Chemical composition and *α*-amylase inhibitory activity of *Apium leptophyllum* essential oils

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Abstract: The objective of the present study was to investigate the chemical composition and α-amylase inhibitory activity of the essential oils extracted from different parts of Apium leptophyllum. The essential oils of the fruits and roots were investigated for the first time by GC-MS analysis. The data were compared with those of the green aerial parts (GAP). A total of 17, 13, 30 and 36 compounds were identified in the GAP, root, unripe and ripe fruit oils, respectively. All the oils are rich in thymohydroquinone dimethyl ether, isothymol methyl ether, thymol methyl ether, p-cymene and γ-terpinene. However, unlike the GAP oil, the other oils contain several aldehydic constituents collectively 8, 4 and 2 in ripe fruit, unripe fruit and root oils, respectively. Cuminaldehyde and γ-terpinene-7-al are common constituents in the three oil samples. The α-amylase inhibitory activity of the fruit oil was stronger than that of the GAP and both were significantly high as compared to that of acarbose; a drug currently used for controlling glucose levels in diabetic patients. Thus both oils need further evaluation for their antidiabetic potential.
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1. Introduction

Apium leptophyllum (Pers.) F. Muell. ex Benth. (Synonym, Cyclospermum leptophyllum (Pers.) Sprague ex Britton and P. Wilson) is native to Central America and naturalized in many tropical and subtropical regions. It is an annual apiaceous tap rooted herb having slender branches reaching up to 60 cm high with finely divided leaves (Boulos, 1999). Numerous traditional uses of this weed and /or its essential oil product were reported. In India, the fruits are used to treat flatulence, dyspepsia, diarrhea, laryngitis, rheumatoid arthritis, bronchitis and asthma (Kumar and Krishna, 2012). In South America, the fruits are used as carminative, antinephritic and antirheumatic (Barboza et al., 2009). In Ethiopian traditional medicine, the leaves are used for the treatment of a disease condition known locally as "Mitch" and characterised mainly by inflammation, sweat and loss of appetite (Asamenew et al., 2008). Some authors have reported the in vitro antimicrobial activity of the essential oil isolated from GAP of the plant using the standard disc diffusion technique. The data indicate that the oil is active against a broad spectrum of pathogens including Gram-positive and Gram-negative bacterial strains (Abd El-Aziz, 1992; Asamenew et al., 2008; Singh et al., 2013) as well as certain fungal strains (Abd El-Aziz, 1992; Asamenew et al., 2008). Everitt et al., (2007), reported that the essential oil of the plant is medicinally used as a mild balm in stomachic complaints and also for diarrhoea. Fruit extracts of A. leptophyllum showed strong antioxidant activity by inhibiting DPPH, hydroxyl, nitric oxide, superoxide radical scavenging activities when compared with standard ascorbic acid (Sahoo et al., 2013). Further study revealed that the methanolic fruit extract has chemopreventive potential on induced skin carcinogenesis in mouse, which may be due to the modulation of cutaneous lipid peroxidation or enhancement of total antioxidant capacity (Sahoo et al., 2014).

Reviewing the current literature, it was evident that the chemical analyses of the essential oils were limited to those steam distilled from GAP, mainly leaves or leaves and stems of the title species grown in different countries around the world, viz: Australia (Park and Sutherland, 1969), Brazil (Brasil et al., 1971), Egypt (Abd El-Aziz, 1992), Japan (Shin'ichi and Kayo, 2002), Ethiopia (Asamenew et al., 2008) and India (Pande et al., 2011). The Brazilian source reported the phenolic ether, apiole as the dominant component while none of the other reports cited even traces of this compound. Instead, they all reported thymohydroquinone dimethyl ether as the dominant component while the major ones were thymol methyl ether, γ-terpinene and p-cymene and in addition either carvacrol methyl ether (Park and Sutherland, 1969; Abd El-Aziz, 1992; Pande et al., 2011) or isothymol methyl ether (Shin'ichi and Kayo, 2002; Asamenew et al., 2008). Surprisingly, although a great part of the traditional uses of the herb is pertinent to its aromatic fruits, the literature is free of any mention of the essential oil analysis of this organ .Therefore the present study aims to thorough investigation of the composition of the essential oils produced by the ripe fruits, unripe fruits and roots and compared with that produced by the vegetative GAP.

Diabetes is a group of metabolic diseases characterized by chronic hyperglycemia resulting from deficiency in insulin secretion or action. One therapeutic approach for treating diabetes is to decrease the postprandial glycaemia by the inhibition of enzymes responsible for the carbohydrate hydrolysis, such as α-amylase and α-glucosidase (Wild et al., 2004). Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma glucose rise (Rhabasa-Lhoret and Chiasson, 2004). Examples of such inhibitors which are in clinical use are acarbose, miglitol and voglibose (Bailey, 2003). Our search for α-amylase inhibitors in several cultivated and wild plants, showed a methanol extracts of A. leptophylum fruits and GAP were among those afforded a positive activity. Thus, it deemed interesting to evaluate the essential oils of this species for their α-amylase inhibitory potential against porcine pancreatic α-amylase using starch as substrate.

2. Material and Methods

2.1. Plant material

Fruits of *A. leptophyllum* were originally collected from plants growing wildly near El-Geddeyah village, Rasheed, El-Behera Governorate, Egypt. The plant was authenticated by Prof. Ibrahim Mashaly, Department of Plant Science, Faculty of Science, Mansoura University and a voucher specimen (I.M.-1112) is deposited in the herbarium of the same Department. The fruits were later cultivated in the Medicinal Plants Experimental Station, Faculty of Pharmacy, Mansoura University in order to get crops sufficient for the present study.

2.2. Chemicals

Porcine pancreatic α-amylase (EC3.2.1.1, type VI) was purchased from Sigma-Aldrich, St. Louis, USA; acarbose from AK Scientific, Inc., CA, USA; thymol from the New York Quinine and Chemical WORK, Inc. USA and CH_3I was purchased from Fluka AG, Chemische Fabrik CH-9470 Buchs, Seelze, Germany. All the other chemicals used were of analytical grade.

Thymol methyl ether was prepared following the procedure of Chao *et al*., (2010) with minor modification: Dry potassium carbonate (34.5 mg, 0.25 mmol) was added to a solution of thymol, (75 mg, 0.5 mmol, 1 equiv.) in dry acetone (1 mL). Methyl Iodide (71 mg, 0.5 mmol, 1 equiv.) was dissolved in 1mL dry acetone then added drop wise. The reaction mixture was stirred for 24 hr. at room temperature and then the reaction was stopped by addition of water to the reaction mixture and acetone was removed under reduced pressure by a rotary evaporator. The obtained aqueous phase was extracted with methylene chloride (3x3 mL). The organic phase was dried over anhydrous Na_2SO_4, and the solvent was partially removed under reduced pressure. The purity of the product was checked by TLC and confirmed by GC-MS.

2.3. Preparation of the essential oils:

Fresh GAP, roots and unripe fruits were collected from a crop harvested in April, while the ripe fruits were collected in late May to early June of the same year. The fresh GAP and roots were chopped into small pieces. The essential oil was then isolated from each part; GAP, root, unripe and ripe fruits by hydro-distillation for 4 hrs using a Clevenger-type all glass apparatus. The oils were transferred to screw-capped glass vials and stored at -10^{o}C in the dark until analysis. The yields were 0.4%, 0.1%, 0.8% and 1.1% for GAP, root, unripe fruit and ripe fruit oils, respectively.

2.4. Gas Chromatography-mass spectrometry analyses (GC-MS)

The GC-MS analyses were executed on Thermo Scientific Focus DSQ GC-MS unit. The GC was equipped with a capillary column Thermo TR-5ms SQC (25 m × 0.25 mm i.d. 0.25 µm film thickness). Helium was used as carrier gas at flow rate of 1 mL/min. Injector temperature was 220°C and the injection volume 0.1µL in methylene dichloride (1 : 10), with a split ratio of 1 : 20. Oven temperature was programmed from 60^{o}C to 240^{o}C at 3^o/min and then held isothermally for 10 min. Mass spectral analyses were run by eI technique at 70 eV. The mass range was adjusted from 40 to 350 amu. Mass spectra correlations were done using Wiley, NIST library search, our own MS filed data and the published MS data (Adams, 2007).

2.5. α-Amylase Inhibition Assay

It was performed using the chromogenic method adopted from Sigma–Aldrich, which was originally proposed by Bernfeld (1955). Porcine pancreatic α-amylase was dissolved in ice-cold distilled water to give a concentration of 4 unit/ml solution. Potato starch (0.5%, w/v) was dissolved in 20mM phosphate buffer (pH 6.9) containing 6.7mM sodium chloride, and used as a substrate solution. Dinitrosalicylic acid color reagent (DNSA) was prepared by dissolving 1 gm of 3, 5-dinitrosalicylic acid in 50 ml of distilled water. 30 gm of sodium potassium tartrate tetrahydrate was added slowly followed by 20 ml of 2 N NaOH and the solution was diluted to a final volume of 100 ml with distilled water. The oils were first dissolved in a volume of

DMSO to provide stock solutions which were used to prepare various concentrations of the oils. 200 µl of enzyme solution and 40 µl of oil of varying concentration were mixed thoroughly in a tube and incubated for 5 min. Then 400 µl of starch solution and 160 µl distilled water were added to each tube and incubated at 25 °C for 3 min. An aliquot of 400 µl of the mixture was transferred to a separate tube containing 200 µl DNSA color reagent and left in a water bath at 85 °C for 15 min. The mixture was then diluted with 1800 µl distilled water and the α-amylase activity was determined by measuring the absorbance at 540 nm. Control incubations, representing100% enzyme activity were conducted in an identical fashion replacing oil with DMSO (40 µl). For blank incubations (to allow for absorbance produced by the oil), the enzyme solution was replaced with distilled water and the same procedure was carried out as above. Acarbose solution (diluted in DMSO to 25–200 µL/mL) was used as a positive control. The absorbance (*A*) due to maltose generated was calculated as:

A_{540nm} control or oil = A_{540nm} Test − A_{540nm} Blank

From the net absorbance obtained, the % (w/v) of maltose generated was calculated from the equation obtained from the maltose standard calibration curve (0–0.1%, w/v, maltose). The α-amylase inhibitory activity was expressed as percent inhibition and was calculated using the following equation:

$$\% \text{ α-amylase inhibition} = 100 - \left[\frac{[\text{maltose}]\text{sample} \times 100}{[\text{maltose}]\text{control}}\right]$$

The % α-amylase inhibition was plotted against the sample concentrations and regression curve established for determination of the IC_{50} value using Graph Pad PRISM 2.01 software.

2.6. Statistical Analysis

For the essential oils and standard compound, three samples were prepared for each assay. The data was presented as mean ± standard deviation of three experiments.

3. Results and discussion:

Comparative data of the identified essential oil constituents of *A. leptophyllum* are listed in Table 1. A total of 17, 13, 30 and 36 compounds were identified in the GAP, root, unripe and ripe fruit oils representing 99.82%, 99.65%, 97.65% and 89.51% of the oils composition, respectively. The results indicate that all the oils are still rich in the monoterpene hydrocarbons; *p*-cymene and *γ*-terpinene and the phenolic ethers; thymohydroquinone dimethyl ether and two monomethoxy cymene isomers. The mass spectra of these isomers are very close and reflecting a rather confusing NIST library search data. Their representative peaks at t_R 16.93 min and 17.19 min (Table 1) gave thymol methyl ether as the first hit (SI: 939 and 946) and isothymol methyl ether as the second hit (SI: 881 and 883). So, co-injection of the oil and a sample of thymol methyl ether was essential and the result proved that the peak eluted at 17.19 min represents that compound. Hence the peak eluted earlier represents isothymol methyl ether in accordance with reported data for essential oils analyzed using the same column (Yassaa *et al*, 2003; Collin *et al*, 2014) and thus excluding carvacrol methyl ether as one of the major compounds.

Further investigation of the data revealed that, unlike the GAP oil, the oils prepared from the other parts contain significant amount of the aromatic monoterpene aldehyde, cuminaldehyde in addition to several minor and traces of aldehydic constituents collectively 2, 4 and 8 in the root, unripe fruit and ripe fruit oils, respectively. Cuminaldehyde and *γ*-terpinene-7-al are common constituents in the three oil samples. Oil of the unripe fruit contains, in addition, 2-caren-10-al and 3-methoxy cuminaldehyde. Oil of the ripe fruit adds n-hexanal, n-heptanal, n-decanal and possibly an isomer of 3- methoxy cuminaldehyde.

Cuminaldehyde, 2-caren-10-al and γ-terpinen-7-al are known essential oil constituents. On the other hand and to the best of the authors knowledge, 3-methoxy cuminaldehyde (t_R: 25.99 min., Table 1) was never reported as an oil constituent. However, it was isolated as a new natural product among many diterpenoid compounds from an extract of *Olearia* species (Warning *et al*., 1988). The component tentatively identified as a methoxy cuminaldehyde (t_R: 27.98 min., Table 1) revealed close mass spectral pattern to that of 3-methoxy cuminaldehyde. Unfortunately, the presence of both compounds in minute amount precludes their preparative isolation and identification using more decisive spectral techniques.

It is worth notice that each of the identified cyclic monoterpene aldehydes in the fruits is presumably derived from its corresponding mother component via the selective enzymatic oxidation of the methyl moiety directly attached to the ring and never through those of the isopropyl moiety, *e.g.* 2-caren-10-al, cuminaldehyde, γ-terpinen-7-al, 3-methoxy cuminaldehyde and its isomer from car-3-ene, p-cymene, γ-terpinene, thymol methyl ether and one of its isomers, respectively. Furthermore, the absence of any of the expected intermediate alcoholic representatives among the identified constituents of any of the oils except that of cumin alcohol isovalerate (t_R: 33.76 min.), is surprising.

It is also worth to note that among the six reports that deal with the analysis of the oil of GAP, only the most recent report (Pande *et al*., 2011) added

cuminaldehyde to the list of constituents. This is rather questionable whether this variation is due to different environmental conditions or due to the co-collection of a sizable amount of the fruits either ripe or unripe with the bulky green part sample prior to subjection to the steam distillation process. Nevertheless, the significant differences among the oil components of the different parts necessitate the need to specify the time and stage of collection as well as the exact part or parts collected.

Table 1: Essential oil composition of ripe fruit, unripe fruit, root and GAP of *Apium leptophyllum*

Compound	t_R (min)	Area %			
		Ripe fruit	Unripe fruit	Root	GAP
n-Hexanal	3.04	0.01			
n-Heptanal	4.89	0.01			
α-Thujene	5.34	0.23	0.45	0.06	0.02
α –Pinene	5.55	0.10	0.1		0.01
Sabinene	6.72	0.86	1.49	0.27	0.07
β-Pinene	6.88	0.05	0.22		0.01
Myrcene	7.20	0.52	0.94	0.11	0.02
Car-3-ene	7.87	0.10	0.22	0.02	0.02
α –Terpinene	8.18	0.21	0.44	0.02	0.01
p-Cymene	8.61	7.22	11.55	6.58	3.37
trans-Ocimene	9.23	0.02	0.07		
γ –Terpinene	9.81	6.87	14.51	4.28	3.29
cis-Sabinene hydrate	10.51	0.02			
Terpinolene	10.76	0.02	0.03		
p-Cymenene	11.14	0.02	0.01		
Linalool	11.69	0.02			
Terpinen-4-ol	15.11	0.07	0.22		
n-Decanal	16.05	0.08			
Isothymol methyl ether	16.93	8.95	15.09	10.73	8.85
Thymol methyl ether	17.19	5.87	10.11	5.98	3.94
Carvacrol methyl ether	17.50	0.17	0.52	0.03	0.10
Cuminaldehyde	18.18	9.52	3.05	6.59	
Car-2-en-10-al	19.87	0.01	0.01		
γ -Terpinen-7-al	20.11	3.21	0.48	0.84	
Carvacrol	20.81	0.02	0.09		
α-Copaene	22.95	0.01	0.02		
B-Bourbonene	23.31	0.01			
Thymohydroquinone dimethyl ether	25.37	42.84	38.03	64.14	80.24
3-Methoxy cuminaldehyde* {M^+/z 178(50), 163(100), 135(18), 105(44), 91(40), 77(38), 65(18), 51(14)}	25.99	0.22	0.14		
α-Humulene	26.48	0.06	0.26		0.04
Germacrene D	27.39		0.41		
α-Zingiberene	27.92		0.28		0.01
Methoxy cuminaldehyde isomer* {M^+/z 178(47), 163(100), 135(13), 105(54), 77(48), 55(32)}	27.98	0.05			
Caryophyllene oxide	31.64	0.02	0.04		0.01
Humulene epoxide II	32.76	0.02	0.01		0.01
Cumin alcohol isovalerate* {M^+/z 234(10), 133(100), 117(30), 91(19), 57(40), 41(40)}	33.76	0.05	0.03		
2-Pentadecanone, 6,10,14-trimethyl	40.86	0.15	0.07		
Palmetic acid	46.46	1.90			

*tentatively identified

The *α*-amylase inhibitory data are listed in Table 2. Both oils showed dose-dependent inhibition response. The ripe fruit oil is the most effective (IC_{50}= 1.09µg/ml) as compared to the GAP oil (IC_{50}=18.14 µg/ml). Both are much stronger than acarbose (IC_{50}= 47.8 µg/ml). For the first look one would expect that the high activity of the GAP oil is due mainly to its dominant component *i.e.* thymohydroquinone dimethyl ether. However, the fruit oil, while containing much lesser amount of that compound (Table 1) yet its activity is much higher. Hence, further investigation is needed and should include fractionation of the fruit oil into its hydrocarbon, phenolic methyl ether, and aldehydic fractions and measuring the inhibition value of each. Furthermore, both oils should be subjected to suitable *in vivo* experiments in order to assess and evaluate their antidiabetic potential.

Table 2: α- amylase inhibition activity of essential oils of Apium leptophyllum

Samples	Concentration (µg/ml)	% inhibition*	IC_{50} (µg/ml)
GAP oil	2.5	21.50±0.87	18.14
	5	28.49±1.09	
	10	38.21±0.91	
	20	51.81±1.01	
Fruit oil	0.75	41.00±0.59	1.09
	1.5	52.00±0.78	
	3	62.4±1.03	
	6	71.00±0.98	
Acarbose	25	43.10±2.30	47.8
	50	50.33±2.78	
	100	65.00±3.45	
	200	80.13±3.12	

* Values are expressed as mean ± SD, n=3.

4. Conclusion

Several monoterpene aldehydes, while being absent in GAP, are present in the oils of the fruits (ripe and unripe) and to a lesser extent of the root of *A. leptophyllum*. These compounds represent a higher oxidative level of many of the compounds already present in all the oils. The *in vitro* study reveals that both GAP and fruit oils are capable of inhibiting α-amylase activity. However, further *in vivo* study is essential to assess and ascertain such activity.

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