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Research Article

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A New Ethyl Substituted Glycoside and Lipids from *Nigella sativa* Seeds

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ABSTRACT

Phytochemical investigation of alcohol extract of seeds of Nigella sativa were isolated a new ethyl substituted glycoside, O-β-D-ethyl glucopyranosyl-(2→4)-O-β-D-ethyl glucopyranosyl-(2→4)- β-D-ethyl glucopyranoside (1) along with three new aliphatic compounds heptadecyl docosanoic acid (2), hexatetracontan -22-ol (3), 21-methyl pentacosa-6,15-dione (4) and one known compound 16-methyl heptadecanoic acid (5). The structural elucidation of the isolated compounds was based primarily on 1D- and 2D- NMR analysis, including COSY, HMBC and HMQC correlations. The compound 1 shows significant activity against lipoxygenase enzyme.

Keywords: *Nigella sativa;* Seeds; Alcohol extract; Lipids; Glycoside

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INTRODUCTION

Nigella sativa (Ranunculacae) has many uses in Egyptian systems of medicine [1]. The seeds have a wide variety of application in traditional medicine, especially for the treatment of cough, fever, bronchial asthma and eczema [2]. Recent pharmacological investigation on the seed extract revealed a wide spectrum of activities such as anti-tumor, anti-inflammatory, analgesic, anti-pyretic and gastroprotective [3-5]. Other notable properties are anticestode and antinematode, antidiabetic, cytotoxic and immunopotentiating activities as well as protection against cytotoxic damage from chemotherapeutic drugs and oxidative stress [6-10]. The effects of the seeds have also been evaluated in clinical and animal studies. Various bioactive compounds have been isolated from the seeds of *N. sativa*, such as alkaloids, steroids (ergosterol and cycloartenol), flavonol triglycosides, saponins, aliphatic esters, sugars and an isobenzofuranone derivative [11-23]. In view of its wide spectrum activities, alcohol extract of seeds furnished a new ethyl substituted glycoside, O-β-D-ethyl glucopyranosyl-(2→4)-O-β-D-ethyl glucopyranosyl-(2→4)- β-D-ethyl glucopyranoside (1) along with three new aliphatic compounds heptadecyl docosanoic acid (2), hexatetracontan -22-ol (3), 21-methyl pentacosa-6,15-dione (4) and one known compound 16-methyl heptadecanoic acid (5) (Figure 1).
Lipoxygenase (LOX) is involved in arachidonic acid metabolism, generating biologically active leukotriene that plays an important role in inflammation. The compound 1 showed inhibitory potential against lipoxygenase enzyme, hence showed anti-inflammatory activity.

EXPERIMENTAL SECTION

General Experimental Procedure

Melting points (mp) are uncorrected. ^{1}H NMR spectra were measured on a solution of the glycoside 1 (30 mg) in D_2O and 2, 3, 4 and 5 (28 mg) in $CDCl_3$ at ambient temperature. The high resolution 1D and 2D NMR spectra (^{1}H-^{1}HCOSY, HMQC and HMBC) and ^{13}C-NMR spectral analyses were performed using a JEOL-JNM-300 and 75 MHz spectrometer. All chemical shifts are given in ppm and (tetra methyl silane) TMS was used as an internal standard. The degree of protonation on carbon (CH_3, CH_2 and CH) was determined by DEPT (90, 135) experiments. Conventional pulse sequences were used for COSY, HMQC and HMBC. The Thin layer chromatography was

performed using silica gel G and spots were visualized by exposure to iodine vapors or by spraying with H_2SO_4-vanillin solution followed by heating at 105ºC for 5 min.

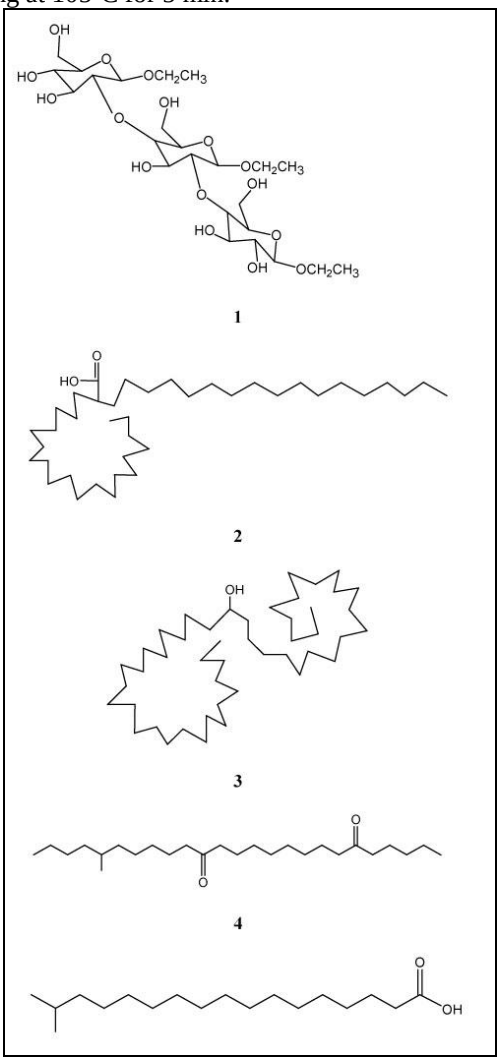

Figure 1: Structures of characterized compounds 1-5

Plant Material

The seeds (10 kg) of *N. sativa* were collected from the local medicinal market of Ujjain city and were authenticated by the authorities at EIMPS, Vikram University, Ujjain.

Extraction and Isolation of the Constituents

Ten kilograms of shade-dried, cleaned and coarse-powdered seeds were extracted by n-hexane, benzene, benzene:acetone and alcohol serially each for 72-75 h in soxhlet extractor. Alcohol extract was concentrated to dryness under reduced pressure by rotary film evaporator to afford a dark brown syrupy residue (450 mg). The dried sample was fractionated on normal phase silica gel column chromatography (1000 cm × 25 cm) by using gradient elution with different solvent mixtures in their increasing order of polarity. A dried portion of the 50% benzene:MeOH (145 mg) elute was subjected to repeated chromatography on silica gel, using a discontinuous gradient from 1:1 benzene : EtOAc to 1:1 benzene : methanol. Fractions 40-50 (2500 mL) of (benzene: EtOAc, v/v, 1:3) afforded colourless gummy solid (60 mg), in pure form designated as 1. Hexane and benzene fractions of alcohol extract further separated by rechromatography over alumina grade III and afforded two compounds in pure form designated as 2 and 3 respectively. (Benzene: EtOAc, v/v 9:1) fraction of alcohol extract further rechromatographed over silica gel and eluate benzene and (benzene: ether, 8:2, v/v) gave compound 4 in pure form and 5 in crystalline form, respectively.

***In vitro* Lipoxygenase Inhibition Assay**

Lipoxygenase inhibiting activity was conveniently measured by modifying the spectrometric method developed by AL Tappel [24]. Lipoxygenase (1.13.11.12) type I-B and linoleic acid were purchased from Sigma. All other chemicals were of analytical grade. The reaction mixture containing 165 µL of 100 mM sodium phosphate buffer (pH 8.0), 5 µL of test-compound solution and 20 µL of lipoxygenase solution were mixed and incubated for 10 min at 25°C. The reaction was then initiated by the addition of 10 µL linoleic acid (substrate) solution, with the formation of (9*Z*, 11*E*)-(13 *S*)-13-hydroperoxyoctadeca-9, 11-dienoate; the change in absorbance at 234 nm was followed for 6 min. The test compound and the control were dissolved in methanol. All the reactions were performed in triplicate in 96-well micro-plate in Spectra Max 384 plus.

O-β-D-ethyl glucopyranosyl-(2→4)-O-β-D-ethyl glucopyranosyl-(2→4)- β-D-ethyl glucopyranoside (1):

($[M+H]^+$)589, (60mg, methanol), mp 185ºC (Found: C, 48.98; H, 7.53; O, 43.478 Calc. for: $C_{24}H_{44}O_{16}$: C, 48.67; H, 7.22; O, 43.12%). $[\alpha]_D$ = +63.2 (*c* 0.04, MeOH), isolated from benzene: EtOAc (1:3,v/v)fraction. On TLC examination it showed single spot using ($CHCl_3$/MeOH, v/v, 9:1) R_f 0.27 as solvent system; UV λ_{max} /nm: 203 (4.2), 192(3.42); IR (KBr) ν_{max} / cm^{-1}: 2938, 2890, 2833, 1460, 1384, 1341, 1279, 1220, 1137 and 1051 cm^{-1}. ^{1}H-NMR (300MHz, DMSO-d_6 TMS, δ): 4.64 (1H, d,-CH, *J* 7.3 Hz), 3.43 (1H, dd, *J* 2.7, 7.3 Hz, -CH), 3.69 (1H, m, -CH), 3.60(1H, m, -CH) 3.56 (2H, ov, CH_2), 3.61 (2H, ov, CH_2), 1.13 (3H, t, *J* 7.6Hz, -CH_3), 3.56-3.60 (2H, ov, -OCH_2). ESI-MS: m/z 589 ($[M+H]^+$), 439, 231, 208, 173 and 150. ^{13}C-NMR (75MHz, DMSO, TMS, ppm): 99.6 (C-1), 72.1(C-2), 70.5(C-3), 69.3(C-4), 69.8(C-5), 63.2(C-6), 61.5(OCH_2) and 15.0 ppm (CH_3).

2-heptadecyldocosanoic acid (2):

m/z 578, White crystalline (35 mg, chloroform), mp 120ºC (Found: C, 80.89; H,13.57; O, 5.52 Calc. for $C_{39}H_{78}O_2$: 80.33, 13.12, 5.12%). On TLC examination it showed single spot using (hexane/CH_2Cl_2, v/v, 2:8) R_f 0.38 as solvent system. IR (KBr) ν_{max} / cm^{-1}: 3439, 1700, 2920, 2840, 1380 1461, 1046, 730-720 cm^{-1}. ^{1}H-NMR (300MHz, $CDCl_3$, TMS, δ): 0.88(6H, t, *J* 7.2Hz, 2 X -CH_3), 2.3 (1H, t, *J* 6.2Hz, -CH), 1.6 (4H, m, 2X -CH_2), 1.20 (66H, br s, 33X -CH_2) 10.5 (1H, br s, -COOH). ^{13}C-NMR (75MHz, $CDCl_3$, ppm): 180.4, 34.1, 31.9, 29.7, 24.6, 24.7, 14.0, 29.6-29.0 as a bunch. ESI-MS m/z: 578, 564, 538, 471, 396, 380, 355, 253, 239, 298, 279, 211, 196, 183, 169, 167, 155, 114 and 99.

Hexatetracontan-22-ol (3):

($[M+2H]^+$) 664, white crystalline (34 mg, benzene), mp111°C (Found: C, 83.30; H,14.28 ; O, 2.41 Calc. for $C_{46}H_{94}O$ C,82.99; H,13.81; O, 2.10%). On TLC examination showed single spot using (hexane/CH_2Cl_2, v/v, 1:9) R_f 0.32 solvent system. IR (KBr) ν_{max} / cm^{-1}: 3460, 1170, 2920, 2845, 1461, 1384, 1040 and 720 cm^{-1}. ^{1}H-NMR (300MHz, $CDCl_3$, TMS, δ): 0.88(6H, t, *J* 7.4Hz, 2X -CH_3), 3.63 (1H, m, CHOH), 1.50 (5H, m, 2 X CH_2OH), 1.26 (82H, s, 41XCH_2). ^{13}C-NMR (75MHz, $CDCl_3$, δ): 62.0, 34.0, 31.8, 29.6, 24.8, 22.6 and. 14.0. ESI-MS m/z: 664, 662, 591, 507, 437, 395 and 367.

21-methyl pentacosa-6, 15-dione (4):

M^+ 394, White amorphous (35 mg, chloroform), mp 80-82°C (Found: C, 79.12; H, 12.77, O, 8.10 Calculated for $C_{26}H_{50}O_2$: C, 78.72; H, 12.23, O, 7.72%). On TLC examination showed single spot using (benzene:ethyl acetate :acetic acid, 9:1:1) R_f 0.49 solvent system. IR (KBr) ν_{max} / cm^{-1}: 1700, 2917, 2955, 2849, 1464, 1432, 1410, 1020, 783 and 722. ^{1}H-NMR (200MHz, $CDCl_3$, TMS, δ): 0.87(9H, t, *J* 7.0Hz, 3X -CH_3), 1.25 (24H, s, 12X -CH_2), 1.60 (9H, br s, 4XCH_2, CH), 2.34 (8H, t, $COCH_2$). ^{13}C-NMR (75MHz, $CDCl_3$, δ): 204.7, 36.5, 32.4, 29.8- 29.2, 23.8, 21.8, 15.4, 14.2 EI-MS m/z: M^+ 394(3.9), 350(2.9), 339(11.6), 309(4.9), 273(2.5), 255(3.7), 254(11.1), 212(6.5), 198(4.7), 184(5.5), 170(4.5), 169(5.6), 156(5.5), 154(3.5), 152(2.5), 144(3.3), 140(2.6), 138(3.6), 128(19.6),96(18.1), 84(24.3), 83(23.7), 82(10.8), 73(37.8), 72(31.1), 71(25.1), 58(43.7), 57(76.1), 56(50.7) and 43(100).

16-methyl heptadecanoic acid (5):

m/z 284, $C_{18}H_{36}O_2$, white crystalline (40 mg, chloroform), mp 69-70°C, TLC (benzene: ether, 8:2), IR (KBr) ν_{max} / cm^{-1}: 3420, 1710, 2929, 2848, 1382, 729. ^{1}H-NMR (200MHz, $CDCl_3$, TMS, δ): 0.87(6H,d, *J* 7.1Hz, 2X -CH_3), 1.25(26H, s, 13X -CH_2), 1.60 (2H, br s, β-CH_2), 2.32(2H, t, CH_2COOH), 10.2 (1H, br s, COOH). ^{13}C-NMR (75MHz, $CDCl_3$, δ): 180.7, 34.17, 32.0, 29.8, 29.4, 29.3, 29.2, 24.8, 22.8, 16.4, 14.2; EI-MS:M^+ 284(34.3), 241(27.3), 185(30.0), 171(11.1), 143(10.1), 129(57.5), 115(17.3), 97(23.4), 83(25.4), 73(100), 71(34.4), 69(31.1), 68(31.0), 61(23.1), 60(79.1), 57(60.1), 55(59.0), 43(83.4), 41(63.3) and 29(28.1).

RESULTS AND DISCUSSION

The natural compounds were identified mainly by their 1D (^{1}H and ^{13}C NMR), 2D (^{1}H-^{1}H COSY), DEPT and mass spectrometry analysis, including comparison with literature data. The ESI-MS and elemental analysis of **1** indicated the molecular ion peak at *m/z* 588 suggesting its molecular formula $C_{24}H_{44}O_{16}$. Its UV spectrum showed only the end absorption bands at λ_{max} 203 and 192 nm. Its IR spectrum displayed typical absorption bands for hydroxyl group at 3327 cm^{-1} and long chain aliphatic nature of the molecule (2938, 2890, 2833, 1384, 1341, 1460 cm^{-1}) [25]. In its ESI mass spectrum, the regular differences of *m/z* 208 mass units were observed which indicating that **1** was the trimer of basic unit of ethyl glucoside ($C_8H_{16}O_6$). Further ehtoxy functionality was also confirmed by observing abundant ion at *m/z* 208([162+46]$^+$) in the spectrum. The other fragment ions at *m/z* 589, 524, 381, 231,173 and 150 justified the proposed structure of 1. The fragmentation pattern was given in Figure 2. Complexities of the signals appeared in ^{1}H NMR at δ 3.33 to 4.64 were due to characteristic of a sugar molecule, which indicated glycosidic nature of the compound. The methyl and methylenoxyl protons resonated as a triplet at δ 1.13 and as multiplet at δ 3.56-3.60, which indicated ethoxy functionality present in the molecule as a substituent [26,27]. The anomeric proton resonated at δ 4.64 as a doublet and other methine and methylene carbinolic protons resonated at δ 3.30 to 3.60, indicating that 1 was an ethyl glucoside. The large value of coupling constant (*J* 7.3 Hz) suggested that it was a β-glucoside derivative [28]. A total of 8 carbon signals were detected in the ^{13}C NMR and DEPT spectra including those for hexose moiety and ethyl substitution. The signals appeared at δ 63.2, 61.5 and 15.0 were due to methylenoxy and methyl group respectively [29,30]. Anomeric carbon resonated at δ 99.6 while other CHOH groups resonated at δ 72.1, 70.5 and 69.3 which revealed the presence of β-D-glucose moiety [31]. Thus the compound was identified as β-D- ethyl glucoside, which was further confirmed by 2D NMR (correlation spectroscopy) ^{1}H-^{1}H COSY and (heteronuclear multiple bond coherence) HMBC spectroscopy.

In ^{1}H-^{1}H COSY spectrum, a multiplet at δ 3.43 was assigned to H-2 on the basis of a cross peak with the anomeric H-1 (δ 4.64, 1H, *J* 7.3 Hz) which also showed a cross peak with a multiplet signal at δ 3.69 and was assigned to H-3. In HMBC spectrum, a proton signal at δ 1.13 was coupled to carbon signals at δ 63.2 (C-7) and 99.6 (C-1) revealed that ethoxy functionality (OCH_2CH_3) was present at C-1 position. Further the sequence of sugar was determined by observing a proton signal at δ 3.43 (H-2) which coupled to C- 4 of other sugar confirm that C-2 position of glucose linked to C-4 position of other glucose, hence glucosidation in 1 is: O-β-D- ethyl glucopyranosyl-(2→4)-O-β-D-ethyl glucopyranosyl----

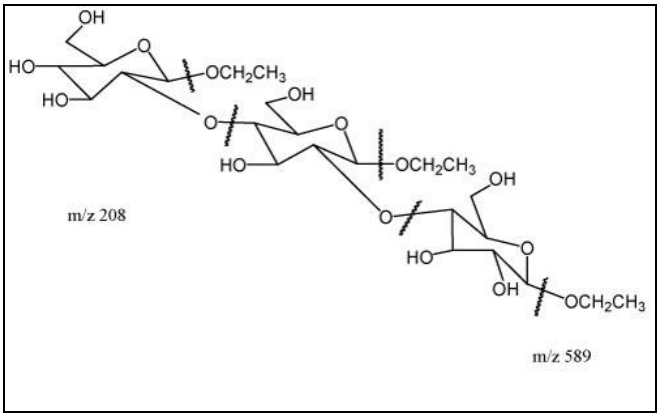

Figure 2: Fragmentation patterns proposed for 1

On the basis of above evidences [32], 1 was identified and characterized as: O-β-D-ethyl glucopyranosyl-(2→4)-O-β-D-ethyl glucopyranosyl-(2→4)-β-D-ethyl glucopyranoside. IR spectrum of 2 showed typical absorption bands for alcohol and carbonyl groups (3439, 1700 cm^{-1}) and for the long chain aliphatic nature of the molecule (2920, 2840, 1380 1461, 1046 and 730-720 cm^{-1}) [33]. The ESI-mass spectral data and elemental analyses associated to the NMR data of **2** are coherent with the molecular formula $C_{39}H_{78}O_2$. The pattern of the ESI-MS spectrum revealed that **2** was a long chain fatty acid. The base peak at *m/z* 298 corresponding to fragment ion ($[C_{19}H_{37}O_2]^+$) and abundant ion at *m/z* 279 was due to ($[C_{20}H_{41}\text{-}2H]^+$), indicated that side chain of ($C_{20}H_{41}$) present at C-2 position to the carboxylic group. The possibility of carboxylic group at terminal position was also ruled out, since abundant ion was not observed at *m/z* 60 in the mass spectrum [34,35]. Other fragment ions were observed at *m/z* 99, 114, 155, 167, 169, 183, 196, 211, 239, 253, 355, 380, 396, 471, 538 and *m/z* 564 attributed to the long chain aliphatic acid. The fragmentation pattern was given in Figure 3. ^{1}H NMR spectrum showed a peak at δ 0.88 (*J* 7.2 Hz) as a triplet for

six protons was due to terminal methyl groups. Methylene protons α- to the carboxyl group resonated at δ 2.3 (*J* 6.2 Hz) while methylene protons β- to carboxyl group resonated at δ 1.6. The remaining methylene groups resonated at δ 1.2 ppm as a broad singlet. A broad singlet appeared at δ 10.5 shows the presence of carboxylic group in the molecule [36]. The ^{13}C NMR spectrum of 2 showed the presence of carboxylic group at δ180.4.Other peaks resonated at δ 34.1, 31.9, 24.6, 22.7 and 14.0 were due to methylene and methyl groups which present in decreasing order of deshielded values with respect to the carboxylic group. Other methylene groups resonated at δ 29.0-29.6 as singlets. Thus on the basis of above spectral evidences, 2 was characterized as 2-heptadecyldocosanoic acid, it is a new compound being reported for the first time by us. IR spectrum of 3 showed absorption bands for alcohol group (3460 cm^{-1}) and for the long chain aliphatic nature of the molecule (2920, 2845, 1461, 1384, 1040 and 730-720 cm^{-1}). The ESI-MS spectrum and combined ^{1}H/^{13}C NMR studies of 3 suggested the molecular formula as $C_{46}H_{94}O$.

In its ESI-MS spectrum, molecular ion peak obtained at *m/z* 664 $[M+2H]^+$) suggesting molecular formula $C_{46}H_{94}O$, to be a long chain aliphatic alcohol. The separation of most of the peaks by 14 and 28 mass units and presence of fragments corresponding to ($[C_nH_{2n+1}]^+$) and ($[C_nH_{2n}]^+$) ion series indicated that it was a long chain aliphatic compound. An abundant peak obtained at *m/z* 367 ($[C_{25}H_{51}O]^+$) was due to α-cleavage from carbinolic group which further gave fragment ion at *m/z* 337, indicating the position of hydroxyl group. Other fragment ions at m/z 395, 437, 507 and 591 were in agreement with the proposed structure. The fragmentation pattern was given in Figure 4. ^{1}H NMR spectrum showed a peak at δ 0.88 (*J* 7.4 Hz) as a triplet for six terminal methyl groups.

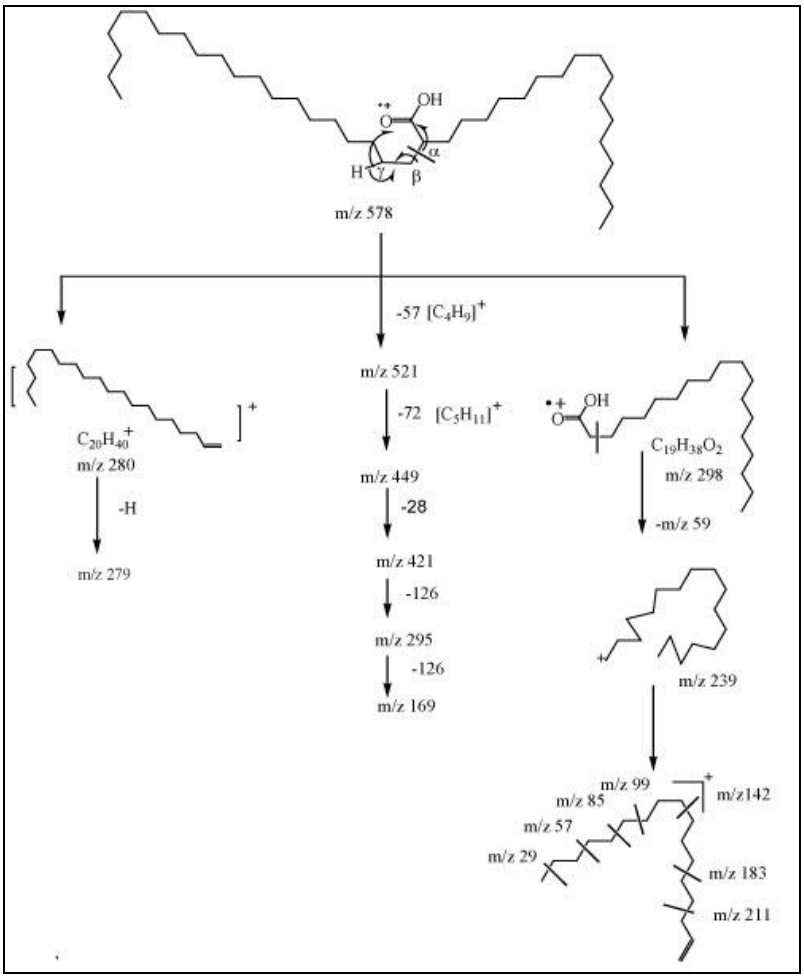

Figure 3: Fragmentation patterns proposed for 2

The peak was resonated at δ 3.63 as a triplet revealed the presence of carbinolic proton while hydroxyl group and methylene proton β- to hydroxyl group was resonated as a broad multiplet at δ 1.5. The remaining methylene protons resonated as a broad singlet at δ 1.26. Thus 3 was proposed to be a long chain aliphatic alcohol. In ^{1}H-^{1}H COSY spectrum, carbinolic proton at δ 3.63 showed cross peaks with at δ 1.50 and 1.26 which indicated connectivity with α- and other methylene groups. Further the peak at δ 1.26 showed a cross peak at 0.88 ppm

showed connectivity with terminal methyl protons. The ^{13}C NMR showed peak at δ 62.0 ppm due to carbinolic carbon atom. The peaks at δ 34.0 and 31.8 ppm were due to α- and β- methylene carbon atoms to alcoholic group respectively. The peaks at δ 14.0, 24.8 and 22.6 were due to terminal methyl carbons, α- and β- methylene carbon atoms to methyl group respectively. All other methylene carbons resonated at δ 29.6. Thus on the basis of above evidences, **3** was characterized as hexatetracontan-22-ol.

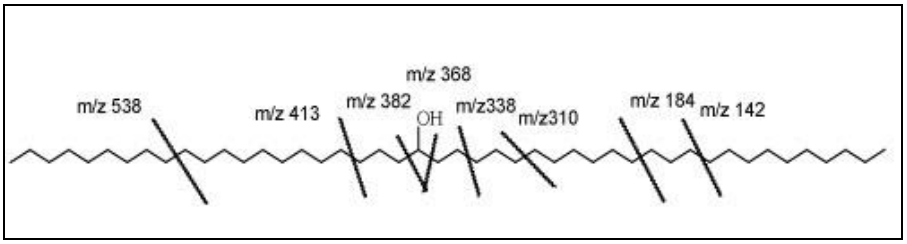

Figure 4: Structure showing the fragmentation patterns proposed for 3

The mass spectral analyses and combined NMR data of 4 suggested the molecular formula as $C_{26}H_{50}O_2$. IR spectrum of 4 showed typical absorption bands for carbonyl group (1700 cm^{-1}) and long chain aliphatic nature (1020 and 722 cm^{-1}). The peak obtained at *m/z* 339 was due to α-cleavage to the carbonyl group, indicating the position of carbonyl group at C-5 in the molecule. Further the position of another carbonyl group at C-15 was justified by fragmentation that takes place α-to the carbonyl group, which is involved through McLafferty rearrangement to give fragment ion ($C_{16}H_{32}O_2$) and other ion at *m/z* 85. Hence EI-MS justified the nature of long chain aliphatic molecule. ^{1}H-NMR spectrum showed a peak at δ 0.87 (*J* 7.0 Hz) as a triplet for nine protons due to terminal methyl groups. The signal resonated at δ 2.34 as a triplet was due to methylene group attached α- to the carbonyl group while signal at δ 1.60 as a multiplet was due to methylene group β- to carbonyl group. Other methylene protons resonated as a broad singlet at δ 1.25. The molecular ion peak obtained at *m/z* 394 in its EI-MS, which shows two degree of unsaturation in the molecule. The ^{13}C NMR showed peak at δ 204.7 ppm due to ketone functional group present in the molecule. The peaks at δ 36.5 and 32.4 ppm were due to α- and β- methylene carbon atoms to the ketone group respectively.

Table 1: LOX activity and ^{1}H[a] and ^{13}C NMR[b] data for compounds

***In vitro* inhibition of LOX**	
Compounds	**IC50 ± SEM[a] [µM]**
1	(38.2 ± 1.2)
2	-
3	-
4	-
Baicalein[b]	(20.5 ± 0.5)

[a]Standard error of means of five assays; [b]Positive control for LOX inhibition activity

^{1}H and ^{13}C NMR[b] data for compounds 1		
S. no.	**H (δ)**	**C(δ)**
1	4.64 d (7.3 Hz)	99.6
2	3.43 dd (2.7, 7.3 Hz,)	72.1
3	3.69 m	70.5
4	3.60 m	69.3
5	3.56 ov	69.8
6	3.61 ov	63.2
	1.13 t (7.6Hz, -CH_3)	15
	3.56-3.60 ov (-OCH_2)	61.5

[a](300MHz, DMSO-d6 TMS, δ); [b](75MHz, DMSO, TMS, ppm)

The peaks at δ 29.8-29.2, 23.8, 21.8, 15.4 and 14.2 were in decreasing order which indicated that molecule to be long chain aliphatic ketone. Thus on the basis of the above evidences, 4 was identified and characterized as 21-methyl pentacosa-6, 15-Dione, it is a new compound and being reported for the first time by us. On the basis of IR, ^{1}H-NMR, ^{13}C-NMR, mass spectral analysis and comparison by literature data, 5 was identified as 16-methyl heptadecanoic acid. It is known compound and found in adipose tissue, milk fat, liver and tobacco smoke and also used as a surfactant [37,38].

CONCLUSION

From the *In vitro* screening of compounds 1-5 against lipoxygenase enzyme, 1 showed inhibitory potential against lipoxygenase enzyme with IC_{50} value (38.2 ± 1.2) µM. The IC_{50} value for the positive control of lipoxygenase was found to be (20.5 ± 0.5) µM (Table 1).

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