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RESEARCH ARTICLE

STRUCTURE ELUCIDATION OF NEW TRITERPENOIDS FROM ETHANOL EXTRACT OF *STEVIA REBAUDIANA (LEAVES)*

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ABSTRACT

Stevia rebaudiana is a South American plant native to Paraguay that traditionally has-been used to sweeten beverages and make tea. Previous phytochemical studies have revealed this genus to be rich in secondary metabolites including glycoside, flavonoids, lactones and terpenoids. A phytochemical study was conducted on the leaves of S. rebaudiana.Lupeol-3(4'-hydroxy) dodecanoate were isolated and identified. The separations of the chemical constituents (triterpenoids) were carried out using different chromatographic techniques including column chromatography and structures of compounds were confirmed by spectroscopic techniques including nuclear magnetic resonance as well as mass spectrometry. Separation, isolation and characterization of novel secondary metabolites (triterpenoid), which may act as prototype for the preparation of new drugs with low side effects and better efficacy.

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INTRODUCTION

Nature has been a source of medicinal agents since times immemorial. The plants are indispensable to man for his life. The three important necessities of life –food, clothing and shelter and host of other products are supplied by the plant kingdom. The knowledge of drug has collected over thousands of years for man's inquisitive nature so that today we possess many effective means of ensuring health care (Kokate *et al*., 2005). Naturally low calorie sweeteners isolated from medicinal plants are gaining a great interest in pharmaceutical and food products as dietary sucrose substitutes. Researchers showed great interest in *S. rebaudiana* (as a low calorie sweetener) not only to characterize, isolate and extract the sweet constituents but also verify the toxicological safety and pharmacological properties. The plant has immense potential and appears to have a broad spectrum of activity on several ailments (Kasai *et al*., 1987; Kinghorn *et al.*, 2010; Liu *et al*., 1997). In the present paper we describes the isolation and structure elucidation of Lupeol-3(4'-hydroxy) - dodecanoate (Figure-1) from ethanol extract. On the basis of IR, NMR and mass spectroscopic data and in comparison of their physical and spectral properties reported from the literature.

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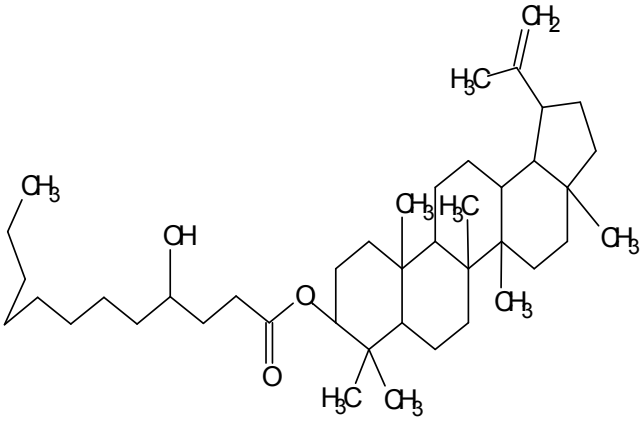


Fig. 1. Lupeol-3(4'-hydroxy) – dodecanoate

MATERIALS AND METHODS

Plant material and Extract preparation

The dried leaves of *S. rebaudiana* were purchased from Sun fruit Pvt. Ltd, Pune. A voucher specimen was deposited at, Department of Pharmacognosy, Mandsaur (MP), India. No: (MIP/PD/HN/Stevia/S-01) The leaves of *S. rebaudiana* were shade dried, powdered and extracted in soxhlet extractor serially with n-hexane, dichloro methane, ethyl acetate, acetone, methanol, ethanol and water. Removal of solvent

under reduced pressure afforded solid extracts. The removal of solvent under reduced pressure by rotary film evaporator yielded 20gm of ethanol extract. This extract further tested for phytochemical screening.

Test for steroid or terpenoids

Libermann-Buchard test: A solution of extract gave a greenish blue colour with cold AC_2O and concentrated H_2SO_4 or compound in $CHCl_3$ treated with AC_2O and concentrated H_2SO_4 also gave the same results, confirming the molecule to be of steroidal or triterpenoidal type.

Salkowasi test: A solution of extract in $CHCl_3$ shaken with concentrated H_2SO_4 gave a red colour in the $CHCl_3$ layer, confirming the molecule to be of steroidal or triterpeniodal type.

Investigation of ethanol extract

The ethanol extract was analysed by thin layer chromatography on silica gel G for the number of compounds presents in the extract. The ethanol extract was subjected to column chromatography on silica gel. The column was eluted with different solvents in their increasing order of polarity. The column was afforded one compounds with some impurities, which on repeated crystallization from chloroform: methanol yielded pure compounds designated as RS-10.

Experimental Work

The ethanol extract of *S. rebaudiana*, was prepared was subjected to column chromatography. The column was yielded mixtures of compounds at various elution stages. The mixtures that revealed clear five or six spots on thin layer chromatography examination were again subjected to rechromatography. The rechromatography resulted in the isolation of compound RS-10 in pure form. The details are given in the table 1:

IR Spectrum (λmax, KBr, cm^{-1})

The absorption band at 1733 cm^{-1} showed the presence of ester group in the molecule. The strong band at 3446 cm^{-1} showed the presence of hydroxyl group in the molecule. The absorption bands at 2920, 2851, 2360, 2341, 1457, 1386, 1247 and 980 cm^{-1}showed to be triterpenoidal molecule (Nakanishi, 1962).

^{1}H NMR Spectrum (300MHz, $CDCl_3$, TMS, δ)

The PMR spectrum is of typical lup-20(29)-ene. It showed sharp singlets for six tertiary methyl groups at δ 0.78, 0.83, 0.85, 0.93, 1.04, 1.25 and vinyl methyl singlet at δ 1.68. A broad singlet at δ1.68, 4.68(^{1}H, J=1.5 Hz) and 4.56 (^{1}H, J=1.5 Hz) confirmed the presence of vinyl group. The appearance of this vinylic group as singlet suggested the compound to be of lupane series. A broad multiplet at δ 2.37 was assigned to C_{19} proton. The H-3proton resonated at δ 4.46. (Jain *et al*.1994). The methylene protons attached to carbonyl group were resonated at δ 2.04 as braod singlet in aliphatic chain and carbinolic proton was resonated δ 3.66 as singlet. Rest of the methylene protons attached to -CHOH were resonated at δ 2.13 as broad singlet, terminal methyl group resonated at δ 0.73 in aliphatic chain.

^{13}C NMR Spectrum (75MHz, $CDCl_3$, ppm)

The chemical shifts in ^{13}C NMR spectra suggested a close resemblance with 20(29) – lupane. The quaternary carbon linked to vinylic carbon (C_{20}) resonated at 151.21 ppm. The vinylic carbon (C_{29}) was resonated at 109.57 ppm. In the ^{13}C NMR spectrum of triterpenoid the peaks at 27.62, 16.71, 16.39, 16.16, 14.70, 18.20, 19.49ppm confirmed the presence of seven methyl's. (Mahato *et al.*1994; Marina *et al.,* 1997, Rosenel *et al*., 1998). The carbon of ester group resonated at 171.28 ppm and carbinolic carbon resonated at 76.21 ppm. The methylene carbons attached to carbonyl group were resonated at 44.37, 39.57 and methene carbons attached to carbinolic group were resonated at 32.99. Rest of the methylene carbons were resonated at 32.14-19.95 ppm, terminal methyl carbon resonated at 14.36ppm in aliphatic chain (Yamaguchi, 1970).

Table 1. Processing of ethanol extract

Fraction no.	Eluent	Ratio(v/v)	Volume collected(ml)	Spots on TLC	Compound code
1	Hexane	Pure	5000	2 spots	
2	Hexane: Dichloromethane	7:3	7000	1 spot	RS-10
3	Hexane: Dichloromethane	1:1	5000	2 spots	
4	Hexane: Dichloromethane	1:3	7000	2 spots	
5	Dichloromethane	Pure	5000	4 spots	
6	$CHCl_3$	Pure	8000	3 spots	
7	$CHCl_3$:MeOH	3:1	2000	5 spots	
8	$CHCl_3$:MeOH	1:1	2000	3 spots	
9	$CHCl_3$:MeOH	1:3	1000	4 spots	
10	Methanol	Pure	800	Steak	

Wt. of the sample-5 gm,
Wt. of silica gel G-30 gm

RESULTS AND DISCUSSION

Characterization of Compound RS-10

The hexane: Dichloromethane (7:3) fraction showed presence of a single compound on TLC examination. All the similar fractions were mixed together and solvent was, removed to yield a solid mass which was recrystallized from methanol as white flakes. It was designated as RS-10 and on the basis of mass spectral analysis its molecular formula was found to be $C_{42}H_{72}O_3$, M^+624, m.p.222^{0}C.

ESI MS Spectrum

Mass spectrum showed characteristic fragmentation pattern of a terpenoid. The molecular ion peak appeared at m/z 624 and the molecular formula was found to be $C_{42}H_{72}O_3$. The important peaks were obtained at m/z 610(M-CH_2), 569 (M-C_4H_8), 537, 478, 450, 409, 358, 323, 288.

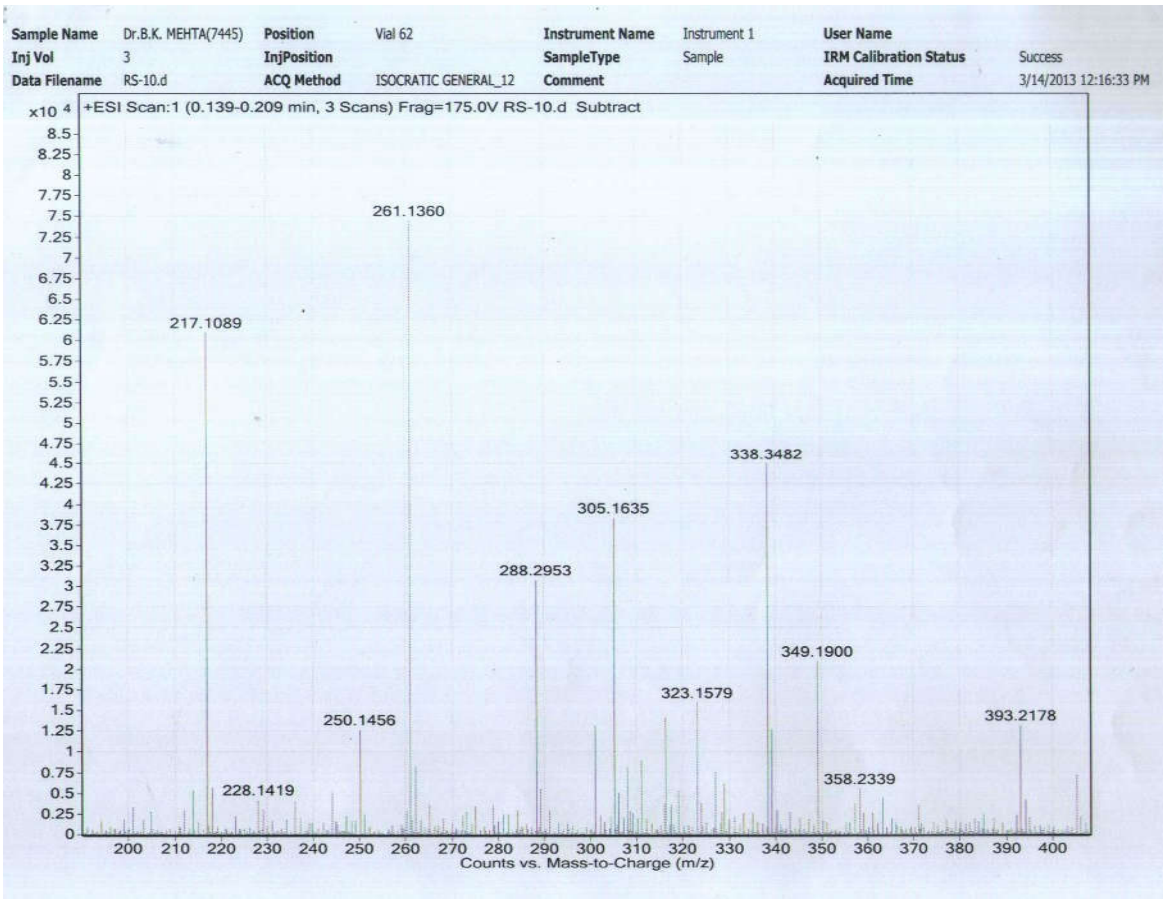


Fig. Mass Spectrum of RS-10

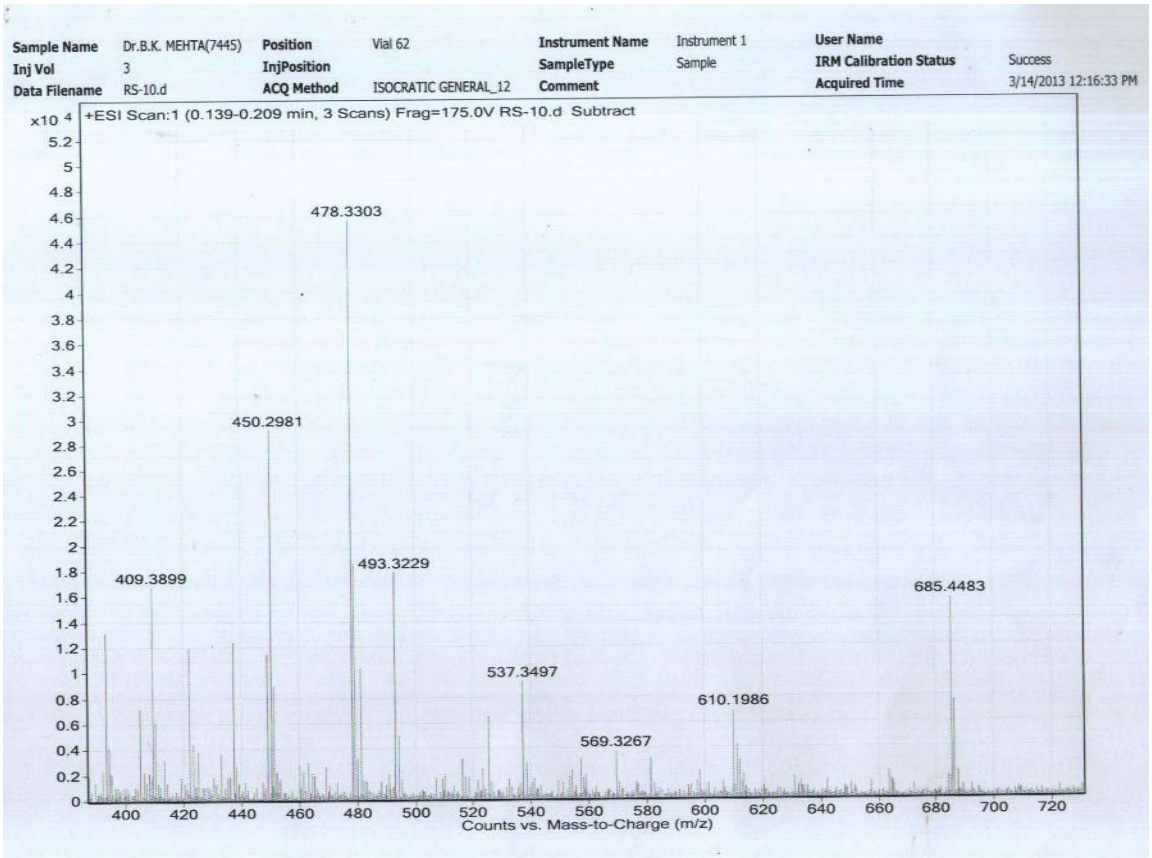


Fig. Mass Spectrum of RS-10

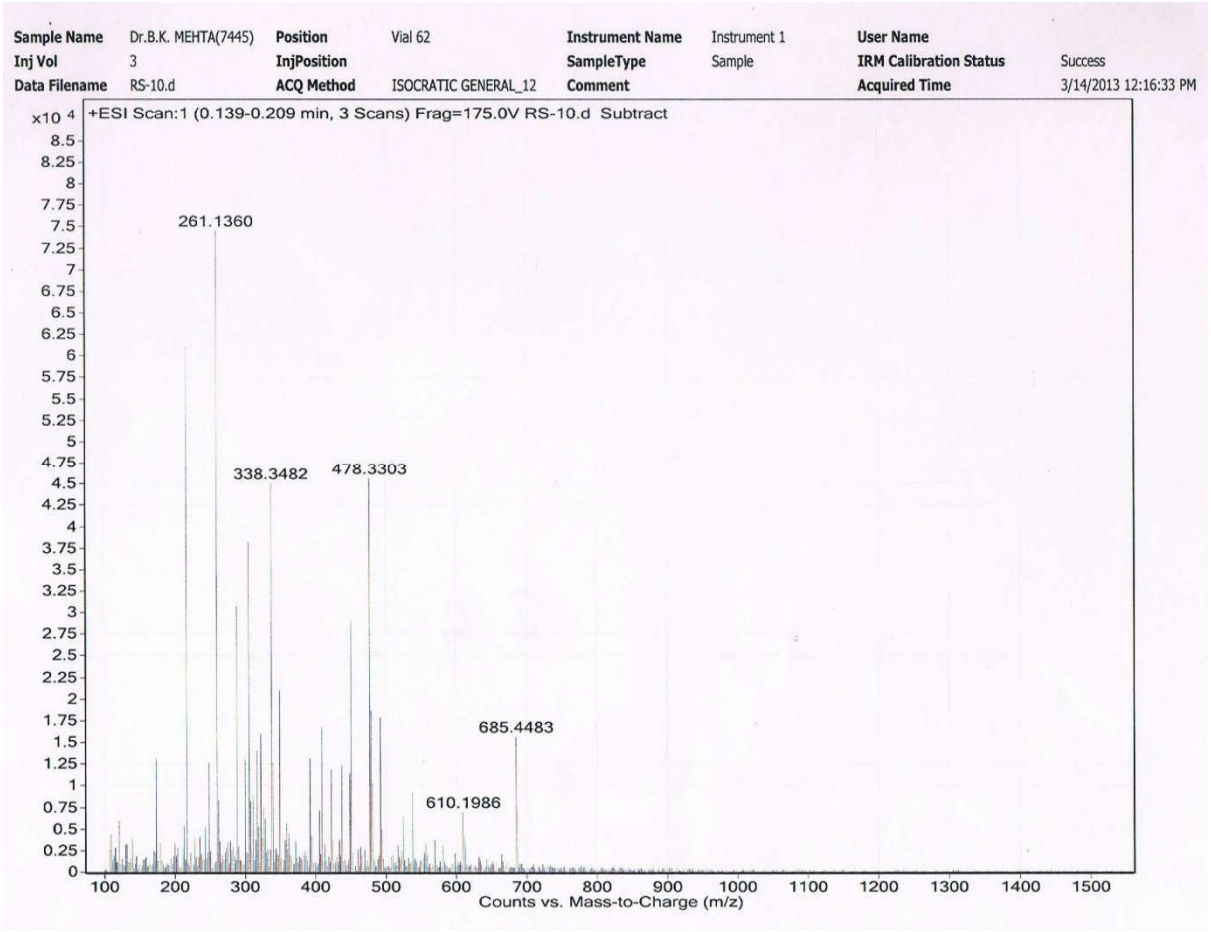


Fig. Mass Spectrum of RS-10

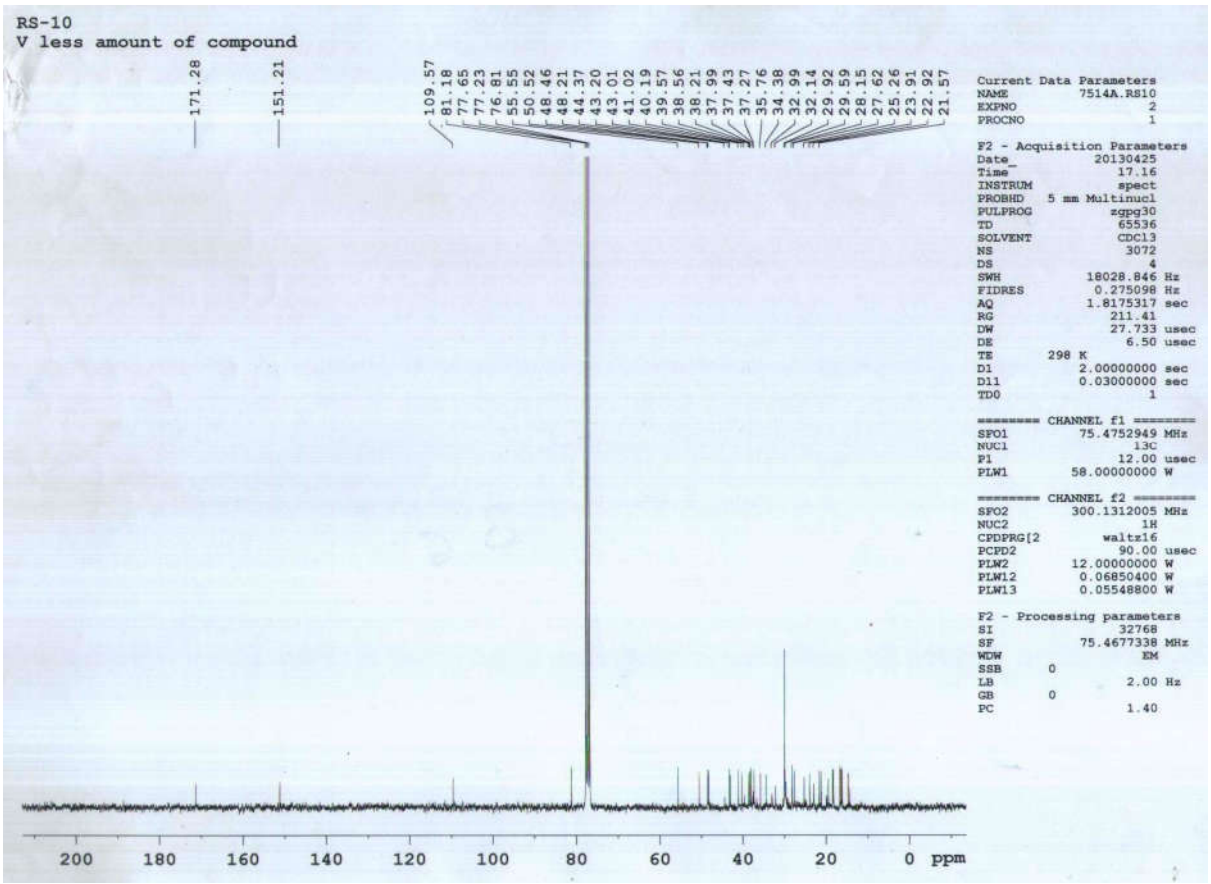


Fig. ^{13}C NMR Spectrum of RS-10

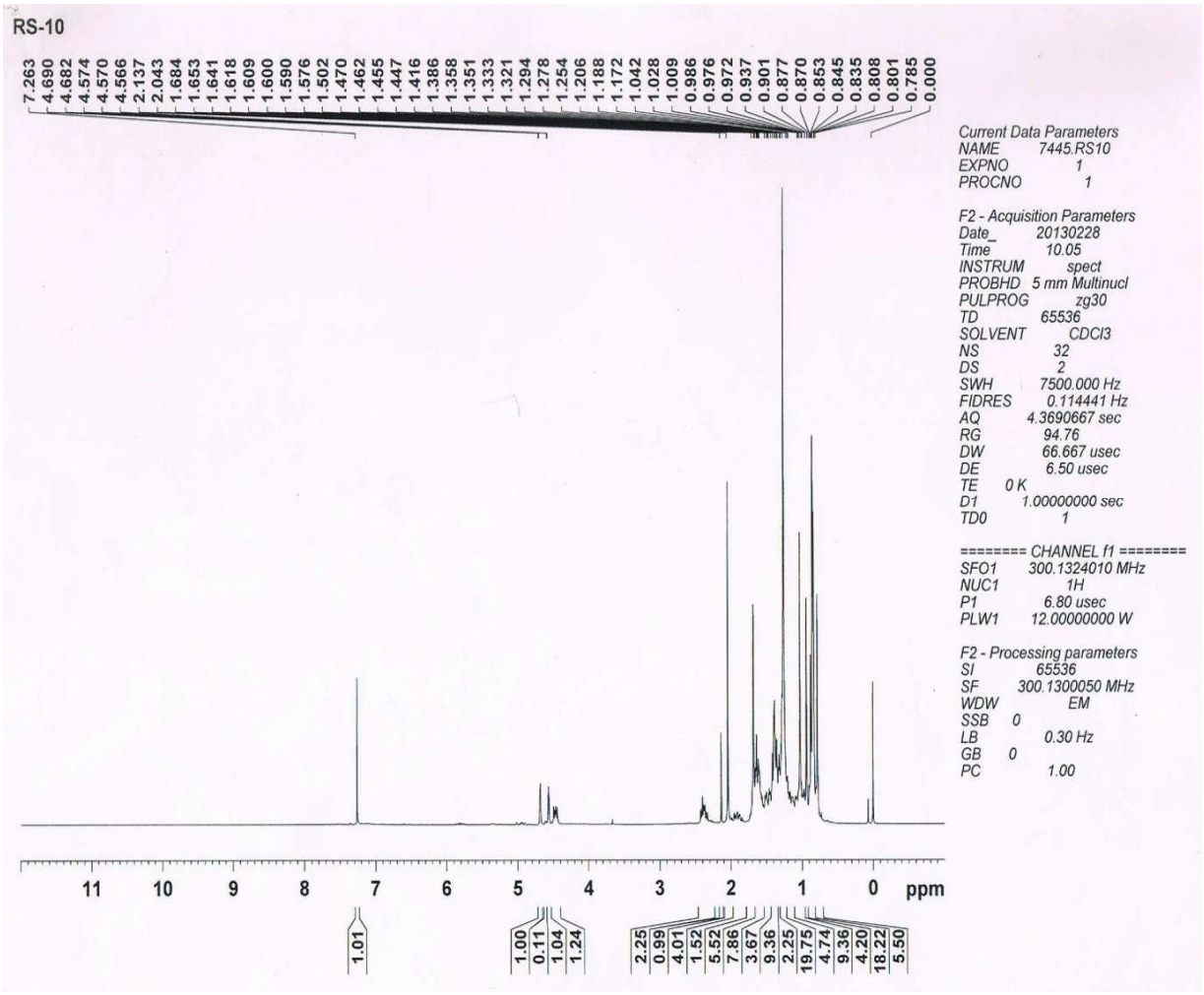


Fig. [1] H NMR Spectrum of RS-10

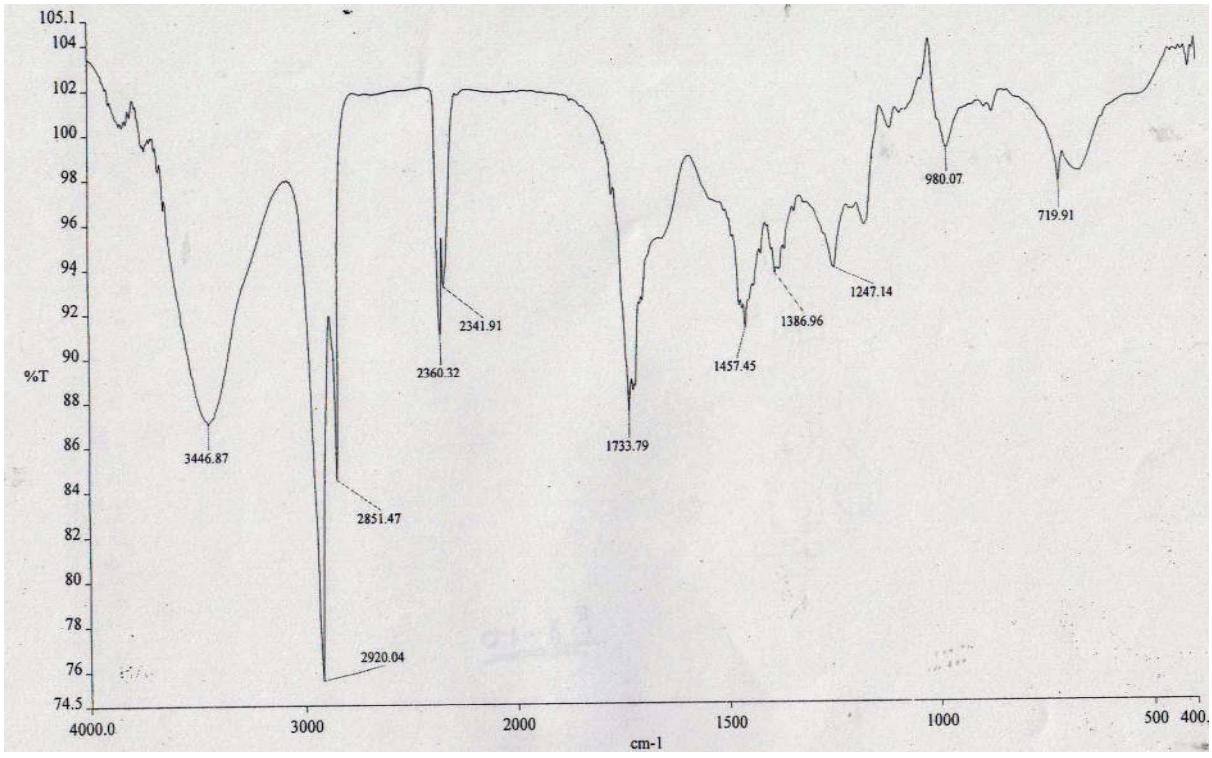
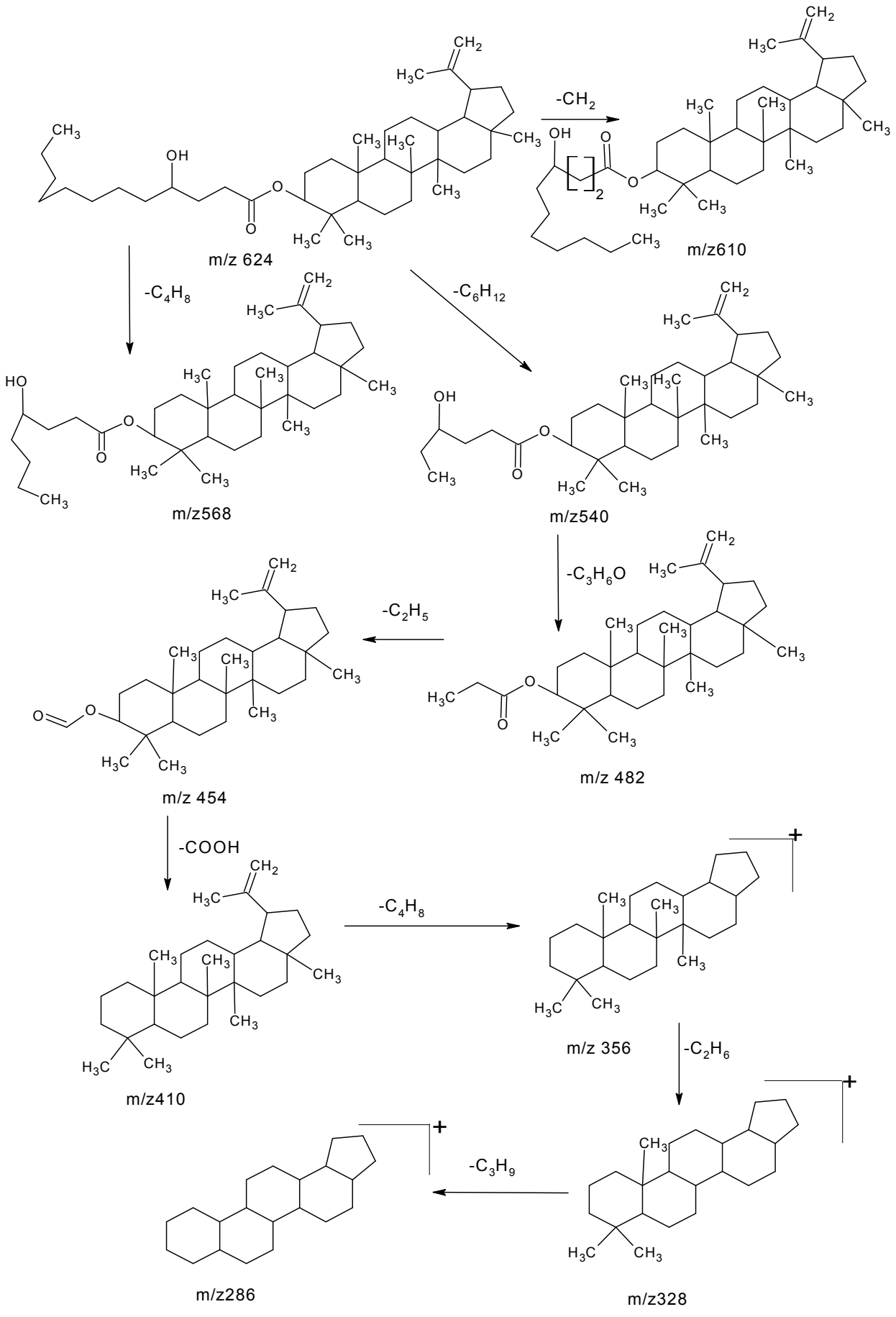


Fig. IR Spectrum of RS-10



Fragmentation of RS-10

^{13}C NMR Spectrum: Result shown in table

Table: ^{13}C NMR, ^{1}H NMR data of RS-10

S.NO.		^{13}CNMR (ppm)	^{1}HNMR(δ)
1	$-CH_2$	38.56	
2	$-CH_2$	21.57	
3	-CH	81.18	4.4dd
4	-C	38.21	
5	-CH	55.55	
6	$-CH_2$	18.39	
7	$-CH_2$	34.38	
8	-C	41.02	
9	-CH	50.52	
10	-C	37.27	
11	$-CH_2$	21.13	
12	$-CH_2$	23.91	
13	-CH	37.99	
14	-C	43.01	
15	$-CH_2$	25.26	
16	$-CH_2$	35.76	
17	-C	43.20	
18	-CH	48.46	
19	-CH	48.21	2.37m
20	-C	151.21	
21	$-CH_2$	29.92	
22	$-CH_2$	40.19	
23	$-CH_3$	27.62	0.93 s
24	$-CH_3$	16.71	0.85s
25	$-CH_3$	16.39	1.25s
26	$-CH_3$	16.16	0.83s
27	$-CH_2$	14.70	0.78s
28	$-CH_3$	18.20	1.04s
29	$-CH_2$	109.57	4.56s,4.68s
30	$-CH_3$	19.49	1.68s
C-1'	-COO	171.28	
C-2'	-CHOH	76.81	3.66s
C-2'	$-CH_2$	44.37	
C-3'	$-CH_2$	39.57	
C-4'	$-CH_2$	32.99	
C-5'	$-CH_2$	32.14	
C-6'	$-CH_2$	29.59	
C-7'	$-CH_2$	28.15	
C-8'	$-CH_2$	22.92	
C-9'	$-CH_2$	21.57	
C-10'	$-CH_2$	19.95	
C-11'	$-CH_3$	14.36	0.73m

Identification of compound RS-10

- Designation: Lupeol-3(4'-hydroxy)–dodecanoate.
- State: Solid
- Molecular formula: $C_{42}H_{72}O_3$
- Molecular ion peak: M^+624
- TLC solvent system: Hexane: Dichloromethane (7:3, v/v)
- Recrystallization: Methanol: chloroform
- m.p.: 222^0C
- Solubility: $CDCl_3$
- I.R. (KBr, cm^{-1}): 3446, 1733, 2920, 2851, 2360, 2341, 1457, 1386, 1247 and 980 cm^{-1}
- ^{1}H NMR Spectrum: δ0.78 (s,3H,CH_3,H-27), 0.83 (s,3H,CH_3,H-26), 0.85 (s,3H,CH_3,H-24), 0.93 (s,3H,CH_3,H-23), 1.04 (s,3H,CH_3,H-28), 1.25 (s,3H,CH_3,H-25), 1.68 (s,3H,CH_3,H-30), 2.37 (1H,m,H-19), 3.66 (S,1H,CHOH,J=10.8Hz), 4.4 (dd,1H,J=4.9Hz and 11.6Hz,H-3) and 4.68 (s,1H, J=1.5Hz,3.0 Hz,H-29) and 4.56 (s,1H,J=1.5Hz, H-29), δ 2.04 (s, 4H, CH_2COO, J=7.0Hz), 0.73 (m,3H,CH_3).
- Mass (m/z, rel.Inte.): M^+624, 610, 569, 537, 478, 450, 409, 358, 323, 288.

Acetylation of compound RS-10

Compound (20mg) was taken in a conical flask and to its (20) ml acetic anhydride and 4ml pyridine was added and kept for overnight. The progress of acetylation was monitored by TLC Using hexane: ether: acetic acid (8:2:0.5, v/v). After acetylation was completed it was poured on ice cool water and stirred, a solid mass was separated which was filtered and then recrystallized from methanol. IR has shown absence of –OH band and a strong band at 1730 cm^{-1} confirming the formation of monoacetate.

Conclusion

The body of existing ethno medical knowledge of plant has led to great developments in healthcare. The work was carried out by means of various physical (solvent extraction, thin layer chromatography, column chromatography and IR, NMR and mass spectral techniques. Thus on the basis of above spectral evidences the compound RS-10 was identified and characterized as Lupeol-3 (4'-hydroxy)-dodecanoate. It has been first time reported from *S. rebaudiana.*

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