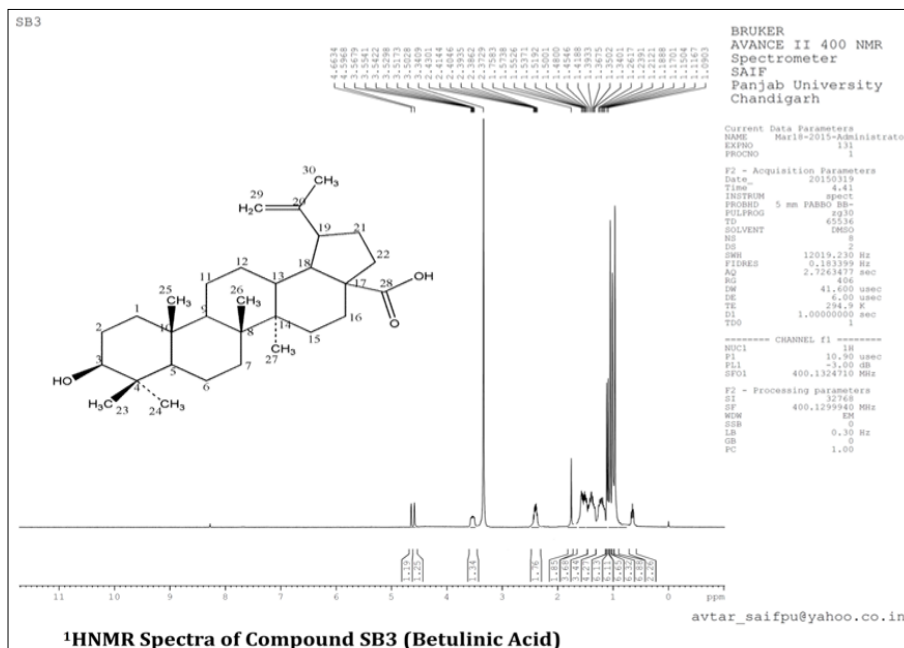


Fig 9: ¹H NMR Spectra of Compound SB3 (Betulinic acid)

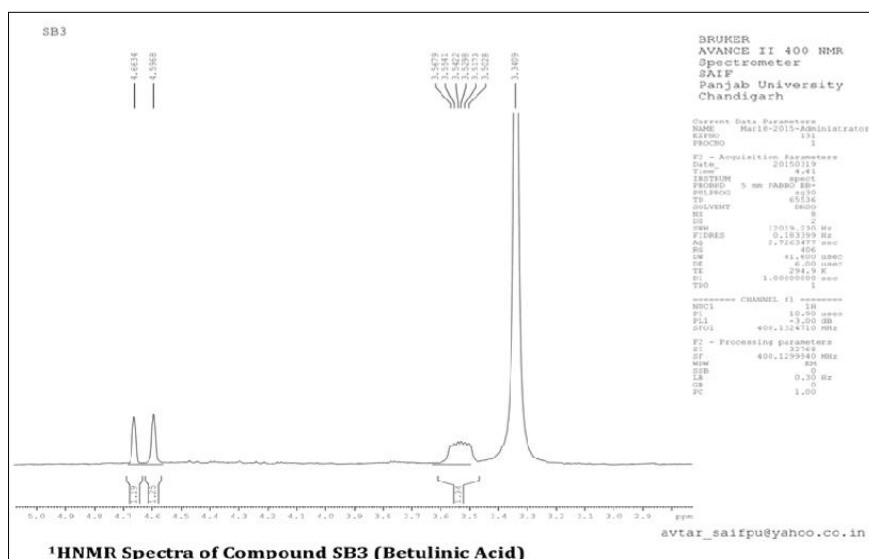


Fig 10: ¹H NMR Spectra of Compound SB3 (Betulinic acid)

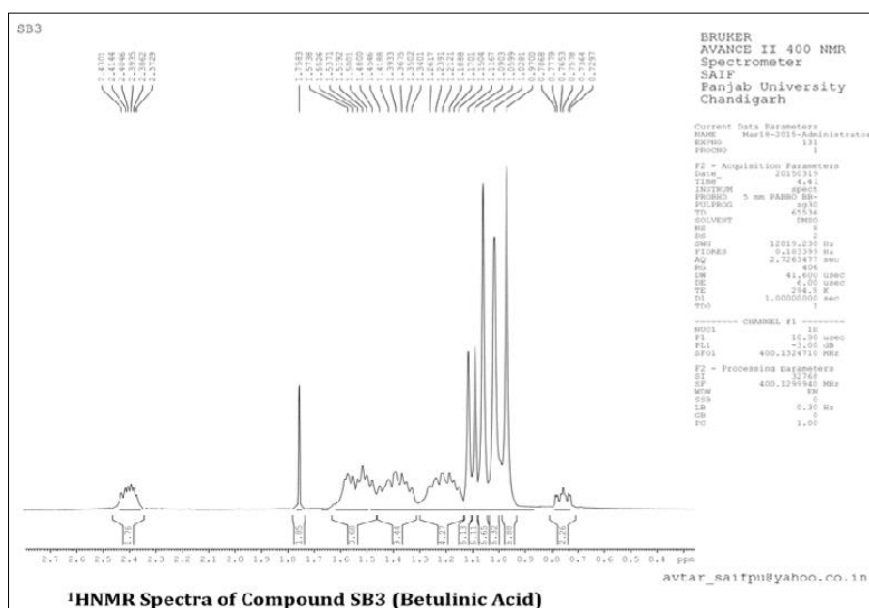
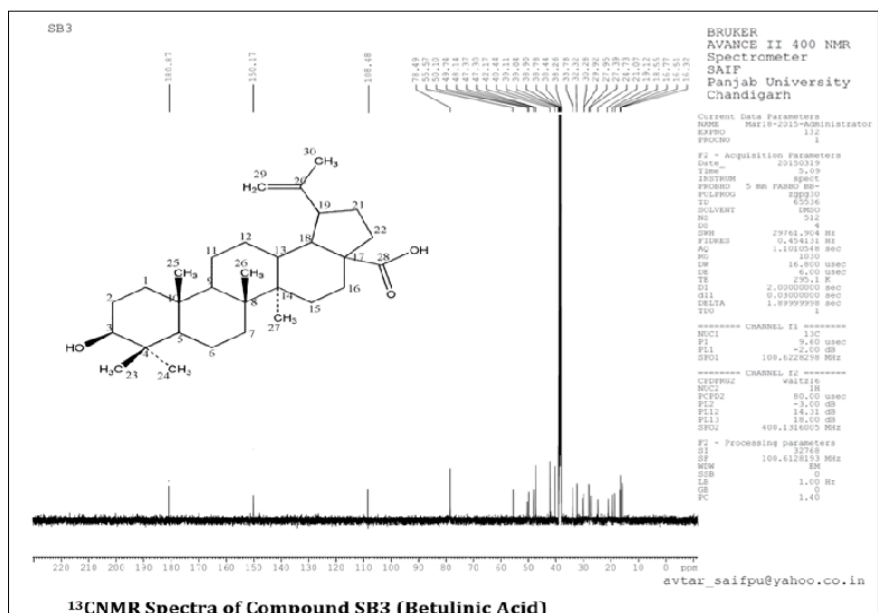
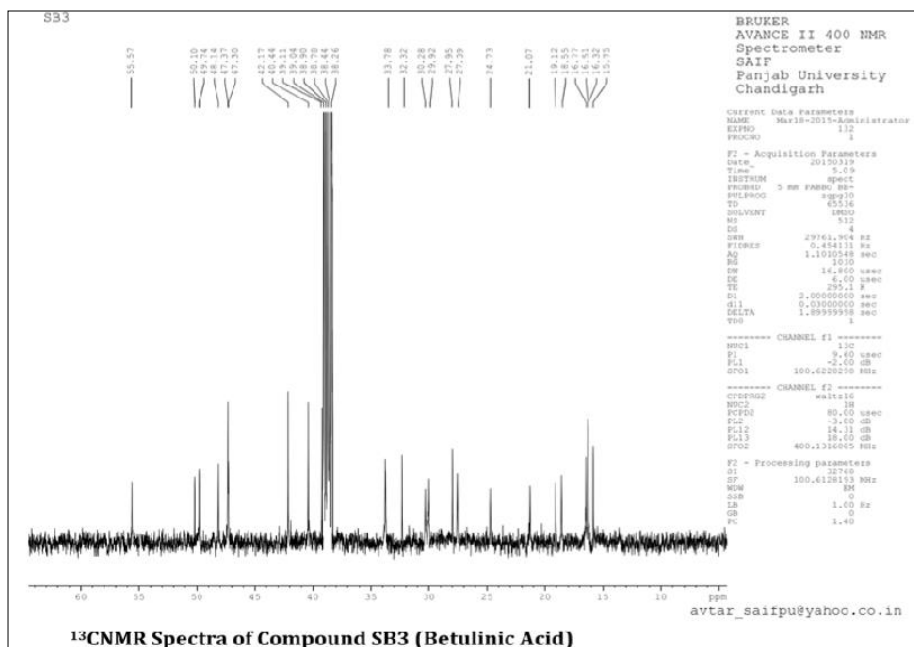


Fig 11: ¹H NMR Spectra of Compound SB3 (Betulinic acid)

Fig 12: ¹³CNMR Spectra of Compound SB3 (Betulinic acid)Fig 13: ¹³CNMR Spectra of Compound SB3 (Betulinic acid)

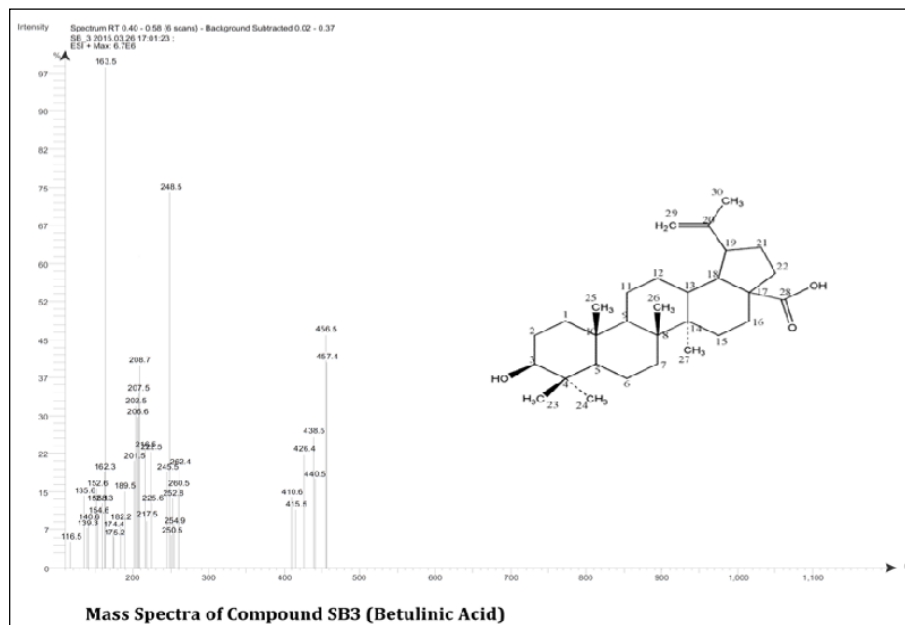


Fig 14: Mass Spectra of Compound SB3 (Betulinic acid)

Analysis of compound SB4: Physical state - Buff coloured Crystalline Powder; Rf value - 0.54 (solvent system EtOAc: MeOH 90:10); Melting Point - 271°C; The compound SB4 gave a positive response for Liebermann- Burchard test for triterpenoids.

Spectral characteristics of compound SB4 - Spectra of SB4 (Fig. 15-21).

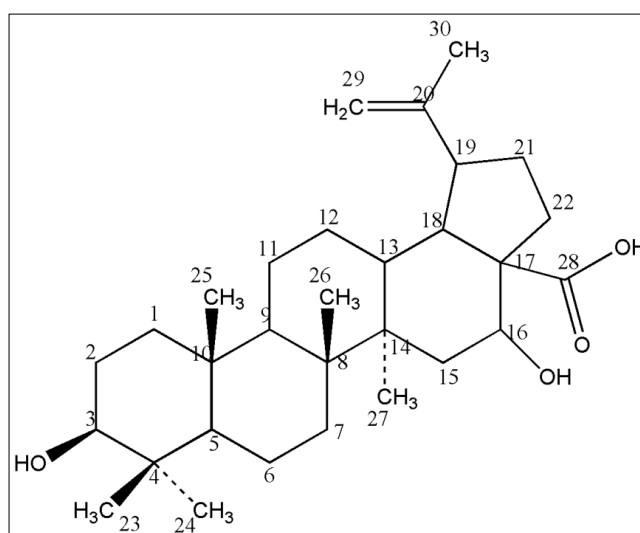
IR (KBr) - 3351.40 cm^{-1} (br, OH); 2952.47 cm^{-1} (C-H str in CH_3); 2846.10 cm^{-1} (C-H str in CH_2); 1712.71 cm^{-1} (C=O str in acid); 1633.37 cm^{-1} (C=C str); 1451.41 cm^{-1} (C-H deformation in geminal dimethyl); 1026.20 cm^{-1} (C-O str in 2° alcohol); 877.17 cm^{-1} (=C-H deformation).

$^1\text{H NMR}$ (DMSO) - δ 0.74 – δ 0.76 (t, 1H, C-5); δ 0.87 (s, 3H, C-25); δ 1.00 (s, 3H, C-24); δ 1.04 (s, 3H, C-27); δ 1.08 (s, 3H, C-26); δ 1.11 (s, 3H, C-23); δ 1.15 – δ 1.26 (m, 5H, C-9, 11, 12); δ 1.36 – δ 1.49 (m, 6H, C-1, 6, 7); δ 1.51 – δ 1.61 (m, 7H, C-2, 18, 21, 22); δ 1.77 (s, 3H, C-30); δ 1.81 – δ 1.83 (d, 2H, C-15); δ 2.35 – δ 2.45 (m, 1H, C-13); δ 3.49 – δ 3.55

(m, 2H, C-3, 19); δ 4.18 – δ 4.22 (dd, 1H, C-16); δ 4.60 (s, 2H, C29a); δ 4.71 (s, 2H, C-29b).

$^{13}\text{C NMR}$ (DMSO) - δ 39.07 (C-1); δ 27.39 (C-2); δ 78.55 (C-3); δ 39.15 (C-4); δ 55.43 (C-5); δ 18.54 (C-6); δ 34.26 (C-7); δ 40.45 (C-8); δ 50.11 (C-9); δ 37.38 (C-10); δ 21.10 (C-11); δ 24.73 (C-12); δ 38.26 (C-13); δ 42.17 (C-14); δ 29.90 (C-15); δ 79.67 (C-16); δ 49.17 (C-17); δ 49.54 (C-18); δ 49.73 (C-19); δ 150.55 (C-20); δ 30.27 (C-21); δ 37.00 (C-22); δ 27.96 (C-23); δ 16.31 (C-24); δ 16.51 (C-25); δ 16.75 (C-26); δ 15.72 (C-27); δ 180.57 (C-28); δ 109.08 (C-29); δ 19.10 (C-30)

Mass spectra: Molecular formula - $\text{C}_{30}\text{H}_{48}\text{O}_4$; Molecular weight - 472; (ESI-MS) (m/z) - 472.5 ($[\text{M}] + \text{C}_{30}\text{H}_{48}\text{O}_4$). The other peaks appeared at 454.9, 246.8, 207.6, 198.3, 189.7 (100%), 125.5. From the melting point, IR, $^1\text{H NMR}$, $^{13}\text{C NMR}$ and Mass spectra, compound SB4 was designated as 16-Hydroxybetulinic acid.



16-hydroxy betulinic acid

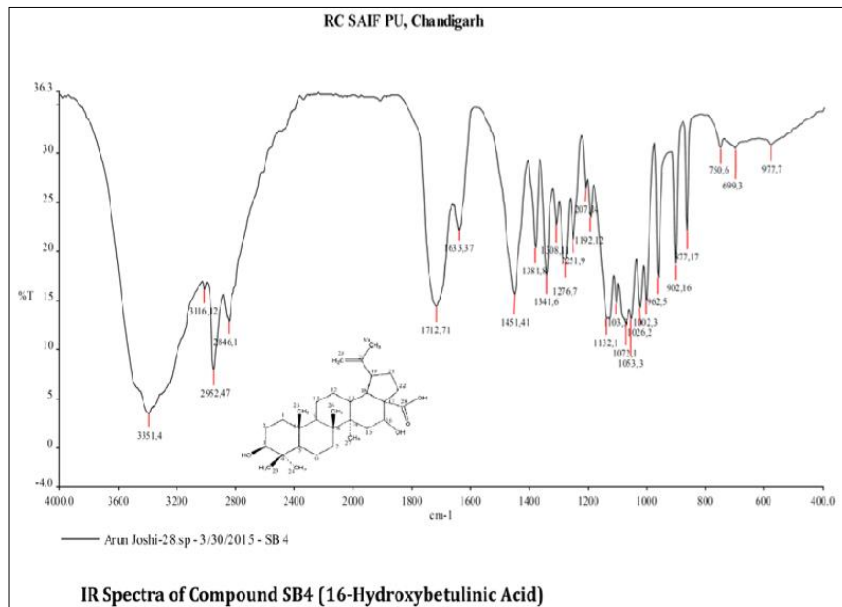


Fig 15: IR Spectra of Compound SB4 (16-Hydroxy betulinic acid)

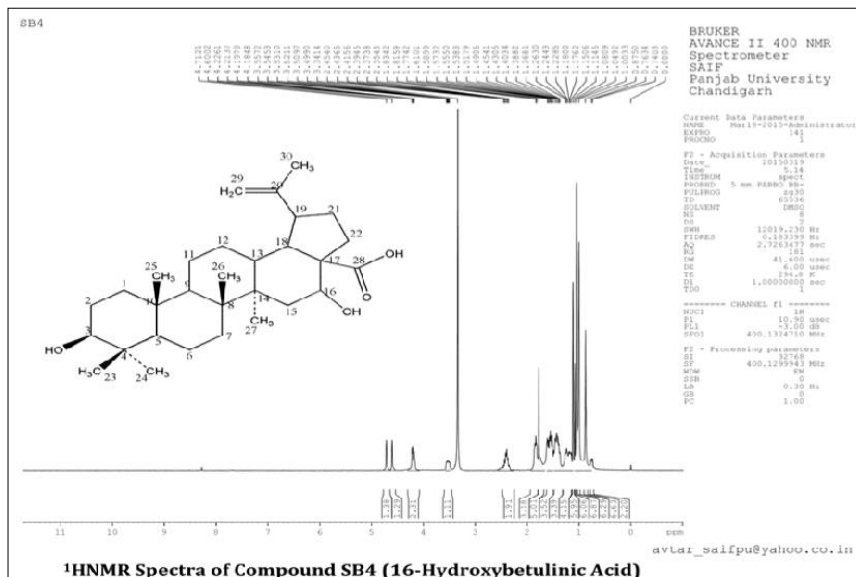


Fig 16: ¹H NMR Spectra of Compound SB4 (16-Hydroxy betulinic acid)

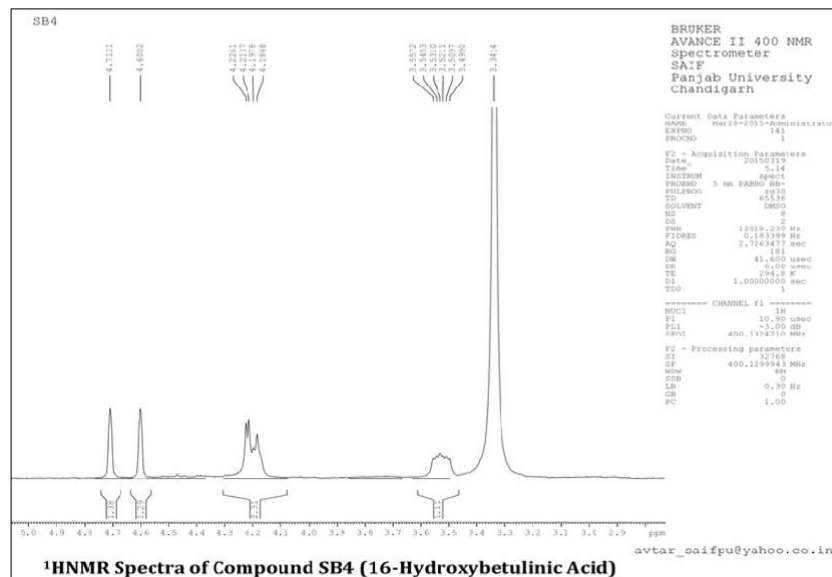


Fig 17: ¹H NMR Spectra of Compound SB4 (16-Hydroxy betulinic acid)

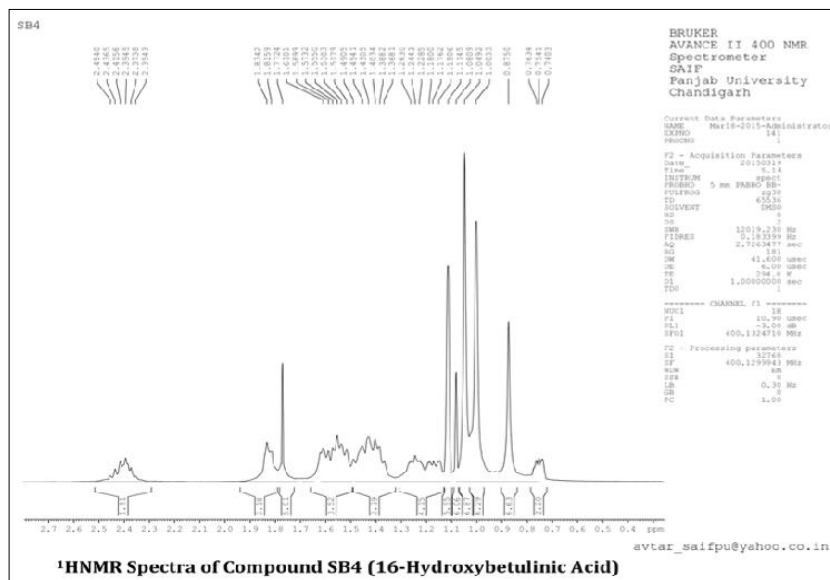


Fig 18: ¹H NMR Spectra of Compound SB4 (16-Hydroxy betulinic acid)

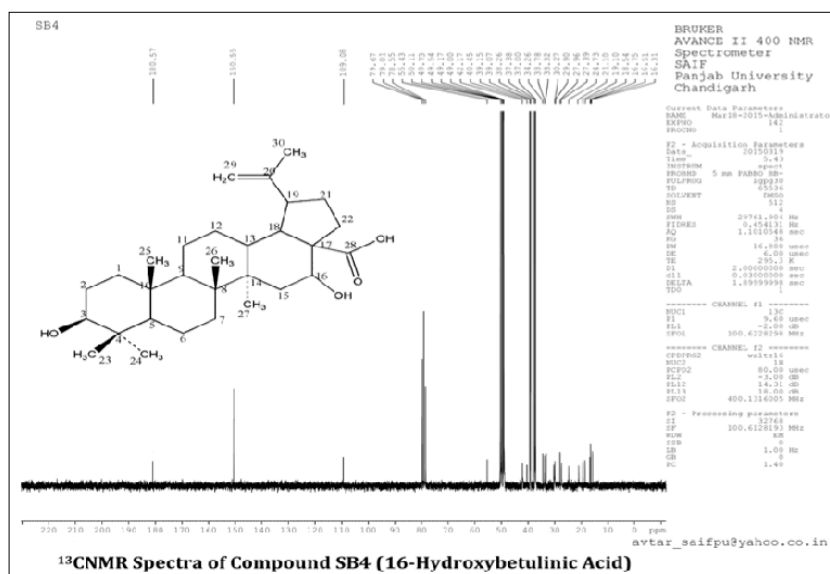


Fig 19: ¹³C NMR Spectra of Compound SB4 (16-Hydroxy betulinic acid)

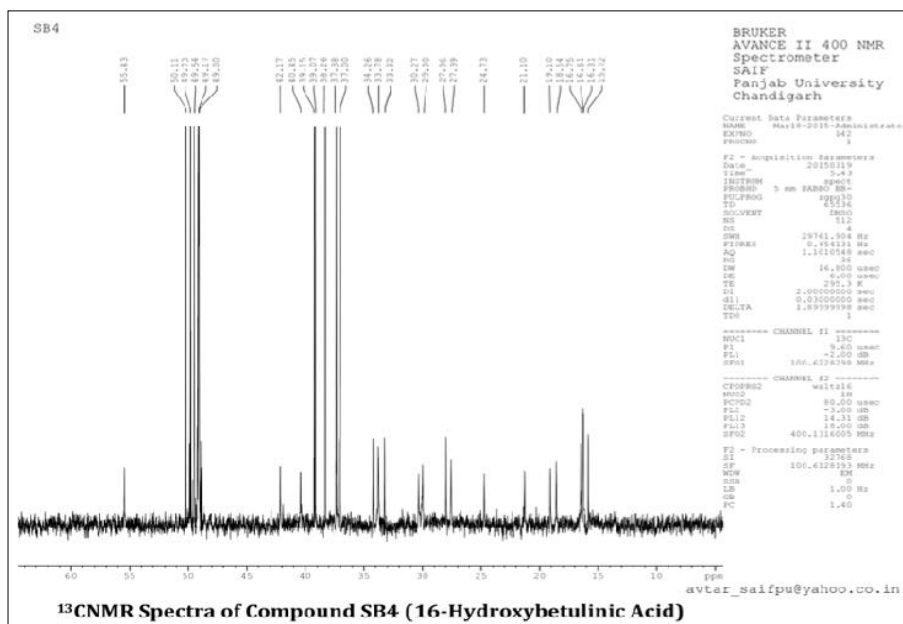


Fig 20: ¹³C NMR Spectra of Compound SB4 (16-Hydroxy betulinic acid)

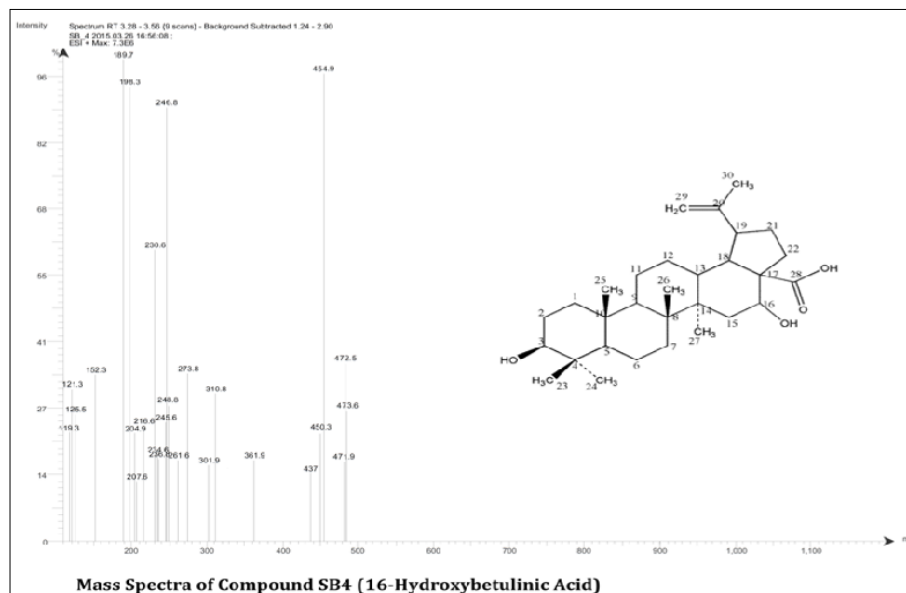


Fig 21: Mass Spectra of Compound SB4 (16-Hydroxy betulinic acid)

Analysis of compound SB5: Physical state - White Crystalline Powder; Rf value - 0.8 (solvent system EtOAc: MeOH 70:30); Melting Point - 210°C; The compound SB5 gave a positive response for Liebermann- Burchard test for triterpenoids.

Spectral characteristics of compound SB5 –Spectra (Figure- 22-28)

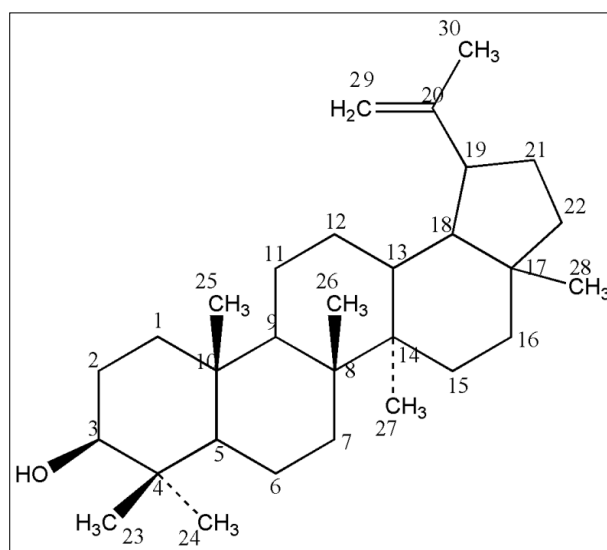
IR (KBr) - 3374.46 cm⁻¹ (br, OH); 2934.35 cm⁻¹ (C-H str in CH₃); 2868.33 cm⁻¹ (C-H str in CH₂); 1635.57 cm⁻¹ (C=C str); 1463.74 cm⁻¹ (C-H deformation in geminal dimethyl); 1034.49 cm⁻¹ (C-O str in 2° alcohol); 884.41 cm⁻¹ (=C-H deformation).

¹HNMR (DMSO) - δ 0.61 – δ 0.62 (t, 1H, C-5); δ 0.75 (s, 3H, C-28); δ 0.77 (s, 3H, C-23); δ 0.81 (s, 3H, C-24); δ 0.92 (s, 3H, C-25); δ 0.95 (s, 3H, C-26); δ 1.02 (s, 3H, C-27); δ 1.06 – δ 1.17 (m, 4H, C-11, 12); δ 1.23 – δ 1.37 (m, 7H, C-7, 9, 16, 22); δ 1.50 – δ 1.62 (m, 8H, C-1, 2, 6, 13, 18); δ 1.67 (s, 3H, C-30); δ 1.83 – δ 1.91 (m, 2H, C-21); δ 2.35 – δ 2.38 (t,

2H, C-15); δ 2.95 – δ 3.00 (m, 1H, C-19); δ 3.07 – δ 3.10 (m, 1H, C-3); δ 4.57 (s, 2H, C-29a); δ 4.65 (s, 2H, C-29b).

¹³CNMR (DMSO) - δ 38.01 (C-1); δ 27.47 (C-2); δ 79.09 (C-3); δ 38.68 (C-4); δ 55.30 (C-5); δ 18.33 (C-6); δ 33.78 (C-7); δ 40.16 (C-8); δ 50.40 (C-9); δ 37.71 (C-10); δ 21.07 (C-11); δ 25.13 (C-12); δ 37.92 (C-13); δ 42.84 (C-14); δ 27.39 (C-15); δ 35.58 (C-16); δ 48.25 (C-17); δ 48.14 (C-18); δ 48.03 (C-19); δ 150.00 (C-20); δ 28.52 (C-21); δ 40.00 (C-22); δ 28.11 (C-23); δ 15.32 (C-24); δ 16.30 (C-25); δ 15.85 (C-26); δ 14.52 (C-27); δ 18.01 (C-28); δ 109.04 (C-29); δ 19.47 (C-30).

Mass spectra: Molecular formula - C₃₀H₅₀O; Molecular weight – 426; (ESI-MS) (m/z) - 26.4 ([M⁺], C₃₀H₅₀O). The other peaks appeared at 411.7, 408.6, 218.4, 207.3, 189.4, 162.8 (100%), 108.4. From the melting point, IR, ¹HNMR, ¹³CNMR and Mass spectra, compound SB5 was designated as Lupeol.



Lupeol

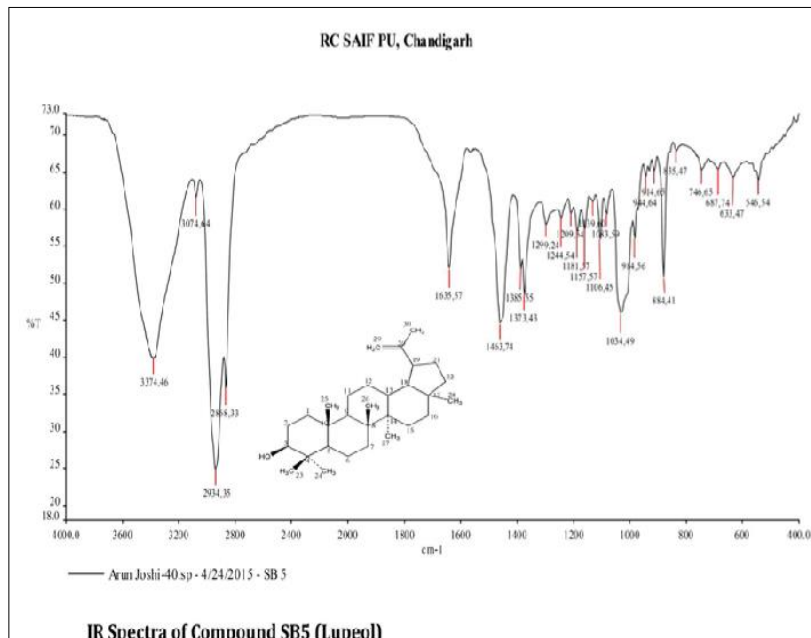


Fig 22: IR spectra of compound SB5 (Lupeol)

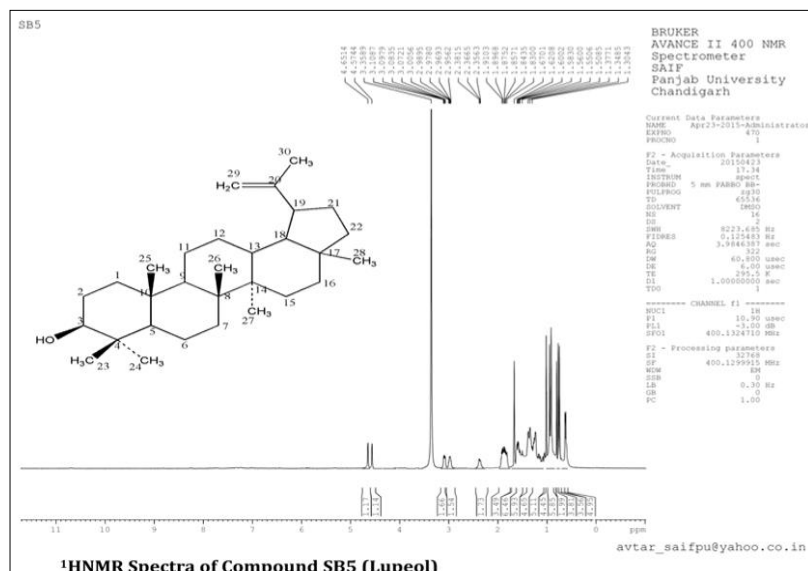


Fig 23: ¹HNMR spectra of compound SB5 (Lupeol)

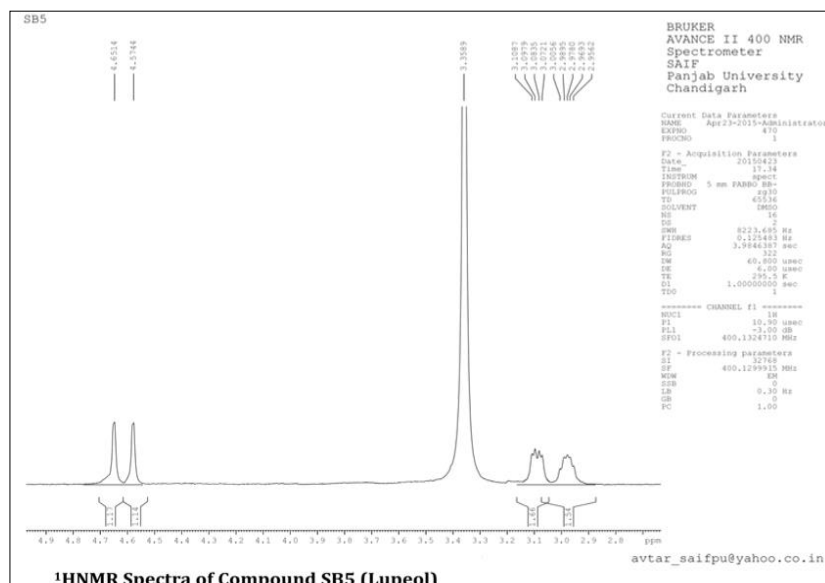


Fig 24: ¹³CNMR spectra of compound SB5 (Lupeol)

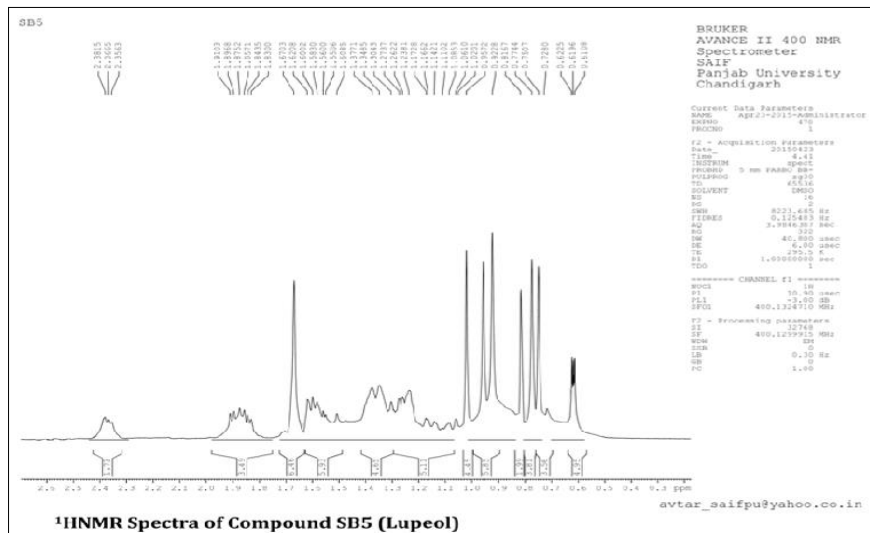


Fig 25: ¹H NMR spectra of compound SB5 (Lupeol)

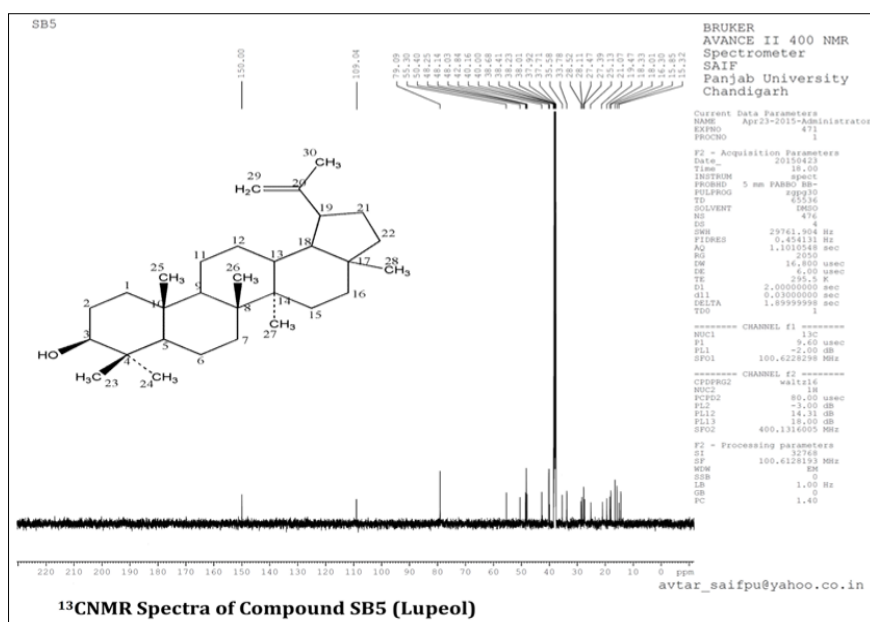


Fig 26: ¹³C NMR spectra of compound SB5 (Lupeol)

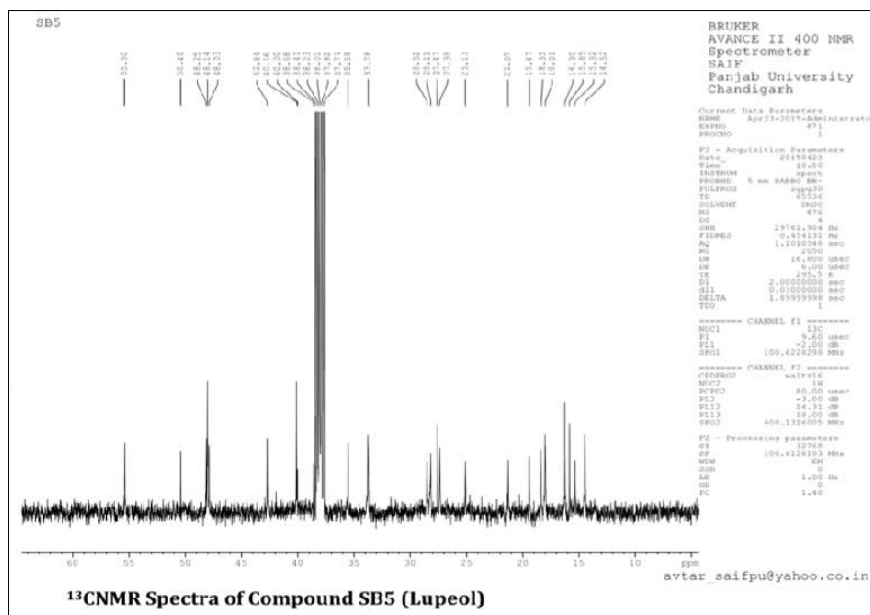


Fig 27: ¹³C NMR spectra of compound SB5 (Lupeol)

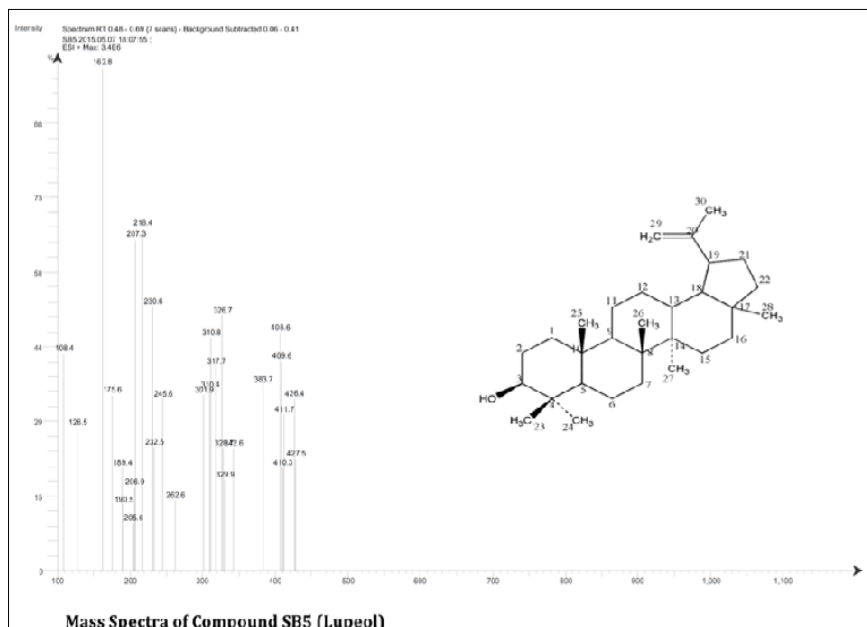


Fig 28: Mass spectra of compound SB5 (Lupeol)

In-vitro antidiabetic studies: The isolated compounds were evaluated for *in-vitro* antidiabetic activities such as α -

amylase, sucrase and α -glucosidase inhibitory assays. The results were shown in Table 1.

Table 1: the IC_{50} values of α -amylase, sucrase and α -glucosidase inhibitory concentration of isolated compounds of *Diospyros malabarica* stem bark.

Compounds	IC ₅₀ in μ g		
	α -amylase	Sucrase	α -Glucosidase
Meglitol	< 50	<50	<50
SB2 (Betulin)	61.8 \pm 1.9	416.8 \pm 8.1	65.2 \pm 1.4
SB3 (Betulinic acid)	56.1 \pm 1.4	87.9 \pm 3.3	47.4 \pm 0.7
SB4 (16-hydroxy Betulinic acid)	279.2 \pm 1.6	278.7 \pm 8.4	112.9 \pm 1.3
SB5 (Lupeol)	435.2 \pm 8.1	428.7 \pm 3.4	271.9 \pm 2.1

MEF – Ethyl acetate fraction of *D. malabarica*, Meglitol was used as positive control. Data presented is the mean \pm SD of samples runs in triplicate.

Discussion

Hyperglycaemia, an abnormal postprandial increase of blood glucose level in type 2 insulin-dependent diabetes mellitus is associated with cardiovascular complications including hypertension. Carbohydrate metabolizing enzymes such as α -amylase and α -glucosidase, such as sucrase are key enzymes involved in starch breakdown and intestinal glucose absorption respectively. The inhibition of these enzymes slow-down the passage of carbohydrates into the bloodstream and significantly decrease the postprandial blood glucose after meals. Therefore can be an important strategy in the management of type 2 diabetes [16]. A main drawback of currently used α -glucosidase inhibitors (acarbose) are abdominal distension, flatulence and possibly diarrhoea. Therefore inhibitors from medicinal plants are useful due to their minimal side effects.

Kavatagimath *et.al.* Reported that ethanol extract of *D. malabarica* stem bark has indicated significant antioxidant and antidiabetic activity in alloxan-induced rat model [18]. Therefore we selected *D. malabarica* stem bark for our research work. The present study was conducted to isolate secondary metabolites from stem bark of *D. malabarica* and they were screened for *in-vitro* antidiabetic activities.

This is the first report for isolation of betulin, betulinic acid and 16-Hydroxy betulinic acid and lupeol from the stem bark

of *D. malabarica*. All the isolated compounds were identified on the basis of IR and [1]. H NMR, [13]. C NMR and mass spectral data and compared with reported literature and confirmed the fractions i.e., SB2, SB3, SB4 and SB5 compounds from the chloroform fraction of the stem bark of *D. malabarica* as betulin, betulinic acid, 16-hydroxy betulinic acid and lupeol.

Betulin and betulinic acid are naturally occurring triterpenes found in birch tree, (*Betula* spp., Betulaceae) is the most widely reported source. They are also isolated from various sources include *Zizi phus* spp. (Rhamnaceae), *Syzygium* spp. (Myrtaceae), *Diospyros* spp. (Ebenaceae) and *Paeonia* spp. (Paeoniaceae). Recently, many studies have been conducted to reveal their pharmacological properties of both betulin and betulinic acid: i.e., antiviral (anti-influenza, anti-HIV), anti-inflammatory, antiallergenic, hepatoprotective, antituberculosis and anti-cancer activity. Betulinic acid was evaluated for PPAR γ antagonist that improves glucose uptake, promotes osteogenesis and inhibits Adipogenesis activities [19]. Anti-tumour-promoting and thermal-induced protein denaturation inhibitory activities of β -sitosterol and lupeol isolated from *Diospyros lotus* L [20].

In the present study, *in-vitro* antidiabetic activities of isolated compounds - indicated that betulin exhibited significant IC_{50} of 61.8 \pm 1.9, 65.2 \pm 1.4 of α -amylase and α -glucosidase enzyme respectively. Betulinic acid exhibited significant IC_{50} of 56.1 \pm 1.4, 47.4 \pm 0.7 of α -amylase and α -glucosidase enzyme respectively. The present study indicated betulin and betulinic acid showed significant enzyme inhibitory activity and may

be useful in managing the postprandial hyperglycaemia in diabetes mellitus.

Conclusion

From the research work, we isolated four known triterpene compounds from *D. malabarica* stem bark and were confirmed as betulin, betulinic acid, 16-hydroxy betulinic acid and lupeol. They were identified by comparing their chemical and spectral data with that of published literatures and screened them for *in-vitro* antidiabetic activities. The compound betulin and betulinic acid had shown significant enzyme inhibitory activity may be helpful in the management of diabetes mellitus. Further preclinical studies may be extended to elucidate their mechanism.

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References

- Ozougwu JC, Obimba KC, Belonwu CD, Unakalamba CB, Review-The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. *J Physiol Pathophysiol.* 2013; 4(4):46-57.
- Diagnosis and Classification of Diabetes Mellitus. American diabetes association Editorial). *Diabetes care.* 2013; 36(1):67-74.
- International Diabetes Federation. IDF Diabetes Atlas-7th Edition Diabetes Atlas, 2015, <http://www.diabetesatlas.org/>
- Holman N, Young B, Gadsby R. Current prevalence of type 1 and type 2 diabetes in adults and children in the UK. *Diabet Med.* 2015; 32:1119-1120.
- Yan Z, Sylvia HL, Frank BH. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nature Reviews Endocrinology.* 2018; 14:88-98.
- Beagley J, Guariguata L, Weil C, Motala AA. Global estimates of undiagnosed diabetes in adults. *Diabetes Res. Clin. Pract.* 2014; 103:150-160.
- Heiko Bugger, Abel ED, Review; Molecular mechanisms of diabetic cardiomyopathy. *Diabetologia.* 2014; 57:660-671.
- Cox PA, The ethnobotanical approach to drug discovery: strengths and limitations. In, ethnobotany and the search for the new drugs. John Wiley and Sons. UK, 1994; 25-36.
- Warrier PK, Nambiar VPK, Cramankutty. Indian medical plants: A compendium of 500 species, Orient Longman Ltd. 1994; (2):339-341.
- Ravikumar A, Vengalrao P, Shobhana K, Kishore S. An overview on *Diospyros malabarica*. *International Journal of Novel Trends in Pharmaceutical Sciences.* 2014; 4(5):93-96.
- Mandal SK, Chakraborty G, Gupta M, Muzumdar UK, In vitro antioxidant activity of *Diospyros malabarica* Kostel bark. *IJEB.* 2006; 44:39-44.
- Dewanjee S, Das AK, Sahu R, Gangopadhyay M. Antidiabetic activity of *Diospyros peregrina* fruit: effect on hyperglycemia, hyperlipidemia and augmented oxidative stress in experimental type 2 diabetes. *Food Chem Toxicol.* 2009; 47(10):2679-85.
- Satish AK, Sunil SJ. Screening of Ethanolic extract of *Diospyros malabarica* Desr. Bark for Anti-diabetic and Antioxidant Potential. *Indian Journal of Pharmaceutical Education and Research.* 2016; 50(1):179-189.
- Gopalakrishna CH, Reddy AK, Sharief N, Sushma KR, Narendra S, A Study on Anti-Ulcer Activity of *Diospyros malabarica* Bark in Ulcer Induced Rats. *International Journal for Pharmaceutical Research Scholars.* 2014; 3(2):2277-7873.
- Laxmikant MP, Suryadevera V, Antistress activity of *Diospyros malabarica* (Desr.) Kostel in mice and rats. *IJPSR.* 2016; 7(8):3299-3305.
- Kamtekar S, Keer V, Patil V. Estimation of Phenolic content, Flavonoid content, Antioxidant and Alpha amylase Inhibitory Activity of Marketed Polyherbal Formulation. *Journal of Applied Pharmaceutical Science.* 2014; 4(9):61-65.
- Hondaa M, Yukihiko Haraa Y. Inhibition of Rat Small Intestinal Sucrase and α -Glucosidase Activities by Tea Polyphenols. *Biosci. Biotech. Biochem.* 1993; 57(1):123-124.
- Kavatagimath SA, Jalalpure SS. Screening of Ethanolic extract of *Diospyros malabarica* Desr. Bark for Anti-diabetic and Antioxidant Potential. *Indian Journal of Pharmaceutical Education and Research.* 2016; 50(1):179-189.
- Gloria B, Roberta M, Davide C, Giulia C, Antonio L, Paolo T, *et.al.* Betulinic acid is a Ppar γ antagonist that improves glucose uptake, promotes osteogenesis and inhibits Adipogenesis. *Scientific Reports.* 2017; 7(5777):1-14.
- Rauf A, Uddin G, Khan H, Raza M, Zafar M, Tokuda H. *et al.* Anti-tumour-promoting and thermal-induced protein denaturation inhibitory activities of β -sitosterol and lupeol isolated from *Diospyros lotus* L. *Nat Prod Res.* 2016; 30(10):1205-7.