Pharmaceutical Sciences
December 2018, 24, 332-339
doi: 10.15171/PS.2018.47
http://ps.tbzmed.ac.ir/

Research Article

Cytotoxic Flavonoids from the Aerial Parts of *Stachys lavandulifolia* Vahl

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Article Info

Article History:
Received: 24 June 2018
Revised: 29 July 2018
Accepted: 5 August 2018
ePublished: 30 December 2018

Keywords:
-*Stachys lavandulifolia* Vahl
-Toxicity
-Essential oil
-Flavonoids
-Brine shrimp lethality test
-MTT assay

ABSTRACT

Background: *Stachys lavandulifolia* Vahl is a herbaceous plant distributed in the west and south western Asia. Despite of the wide medicinal uses, there are some reports on toxicity potential of this plant. In present study we attempted to evaluate the toxicity and to characterize the cytotoxic principles of *S. lavandulifolia*.
Methods: Brine shrimp lethality test (BSLT) was used to evaluate the general toxicity of the extracts and essential oil obtained from the aerial parts of *S. lavandulifolia*. Phytochemical constituents of the active extract were investigated using various chromatographic and spectroscopic methods. GC and GC-MS were also used to analyze the plant essential oil. GC and GC-MS were used to analyze the plant essential oil. Cytotoxic activities of the isolated compounds were also evaluated using MTT assay method.
Results: In brine shrimp lethality test (BSLT), chloroform extract and the plant essential oil exhibited the most toxicity against *Artemia salina* larvae (LD_{50}: 121.8±5.6 and 127.6±14.7 µg ml^{-1}, respectively). GC and GC-MS analyses of essential oil led to the characterization of forty compounds of which α-bisabolol (23.85%) and thymol (17.88%) were identified as the main constituents. Nine flavonoids, Pachypodol (**1**), chrysosplenetin (**2**), kumatakenin (**3**), velutin (**4**), penduletin (**5**), viscosine (**6**), chrysoeriol (**7**), hydroxygenkwanin (**8**) and apigenin (**9**) were isolated from the chloroform extract of *S. lavandulifolia*. Among the isolated compounds, chrysosplenetin (**2**), a polymethoxylated flavonoid, was found as the most toxic compound toward MDA-MB-23 and HT-29 cells, with IC_{50} values of 88.23 and 116.50 µg ml^{-1}, respectively. Furthermore, chrysosplenetin (**2**), kumatakenin (**3**) and viscosine (**6**) with selectivity indices of 2.70, 2.59 and 3.33, respectively, showed higher preferential toxicity against MDA-MB-23 cells in comparison with tamoxifen (SI:2.45).
Conclusion: This study reports methoxylated flavonoids as compounds which could be involved in toxicity of *S. lavandulifolia*. The results of MTT assay also suggest some of these compounds as appropriate candidates for anti-cancer drug development research.

Introduction

The genus *Stachys* L. with about 300 species all over the world is one of the largest genera of Lamiaceae (*alt.* Labiatae) family.[1] *Stachys lavandulifolia* Vahl from this genus is a perennial herbaceous plant distributed in Iran, Turkey, Iraq, Caucasia and central Asia.[2] This species has been described under the name of "Marmazad" in Old Persian medicinal literature, useful in the treatment of liver, stomach and uterus diseases and as liver tonic, gastrotonic and emmenagogue agent.[3] In different parts of Iran and Anatolia the flowering aerial parts of *S. lavandulifolia* is used as a popular herbal tea for its sedative, gastrotonic and spasmolytic properties, as well as for the treatment of some gastrointestinal disorders, colds and flu.[4-6]

So far different biological and pharmacological effects such as antioxidant,[7] antimicrobial,[7,8] anxiolytic,[9,10] wound healing,[11] gastroprotective,[12] analgesic,[13] anti-inflammatory,[13] anti-tyrosinase[14] and acetylcholinesterase inhibitory[14] activities have been documented for the various extracts obtained from the aerial parts of *S. lavandulifolia*. The results of clinical trials have also confirmed the therapeutic value of this species in abnormal uterine bleeding caused by polycystic ovary syndrome and as a useful supplement in management of diseases related to oxidative stress.[15,16]

Previous phytochemical investigations on *S. lavandulifolia* have led to the isolation of four phenylethanoid glycosides; lavandulifoliosides A and B, verbascoside and leucosceptoside A, three iridoid

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glycosides; monomelittoside, melittoside, 5-allosyloxy-aucubin, a diterpenoid; stachysolone and a phenolic glycoside; arbutin from its aerial parts.[14,17] Two flavonoid derivatives, apigenin and luteolin, have also been determined using HPLC in the hydroalcoholic extract of *S. lavandulifolia*.[18] Furthermore, chemical constituents of this plant essential oil have been reported from different regions of Iran.[19-23]

Despite of medicinal uses and beneficial health effects of *S. lavandulifolia*, there are some reports on toxicity potential of this plant species.[24-26] It has been demonstrated that peroral administration of hydroalcoholic extract of *S. lavandulifolia* with doses higher than 140 mg/kg/day could be resulted in significant subacute and subchronic toxicity in female mice.[26] In another study, the hydroalcoholic extract of *S. lavandulifolia* aerial parts was found hepatotoxic in a dose dependent manner, following administration at the doses 50-200 mg/kg, intraperitoneally for 28 days.[24] Regarding to the lack of information about compounds involved in the toxicity properties of *S. lavandulifolia*, the present study was designed to assess toxicity and to identify of cytotoxic principles of this species using a bioassay guided approach.

Materials and Methods

Plant material

The aerial parts of *S. lavandulifolia* Vahl were collected during its flowering stage from Ghotour region (Khoy, West-Azerbaijan, Iran) in Jun 2014. The plant sample was authenticated by botanist Dr. Yousef Ajani and its specimen was deposited at the herbarium of Institute of Medicinal Plants, ACECR, Karaj, Iran (Voucher no. 2014 MPIH).

Essential oil preparation

The essential oil of the plant was extracted by hydrodistillation of the air-dried and powdered aerial parts (100 g) using a Clevenger-type apparatus for 4 h. The obtained essential oil was dried over the anhydrous sodium sulfate and stored in 4 °C until analysis.

Extraction

The air-dried and comminuted plant (1.2 kg) was macerated with petroleum ether, chloroform, ethyl acetate and methanol:water (8:2), successively (3× 15 L each) at the room temperature. The four obtained extracts were concentrated using a rotary evaporator under a low pressure at 45 °C.

Brine shrimp lethality test

General toxicity potentials of the plant extracts and essential oil were evaluated in brine shrimp lethality test (BSLT).[27] The brine shrimp (*Artemia salina* L.) eggs were hatched in sterile artificial seawater (38 g L^{-1}, adjusted to pH 9 with $NaHCO_3$) under the constant aeration for 48 hours at 30 °C. the plant samples (50 mg) were dissolved in DMSO (200 µl) and tween 80 (~50 µl) and then diluted by freshly prepared artificial sea water to obtain the solutions with 10, 100, 300, 500, 700 and 1000 µg ml^{-1} concentrations in a series of tubes containing about 20 active nauplii in each. The surviving nauplii in test and control tubes were counted following the incubation of the tubes at 30 °C for 24 hours under light to achieve the LD_{50} value, expressing the concentration causing 50% lethality. Podophyllotoxin, a natural compound with known cytotoxic activity, was applied as positive control. The assays were performed in triplicate and the LD_{50} values were reported as Mean ± SD.

Phytochemical analyses

Chloroform extract and essential oil of the plant with the highest toxicity effects (Table 1) were subjected to phytochemical analyses using various chromatographic and spectroscopic methods.

Table 1. Toxicity of the extracts and essential oil of *S. lavandulifolia* in BSLT.

Samples	Brine Shrimp lethality LD_{50} (µg ml^{-1})
Petroleum ether extract	685.4 ± 12.2
Chloroform extract	121.8 ± 5.6
Ethyl acetate extract	704.4 ± 16.8
Hydroalcoholic extract	1000 <
Essential oil	127.6 ± 14.7
Podophyllotoxin (Positive control)	3.1 ± 0.6

GC and GC-MS analyses

The essential oil was analysed on a HP 6890 gas chromatograph equipped with HP-5MS column (30m ×0.25 mm id, 0.25µm), connected to HP 5973 mass detector (70 eV) under the following conditions; carrier gas: helium (1 ml min^{-1}), temperature program: 40 °C to 250 °C at 3 °C per min, injector temperature: 250 °C, injection volume: 1 µl, split ratio: 1:90. The Kovats retention indices (KI) of the compounds were calculated using a homologous series of *n*-alkanes injected in conditions equal to the sample. Identification of the compounds was carried out based on computer matching with the Wiley7n.L library, as well as by comparison of KIs and fragmentation pattern of the mass spectra with those published for standard compounds.[28] For quantitative purposes, the essential oil was also analysed by GC-FID with the same conditions described above for GC-MS.

Isolation and purification of the compounds

Chloroform extract (12.6 g) was moved on a silica gel column (230-400 mesh, Merck, Germany) and eluted by $CHCl_3$-EtOAc (10:0-5:5) to three main fractions (C_1-C_3). Silica gel column chromatography of a portion of the fraction C_2 (1.5 g) with $CHCl_3$-EtOAc (9:1-7.5:2.5) yielded nine fractions (C_{2A}-C_{2I}). Compound **1** (5 mg) was purified using preparative thin layer chromatography (PTLC) (Handmade plates, Silica gel 60 GF_{254}, Merck, Germany) from the fraction C_{2D} (18 mg) with $CHCl_3$-EtOAc (8:2), as a solvent system. Fraction C_{2E} (320 mg) was chromatographed over the silica gel column and eluted with $CHCl_3$-EtOAc (7.5:2.5) to get three fractions (C_{2E1}-C_{2E3}). Elution of the fraction C_{2E3} (135 mg) over a

Sephadex LH-20 column (Fluka, Switzerland) with MeOH-EtOAc (8:2) resulted in isolation of compounds **2** (23 mg), **3** (18 mg) and **4** (20 mg). Fraction C_{2G} afforded 175 mg of yellowish powder which was purified on a PTLC plate with $CHCl_3$-EtOAc (8:2) to obtain compound **5** (51 mg). Compound **6** (43 mg) was also obtained from the faction C_{2I} (160 mg) over a Sephadex LH-20 column, eluted with MeOH. Silica gel column chromatography of the fraction C_3 (270 mg) with $CHCl_3$-MeOH (9.5:0.5) yielded three fractions (C_{3A}-C_{3C}). Fractions C_{3B} (42) and C_{3C} (28) were individually moved on Sephadex LH-20 columns and eluted with MeOH-EtOAc (8:2) to get a mixture of the compounds **7** and **8** (9 mg) (5.3:4.7), as well as compound **9** (6 mg), respectively.

In all steps, chromatography was monitored by thin layer chromatography (TLC) on Pre-coated Silica gel GF_{254} sheets (Merck, Germany) and the spots were detected under UV (254 and 366 nm) and by spraying anisaldehyde/H_2SO_4 reagent. ^{1}H-NMR and^{13} C-NMR spectra of the isolated compounds were obtained on a Bruker Avance DRX 300 and 500 spectrometers. UV spectra were recorded on a CECIL 7250 spectrophotometer in methanol and after the addition of shift reagents (NaOMe, NaOAc and $AlCl_3$).[29] EIMS spectra were also acquired on a HP 5973 system.

MTT assay

Two cancerous cell lines, MDA-MB-231 (Human breast cancer) and HT-29 (Human colon adenocarcinoma) and one normal cell line, MRC-5 (Human fetal lung fibroblasts) were obtained from Pasture Institute, National Cell Bank of Iran Tehran, Iran. All cell lines were cultured for 24 hours in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS) (Invitrogen, USA), and 1% penicillin/streptomycin (Invitrogen, USA) in a humidified atmosphere at 37°C in a 5% CO_2 incubator.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxic activity of the isolated flavonoids on two cancer cell lines, MDA-MB-231 and HT-29, and one normal cell line, MRC-5.[30] Cells were seeded in 96-well plates containing RPMI medium and incubated for 24 h at 37°C. The cells were then treated with fresh medium containing different concentrations of test compounds in triplicates. After 24 h the medium was changed by phosphate-buffered saline (PBS) medium containing MTT (10µl, 5mg/ml) and incubated for additional 3 h. Afterwards, the formazan crystals produced from MTT were completely dissolved in DMSO, and their absorbance was recorded at 630nm using microplate reader (Anthons 2020, version 1.8.3, UK). The cell viabilities were calculated by the following formula: Relative viability (%) = (Absorbance of the test /Absorbance of the control) ×100. IC_{50} value was defined as the concentration of the compounds (in µg ml^{-1}) which caused a 50% reduction in the number of viable cells relative to the negative control. Tamoxifen was used as positive control.

Results

General toxicity

The results of general toxicity assay of the plant samples in brine shrimp lethality test have been shown in Table 1. Among the tested samples, chloroform extract and the plant essential oil with LD_{50} values of 121.8 ± 5.6 and 127.6 ± 14.7 µg ml^{-1}, respectively, exhibited the most toxicity effects against *Artemia salina* larvae, in comparison with podophyllotoxin (LD_{50} value; 3.1 ± 0.6 µg ml^{-1}).

Essential oil composition

Hydrodistillation of the plant aerial parts led to the extraction of a pale yellowish oil with a yield of 0.3 % (V/W). Forty compounds representing 88.15% of the total oil were characterized as a result of GC and GC-MS analyses of the essential oil (Table 2).

Isolation and purification of compounds

Phytochemical investigation of the chloroform extract, as the most toxic extract of *S. lavandulifolia* aerial parts, resulted in the isolation of nine compounds (**1-9**). The structures of the isolated compounds were identified as pachypodol (**1**), chrysosplenetin (**2**), kumatakenin (**3**), velutin (**4**), penduletin (**5**), viscosine (**6**), chrysoeriol (**7**), hydroxygenkwanin (**8**) and apigenin (**9**), using ^{1}H-NMR,^{13} C-NMR, UV and EIMS spectral analyses, as well as by comparison of their spectroscopic data with those published in literature (Figure 1).

Chromatographic and spectroscopic data of the isolated compounds

Compound **1**; Pachypodol (Quercetin 3,7,3'-trimethyl ether): Yellow needles: $R_{f=}$0.67 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 12.63 (1H, *s*, OH-5), 7.70 (1H, *br s*, H-2'), 7.67 (1H, *br d*, *J*= 8.5 Hz, H-6'), 7.04 (1H, d, *J*= 8.5 Hz, H-5'), 6.44 (1H, *br s*, H-8), 6.36 (1H, *br s*, H-6), 3.98 (3H, *s*, OCH_3), 3.88 (3H, *s*, OCH_3), 3.86 (3H, *s*, OCH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 178.72 (C-4), 165.45 (C-7), 162.08 (C-5), 155.82 (C-2), 155.76 (C-9), 148.37 (C-4'), 138.84 (C-3), 122.69 (C-6'), 114.58 (C-5'), 110.91 (C-2'), 122.46 (C-1'), 97.84 (C-6), 92.20 (C-8), 60.16 (OCH_3), 56.12 (OCH_3), 55.81 (OCH_3); UV (MeOH) λ_{max}: 270, 355., +NaOMe: 266, 405.[31]

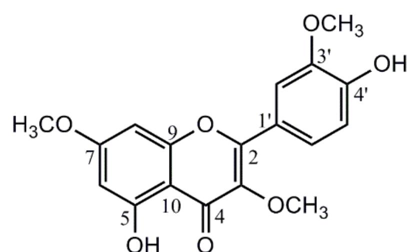
Compound **2**; Chrysosplenetin (Quercetagetin 3,6,7,3'-tetramethyl ether): Yellow needles: $R_{f=}$ 0.62 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 12.62 (1H, *s*, OH-5), 7.66 (1H, *br s*, H-2'), 7.62 (1H, *br d*, *J*= 8.7 Hz, H-6'), 6.96 (1H, d, *J*= 8.7 Hz, H-5'), 6.89 (1H, *s*, H-8), 3.91 (3H, *s*, OCH_3), 3.87 (3H, *s*, OCH_3), 3.80 (3H, *s*, OCH_3), 3.72 (3H, *s*, OCH_3); UV (MeOH) λ_{max}: 256, 269 (sh), 355., +NaOMe: 269, 411., +NaOAc: 266, 414.[32,33]

Compound **3**; Kumatakenin (Kaempferol 3,7-dimethyl ether): Yellow needles: $R_{f=}$0.67 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 7.95 (2H, *d*, *J*= 8.1 Hz, H-2',6'), 6.94 (2H, *d*, *J*= 8.1 Hz, H-3',5'), 6.70 (1H, *br s*, H-8), 6.33 (1H, *br s*, H-6), 3.84 (3H, *s*, OCH_3), 3.78 (3H, *s*, OCH_3); UV (MeOH) λ_{max}: 266, 355., +NaOMe: 259, 395., +NaOAc: 266, 368.[34]

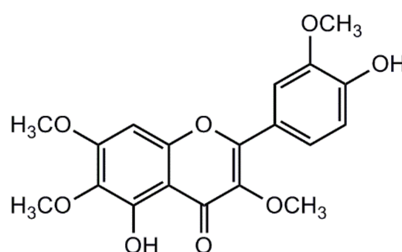
Table 2. Essential oil composition of the aerial parts of *S. lavandulifolia* from north-west of Iran.

No	Compounds[a]	KI[b]	%	No	Compounds	KI	%
1	α-Thujene	927	0.23	**25**	β-Bourbonene	1389	0.56
2	α-Pinene	939	0.70	**26**	(E)-Caryophyllene	1419	1.29
3	Camphene	949	0.10	**27**	(E)-β-Farnesene	1456	0.92
4	Benzaldehyde	955	0.40	**28**	ar-Curcumene	1482	5.00
5	Sabinene	972	0.29	**29**	Germacrene D	1487	1.17
6	β-Pinene	977	0.29	**30**	β-Bisabolene	1506	2.82
7	1-Octene-3-ol	990	0.23	**31**	δ-Cadinene	1526	1.92
8	Myrcene	991	1.48	**32**	Spathulenol	1579	**7.21**
9	α-Phellandrene	1005	0.12	**33**	α-Bisabolol	1688	**23.85**
10	δ-3-Carene	1011	0.64	**34**	Methyl hexadecanoate	1923	1.33
11	α-Terpinene	1017	0.46	**35**	Hexadecanoic acid	1963	4.34
12	o-Cymene	1025	1.79	**36**	Linoleic acid	2135	1.68
13	β-Phellandrene	1028	1.47	**37**	Sclareol	2225	1.31
14	1,8-Cineol	1029	0.82	**38**	Pentacosane	2500	0.22
15	(E)-β-Ocimene	1047	0.04	**39**	Heptacosane	2700	0.77
16	γ-Terpinene	1057	1.52	**40**	Nonacosane	2900	0.75
17	(Z)-Sabinene hydrate	1068	0.15				
18	Terpinolene	1089	0.17		Monoterpene hydrocarbons		9.30
19	Linalool	1098	0.60		Oxygenated monoterpenes		22.30
20	Borneol	1168	0.31		Sesquiterpene hydrocarbons		14.46
21	Terpinen-4-ol	1178	1.73		Oxygenated sesquiterpenes		31.06
22	Carvacrol methyl ether	1244	0.81		Diterpenoids		1.31
23	Thymol	1291	**17.88**		Non-terpenes		9.72
24	α-Copaene	1376	0.78		Total identified		88.15

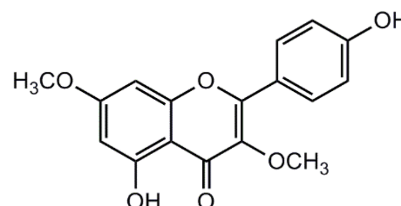
[a] Identified compounds listed in order of elution from HP-5MS column; [b] Kovats retention indices to C_8-C_{30} *n*-alkanes on HP-5MS column.



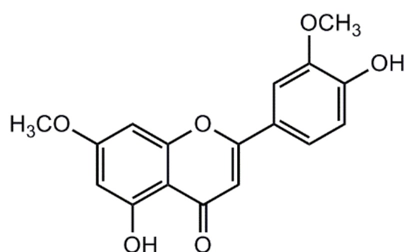
Pachypodol (**1**)



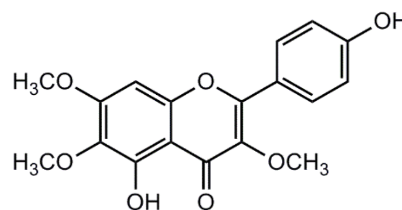
Chrysosplenetin (**2**)



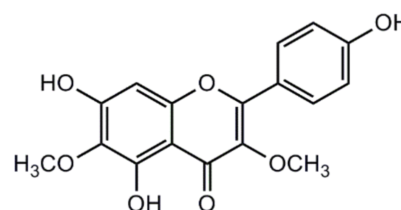
Kumatakenin (**3**)



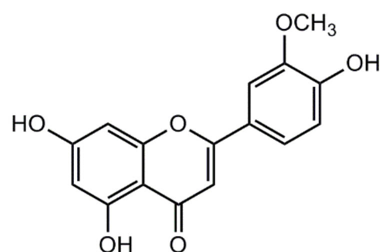
Velutin (**4**)



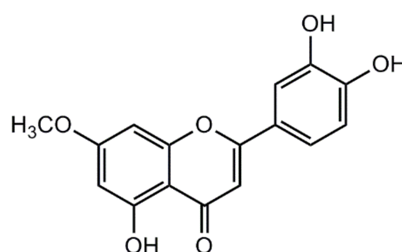
Penduletin (**5**)



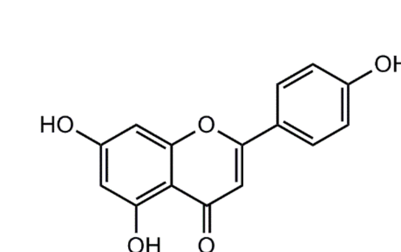
Viscosine (**6**)



Chrysoeriol (**7**)



Hydroxygenkwanin (**8**)



Apigenin (**9**)

Figure 1. Structures of the isolated compounds from the aerial parts of *S. lavandulifolia.*

Compound **4**; Velutin (Luteolin 7,3'-dimethyl ether): Yellow needles: R_{f} = 0.62 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 12.98 (1H, *s*, OH-5), 7.60 (1H, *br d*, *J*= 8.1 Hz, H6'), 7.59 (1H, *br s*, H-2'), 6.95 (1H, *d*, *J*= 8.1 Hz, H-5'), 6.97 (1H, *s*, H-3), 6.81 (1H, *br s*, H-8), 6.37 (1H, *br s*, H-6), 3.90 (3H, *s*, OCH_3), 3.87 (3H, *s*, OCH_3); UV (MeOH) λ_{max}: 250, 264 (sh), 345., +NaOMe: 259, 404., +NaOAc: 256, 355.[34]

Compound **5**; Penduletin (5,4′-dihydroxy-3,6,7-trimethoxyflavone): Yellow needles: R_{f} = 0.51 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.92 (2H, *d*, *J*= 8.6 Hz, H-2',6'), 6.91 (2H, *d*, *J*= 8.6 Hz, H-3',5'), 3.89 (3H, *s*, OCH_3), 3.76 (3H, *s*, OCH_3), 3.73 (3H, *s*, OCH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 176.65 (C-4), 158.72 (C-4'), 156.87 (C-7), 154.48 (C-9), 150.35 (C-2), 150.24 (C-5), 136.00 (C-3), 129.96 (C-6), 128.34 (C-2',6'), 118.83 (C-1'), 113.96 (C-3',5'), 104.16 (C-10), 89.12 (C-8), 58.44 (OCH_3), 57.92 (OCH_3), 54.59 (OCH_3),; UV (MeOH) λ_{max}: 271, 338., +NaOMe: 272, 392., +NaOAc: 272, 344 (sh), 396; EIMS (40 eV) *m/z*: 344 $[M]^+$, 329 $[M-Me]^+$, 181 $[A_1-Me]^+$, 121 $[B_2]^+$.[35]

Compound **6**; Viscosine (5,7,4'-trihydroxy-3,6-dimethoxyflavone): Yellow needles: R_{f} = 0.44 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.93 (2H, d, *J*= 8.2, H-2',6'), 6.94 (2H, d, *J*= 8.2, H-3',5'), 6.54 (1H, *s*, H-3), 3.78 (3H, *s*, OCH_3), 3.75 (3H, *s*, OCH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 178.62 (C-4), 160.60 (C-4′), 158.77 (C-2), 156.12 (C-9), 152.84 (C-5), 152.03 (C-7), 137.72 (C-3), 131.60 (C-6), 130.59 (C-2′,6'), 121.07 (C-1′), 116.09 (C-3',5′), 105.01 (C-10), 94.43 (C-8), 60.42 (OCH_3), 60.13 (OCH_3); UV (MeOH) λ_{max}: 268, 338., +NaOMe: 272, 331 (sh), 397., +NaOAc: 277, 350., +$AlCl_3$: 406 (sh), 361, 310 (sh), 272; +HCl-$AlCl_3$; 404 (sh), 362, 306 (sh), 281.; EIMS (40 eV) *m/z*: 330 $[M]^+$, 315 $[M-Me]^+$, 287 $[M-MeCO]^+$, 153 $[A_1]^+$, 121 $[B_2]^+$.[35]

Compound **7**; Chrysoeriol (Luteolin 3'-methyl ether): Yellow solid: R_{f} = 0.40 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 12.96 (1H, *s*, OH-5), 7.89 (1H, *br d*, *J*= 8.0 Hz, H-6'), 7.52 (1H, *br s*, H-2'), 6.90 (1H, *s*, H-3), 6.89 (1H, *d*, *J*= 8.0 Hz, H-5'), 6.56 (1H, *br s*, H-8), 6.18 (1H, *br s*, H-6), 3.74 (3H, *s*, OCH_3).[36]

Compound **8**; Hydroxygenkwanin (Luteolin 7-Methyl ether): Yellow solid: R_{f} = 0.40 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 13.06 (1H, *s*, OH-5), 7.89 (1H, *br d*, *J*= 8.0 Hz, H-6'), 7.52 (1H, *br s*, H-2'), 6.90 (1H, *s*, H-3), 6.89 (1H, *d*, *J*= 8.0 Hz, H-5'), 6.74 (1H, *br s*, H-8), 6.48 (1H, *br s*, H-6), 3.88 (3H, *s*, OCH_3).[37]

Compound **9**; Apigenin (5,7,4'-trihydroxyflavone): Yellow solid: R_{f} = 0.38 ($CHCl_3$-EtOAc, 8:2): ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 12.95 (1H, *s*, OH-5), 7.91 (2H, *d*, *J*= 8.0 Hz, H-2',6'), 6.91 (2H, *d*, *J*= 8.0 Hz, H-3',5'), 6.77 (1H, *s*, H-3), 6.46 (1H, *br s*, H-8), 6.17 (1H, *br s*, H-6); UV (MeOH) λ_{max}: 266, 335., +NaOMe: 273, 325 (sh), 394., +NaOAc: 272, 384.[38]

Cytotoxic activity

The results of cytotoxic activity of isolated compounds on MDA-MB-23, HT-29 and MRC-5 cell lines by MTT assay have been summarized in Table 3.

Discussion

The results GC and GC-MS analyses showed that the plant essential oil was rich in oxygenated sesquiterpenes (31.06%) and oxygenated monoterpenes (22.30%) with α-bisabolol (23.85%), thymol (17.88%) and spathulenol (7.21%) as the main compounds. A review of previous reports on essential oil of *S. lavandulifolia* from different parts on Iran revealed wide variations in their chemical constituents.[19-23] In a comparative study, Aghaei *et al.* characterized five chemotypes; I (germacrene D/bicyclogermacrene), II (germacrene D/spathulenol), III (limonene/δ-cadinene), IV (pulegone), and V (α-zingiberene) based on chemical diversity observed in essential oils of *S. lavandulifolia* collected from ten different locations in north, north-west and north-east of Iran.[20] Beside possible genetic diversities within different population of plants classified as one phenotype, some extrinsic factors such as geographic and climate conditions, stresses caused by drought, insects or microorganisms, plant harvesting time, drying and storage conditions, as well as essential oil extraction method can affect the essential oil composition.[39]

Because of lipophilic nature of their constituents, essential oils generally exert cytotoxic activity via disturbance of cell membrane permeability.[40] Essential oils rich in phenols, aldehydes and alcohols have been reported to possess more cytotoxic effects in comparison with others.[40] The cytotoxic activities of α-bisabolol and thymol, two major compounds of our analysed oil sample, have been reported during previous studies.[41,42]

Table 3. Cytotoxic activity of the flavonoids from *S. lavandulifolia* on different cell lines.

Samples	Cells				
	MDA-MB-231		HT-29		MRC-5
	IC_{50} (µg ml^{-1})	SI[a]	IC_{50} (µg ml^{-1})	SI	IC_{50} (µg ml^{-1})
Pachypodol (**1**)	165.21	1.74	235.76	1.21	287.16
Chrysosplenetin (**2**)	88.23	2.70	116.50	2.04	238.04
Kumatakenin (**3**)	130.93	2.59	370.15	0.91	335.07
Velutin (**4**)	289.64	0.83	205.27	1.17	240.98
Penduletin (**5**)	156.14	2.04	163.63	1.95	319.20
Viscosine (**6**)	148.15	3.33	150.01	3.29	493.17
Chrysoeriol (**7**) & Hydroxygenkwanin(**8**)	112.89	0.72	145.20	0.56	80.92
Apigenin (**9**)	141.99	0.25	168.70	0.21	35.67
Tamoxifen	4.61	2.45	2.5	4.52	11.31

[a] Selectivity index

Chen *et al*. demonstrated cytotoxic activity of α-bisabolol on PC-3, Hela, ECA-109 and HepG2 cell lines. Moreover, they showed α-bisabolol induces apoptosis in HepG2 cells in a dose- and time-dependently manner through both Fas- and mitochondrial-related pathway with involvement of p53 and NFκB either.[41] It has also been reported the exposure of permanent colonic cell line, Caco-2, to thymol and its mixture with carvacrol could be led to the mitochondrial damage, lipid degeneration, chromatin condensation and finally cell death through apoptosis and necrosis.[42]

This study reports the isolation and identification of compounds **1**-**8** from the aerial parts of *S. lavandulifolia* for the first time. Furthermore, our literature review indicates this is the first report on the isolation of Pachypodol (**1**), kumatakenin (**3**), velutin (**4**), viscosine (**6**) and hydroxygenkwanin (**8**) from the genus *Stachys*.

The isolated flavonoids (**1**-**9**) exhibited a moderate cytotoxic activity on MDA-MB-23, HT-29 and MRC-5 cell lines, of which chrysosplenetin, was found as the most toxic compounds toward MDA-MB-23 and HT-29 cells, with IC_{50} values of 88.23 and 116.50 μg ml^{-1}, respectively. Furthermore, most of the examined methoxylated flavonoids inhibited the growth of mentioned cancerous cell lines with higher selectivity in comparison with MRC-5 normal cells (Table 3). Among the tested compounds, chrysosplenetin, kumatakenin and viscosine with selectivity indices of 2.70, 2.59 and 3.33, respectively, showed higher preferential toxicity against MDA-MB-23 cells than tamoxifen (SI: 2.45).

Previous studies demonstrated that methoxylation of hydroxyl residue increases the cytotoxic potential of flavonoids and their selective activity against tumor cells, either.[43-45] The results of recent investigation on cytotoxicity of dietary flavonoids on different human cancer types have been reviewed by Sak.[46] In agreement with our findings, Kawaii and colleagues found that higher degrees of methoxylation of the A-ring and also the presence of a 3'-methoxyl residue are associated with enhanced antiproliferative activity of some synthesized polymethoxylated flavonoids against the leukemic HL60 cells.[47]

A mechanistic study on HepG2 and MCF-7 cells treated with two polymethoxylated flavonoid derivatives isolated from the aerial parts of *Euryops arabicus* indicated that antiproliferative activity of tested compounds was attributed to S-phase cell cycle arrest.[48] In another study, Saito *et al*. demonstrated nobiletin, a citrus polymethoxylated flavonoid, potentiated the cytolytic activity of KHYG-1 cells (a natural killer leukemia cell line) by induction of the expression of granzyme B gene, a serine protease which acts as a cytotoxic effector.[49]

Polymethoxylated flavonoids have also received great attention because of their antiangiogenic activity.[50] Potent antiangiogenic activity of xanthomicrol and calycopterin from the aerial parts of *Dracocephalum kotschi* and its relation with inhibition of VEGF (vascular endothelial growth factor) expression has been shown during previous investigations.[51]

Beside cytotoxic activity, viscosine has been previously reported for its anxiolytic and anticonvulsant effects via $GABA_A$ receptor modulation.[52] Therefore, this major flavonoid isolated from the chloroform extract of *S. lavandulifolia*, could be considered as an active compound involved in anxiolytic activity of *S. lavandulifolia* extracts documented by Rabbani *et al*.[10] Some hormone induced effects followed by administration of the aerial parts of *S. lavandulifolia* may be associated with the presence of penduletin and apigenin, flavonoids with confirmed estrogen receptor-β (ERβ) selective activity.[53]

Conclusion

In conclusion, flavonoids (**1**-**9**) identified in the chloroform extract of *S. lavandulifolia* could be assumed as toxic principles of this species. However, future *in vivo* studies need to confirm this finding. Furthermore, this study suggests some of the isolated methoxylated flavonoids such as chrysosplenetin (**2**), kumatakenin (**3**) and viscosine (**6**) as appropriate candidates for anti-cancer drug development research.

Acknowledgements

This research was supported by Tehran University of Medical Sciences and Health Services grant (No. 15951).

Conflict of interests

The authors claim that there is no conflict of interest.

References

1. Tundis R, Peruzzi L, Menichini F. Phytochemical and biological studies of Stachys species in relation to chemotaxonomy: a review. Phytochemistry. 2014;102:7-39. doi:10.1016/j.phytochem.2014.01.023
2. Jamzad Z. Flora of Iran, No.76: Lamiaceae. Tehran: Research Institute of Forests and Rangelands; 2012.
3. Minae B, Sardari M, Sharifi H, Abadi MSR, Sadeghpour O. *Stachys lavandulifolia* Vahl. and its Relation With Marmazad Activities in Traditional Manuscripts. Iran Red Crescent Med J. 2015;17(11):1-4. doi:10.5812/ircmj.19932
4. Zargari A. Medicinal Plants. Vol. 4. Tehran: University of Tehran Press; 1987.
5. Amin GR. *Popular medicinal plants of Iran*. Tehran: Tehran University of Medical Sciences Press; 2005.
6. Polat R, Cakilcioglu U, Satil F. Traditional uses of medicinal plants in Solhan (Bingol-Turkey). J Ethnopharmacol. 2013; 148(3):951-63. doi:10.1016/j.jep.2013.05.050
7. Iscan G, Demirci B, Demirci F, Goger F, Kirimer N, Kose Y, et al. Antimicrobial and antioxidant activities of *Stachys lavandulifolia* subsp. *lavandulifolia* essential oil and its infusion. Nat Prod Commun. 2012:7(9):1241-4.
8. Moghim H, Taghipoor S, Shahinfard N, Kheiri S, Heydari Z, Rafieian S. Antifungal effects of *Allium ascalonicum*, *Marticaria chamomilla* and *Stachys*

lavandulifolia extracts on *Candida albicans*. J HerbMed Pharmacol. 2014;3(1): 9-14.
9. Rabbani M, Sajjadi SE, Zarei HR. Anxiolytic effects of *Stachys lavandulifolia* Vahl on the elevated plus-maze model of anxiety in mice. J Ethnopharmacol. 2003;89(2-3):271-6. doi:10.1016/j.jep.2003.09.008
10. Rabbani M, Sajjadi SE, Jalali A. Hydroalcohol extract and fractions of *Stachys lavandulifolia* vahl: effects on spontaneous motor activity and elevated plus- maze behaviour. Phytother Res. 2005;19(10):854-8. doi:10.1002/ptr.1701
11. Ghasemi Pirbalouti A, Koohpyeh A. Wound healing activity of extracts of *Malva sylvestris* and *Stachys lavandulifolia*. Int J Biol. 2011;3(1):174-9. doi:10.5539/ijb.v3n1p174
12. Nabavizadeh F, Alizadeh AM, Adeli S, Golestan M, Moloudian H, Kamalinejad M. Gastroprotective effects of *Stachys Lavandulifolia* extract on experimental gastric ulcer. Afr J Pharm Pharmacol. 2011;5(2):155-9. doi:10.5897/ajpp10.296
13. Hajhashemi V, Ghannadi A, Sedighifar S. Analgesic and anti-inflammatory properties of the hydroalcoholic, polyphenolic and boiled extracts of *Stachys lavandulifolia*. Res Pharm Sci. 2006;2:92-8.
14. Tundis R, Bonesi M, Pugliese A, Nadjafi F, Menichini F, Loizzo MR. Tyrosinase, acetyl- and butyryl-cholinesterase inhibitory activity of *Stachys lavandulifolia* Vahl (Lamiaceae) and its major constituents. Rec Nat Prod. 2015;9(1):81-93.
15. Jalilian N, Modarresi M, Rezaie M, Ghaderi L, Bozorgmanesh M. Phytotherapeutic Management of Polycystic Ovary Syndrome: Role of Aerial Parts of Wood Betony (*Stachys lavandulifolia*). Phytother Res. 2013;27(11):1708-13. doi:10.1002/ptr.4921
16. Rahzani K, Malekirad AA, Zeraatpishe A, Hosseini N, Seify SMR, Abdollahi M. Anti-oxidative stress activity of *Stachys lavandulifolia* aqueous extract in human. Cell J. 2013;14(4):314-7.
17. Delazar A, Delnavazi MR, Nahar L, Moghadam SB, Mojarab M, Gupta A, et al. Lavandulifolioside B: a new phenylethanoid glycoside from the aerial parts of *Stachys lavandulifolia* Vahl. Nat Prod Res. 2011;25(1):8-16. doi:10.1080/14786411003754330
18. Safaei A. Identification and quantitative determination of luteolin and apigenin in the aerial parts and an extract of *Stachys lavandulifolia* by HPLC. Iran J Pharm Res. 2004;3(S2):90.
19. Ghasemi Pirbalouti A, Mohammadi M. Phytochemical composition of the essential oil of different populations of *Stachys lavandulifolia* Vahl. Asian Pac J Trop Biomed. 2013;3(2):123-8. doi:10.1016/s2221-1691(13)60036-2
20. Aghaei Y, Hossein Mirjalili M, Nazeri V. Chemical diversity among the essential oils of wild populations of *Stachys lavandulifolia* Vahl (Lamiaceae) from Iran. Chem Biodivers. 2013;10(2):262-73. doi:10.1002/cbdv.201200194
21. Meshkatalsadat MH, Sajjadi M, Amiri H. Chemical constituents of the essential oils of different stages of the growth of *Stachys lavandulifolia* Vahl. from Iran. Pak J Biol Sci. 2007;10(16):2784-6. doi:10.3923/pjbs.2007.2784.2786
22. Feizbaksh A, Tehrani MS, Rustaiyan A, Masoudi S. Composition of the essential oil of *Stachys lavandulifolia* Vahl. from Iran. J Essent Oil Res. 2003;15(2):72-3. doi:10.1080/10412905.2003.9712068
23. Javidnia K, Mojab F, Mojahedi S. Chemical constituents of the essential oil of *Stachys lavandulifolia* Vahl from Iran. Iran J Pharm Res. 2004;3(1):61-3.
24. Taghikhani A, Afrough H, Ansari-Samani R, Shahinfard N, Rafieian-Kopaei M. Assessing the toxic effects of hydroalcoholic extract of *Stachys lavandulifolia* Vahl on rat's liver. Bratisl Lek Listy. 2013;115(3):121-4. doi:10.4149/bll_2014_026
25. Taghikhani M, Nasri H, Asgari A, Afrough H, Namjoo A, Ansari-Samani R, et al. The renal toxicity of hydroalcoholic extract of *Stachys lavandulifolia* Vahl in Wistar rats. Life Sci J. 2012;9(4):3025-31.
26. Monji F, Hosein Tehrani H, Halvaei Z, Arbabi Bidgoli S. Acute and subchronic toxicity assessment of the hydroalcoholic extract of *Stachys lavandulifolia* in mice. Acta Med Iran. 2011;49(12):769-75.
27. Mohammadifar F, Delnavazi MR, Yassa N. Chemical analysis and toxicity screening of *Phlomis olivieri* Benth. and *Phlomis persica* Boiss. essential oils. Pharm Sci. 2015;21(1):12-7. doi:10.15171/ps.2015.11
28. Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry. Carol Stream: Allured Publishing Corporation; 2007.
29. Mabry T, Markham KR, Thomas MB. The systematic identification of flavonoids. New York: Springer-Verlag; 1970.
30. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, et al. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res. 1988;48(3):589-601.
31. Ali HA, Chowdhury A, Rahman AK, Borkowski T, Nahar L, Sarker SD. Pachypodol, a flavonol from the leaves of *Calycopteris floribunda*, inhibits the growth of CaCo 2 colon cancer cell line in vitro. Phytother Res. 2008;22(12):1684-7. doi:10.1002/ptr.2539
32. He F, Aisa H, Shakhidoyatov KM. Flavones from *Artemisia rupestris a*. Chem Nat Compd. 2012;48(4):685-6. doi:10.1007/s10600-012-0350-x
33. Alwahsh MAA, Khairuddean M, Chong WK. Chemical Constituents and Antioxidant Activity of *Teucrium barbeyanum* Aschers. Rec Nat Prod. 2015;9(1):159-63.
34. Citoglu G, Sever B, Antus S, Baitz-Gacs E, Altanlar N. Antifungal flavonoids from *Ballota glandulosissima*. Pharm Biol. 2003;41(7):483-6. doi:10.1080/13880200308951339
35. Sachdev K, Kulshreshtha DK. Flavonoids from *Dodonaea viscosa*. Phytochemistry. 1983;22(5):1253-6. doi:10.1016/0031-9422(83)80234-9

36. Awaad Amani AS, Maitland DJ, Soliman GA. Antiulcerogenic Activity of *Alhagi maurorum*. Pharm Biol. 2006;44(4):292-6. doi:10.1080/13880200600714160
37. Azimova S, Vinogradova V. Natural Compounds; Flavonoids; Plant Sources, Structure and Properties. New York: Springer; 2013.
38. Loizzo MR, Said A, Tundis R, Rashed K, Statti GA, Hufner A, et al. Inhibition of angiotensin converting enzyme (ACE) by flavonoids isolated from *Ailanthus excelsa* (Roxb) (Simaroubaceae). Phytother Res. 2007;21(1):32-6. doi:10.1002/ptr.2008
39. Baser KHC, Buchbauer G. Handbook of essential oils: science, technology, and applications. New York: CRC Press; 2015.
40. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils-a review. Food Chem Toxicol. 2008;46(2):446-75. doi:10.1016/j.fct.2007.09.106
41. Chen W, Hou J, Yin Y, Jang J, Zheng Z, Fan H, et al. α-Bisabolol induces dose-and time-dependent apoptosis in HepG2 cells via a Fas-and mitochondrial-related pathway, involves p53 and NFκB. Biochem Pharmacol. 2010;80(2):247-54. doi:10.1016/j.bcp.2010.03.021
42. Llana-Ruiz-Cabello M, Gutierrez-Praena D, Pichardo S, Moreno FJ, Bermudez JM, Aucejo S, et al. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. Food Chem Toxicol. 2014;64:281-90. doi:10.1016/j.fct.2013.12.005
43. Plochmann K, Korte G, Koutsilieri E, Richling E, Riederer P, Rethwilm A, et al. Structure-activity relationships of flavonoid-induced cytotoxicity on human leukemia cells. Arch Biochem Biophys. 2007;460(1):1-9. doi:10.1016/j.abb.2007.02.003
44. Moghaddam G, Ebrahimi SA, Rahbar- Roshandel N, Foroumadi A. Antiproliferative activity of flavonoids: influence of the sequential methoxylation state of the flavonoid structure. Phytother Res. 2012;26(7):1023-8. doi:10.1002/ptr.3678
45. Berim A, Gang DR. Methoxylated flavones: occurrence, importance, biosynthesis. Phytochem Rev. 2016;15(3):363-90. doi:10.1007/s11101-015-9426-0
46. Sak K. Cytotoxicity of dietary flavonoids on different human cancer types. Pharmacogn Rev. 2014;8(16):122-46. doi:10.4103/0973-7847.134247
47. Kawaii S, Ikuina T, Hikima T, Tokiwano T, Yoshizawa Y. Relationship between structure and antiproliferative activity of polymethoxyflavones towards HL60 cells. Anticancer Res. 2012;32(12):5239-44.
48. Alarif WM, Abdel-Lateff A, Al-Abd AM, Basaif SA, Badria FA, Shams M, et al. Selective cytotoxic effects on human breast carcinoma of new methoxylated flavonoids from *Euryops arabicus* grown in Saudi Arabia. Eur J Med Chem. 2013;66:204-10. doi:10.1016/j.ejmech.2013.05.025
49. Saito T, Abe D, Nogata Y. Polymethoxylated flavones potentiate the cytolytic activity of NK leukemia cell line KHYG-1 via enhanced expression of granzyme B. Biochem Biophys Res Commun. 2015;456(3):799-803. doi:10.1016/j.bbrc.2014.12.027
50. Lam IK, Alex D, Wang YH, Liu P, Liu AL, Du GH, et al. In vitro and in vivo structure and activity relationship analysis of polymethoxylated flavonoids: identifying sinensetin as a novel antiangiogenesis agent. Mol Nutr Food Res. 2012;56(6):945-56. doi:10.1002/mnfr.201100680
51. Abbaszadeh H, Ebrahimi SA, Akhavan MM. Antiangiogenic activity of xanthomicrol and calycopterin, two polymethoxylated hydroxyflavones in both in vitro and ex vivo models. Phytother Res. 2014;28(11):1661-70. doi:10.1002/ptr.5179
52. Karim N, Irshad S, Khan I, Mohammad A, Anis I, Shah MR, et al. $GABA_A$ receptor modulation and neuropharmacological activities of viscosine isolated from *Dodonaea viscosa* (Linn). Pharmacol Biochem Behav. 2015;136:64-72. doi:10.1016/j.pbb.2015.07.006
53. Jarry H, Spengler B, Porzel A, Schmidt J, Wuttke W, Christoffel V. Evidence for estrogen receptor β-selective activity of *Vitex agnus-castus* and isolated flavones. Planta Med. 2003;69(10):945-7. doi:10.1055/s-2003-45105