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Research Article

PHYTOCHEMICAL STUDY OF BIOACTIVE CONSTITUENTS FROM *SATUREJA MONTANA* L. GROWING IN EGYPT AND THEIR ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES

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ABSTRACT

Objective: This work aimed to investigate the lipid constituents and flavonoidal compounds of *Satureja montana*, in addition to evaluation of different extracts and/or isolated compounds as antimicrobials and antioxidants.

Methods: The volatile and lipid constituents were extracted with n-hexane by partition from hydroalcoholic extract of *S. montana* L. aerial parts, after then were fractionated to unsaponifiable matters and fatty acid methyl esters which were identified by gas–liquid chromatography and/or gas chromatography–mass spectrometry. The phenolic constituents were isolated from the ethyl acetate fraction of the aqueous methanolic extract of the aerial parts of the plant. The antimicrobial activity of different extracts and the isolated compounds was evaluated against Gram-positive, Gram-negative bacteria, yeast, and fungus using a modified Kirby-Bauer disc diffusion method.

Results: The identified compounds are luteolin-7-rhamnoside-4′-*O*-β-glucopyranoside (1), quercetin-3-*O*-α-L-rhamnopyranoside (2), quercetin-7-*O*-glucopyranoside (3), luteolin-7-*O*-glucopyranoside (4), 5-hydroxy-6,7,8,4′-tetramethoxy flavone (5), gallic acid (6), 2,3-hexahydroxydiphenoyl 1-galloyl glucopyranoside (7), and quercetin (8). The structure of all isolated compounds was established using different chromatographic and spectroscopic measurements (PC, thin-layer chromatography, ultraviolet [UV], 1D, 2D-nuclear magnetic resonance, and MS). Compound-2 showed the highest antibacterial activity against all the tested microorganisms. Hydroalcoholic extract exhibited high antioxidant activity (87.7%). On the other hand, hexane fraction showed a low antioxidant activity (46.4%), in addition to the compound-8 showed the highest antioxidant activity (96.27%) in 2,2-diphenyl-1-picrylhydrazyl assay.

Conclusion: It can be concluded that the hydroalcoholic extract of *S. montana* showed significant antimicrobial and antioxidant activity.

Keywords: *Satureja montana*, Family Lamiaceae, Lipid constituents, Flavonoids, Antimicrobial, Antioxidant activity.

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INTRODUCTION

The genus *Satureja* L. belonging to the family Lamiaceae contains about 200 species of aromatic herbs and shrubs that are native to warm temperate regions and may be annual or perennial grow in the Middle East, Mediterranean region to Europe, West Asia, and North Africa. Over 30 species of this genus are distributed in eastern parts of the Mediterranean area [1]. Many of these species have different biological activities such as antibacterial, antifungal, antioxidant, cytotoxic, insecticidal, antidiabetic, anti-leishmanial, insect repellant, hepatoprotective, antiviral, anticholinesterase, hypolipidemic-hypoglycemic, anti-inflammatory, antinociceptive, nematicidal, anti-proliferative, genotoxic, anti-genotoxic, neuroprotective, ovicidal, anti-biofilm, molluscicidal, antihelmintic, herbicidal, anti-epilepsy, anti-Alzheimer, amebicidal, nephroprotective, anti-lipase, wound healing, trypanocidal/anti-protozoal, enzyme inhibition, anti-spasmodic, vasodilatory-vasorelaxant, anti-tumoral, and diuretic activities [2-9]. Previously, phytochemical analysis of *Satureja* species revealed the presence of volatile oils, phenolic acids, anthocyanins, flavones, diterpenes, triterpenes, and sterols [10-14].

The major constituents of hydrodistilled volatile oils from the aerial parts of *Satureja montana* and *Saxifraga cuneifolia* growing in Croatia were identified by gas chromatography–mass spectrometry (GC–MS) and found carvacrol (17.7%) and spathulenol (13.2%) as the major compounds [15]. Twenty one compounds in the oil of *Satureja montana* essential oil were identified by GC-FID analyses and carvacrol was the main (60 %) [9]. Many flavonoids were isolated from *Satureja khuzistanica* [16]. The identified flavonoids are aromadendrin, taxifolin, naringenin, 5,7,3′,5′-tetrahydroxy flavanone, xanthomicrol, acacetin, cirsimaritin, 7-methoxyluteolin, apigenin, cirsilineol, diosmetin, and 6-hydroxyluteolin 7,3′- dimethyl ether, in addition to a new monoterpene–flavonoid known as saturejin, while Matloubi-Moghaddam *et al*., in 2007, isolated β-sitosterol, β-sitosterol-3-*O*-β-D-glucoside, ursolic acid, and 4′,5,6-trihydroxy-3′,7-dimethoxyflavone from the dichloromethane extract of the same plant.

Ahamad *et al*., in 2005 [17], isolated thymol, oleanolic acid, ursolic acid, and caryophyllene oxide from the aerial parts of *S. macrantha* and these compounds were effective against *Artemia salina* larvae. While luteolin, oleanolic acid, β-sitosterol, and diosmetin were isolated from the ethyl acetate and methanol extracts of *Satureja sahendica* [13].

The surface flavonoids in *Satureja thymbra* and *Satureja spinosa* were studied [18] and identified the following compounds: Naringenin, aromadendrin, eriodictyol, taxifolin, apigenin, genkwanin, ladanein, cirsimaritin, thymusin, xanthomicrol, luteolin7-methyl ether, 6-hydroxyluteolin 7,3′-dimethyl ether, 6-hydroxyluteolin 7,3′,4′-trimethyl ether, cirsilineol, thymonin, and 8-methoxycirsilineol.

METHODS

Plant material

S. montana L. was cultivated and grown in the farm of National Research Center (NRC), Giza, Egypt, and it was collected in May 2015. The authentication of plant sample was achieved by Dr. Mohammed Algebaly a taxonomist at NRC. A voucher specimen was deposited in the herbarium of the NRC. The aerial parts were air-dried for 2 weeks under laboratory conditions at 28 ± 2°C. The dried material was ground using a domestic blender to fine powder.

Instruments and chemicals

UV spectra were recorded on Shimadzu model UV-240 and 2401 PC spectrophotometer (Shimadzu Inc., Tokyo, Japan). Buchi apparatus was used to determine the melting points of the isolated pure compounds in open capillaries. Nuclear magnetic resonance (NMR) experiments were recorded on Bruker spectrometer (Switzerland) 600 (^{1}H NMR spectra: 600 MHz; ^{13}C NMR spectra: 150 MHz). The chemical shifts are given in δ (ppm) relative to tetramethylsilane (Me_4Si). Column chromatography (CC) was carried out on Polyamide S6: (Riedel-De-Haen AG, Seelze Haen AG, Seelze Hanver, Germany) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Paper chromatography (PC, descending) Whatman No. 1 and 3 mm papers, using solvent systems (1) H_2O, (2) 15% HOAc (H_2O: HOAc, 85:15), (3) BAW (n-BuOH: HOAc:H_2O, 4:1:5, upper layer), and (4) BBPW (C_6H_6:n-BuOH: Pyridine:H_2O, 1:5:3:3, upper layer).

Isolation and purification of the chemical constituents

The powder of the aerial parts (2 kg) was extracted 3 times at room temperature with 70% methanol. The aqueous-methanol extract was evaporated under reduced pressure and temperature to obtain a residue of 350 g. The residue was defatted by n-hexane giving (80 g) n-hexane extract and (270 g) methanol extract.

Fractionation of lipid constituents

A fraction (1 g) of the n-hexane extract was analyzed for volatile constituents by GC/MS and after then, the rest of the n-hexane extract was passed over fuller's earth (to remove the colored pigments). The solvent was evaporated under reduced pressure at 35°C; the obtained residue (20 g) was saponified to give unsaponifiable matters and the fatty acid methyl esters [19], which were analyzed by GC/MS and/or gas–liquid chromatography (GLC) as follows.

GC/MS analysis of volatile constituents of n-hexane extract

The analysis of n-hexane fraction of *S. montana* was performed using a Thermo Scientific capillary gas chromatography (model Trace GC ULTRA) directly coupled to ISQ Single Quadruple MS and equipped with TG-5MS, non-polar 5% phenyl methylpolysiloxane capillary column (30 m × 0.25 mm ID × 0.25 μm). The operating condition of GC oven temperature was maintained as initial temperature 40°C for 3 min, programmed rate 5°C/min up to final temperature 280°C with isotherm for 5 min. For GC/MS detection, an electron ionization (EI) system with ionization energy of 70 eV was used. Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. 1 μl of the extract was injected automatically in the splitless mode. The quantification of the components was based on the total number of fragments (total ion count) of the metabolites as detected by the mass spectrometer. Identification of the constituents was carried out by comparison of their retention times and fragmentation patterns of mass with those of published data and/or with those of the Wiley and NIST mass spectra libraries.

GLC analysis of unsaponifiable matters and GC/MS of fatty acid methyl esters

The GLC analysis was carried out for unsaponifiable matters using the following conditions; Instrument: Varian model 3700 GC. Column for unsap.: 10% OV-101 on chromosorb W/HP, 80/100, (2 m stainless steel, 0.25 mm i.d.), column for unsap.: Column: 70°C up to 270°C, 4°C/min., injector: 280°C., detector (FID): 290°C., temperature for unsap.: Column:70°C up to 270°C, 4°C/min., injector: 280°C., detector (FID): 290°C. While the conditions for FAMEs are instrument: A TRACE GC ultragas chromatographs (THERMO Scientific Corp., USA) coupled with a thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC–MS system was equipped with a TG-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10 using the following temperature program: 80°C for 1 min; rising at 4.0°C/min to 300°C and held for 1 min. The injector and detector were held at 240°C. Diluted samples (1:10 hexane, v/v) of 0.2 μL of the mixtures were always injected. Mass spectra were obtained by EI at 70 eV, using a spectral range of m/z 40–450.

Isolation of the phenolic constituents

The methanol extract was subjected on polyamide S6 CC, eluting with H_2O followed by MeOH/H_2O mixtures of decreasing polarity which yielded four fractions 40, 60, 80, and 100% methanol. Each fraction was subjected to further purification using columns chromatography (small polyamide and Sephadex LH-20) and preparative paper chromatography (Whatman 3MM, with different solvent systems). The fraction 40% MeOH/H_2O yielded compound 1 (14 mg), followed by the fraction 60% MeOH/H_2O which yielded compound 2 (16 mg), compound 3 (17 mg), compound 4 (13 mg), and compound 5 (15 mg). On the other hand, compound 6 (13 mg) and compound 7 (18 mg) were isolated from the fraction 80% MeOH/H_2O, while 100% MeOH fraction yielded compound 8 (19 mg).

Acid hydrolysis

It was carried out for 2 h at 100°C using 2N HCl; the hydrolyzed part was then extracted with ethyl acetate; the extract being subjected to PC investigation to detect the aglycones. Sugars were identified by PC using (benzene:n-butanol:pyridine:water, 1:5:3:3) with authentic sugars. The dried chromatograms were visualized by aniline phthalate reagent, the sugar spots were observed in daylight. The tested sugars were compared with reference sugars. Identification of the aglycones was based on direct PC using authentic samples and/or spectral analyses.

Antimicrobial activity study

The antimicrobial activity of the tested extracts and/or isolated compounds against some microorganisms was determined using a modified Kirby-Bauer disc diffusion method [20-22].

Plates inoculated with filamentous fungi as *Aspergillus flavus* at 25°C for 48 h; Gram (+) bacteria as *Staphylococcus aureus* and *Bacillus subtilis*; Gram (-) bacteria as *Escherichia coli* and *Pseudomonas aeruginosa*, they were incubated at 35–37°C for 24–48 h and yeast as *Candida albicans* incubated at 30°C for 24–48 h, and then, the diameters of the inhibition zones were measured in millimeters [20]. The diameter of the inhibition zone surrounding the sample is taken as a measure of the inhibitory power of the sample against the particular test organism. Standard discs of ampicillin (antibacterial agent), amphotericin B (antifungal agent) served as positive controls for antimicrobial activity.

Calculations

% inhibition = sample inhibition zone (cm)/plate diameter ×100. The filter discs impregnated with 10 μl of solvent (distilled water, chloroform, DMSO) were used as a negative control. The test was carried out in triplicates [23].

Antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging method

DPPH scavenging activity of the tested extracts and/or isolated compounds was measured at 517 nm by spectrophotometer method [24]. A 1 mL solution of 0.004% DPPH solution was added to the tested extracts and/or isolated compounds. The solution in the test tubes was shaken well and incubated in the dark for 30 min at room temperature. The disappearance of violet color of methanolic DPPH solution indicates scavenging capacity of the extract considered as the positive reaction.

RESULTS AND DISCUSSION

The GC/MS analysis was used to identify the volatile constituents from n-hexane fraction of *S. montana*, 39 compounds belonging to many classes such as monoterpenes (30.7%) with carvacrol as the major one (24.23%), sesquiterpenes (4.06%) in which farnesyl acetone is the main, diterpenes (17.67%), one saturated fatty acid (palmitic acid, 17.1%), esters of fatty acids (10.14%) with methyl hexadecatrienoate as a main ester, phthalates constituted about 2.9%, and a sterol fraction of about 2.7% were identified (Table 1). These data were in accordance with that reported [15], where they identified the major constituents of hydrodistilled volatiles from the aerial parts of *S. montana* growing in Croatia and they found carvacrol (17.7%) as the major compound. Furthermore, Hanene *et al.*, 2013, identified carvacrol (53.17%) as a main in the winter *S. montana* [25].

The fatty acids (Table 2) were found to contain a mixture of nine fatty acids from which six acids were identified and constituted about 91.28%. The saturated fatty acids four acids with palmitic acid as major (40.19%) and the unsaturated acids constitute about 47.64%, in which linolenic acid is the main acid. These data are very near from that reported by Ahmet *et al.,* in 2003 [19], where they studied the fatty

Table 1: GC/MS analysis of n-hexane fraction of *S. montana*

Peak No.	Rt (min.)	Rel. %	Molecular weight	Molecular formula	Compounds
1	19.93	0.41	164	$C_{10}H_{12}O_2$	Thymoquinone
2	21.33	24.23	150	$C_{10}H_{14}O$	Carvacrol
3	21.48	0.46	182	$C_{11}H_{18}O_2$	1,3-Dioxolane, 2,2-dimethyl-4,5-di-1-propenyl
4	21.99	0.6	182	$C_{10}H_{18}O_2$	2 (1H)-Naphthalenone, octahydro-8a-hydroxy-4a-methyl
5	23.38	0.62	192	$C_{11}H_{12}O_3$	3-Isopropoxyphthalide
6	25.62	0.56	180	$C_{10}H_{16}OSi$	Silane, (4-methoxyphenyl) trimethyl
7	26.37	2.1	220	$C_{13}H_{16}O_3$	Ageratochromene
8	27.47	1.99	180	$C_{11}H16O_2$	Dihydroactinidiolide
9	28.37	0.78	220	$C_{15}H_{24}O$	Spathulenol
10	29.54	0.59	190	$C10H_{12}O_2$	Megastigmatrienone
11	29.86	0.61	222	$C_{15}H_{26}O$	α-Bisabolol
12	30.2	0.51	220	$C_{15}H_{24}O$	Caryophyllene oxide
13	30.31	3.87	178	$C_{10}H_{10}O_3$	(S, S)-4-(2-Hydroxyproplonyl) benzaldehyde
14	30.56	0.41	290	$C_{19}H_{30}O_2$	Methyl, 2,5-octadecadiynate
15	30.64	0.48	220	$C_{12}H_{14}O_4$	Apiol
16	31.75	0.78	192	$C_{13}H_{20}O$	(4R, S) 4 (2Butyl) cis Bicyclo[4.3.0] 2nonen8one
17	31.91	2.89	108	C_7H_8O	2-(2-Propenyl)-furan
18	32.63	0.42	191	$C_{11}H_{13}NO_2$	6,7-Dimethoxy-3,4-dihydroisoquinoline
19	32.81	0.74	212	$C_{10}H_{12}O_5$	3,4,5-Trimethoxy benzoic acid
20	33.89	1.84	268	$C_{18}H_{36}O$	Hexahydrofarnesyl acetone
21	35.35	1.11	238	$C_{15}H_{26}O_2$	4,4,8-Trimethyltricyclo [6.3.1.0 (1,5)] dodecane-2,9-diol
22	35.62	12.5	270	$C_{20}H_{14}O$	8,8-Diphenylbicyclo [4.2.0]octa-1,3,5,-triene-7-one
23	36.43	1.08	278	$C_{16}H_{22}O_4$	Butyl phthalate
24	37.33	17.1	256	$C_{16}H_{32}O_2$	Palmitic acid
25	37.57	0.47	208	$C_{14}H_{24}O$	2,5,5,8a tetramethyl1,2,3,5,6,7,8,8a octahydro naphthalen1ol
26	38.89	2.08	294	$C_{19}H_{34}O_2$	Methyl linoleate
27	39.04	7.64	364	$C_{17}H_{28}O_2$	Methyl hexadecatrienoate
28	39.19	2.68	296	$C_{20}H_{14}O$	Phytol
29	39.46	0.42	298	$C_{19}H_{38}O_2$	Methyl isostearate
30	39.68	0.76	300	C20H28O2	Retinoic acid
31	39.76	1.73	318	$C_{21}H_{34}O_2$	Pregnanolone
32	40.02	0.6	300	$C_{19}H_{24}O_3$	4-Androst-4-one-3, 6,17-trione
33	40.13	0.43	408	$C_{23}H_{36}O_2S_2$	8,16,16 trimethyldithio Androst5ene3,17àdiol
34	40.6	2.06	256	$C_{14}H_{19}ClN_2O$	3-(2-chlorophenyl) quinazolin-4 (3H)-one
35	40.96	0.95	414	$C_{29}H_{50}O$	Clionasterol
36	42.29	0.45	412	$C_{29}H_{48}O$	Stigmasterol
37	42.42	0.64	334	$C_{21}H_{34}O_3$	3,17dihydroxy pregnan 20 one
38	44.8	0.6	306	$C_{19}H_{30}O_3$	11-a-Hydroxyandrosterone
39	46.33	1.81	390	$C_{24}H_{38}O_4$	Di (2ethylhexyl) phthalate

GC-MS: Gas chromatography–mass spectrometry, *S. montana: Satureja montana*

Table 2: GC/MS data of FAME of *S. montana*

Peak No.	Rt (min.)	Rel. %	Mol. Formula	Mol. Wt.	Compounds
1	15.86	40.19	$C_{17}H_{34}O_2$	270	Methyl Palmitate
2	19.74	5.68	$C_{19}H_{34}O_2$	294	Methyl Linolelaidate
3	19.98	40.59	$C_{19}H_{32}O_2$	292	Methyl Linolenate
4	20.44	3.01	$C_{19}H_{38}O_2$	298	Methyl Stearate
5	24.77	1.37	$C_{21}H_{42}O_2$	326	Methyl Arachidate
6	28.78	0.44	$C_{23}H_{46}O_2$	354	Methyl Behen
7	8.72		Unknowns		

GC-MS: Gas chromatography–mass spectrometry, *S. montana: Satureja montana*

acids in *S. thymbra* and *S. cuneifolia* and found both palmitic acid and linolenic acid are major in both species.

The GLC analysis (Table 3) proved that the unsaponifiable fraction consists of a mixture of hydrocarbons (89.97%) in which n-tricosane is the most abundant (27.7%) and a sterol fraction of 10.03% with stigmasterol as a main.

Identification of the phenolic compounds: Investigation of the phenolic constituents resulted in isolation of seven flavonoidal compounds, in addition to gallic acid, their structures were carried out through color reactions, R_f values, chemical investigations (acid hydrolysis), and spectral measurements (UV spectral analysis in methanol and on addition of shift reagents confirmed the aglycone and glycosidic linkage, 1D and 2D^{1}H NMR, ^{13}C-NMR). Further, authentication was carried out by comparison of their spectroscopic data with previously published values. These compounds were identified as luteolin-7-rhamnoside-4'-*O*-β-glucopyranoside (1), quercetin-3-*O*-α-L-rhamnopyranoside (2), quercetin-7-*O*-glucopyranoside (3), luteolin-7-*O*-glucopyranoside (4), 5-hydroxy-6,7,8,4'-tetramethoxy flavone (5), gallic acid (6), 2,3-hexahydroxydiphenoyl 1-galloyl glucopyranoside (7), and quercetin (8). Complete acid hydrolysis indicated that compounds 1-4 and 7 were in *O*-glycosidic linkage [26,27] as follows.

Compound (1): Luteolin-7-rhamnoside-4'-O-β-glucopyranoside: It was obtained as yellow amorphous powder (14 mg), m.p. 320-324°C. UV spectral data λ_{max} (nm) MeOH: 270, 340; NaOMe: 270, 377; $AlCl_3$: 276, 300sh, 348, 388; $AlCl_3$/HCl: 277, 300sh, 348, 388; NaOAc: 269, 337, 348; NaOAc/H_3BO_3: 269, 237, 339. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) at 7.49, (1H, dd, J=8 Hz, H-6'), 7.51 (1H, d, J=2 Hz, H2'), 7.25 (1H, d, J=8 Hz, H-5'), 6.56 (1H, s, H-3), 6.87 (1H, d, J=2.5 Hz, H-8), 6.55 (1H, d, J=2.5 Hz, H-6). Sugar moiety, at 5.5 (1H, d, J=7.5 Hz, H-1'' of glucose), 4.55 (1H, d, J=2 Hz, H-1''' of rhamnose), 3.01→3.5 (complex signals, m, due to rest sugar protons), 0.98 (3H, d, J=6.1 Hz, CH_3 of rhamnose moiety).

^{13}C-NMR spectral measurement (150 MHz, DMSO-d_6): Aglycone moiety δ (ppm) at 161.92 (C-2), 104.20 (C-3), 182.3 (C-4), 163.9 (C-5), 98.3 (C-6), 165.5 (C-7), 94.49 (C-8), 157.78 (C-9), 104.3 (C-10), 121.5 (C-1'), 118.64 (C-2'), 147.90 (C-3'), 151.98 (C-4'), 116.23 (C-5'), 124.70 (C-6'). Sugar moiety: 4'-*O*-glucoside, at 100.9 (C-1''), 72.49 (C-2''), 77.3 (C-3''), 71.1 (C-4''), 77.4 (C-5''), 60.07 (C-6''). 7-*O*-rhamnoside, at 100.1 (C-1'''), 70.3 (C-2'''), 70.7 (C-3'''), 72.01 (C-4'''), 69.07 (C-5'''), and 18.4 (C-6''').

Complete acid hydrolysis yielded luteolin, glucose, and rhamnose, which was compared with authentic samples. Hence, the compound 1 was identified as luteolin-7-rhamnoside-4'-O-β-glucopyranoside.

Table 3: GLC data of unsaponifiable of *S. montana*

Peak No.	Rt (min.)	Rel. %	Molecular formula	Compounds
1	6.48	2.87	C_9H_{20}	n-nonane
2	9.72	1.58	$C_{11}H_{24}$	n-undecane
3	11.96	0.84	$C_{15}H_{32}$	n-pentadecane
4	13.29	4.98	$C_{17}H_{36}$	n-heptadecane
5	15.42	11.27	$C_{18}H_{38}$	n- octadecane
6	16.46	0.9	$C_{19}H_{40}$	n-nonadecane
7	17.36	12.32	$C_{20}H_{42}$	n-eicosane
8	18.45	9.46	$C_{21}H_{44}$	n-heneicosane
9	19.82	4.53	$C_{22}H_{46}$	n-docosane
10	20.03	27.7	$C_{23}H_{48}$	n-tricosane
11	21.39	4.99	$C_{24}H_{50}$	n-tetracosane
12	22.71	1.26	$C_{25}H_{52}$	n-pentacosane
13	23.47	6.12	$C_{26}H_{54}$	n-hexacosane
14	24.41	1.15	$C_{27}H_{56}$	n-heptacosane
15	26.01	0.98	$C_{27}H_{46}O$	Cholesterol
16	27.51	1.18	$C_{28}H_{48}O$	Campasterol
17	28.04	5.64	$C_{29}H_{48}O$	Stigmasterol
18	29.95	2.23	$C_{29}H_{50}O$	β-Sitosterol

S. montana: Satureja montana, GLC: Gas–liquid chromatography

Compound (2): *Quercetin-3-O-α-L-rhamnopyranoside***:** Yellow amorphous powder (16 mg), m.p. 316-318°C. UV spectral data λ_{max} (nm) MeOH: 258, 269sh, 360; NaOMe: 272, 328sh, 405; $AlCl_3$: 275, 305sh, 332sh, 435; $AlCl_3$/HCl: 275, 305sh, 361, 403; NaOAc: 269, 323sh, 380; NaOAc/H_3BO_3: 262, 300sh, 387; ^{1}H-NMR (600 MHz, DMSO-d_6): Aglycone moiety: δ ppm: 7.6 (1H, d, J=2.5 Hz, H-2'), 7.5 (1H, dd, J=2.5 Hz and J=8 Hz, H-6'), 6.77 (1H, d, J=8Hz, H-5'), 6.24 (1H, d, J=2.0 Hz, H-8), 6.04 (1H, d, J=2.0 Hz, H-6) ppm. Sugar moiety: 5.5 (1H, d, J=2.0 Hz, H-1'' of rhamnose), 3.1→3.5 (m, sugar protons), 0.98 (3H, d, J=6.1 Hz, CH_3 of rhamnosyl) ppm. ^{13}C-NMR (150 MHz, DMSO-d_6): 157.04 (C-2), 134.5 (C-3), 177.9 (C-4), 161.7 (C-5), 99.5 (C-6), 161.2 (C-7), 94.3 (C-8), 157.4 (C-9), 103.9 (C-10), 121.1 (C-1'), 115.9 (C-2'), 145.76 (C-3'), 149.1 (C-4'), 115.99 (C-5'), 121.52 (C-6'). Sugar moiety: 101.1 (C-1''), 70.6 (C-2''), 70.81 (C-3''), 71.66 (C-4''), 70.5 (C-5''), and 17.9 (C-6''). The acid hydrolysis of compound 2 yielded both quercetin and rhamnose, which was identified by Co-PC, using authentics in different solvents. Thus, the structure of compound 2 was proved as quercetin-3-O-α-L-rhamnopyranoside.

Compound (3): Quercetin-7-O-glucopyranoside: The compound was obtained as yellow amorphous powder (17 mg), m.p. 318-320°C. UV spectral data λ_{max}(nm) MeOH: 257, 294 sh, 357; NaOMe: 266, 314, 406; $AlCl_3$: 273, 301sh, 335sh, 433; $AlCl_3$/HCl: 269, 300sh, 358, 402; NaOAc: 259, 396sh, 367, 415; NaOAc/H_3BO_3: 261, 295sh, 378. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) at 12.60 (1H, s, OH-5), 7.61 (1H, d, J=2.2 Hz, H-2'), 7.58 (1H, dd, J=2.2, 8.0 Hz, H-6'), 6.86 (1H, d, J=8 Hz, H-5'), 6.7 (1H, d, J=2.0 Hz, H-8), 6.48 (1H, d, J=2.0 Hz, H-6). The sugar protons at 5.5 (1H, J=7.5 Hz, H-1''), 3.1→3.6 (complex m).

^{13}C-NMR: 159.1 (C-2), 133.6 (C-3), 177.4 (C-4), 162.2 (C-5), 98.6 (C-6), 165.6 (C-7), 94.4 (C-8), 159.1 (C-9), 104.6 (C-10), 122.1 (C-1'), 116.1 (C-2'), 145.2 (C-3'), 150.2 (C-4'), 116.9 (C-5'), 122.7 (C-6'), 100.6 (glc, C-1), 74.2 (glc, C-2), 77.9 (glc, C-3), 70.2 (glc, C-4), 76.4 (glc, C-5), 61.7 (glc, C-6). Complete acid hydrolysis of compound 3 gave quercetin and glucose by comparing with Co-PC with authentic samples. Compound 3 was identified as quercetin-7-O-glucopyranoside.

Compound (4): Luteolin-7-O-glucopyranoside: Off white amorphous powder (13 mg), m.p.324–326°C. UV spectral data λ_{max} (nm) MeOH: 258, 347; NaOMe: 262.5, 298sh, 398; $AlCl_3$: 275, 302, 345, 385, 421; $AlCl_3$/HCl: 269, 280, 295sh, 340, 282, 387; NaOAc: 267, 379, 423; NaOAc/H_3BO_3: 261.5, 370. ^{1}H-NMR (DMSO-d_6):7.7 (1H, dd, J=2.5 Hz, and J=8 Hz, H-6'), 7.55 (1H, d, J=2.5 Hz, H-2'), 6.85 (1H, d, J=8 Hz, H-5'), 6.42 (1H, s, H-3), 6.72 (1H, d, J=2.0 Hz, H-8), 6.61 (1H, d, J=2.0 Hz, H-6), 5.2 (1H, d, J=7.5 Hz, H-1''), 3.2→3.8 (m, of sugar protons). ^{13}C-NMR: 161.5 (C-2), 103.5 (C-3), 182.3 (C-4), 163.39 (C-5), 99.9 (C-6), 164.5 (C-7), 95.16 (C-

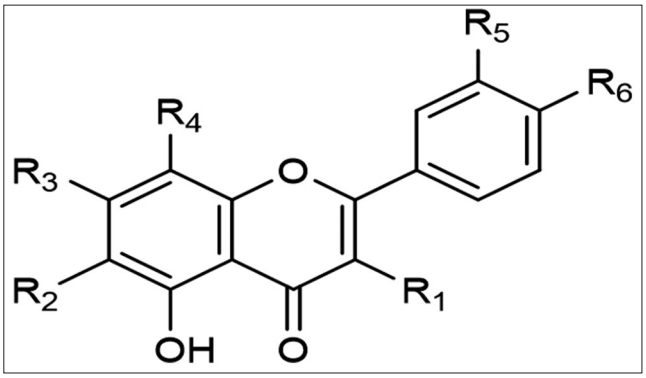

Fig.1: Phenolic compounds isolated from *S. montana* L.

Compound 1 R1, R2, R4 = H, R3= O-rhamnose, R5=OH, R6 =O-glucose
Compound 2 R1=O-rhamnose, R2,R4= H, R3, R5, R6= OH
Compound 3 R1, R5, R6 = OH, R2, R4 = H, R3= O-glucose
Compound 4 R1, R2, R4 =H , R3= O-glucose, R5, R6 = OH
Compound 5 R1, R5 = H, R2, R3, R4, R6 = OCH3
Compound 8 R1, R3, R5, R6 = OH , R2, R4 = H

Table 4: Antimicrobial activity of *S. montana* (Dry DMSO as solvent)

Sample name	Inhibition zone diameter in mm.					
n-Hexane extract	***B. subtilis* (G+)**	***S. aureus* (G+)**	***E. coli* (G-)**	***P. aeruginosa* (G-)**	***A. flavus* (Fungus)**	***C. albicans* (Fungus)**
	12	**10**	**11**	**11**	**5**	**6**
Methanol extract	21	18	20	21	10	12
Compound (1)	15	16	16	17	7	8
Compound (2)	22	19	21	22	10	11
Compound (3)	17	16	17	18	7	9
Compound (4)	20	17	19	20	8	9
Compound (5)	14	15	14	15	5	7
Compound (6)	13	12	13	14	6	7
Compound (7)	14	13	13	14	7	9
Compound (8)	21	17	19	20	9	10
Ampicillin	26	21	25	26	-	-
Amphotericin B	-	-	-	-	16	19

P. aeruginosa: Pseudomonas aeruginosa, A. flavus: Aspergillus flavus, B. subtilis: Bacillus subtilis, S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, C. albicans: Candida albicans

8), 157.4 (C-9), 105.78 (C-10), 121.66 (C-1'), 119.6(C-2'), 146.3 (C-3'), 150.4 (C-4'), 116.42 (C-5'), 122.1 (C-6'). Sugar moiety δ ppm: 100.3 (C-1''), 72.3 (C-2''), 77.6 (C-3''), 69.99 (C-4''), 76.83 (C-5''), 61.06 (C-6''). Acid hydrolysis of compound 4 led to identification of both luteolin and glucose by Co-PC using authentic markers in different solvents. The compound 4 could be identified as luteolin-7-O-glucopyranoside.

Compound (5): 5-Hydroxy-6,7,8,4'-tetramethoxy flavone: It was obtained as white yellowish amorphous powder (15 mg), m.p. 224-226°C. UV spectral data λ_{max} (nm) MeOH: 254 sh, 277, 342, NaOMe: 268, 406; $AlCl_3$: 272sh, 287, 309, 362, 410sh; $AlCl_3$/HCl: 262sh, 287, 310, 360, 408sh; NaOAc: 259sh, 276, 342, 406; NaOAc/H_3BO_3: 254sh, 277, 339; ^{1}H-NMR(600 MHz, DMSO-d_6): δ (ppm) at 7.9 (2H, dd, J=2.5 Hz, 8 Hz, H-2' and H-6'), 7.01 (2H, dd, J=2.5 Hz, 8 Hz for H-3' and H-5'), 6.9 (1H, s, H-3), 4.1 (s, OCH_3-4'), 4.02 (s, OCH_3-6), 3.9 (s, OCH_3-7), 3.85 (s, OCH_3-8).^{13}C-NMR: 162 (C-2), 103.1 (C-3), 183.02 (C-4), 148.54 (C-5), 136.30 (C-6), 152.92 (C-7), 133.07 (C-8), 145.68 (C-9), 106.66 (C-10), 121.50 (C-1'), 110.48 (C-2'), 116.4 (C-3'), 154.6 (C-4'), 116.40 (C-5'), 128.98 (C-6'), 62.31 (OCH_3-6), 62.37 (OCH_3-7), 61.95 (OCH_3-8), 55.24 (OCH_3-4'). The above data showed that compound 5 is 5-Hydroxy-6,7,8,4'-tetramethoxy flavone.

Compound (6): Gallic acid: White amorphous powder. m.p. 258-260°C UV spectral data λ_{max} (nm) MeOH: 272 nm. ^{1}H-NMR (600 MHz, DMSO-d_6) δ (ppm) at 6.83 (2H, s, H-2, and H-6). ^{13}C-NMR at 120.6 (C- 1), 108.8 (C-2 and C-6), 145.5 (C-3 and C-5), 138.1 (C-4), 167.7 (C-7). Hence, compound **6** was identified as gallic acid [28].

Compound (7): 2,3-hexahydroxydiphenoyl 1-galloyl glucopyranoside; it was obtained as yellowish powder (18 mg), m.p. 226-228°C. UV spectral data λ_{max} (nm) MeOH: 220, 226, 277. ^{1}H-NMR: 7.1 (2H, s, H-2, 6 of galloyl proton), 6.8 (1H, s, H-5 of hexahydroxydiphenoyl portion (HHDP) ring C), 6.74 (1H, s, HHDP H-5 ring B), 6.4 (1H, d, J=8 Hz, glucose (Glc) H-1), 5.7 (1H, d, J=8 Hz, Glc, H-2), 5.6 (1H, d, J=8 Hz, Glc, H-3), 4.3 (1H, d, J=8 Hz, Glc, H-4), 4.2 (1H, t, J=9.4 Hz, Glc, H-5), 4.1 (2H, t, J=11, dd, J=2.8, 8 Hz, CH_2-Glc). ^{13}C-NMR: 93.95 (Glc, C-1), 72.77 (Glc, C-2) 75.5 (Glc, C-3), 69.1 (Glc, C-4), 74.1 (Glc, C-5), 61.5 (Glc, C-6), 120.72 (gallic acid (Gal), C-1), 110.7 (Gal, C-2, 6), 145.71 (Gal, C-3, 5), 139.04 (Gal, C-4), 164.85 (Gal, C=O), 115.76 (HHDP ring B, C-1), 116.27 (HHDP ring C, C-1), 144.55, 144.65, 144.66, 145.32 (HHDP ring B, C-2, 4; ring C, C-2, 4), 136.62 (HHDP ring B, C-3), 137.5 (HHDP ring C, C-3), 107.88 (HHDP ring B, C-5), 109.66 (HHDP ring C, C-5), 125.40 (HHDP ring B, C-6), 125.54 (HHDP ring C, C-6), 167.22 (HHDP ring C, C=O), 168.24 (HHDP ring B, C=O). Compound 7 was subjected to acid hydrolysis, yielding glucose, gallic acid, and hexahydroxydiphenic acid, the latter has high tendency to spontaneously undergo lactonization to afford ellagic acid (EA) [27,28] and identified by Co-PC using authentic markers in different solvents. Hence, compound 7 could be identified as 2,3-hexahydroxydiphenoyl-1-galloyl glucopyranoside.

Compound (8): Quercetin: Yellow crystals (19 mg), m.p. 322-324°C. UV spectral data λ_{max}(nm) MeOH: 256, 268sh, 371; NaOMe: 249, 424; $AlCl_3$: 273, 305sh, 335sh; $AlCl_3$/HCl: 266, 303sh, 350, 414; NaOAc: 264, 325, 390; NaOAc/H_3BO_3: 262, 302sh, 386.^{1}H-NMR (600 MHz, DMSO-d_6): 7.8 (1H, dd, J=2.5 Hz and J=8Hz, H-6'), 7.6 (1H, d, J=2.5 Hz, H-2'), 6.88 (1H, d, J=8.5 Hz, H-5'), 6.4 (1H, d, J=2.5 Hz, H-8), and 6.2 (1H, d, J=2.5 Hz, H-6) ppm.^{13}C-NMR: 147.25 (C-2), 136.18 (C-3), 176.29 (C-4), 161.17 (C-5), 98.63 (C-6), 164.35 (C-7), 93.81 (C-8), 156.59 (C-9), 103.46 (C-10), 122.40 (C-1'), 115.3 (C-2'), 154.51 (C-3'), 148.15 (C-4'), 115.9 (C-5'), 120.43 (C-6'). From the above data, compound **8** was identified as quercetin [26,27,29,30].

The antimicrobial activity of different extracts and isolated compounds

The antimicrobial activity of different extracts and isolated compounds data was summarized in Table 4, which proved that the methanolic extract showed significant antimicrobial activity against *S. aureus* (85.7%), while, it showed a moderate antifungal activity against *A. flavus* (62.5%) and *C. albicans* (63.2%). On the other hand, the n-hexane extract showed a relatively low activity against Gram-positive *B. subtilis* (46.2%), while, it showed a low antifungal activity against *A. flavus* (31.3%).

Compound 2 showed the highest activity against Gram-positive *B. subtilis* and *S. aureus* (84.6% and 90.5%), respectively. Compound 8 exhibited a significant inhibition against the tested Gram-positive *B. subtilis* and *S. aureus* (80.8% and 81%), respectively, Gram-negative *E. coli* (76%) and *P. aeruginosa* (77%), while it showed a moderate antifungal activity against *A. flavus* and *C. albicans* (56.3% and 52.6%), respectively. Compound 4 exhibited a significant inhibition against *B. subtilis* (77%), Gram-negative *E. coli* and *P. aeruginosa* (76% and 77%), respectively, and relatively moderate antifungal activity against *A. flavus* and *C. albicans* (50% and 47.4%).

Furthermore, compound 3 exhibited a moderate activity against Gram-negative *E. coli* and *P. aeruginosa* (68% and 69.2%), respectively. Compounds 1 and 5 exhibited a significant inhibition against Gram-positive *S. aureus* (76.2% and 71.4%), respectively, and *B. subtilis* (57.7% and 53.8%), respectively, Gram-negative *P. aeruginosa* (65.4% and 57.7%), respectively, and *E. coli* (64% and 56%), respectively. On the other hand, both compounds showed a low antifungal activity against *A. flavus* (43.7% and 31.2%), respectively, and *C. albicans* (42.1% and 36.8%), respectively.

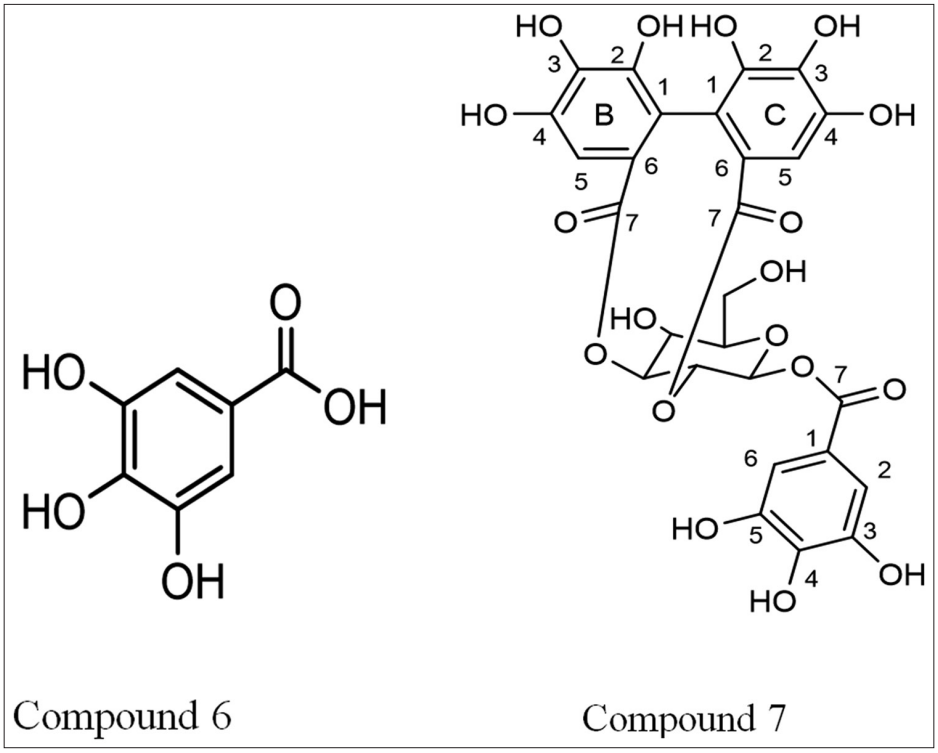

Fig. 2: Phenolic compounds isolated from Satureja montana L.

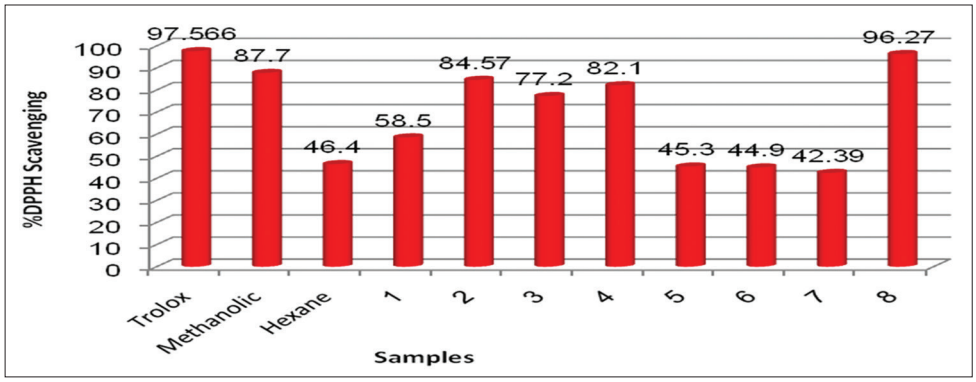

Fig. 3: Antioxidant activity of extracts and compounds from *Satureja montana*

Compounds 6 and 7 showed a moderate activity against Gram-positive *S. aureus* (57.1% and 61.9%), respectively, *B. subtilis* (50% and 53.8%), respectively, Gram-negative *P. aeruginosa* (53.9% both) and *E. coli* (52% both).

The observed results in this study might be due to the presence of flavonoidal compounds in methanol extract and some volatile constituents beside the high percentage of unsaturated fatty acids in the hexane extract [31,32].

Antioxidant activity

The antioxidant activity study (Fig. 3) proved that the compound 2 and the methanolic extract exhibited the highest activity (inhibition % = 96.27 and 87.7, respectively). On the other hand, n-hexane extract and compounds 5-7 showed low activity, while compounds 2-4 gave moderate activity.

These data can be explained in light of the structure of different flavonoids play an important role in their activity as antioxidant where the structure of the flavonoids is the most significant determinant of radical scavenging [33,34]. The OH groups on the ring-B give hydrogen and an electron to OH and peroxyl radicals, which led to more flavonoidal radical stability and the activity increases linearly, according to the total number of OH groups [35]. When 3',4'-dihydroxy structure in ring-B, it increases lipid peroxidation inhibition effectively [36]. Hence, the peroxyl radical scavenging ability of luteolin substantially exceeds kaempferol [37]. Free radical scavenging of flavonoids is strongly dependent on the presence of a free OH group at C-3 in the flavonoid nucleus [38]. Flavonoids with a C-3-OH and 3',4'dihydroxy are reported to be 10-fold more potent against peroxynitrite. The superiority of quercetin in inhibiting both metal and non-metal-induced oxidative damage is partially attributed to its free 3-OH substituent, which is thought to increase the stability of the flavonoid radical [39].

CONCLUSION

The findings of this study showed that the hydroalcoholic extract of *S. montana* showed significant antimicrobial and antioxidant activity. On the other hand, compound 2 showed the highest antibacterial activity against all the tested microorganisms, and compound 8 showed the highest antioxidant activity (96.27%) in DPPH assay.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interests regarding the publication of this article.

AUTHOR CONTRIBUTIONS

Ali M. El-Hagrassi, designed the experiments, extraction, separate pure compounds and lipid constituents and collect results, make the laboratory and store experiments and writing the manuscript. Walid E. Abdallah, share in isolation and separate pure compounds, volatile and lipid constituents, and writing the manuscript. Abeer F. Osman, share in extraction, isolation, separate pure compounds, volatile and lipid constituents, antioxidant and antimicrobial activity and writing the manuscript. Khaled A. Abdelshafeek, share in compounds identification, auditing, writing the research and reviewed the manuscript.

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