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Isolation and characterization of triterpenoids and fatty acid ester of triterpenoid from leaves of *Bauhinia variegata*

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ABSTRACT

Indian traditional medicinal plant Bauhinia variegata were extracted with ethanol and fractionated successively with petroleum ether (60 – 80°C), chloroform, n-butanol and methanol. The preliminary phytochemical studies of ethanolic extract of plant material showed the presence of carbohydrate, protein, glycosides, triterpenoids, saponins and steroids. The detailed phytochemical investigation of petroleum ether fraction of ethanolic extract of this plant isolated three triterpenoids and a fatty acid ester of triterpenoid, which were identified by spectroscopical analysis as α- amyrin caprylate, Lupeol, nor-α-amyrin and 3β, 28-di hydroxyl olean-12-enyl-palmitate, respectively.

Keywords: *Bauhinia variegate,* Fatty acid ester of triterpenoid, Phytochemical investigation, Phytoconstituents, Triterpenoids.

INTRODUCTION

Bauhinia variegata Linn. (Caesalpiniaceae), which is commonly known as 'Kachnar' in Hindi, is a medium sized deciduous tree and distributed in India, Burma and China. Traditionally this plant is used in cough conditions, asthma, abdominal distention also acts as a gargle for sore throats, prevent from skin diseases, or internally as a remedy for diarrhoea. It is helpful in managing skin discoloration, veiling, baldness and conditions involving bilious [1]. Previous

studies have reported various biological activities such as antimicrobial, anti-inflammatory, antitumor, cytotoxic and hepatoprotective activities of B. variegata [2-4]. Many phytochemical constituents of this plant has also been reported, such as, kaempferol, ombuin, kaempferol 7,4'-dimethyl ether 3-O-beta-d-glucopyranoside, kaempferol 3-O-beta-d-glucopyranoside, isorhamnetin 3-O-beta-d-glucopyranoside, hesperidin, triterpene caffeate, 3beta-trans-(3,4-dihydroxycinnamoyloxy)olean-12-en-28-oic acid, flavanone, dihydrodibenzoxepin, flavonol glycoside 5, 7, 3', 4' -tetrahydroxy-3-methoxy-7-O-alpha-L-rhamnopyranosyl(1-->3)-O-beta-galactopyranoside insulin like protein, triterpenoid saponins and flavonoids [5-9]. In the present study, we have isolated and identified a fatty acid ester of triterpenoid and three triperpenoids. The fatty acid ester of triterpenoid from this plant has been isolated for first time.

MATERIALS AND METHODS

Equipment and chemicals

All the melting points were recorded in a Toshniwal melting point apparatus and were uncorrected. IR spectra of the compounds were recorded using the KBr pellet method on a Perkin Elmer 700 IR spectrophotometer. NMR spectra of the compound were taken on Bruker Avance II 400 NMR spectrometer in $CDCl_3$, at 400 MHz for ^{1}HNMR and 100.62 MHz for ^{13}CNMR with tetramethylsilane (TMS) as internal standard. ESI (Electrospray ionization) / APCI (Atmospheric pressure chemical ionization) Mass spectra (positive-ion-mode) were taken on a SHIMADZU LCMS-2010A. TLC was carried out using Silica gel 60 F_{254} plates (Merck). Column chromatography was carried out on neutral alumina of 70-300 mesh from S.D. fine chemicals Pvt. Ltd., Bombay. All the chemicals and reagents used were obtained in high purity either from S.D. fine chemicals Pvt. Ltd; Bombay, India or E. Merck Pvt. Ltd., Bombay, India.

Plant material

The leaves of *Bauhinia variegata* were collected from Paneer, India during August 2008 and its botanical authentication voucher specimen (voucher no. 324 b) has been deposited in NGSM Institute of Pharmaceutical Sciences, Derelakatte, Mangalore, India.

Extraction and isolation of plant material

The shade dried powdered leaves (5 kg) soaked in ethanol (95%) and kept aside for four days. After four days, the ethanolic layer was decanted off. The process was repeated for four times. The solvent from the total extract was distilled off and the concentrate was evaporated on a water bath to a syrupy consistency and then evaporated to dryness.

Preliminary Phytochemical screening

The preliminary phytochemical studies [10] were performed on the ethanolic extract of the leaves of *Bauhinia variegata* and the results are shown in table 1.

1. Alkaloids

(a) Dragendorff's test

To 2 mg of the ethanolic extract 5 ml of distilled water was added, 2M Hydrochloric acid was added until an acid reaction occurs. To this 1 ml of Dragendorff's reagent was added. Formation of orange or orange red precipitate indicates the presence of alkaloids.

(b) Hager's test
To 2 mg of the ethanolic extract taken in a test tube, a few drops of Hager's reagent were added. Formation of yellow ppt confirms the presence of alkaloids.

c) Wagner's test
2 mg of ethanolic extract was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner's reagent were added. A yellow or brown ppt. indicates the presence of alkaloids.

(d) Mayer's test
To a few drops of the Mayer's reagent, 2 mg of ethanolic extract was added. Formation of white or pale yellow precipitate indicates the presence of alkaloids.

2. Carbohydrates
(a) Anthrone test
2 mg of ethanolic extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 2ml of anthrone reagent solution was added. Formation of green or blue colour indicates the presence of carbohydrates.

(b) Benedict's test
2 mg of ethanolic extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 5 ml of Benedict's solution was added and boiled for 5 minutes. Formation of brick red coloured ppt indicates the presence of carbohydrates.

(c) Fehling's test
2 mg of ethanolic extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 1 ml mixture of equal parts of Fehling's solution A and B were added and boiled for few minutes. Formation of red or brick red coloured precipitate indicates the presence of reducing sugar.

(d) Molisch's test
2 mg of ethanolic extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 2 drops of freshly prepared 20% alcoholic solution of α- naphthol was added. 2 ml of conc. sulphuric acid was added so as to form a layer below the mixture. Red-violet ring appear, indicating the presence of carbohydrates which disappear on the addition of excess of alkali.

3. Proteins
(a) Biuret's test
To 1 ml of hot aqueous extract, 5-8 drops of 10% w/v sodium hydroxide solution, followed by 1 or 2 drops of 3% w/v copper sulphate solution were added. Formation of violet red colour indicated the presence of proteins.

(b) Millon's test
1 ml of aqueous extract was dissolved in 1 ml of distilled water and 5-6 drops of Millon's reagent were added. Formation of white precipitate, which turns red on heating, indicated the presence of proteins.

4. Flavonoids

(a) Shinoda's test

2 mg of ethanolic extract was dissolved in 5ml of ethanol and to this 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish or brown colour indicates the presence of flavonoids.

5. Glycosides

Molisch's test

2 mg of ethanolic extract was shaken with 10ml of water, filtered and the filtrate was concentrated. To this 2-3 drops of Molisch's reagent was added, mixed and 2ml of concentrated sulfuric acid was added carefully through the side of the test tube. Reddish violet ring appear, indicating the presence of glycosides.

6. Triterpenoids

Liebermann - Burchard's test

2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of a pink colour indicates the presence of triterpenoids.

7. Resins

1 ml of ethanolic extract was dissolved in acetone and the solution was poured in distilled water. Turbidity indicates the presence of resins.

8. Saponins

In a test tube containing about 5 ml of an ethanolic extract, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 minutes. Formation of honeycomb like froth indicates the presence of saponins.

1. Steroids

(a) Liebermann-Burchard's test

2 mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of green colour indicates the presence of steroids.

(b) Salkowski reaction

2 mg of dry extract was shaken with chloroform, to the chloroform layer sulphuric acid was added slowly by the sides of test tube. Formation of red colour indicated the presence of steroids.

10. Tannins

To 1-2 ml of the ethanolic extract, few drops of 5% w/v $FeCl_3$ solution was added. A green colour indicated the presence of gallotannins, while brown colour indicates the presence of pseudotannins.

11. Starch

0.01 g of Iodine and 0.075 g of potassium iodide were dissolved in 5 ml of distilled water and 2-3 ml of ethanolic extract was added. Formation of blue colour indicated the presence of starch.

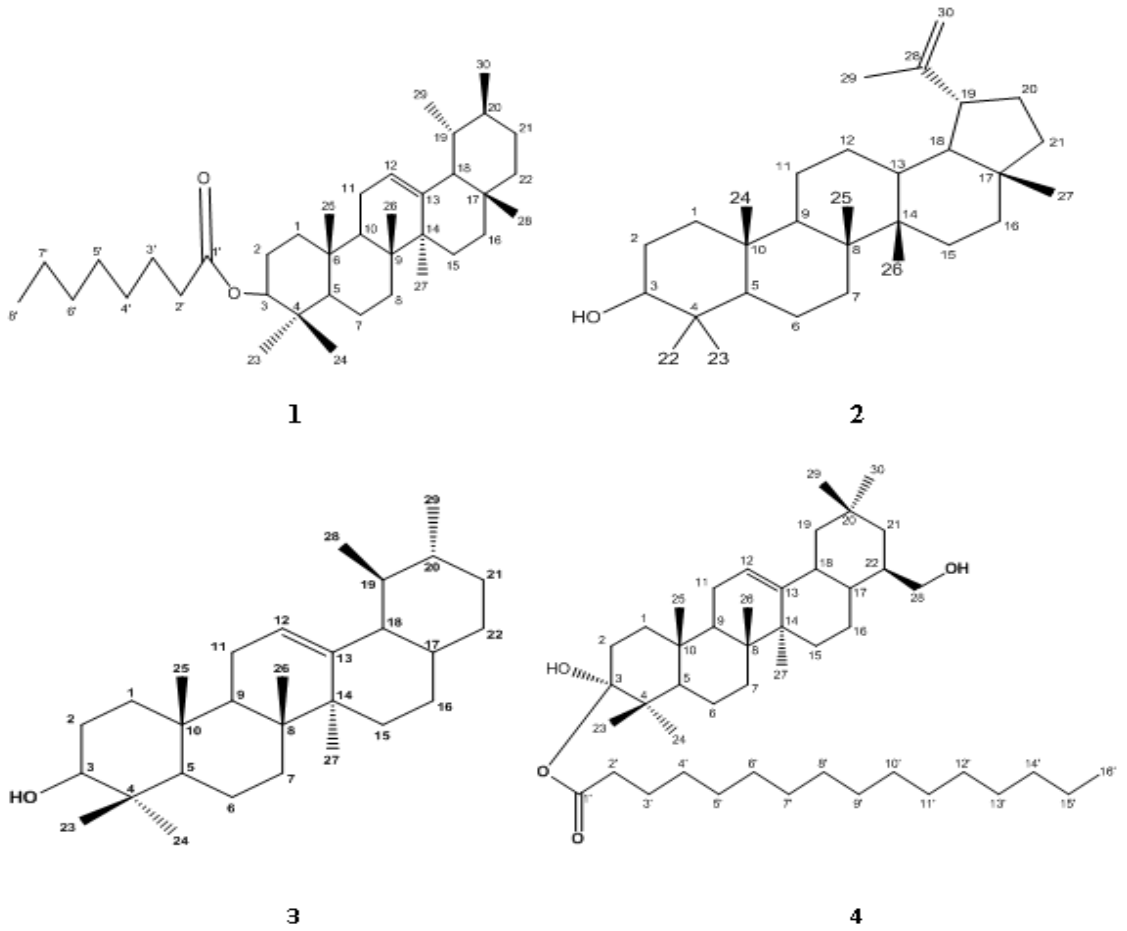

Fig. 1: Structures of compounds 1-4 from petroleum ether fraction of ethanolic extract of leaves of *Bauhinia variegata*

Fractionation of ethanolic extract and isolation of compounds

The ethanolic extract (400g) was suspended in distilled water (1,500 ml) and then fractionated successively with petroleum ether (60 – 80°C, 8×500ml), chloroform (8×500ml), n-butanol (8×500ml) and methanol (8×500ml). All the fractions were then washed with distilled water (30 ml), dried over anhydrous sodium sulphate and freed of solvent by distillation. The ethanolic extract was thus fractionated into petroleum ether soluble extract (40g), chloroform soluble extract (45g), n-butanol soluble extract (55g) and methanol soluble extract (60g). The petroleum ether soluble extract (40g) was dissolved in $CHCl_3$ (20ml) and adsorbed onto neutral alumina (20g). After evaporation of the solvent it was loaded onto a neutral alumina column (150g) prepared in petroleum ether (60–80°C). The column was eluted with petroleum ether (60-80°C) followed by petroleum ether (60-80°C): chloroform (95:5, 90:10, 80:20 and 50:50), and finally chloroform : methanol (95:5, 90:10, 80:20 and 50:50). The elution was monitored by TLC (Silica gel G; visualization: vanillin-sulphuric acid reagent heated at 110°C). Each time 10 ml were collected in a test tube and identical elutes (TLC monitored) were combined and concentrated to 10 ml and kept in a refrigerator.

The column chromatography led to isolate four pure compounds 1-4 (Fig. 1) with petroleum ether (60-80°C): chloroform grade mixtures (95:5) (70mg), chloroform: methanol (95:5) (45

mg), chloroform: methanol (90:10) (45 mg) and chloroform: methanol (80:20) (50 mg), respectively. These four compounds were identified and characterized by IR, NMR and mass spectrometry.

α- amyrin caprylate (1): Compound 1 (70 mg) was saponified with 20% ethanolic KOH (100 ml) for 2 h. This was then evaporated to remove all traces of EtOH, the lost volume being replaced by water from time to time. The unsaponifiable portion was then extracted with ether (4 X 50 ml). Each extract was then washed with distilled water (20 ml) and dried over anhydrous sodium sulphate. All the ethereal fractions were combined and evaporated leading to the isolation of triterpene alcohol (40mg) and m.p. of which (184°C) [11] also supported the finding of compound as α – amyrin by spectroscopy. Compound was obtained as Off-white crystal; R_f value: 0.82 (Petroleum ether: Chloroform, 90:10), m. p.: 160°C, IR ν_{max} (KBr) cm^{-1}: 2917.9 cm^{-1} (C-H str. in CH_3), 2847.7 cm^{-1} (C-H str. in CH_2), 1724.1 cm^{-1} (C=O str.), 1465.2 cm^{-1} (C-H def. in CH_3), 1377.2 cm^{-1} (C-H deformation in gem dimethyl), 723.6 cm^{-1} (=C-H out plane bending), ^{1}HNMR ($CDCl_3$, 400MHz): Table 2, ^{13}CNMR ($CDCl_3$, 100.62MHz): Table 3, ESI-MS (m/z): 552(M^+, $C_{38}H_{64}O_2$), 537, 506, 445, 408, 333, 324 (100%), 310, 254, 218, 190, 144.

Lupeol (2): Compound 2 (45 mg) was taken up in dry pyridine (0.2 ml) and freshly distilled AC_2O (1 ml) was added to it. The mixture was kept at room temperature overnight, then added to ice water, stirred, kept for 2 h, filtered and dried. The solid obtained was crystallized from C_6H_6 as white flakes. m.p. 217 - 219°C (218°C) [12]. Compound 2 was obtained as Pearl white crystal; R_f value: 0.71 (Chloroform : Methanol, 80:20), m. p.: 215°C, IR ν_{max} (KBr) cm^{-1}: 3431.6 cm^{-1} (br, OH), 2927.2 cm^{-1}, 2864.1 cm^{-1} (C – H str.in CH_3 and CH_2), 1657.1 cm^{-1}(C=C str.), 1461.2 cm^{-1}(C-H deformation in CH_2/ CH_3), 1377.8 cm^{-1}(C-H deformation in gem dimethyl), 1057.0 cm^{-1}(C – O str. of secondary alcohol), 883.6 cm^{-1}(exocyclic CH_2), ^{1}HNMR ($CDCl_3$, 400MHz): Table 2, ^{13}CNMR ($CDCl_3$, 100.62MHz): Table 3 , APCI-MS(m/z): 426 (M^+, $C_{30}H_{50}O$), 411, (M^+-CH_3), 409, 396(100%), 383, 217, 181.

nor α-Amyrin (3): Orange crystal; R_f value: 0.48 (Chloroform : Methanol, 60:40), m. p.: 148 °C, IR ν_{max} (KBr) cm^{-1}: 3426.2 cm^{-1} (br, OH), 2921.0 cm^{-1}, 2857.2 cm^{-1} (C-H str. in CH_3 and CH_2), 1595.7 cm^{-1} (C=C str. in aromatic ring), 1461.3 cm^{-1} (C-H def. in CH_3), 1375.8 cm^{-1} (C-H deformation in gem dimethyl), 1058.3 cm^{-1} (C-O str. of secondary alcohol), 729.0 cm^{-1} (=C-H out plane bending), 602 cm^{-1} (rocking vibration of CH_2), ^{1}HNMR ($CDCl_3$, 400MHz): Table 2, ^{13}CNMR ($CDCl_3$, 100.62MHz): Table 3, APCI-MS(m/z): 412 (M^+, $C_{29}H_{48}O$), 396 (100%), 382.

3β, 28-di hydroxyl olean-12-enyl-palmitate (4): Orange crystal; R_f value: 0.67 (Methanol: Chloroform, 90:10), m. p.: 146 °C, IR ν_{max} (KBr) cm^{-1}: 3426.2 cm^{-1} (br, OH), 2930.0 cm^{-1}, 2854.6 cm^{-1} (C-H str. in CH_3 and CH_2), 1725.3 cm^{-1} (C=O str.), 1657.0 cm^{-1} (C=C str.), 1595.7 cm^{-1} (C=C str. in aromatic ring), 1461.8 cm^{-1} (C-H def. in CH_3), 1376.5 cm^{-1} (C-H deformation in gem dimethyl), 1058.8 cm^{-1} (C-O str. of secondary alcohol), 734.0 cm^{-1} (=C-H out plane bending), 591 cm^{-1}(rocking vibration of CH_2). ^{1}HNMR ($CDCl_3$, 400MHz): Table 2, ^{13}CNMR ($CDCl_3$, 100.62MHz): Table 3, APCI-MS(m/z): 696 (M^+, $C_{46}H_{80}O_4$), 662 (on loss of two hydroxy molecules), 425, 410, 396(100%), 383, 203.

RESULTS AND DISCUSSION

Indian medicinal plant *Bauhinia variegata* (leaves) was extracted with ethanol and the preliminary phytochemical studies revealed the presence of carbohydrate, protein, glycosides, triterpenoids, saponins and steroids in that ethanolic extract.

Table 1: Results of Qualitative Tests for Phytoconstituents

Sl. No	Tests	Inference
1	Alkaloids	-ve
2	Carbohydrates	+ve
3	Proteins	+ve
4	Flavanoids	-ve
5	Glycosides	+ve
6	Triterpenoids	+ve
7	Resins	-ve
8	Saponins	+ve
9	Steroids	+ve
10	Tannins	-ve
11	Starch	-ve

Table 2: ^{1}HNMR spectroscopic data for compounds 1, 2, 3 and 4

Proton	Compounds			
	1	2	3	4
H-3	3.73 m	4.16 s	4.16 m	-
H-11	-	-	5.04 m	5.14 m
H-12	5.34 m	-	5.15 m	5.35 m
H-23	1.20 s	-	0.93 s	0.99 s
H-24	1.20 s	-	0.93 s	0.99 s
H-25	1.04 s	-	1.04 s	1.04 s
H-26	1.04 s	-	1.04 s	1.04 s
H-27	1.30 s	-	1.33 m	1.30 s
H-28	1.04 s	-	1.25 d	-
H-29	0.89 d	-	1.25 d	0.99 s
H-30	0.89 d	4.68 s	-	0.99 s
H-2'	2.1 m	-	-	2.36 m
H-8'	0.88 m	-	-	-
H-16'	-	-	-	0.88 m
OH	-	3.56 d	3.52 d	-
$CH_3 - C=CH_2$	-	2.04 s	-	-
3'-OH	-	-	-	3.55 s
28'-OH	-	-	-	3.49 s

The ethanolic extract was fractionated successively with petroleum ether (60 – 80°C), chloroform, n-butanol and methanol. The petroleum ether fraction was subjected to column chromatography which led to isolate four pure compounds. α- amyrin caprylate was isolated as compound 1 by petroleum ether (60-80°C): chloroform grade mixtures (95:5). The melting point of compound was found to be 158-160° C (160° C) [13] and gave a green colour with Liebermann Burchard's test. The mass fragmentation was typically that of α -amyrin skeleton. An ester linkage was discernible from the characteristic IR adsorption at 1724.1 cm^{-1}.

Table 3: ^{13}CNMR spectroscopic data for compounds 1, 2, 3 and 4

Carbon	Compounds			
	1	**2**	**3**	**4**
C-1	39.3	37.4	37.4	31.6
C-2	22.7	27.9	28.2	28.2
C-3	209.5	77.3	77.4	111.8
C-4	37.1	39,8	37.4	39.7
C-5	57.6	56.0	56.0	45.8
C-6	19.6	36.2	19.0	18.7
C-7	32.2	42.3	33.9	33.9
C-8	39.2	45.5	39.7	39.7
C-9	46.3	37.3	45.8	45.8
C-10	35.5	37.5	36.5	37.2
C-11	23.3	21.0	23.0	23.0
C-12	123.2	25.1	121.7	121.7
C-13	135.7	37.4	144.7	140.7
C-14	41.4	42.1	42.3	42.3
C-15	46.3	26.7	31.6	31.6
C-16	24.8	36.5	26.0	26.0
C-17	45.5	45.8	45.8	45.8
C-18	30.7	50.1	39.8	37.2
C-19	30.7	50.2	42.3	50.1
C-20	33.9	29.7	37.3	29.1
C-21	32.3	39.3	32.8	42.3
C-22	32.2	23.0	28.2	36.5
C-23	15.5	23.0	23.0	22.7
C-24	16.4	16.2	23.0	22.7
C-25	17.6	19.1	16.2	16.2
C-26	17.8	16.0	18.7	18.7
C-27	25.5	19.1	26.0	26.0
C-28	26.5	148.0	16.2	71.8
C-29	32.3	20.2	21.2	31.6
C-30	23.5	111.7	-	31.6
C-1'	173.5	-	-	173.3
C-2'	34.8	-	-	33.9
C-3’	25.1	-	-	29.7
C-4'	29.2	-	-	28.5
C-5’	29.3	-	-	26.3
C-6'	32.5	-	-	24.3
C-7’	23.5	-	-	22.5
C-8'	15.2	-	-	21.8
C-13’	-	-	-	29.7
C-15'	-	-	-	22.7

The -CH_2COO- of the ester was indicated by the characteristic singlet at δ 2.35. Furthermore mass fragment m/z 408 is suggestive of the loss of caprylic acid (144) from the molecular ion (M^+) 552. The peaks at 333 and 218 (Base peak) m/z were due to Retro-Dials -Alder fragmentation with the usual hydrogen transfer, characteristic of the left and right half arising from triterpene having δ^{12}-oleanane /ursane structure. The peak at 190 (333-143) m/z was due to loss of ester moiety from the left half providing conclusive proof for the attachment of the ester

grouping at C-3. The fact that it is the acetate of α amyrin was established by saponification leading to the isolation of the triterpene alcohol, melting point of which (184°C) was in good agreement with that reported for α-amyrin (186°C) [11].

Compound 2 (Lupeol) was isolated by chloroform: methanol (95:5) and melting point was found 213 –215°C (215°C) [12]. The presence of characteristic diagnostic absorption of vinylidine (>C=CH_2) at 883.6 cm^{-1} indicated the possibility of lupane skeleton. The finding has been supported by the presence of the in plane bending of =CH_2 at 1461.2 cm^{-1}. Further the acetate melted at 217 –219°C (218°C) [12]. Its identity as lupeol was confirmed by direct comparison of spectral characters, by co-chromatography with an authentic sample (Sigma Chemical Company, USA). The isolated compound 3 (nor-α-amyrin), with chloroform: methanol (90:10), gave characteristic colour reaction for triterpenes. The peak at 3426.2 cm^{-1} indicated the presence of OH group. The ^{1}H NMR Signal at δ 5.15 indicates the vinylic proton. The ESI-MS showed the peak at 412 that confirms the compound as nor-α-amyrin. The compound (4) 3β, 28-di hydroxyl olean-12-enyl-palmitate was isolated with chloroform: methanol (80:20) and melting point was found 145-147°C (146°C) [14]. The peak at 3426.2 cm^{-1} indicates the presence of OH group. The ^{1}H NMR Signal at δ 5.35 indicates the vinylic proton. The APCI-MS showed the peak at 696 (M^+) and 662 which further supported the characterization of compound as 3β, 28-di hydroxyl olean-12-enyl-palmitate.

CONCLUSION

The preliminary phytochemical studies identified the presence of carbohydrate, protein, glycosides, triterpenoids, saponins and steroids in that ethanolic extract. Whereas, further phytochemical investigation led to isolate four pure compounds from petroleum ether fraction of ethanolic extract of leaves of *Bauhinia variegata*. These compounds were identified as triterpenoids and fatty acid ester of triterpenoid, and by spectroscopical data compound 1-4 characterized as α- amyrin caprylate, Lupeol, nor-α-amyrin and 3β, 28-di hydroxyl olean-12-enyl-palmitate, respectively.

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