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COMPARISON OF DIFFERENT METHODS IN DETERMINATION OF ESSENTIAL OIL COMPOSITION OF *ORIGAUM SYRIACUM* L. FROM JORDAN AND ITS MODULATION OF PANCREATIC ENZYMES

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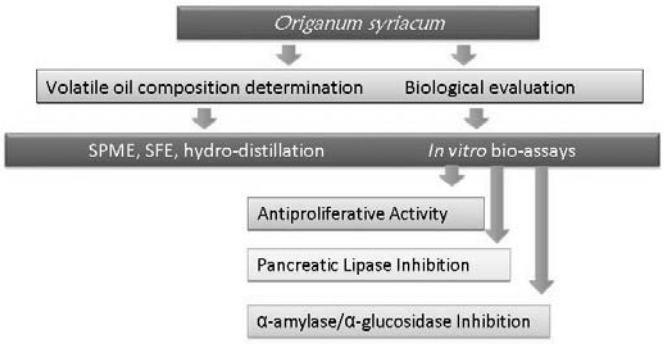
The volatiles emitted from the fresh and dry leaves, determined by solid-phase-micro-extraction (SPME), the essential oils obtained by hydro-distillation and supercritical-fluid-extraction (SFE) from the leaves of *Origanum syriacum* L. (Lamiaceae), grown in Jordan, were analyzed by GC-MS. In all applied methods, oils were rich in monoterpenes, while sesquiterpenes were found in very low concentrations. Thymol was the main component of the hydrodistilled oils of fresh and dry leaves of *O. syriacum* (84.8 and 70.5%, respectively) while carvacrol dominated the oil of the fresh leaves (61.1%) in SFE experiment. The aroma profile of the fresh and dry leaves, obtained through SPME was composed of *p*-cymene, α-terpinene and γ-terpinene. Biologically, the dual alpha-amylase/alpha glucosidase- and pancreatic lipase-inhibitory activities as well as anti-proliferative potential of the aqueous extract (AE) were screened. *O. syriacum* AE as well as its volatile constituents, *p*-cymene and thymol inhibited in a dose dependent trend pancreatic lipase (PL) *in vitro* ($p<0.001$, n=3), similar to orlistat. The PL- IC_{50} values were: orlistat; 114.0 ± 4.0 ng/mL, *O. syriacum* AE; 1.1 ± 0.2 mg/mL, thymol; 1.06 ± 1.2 µg/mL, and *p*-cymene; 0.03 ± 0.0 (% V/V). Comparable to acarbose, *O. syriacum* leaves AEs were identified as *in vitro* dual inhibitors of α-amylase and α-glucosidase with an IC_{50} value of 2.0 ± 0.1 mg/mL ($p<0.001$, n = 3). No antiproliferative effects against colorectal cancer cell lines HT29, HCT116, and SW620 were detected for *O. syriacum* AE (25 µg/mL).



INTRODUCTION

The genus *Origanum* (Lamiaceae) with 38 species consists of annual, perennial and shrubby herbs and is widely spread in upper Jordan Valley, Italy, Greece and Turkey.[1] Members of the genus *Origanum* are among the most important aromatic plants worldwide. *O. syriacum* L. (*Majorana syriaca* (L.) Rafin.) is one of the few wild growing species of this genus in Jordan, widely used as a herbal remedy, and in the food and beverage industry.[2,3] It can be classified as an edible, aromatic and medicinal plant, referred in English with the common names "Syrian oreganum", "hyssop" or "oregano" and in Arabic as "bardaqush suri" or "za'ter". The leaves of *O. syriacum* are used nearly on a daily basis in Jordan. Dried and powdered leaves mixed with sumac (*Rhus*

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coriaria) and sesame seeds (*Sesamum indicum*), referred simple as "za'ter", eaten with bread and dipped in olive oil, is a culinary specialty of Jordan. In the local traditionally medicine, infusions of the leaves are used for eye ailments, burns, as carminative, digestive, diaphoretic, common cold and flu, for the relief of arthritic joints and easing of coughs. It has been suggested for reducing memory loss in Alzheimer's disease, relief from kidney sand and stones, lowering of blood sugar and in nervous conditions. Seeds are used as sedative.[4-6]

The chemical composition of the volatile oil obtained from *O. syriacum* has been determined by several researchers from the Middle Eastern and Mediterranean countries. These studies, using hydro-distilled oils, exhibited great varieties in the composition of major and minor components. In addition to thymol and carvacrol, γ- terpinene and *p*-cymene were reported as major components depending on the chemotype, time of collection and methods of drying.[7-10] Nearly equal amounts of thymol and carvacrol were identified as the most abundant components of the essential oil of *O. syriacum*, collected in Lebanon.[7] *O. syriacum* volatile oil, obtained from wild growing and cultivated samples from El Arish- Egypt, yielded thymol together with *cis*-sabinene hydrate and γ-terpinene as major components.[8] Recently, El-Gandy et al. (2015) compared the composition of the essential oils of the wild growing and cultivated *O. syriacum* samples from Egypt and reported that carvacrol dominated the oil of the cultivated type while thymol together with γ-terpinene and linalool constituted about 50% of the volatile oil of the wild population.[9] The composition of the volatile oil of several Syrian populations of *O. syriacum* was dominated by carvacrol and/or thymol with a high degree of polymorphism in the occurrence of these two compounds.[10] In another Mediterranean country, in Turkey, the samples collected from the southern part of the country yielded carvacrol and γ-terpinene as the two major constituents while thymol was quantified only in concentrations as low as 2.1%.[11]

The essential oil and the crude extracts (aqueous and hydroalcoholic) of different *Origanum* species have been biologically evaluated to justify the traditional claims of their medicinal value in Jordan and in many Mediterranean countries. Their essential oils and extracts are reported to have ovicidal, bioherbicidal, insecticidal, antifungal, antimycotic, antibacterial, amoebicidal, cytotoxic, antioxidant, and anti-inflammatory activities.[11-18] Alleviation of respiratory and neurological disorders was ascribed to *O. syriacum*.[6,18] *O. syriacum* ethanol extract exhibited antiproliferative activity towards MCF7 breast cancer cell lines with an IC_{50} value of 6.40 μg/mL.[19]

The present study aimed to determine the chemical composition of the essential oil of *O. syriacum* using hydro-distillation and SFE and to compare these findings to the aroma profile of the fresh and dry leaves obtained by SPME. Biologically, the claimed hypoglycemic activity and the possible pancreatic lipase (PL) inhibitory and antiproliferative efficacy against colorectal cancer cell lines (HT29, HCT116, and SW620) were determined for the aqueous extract (AE) and for some pure volatile compounds. In accordance with the traditional use of *O. syriacum* as an infusion in Jordan, AE was selected for the screening of the biological activities. To the best of our knowledge this is the first report applying SPME and SFE for *O. syriacum*.

RESULTS AND DISCUSSION

Phytochemical determinations

The hydro-distillation of the fresh and coarsely chopped air dried leaves of *O. syriacum* afforded a colorless oil (1.4% and 1.6%, v/w, respectively) while the yield by SFE was 0.6%. The essential oil components were identified in GC-MS analysis based on the built-in libraries (NIST Co. and Wiley Co., USA) and by comparison of the obtained RI relative to (C_8-C_{20}) *n*-alkanes literature values measured with columns of identical polarity and MS fragmentation patterns to those of standard compounds.[20] The identification of *α*- and *β*-pinenes, *p*-cymene, limonene, linalool (Fluka, Buchs, Switzerland), α-and γ-terpinenes and sabinene hydrate (Sigma-Aldrich, Buchs, Switzerland) were further confirmed using reference substances. The obtained results are presented in Table 1.

Analysis of the SPME obtained volatiles of *O. syriacum,* using fresh and dried leaves, resulted in the identification of each twenty two compounds amounting to 98.6% and 96.4% of the total oil content, respectively. Low molecular weight monoterpene hydrocarbons, with γ-terpinene (31.6%) and *p*-cymene (39.1%) as the major components, dominated the aroma profile of the fresh and dry plants (87.9% and 83.9%, respectively). The less volatile sesquiterpene hydrocarbons hardly reached 2.0%.

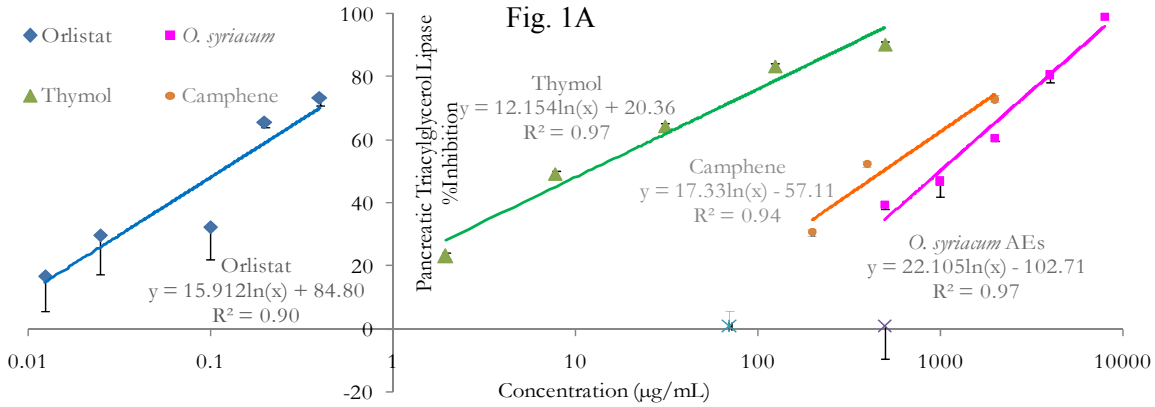
GC/MS analysis of the hydro-distilled oil obtained from fresh and dried leaves led to the identification seventeen components, accounting for 98.0% and 96.8%, respectively, of the total oil content. In contrast to the SPME volatiles, both oils were strongly characterized by the presence of oxygenated monoterpenes, comprising 89.6% of the oil content of fresh leaves and 84.6% of the oil obtained from the dried leaves with thymol dominating this fraction (84.8% and 70.5%, respectively). Monoterpene hydrocarbons and sesquiterpenes were poorly represented.

Table 1

Comparison of the essential oil composition of fresh and dry leaves of *Origanum syriacum* using SPME, hydro-distillation and SFE

RI Lit[a].	RI Exp[b]	Compound	*OS 1 %[c]	*OS 2 %	*OS 3 %	*OS 4 %	*OS 5 %
859	862	Hexenol<3Z >	0.6	-	-	-	-
930	931	α- Thujene	6.1	5.4	0.4	-	0.3
0939	939	α-Pinene	2.7	2.5	-	0.1	0.3
954	957	Camphene	0.4	0.5	-	-	0.1
975	979	Sabinene	1.0	0.4	-	-	0.1
979	982	β- Pinene	0.1	0.5	-	-	0.4
979	979	Octen-3-ol	-	-	1.5	6.7	-
991	994	Myrecene	8.1	6.3	0.3	0.3	-
1003	1005	α-Phellandrene	2. 4	1.5	-		0.2
1017	1020	α-Terpinene	11.5	7.1	0.2	0.5	0.9
1025	1030	*p*-Cymene	20.3	39.1	1.1	2.6	4.5
1026	1029	*o*-Cymene	2.1	0.5	-	-	0.1
1029	1030	Limonene	0.9	0.3	-	-	-
1050	1052	β-Ocimene (E)	0.5	0.2	-	-	-
1060	1061	γ-Terpinene	31.6	19.4	1.9	0.8	1.0
1070	1073	*cis*-Sabinene hydrate	0.6	1.0	0.4	1.6	0.9
1089	1092	Terpinolene	0.2	0.2	-	-	-
1097	1100	Linalool	-	-	-	-	0.2
1098	1100	*t*-Sabinene hydrate	-	-	0.3	0.4	0.2
1129	1129	Menth-2-en-1-ol	-	-	-	-	-
1169	1168	Borneol	-	0.1	0.4	0.3	0.3
1177	1182	Terpinene-4-ol	-	-	0.4	0.4	0.2
1189	1191	α-Terpineol	-	0.2	-	-	0.4
1199	1203	γ-Terpineol	-	-	0.2	0.2	-
1216	1219	Linalyl formate	0.1	-	-	-	-
1235	1238	Thymol methyl ether	-	-	-	-	0.4
1245	1247	Carvacrol methyl ether	-	-	-	-	0.1
1252	1254	Thymoquinone	0.6	1.7	0.9	2.2	2.4
1290	1294	Thymol	0.3	0.4	84.8	70.5	20.5
1299	1300	Carvacrol	6.8	7.1	2.2	9.0	61.1
1419	1422	Caryophyllene (E)	1.4	1.6	1.1	0.3	1.5
1441	1442	Aromadendrene	-	-	-	-	0.1
1500	1502	Bicyclogermacrene	-	-	-	-	0.5
1506	1510	β-Bisobolane	0.3	0.4	1.2	0.4	-
1583	1588	Caryophyllene oxide	-	-	-	0.2	0.1
1749	1753	α-Bisabolol oxide A	-	-	0.7	-	-
		Terpenoids	98.0	96.4	96.5	90.1	96.8
		Monoterpenes	96.3	94.4	93.5	88.9	94.6
	Monoterpene hydrocarbons		87.9	83.9	3.9	4.3	7.9
	Oxygenated monoterpenes		8.4	10.5	89.6	84.6	86.7
		Sesquiterpenes	1.7	2.0	3.0	1.2	2.2
	Sesquiterpene hydrocarbons		1.7	2.0	2.3	0.7	2.1
	Oxygenated sesquiterpenes		-	-	0.7	0.5	0.1
		Miscellaneous	0.6	-	1.5	6.7	-
		Total identified	98.6	96.4	98.0	96.8	96.8

OS 1:*O. syriacum* fresh SPME OS 2: *O. syriacum* dry SPME OS 3: *O. syriacum* fresh hydro-distilled OS 4: *O. syriacum* dry hydro-distilled OS 5: *O. syriacum* fresh SFE [a]RI Lit., Reported Retention Index [20]; [b]RI Exp., Retention index relative to (C_8-C_{20}) *n*-alkanes; [c]The percentage composition based on the GC peak areas.



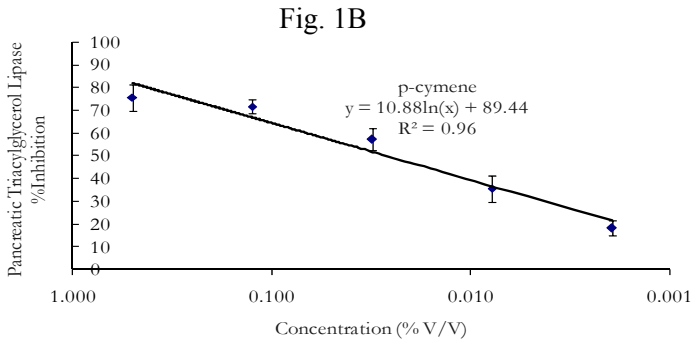


Fig. 1A-B – *In vitro* inhibitory effects of *O. syriacum* leaves AE, some of their volatile principles, and orlistat on PL activity. Results are mean ± SEM (n = 3 independent replicates).

The comparison of the composition of the volatile components detected by both methods revealed that thymol and/or carvacrol, reported as the two main constituents of the hydro-distilled oregano oil in the majority of the studies, are found in very low concentration in the emitted aroma of *O. syriacum* using SPME. This indicates that they are produced from their non-oxygenated biogenetic precursors, terpinenes and cymenes, on exposure to heat during hydro-distillation. On the other hand, since SPME reflects the actual composition of the volatile substances in the aromatic plants, the highly volatile monoterpene hydrocarbons were identified as major components. The present study, with the best of our knowledge, is the first evaluation of *O. syriacum* volatile components using the latter method hence no similar data were available for qualitative and quantitative comparison of the findings.

Supercritical-fluid-extraction is considered as an alternative method for the production of flavors and fragrances in the food industry preventing thermal degradation, hydrolysis and solvent contamination of genuine compounds in the plants. The advantages of SFE over hydro-distillation are described by several researchers especially using *Salvia* species.[21-25] In the present study, carvacrol was found as the major oxygenated terpene in the SFE method, while SPME indicated very low concentration of the oxygenated monoterpenes. This difference between these two sensitive methods, SPME and SFE, might be influenced by the time factor, since the oil obtained by SFE has been refrigerated for some time before GC-MS analysis while the SPME method with the fresh samples was applied immediately after collection of the plant material.

In vitro inhibitory effects of *O. syriacum* AE and volatile oil constituents on PL activity

The PL inhibitory profiles of the AE of *O. syriacum* leaves and two of its volatile constituents are shown in Figure 1A-B. Orlistat PL-IC_{50} value of 114.0 ± 4.0 ng/mL, equivalent to 0.2 ± 0.0 µM, is comparable to reported PL-IC_{50} values elsewhere.[26] Like orlistat, a marked concentration-dependent PL inhibition trend was obtained for *O. syriacum* AE as well as its volatile components. PL-IC_{50} values obtained for a minimum of triple independent determinations are also enlisted (Table 2).

In vitro inhibitory effects of *O. syriacum* leaves AE on enzymatic starch digestion

With acarbose (0.1 mg/mL) as the reference drug, glucose liberation from starch was inhibited by 97.6% highly substantially ($p<0.001$, vs. drug-free control incubations, n=3, Fig. 2). Fig. 2 demonstrates that *O. syriacum* leaves AEs concentrations 0.1-10 mg/mL had highly substantial dose-related reductions in aldohexose release from culinary polymeric cornstarch ($p<0.001$ vs. plant-free control determinations, n=3). The IC_{50} value of *O. syriacum* leaves was 2.0±0.1 mg/mL. Various studies were conducted to explore medicinal plants as potential therapeutic agents for dual management of diabetes and hyperlipidemia via digestive enzymes' inhibition, namely pancreatic alpha-amylase, intestinal alpha-glucosidase and pancreatic triacylglycerol lipase.[26-28] This is the first report on *O. syriacum* inhibitory capacities of starch hydrolases and intestinal lipase.

Antiproliferative activity of *O. syriacum* in colorectal cancer cell lines

Table 3 illustrates the lack of *O. syriacum* AE (25 μg/mL) antiproliferative efficacies in any of the colorectal carcinomas panel incubations; despite their notable activity at the concentration 200 μg/mL. Doxorubicin respective IC_{50} (μg/mL) values for the tested cell lines were 0.09±0.014 (HT29); 0.11±0.017 (HCT116), and 0.7±0.011 (SW620).

Table 2

Pancreatic Lipase IC_{50} values for tested concentrations of *O. syriacum* leaves AEs, its volatile principles and orlistat

Extract/pure compounds	PL-IC_{50}
O. syriacum AE	1.1 ± 0.2 mg/mL
Thymol	10.6 ± 1.2 (x10^{-3}) mg/mL
p-cymene	0.03 ± 0.0% V/V
Orlistat	0.114 ± 0.0 (x10^{-3}) mg/mL

Results are mean ± SEM (n = 3 independent replicates)

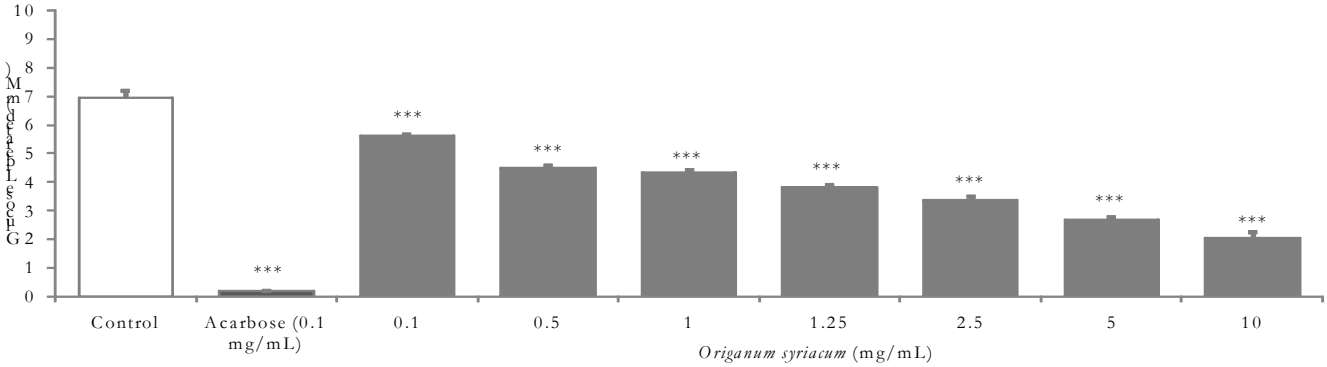


Fig. 2 – *In vitro* inhibitory effects of *O. syriacum* leaves AE on enzymatic starch digestion. Results are mean ± SEM (n = 3 independent replicates). Statistical significance of difference from corresponding control incubations' values: ***P<0.001.

Table 3

Lack of *in vitro* antiproliferative activity of *O. syriacum* AE (25 μg/mL) on colorectal cancer cell lines

Treatment	Cytotoxicity (As%Control)		
	HT29	HCT116	SW620
O. syriacum 200 μg/mL	87.6±1.0	95.6±0.3	80.8±1.7
O. syriacum 25 μg/mL	1.1±2.0	12.2±3.0	15.3±2.7

Results, representing% inhibition of cell proliferation in comparison to non-induced basal 72 h incubations, are mean ± SEM (n = 4 independent replicates).

EXPERIMENTAL

General experimental procedure

Unless stated otherwise, all reagents and chemicals were from Sigma (Dorset, UK). Glucose GOD-PAP kit was obtained from BioLabo Reagents (France). GC-grade hexane and analytical reagent grade anhydrous Na_2SO_4 were purchased from Scharlau (Barcelona, Spain) and UCB (Bruxelles, Belgium), respectively. Reference substances for GC-MS were obtained from Fluka (Buchs, Switzerland). UV-VIS spectrophotometery was determined by SpectroScan 80D (UK). In the SPME experiments fiber assemblies (PDMS/DVB; d_f 65 µm, length 1 cm) for manual sampling (Supelco, USA) were used. The GC-MS analysis was performed using Varian chrompack CP-3800 GC/MS/MS-200 (Saturn, Netherlands) fitted with DP-5 (5% diphenyl, 95% dimethyl polysiloxane) GC capillary column (30 m ×0.25 mm i.d., 0.25 µm film thicknesses). For the quantitative analysis (% area), a Hewlett-Packard HP-8590 gas chromatograph equipped with a split-splitless injector (split ratio 1:50) and an FID detector and an optima-5 (5% diphenyl, 95% dimethyl polysiloxan) fused silica capillary column (30 m × 0.25 mm, 0.25 µm film thickness) were used.

Plant material and extract preparation

Leaves of *O. syriacum* were collected from the vicinity of the University of Jordan campus during early flowering period in 2014 and were identified by one of the authors (A. Gabbiesh). One part of the collected leaves was on the day of collection submitted to hydro-distillation and SPME analysis as fresh leaves. The other part was air dried at room temperature (RT) in the shade until constant weight and was assayed for essential oil composition. Additionally supercritical-fluid-extraction (SFE) was done with fresh *O. syriacum* leaves. Voucher specimens (LAM 101/FMJ) have been deposited in the Department Pharmaceutical Sciences, School of Pharmacy, The University of Jordan, Amman, Jordan. AE for *in vitro* experiments were prepared as described elsewhere.[29] For pancreatic lipase (PL) experimentation; solid residue, obtained after evaporation of the AE, was used.

Hydro-distillation and solid phase micro-extraction (SPME) of the volatile constituents from *O. syriacum* GC-MS and GC-FID analysis

Both experiments were performed under the same conditions as described by Afifi et al.[30] The extractions and analytical experiments were repeated twice. Identification of compounds was based on the built in libraries (NIST Co and Wiley Co, USA) and by comparing their calculated retention indices (RI) relative to (C_8-C_{20}) *n*-alkanes literature values measured with columns of identical polarity, or with authentic samples.[20]

Supercritical fluid extraction (SFE)

The extraction experiments were carried out in a laboratory SFE plant whose P&I diagram has been previously described.[21-22] In the SFE experiment, 10 g of plant material, that work alternatively provided an uninterrupted flow of CO_2 (liquid CO_2; P99.9%) compressed up to the desired operating pressure (40.0±0.2 MPa). The pressurized solvent was preheated to the desired extraction temperature (40 ^oC) before entering the extractor. The extractor was held in an oven whose temperature is controlled within an accuracy of ±0.5 ^oC. The CO_2 flow was set to 1.5±0.2g/min. Depressurized CO_2 was quantified with a totalizer flow meter. Extraction yield was determined gravimetrically by weighing the extract at the end of extraction run. Extraction was repeated three times.

Spectrophotometric quantification of Pancreatic Lipase (PL) activity and assaying PL inhibition of test extract

Tested aqueous extracts were initially dissolved in Tris-HCl buffer (2.5 mM (Promega, USA), pH 7.4 with 2.5 mM NaCl) to give five initial stock solutions with a concentration range of 25 – 400 mg/mL. Subsequently, 20 µL aliquot of each stock solution was used in the reaction mixture to give a final concentration range of 0.5 – 8 mg/mL. Dissolved in DMSO; thymol (mg/mL) and *p*-cymene (V/V%) were prepared into five stock solutions with an initial concentration range of 0.098 - 100. Thereafter, 20 µL aliquot of each stock solution was used in the reaction mixture to give a final concentration range of 0.002 – 2. Finally, orlistat, the reference drug (in DMSO; 1 mg/mL), was prepared into six different stock solutions with a concentration range of 0.625 – 20 µg/ mL. Thereafter, 20 µL aliquot of each stock solution was used in the reaction mixture to give a final concentration range of 0.0125 – 0.4 µg/mL. *In vitro* enzymatic PL activity was assayed according to Al-Hallaq et al.[26] Subsequent determinations were undertaken for the tested extracts and pure compounds in comparison to control evaluations, to calculate the concentration required for PL 50% inhibition (IC_{50}).

In vitro enzymatic starch digestion assay

The extent of polysaccharide breakdown into glucose was evaluated in a concentration range of plants' AEs 1, 5, 10, 12.5, 25, 50 and 100 mg/mL. The effect of the reference drug acarbose at 1000 µg/mL concentration was evaluated as well. Control samples contained neither acarbose nor plant extract.[26-27]

In vitro antiproliferative assay

The cytotoxicity measurements with the colorectal cell lines HT29, HCT116 and SW620 (generously provided by Dr Nizar Mhaidat, Jordan University of Science and Technology, Jordan) and doxorubicin as reference antineoplastic drug were determined using Sulforhodamine B (SRB) colorimetric assay as described previously.[30] Doxorubicin IC_{50} values, in comparison to non-induced basal 72 h incubations, were calculated within treatment concentration range 0.1-50 µg/mL.

Statistical analysis

The values are presented as mean ± S.E.M. (Standard Error of the Mean) of 3-4 independent experiments. Statistical differences between control and different treatment groups were determined using Graphpad Prism one way analysis of variance (ANOVA) followed by Dunnett post test whenever appropriate (version 3.02 for windows; GraphPad Software, San Diego, CA, USA). Values were considered significantly different if $P<0.05$ and highly significantly different if $P<0.01$ and $P<0.001$.

CONCLUSION

The SPME technique was found to be more sensitive in identification of volatile terpenoid compounds, suitable for the quick screening of big

number of plant specimens in determination of their aroma profile. It is a very simple, rapid, solvent-free and inexpensive method that deserves to be considered as an alternative technique in analysis of volatile compounds from plants. Still, for the determination of the volatile components of the aromatic plants hydro-distillation is essential since in the traditional medicine these plants are used in form of teas. Biologically, *O. syriacum* phytochemicals can inhibit crucial gastrointestinal enzymes involved in carbohydrate and lipid digestion and absorption thus advocating a dual-target management strategy in metabolic syndrome and obesity-diabetes (diabesity).

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