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Isolation, characterization and biological activity of compounds isolated from the seeds of *Nigella sativa* L

B.K.Mehta, Meenal Gupta*

School of Studies in Chemistry and Biochemistry, Vikram University, Ujjain-456010 (M.P.), (INDIA)

E-mail: meenal_30_apr@yahoo.co.in

ABSTRACT

New aliphatic compounds and glycosides were isolated from the alcohol extract of seeds of *Nigella sativa* L., is a medicinal important plant. They were identified as tri-[(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(3→4)]-α-D- glucopyranoside 1, tetra-[([O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(4→4)]-α-D-glucopyranosyl-(1→2)- β-D-fructofuranoside 2, 4-tricosanol 3, 22-methyl-12- hexacosanone 4 and 6-methyl pentadecanoic acid 5 along with known compounds 5-nonadecanone 6 and sucrose octa acetate 7. They were characterized on the basis of spectral analysis and comparison by literature data. The antimicrobial activity of alcohol extract was also studied and found to be active against gram positive bacteria.

KEYWORDS

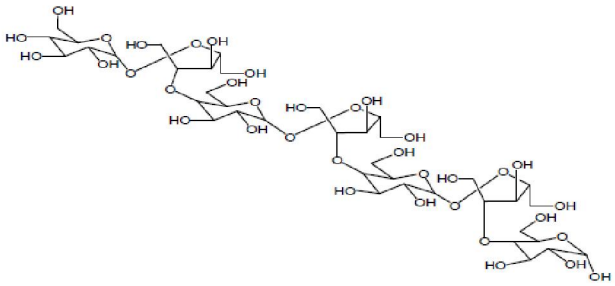
Nigella sativa L;
Ranunculaceae;
Alcohol extract;
Glycosides;
Aliphatic compounds;
Antimicrobial activity.

INTRODUCTION

The plant *Nigella sativa* Linn (Rannunculaceae), its seeds are commonly known as 'Kalaungi' and 'Kalajira' in Hindi, is distributed in India, Middle Eastern countries and reported to be a medicinally important plant[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, antipyretic and gastroprotective and other valuable activities[3-5]. Various bioactive compounds have been isolated from its seeds; such as alkaloids, flavonol triglycosides, saponins and an isobenzofuranone derivative[11-23]. Bioactive principle α-hederin had been isolated from the seeds and reported in vitro anti tumor activity[8]. Here in we report on the isolation and characterization of glycosides: tri-[(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(3→4)]-α-D- glucopyranoside 1, tetra-[(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(4→4)]-α-D-glucopyranosyl-(1→2)- β-D-fructofuranoside 2 and aliphatic compounds: 4-tricosanol 3, 22-methyl-12- hexacosanone 4, 6-methyl pentadecanoic acid 5 along with known compounds 5-nonadecanone6 and sucrose octa acetate7 from alcohol extract of seeds. The antimicrobial spectrum of alcohol extract of black cumin seeds showed inhibition potential against gram positive bacteria.

RESULTS AND DISCUSSION

IR spectrum of 1 showed strong and broad band



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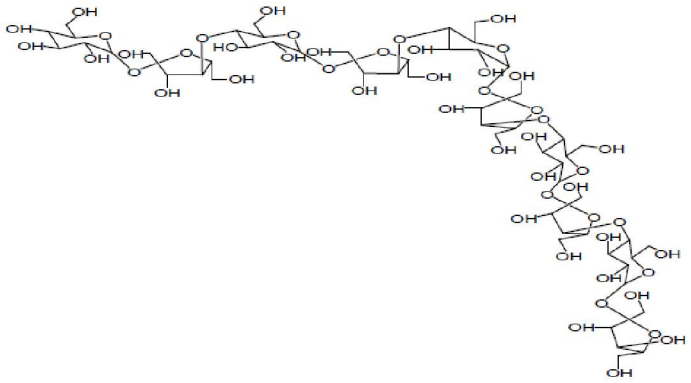

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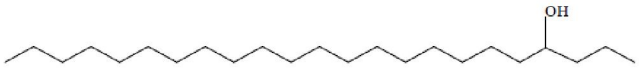

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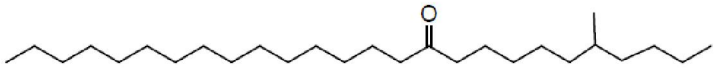

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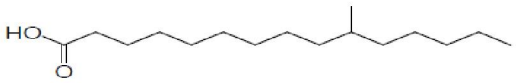

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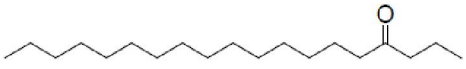

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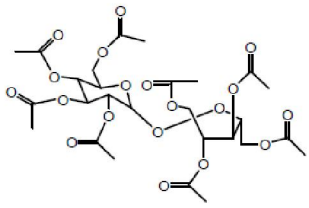

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Structure of compounds

at 3400-3200 cm^{-1} due to presence of OH groups the intensity of the band indicating that compound may be polyol[24]. Weak bands observed at 2929, 2870, 1417 and 1384 cm^{-1} were attributed to the aliphatic C-H stretching and bending vibration. Strong bands observed between 1051 and 1137 cm^{-1} were due to C-O-C out of phase stretching modes of ether. Its UV spectrum showed only the end absorption bands

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at λ_{max} 203 and 192 nm.

The ^{1}H NMR spectrum was characteristic for glycoside in which all protons were resonated in between δ 3.29 to 5.46. A doublet at δ 5.46 was due to anomeric proton of α- D-glucose moiety and only one anomeric signal was observed which indicated that other sugar having in five member rings. It was also ascribable that H-2' of β-D-fructofuranoside was involved in glycosidation with H-1 of α-D-glucopyranose, hence the basic units of oligosaccharide was glucose and fructose. The signals of sucrose were similar to those reported in literature[25,26]. Thus the compound was identified as sucrose. The integration area in ^{1}H NMR spectrum indicated that more than one unit of sucrose was present in the molecule i.e. it may be an oligomer.

In ^{13}C-NMR spectrum, the anomeric carbon appeared at 90.8 ppm, due to α-D-glucose unit and it was linked by C-2' of β-D-fructose which was resonated at 102.4 ppm as quaternary carbon as observed in DEPT 90 and 135[27-30]. There was no other anomeric signals observed; it is indicated that 1 was made up of repeating units of glucopyranosyl and fructofuranosyl moieties present in oligosaccharide.

By ^{1}H-^{1}H COSY spectrum, the cross peaks observed between (H-1) proton of glucose (δ 5.46) to others δ 4.27, δ 3.64 and δ 3.32 proton signals of glucose and between proton (H-1') of fructose to (H-3') δ 3.67 and H-6' to (H-5') δ 3.55. In HMBC spectrum, the cross peaks observed between H-1 of glucose (δ 5.46) and C-2' (δ 102.4) of fructose and between H-4 proton signal of glucose (δ 3.32) to C-3' (δ 71.3) of fructose, indicated that the H-1 and H-4 protons of glucose were involved in glucosidation at C-2' and C-3' of fructose respectively. Further the deshield signals of C-3' of fructose and C-4 of glucose, also suggested that compound 1 was oligosaccharide in which C-3' of fructose and C-4 of glucose involved in glucosidation. Thus the basic skeleton of 1:

[(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(3→4)]-α-D-glucopyranoside

It was also supported by C-2 and C-3 both position of fructose take part in glucosidation in melezitose which was trisaccharide[31]. The NMR data of 1 are shown in TABLE-1. Further the degree of oligomerization of compound 1 was confirmed by mass spectrum.

In the positive ion FAB-MS, molecular ion peak was observed at m/z 1198 [M+2Na$^+$] ascribable the molecular formula as $C_{42}H_{72}O_{36}$.2Na. Fragmentation pattern and analysis of the other abundant ions were indicated that it was an oligosaccharide consisted of seven monosaccharide units of glucose and fructose.

Other significant peaks at m/z995 [M+Na$^+$-162], m/z 833 [M+Na$^+$-162X2], m/z 671 [M+Na$^+$-162X3], m/z 509 [M+Na$^+$-162X4] and 347 [M+Na-162X5] were all are in agreement with the proposed

TABLE 1 : The NMR data of compound 1 (D_2O) and 2 (DMSO) (internal Me_4Si)

	Compound 1		Compound 2	
Atom	δ_H	δ_C	δ_H	δ_C
1	5.46 d (3.6 Hz)	90.8	5.01 d (3.7Hz)	91.9
2	4.27 d	70.9	3.49 d	72.9
3	3.64 ov	71.3	3.66 m	73.0
4	3.32 t (9.0 Hz)	67.9	3.88 t	70.8
5	3.67 m	69.8	3.29 m	71.7
6	3.72 m, 3.56 ov	58.8	3.80 m	60.6
1'	3.96 dd, 3.86dd	61.09	5.14 dd, 4.32dd	62.2
2'		102.4		104.1
3'	3.67 d	71.3		77.2
4'	4.07 m	80.0		82.6
5'	3.55 d	73.0		74.4
6'	4.11 dd, 4.25 dd	60.1	4.74 dd, 4.30dd	62.2

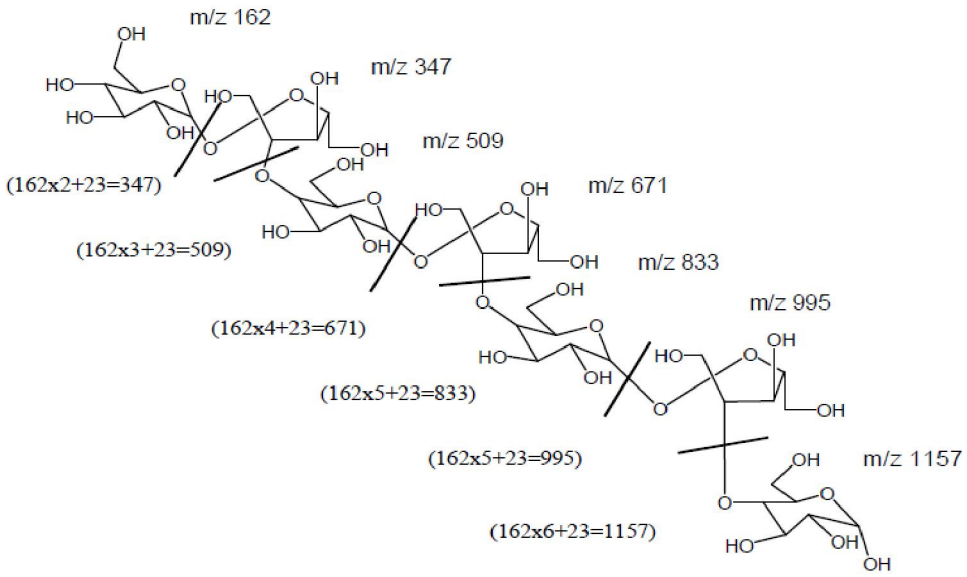

Figure 1 : Fragmentation pattern of 1

structure(Figure 1).

Thus on the basis of spectral techniques FAB-MS, ^{1}H NMR, ^{13}C NMR and DEPT 90, 135, COSY and HMBC compound 1 was to be a heptamer of glucose and fructose and characterized as: tri-[(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(3→4)]-α-D- glucopyranoside.

The IR spectrum of 2 showed characteristic absorptions at (3562, 3335, 2984, 2942, 1475, 1365, 1236, 1104, 1116, 1004 and 942 cm^{-1}) corresponding to sucrose.

A doublet at δ 5.01 was indicated anomeric proton of a sugar. The integration area of the signals between at δ 3.11 to 5.18 showed that sugar was oligosaccharide. In the spectrumonly one anomeric signal was observed, it indicated the presence of only one α-D- glucopyranose unit (pyranoid ring) and other (furanoid ring) was present. The double doublets at δ 5.14, 4.74 and 4.30 indicated that three oxomethylene groups were present.

Rests of the overlapped signals were resonated between δ 3.11 to 3.90due to hydroxyl methine protons.

CMR and DEPT spectra confirmed the presence of 12 carbon signals, indicated that it was made up12 carbons only. Among these three were hydroxyl methylene and one quaternary carbon, eight methine carbon atoms, ascribable disaccharide nature of sugar. The signal at 91.9 ppm was due to anomeric carbon of α-D-glucose linked to the C-2' of β-D-fructose, which was resonated at 104.1 ppm[30]. Other signals of compound were indicated that sucrose is the basic unit, and it was confirmed by literature value also[27-29]. Since C-4'position of fructose (82.6 ppm) and C-4 position of glucose (70.8 ppm) were slightly deshielded, indicating C-4 position of both involved in glucosidation, with different units.

As no other value observed rather than sucrose, it was also indicated symmetrical nature of oligosaccharide. The NMR data of 2 are shown in (TABLE 1).

A quasimolecular ion peak was observed at m/z 1626 in ESI-MS indicated that oligosaccharide was consisting of ten units of glucose and fructose. Other abundant peaks were observed at m/z 163, 343, 532, 685, 865, 1045 and 1392, indicated that repeating units of glucose and fructose were present in 2 (Figure 2)[32].

Thus on the basis of spectral techniques 2 was a decamer of glucose and fructose and it was isolated first time by us and identified as: tetra-[(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(4→4)]-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside.

Mass spectrum and elemental analysis of compound 3 indicated the molecular ion peak at m/z 340 suggesting its molecular formula $C_{23}H_{48}O$.

IR spectrum of 3 showed absorption bands for alcohol group (3430 cm^{-1}) and long chain aliphatic

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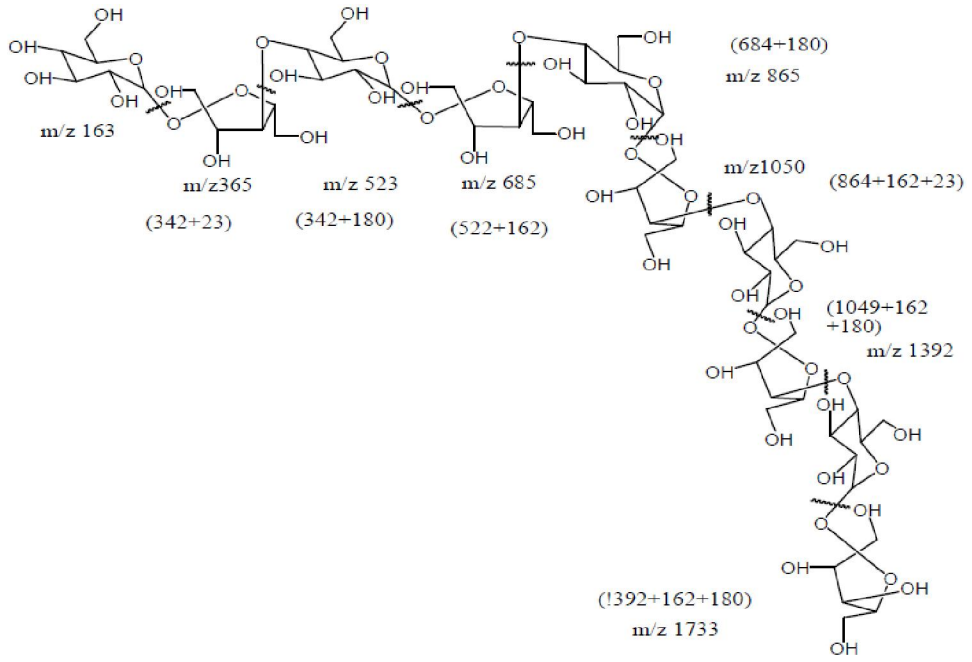

Figure 2 : Fragmentation pattern of 2

nature[33] (1020, 730-720 cm^{-1}). ^1H NMR spectrum showed a triplet and singlet at δ 3.60 (J = 2.4 Hz) and δ 1.6, each for one proton was assigned to carbinolic and hydroxyl group respectively. A triplet for six protons observed at δ 0.93 (J = 6.0 Hz), due to terminal methyl groups and rests of the methylenes were resonated at δ 1.25 as an intense singlet. The ^{13}C NMR spectrum showed the presence of terminal methyl carbon at δ 14.9 ppm. The carbon of alcohol group was resonated at δ 70.0 ppm and rests of the carbons were resonated at 34.2, 32.0, 31.4, 29.7, 29.4, 24.0 and 22.7 ppm[35]. The coupling exhibited in the COSY spectrum between a hydrogen at δ 3.60 (t) connected to the hydroxyl protons at δ 1.60 and δ 1.25 indicated the long chain aliphatic alcohol there was no other substituent present in the molecule. The base peak at m/z 74 was obtained by β- cleavage from the alcohol group and abundant ion was formed due to m/z 267 indicated the position of alcohol group at C-4 position in the molecule.

Most of the peaks were found at the difference of 14 and 28 mass units, which indicated aliphatic nature of the molecule. Hence on the basis of above evidence the compound 3 was characterized as 4-tricosanol and it was reported first time.

Mass spectral analysis gave the molecular formula of compound 4 as $C_{27}H_{54}O$.

IR Spectrum showed absorption bands for carbonyl group (1709 cm^{-1}) and long chain aliphatic nature (730-720 cm^{-1}). ^1H NMR spectrum[33], showed a peak at δ 0.88 as triplet for nine protons due to the terminal methyl groups. The methylene protons were resonated at δ 2.30 and δ 1.60 characteristic of α- and β-position of the carbonyl group. The rest of the methylene protons were merged as a singlet at δ 1.26. The ^{13}C NMR spectrum showed the presence of terminal methyl carbon at 19.9 ppm and carbonyl group at 175 ppm.

The mass spectrum showed aliphatic nature of the molecule[34]. The abundant ion observed at m/z 254 and 212, was due to Mc Lafferty rearrangement confirmed the position of carbonyl group at position C-12. Another abundant ion at m/z 337 indicated the position of methyl group in the side chain at C-22. The other abundant peaks obtained at m/z 253, 227, 195, 166, 145, 55 and 41 were inconsistent with the proposed structure.

Thus on the basis of above evidences the compound 4 was characterized as a 22-methyl-12-hexacosanone.

Mass spectral analysis gave the molecular formula of compound 5 as $C_{16}H_{32}O_2$.

IR Spectrum showed absorption bands for car-

bonyl group (1710 cm^{-1}), alcohol group (3000) and long chain aliphatic nature (730-720 cm^{-1}), indicated that long chain aliphatic carboxylic acid nature of the molecule. ^{1}H NMR spectrum[35], showed a peak at δ 0.87 as triplet for six protons due to the terminal methyl groups. The methylene protons were resonated at δ 2.32 and δ 1.60 characteristic of α- and β-position of the carboxylic group. The rest of the methylene protons were merged as a singlet at δ 1.26. The ^{13}C NMR spectrum showed the terminal methyl carbons were resonated at 14.9 ppm and carboxylic group was resonated at 175.2 ppm. Rests of the carbons on basis of deshielding order were resonated as 34.24, 32.0, 29.5, 29.4, 29.3, 29.2 and 24.99 ppm. The mass spectrum showed characteristic pattern of aliphatic long chain compound. The abundant fragment ion at m/z 60 and 129 revealed that methyl group was present at 6 position and carboxylic group as terminal position. Rests of the peaks were at the difference of m/z 28. Thus on the basis of above evidences the compound is characterized 6- methyl pentadecanoic acid. It is a new compound and is being reported for the first time by us.

On the basis of spectral analysis and reported literature studies suggested that compound 6 and 7 are known; 6 was isolated from alcohol extract while 7 from acetylated fraction of alcohol extract[36]. Alcohol extract shows antimicrobial inhibitory potential against Gram positive bacteria than Gram negative[37, 38]. The results are shown in the (TABLE-2).

TABLE 2: Antimicrobial activity of alcohol extract

Antimicrobial	activity Alcohol extract
Staphylococcus aureus	+++
Solmonella typhae	–
Escherichia coli	–
Bacillus subtilius	++
Sarcina lutea	–
Klebsiella pneumoniae	+
Aspergillus niger	–
Penicillium notatum	+
Alternaria alternate	–
Candida albicans	–
Cunninghamella spp.	–
Aspergillus flavus	-

Disc diameter = 4.0 mm, = No activity, + = 6.8 - 8.0 mm, ++ = 9.0 - 11mm, +++ = 12 - 14mm, ++++ = 16 - 20mm, +++++ = 18 - 14mm, For antibacterial activity: The standards are in the form of sterile Hi-Disc cartridges, each disc containing 10 mcg of the respective drug, For antifungal activity: Amphotericin B was used as standard.

EXPERIMENTAL SECTION

General

NMR spectra were measured on a solution of the glycoside 1 and 2 (25mg) in D_2O 3, 4, 5 (28mg) and 6, 7 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 analysis 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 column chromatography was carried out on silica gel G(60-120Mesh,Merck,India) and TLC using silica gel G (Merck, India) spots were visualized by exposure to iodine vapors or by spraying with H_2SO_4-vanillin solution followed by heating at 105 °C for 5 min. Sugar analysis was performed on paper chromatography by taking Whatman filter paper No. 1 and spraying agent as aniline hydrogen phthalate.

Plant material

The seeds (5 Kg) of *N. sativa* were collected from the local medicinal market of Ujjain city and were identified by the authorities at IEMPS, Vikram University, Ujjain.

Extraction, Isolation and purification

The seeds were shade dried, cleaned, powdered and extracted by n-hexane, benzene, benzene:acetone and ethanol serially each for 72-75 h in soxhlet extractor. From ethanol extract solvent was removed under reduced pressure by rotary film evaporator to obtain a dark brown syrupy residue (450mg). The dried sample was fractionated on normal phase silica gel column, by using gradient elution with different solvent mixtures in their increasing order of polarity. A dried portion of the 40% benzene:MeOH (125mg) elute was subjected to repeated chroma-

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tography on silica gel, using a discontinuous gradient from 1:1 benzene: EtOAc to 1:1 benzene:methanol. Fractions 30-40 (2000mL) of (1:2) benzene: EtOAc and 20-30 (1500 mL) of (1:3) benzene: EtOAc afforded colourless gummy solid in impure form of 1 and 2. They further recrystallized by acetone and methanol. On performing rechromatography of benzene fraction of alcohol extract over silica gel, the hexane:benzene (9:1,v/v) and (7:3,v/v) eluates separated the compound 3 and 4 in pure form respectively.

Benzene fraction of alcohol extract separated by rechromatography over silica gel and afforded two compounds from eluate hexane:benzene (6:4,v/v) and hexane:benzene (1:1,v/v) in pure form designated as 5 and 6 respectively. Benzene fraction of alcohol extract further acetylated and rechromatographed over silica gel and eluate hexane: benzene (2:8, v/v) gave compound 7 in pure form.

Antimicrobial activity

Agar diffusion technique was used for screening of antibacterial and antifungal activity using paper disk method.

Tri-[(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(3→4)]-α-D- glucopyranoside, 1: Colorless gum (60mg): $C_{42}H_{72}O_{36}$.2Na; m/z 1198; elemental analysis %: calc. C 43.7, H 6.29,O 49.9, obs. C 43.5, H 6.0,O 50.0; R_f =0.37 ($CHCl_3$/MeOH/AcOH 9:1:0.5); m.p.280 ^{0}C; IR (Smear on KBr): ν = 3400-3200 cm^{-1} (OH), 2929, 2870, 2833, 1417, 1384, cm^{-1}(C-H), 1279, 1220, 1137 and 1051 cm^{-1} (C-O);^{1}H-NMR (300MHz, D_2O, 25°C, TMS): see TABLE1.ESI-Ms:$[M+2Na]^+$ 1198, 1155, 1139, 1065, 1037, 995, 841, 833, 784, 768, 671, 575, 509, 496, 402, 351, 342, 277, 213, 165,136.

Tetra-[(O-α-D-glucopyranosyl-(1→2)-O-β-D-fructofuranosyl-(4→4)]-α-D-glucopyranosyl-(1→2)- β-D-fructofuranoside, 2: White crystalline (35 mg): $C_{60}H_{102}O_{51}$; m/z 1638; m.p.290 ^{0}C; elemental analysis %: calc. C 43.9, H 6.27,O 49.7, obs. C 43.7, H 6.2,O 51.5; IR ν_{max} (Smear on KBr): ν = 3562, 3335, 1720 cm^{-1}(C=O), 2920, 2848, 1384,1464,1063,730-720 cm^{-1} (C-H); ^{1}H-NMR (300MHz, DMSO, 25°C, TMS): δ = See TABLE 1; ESI-MS: $[M+2Na]^+$ 1638, 1583, 1392, 1052, 1050, 945, 865, 707, 685, 523, 365, 343, 203, 163, 85.

4-tricosanol, 3: White crystalline (34 mg): $C_{23}H_{48}O$; m/z 340; R_f =0.32 (hexane/benzene9:1); m.p.112-114 ^{0}C; IR ν_{max} (Smear on KBr,cm^{-1}): ν = 3430 cm^{-1} (O-H), 2965, 2860,1465,1384,1050 and 730-720 cm^{-1} (C-H); ^{1}H-NMR (300MHz, $CDCl_3$, TMS): δ = 0.93(t, ^3J(H,H)=7.4Hz, 6H;2XCH_2), 3.60ppm (t, 1H;CHOH, J= 2.4 Hz), 1.25ppm (s,40H; 20CH_2), 1.60 (s, 1H,OH); ^{13}C-NMR (75MHz, $CDCl_3$):δ = 70.0, 34.24, 32.00, 31.42, 29.75, 29.43, 24.9, 22.76 and 14.90; MS: (70eV,EI): m/z (%):340(1), 327(10.1), 298(5.3), 285(12.2), 284(8.3), 268(6.1), 267(31.9), 266 (7.0), 255(2.1), 224(2.2), 210(8.1), 168(5.9), 154(13.9), 148(10), 129(12.5), 115(13.1), 112(29.7), 111(15.0), 110(2.4), 109(7.0), 99(11.7), 98(100), 97(22.1), 95(13.2), 84(29.8), 83(12.5), 74(55.5), 57(65.1), 55(48.0), 43(66.2), 41(36.1), 29(13.4).

22-methyl-12- hexacosanone, 4: White amorphous(35 mg):$C_{27}H_{54}O$; m/z394; m.p.154-155 ^{o}C; R_f = 0.49 (benzene/ether/acetic acid, 9:1:1); IR ν_{max} (Smear on KBr): ν = 1709 cm^{-1} (C=O), 2927, 2855, 1465, 1215, 1410, 758 and 722 cm^{-1} (C-H) ; ^{1}H-NMR (200MHz, $CDCl_3$, TMS):δ = 0.88(t, ^3J(H,H) =6.0Hz, 9H;3XCH_3), 1.26 ppm (s, 36H, 18X CH_2), 1.60 (br s, 5H; 2XCH_2, CH), 2.30 ppm (t, 4H;2XCH_2); MS: (70eV,EI): m/z (%): 394(3.9), 393(3.6), 392(4.0), 350(2.1), 349(2.0), 254(17.1), 212(6.5), 198(2.3), 184(5.6), 170(4.9), 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(43.1), 83(23.7), 82(10.8), 73(37.8), 72(31.1), 71(73.1), 56(100) and 43(60.6).

6-methyl pentadecanoic acid, 5: White crystalline (40 mg): $C_{16}H_{32}O_2$; m/z256; m.p.69-70 ^{0}C; TLC (benzene/ether, 9:1); IR ν_{max} (Smear on KBr): ν = 3000, 1710 cm^{-1}(COOH), 2929, 2848, 1382, 720 cm^{-1}(C-H); ^{1}H-NMR (200MHz, $CDCl_3$, TMS):δ = 0.87(t, ^3J (H,H)=7.1Hz,6H; 2XCH_3), 1.25(s, 22H; 11X CH_2), 1.60 (br s, 2H; β-CH_2), 2.32(t, 2H;CH_2COOH), 10.2 (br s, 1H; COOH); ^{13}C-NMR (75MHz, $CDCl_3$):δ = 180.7, 34.17, 32.02, 29.77, 29.44, 29.33, 29.16, 24.79, 22.77, 16.4, 14.18; MS: (70eV,EI): m/z (%): 256(30.3), 213(19.7), 199(8.9), 185(20.1), 179(19.1), 157(22.5), 129(51.3), 97(23.4), 89(22.4), 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).

4-nonadecanone,6: White amorphous(30 mg):$C_{19}H_{38}O$; m/z282; m.p.51-52 ^oC; R_f= 0.39 (benzene/ether/acetic acid, 9:1:1); IR ν_{max} (Smear on KBr): ν = 1709 cm^{-1} (C=O), 2928, 2855, 1466, 1215, 1410, 758 and 722 cm^{-1} (C-H) ; ^{1}H-NMR (200MHz, $CDCl_3$, TMS):δ = 0.86(t, ^3J(H,H) =6.0Hz, 6H;2XCH_3), 1.26 ppm (s, 24H, 12X CH_2), 1.60 (br s, 4H; 2XCH_2), 2.34 ppm (t, 4H;2XCH_2); MS:(70eV,EI):m/z(%):282(21.3), 281(20.4), 265(2.6), 264(11.1), 254(80.1), 253(62.3), 237(40.1), 212(6.5), 198(2.3), 184(5.6), 169(15.9), 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(43.1), 83(23.7), 82(10.8), 73(37.8), 72(31.1), 71(73.1), 56(100) and 42(99.0).

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