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Bioactive Constituents from *Apium leptophyllum* Fruits

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Authors' contributions

All the authors participated in the design of the work, writing the protocol, managing the literature search, analyses of the study and interpretation of the data. All the authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was undertaken to characterize the bioactive constituents of *Apium leptophyllum* fruits and to evaluate their cytotoxicity, anti-inflammatory and alpha-amylase inhibitory effects.
Study Design: Isolation and identification of the phytochemicals from the petroleum ether and methanol extracts and investigation of the cytotoxicity, anti-inflammatory and *α*-amylase inhibitory activities.
Place and Duration of Study: Faculty of Pharmacy, Mansoura University, Egypt and College of Pharmacy, University of Louisiana at Monroe, USA, between June 2013 and January 2016.
Methodology: In the course of our study on *Apium leptophyllum* fruits, six compounds were isolated and purified using different chromatographic techniques and their structures were determined on the basis of their spectroscopic data (IR, NMR, MS). The cytotoxic, anti-inflammatory and *α*-amylase inhibitory activities of the prepared fruit essential oil as well as the isolated compounds were performed.
Results: Pentacosanol, 1-nonadecanol and 7*α*-hydroxy stigmasterol are reported for the first time from family Apiaceae, corosolic acid from genus *Apium* and *β*-sitosterol from *Apium leptophyllum* fruits along with the previously reported compound 8-hydroxy cuminic acid. The essential oil and 8-hydroxy cuminic acid exhibited the highest cytotoxic activity against cell lines of human

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hepatocellular carcinoma (HePG-2) with IC_{50} = 28.3 and 29.0 µg/mL and human breast carcinoma (MCF-7) with IC_{50} = 10.4 and 12.6 µg/mL, respectively. They also revealed a remarkable anti-inflammatory activity with 49.54% and 34.83% inhibition, respectively. The essential oil activity on tested cell lines of other types of human breast carcinoma alongside with that of an immortalized, non-tumorigenic human mammary epithelial (MCF10A) suggests a highly selective cytotoxic effect against MCF-7 cell line. Moreover, 8-hydroxy cuminic acid showed a good *α*-amylase inhibitory activity with 54.21% inhibition (IC_{50} = 43.75 µg/mL).

Conclusion: A new scientific evidence for the ethnopharmacological use of the herb in inflammatory conditions and describe, for the first time, the selective cytotoxic activity of the fruit essential oil.

Keywords: Apium leptophyllum; cytotoxicity; anti-inflammatory; α-amylase inhibitor.

1. INTRODUCTION

A. leptophyllum (Pers.) F. Muell. ex Benth. (Synonym, *Cyclospermum leptophyllum* (Pers.) Sprague ex Britton and P. Wilson) belongs to family Apiaceae [1]. The fruits are used in folk medicine for the treatment of flatulence, dyspepsia, diarrhea, laryngitis, rheumatoid arthritis, bronchitis and asthma [2,3], while its leaves are used in Ethiopia to cure a disease known as "Mitch" characterized by sweat inflammation and loss of appetite [4]. Research interest in *A. leptophyllum* has been focused on its antimicrobial activities [4-6]. It was also found that the fruit extracts exhibited a strong antioxidant activity [7] and chemopreventive potential on induced skin carcinogenesis in mouse [8]. Previous phytochemical investigation of the fruits revealed the isolation of flavonoids [9,10], coumarins [11-14], D-mannitol [12], 2,3-dihydro-2-methyl-6-hydroxybenzofuran-5-carboxylic acid [15] and 8-hydroxy cuminic acid [16]. In our previous study, the chemical composition and *α*-amylase inhibitory activity of the fruit essential oil were extensively studied and 36 compounds representing 89.51% of the oil composition were identified; including thymohydroquinone dimethyl ether, isothymol methyl ether, thymol methyl ether, cuminaldehyde, *p*-cymene and *γ*-terpinene [17]. In the course of our study on *A. leptophyllum* fruits, six compounds were isolated (1-6) and their structures were determined on the basis of their spectroscopic data (IR, NMR, MS). The cytotoxic, anti-inflammatory and *α*-amylase inhibitory activities of some of the isolated compounds as well as the prepared fruit essential oil were performed.

2. MATERIALS AND METHODS

2.1 General Methods

^{1}H and ^{13}C NMR spectra were recorded on a JEOL FT-NMR spectrometer (400 and 100 MHz for ^{1}H and ^{13}C respectively). Electrospray Mass Spectra (ESI-MS) negative mode were carried out on ABI 3200Q Trap high performance triple stage MS/MS mass spectrometer with ESI and atmospheric pressure chemical ionization (APCI) hyphenated with Agilent 1100 HPLC system. HR-ESI-MS was carried out on Synapt G2 HDMS mass spectrometer, Waters Corp, Milford, MA, USA. The melting points were measured on melting point apparatus Fisher-Johns Scientific Co., USA. and were uncorrected. IR v_{max} (KBr, cm^{-1}): were recorded using Infra-red spectrophotometer, Mattson 5000 FTIR (England).

2.2 Cell Lines

The human hepatocellular carcinoma (HepG-2), colorectal carcinoma (HCT-116), human prostate carcinoma (PC-3) and mammary gland breast carcinoma (MCF-7) cell lines were obtained from ATCC via Holding Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt. The human mammary gland breast epithelial tumor (SKBR-3), human breast ductal carcinoma (BT-474), human ductal breast epithelial tumor (T-47D) and MCF-7cell lines were purchased from American Type Culture Collection (Rockville, MD).The cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37ºC. Non-tumorigenic human mammary epithelial (MCF10A) was purchased from ATCC and maintained in DMEM/F12 supplemented with 5% horse serum, 0.5 mg/mL hydrocortisone, 20 ng/mL EGF, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10 mg/mL insulin.

2.3 General Experimental Materials

MTT; The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, RPMI-1640

medium, Dimethyl sulfoxide, Indomethacin and 5-Fluorouracil (5-FU) are from Sigma Chemical Co., St. Louis, MO, USA. Fetal bovine serum (GIBCO, UK). Penicillin and Streptomycin were purchased from Amresco (Solon, OH, USA). *λ*-Carrageenan and porcine pancreatic *α*-amylase (EC3.2.1.1, type VI) were purchased from Sigma Aldrich, St. Louis, USA; acarbose from AK Scientific, Inc., USA. All other chemicals used were of analytical grade.

2.4 Plant Material

Fresh fruiting aerial parts were collected from private farms in El-Geddeyah village, Rasheed, Egypt. A voucher specimen was kept in the herbarium of Dept. of Plant Science, Mansoura University under the code number (I.M.-1112) after being authenticated by Dr. I. Mashaly, Prof. of Botany. To get crops sufficient for the present study, the ripe fruits were later on cultivated in the Medicinal Plants Experimental Station, Faculty of Pharmacy of the same University.

2.5 Extraction and Isolation

2.5.1 Preparation of the essential oil

Fresh fruits were collected in late May to early June. The essential oil was then isolated from fruits by hydro-distillation for 4 hrs. using a Clevenger-type all glass apparatus 1.1% v/w yield. The oil was transferred to a screw capped glass vial, dried over anhydrous sodium sulfate and stored at -10ºC in the dark.

2.5.2 Extraction and isolation of compounds

Powdered fruits (1750 g) of *A. leptophyllum* were extracted using petroleum ether (20 L) then methanol (15 L). Each extract was concentrated under reduced pressure at 45ºC to yield 24 g and 40 g respectively. The petroleum ether extract was subjected to silica gel column chromatography and eluted with pet. ether - EtOAc (100 → 0 to 50 → 50, gradient). Fractions of matched TLC patterns were pooled and purified by re-chromatography on silica gel columns and by repeated crystallization to afford pentacosanol **1** (30 mg) and *β*- sitosterol **2** (250 mg). The methanol extract was subjected to chromatography on silica gel column and applying gradient elution with CH_2Cl_2 - EtOAc (100→0 to 0 →100) and then EtOAc - MeOH (100→0 to 0 →100). Fractions of matched TLC patterns were pooled and subjected to purification through column chromatography and repeated crystallization to afford 1-nonadecanol **3** (20 mg), 8-hydroxy cuminic acid **4** (300 mg), corosolic acid **5** (15 mg) and 7*α*-hydroxy stigmasterol **6** (10 mg).

2.6 Spectral Analysis

Pentacosanol (**1**): colorless needle crystals; mp 83-84ºC. IR (KBr): 3314, 1061, 719 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) *δ*: 3.62 (t, *J* = 6.6 Hz, H-1), 1.56 (m, H-2), 1.23 (m, H-3 to H-24), 0.85 (t, *J* = 6.6 Hz, H-25). ^{13}C-NMR ($CDCl_3$, 100 MHz) *δ*: 63.2 (C-1), 32.9 (C-2), 32.0 (C-3), 29.5 – 29.8 (C-4 : C-22), 25.8 (C-23), 22.7 (C-24), 14.2 (C-25). ESI-MS *m/z*: 367 $[M-H]^+$ ($C_{25}H_{52}O$).

1-Nonadecanol (**3**): colorless needle crystals; mp 60-61°C. IR (KBr): 3316, 1062, 720 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) *δ*: 3.62 (*t*, *J* = 6.6 Hz, H-1), 1.55 (*m*, H-2), 1.23 (*m*, H-3 to H-18), 0.85 (*t*, *J* = 6.6 Hz, H-19).^{13}C-NMR ($CDCl_3$, 100 MHz) *δ*: 63.2 (C-1), 32.9 (C-2), 31.9 (C-3), 29.5-29.8 (C-4 : C-16), 25.8 (C-17), 22.7 (C-18), 14.2 (C-19). ESI-MS *m/z*: 283 $[M-H]^+$ ($C_{19}H_{40}O$).

8-Hydroxy cuminic acid (**4**): white needle crystals; mp 156-157°C. IR (KBr): 3265, 2970, 1694, 1639 cm^{-1}.^{1}H-NMR (DMSO, 400 MHz) *δ*: 7.88 (d, *J* = 6.0 Hz, H-2 & H-6), 7.58 (d, *J* =5.48, H-3 & H-5), 1.44 (*s*, H-9 & H-10).^{13}C-NMR (DMSO, 100 MHz) *δ*: 129.3 (C-1), 129.3 (C-2 & 6), 125.1 (C-3 & 5), 155.9 (C-4), 167.9 (C-7), 71.2 (C-8), 32.1 (C-9 & 10).

Corosolic acid (**5**): white needles; mp 254-256°C. ^{1}H-NMR (CD_3OD, 400 MHz) *δ*: 5.20 (*br. s*, H-12), 3.61 (*m*, H-2), 2.88 (*d*, *J* = 9.6, H-3), 2.19 (*d*, *J* =11, H-18), 0.78 (s, H-24), 0.83 (*s*, H-26), 0.84 (*br. s*, H-29), 0.86 (*br. s*, H-30), 0.98 (*s*, H-23 and H-25), 1.09 (*s*, H-27). ^{13}C-NMR (CD_3OD, 100 MHz) *δ*: 68.7 (C-2), 83.8 (C-3), 39.9 (C-4), 56.5 (C-5), 18.8 (C-6), 33.6 (C-7), 40.2 (C-8), 48.3 (C-9), 38.6 (C-10), 23.7 (C-11), 125.7 (C-12), 139.0 (C-13), 42.7 (C-14), 28.4 (C-15), 24.8 (C-16), 53.7 (C-18), 40.0 (C-19), 39.6 (C-20), 31.3 (C-21), 37.6 (C-22), 28.6 (C-23), 16.9 (C-24), 16.7 (C-25), 17.0 (C-26), 23.4 (C-27), 178.0 (C-28), 17.4 (C-29), 21.0 (C-30), C-1 and C-17 signals were masked by the solvent. HR-ESI-MS *m/z*: 471.3468 $[M-H]^+$ (calculated = 471.3473, $C_{30}H_{48}O_4$).

7*α*-Hydroxy stigmasterol (**6**): white needles; mp 202-203°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) *δ*: 5.58 (br. s, H-6), 5.13 (dd, H-22), 4.99 (dd, H-23), 3.83 (br. s, H-7), 3.58 (m, H-3), 0.97 (s, H-19), 0.91 (d, *J* =5.9, H- 21), 0.82 (d, *J* =7.2, H-26), 0.80 (t, H-29), 0.68 (d, *J* =7.3, H-27), 0.66 (s, H-18). ^{13}C-NMR ($CDCl_3$, 100 MHz) *δ*: 37.0 (C-1), 31.4 (C-2),

71.4 (C-3), 42.0 (C-4), 146.3 (C-5), 123.9 (C-6), 65.4 (C-7), 37.5 (C-8), 42.3 (C-9), 37.4 (C-10), 20.7 (C-11), 39.1 (C-12), 42.0 (C-13), 49.2 (C-14), 24.4 (C-15), 28.3 (C-16), 55.8 (C-17), 11.7 (C-18), 18.3 (C-19), 45.8 (C-20), 21.3 (C-21), 138.3 (C-22), 129.3 (C-23), 51.5 (C-24), 32.0 (C-25), 19.0 (C-26), 21.3 (C-27), 25.6 (C-28), 12.0 (C-29).

2.7 Biological Assays

2.7.1 Cytotoxic assay

The MTT colorimetric assay was used to determine the cytotoxic activity against different cell lines. The cell lines were seeded in a 96-well plate at a density of 1 x 10^4 cells/ well at 37℃ for 24 hr under 5% CO_2.Tested materials were dissolved in DMSO and diluted with phosphate buffer solution. Different concentrations were added to the tested cell lines and cultured for 48 hr. [18]. Cytotoxic activity of the essential oil and compounds **2**-**4** were performed against HePG-2, MCF-7, PC-3 and HCT-116 cell lines. The cytotoxic activity of the essential oil was further performed against SKBR-3, BT-474, T-47 D, MCF-7 and MCF10A.

2.7.2 Anti-inflammatory assay

The anti-inflammatory activity of the essential oil in addition to compounds **2** and **4** were carried out using carrageenan-induced rat paw oedema model [19]. Five groups of male Wistar albino rats weighing 150–180 g, each consisting of six rats, were used. The control group received the vehicle (i.p.,1% DMSO in normal saline) while the standard drug, indomethacin (10 mg/kg) and each of the tested compounds (25 mg/kg) were injected i.p. as suspension in the same vehicle. *λ*-carrageenan (0.1 mL of a 1% suspension in normal saline) is used as the phlogistic agent.

2.7.3 *α*-Amylase assay

α-Amylases are enzymes that hydrolyze carbohydrates at 1, 4 linkages to glucose and maltose [20]. The *α*-amylase inhibition activity of the isolated compounds **2**-**4** (1 mg/mL in DMSO) was performed twice using chromogenic method adopted from Sigma Aldrich [21,22]. Control incubations, representing 100% enzyme activity were conducted in an identical fashion replacing compounds with DMSO.

2.7.4 Statistical study

In the cytotoxic assay, data are presented as mean ± S.E.M of at least three independent experiments. The IC_{50} (concentrations that induce 50% cell growth inhibition) values were determined by using non-linear regression curve fit analysis using Graph Pad Prism software (Graph Pad software Inc. V4.03, San Diego, Ca, USA). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. Differences were considered significant at $P < 0.05$ when compared with the vehicle-treated control group.

3. RESULTS AND DISCUSSION

3.1 Identification of Compounds, Fig. 1

From the petroleum ether and methanol extracts of the fruits, six known compounds (**1**-**6)** were isolated and identified as pentacosanol **1,** *β*-sitosterol **2**, 1-nonadecanol **3**, 8-hydroxy cuminic acid **4** [16], corosolic acid **5** [23,24] and 7*α*-hydroxy stigmasterol **6** [25] by comparing their physical and spectral (IR, MS, NMR) data with those previously reported.

3.2 Cytotoxic Assay

The cytotoxicity of the essential oil as well as *β*-sitosterol **2**, 1-nonadecanol **3** and 8-hydroxy cuminic acid **4** were evaluated, by the MTT assay, on human hepatocellular carcinoma (HePG-2), colorectal carcinoma (HCT-116), human prostate carcinoma (PC-3) and mammary gland carcinoma (MCF-7) cell lines. The results showed that, the essential oil and 8-hydroxy cuminic acid **4** exhibited the highest cytotoxic activity, as compared to 5-FU, against HePG-2 (IC_{50} = 28.3 and 29.0 µg/mL) and MCF-7 (IC_{50} = 10.4 and 12.6 µg/mL) cell lines and they were less active against other cell lines (Table 1).The above data initiated further investigation of the cytotoxic effect of the essential oil on other human breast cancer cell lines viz. human mammary gland breast epithelial tumor cell line (SKBR-3 & MCF-7), human breast ductal carcinoma cell line (BT-474) and human ductal breast epithelial tumor cell line (T-47 D) as well as an immortalized, non-tumorigenic normal human mammary epithelial (MCF10A) cell line. Acute exposure of breast cancer cells to the fruit essential oil caused rather weak effect on cell viability of SKBR-3, BT-474 and T-47 D in a dose-dependent manner with IC_{50} of 38.49, 61.45 and 75.41 µg/mL, respectively as compared to simultaneously experimented MCF-7 with IC_{50} 10.42. On the other hand, the treatment effect of the essential oil (0–30 µg/mL) on the growth of

normal human mammary epithelial cells (MCF10A) for 48 hr had no effect on the (MCF10A) cells viability as compared to their respective vehicle-treated control groups. This clearly demonstrates the selectivity of oil cytotoxic activity against human mammary gland cancer cells (MCF-7) and no effect on the growth and/or viability of the normal human mammary epithelial cells (MCF10A).

3.3 Anti-inflammatory Assay

The anti-inflammatory activity results of the tested compounds (Table 2) indicate that, the essential oil revealed a stronger anti-inflammatory activity (49.54%) comparable to Indomethacin (42.29%), while those of *β*-sitosterol **2** and 8-hydroxy cuminic acid **4** were weaker.

3.4 Alpha-amylase Inhibitory Assay

The inhibition of the carbohydrate hydrolyzing enzyme such as *α*-amylase leads to lower the post prandial blood glucose level and this is one of the therapeutic approaches to treat Type II Diabetes [26].The *α*-amylase inhibitors act as an anti-nutrient that obstructs the digestion and absorption of carbohydrates and potentially useful in control of obesity and diabetes [27]. The

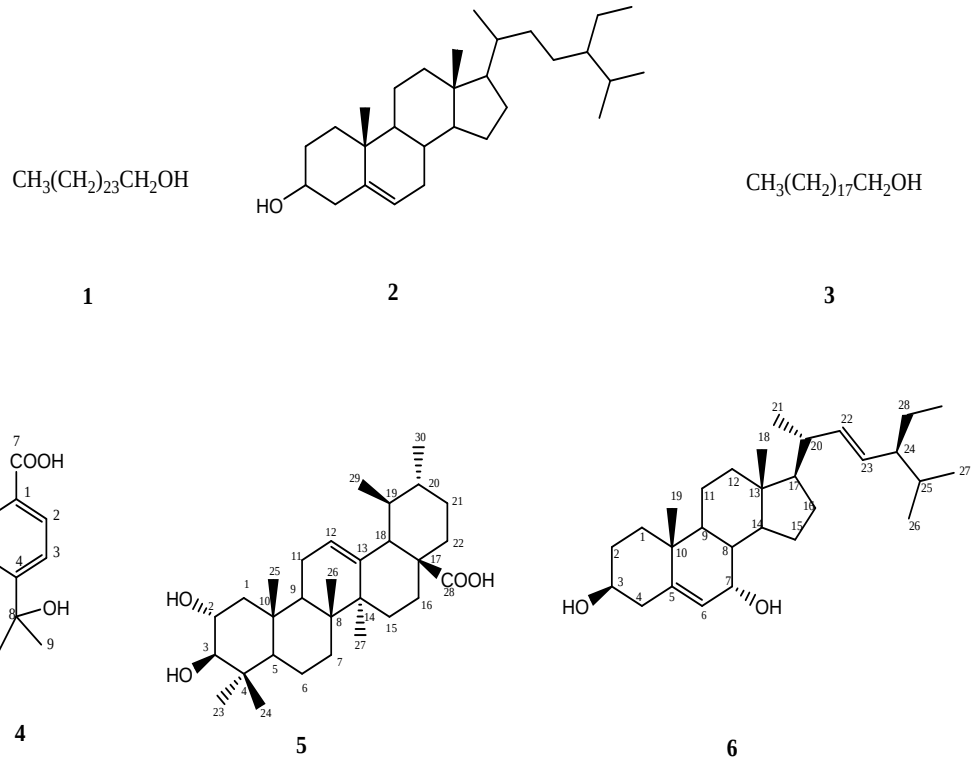

Fig. 1. Structures of isolated compounds

Table 1. Results of cytotoxic activities of tested constituents

Tested materials	IC_{50} (µg/mL)			
	HePG-2	HCT-116	PC-3	MCF-7
5-FU	20.3±1.2	9.1±0.9	20.5±1.5	5.1±0.7
Essential oil	28.3±1.3	19.9±1.3	38.0±2.3	10.4±1.3
2	48.9±2.4	43.7±2.6	69.6±3.8	28.3±2.5
3	66.3±3.5	58.2±3.7	84.6±4.8	43.2±3.5
4	29.0±1.7	31.7±1.9	42.2±2.5	12.6±1.3

Values are expressed as mean ± SEM of three independent study, P <0.05 as compared with the control group

Table 2. Results of anti-inflammatory activities

Tested materials	Increase in paw thickness (%)	Inhibition of oedema (%)
Vehicle control	143.76 ± 2.9	-
Indomethacin	81.89 ± 1.34*	42.29 ± 0.59
Essential oil	73.79 ± 0.65*	49.54 ± 0.59
2	97.33 ± 0.55*	31.77 ± 0.64
4	96.44 ± 2.49*	34.83 ± 0.52

Values are expressed as mean ± SEM, n = 6 in each group, P<0.01
**Significantly different from the mean value of the control group*

α-amylase inhibitory activity of *β*-sitosterol **2**, 1-nonadecanol **3** and 8-hydroxy cuminic acid **4** revealed that the latter was the most active with 54.21% inhibition (IC_{50} = 43.75 µg/mL) comparable to acarbose (IC_{50}= 47.80 µg/mL), while the others exhibited no activity with 3.20 and 6.20% inhibition, respectively.

4. CONCLUSION

The present study provided a new scientific evidence for the ethnopharmacological use of *A. leptophyllum* in inflammatory conditions and describe, for the first time, the selective cytotoxic activity of the fruit essential oil against mammary gland carcinoma (MCF-7) cell line. Moreover, 8-hydroxy cuminic acid **4** showed a good *α*-amylase inhibitory activity. It is worth to note that, pentacosanol, 1-nonadecanol and 7*α*-hydroxy stigmasterol are reported in the present study for the first time from the Apiaceae as well as corosolic acid from genus *Apium* and *β*-sitosterol from *A. leptophyllum* fruits.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Experimental procedures have been performed following the rules of the Research Ethics Committee, Faculty of Pharmacy, Mansoura University and in accordance with the recommendations of the proper care and use of laboratory animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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