Chemical investigations of male and female leaf extracts from Schinus molle L.

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Keywords:	Schinus molle L., extracts, GC/MS, elemol

Chemical investigations of male and female leaf extracts from *Schinus molle* L.

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Abstract

The pepper-tree Schinus molle is an evergreen ornamental plant with various and diversified list of medical uses. In this article we analysed the chemical composition of male and female leaves of this plant during the off-flowering and flowering seasons. The leaf extracts were obtained by using a sequential extraction with solvents of different polarities and the chemical composition was investigated by GC-MS. The results showed a total of twenty-three components, **in which elemol is the most abundant constituent followed by bicyclogermacrene, γ-eudesmol, α-eudesmol, β-eudesmol and isocalamendiol**. The petroleum ether and diethyl ether extracts from male and female flowering and off-flowering leaves consisted of sesquiterpene hydrocarbons as a major constituent followed by monoterpene hydrocarbons, while the acetone extracts showed a different composition. The obtained results show differences in the chemical composition between male and female and flowering and not flowering.

Keywords: *Schinus molle* L.; Extracts; GC/MS; Elemol

1. Introduction

Schinus molle L. is an evergreen plant belonging to the Anacardiaceae family. Commonly called "pink" or "false pepper", *S. molle* is a dioecious plant with compounds, imparipinnate and lanceolate leaves having a peppery smell when crushed. It has pendulous branches with yellowish-white flowers arranged in clusters; the fruits are coral-red in the size of peppercorns (Kasimala and Kasimala 2012).

Essential oils purified from *S. molle* are mainly constituted by monoterpenoids and sesquiterpenoids, among which the major components were α-phellandrene and sylvestrene (Bendaoud et al. 2010).

Different studies carried out on essential oils have shown that the sexual differences in dioecious plant (Sharma et al. 2015), the vegetative cycle or physiological variations (Figueiredo et al. 2001), the seasonal variations (Choudhry et al. 2014) and the geographical and environmental factors (Borges et al. 2017) can affect the chemical variability concerning plant composition and/or secondary metabolites productions (Figueiredo et al. 2008).

Based on these evidences, we aimed to investigate the chemical composition of leaf extracts from male and female *S. molle* plants collected during the off-flowering season and the flowering season. The samples were obtained by using a sequential extraction with solvents of different polarities (petroleum ether, diethyl ether and acetone), with the purpose to achieve a selectivity for the extracted phytochemicals polarity. The extracts were then chemically analyzed by using GC-MS.

2. Results and Discussion

The composition of the male and female *S. molle* petroleum ether extract from leaves in the flowering and off-flowering periods reveals that the main component of the considered stages was elemol (53.68% and 65.83%) for the male and (69.53% and 54.82%) for female, respectively (Table S1). The second most abundant component was bicyclogermacrene (9.73% and 11.59%) for the male and germacrene D (15.58% and 15.23%) was for the female. Elixene (1.21% and 1.71%) and spathulenol (0.64% and 2.22%) were present only in the male leaves while α-thujene (0.72% for male and 0.99% for female), germacrene D-4-ol (5.20% for male and 8.31% for female) and n-hexadecanoic acid (0.24% for male and 0.32% for female) were present only in the off-flowering season's leaves. Viridiflorol (4.85%) was present only in the female leaves of off-flowering season. The composition of the male and female *S. molle* diethyl ether

extract from leaves in the flowering and off-flowering periods are reported in Table 2. As for petroleum ether extracts, elemol (57.57% and 72.58%) for the male and (71.37% and 60.80%) for the female was the main component and bicyclogermacrene (9.56% and 7.76%) was the second most abundant component for the male while the germacrene D (11.39% and 10.86%) was for the female. Elixene (1.46% and 1.11%) and spathulenol (3.22% and 0.65%) were present only in the male leaves. α-thujene (0.67% for male and 0.72 for female), germacrene D-4-ol (1.65% for male and 3.83% for female) and n-hexadecanoic acid (0.89% for male and 10.41% for female) were present only in the leaves of off-flowering season. In acetone extract the number of identified components was lower than the other two extracts. 2-pentanone, 4-hydroxy-4-methyl (57.47% for the male and 59.92% for the female) was the most abundant component of extract obtained from off-flowering leaves (Table S3). N-hexadecanoic acid (49.77% for the male), was the most abundant component of extract obtained from flowering male leaves and absent in the female flowering leaves. The percentage (9.98% and 17.42%) of elemol from the male was lower if compared to the extract in diethyl ether and in petroleum ether and remained high for the female (51.89% and 25.72%). In the male flowering season leaves β-eudesmol (24.68%) was the most abundant component. Sabinene (0.75% and 0.39%), humulene (1.07% and 0.30%), δ-cadinene (0.96% and 0.84%) and 2-propanone, 1-hydroxy (1.04% and 0.32%) were present only in the female leaves.

The GC/MS analysis of the extracts showed elemol as the most abundant component in the petroleum ether and diethyl ether extracts while its percentage was lower in the acetone extract. A total of twenty-three components were identified by GC-MS. The petroleum ether and diethyl ether extracts from male and female flowering and off-flowering leaves consisted of sesquiterpene hydrocarbons as a major constituent followed by monoterpene hydrocarbons. The acetone extracts showed different composition and the extracts from the male flowering and off-flowering samples were characterized by the presence of n-hexadecanoic acid (49.77%) and 2-pentanone, 4-hydroxy-4-methyl (57.47%), respectively. On the other hand, the extracts from the female flowering and off-flowering leaves were mainly characterized by the presence of 2-pentanone, 4-hydroxy-4-methyl (17.86% and 59.92%).

Taken together, the obtained results suggest that the use of solvents having different polarity grade is very important for investigations of the extracts from *S. molle* and

clearly show differences in the chemical composition between male and female flowering and off-flowering leaves. Further investigations will need to point out if such chemical differences could be related to the different role of male and female plants in the fertilization process.

There are many studies related to the extracts composition of *S. molle* leaves, berries and aerial parts from different regions of the world (Bendaoud et al. 2010; Salem et al. 2016). As reported by dos Santos Cavalcanti et al. (2015), the different chemical composition can be related to various factors. The main factor that determines the concentration of each compound is its genotype (Gomes et al. 2013). However, variations in the extract contents from plant tissues should be also dependent from different factors, some of them intrinsic and controlled by the plant's genetic variability and others dependent on the extraction method applied. Furthermore, quantitative profiles are susceptible to climatic conditions, such as seasonality, water availability and soil nutrients (dos Santos Cavalcanti et al. 2015).

3. Experimental (Supplied as supplementary materials)

4. Conclusions

Our investigations confirm that the extracts of *S. molle* are an important source of chemical constituents and in this view further studies need to address such compounds for possible uses in aromatherapy and pharmacy.

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Abstract

The pepper-tree Schinus molle is an evergreen ornamental plant with various and diversified list of medical uses. In this article we analysed the chemical composition of male and female leaves of this plant during the off-flowering and flowering seasons. The leaf extracts were obtained by using a sequential extraction with solvents of different polarities and the chemical composition was investigated by GC-MS. **The results showed a total of twenty-three components, in which elemol is the most abundant constituent followed by bicyclogermacrene, γ-eudesmol, α-eudesmol, β-eudesmol and isocalamendiol**. The petroleum ether and diethyl ether extracts from male and female flowering and off-flowering leaves consisted of sesquiterpene hydrocarbons as a major constituent followed by monoterpene hydrocarbons, while the acetone extracts showed a different composition. The obtained results show differences in the chemical composition between male and female and flowering and not flowering

Experimental section

Plant collection and extracts preparation

Fresh leaves of male and female *S. molle* plants, **included in the internal management system and identified by cod. AS21 (male individual) and cod. AS22 (female individual),** were collected at the "Angelo Rambelli" Botanical Garden (Tuscia University, Viterbo, Italy) in October 2017 for the off-flowering samples and in June 2017 for the flowering samples. The plant materials were washed with distilled water, air-dried in a shaded area for 2-4 days and carefully hand-selected to separate the leaves, which were kept in a plastic container and frozen at –80°C for freeze-drying. After lyophilisation, the dried material was chopped and stored at 4°C until use. Dried leaves were extracted using three solvents (100 mL each one) of increasing polarity (petroleum ether 40-60, diethyl ether and acetone) in a Soxhlet apparatus. After 6 extraction cycles for each solvent, the solid extracts were obtained by rotary evaporation (RV 08-VC, IKA, USA).

GC-MS analysis:

GC-MS Perkin Elmer Clarus 500 instrument equipped with flame ionization detector (FID) and a Restek Stabilwax fused-silica capillary column (length 60 m x 0.25 mm ID) was employed. Helium was used as the carrier gas with a flow rate of 1 mL/min, and the oven temperature program was as follows: 5 minutes at 60°C then a gradient of 5°C/minute to 220°C. 1 µl of sample was diluted in 1 ml of CH_3OH and 1 µl of the solution was manually injected at 280°C into the GC injector in the splitless mode. All mass spectra were recorded in the electron impact ionization (EI) at 70 eV. The mass spectrometer was scanned from m/z 30-350 amu with scan time 0.2 sec.

Relative percentages for quantification of the components were calculated by electronic integration of the GC-FID peak areas. Identification of the constituents was performed on MS library search (Wiley and Nist). Linear retention indices (LRIs) of each compound were calculated using a mixture of aliphatic hydrocarbons (C8-C30, Ultrasci) injected directly into GC injector at the same

temperature program reported above. Analyses were repeated twice.

# [1]	Component[2]	LRI[3]	LRI[lit] [4]	Male		Female	
				Leaves (flowering)	Leaves (off-flowering)	Leaves (flowering)	Leaves (off-flowering)
1	α-thujene	1037	1038	-	0.72	-	0.99
2	sabinene	1133	1133	0.70	2.58	0.18	2.19
3	2-propanone, 1-hydroxy	1331	1326	-	-	-	-
4	2-pentanone, 4-hydroxy-4-methyl	1360	1369	-	-	-	-
5	elixene	1510	1514	1.71	1.21	-	-
6	α-gurjunene	1556	1549	0.56	0.31	0.91	0.74
7	β-elemene	1608	1598	1.27	0.74	2.11	1.15
8	4-terpineol	1610	1621	0.31	-	-	-
9	β-caryophyllene	1637	1634	1.80	0.68	2.18	2.44
10	humulene	1705	1693	0.63	0.32	0.97	0.80
11	germacrene D	1732	1726	9.63	6.55	15.23	15.58
12	bicyclogermacrene	1774	1769	11.59	9.73	0.58	0.56
13	δ-cadinene	1788	1788	1.16	0.64	1.76	1.44
14	germacrene D-4-ol	2077	2069	-	5.20	-	8.31
15	elemol	2093	2090	53.68	65.83	69.53	54.82
16	viridiflorol	2117	2110	-	-	-	4.85
17	dehydroxyisocalamendiol	2141	*	-	0.87	2.02	2.07
18	spathulenol	2142	2136	2.22	0.64	-	-
19	γ-eudesmol	2191	2185	2.00	0.49	-	0,63
20	α-eudesmol	2240	2232	3.94	1.36	1.93	1,34
21	β-eudesmol	2258	2249	6.69	1.79	1.95	1.69
22	isocalamendiol	2510	2500	2.11	-	0.65	-
23	dodecanoic acid	2887	2900	-	0.34	-	0.40
	Monoterpene hydrocarbons			10.33	9.85	15.41	18.76
	Monoterpenes alcohol			0.31			
	Sesquiterpene hydrocarbons			71.64	72.69	79.16	62.61
	Oxygenated sesquiterpenes				0.87	2.02	2.07
	Tricyclic sesquiterpenes			2.22	0.64		
	Sesquiterpene alcohol				5.20		13.16
	Bicyclic sesquiterpenes			13.39	10.41	2.76	3.0
	Others			**2.11**	**0.34**	**0.65**	**0.40**

[1]# indicates the compound identification number; [2]the components are reported according their eluition order on polar column.[3]Linear Retention Indices measured on polar column; [4]Linear Retention Indices from literature; *LRIs[lit] not available

Table S1. Chemical composition (%) of petroleum ether extract from male and female *S. molle*

# [1]	Component[2]	LRI[3]	LRI[lit] [4]	Male		Female	
				Leaves (flowering)	Leaves (off-flowering)	Leaves (flowering)	Leaves (off-flowering)
1	α-thujene	1037	1038	-	0.67	-	0.72
2	sabinene	1133	1133	1.46	1.84	2.42	1.66
3	2-propanone, 1-hydroxy	1331	1326	-	-	-	-
4	2-pentanone, 4-hydroxy-4-methyl	1360	1369	-	-	-	-
5	elixene	1510	1514	1.46	1.11	-	-
6	α-gurjunene	1556	1549	0.67	0.22	0.90	0.54
7	β-elemene	1608	1598	0.95	0.61	1.32	1.22
8	4-terpineol	1610	1621	0.37	-	-	-
9	β-caryophyllene	1637	1634	1.73	0.55	1.92	2.08
10	humulene	1705	1693	0.56	0.25	0.77	0.60
11	germacrene D	1732	1726	7.61	5.14	11.39	10.86
12	bicyclogermacrene	1774	1769	9.56	7.76	0.38	0.41
13	δ-cadinene	1788	1788	1.05	0.52	1.28	1.07
14	germacrene D-4-ol	2077	2069	-	1.65	-	3.83
15	elemol	2093	2090	57.57	72.58	71.37	60.80
16	viridiflorol	2117	2110	-	-	-	-
17	dehydroxyisocalamendiol	2141	*	-	1.83	1.65	1.26
18	spathulenol	2142	2136	3.22	0.65	-	-
19	γ-eudesmol	2191	2185	1.83	0.46	-	0.64
20	α-eudesmol	2240	2232	3.13	1.45	2.57	1.73
21	β-eudesmol	2258	2249	4.95	1.82	2.60	2.17
22	isocalamendiol	2510	2500	3.88	-	1.43	-
23	dodecanoic acid	2887	2900	-	0.89	-	10.41
	Monoterpene hydrocarbons			9.07	7.65	13.81	13.24
	Monoterpenes alcohol			0.37			
	Sesquiterpene hydrocarbons			76.05	79.02	82.24	68.77
	Oxygenated sesquiterpenes				1.83	1.65	1.26
	Tricyclic sesquiterpenes			3.22	0.65	-	
	Sesquiterpene alcohol				1.65		3.83
	Bicyclic sesquiterpenes			11.29	8.31	2.3	2.49
	Others				0.89		10.41

[1]# indicates the compound identification number; [2]the components are reported according their elution order on polar column.[3]Linear Retention Indices measured on polar column; [4]Linear Retention Indices from literature; *LRIs[lit] not available.

Table S2. Chemical composition (%) of diethyl ether extract from male and female *S. molle*

#[1]	Component[2]			Male		Female	
		LRI[3]	LRI[lit 4]	Leaves (flowering)	Leaves (off-flowering)	Leaves (flowering)	Leaves (off-flowering)
1	α-thujene	1037	1038	-	-	-	-
2	sabinene	1133	1133	-	-	0.75	0.39
3	2-propanone, 1-hydroxy	1331	1326	-	-	1.04	0.32
4	2-pentanone, 4-hydroxy-4-methyl	1360	1369	-	57.47	17.86	59.92
5	elixene	1510	1514	-	-	-	-
6	α-gurjunene	1556	1549	-	-	-	-
7	β-elemene	1608	1598	-	-	-	-
8	4-terpineol	1610	1621	-	-	-	-
9	β-caryophyllene	1637	1634	1.57	1.46	3.33	1.54
10	humulene	1705	1693	-	-	1.07	0.30
11	germacrene D	1732	1726	1.34	6.52	9.21	3.70
12	bicyclogermacrene	1774	1769	1.14	11.07	0.66	0.34
13	δ-cadinene	1788	1788	-	-	0.96	0.84
14	germacrene D-4-ol	2077	2069	-	2.07	-	1.58
15	elemol	2093	2090	17.42	9.98	51.89	25.72
16	viridiflorol	2117	2110	-	-	-	-
17	dehydroxyisocalamendiol	2141	*	-	1.02	2.23	-
18	spathulenol	2142	2136	0.06	0.22	-	-
19	γ-eudesmol	2191	2185	0.08	0.98	-	0.37
20	α-eudesmol	2240	2232	0.16	3.69	1.92	1.34
21	β-eudesmol	2258	2249	24.68	3.78	2.33	1.56
22	isocalamendiol	2510	2500	3.78	-	6.75	-
23	n-hexadecanoic acid	2887	2900	49.77	1.74	-	2.08
	Monoterpene hydrocarbons			1.34	6.52	9.96	4.09
	Monoterpenes alcohol			-	-	-	-
	Sesquiterpene hydrocarbons			46.12	18.43	64.92	30.13
	Oxygenated sesquiterpenes			-	1.02	2.23	-
	Tricyclic sesquiterpenes			0.06	0.22	-	-
	Sesquiterpene alcohol			-	2.07	-	1.58
	Bicyclic sesquiterpenes			2.71	12.53	3.99	1.88
	Others			**49.77**	**59.21**	**18.9**	**62.32**

[1]# indicates the compound identification number; [2]the components are reported according their elution order on polar column[3]Linear Retention Indices measured on polar column; [4]Linear Retention Indices from literature; [*]LRIs[lit] not available.

Table S3. Chemical composition (%) of acetone extract from male and female *S. molle*

Reviewer: 1

Comments to the Author
Q1: It is necessary to add a relevant detail. The studied organism(s) should be carefully identified and the voucher specimen number(s) should be reported.

A1: *Schinus molle* plants, present in the Botanical Garden of Tuscia University (A. Rambelli Botanical Garden), are included in the internal management system and identified by cod. AS21 (male individual) and cod. AS22 (female individual). Such data have been added in the text.

Reviewer: 2
Comments to the Author
The article can be published after major revision.

Q1: The graphical abstract needs good resolution.

A1: It has been modified as requested following the Author's instructions

Q2: How did you differentiate between male and female leaf?

A2: Male and female leaf were collected from two separate trees, one male and one female being *Schinus molle* a dioecius plant

Q3: In the abstract please show the main chemical components of extracts and essential oils.

A3: It has been done as requested

Q4: The After lyophilisation can be done using petroleum ether and you extracted some extracts using petroleum ether, how can you differentiate between them.

A4: Lyophilization was carried out by a Freezone 2.5 L Benchzone Freeze Dryer and not using petroleum ether that was employed in Soxhlet extraction

Q5: Antibacterial or antifungal activities are missing.

A5: The paper deals with chemical investigations of male and female leaf extracts from *Schinus molle*; further investigations on biological activities are in progress

Reviewer: 3
Comments to the Author
Q1: Using Soxhlet apparatus can be decomposition the chemical components of extracts, since you use heating.

A1: Yes it's possible, however many other papers reported the use of the soxhlet apparatus. Boiling points for the used solvents are not high: petroleum ether 42-62°C; diethyl ether 34,6 °C; acetone 56,05 °C. They are continuously checked during all the extraction time.

Q2: Graphical abstract is poor.

A2: It has been modified as requested

Q3: Where is the antimicrobial activity of extracts?

A3: The paper deals with chemical investigations of male and female leaf extracts from *Schinus molle*; further investigations on biological activities are in progress

Q4: Most of the identified compounds are related to essential oils, where other groups of components

A4: The aim of this preliminary paper is to investigate the chemical composition of male and female *S. molle* leaf extracts obtained by using a sequential extraction with solvents of different polarities (petroleum ether, diethyl ether and acetone); such results could represent a contribution for establishing possible chemical differences between the genders. Studies are running in order to investigate chemical composition of essential oils.

Q5: Figures S1 and S2 are not necessary.

A5: Thanks of your suggestion; they have been deleted.

Reviewer: 4

Comments to the Author

Q1: The subject is relevant, however, there are many works on the composition of the S. molle plants with similar results. The novel information provided was the comparison between male and female plants in two stages. However, the compounds found in this work were already published for the essential oils of S. molle with a few differences. The plant growth conditions was not provided and this is a major factor for the composition of *S. molle* plants. The major problem with the manuscript was the absence of statistical treatment. The comparison reported above will provide a two-way ANOVA experimental design. Therefore, proper analysis is need with replicates to support the results as well as the conclusion. There are a few language mistakes making some sentences difficult to understand (as the last phrase of the abstract, for example). The work is poorly discussed and the conclusion seems not to fit the objectives, as well as it was not supported by the results. Therefore, I sorry but I could not recommend the work to publish the way it is.

A1: We are sorry if the reviewer does not consider our work for publication. The aim of the paper is to investigate the chemical composition of leaf extracts from male and female *S. molle* plants collected during the off-flowering season and the flowering season. Concerning the plant growth conditions, the *S. molle* trees objects of our investigation are cultured in the natural environment of the Botanical Garden of Tuscia University and treatments or specific culture conditions are not supplied.

The molecules percentages refer to the relative molecules abundances in each extract and not to the absolute concentrations. For this reason, the comparison between the molecules found in the different extracts couldn't be done unless having the possibilities to normalize the obtained data.

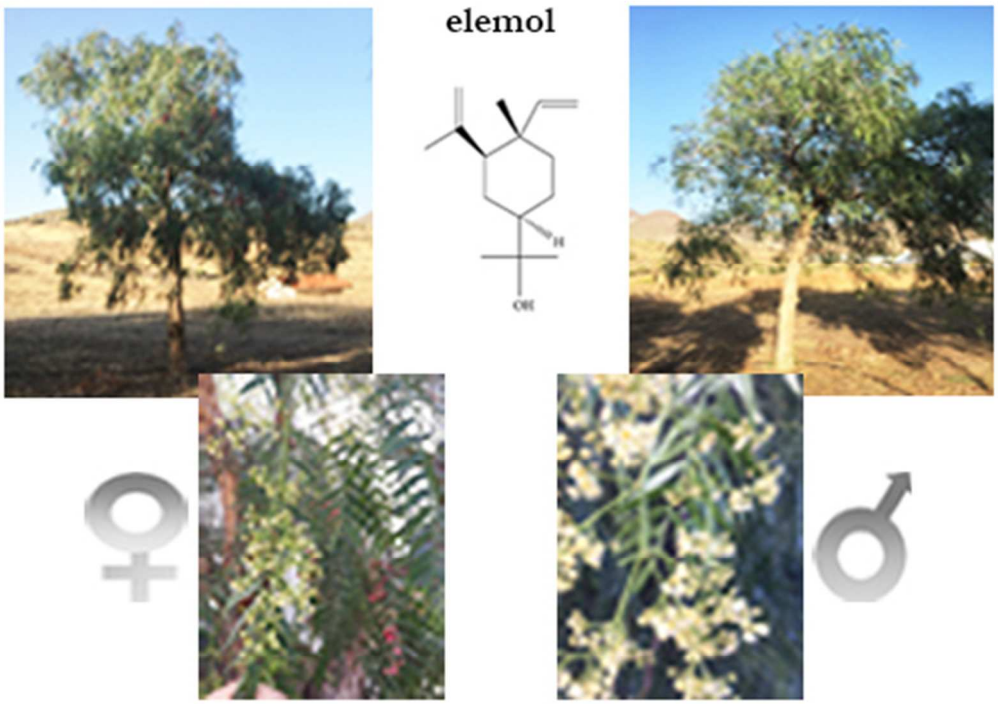
Furthermore different paper deals with this kind of analysis (GC-MS and relative abundance). Few examples:

- Yuan, J., T. Gan, et al. (2018). "Composition and antimicrobial activity of the essential oil from the branches of *Jacaranda cuspidifolia* Mart. growing in Sichuan, China." Natural Product Research 32(12): 1451-1454.

- Garzoli, S., M. Božović, et al. (2018). "Essential oil extraction, chemical analysis and anti-Candida activity of *Foeniculum vulgare* Miller – new approaches." Natural Product Research 32(11): 1254-1259.

- Ali Rostaefar, Abbas Hassani & Fatemeh Sefidkon (2017): Seasonal variations of essential oil content and composition in male and female plants of *Juniperus communis* L. ssp. *hemisphaerica* growing wild in Iran, Journal of Essential Oil Research

- Ammar, I., M. Ennouri, et al. (2012). "Variation in chemical composition and biological activities of two species of Opuntia flowers at four stages of flowering." Industrial Crops and Products 37(1): 34-40.



176x172mm (72 x 72 DPI)