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Research Article

ISOLATION OF PHYTOCHEMICAL CONSTITUENTS FROM STEM BARKS OF *MADHUCA LONGIFOLIA*

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ABSTRACT
The stem barks of *Madhuca longifolia* (Sapotaceae,) are reported to be of great medicinal importance. Isolation of chemical constituents was carried out from the leaves of *Madhuca longifolia*, an evergreen member of the family Sapotaceae, using column chromatography. Identification of chemical constituents was done by coloumn chromatography, IR and NMR techniques. Five compounds ie β-amyrin acetate, 21-Hydroxy-3-oleanyl myricitate, Ursolic Acid, n-hexyl-3-acetyl betulinate and 3-(27-Carboxy oleanyl) – Octanate were isolated from the ethyl acetate extracts. These findings are useful in establishing a relationship between chemical composition of the stem bark extract and previously reported activities of Madhuca longifolia and also may assign a new potential role of Madhuca longifolia extract in human health care.

KEY WORDS: *Madhuca longifolia*, Chemical constituents, Column chromatography, IR, NMR

INTRODUCTION

Madhuca longifolia is a folklore medicinal plant; it is commonly used for the treatment of snakebite as antidote in Southern part of Tamil nadu, India.[1] The seed oil is used for cooking food. Its flower is widely used for making local liquor and leaves are used in headache [2]. Decoction of stem bark and leaf of Madhuca longifolia is used to cure hydrocele, stomach ache and skin disease [3, 4]. Powdered bark is employed for the treatment of scabies and rheumatism, Roasted flowers are eaten with salt to cure cough, and roasted fruit is used orally to

treat asthma and phthisis [5]. Paste of flowers was applied on wounds to cure it and to prevent formation of abscess [6]. The major chemical compounds like vitamin A and C, ethyl cinnamate, histidine, glutamic acid and arabinose [7], flavones, chalcones-mallotus AB, tannins, cardenolide, srottlerin, isorottlerin, tannic acid, gum, volatile oil [8], new oleanene, triterpenoid and saponins are identified in this plant. In addition, alkaloids were found to strongly inhibit lipid peroxidation induced in isolated tissues via antioxidant activity. Traditionally, Madhuca bark has been used against diabetes, rheumatism, ulcers, bleeding and tonsillitis [9, 10].

MATERIALS AND METHODS

Plant materials

The stems of Madhuca longifolia was collected in and around Andhra University, Visakhapatnam in the month of April, 2006. The authentications of the plant were done by Prof. Dr. M. Venkaiah, Department of Botany, Andhra University, Visakhapatnam.

Instruments

Rotary Flash Evaporator (Medica Instrument Manufacturing Company, Mumbai), Model: Roteva Equitron, IR and NMR.

Source of Chemicals and Drugs

All chemicals and solvents were of the analytical grade obtained from S.D. Fine Chemical Pvt. Ltd., Mumbai, Sigma Chemical Company, U.S.A., Loba Chemic, Mumbai.

Extraction and Isolation procedure

Freshly collected plant materials (Madhuca longifolia stem bark) were shade dried at room temperature and coarsely powdered in Wiely mill. The powdered material of Madhuca longifolia (1kg) was successively extracted with hexane, ethyl acetate and methanol by Soxhlet extraction. The crude extracts were evaporated to dryness in a rotary film evaporator.

Yield of extracts

Hexane extract - 5.5gm

Ethyl acetate extract – 25.3gm

Methanolic extract - 14.8 gm

Since the yield of hexane and methanolic extract was very low from all the three extracts, these extracts were not used in this study.

RESULTS AND DISCUSSION

Total five compounds were isolated. Their structures were elucidated by IR and NMR spectroscopic methods. Results of qualitative phytochemical screening of plant extracts are shown in Table-1.

Table-1: Nature of Phytoconstituents present in different extracts of *M. Longifolia* stem bark

Phytoconstituents	*Madhuca longifolia*	
	Ethyl acetate extract	Methanolic extract
Phytosterols	+	-
Triterpenes	+	-
Glycosides	-	+
Saponins	-	+
Flavonoids	-	-
Tannins	-	+
Carbohydrates	-	+
Alkaloids	-	-

Column Chromatography for ethyl acetate extract

Column chromatography was done by standard procedure. Silica gel for CC (Qualigens), 60-20 mesh was used as adsorbent. The column was eluted with n-hexane: ethyl acetate by step gradient and the course of chromatography is shown in Table-2. Weight of residue-10 g, weight of the silica gel-400 g and volume of each eluant-500 ml.

Table 2: Column chromatography of ethyl acetate extract of *Madhuca longifolia.*

Fraction No.	**Eluant composition**	**Weight**	**Compound Isolated**
1-15	n-Hexane	Fatty matter	
15-25	5% Ethyl acetate in hexane	30mg	ML-1
25-35	10% Ethyl acetate in hexane	20mg	ML-2
35-45	20% Ethyl acetate in hexane	15 mg	ML-3
45-55	30% Ethyl acetate in hexane	residue	
55-65	40% Ethyl acetate in hexane	25mg	ML-4
65-75	50% Ethyl acetate in hexane	35mg	ML-5
75-85	60% Ethyl acetate in hexane	residue	

85-95	70% Ethyl acetate in hexane	residue	
85-95	80% Ethyl acetate in hexane	residue	
95-105	Pure Ethyl acetate	residue	

STRUCTURE ELUCIDATION OF COMPOUND ML-1:

This compound was obtained from hexane – ethyl acetate (95:5) fraction as colourless needles, m.p. 236-238 °C. The compound gave a positive LB test for tri terpenoids. The IR (KBr, Fig.2) spectrum of ML-1 showed bands at V_{max} 3430.6 cm^{-1} (hydroxyl) and 1713 cm^{-1}(carbonyl). The ^1H NMR spectral (400MHz, $CDCl_3$, Fig-3, Table-3) data showed the presence of a downfield methine proton at δ 3.3 (1H, m) characteristic of H-18 of oleanene derivatives (Chang *et al.*, 2004), an olefinic proton signal at δ 5.4 (br, s, H-12). The ^1H NMR spectra also showed the presence of eight tertiary methyls in the region δ 1.25-0.78 indicative of oleanene skeleton in ML-1 (Abramovitch and Micetich, 1963). Literature survey on oleanene derivatives revealed that the above physical and spectral data of ML-1 was in good agrement with β-amyrin acetate. Based on the foregoing, the structure of ML-1 was deduced as β-amyrin acetate. This compound has been reported for the first time from *M.longifolia.*

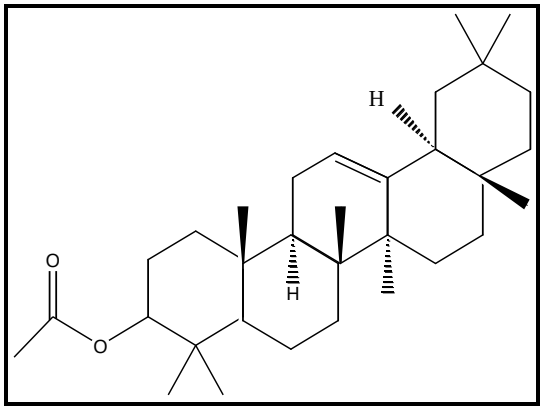


Figure 1: β-amyrin acetate

Table 3: ^{1}H NMR spectral data of ML-1 (400MHz, δ, $CDCl_3$) (β-amyrin acetate)

Chemical shift(δ)	Proton integration	Multiplicity	Assignment
2.00	3H	Br, s	$OCOCH_3$
3.3	1H	Br, m	H-18
0.84	3H	S	H-30
0.88	3H	S	H-29
0.78	3H	S	H-28
0.78	3H	S	H-26
1.00	3H	S	H-23
1.00	3H	S	H-24
1.10	3H	S	H-27
1.06	3H	S	H-25
5.4	1H	S	H-12

Figure 2: IR spectrum of β-amyrin acetate

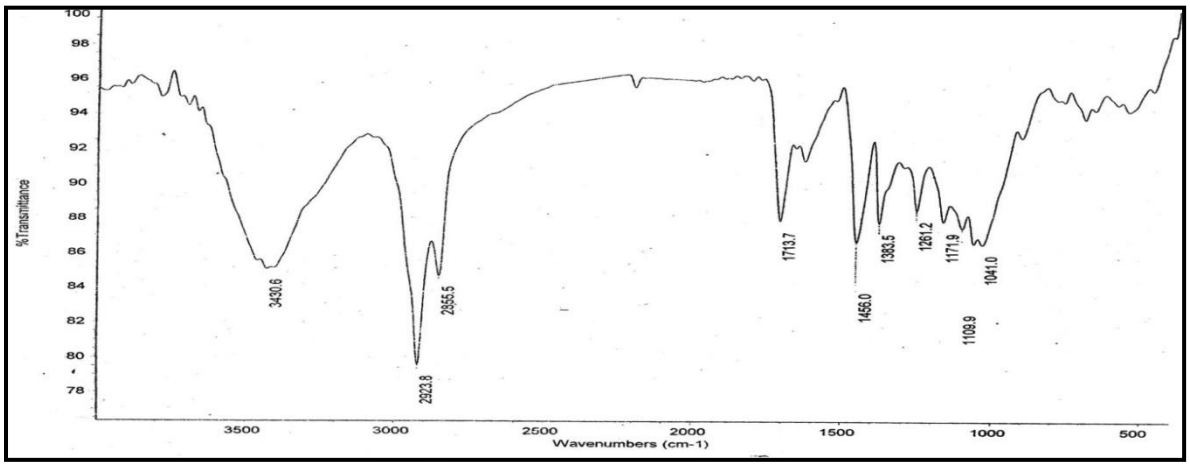
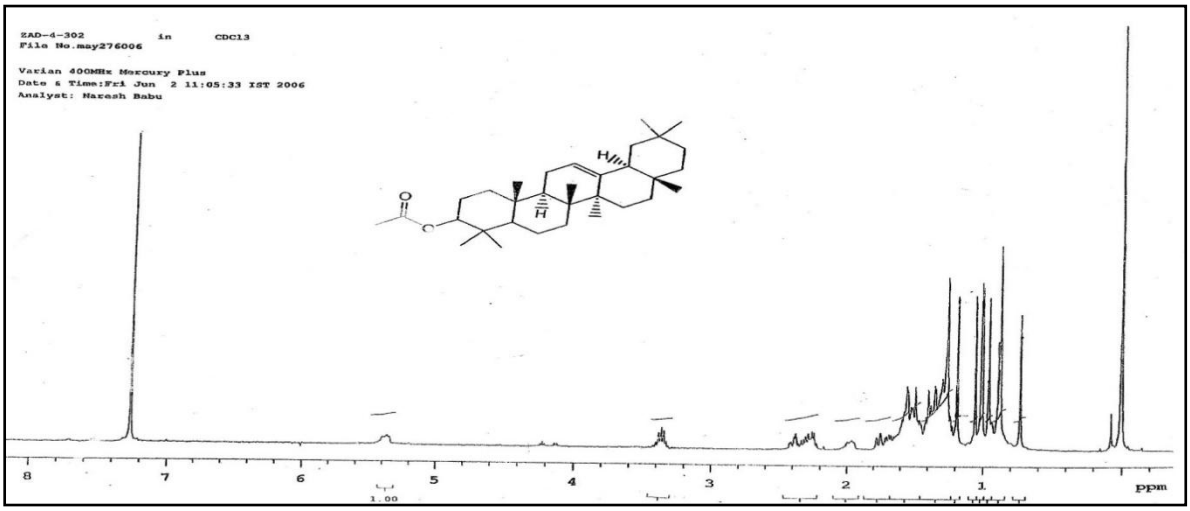


Figure 3: ^{1}H NMR spectrum of β-amyrin acetate



STRUCTURE ELUCIDATION OF COMPOUND ML-2

This compound was isolated from n-hexane – ethyl acetate (90:10) fraction as colourless needles, m.p. 115-117 °C. According to LC-MS spectral data molecular formula of this compound was analysed as $C_{43}H_{74}O_3$. It gives a positive L.B. test for tri terpenoids. The IR spectrum (Kbr, Fig.5) of this compound showed a broad band at 3400 cm^{-1} for the hydroxyl group, the characteristic absorption band of ester carbonyl was observed at 1730 cm^{-1}. The bands appearing at 1698cm^{-1} corresponds and to C=C double band, the aliphatic C-H stretchings appeared at 2923, 2854, 1219,772.0 cm^{-1}. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$, Fig.6) of ML-2 showed signals at δ 0.86, 0.87, 0.90, 0.92, 0.93, 1.25, 1.26, and 1.28 indicating the presence of 8 tertiary methyl groups. A multiplet at 0.99 is due to terminal methyl group of long chain carbon atoms. The signals are in between δ 0.80 to 2.30 indicating that the compound is tri terpenoid in nature. A broad singlet at δ 5.3 indicates the presence of C=C unsaturated proton. The signals at δ 4.5 and 3.5 reveal that the oxygen atom in ester function is attached at C-3 carbon and the hydroxyl group is attached at C-21 carbon. It was further confirmed by ^{13}C NMR. ^{13}C NMR spectrum (100 MHz, $CDCl_3$, Fig-6; Table-4) of ML-2 showed signals at δ 173.70, corresponding to ester carbonyl group. The signals absorbed at δ 122.64 and 143.60 reveal the presence of unsaturation. Two signals were observed at δ 80.23 and 80.59 indicating the presence of two carbon atoms with attached oxygen atoms. The ^{13}C-NMR signal for C-21 is at δ 80.23 which suggests that it is adjacent to a quaternary carbon. The proton signal in ^{1}H NMR (H-21) appears as a doublet of doublet at δ 3.05 suggesting that it is adjacent to a methylene group. Further the proton signal for H-18 appears as a doublet of doublet at δ 2.82 which indicates that C-19 is a methylene carbon without any substitution. These facts place the hydroxyl group at C-21. The carbon signals at 31.67, 30.65, 28.91, 28.00, 27.02 and 15.36 were due to the long chain carbon atoms. Based on the foregoing, the structure of ML-2 was deduced as 21-hydroxy-3-olenyl myricitate. This compound has been reported for the first time from *M.longifolia.*

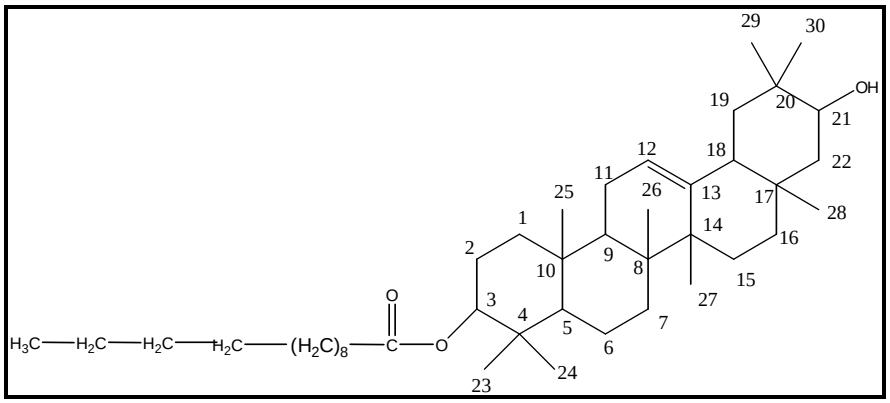


Figure 4: 21-hydroxy-3-oleanyl myricitate

Table 4: ^{13}C –NMR spectral data of compound ML-2: 21-Hydroxy-3-oleanyl myricitate

Carbon No.	**^{13}C NMR δ (100 MHz, $CDCl_3$)**
1	33.03
2	27.69
3	80.59
4	41.21
5	55.35
6	22.57
7	34.84
8	38.12
9	45.87
10	37.77
11	25.16
12	122.64
13	143.60
14	41.85
15	29.67
16	33.82
17	29.15
18	55.35
19	32.61
20	36.99
21	80.23
22	32.45
23	18.82
24	25.87
25	14.02
26	18.20
27	17.09
28	16.72
29	23.00
30	23.56
C=O	173.7

Figure 5: IR spectrum of 21-Hydroxy-3-oleanyl myricitate

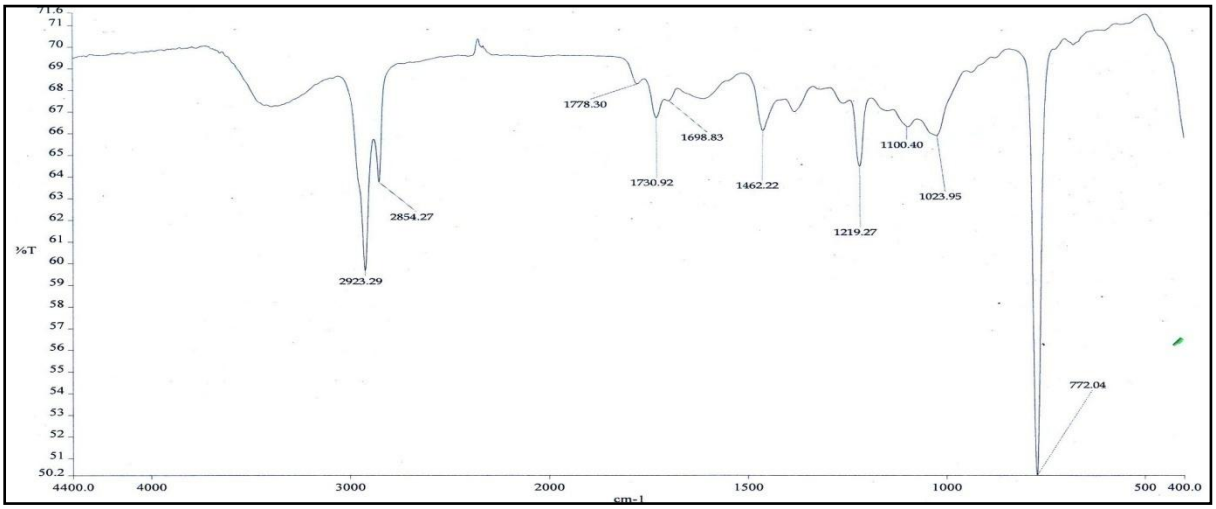
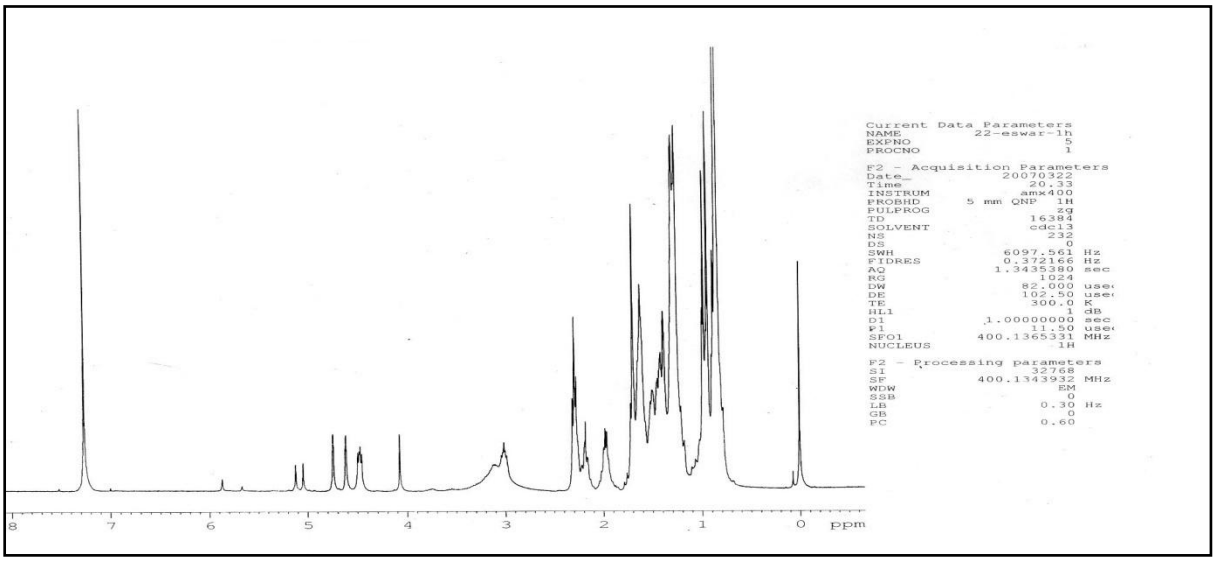


Figure 6: ^{1}H NMR spectrum of 21-Hydroxy-3-oleanyl myricitate



STRUCTURAL ELUCIDATION OF COMPOUND ML -3

It was obtained as a white amorphous powder from methanol, melting point 285-287 °C. Its molecular formula, $C_{30}H_{48}O_3$, was deduced based on elemental analysis and LC-MS data. It showed positive LB test for triterpenoids. The IR (KBr, Fig.8) spectrum of ML-3 showed bands at 3390 cm^{-1} (hydroxyl) and 1696 cm^{-1}(COOH). The ^{1}H NMR spectral data (400MHz, DMSO, Fig.9, Table-5), showed the presence of a hydroxyl methine proton at δ 5.4,1 (H, Br, s, H-12) and a methine proton at δ 2.7 (1H, m) attributable to H-18 of ursane derivatives. In addition, the ^{1}H NMR spectrum showed the presence of

five tertiary methyl groups δ 0.88-1.1, (each 3H, s) and two secondary methyl groups at δ 0.98, (3H, d, *J*=7.0 Hz and 0.91, 3H d, *J* = 6.5 Hz) characteristic of ursane skeleton. Literature survey on ursane derivatives revealed that the physical and spectral data of ML-3 were in good agreement with those recorded for ursolic acid (3β-hydroxy -12-en28-oic acid) isolated earlier (Alves *et al*., 2000).

The identity of ML-3 as Ursolic acid was ascertained further by direct comparison with authentic sample (co-TLC and mmp). Based on the foregoing, the structure of ML-3 was established as ursolic acid.

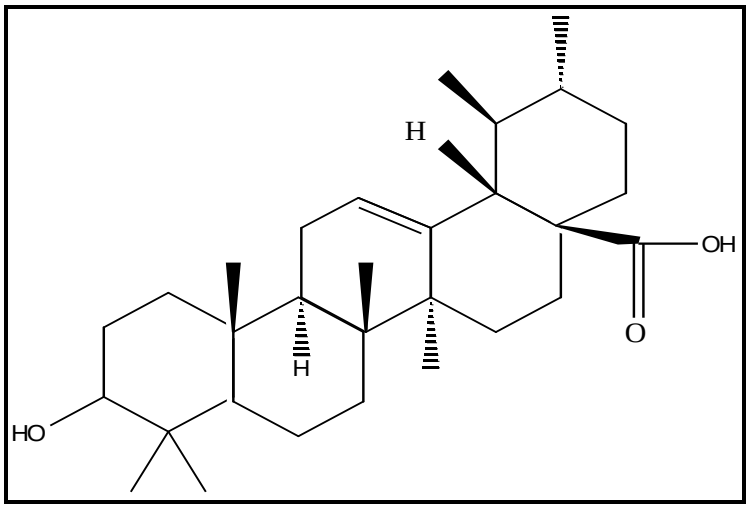


Figure 7: Ursolic acid

Table 5: ^{1}H NMR, spectral data of the compound ML -3 (Ursolic acid)

Proton Number	δ (400 MHz, DMSO,)
3	3.20 (br, s)
12	5.4 (br, s)
23	2.7 (1H, m)
24	1.26 (3H, s)
25	1.01 (3H, s)
26	0.84 (3H, s)
27	1.26 (3H, s)
29	0.98 (3H, d)
30	0.91 (3H, d)

Figure 8: IR spectrum of Ursolic acid

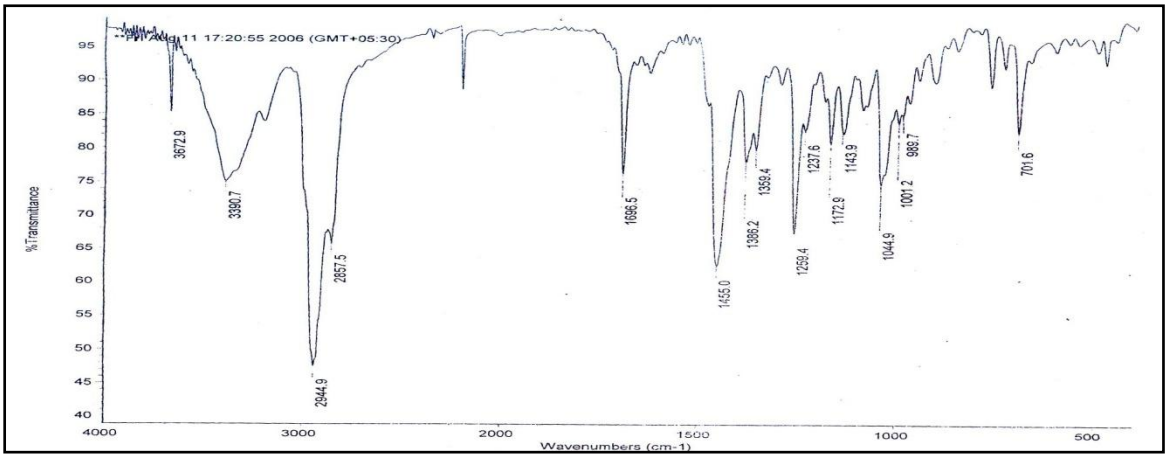
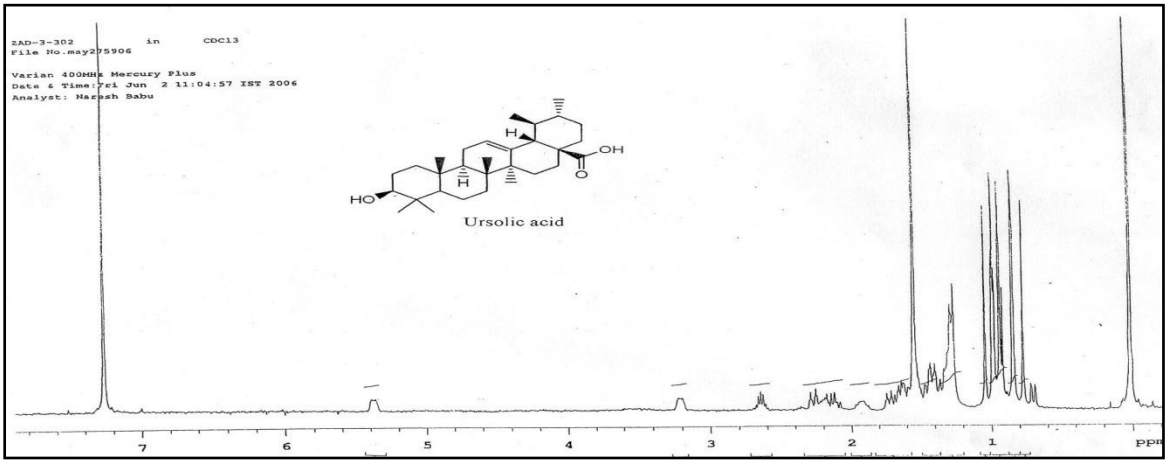


Figure 9: ^{1}H NMR spectrum of Ursolic acid



STRUCTURAL ELUCIDATION OF COMPOUND ML-4

This compound was isolated from hexane – ethyl acetate (60:40) fraction as colorless needles, m.p. 209-210 ºC. The compound gives positive L.B. test for triterpenoids. According to LC-MS data molecular formula of this compound was analysed as $C_{38}H_{62}O_4$. The IR spectrum (Kbr, Fig.11) of ML-4 showed two carbonyl bonds at 1729 cm^{-1} and 1698 cm^{-1} which indicates the presence of two ester groups. The band observed at 1600 cm^{-1} indicates C=C. The C-H stretchings were observed at 2933.16, 2863.15, 1729.39, 1698.71 and 757.32 cm^{-1}. The ^{1}H- NMR spectrum of ML-4 (400MHz, $CDCl_3$, Fig-61) showed two olefinic protons of the exocyclic methylene group at δ 4.75 (1H, br, S) and 4.60 (1H, br S) and the doublet of doublet at δ 4.45 for two protons of the methylene group attached to oxygen atom. The singlet observed at δ 4.00 indicates that proton is directly attached to exocyclic group. This spectrum also showed singlet at δ 2.3 for a exocyclic methylene group,

singlet at δ 1.70 for a methyl group attached to a double bond and singlets at δ 0.83, 0.84, 0.85, 0.97, 0.98 for the tertiary methyl groups. The above facts suggest that the ML-4 was a triterpenoid containing lupen (Mahato and Kundu, 1994) system with both the oxygen function esterified at C-3 and C-28 positions. This was further confirmed by the ^{13}C-NMR spectrum of ML-4 (100 MHz, $CDCl_3$, Fig.12, Table-6). It showed two carbonyl carbon at δ173.72 and 181.87.The singlet at δ 150.30 and 109.72 indicates the tetra substituted and disubstituted unsaturated carbons. The exocyclic carbon resonated at δ 80.61 and is placed in C-3 for biogenetic reasons. The rest of the carbon signals were in accordance with the structure.The signals at δ 55.45, 37.08, 32.17, 29.69, 14.04, 16.13, 25.17, 28.92 represent methylene carbons of the side chains. From the above physical and spectral data the compound was named as n- hexyl-3-acetyl betulinate. This compound has been reported for the first time from this *M.longifolia.*

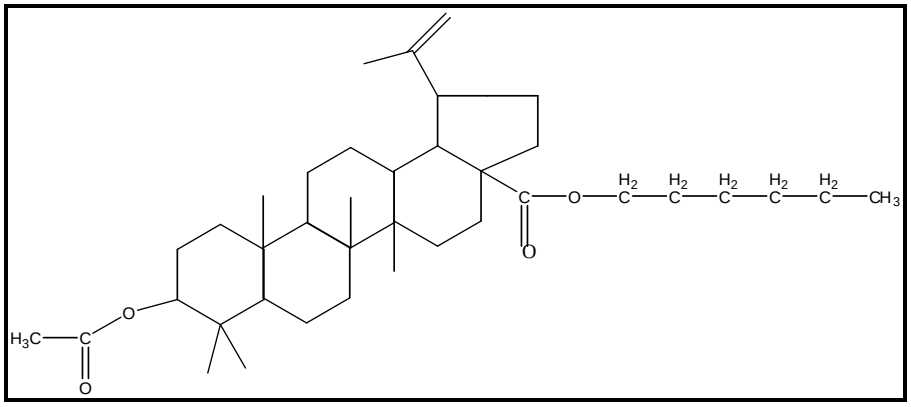


Figure 10: n-hexyl-3-acetyl betulinate

Table 6: ^{13}C NMR spectral data of compound ML-4: n-hexyl-3-acetyl betulinate

Carbon No.	^{13}C NMR δ (100 MHz, $CDCl_3$)
1	34.27
2	27.97
3	80.61
4	40.73
5	55.45
6	22.58

7	25.47
8	37.86
9	49.31
10	38.42
11	25.17
12	23.74
13	46.96
14	42.45
15	34.85
16	30.60
17	56.41
18	50.42
19	38.42
20	28.92
21	31.67
22	150.36
23	20.88
24	19.35
25	18.17
26	16.53
27	14.13
28	29.15
29	29.69
30	109.72
C=O	173.72 181.87

Figure 11: IR spectrum of n-hexyl-3-acetyl betulinate

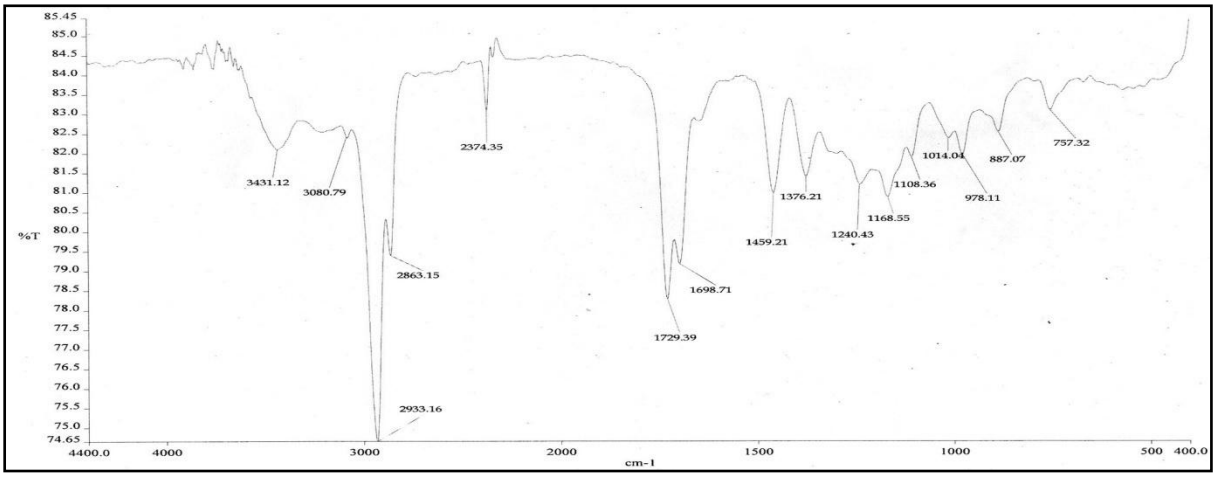
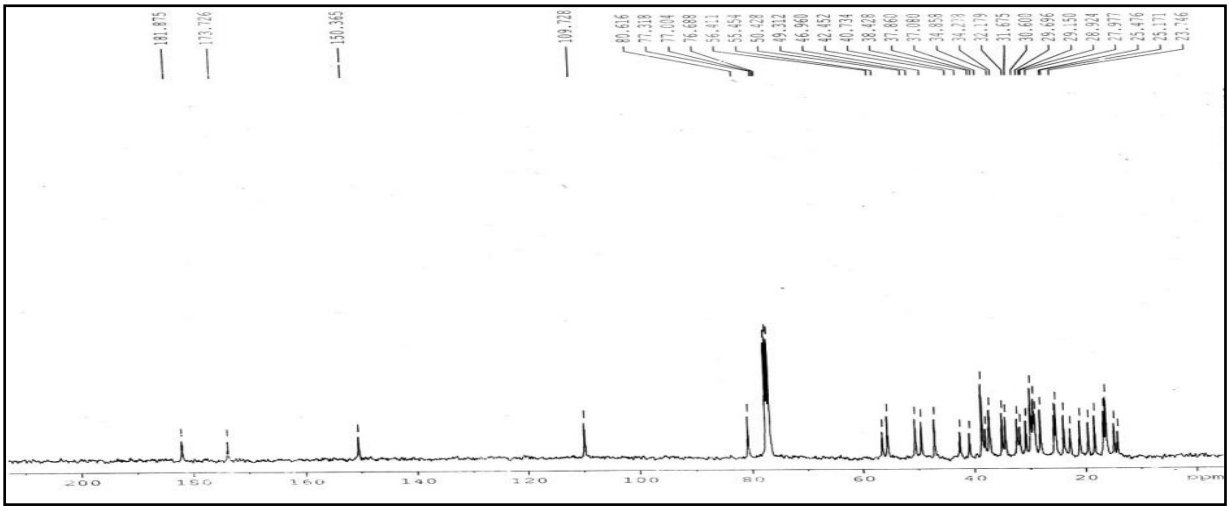


Figure 12: ^{1}H NMR spectrum of n-hexyl-3-acetyl betulinate



STRUCTURAL ELUCIDATION OF COMPOUND ML-5

This compound was obtained from n-hexane – ethyl acetate (70:30) fraction as colorless solid from hexane and methanol, melting point 212-213 °C. From LC-MS data the molecular formula was analysed as $C_{38}H_{62}O_4$. The compound gave a positive LB test for triterpenoids. IR spectrum (Kbr, Fig.63) showed a broad band from 2500 to 3500 cm^{-1} characteristic of a carboxylic acid group and two carbonyl bands at 1782 cm^{-1} and 1692 cm^{-1}. The unsaturation peak at 1600 cm^{-1} gets masked into the carbonyl band. The bands at 1460cm^{-1}, 1377 cm^{-1} were due to the long chain methylene groups. The ^{1}H-NMR spectrum (400MHz, $CDCl_3$, Fig.64) of ML-5 showed the broad singlet at δ 11.96 due to carboxylic proton. The broad singlet at δ 5.16 shows the presence of one unsaturated proton. The doublet of doublet downfield signal at δ 4.39 is due to the proton under oxygen function which was esterified with an acid. The oxygen atom was placed in C-3 due to biogenetic reasons. It shows 7 singlets at δ 0.72, 0.81, 0.85, 0.87, 0.89, 1.11 and 1.21 for seven tertiary methyl groups. The above data suggests that it is a triterpenoid. The multiplet signal at δ 2.29 is due to the H-18 proton. The above observations are strongly supported by the ^{13}C-NMR spectra (100MHz, $CDCl_3$, Fig.65; Table-46) showing the presence of two carbonyl groups at δ 178.49 and 172.50; signals due to one double bond at

121.38 and 143.85 (suggesting one carbon trisubstituted and the other carbon as tetra substituted) and confirms the oleanene skeleton (Mahato and Kundu, 1994); one carbon under oxygen function at δ 79.72. All the other signals are in agrement with the structure of 3- (27- carboxyl oleanyl-octanate). This compound has been reported for the first time from *M.longifolia.*

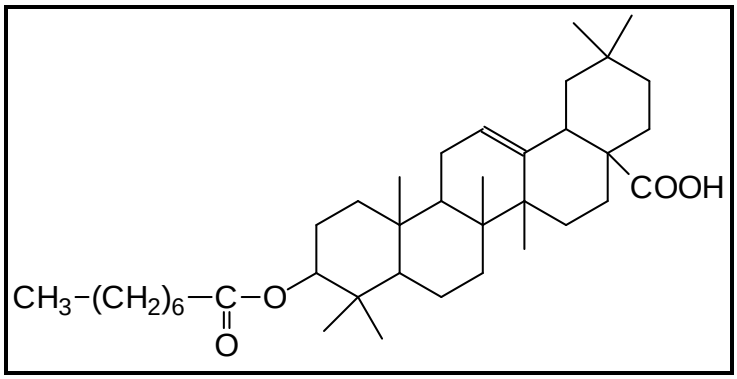


Figure 13: 3-(27-carboxy oleanyl) – octanate

Table 7: ^{13}C-NMR spectral data of compound ML-5: 3-(27- carboxy oleanyl) –octanate)

Carbon No.	^{13}C-NMR, δ (100MHz, $CDCl_3$)
1	33.93
2	24.58
3	79.72
4	37.52
5	54.53
6	21.93
7	36.49
8	40.20
9	45.45
10	37.27
11	25.53
12	121.88
13	143.85
14	41.35
15	30.35
16	27.20
17	40.82
18	39.98
19	45.69
20	31.05
21	40.20
22	28.96
23	22.87
24	22.61

25	15.12
26	16.80
27	16.61
28	178.49
29	23.22
30	23.35
1’	172.50
2’	33.32
3’	28.22
4’	28.35
5’	28.55
6’	32.07
7’	32.34
8’	15.00

Figure 13: IR spectrum of 3-(27- carboxy oleanyl) –octanate.

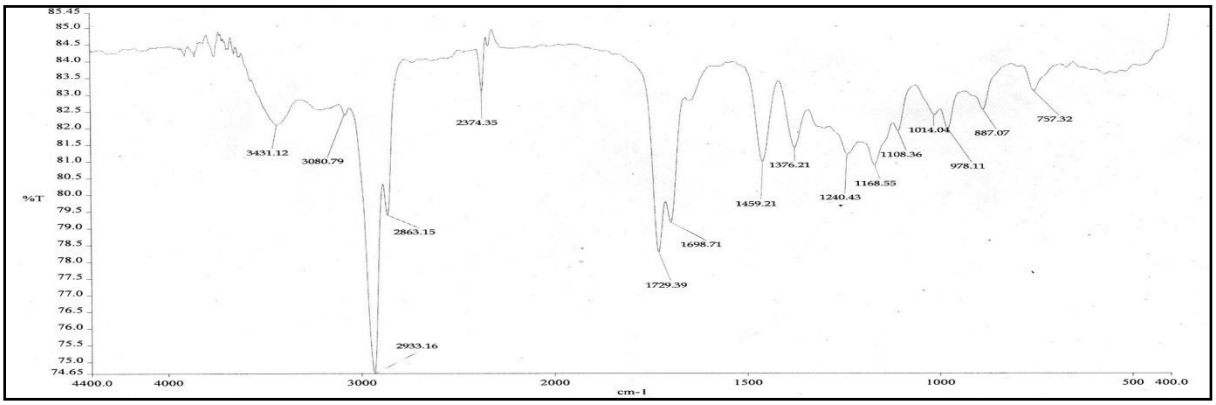
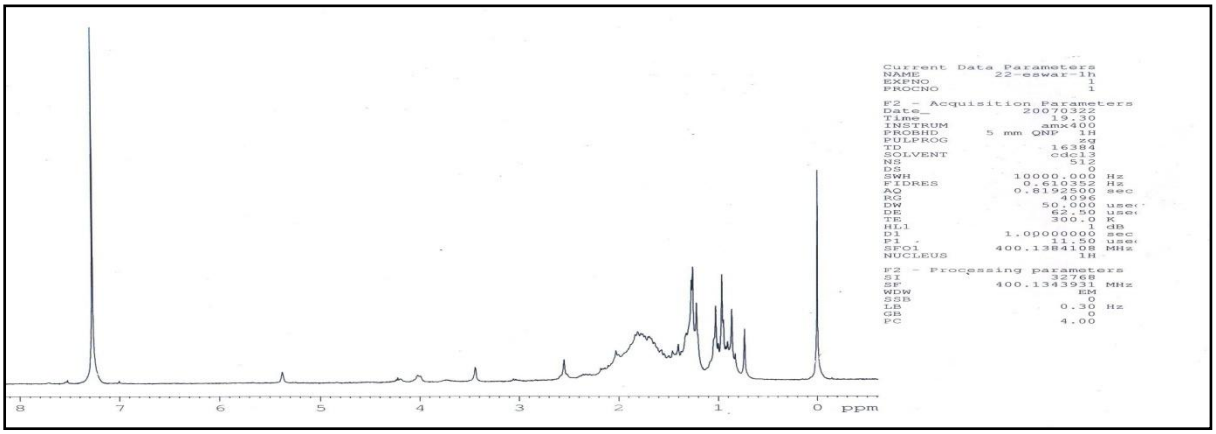


Figure 14: ^{1}H NMR spectrum of 3-(27- carboxy oleanyl) –octanate.



DISCUSSION

Compound ML-1 (β-amyrin acetate)

Fractions (15-25) of ethyl acetate extract were combined as they were homogenous on TLC and crystallization from hexane – chloroform gave colourless needles. m.p. 236-237°C.

Found : C, 81.86%; H, 11.40%;

Calculated for $C_{43}H_{74}O_3$: C, 82.05%; H, 11.11%;

R_f Value : ethyl acetate: methanol (9:1)

Yield : 30mg

IR (KBr, cm^{-1}): V_{max} 3430, 2923, 2854 and 1219.

^1H NMR: Fig, 3; Table-3

Compound ML-2 (21-Hydroxy-3-oleanyl myricitate)

Fractions (25-35) were combined as they were homogenous on TLC and crystallization from hexane- ethyl acetate gave colourless needles m.p. 115-117 °C.

Found : C, 80.86%; H, 11.64%; O, 7.54%

Calculated for $C_{43}H_{74}O_3$: C, 80.87%; H, 11.59%; O, 7.52%

R_f Value :ethyl acetate: methanol (8:2)

Yield: 20 mg

IR (KBr, cm^{-1}): V_{max} 3400, 1698, 2923, 2854, 1219,772.

^1H NMR: Fig.6, Table-4

Compound ML-3 (Ursolic Acid)

Fractions (35-45) were combined as they were homogenous on TLC and crystallization from ethyl acetate-methanol gave colorless powder m.p. 285-287ºC.

Found : C, 79.10%; H, 10.72%

Calculated for $C_{30}H_{48}O_3$: C, 78.94%; H, 10.52%

IR (KBr, cm^{-1}): V_{max} 3390, 2944, 1696, and 2857.

Rf :chlorofom: ethyl acetate (8:2)

Yield : 15 mg

^1H NMR: Fig.9; Table-5

Compound ML-4 (n-hexyl-3-acetyl betulinate)

Fractions (55-65) were combined as they were homogenous on TLC and crystallization from hexane- ethyl acetate gave colorless needles m.p. 209-210ºC. The compound gave positive L.B. test for triterpenoids.

Found: C, 78.34%; H, 11.58%; O, 10.99%

Calculated for $C_{43}H_{74}O_3$: C, 78.35%; H, 11.52%; O, 10.98%

R_f Value : hexane: ethyl acetate (8.5:1.5)

Yield : 25mg

IR (KBr, cm^{-1}):V_{max}.2933.16, 2863.15, 1729.39, 1698.71 and 757.32cm^{-1}.

^{1}H- NMRFig.12; Table-6

Compound ML-5 (3-(27-Carboxy oleanyl) – Octanate)

Fractions (65-75) were combined as they were homogenous on TLC and crystallization from hexane- ethyl acetate gave colorless solid m.p. 218-219ºC. The compound gives positive L.B. test for triterpenoids.

Found : C, 78.36%; H, 11.59%; O, 11.00%

Calculated for $C_{43}H_{74}O_3$: C, 78.34%; H, 11.52%; O, 10.99%

R_f Value: hexane: ethyl acetate

Yield : 35mg

IR (KBr, cm^{-1}): V_{max}.1460, 1377 cm^{-1}.

^{1}H NMR: Fig.15, Table-7

The isolated compounds from *Madhuca longifolia* can be use by pharmaceutical firms for drug formulation.

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