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Chemical and biological study of *Mentha suaveolens* Ehrh. cultivated in Egypt

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Investigation of the different fractions of the ethanolic extract of the aerial parts of *M. suaveolens* growing in Egypt yielded nine compounds; two new triterpenes [3*β*-acetyl -22α-hydroxy ursa-12,20-diene (compound 1) and 2*α*, 3*β*-dihydroxy-olean-18-en-29-oic acid (compound 7)] and nine known compounds: a sterol and its glucoside; *β*-sitosterol (compound 2) and *β*-sitosterol-3-O- β-D-glucoside (compound 4), a triterpene; oleanolic acid (compound 3), two monoterepenes; dihydrolimonene (compound 5) and 7-hydroxy-*p*-cymene (compound 6), two flavonoids; isoquercitrin (compound 8) and rutin (compound 9) which were isolated from *M. suaveolens* Ehrh. for the first time. The structures of the isolated compounds were identified by spectral data (UV, MS, 1D and 2D-NMR) and comparison with authentic samples. Moreover, the ethanolic extract showed potent analgesic activity as compared to indomethacin. The ethyl acetate fraction was the most potent as anti-inflammatory (88%), followed by the ethanolic extract (82.9%) as compared with indomethacin. The ethanolic extract and its four subfractions showed a moderate inhibitory activity against the tested human pathogenic bacteria.

Key words: *Mentha suaveolens*, anti-inflammatory, analgesic, flavonoids, sterols, triterpenes.

INTRODUCTION

The genus *Mentha*, one of the important members of the Lamiaceae family, is represented by about 19 species and 13 natural hybrids. *Mentha suaveolens* Ehrh. is native of Africa, temperate Asia and Europe (Abbaszadeh et al., 2009).

M. suaveolens has been used in the traditional medicine of Mediterranean areas and has a wide range of effects: tonic, stimulating, stomachic, carminative, analgesic, choleretic, antispasmodic, sedative, hypotensive and insecticidal. It shows depressor activity, analgesic and anti-inflammatory activities (Moreno et al., 2002). On reviewing the current literature on *M. suaveolens,* flavonoids were the major constituents isolated from this species (Tomas-Barberan et al., 1998; Zaidi et al., 1998). Concerning the biological activities, it was found that *M. suaveolens* has antihypertensive (Bello et al., 2001), antioxidant and acetylcholinesterase inhibitory activities (Ferreira et al., 2006) and a monoamine oxidase inhibitory activity (López et al., 2010). Also, the essential oil of *M. suaveolens* was found to have a candidacidal activity

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(Donatella et al., 2011). Moreover, in a previous publication, the authors proved that the essential oil of the aerial parts of *M. suaveolens* has analgesic, anti-inflammatory, antioxidant, cytotoxic, hepatoprotective, antioxidant and antifungal activities (El-Kashoury et al., 2012).

The aim of this study was to isolate and identify the major constituents of the aerial parts of *M. suaveolens* Ehrh. cultivated in Egypt and to investigate the possibility of introducing it as a new medicinal plant after screening for some of its biological activities such as analgesic, anti-inflammatory and antimicrobial activities.

MATERIALS AND METHODS

Chemicals

Sterols, triterpenes, phenolics and sugars used as references in co-chromatography (PC, TLC and HPLC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Authentic reference samples used in GLC analyses of the unsaponifiable matters (USM) and those of fatty acids methyl esters (FAME) were provided by the central laboratory at the Faculty of Agriculture, Cairo University. Carrageenan and ascorbic acid were purchased from Sigma, St. Louis, MO, USA. Indomethacin was purchased from EIPICO, Pharmaceutical Co., 6 October City, Egypt. Tetracycline was purchased from Sedico Pharmaceutical Co., 6 October City, Egypt. Diaion HP-20 AG for column chromatography was purchased from 75 to 150 µ, Mitsubishi Chemical Industries Co. Ltd. Silica gel H for vacuum liquid chromatography (VLC) was purchased from E-Merck (Darmstadt, Germany). Silica gel 60 and silica gel RP-18 (70-230 mesh) for column chromatography were obtained from Fluka. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Thin-layer chromatography (TLC) was performed on silica gel GF_{254} and silica gel RP-18 precoated plates (Fluka, Germany). The chromatograms were visualized under UV light (at 254 and 366 nm) before and after exposure to ammonia vapor, as well as spraying with *p*-anisaldehyde-sulfuric acid or natural products-polyethylene glycol (NP/PEG) spray reagents.

Apparatus and equipment

UV spectra were measured using a Shimadzu UV 240 (P/N 204-58000) spectrophotometer (Kyoto, Japan). Mass spectra were measured using Shimadzu QP-2010 Plus (Kyoto, Japan). NMR spectra were recorded at 300 (^{1}H) and 75 MHz (^{13}C) on a Varian Mercury-300 instrument (Palo Alto, CA, USA). The NMR spectra were recorded in $CDCl_3$ or DMSO-d_6, and chemical shifts were given in δ (ppm) relative to trimethylsulphoxide (TMS) as internal standard. Electrothermal 9100 (Labequip, Markham, Ontario, Canada) was used for the determination of melting points (mp) (uncorrected). Hewlett-Packard HP 6890 N network GC system equipped with an MSD detector was used for analysis of unsaponifiable matters. Pye Unicam 304 series GC equipped with a dual flame ionization detector and a dual channel recorder was used for analysis of fatty acid methyl esters. The unsaponifiable matter of the *n*-hexane fraction was analyzed on TR-5-MS column (5% phenyl-polysil phenylene siloxane, 30 m × 0.25 mm D × 0.25 µm film thickness), injector temperature 270°C, the initial temperature was 70°C, kept isothermal for 5 min, increased to 280°C by the rate of 4°C min^{-1}, then kept isothermal for 10 min, using helium as a carrier gas at a flow rate 1 ml/min and MSD detector. The fatty acid methyl esters were analyzed on a coiled glass column (1.5 m × 4 mm D) packed with diatomite (100 to 120 mesh) and coated with 10% polyethylene glycol adipate (PEGA), the injector temperature was 250°C. Initial temperature, 70°C increased to 190°C by the rate of 8°C min^{-1}, then kept isothermal for 25 min, using nitrogen as a carrier gas at a flow rate 30 ml/min and FID detector.

Plant

The fresh aerial parts of *M. suaveolens* Ehrh. cultivated in Egypt were collected during the years 2009 to 2011 from plants cultivated in the Experimental Station of Medicinal and Aromatic Plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza, Egypt. The plant was kindly authenticated by Dr. Gemma L. C. Bramley, curator of the Lamiaceae collections, Herbarium Department, Library, Art and Archives, Royal Botanic Gardens, Kew, Richmond, Surrey, United Kingdom. Voucher specimen (M-20/313) was kept at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University. The plants were left to dry under normal air at ambient temperature (mean temperature, 28°C) in a dark, well-ventilated room for 4 to 5 days, reduced to powder and then subjected to extraction.

Preparation of extracts and fractions

The air-dried powdered aerial parts of *M. suaveolens* Ehrh. (1.5 kg) were exhaustively extracted with 90% ethanol by cold maceration. The total extract was evaporated under reduced pressure to yield a brownish green semi-solid residue (407 g). The residue (400 g) was suspended in water (800 ml) and successively subjected to liquid-liquid fractionation with *n*-hexane (8 × 400 ml), chloroform (10 × 400 ml), ethyl acetate (8 × 400 ml) and *n*-butanol saturated with water (8 × 400 ml). The solvents were evaporated under reduced pressure, yielding 14.0, 7.0, 11.4 and 54.0 g from the *n*-hexane, chloroform, ethyl acetate and *n*-butanol, respectively.

Isolation of the major constituents

Spots from the essential oil were neglected as it was studied separately. *n*-Hexane fraction: 10 g of the *n*-hexane fraction were chromatographed over a VLC column (Silica gel H, 150 g, 13 cm L × 7 cm D). Gradient elution was carried out using *n*-hexane, *n*-hexane/chloroform mixtures, chloroform, chloroform/ethyl acetate mixtures and ethyl acetate. Fractions (200 ml, each) were collected and monitored by TLC (precoated silica gel plates) to yield 3 main subfractions (A to C). Subfraction A (eluted with 100% *n*-hexane, 25% chloroform in *n*-hexane, 0.86 g) was rechromatographed over a silica gel 60 column using *n*-hexane as eluent to give compound 1 (60 mg). Subfraction B (eluted with 70-95% chloroform in *n*-hexane, 1.3 g) was rechromatographed over a silica gel 60 column using *n*-hexane/ethyl acetate mixture (95:5 v/v) as eluent to give compound 2 (17 mg) and compound 3 (85 mg). Subfraction C (eluted with 50 to 95% ethyl acetate in chloroform, 2 g) was rechromatographed over a silica gel 60 column using chloroform/methanol mixture (97:3 v/v) as eluent to give compound 4 (45 mg).

Chloroform fraction: 5 g were chromatographed over a VLC column (Silica gel H, 100 g, 9 cm L × 7 cm D) in the same manner as the *n*-hexane fraction. The collected fractions were monitored by TLC (precoated silica gel plates) to yield 2 main subfractions (D and E). Subfraction D (eluted with 100% *n*-hexane -45% chloroform in *n*-hexane, 2.2 g) was rechromatographed over a silica gel 60 column using *n*-hexane/ethyl acetate mixture (95:5 v/v) as eluent to give compound 5 (20 mg) and compound 6 (61 mg). Subfraction E(eluted with 15% ethyl acetate in chloroform -100% ethyl acetate, 1.5 g) was rechromatographed over a silica gel 60 column using n-

hexane/ethyl acetate mixture (80:20 v/v) as an eluent to give compound 7 (42 mg).

n-Butanol fraction: 15 g were fractionated over a VLC column (Silica gel H, 300 g, 12 cm L × 7 cm D). Gradient elution was carried out using chloroform, chloroform/ethyl acetate mixtures, ethyl acetate, ethyl acetate/methanol mixtures and methanol. Fractions (200 ml, each) were collected and monitored by TLC (precoated silica gel plates) to yield 2 main subfractions (F to G). Subfraction F (eluted with 100% ethyl acetate till 30% methanol in ethyl acetate, 0.5 g) was rechromatographed over a RP-18 column using water as eluent to give compound 8 (40 mg). Subfraction G (eluted with 40% methanol in ethyl acetate till 100% methanol, 5 g) was rechromatographed over a Diaion column using water/methanol mixtures as eluent. A semi-purified subfraction was obtained. Further purification on a Sephadex LH-20 column using methanol as eluent, it gave compound 9 (40 mg). Purification of the ethyl acetate fraction is under publication in another article (Figure 1).

Compound 1

Oily yellow liquid, R_f value (0.83, chloroform-methanol 98:2 v/v); color in *p*-anisaldehyde/H_2SO_4 (purple). EI-MS (70 ev, relative intensity, m/z): m/z at 482 (12.5%), 467 (27.5%), 422 (39.11%), 232 (5.32%), 250 (10.04%) 218 (100%), 207 (5.1%), 204 (15.08%), 203 (14%) and 189 (37.61%). ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.80 (3H, s, Me-28), 0.83 (3H, s, Me-25), 0.85 (3H, d, J= 6.9 Hz, Me-29), 0.87 (3H, s, Me-23), 0.98 (3H, s, Me-24), 1.01 (3H, s, Me-26), 1.07 (3H, s, Me-27), 1.68 (3H, s, Me-30), 1.99 (1H, m, H-18), 2.28 (3H, s, CH_3COO), 4.57 (1H, s, H-3), 4.59 (1H, s, H-22), 5.12 (1H, br. s, H-12), 5.33 (1H, m, H-21) ppm.^{13} C-NMR: δ (75 MHz, $CDCl_3$) 16.2 (C-24), 16.7 (C-25), 16.7 (C-26), 18.1 (C-6), 21.3 (C-32), 22.6 (C-11), 23.2 (C-29), 23.5 (C-30), 24.7 (C-27), 25.0 (C-15), 27.9 (C-2), 29.3 (C-23), 29.4 (C-28), 34.2 (C-7), 34.7 (C-17), 36.6 (C-16), 37.2 (C-10), 37.3 (C-4), 38.3 (C-1), 39.9 (C-19), 41.4 (C-8), 41.9 (C-14), 47.5 (C-9), 55.1 (C-5), 58.9 (C-18), 61.0 (C-22), 80.4 (C-3), 118.1 (C-21), 124.1 (C-12), 139.4 (C-13), 142.3 (C-20), 173.7 (C-31).

Compound 2

White needle crystals, m.p. 140 to 141°C, R_f value (0.62, Chloroform-Methanol 98:2 v/v); color in *p*-anisaldehyde/H_2SO_4 (violet). EI-MS (70 ev, relative intensity), m/z at 414 $[M]^+$ (100%), 396 (51.2%), 329 (41.8%), 303 (43.7%), 273 (59.6%) and 255 (82.5%). ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.72 (3H, d, J=5.4, Me-21), 0.86 (3H, t, J=6.3, Me-29), 0.91 (3H, d, J=6.3, Me-26), 0.95 (3H,d, J=6.3, Me-27), 1.04 (3H, s, Me-18), 1.56 (3H, s, Me-19), 3.52 (1H, m, H-3), 5.38 (1H, br.s., H-6) ppm.

Compound 3

White amorphous powder, R_f value (0.56, chloroform-methanol 98:2 v/v); color in *p*-anisaldehyde/H_2SO_4 (purple). EI-MS (70 ev, relative intensity, m/z): m/z at 456 $[M]^+$(1.59 %), 411 (0.33%), 438 (2.51%), 248 (100%). 208 (4.80%), 203 (53.14%) and 190 (11.37%). ^{1}H-NMR: δ (300 MHz, DMSO) 0.67 (3H, s, Me-25), 0.71 (3H, s, Me-30), 0.85 (3H, s, Me-29), 0.87 (3H, s, Me-24), 0.89 (3H, s, Me-27), 1.09 (3H, s, Me-26), 1.23 (3H, s, Me-23), 3.00 (1H, m, H-18), 4.25 (1H, br.d, H-3) and 5.16 (1H, br.s, H-12) ppm. ^{13} C-NMR: δ (75 MHz, DMSO) 15.1 (C-25), 16.0 (C-24), 16.9 (C-26), 18.0 (C-6), 22.6 (C-30), 22.9 (C-11), 23.4 (C-16), 25.6 (C-27), 27.2 (C-2), 28.2 (C-15), 29.0 (C-23), 30.4 (C-20), 32.1 (C-21), 32.4 (C-22), 32.8 (C-29), 33.3 (C-7), 36.6 (C-10), 38.4 (C-1), 38.7 (C-4), 40.4 (C-8), 40.8 (C-14), 41.3 (C-18), 45.5 (C-19), 45.7 (C-17), 47.1 (C-9), 54.8 (C-5), 76.8 (C-3), 121.5 (C-12), 143.8 (C-13), and 178.5 (C-28).

Compound 4

White amorphous powder, R_f value (0.21, chloroform-methanol-formic acid 95:5:0.2 v/v); color in *p*-anisaldehyde/H_2SO_4 (violet). EI-MS (70 ev, relative intensity), m/z at 415 (8.71%), 414 (26.74%), 399 (9.83%), 396 (14.45%), 381 (8.36%), 329 (12.76%), 303 (11.04%), 275 (4.91%), 273 (11.97%), 255 (20.20%), 246 (3.77%), 231 (12.73%), 229 (7.43), 218 (12.48%), 213 (18.60%), 57 (100%). ^{1}H-NMR: δ (300 MHz, DMSO) 0.66 (3H, d, J=5.5 Hz, Me-21), 0.78 (3H, t, J=6.3,Me-29), 0.83 (3H, d, J=6.2 Hz, Me-26), 0.90 (3H, d, J=6.3 Hz, Me-27), 0.92 (3H, s, Me-18), 0.96 (3H, s, Me-19), 3.03 (1H, m, H-3), 4.21 (1H, d, J=7.5, H-1`), 5.33 (H, br.s, H-6) ppm.

Compound 5

Colorless oily liquid, R_f value (0.57, chloroform-methanol 95:5 v/v); color in *p*-anisaldehyde/H_2SO_4 (violet). EI-MS (70 ev, relative intensity, m/z): 138 (3.62%), 125 (21.76%), 123 (5.94%), 111 (42.96%), 97 (69.49%), 95 (14.98%), 83 (64.42%), 80 (2.49%), 70 (33.74%), 68 (12.67%), 67 (20%), 57 (100%), 55 (80.01%) and 54 (10.46%). ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.87 (3H, br.s, Me-10), 0.89 (3H, br.s, Me-9), 1.56 (1H, br.s, H-5a), 1.73 (H, br.s, H-5b), 2.02 (3H, s, Me-7), 2.04 (2H, br.s, H-6), 2.06 (1H, br.s, H-4), 2.08 (2H, br.s, H-3), 2.29 (1H, br.s, H-8), 5.81 (1H, m, H-2). ^{13} C-NMR: δ (75 MHz, $CDCl_3$) 22.7 (C -9,10), 28.9 (C-7), 29.7 (C-5), 30.0 (C-3), 30.1 (C-6), 31.9 (C -8), 33.8 (C-4), 114.0 (C -2), 139.2 (C-1).

Compound 6

Oily yellow liquid, R_f value (0.46, chloroform-methanol 95:5 v/v); color in *p*-anisaldehyde/H_2SO_4 (violet). ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.89 (3H, d, J= 7.8, Me-10), 0.92 (3H, d, J= 7.8, Me-9), 1.32 (1H, m, H-8), 4.20 (2H, s, H-7), 7.53 (2H, d, J=8.4 Hz, H-2,6), 7.69 (2H, d, J=8.4 Hz, H-3,5) ppm. ^{13} C-NMR: δ (75 MHz, $CDCl_3$) 22.9 (C -10), 23.6 (C -9), 38.6 (C-8), 68.0 (C-7), 128.6 (C-3,5), 130.7 (C -2,6), 132.2 (C -1), 139.0 (C-4).

Compound 7

White powder, R_f value (0.23, chloroform-methanol 95:5 v/v); color in *p*-anisaldehyde/H_2SO_4 (purple). EI-MS (70 ev, rel. int., m/z): 472 (5.73%), 457 (73.4%), 395 (1.46%), 248 (100%), 218 (12.3%), 203 (49.3%), 189 (22.3%). ^{1}H-NMR: δ (300 MHz, DMSO) 0.76 (3H, s, Me-27), 0.80 (3H, s, Me-23), 0.82 (3H, s, Me-24), 0.875 (3H, s, Me-25), 0.90 (3H, s, Me-26), 1.04 (3H, s, Me-28), 1.24 (3H, s, Me-30), 3.00 (1H, m, H-2), 4.20 (1H, m, H-3), 5.14 (1H,br.s, H-19) ppm.^{13} C-NMR: δ (75 MHz, DMSO) 18.9 (C -26),19.8 (C -24), 21.9 (C-25), 27.4 (C -30), 26.3(C-28), 25.5 (C -27), 28.0 (C -23), 33.2 (C-22), 32.3 (C-21), 24.6 (C-11), 20.1 (C-6), 26.4 (C-12), 30.5 (C-15), 36.4 (C-16), 37.7 (C-17), 35.3 (C-10), 36.9 (C-7), 40.4 (C-8), 39.0 (C-4), 56.4 (C-20), 49.7 (C-13), 51.0 (C-14), 38.4 (C-1), 54.2 (C-9), 56.2 (C-5), 64.2 (C-2), 86.2 (C-3), 134.0 (C-19), 147.6 (C-18), 187.7 (C -29).

Compound 8

Yellow powder, R_f value (0.58, in ethyl acetate- formic acid -glacial acetic acid- water 100:11:11:10 v/v), Color of the spot in UV (yellow) and in NP-PEG/ UV (orange-yellow). ^{1}H-NMR: δ (300 MHz, DMSO) 4.12 (1H,d, J= 6.9 Hz, H-2``), 5.43 (1H,d, J= 7.5 Hz, H-1``),6.19 (1H, d, J=1.8, H-6), 6.39 (1H, d, J=2.1, H-8), 6.82 (1H, d, J=9 Hz, H-5`), 7.13 (1H, br.s., H-2`), 7.56 (1H, dd, J=2.7,8.1 Hz, H-6`).UV (nm): MeOH 257, 273sh, 365 (3-OH substituted flavonol) NaOMe 279, 329, 410 (free OH on ring A and B) $AlCl_3$ 279, 303sh,

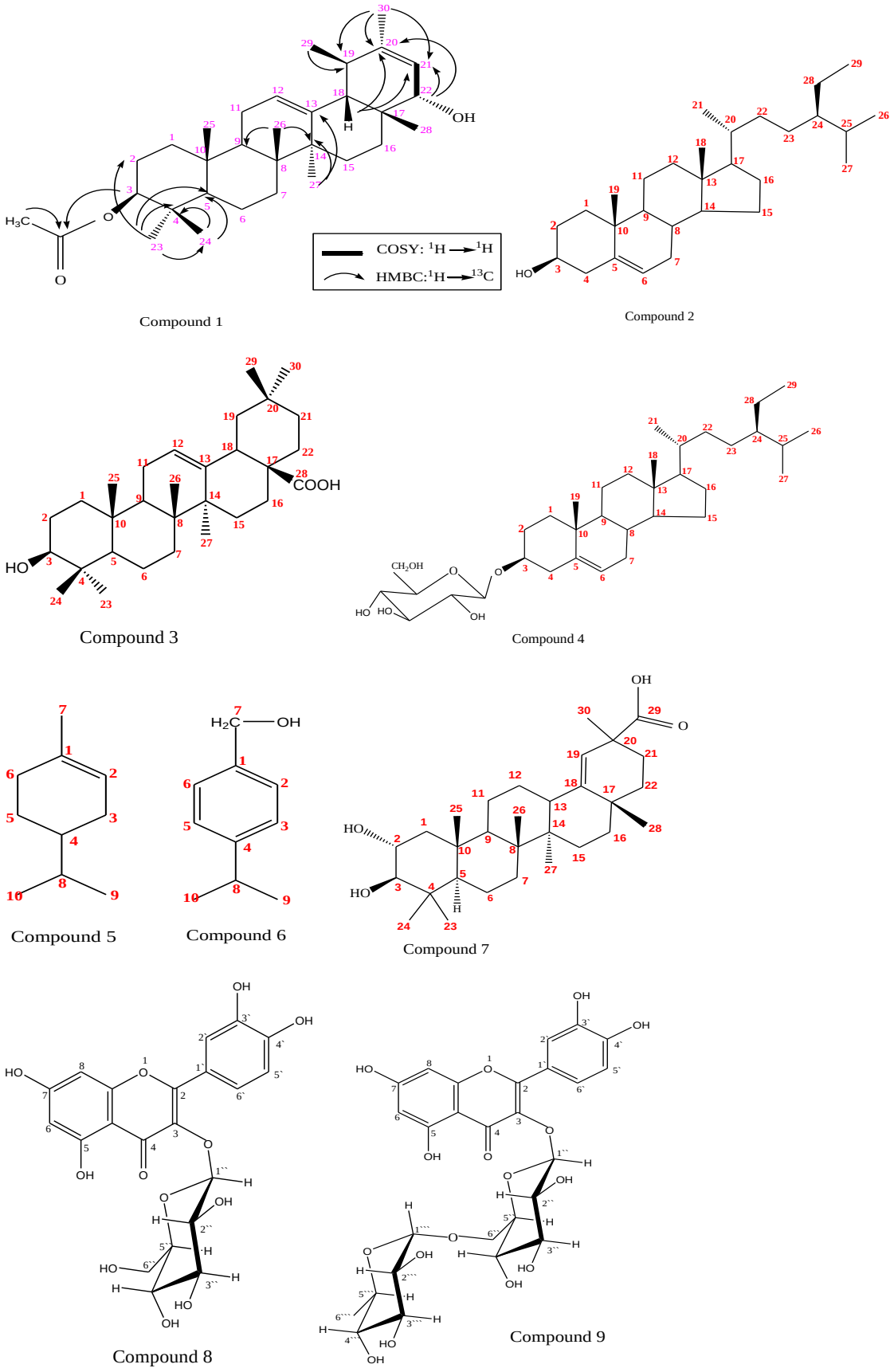

Figure 1. Structures of the isolated compound.

Table 1. Components identified by GLC analysis of the USM of the *n*-hexane fraction of *M. suaveolens* Ehrh.

Peak	RR_t* (min)	Identified components	Number of carbons	Relative percentage
1	0.810	*n*-Dodecane	C_{12}	9.70
2	0823	*n*-Tridecane	C_{13}	7.49
3	0.826	*n*-Tetradecane	C_{14}	2.61
4	0.839	*n*-Pentadecane	C_{15}	4.66
5	0.867	*n*-Hexadecane	C_{16}	4.80
6	0.873	*n*-Heptadecane	C_{17}	3.16
7	0.889	*n*-Octadecane	C_{18}	1.08
8	0.922	*n*-Nonadecane	C_{19}	2.86
9	0.936	*n*-Eicosane	C_{20}	2.65
10	0.991	*n*-Heneicosane	C_{21}	3.50
11	1	*n*-Docosane	C_{22}	13.76
12	1.015	*n*-Tricosane	C_{23}	7.87
13	1.028	*n*-Tetracosane	C_{24}	3.22
14	1.282	*n*-Pentacosane	C_{25}	3.64
15	1.286	*n*-Hexacosane	C_{26}	2.27
16	1.291	*n*-Heptacosane	C_{27}	3.98
17	1.294	*n*-Octacosane	C_{28}	1.21
18	1.295	*n*-Nonacosane	C_{29}	2.24
19	1.299	*n*-Triacontane	C_{30}	1.03
20	1.472	*n*-Hentriacontane	C_{31}	0.73
21	1.623	*n*-Dotriacontane	C_{32}	0.99
22	1.680	Stigmasterol	C_{27}	1.90
23	1.705	β-Sitosterol	C_{27}	5.61
% Total identified components				90.96
% Identified hydrocarbons				83.45
% Identified sterols				7.51

*Retention time relative to *n*-Docosane (R_t = 38.21 min).

435 (free OH on ring A and B) $AlCl_3$/HCl 276, 365sh, 408 (free OH at C5) CH_3COONa 278, 328sh, 398 (free OH at C7 and ring B) CH_3COONa/H_3BO_3 267, 291sh, 390 (ortho OH at ring B).

Compound 9

Pale buff powder, R_f value (0.4, ethyl acetate- formic acid -glacial acetic acid- water 100:11:11:10 v/v), Color of the spot in UV (brown) and in NP-PEG/ UV (orange). ^{1}H-NMR: δ (300 MHz, DMSO) Aglycone: 6.43 (1H, br.s, H-6), 6.69 (1H, br.s, H-8), 6.83 (1H, d, J=8.4 Hz, H-5'), 7.38 (1H, br.s, H-2'), 7.42 (1H, dd, J=1.2, 8.4, H-6'). Sugar: 1.02 (3H, d, J =6.3Hz, Me-6```), 4.54 (1H, br.s, H-1'''), 5.04 (1H, d, J=7.8Hz, H-1''). UV (nm): in CH_3OH 258, 300sh, 358 (flavonol) CH_3ONa 268, 328sh, 410(free OH on ring A & B) $AlCl_3$ 270, 306sh, 426 (free OH on ring A & B) AlCl3/HCl 268,298sh, 366, 400 (free OH at 5 and ortho OH at ring B) CH_3COONa 264, 300sh, 382 (free OH at 7 & ortho OH at ring B) CH_3COONa/H_3BO_3 262, 308sh, 378 (ortho OH at ring B).

GLC analysis of the lipoidal matter

The saponifiable and unsaponifiable fractions were obtained from the *n*-hexane fraction (1 g) and the separated fatty acids were methylated by adopting the method described by Vogel (1975). Each of the unsaponifiable matter and fatty acid methyl esters was subjected to GLC analysis. Components were identified by GLC analysis and shown in Table 1 and 2.

Biological evaluation

Animals

Adult male rats of Sprague-Dawley strain [130 to 150 g body weight] and Swiss male Albino mice (20 to 25 g) were obtained from the animal house of the National Research Centre, Giza, Egypt. They were kept under the same hygienic conditions and were fed by the basal diet recommended by the American Institute of Nutrition (AIN, 1977). All experimental procedures were performed in accordance with internationally accepted principles for laboratory animal use and were approved by the Ethics Committee of the National Research Centre (No. 9-031).

Analgesic activity

Analgesic activity of the oral administration of the ethanolic extract and its subfractions (*n*-hexane, choloform, ethyl acetate and *n*-butanol) were evaluated using acetic acid induced writhing test in mice (Koster et al., 1959) using indomethacin as a standard drug.

Table 2. Components identified by GLC analysis of the FAME of the *n*-hexane fraction of *M. suaveolens* Ehrh.

Peak	R_t* (min)	Fatty acids corresponding to identified FAME	Relative percentage
1	0.72	Lauric acid (C_{12})	0.84
2	0.896	Myristic acid (C_{14})	2.43
3	1	Palmitic acid (C_{16})	45.27
4	1.039	Palmitoleic acid ($C_{16:1}$)	11.21
5	1.246	Stearic acid (C_{18})	5.67
6	1.284	Oleic acid ($C_{18:1}$)	5.30
7	1.361	Linoleic acid ($C_{18:2}$)	7.86
8	1.459	Linolenic acid ($C_{18:3}$)	19.26
% Total identified components			97.84
% Saturated fatty acids			54.21
% Unsaturated fatty acids			43.63

*Retention time relative to palmitic acid (R_t = 12.833 min).

Table 3. Analgesic activity of *M. suaveolens* Ehrh. ethanolic extract and its fractions using acetic acid induced writhing test.

Animal group	Body weight (mg/kg)	Number of writhes (Mean ± SE)	Inhibition as compared to the control (%)	Potency as compared to the standard (%)
Control	1 ml saline	46.8±1.2	00.0	00.0
Ethanolic extract	100	24.9±0.7*	46.8	78.5
n-Hexane fraction	100	36.5±1.4	22.0	36.9
Choloform fraction	100	39.7±1.3	15.0	25.2
Ethyl acetate	100	28.3±0.6*	39.5	66.3
n-Butanol fraction	100	31.2±1.1*	32.7	54.7
Indomethacin	20	18.9±0.3*	59.6	100.0

*Statistically significant difference from zero time at P<0.01, n=6.

Table 4. Acute anti-inflammatory activity of *M. suaveolens* Ehrh. ethanolic extract and its fractions using carrageenan-induced rat paw oedema test

Animal group (Dose)	Body weight (mg/kg)	Percentage of oedema (Mean ± SE)	Inhibition as compared to the control (%)	Potency as compared to the standard (%)
Control	1 ml saline	59.7± 1.8	0	0
Ethanolic extract	100	28.7± 0.6*	51.9	82.9
n-Hexane fraction	100	48.6± 1.7	18.6	29.7
Choloform fraction	100	47.5± 1.2	20.4	32.6
Ethyl acetate	100	26.8± 0.6*	55.1	88.0
n-Butanol fraction	100	36.3± 1.4*	39.2	62.6
Indomethacin	20	22.3± 0.4*	62.6	100.0

*Statistically significant difference from zero time at P<0.01, n=6.

The number of writhes was counted over a period of 30 min after acetic acid injection. Results are as shown in Table 3.

Acute anti-inflammatory activity

Acute anti-inflammatory activity of the oral administration of the ethanolic extract and its subfractions were evaluated and compared with that of indomethacin as a standard drug using carrageenan-induced rat paw oedema test (Winter et al., 1962). The paw oedema was measured 3 h after injection of carrageenan. Results are recorded as shown in Table 4.

Agar disc diffusion method

The antimicrobial screening of the ethanolic extract and its sub-fractions were performed by the agar disc diffusion method (Bauer

Table 5. Antimicrobial activity of *M. suaveolens* Ehrh. ethanolic extract and its fractions using agar disc diffusion method.

Tested microorganism	Diameter of inhibition zone in mm (percentage of efficiency)					
	Ethanolic extract	*n*-Hexane fraction	Chloroform fraction	Ethyl acetate fraction	*n*-Butanol fraction	Tetracycline
Gram-positive bacteria *Staphylococcus aureus* (ATCC12600)	12 (43)	12 (43)	12 (43)	11 (39)	10 (36)	28 (100)
Streptococcus faecalis (ATCC19433)	12 (39)	13 (42)	15 (48)	11 (35)	11 (35)	31 (100)
Bacillus subtilis (ATCC6051)	10 (31)	13 (41)	13 (41)	13 (41)	10 (31)	32 (100)
Gram-negative bacteria *Escherichia coli* (ATCC11775)	12 (40)	13 (43)	12 (40)	12 (40)	10 (33)	30 (100)
Neisseria gonorrhoeae (ATCC19424)	12 (35)	10 (29)	12 (35)	12 (35)	10 (29)	34 (100)
Pseudomonas aeruginosa (ATCC10145)	12 (39)	11 (35)	13 (42)	12 (39)	10 (32)	31 (100)

et al., 1966). Standard discs of tetracycline (5 µg/disc) served as a postive control. The test samples were dissolved in DMSO at a concentration of 20 mg/ml. Aliquots of 50 µl (equivalent to 1 mg of the tested extracts) were aseptically added to the cups of the inoculated plates. Results are shown in Table 5.

Statistical analysis

The data obtained were presented as mean ± standard error and the significance of difference between test and control groups was statistically analyzed using student's t-test. P values of 0.05 or less was considered as criteria for significance.

RESULTS AND DISCUSSION

The EI-MS spectrum of compound 1 showed a molecular ion peak at m/z 482 calculated for the molecular formula $C_{32}H_{50}O_3$. In addition to the peak at m/z 467 $[M\text{-}CH_3]^+$. A prominent peak appeared at m/z 422 which is the characteristic for $[M\text{-}CH_3COOH]^+$. The EI-MS spectrum of this compound was similar to pentacyclic triterpenes of the ursane series in which ring C is unsaturated at C_{12} (Goad and Akihisa, 1997). The retro-Diels-Alder fragmentation generated two fragments, a (at m/z 232) and b (at m/z 250). The fragment at m/z 232 gives the base peak at m/z of 218 (by the loss of methylene group). In addition to peaks at m/z of 204 and 189 which are characteristic to pentacyclic triterpenes of the ursane nucleus (Shiojima et al., 1992). The ^{1}H-NMR spectrum showed signals for eight methyl groups, seven of which were positioned at quaternary carbons corres-ponding to the singlets at δ 1.68 ppm (CH_3-30), 1.07 ppm (CH_3-27), 1.01 ppm (CH_3-26), 0.98 ppm (CH_3-24), 0.87 ppm (CH_3-23), 0.83 ppm (CH_3-25) and 0.80 ppm (CH_3-28). The CH_3-29 methyl appeared as three-proton doublet at δ 0.85 ppm (J=6.9 Hz). So, the ^{1}H-NMR spectrum suggested that it is urasne type triterpene with the olefinic proton at C-12 and extra double bond at C-21 (Goad and Akihisa, 1997; Mahatao and Kundu, 1994). The assignments of protons were supported by the correlations in ^{1}H-^{1}H COSY. The ^{1}H-NMR spectrum also showed, two downfield protons at δ 5.33 and 5.12 ppm which were assigned to H-21 and H-12, respectively. The two protons at δ 4.59 and 4.57 ppm were assigned to H-22 and H-3, respectively. In addition to the signal at δ 2.28 ppm methyl were assigned to acetate moiety. The proton at δ 1.99 ppm was ascribed to 18-*β* proton (Goad and Akihisa, 1997). On the other hand, the ^{13}C-NMR of compound 1 showed 30 carbon signals corresponding to those of the urs-12-ene type framework of the molecule with extra double bond at C-21 (Goad and Akihisa, 1997; Mahatao and Kundu, 1994). The signals of vicinyl carbons appeared at δ 124.1 (C-12), 139.4 (C-13), 142.3 (C-20) and 118.1 ppm (C-21). These assignments were deduced from HSQC correlations. The first double bond was established at C-12/C-13 from the HMBC correlations of CH_3-27 with C-13. The second double bond was established at C-20/C-21 from the HMBC correlations of H_1-22, H_1-18 and CH_3-30 with C-20 and C-21. The signal at δ 173.7 ppm was assigned for the carbonyl of acetate group. The acetyl group was established to C-3 position based on the HMBC correlations of protons of the acetate group with C-3. The signals at δ 80.4 and 61.0 ppm were assigned for the oxymethine groups of C-3 and C-22, respectively. From the aforementioned data, this compound was identified as 3β-acetyl -22α-hydroxy ursa-12,20-diene which is a new natural product.

Compounds 2 to 4 were identified as *β*-sitosterol (compound 2), oleanolic acid (compound 3) and *β*-sitosterol -3-O- β-D-glucoside (compound 4) from their mass spectra, ^{1}H and ^{13}C-NMR, direct

comparison of melting points and co-chromatography with authentic samples as well as the available literature (Goad and Akihisa, 1997; Hu et al., 1995; Shiojima et al., 1992).

The EI-MS of compound 5 represents the presence of a molecular ion peak at m/z 138, calculated for the molecular formula $C_{10}H_{18}$. In addition to the peak at m/z 123 $(M-15)^+$. The two peaks at m/z 70 and 68 are characteristic of a retro-Diels-Alder fragmentation of Δ^1-*P*-menthene. Moreover, the peak at m/z 67 (loss of proton from fragment at m/z 68) and the peak at m/z 95 represents the loss of isopropyl group $(M-43)^+$. The characteristic peaks at m/z 80 represents $(95-CH_3)^+$, the peak at m/z 55 represents a retro-Diels-Alder fragmentation of fragment at m/z 80 and the peak at m/z 54 represents the loss of proton from fragment at m/z 55 (Crews et al., 1998; Fang et al., 2010; Silverstein and Webster, 1996). ^{13}C-NMR spectrum of compound 5 showed 10 carbons indicating that it is a monoterpene. This suggestion was confirmed from ^{1}H-NMR spectrum by the presence of two signals at δ 0.87 and 0.89, each integrated as three protons and assigned to the two methyls of the isopropyl group. The presence of a signal at δ 5.81 ppm (1H, m, H-2) indicates the presence of a double bond, which is most probably at C-1, that was confirmed from the downfield shift of Me-7 (δ 2.02 ppm). The ^{13}C-NMR spectrum of the compound confirmed the presence of a double bond by the two olefinic carbons at δ 114.0 and 139.2 ppm assigned to C-2 and C-1, respectively (Fang et al., 2010). From the aforementioned data, compound 5 was identified as dihydrolimonene.

^{13}C-NMR spectrum of compound 6 showed 10 carbons indicating that it is a monoterpene (Crews et al., 1998; Silverstein and Webster, 1996). This suggestion was confirmed by the presence of two signals at δ 0.89 and 0.92 ppm, each integrated as three protons and assigned to the two methyls of the isopropyl group. The presence of two doublets at δ 7.53 and 7.69 ppm in its ^{1}H-NMR spectrum with a coupling constant (J= 8.4 Hz) indicating an ortho coupling, each integrated as two protons assigned to H-2,6 and H-3,5 suggests the presence of a *p*-disubstituted benzene ring. The presence of two signals at δ 128.6 and 130.7 ppm assigned to C-3,5 and C-2,6, in addition to two signals at δ 132.2 and 139.1 ppm assigned to C-1 and C-4, respectively confirmed the presence of a *p*-disubstituted aromatic system. The presence of a singlet at δ 4.20 ppm also integrated as two protons suggest the presence of a benzylic moiety at H-7. The downfield shift of C-7 (δ 68.0 ppm) indicated that it is a hydroxylated carbon (Crews et al., 1998; Silverstein and Webster, 1996). From the aforementioned data, compound 6 was identified as 7-hydroxy-*p*-cymene.

The EI-MS spectrum of compound 7 showed a molecular ion peak at m/z of 472, a base peak at m/z 248 in addition to peaks at m/z 203 and 189. In addition to other characteristic peaks of olean-18-ene skeleton (at m/z 395, 218, 203 and 189) (Shiojima et al., 1992). The ^{1}H-NMR spectrum of compound 7 showed the seven singlets assigned to the seven methyl groups of a pentacyclic triterpene and the ^{13}C-NMR spectrum also showed in addition a signal at δ 187.7 ppm indicating that it is a pentacyclic triterpene acid. Data of this compound showed signals characteristic of an olean-18-en skeleton, especially the two signals at δ 134.0 and 147.6 ppm assigned to C-19 and C-18, respectively (Goad and Akihisa, 1997; Mahatao and Kundu, 1994). In addition, the downfield shift of C-3 (δ 86.2 ppm with a downfield shift of about 6 ppm) indicated the presence of α-hydroxy group at C-2 in the ^{13}C-NMR spectrum (Mahatao and Kundu, 1994). This was confirmed from the downfield shift of C-2 (δ 64.2 ppm) also the downfield shift of C=O of the carboxylic acid group (δ 187.7 ppm) suggests its presence at C-29 (Mahatao and Kundu, 1994; Nakano et al., 1997). ^{1}H and ^{13}C-NMR spectra indicated that this compound was a Δ^{18} oleanane-type triterpene by the chemical shifts of the two olefinic carbons at δ 147.6 (C18) and 134 (C19). The position of the two hydroxy methine carbons at C-2 and C-3 and their configurations were confirmed as *α*- and *β*-oriented, respectively, by comparing their values with previously reported data (Mahatao and Kundu, 1994). From the aforementioned data, it can be concluded that compound 7 was identified as 2*α*, 3*β*-dihydroxy-olean-18-en-29-oic acid which is a new natural product.

Compounds 8 and 9, isolated from the *n*-butanol fractions, were identified as isoquercitrin and rutin, respectively, from their UV, ^{1}H and ^{13}C-NMR and co-chromatography with authentic samples as well as comparing with the available literature (Grace et al., 1998; Markham, 1982).

To the best of our knowledge, this is the first report on the isolation of these compounds from *M. suaveolens* Ehrh. In addition, compounds 1 and 7 are new compounds.

Concerning the biological screening, it was clear that the ethanolic extract showed the most potent analgesic activity as it caused the least number of writhes (24.9) (78.5% potency) as compared to indomethacin. It was followed by the ethyl acetate and n-butanol fractions whose potency percentages were 66.3 and 54.7%, respectively. While, the ethyl acetate fraction was the most potent anti-inflammatory (88% potency) as compared to indomethacin, followed by the ethanolic extract (82.9% potency) followed by *n*-butanol fraction which exhibited 62.6% potency. From the results, it is obvious that both the interesting analgesic and anti-inflammatory activities of *M. suaveolens* were exerted by the ethanol extract, the ethyl acetate and n-butanol fractions. So, it could be concluded that these activities may be attributed to their phenolic contents.

Concerning the antimicrobial activity, it was found that all the tested samples showed a moderate inhibitory activity against human pathogenic bacteria.

Results of GLC analysis of the unsaponifiable matter

(Table 1) revealed that the total identified hydrocarbon and sterol components represented 90.96% of the total unsaponifiable matter. Hydrocarbons constituted 83.45% of the total composition; among which, the major constituents were *n*-docosane (13.76%) and *n*-dodecane (9.70%). Sterols were represented only by β-sitosterol and stigmasterol (5.61 and 1.90%, respectively). GLC analysis of the fatty acid methyl esters (Table 2) revealed that the total identified fatty acids represented 97.84% of the total saponifiable matter. The saturated fatty acid constituted 54.21% of the total fatty acid composition; among which palmitic acid (45.27%) was the major constituent. The unsaturated fatty acids amounted to 43.63% of the total fatty acid composition. Linolenic acid was the major component (19.26%) followed by palmitoleic (11.21%), linoleic (7.86%) and oleic (5.30%) acids. The potent analgesic and anti-inflammatory activities of the ethanolic extract may be due to its content of sterols, triterpenes, phenolic acids and flavonoids which have been proved to exert anti-inflammatory activity. *β*-Sitosterol and its glucoside have profound anti-inflammatory activity (Bouic et al., 1996). In addition, flavonoids have been discovered to have anti-inflammatory activity (Ziaullah et al., 2013). More specifically, rutin is widely used in treating ailments through its anti-inflammatory activities (Yang et al., 2008). Oleanolic acid has also been long-recognized to have anti-inflammatory properties in laboratory animals (Liu, 1995). *β*-Sitosterol has been reported to reduce carcinogen-induced cancer in rats (Bouic et al., 1996). During the last two decades, pharmacological studies of oleanolic acid, the major triterpenoid compound isolated from the *n*-hexane fraction, indicated that this triterpenoid have many beneficial effects, notably hepatoprotection, antiinflammation and antitumor-promotion (Liu, 1995).

Conclusion

The present work showed that *M. suaveolens* cultivated in Egypt is a rich source of the important isolated compounds, described here for the first time from the plant. In addition, the plant revealed remarkable bio-logical activities which need further clinical investigation.

Conflict of Interest

Authors declare no conflict of interest.

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