Chemical characterization and nematicidal activity of the essential oil of Nepeta nuda L. ssp. pubescens and Nepeta curviflora Boiss. from Lebanon

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Abstract

The chemical characterization and the nematicidal activity of the essential oil from *Nepeta nuda* L. ssp. *pubescens* growing wild in Lebanon are reported. A comparative study with the essential oil of *Nepeta curviflora* Boiss. growing in Lebanon as well, was carried out. The most abundant (> 5%) components of *N. nuda* ssp. *pubescens* essential oil were pinane (12.89%), 1-ethyl-1H-pyrrole (12.67 %), 1-cycloethyl-1-(2-methylenecyclohexyl)ethanol (10.37%), 3-methyl-2-cyclohexen-1-one (9.17%) and 2,3-dimethyl-3-hexanol (5.88%). Among oxygenated monoterpenes, two nepetalactones were identified, *i.e.* (*E,Z*)-nepetalactone (2.24%) and (*Z,E*)-nepetalactone (0.31%). The major constituents (> 5 %) of *N. curviflora* essential oil were 2-isopropyl-5-methyl-3-cyclohexen-1-one (12.51%), (-)-spathulenol (11.73%), *cis*-*Z*-α-bisabolene epoxide (8.07%), widdrol (7.0 %), (*E,Z*)-5,7-dodecadiene (6.93%), dihydronepetalactone (5.57%) and 4-propyl-cyclohexene (5.43%). The essential oil of *N. curviflora* was more active than the *N. nuda* ssp. *pubescens* one against the nematode *Panagrolaimus rigidus*. According to the motility assay, LD_{50} was 0.5 mg/mL and 2.5 mg/mL 24 h after treatment with *N. curviflora* and *N. nuda* ssp. *pubescens* essential oil, respectively. To the best of our knowledge, *N. nuda* ssp. *pubescens* has not been investigated to date.

Keywords: *Lamiaceae, Panagrolaimus rigidus*, terpenes, nepetalactones, biopesticides, anthelmintics.

Introduction

The genus *Nepeta* (Lamiaceae) consists of around 300 herbaceous, perennial, rarely annual species. This genus is distributed over a wide area including Europe, Asia, Africa and the Middle East (Formisano et al., 2011). Some of these species are widely used in traditional medicine and are endowed with a number of pharmacological properties, usually attributed to their essential oils (Sharma et al. 2013). The main bioactive compounds are a variety of secondary metabolites, including monoterpenes (iridoids and their glycosides), diterpenes, triterpenes as well as phenols.

Nepeta nuda L. is one of the most common species of the genus *Nepeta*. It is widespread from central and southeast Europe to Russia and towards southwest and central Asia. The essential oil composition of *N. nuda* was studied in a number of investigations (De Pooter et al. 1987; Alim et al. 2009; Turkey et al., 2011; Bozari et al., 2013; Gormez et al., 2013).

As part of a continuing study on the essential oils of some *Nepeta* species growing in Lebanon, we were interested in studying the chemical composition and the nematicidal activity of the essential oil of *N. nuda* ssp. *pubescens*. To the best of our knowledge, this plant has not been investigated to date.

Nematodes or roundworms are parasites of humans, vertebrates, insects and plants. Plant-parasitic nematodes are soil-inhabiting organisms mostly 0.5 to 1 mm long. These soil invertebrates have a hollow spear, called stylet, that penetrates plant cells and withdraws the cytoplasmic contents, thus damaging root tissues, with typical root and foliar symptoms. Nematodes can be grouped by feeding habit as endoparasitic (entire body inside the root), ectoparasitic (entire body outside the root) and semi-endoparasitic (part of body inside the root), whereas, by movement when feeding, they are divided into sedentary (mostly immobile during their life cycle) and migratory (mobile for all their life). In addition, nematodes can be vectors of plant viruses. Currently, plant diseases caused by nematodes threaten the world agriculture because of their severity and economic burden: in the developing countries, crop production losses attributable to nematodes are estimated at around 15%, compared with around 9% in developed countries. Not least, chemical control of nematodes is troublesome after the ban of soil fumigants such as methyl bromide, and, in addition, non-volatile nematicides, organophosphates and carbamates, can represent environmental and human threats due to their toxicological and ecotoxicological risks (Perry et al., 2006; Thompson et al., 2005; De Waele et al., 2007; Lewis et al., 2009; Tritten et al., 2012). Therefore, environmentally friendly and low-cost alternatives to chemical control measures for phytoparasitic nematodes are urgently needed. In this context, essential oils from plants could be attractive nematicide compound sources, possibly safe for non-target organisms.

In this paper, we report, for the first time, the chemical characterization and the nematicidal activity of the essential oil from *N. nuda* ssp. *pubescens* growing in Lebanon. A comparative study with the essential oil of *Nepeta curviflora* Boiss. (Senatore et al., 2005; Mancini et al., 2009; Al-Qudah 2016), growing in Lebanon as well, was carried out.

Materials and methods

Plant material and essential oil production. The dried parts of *Nepeta nuda* ssp. *pubescens* (flowering tops, seeds and leaves) were collected between 2005 and 2009 in a mountain area (1500 m above sea level) near to Tannourine, Lebanon. The dried parts of *Nepeta curviflora* (flowering tops, seeds, and leaves) were collected in 2005 in Lebanon, near to the St. Antonio of Qozhaya convent, Ehden region at 664 m above sea level.

Essential oils were obtained by steam distillation (250 mL water) from a sample (5 g) of air-dried aerial parts of the plant. The collected aqueous phase (150 mL) containing the essential oil was extracted with diethyl ether (40 mL × 5). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to obtain 12 mg (0.2%) of a light yellow oil from *N. nuda* and 19 mg (0.4%) from *N. curviflora.* The extracted EO samples were stored in sealed vials at low temperature (4°C) before gas chromatography coupled with mass spectrometry (GC-MS) analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis. Oil compositions were analysed by SPME/GC-MS, as reported by Scaglia et al. (2011) with some modifications. A manual SPME device and divinylbenzene (DVB)/Carboxen/polydimethylsiloxane (PDMS) 50-30 μm fiber - Supelco, Bellefonte, PA, USA) was used. The compounds were adsorbed from the gas-phases by exposing the fiber, preconditioned for 3 h at 250°C as suggested by the Supplier, to the oils on 20 ml vials. The fibers were incubated at 40°C for 60 min under agitation condition (speed of 500 rpm with on time of 5 seconds and off time of 2 seconds).

Oils analysis was performed using an Agilent 5975C Series GC/MSD and FID as detector. Volatiles were separated using a capillary column Zebron-WAX (Zebron, Phenomenex, USA) of 30 m x 0.25 mm (ID) and a film thickness of 0.25 μm. Carrier gas was helium at a flow rate of 1 ml min^{-1}. Molecules were desorbed exposing the SPME in the GC injection port in splitless condition for 600 seconds at 250 °C. The temperature program was isothermal for 3 min at 40 °C, then three different temperature ramps were done to raise 115°C, 150°C and 220°C at a rate of 3 °C min^{-1}, 5°C min^{-1} and 7°C min^{-1} respectively. Finally an isothermal at 220°C for 15 min was done. The transfer line to the mass spectrometer was maintained at 150 °C. The mass spectra were obtained by electronic impact at 70 eV, a multiplier voltage of 1294 V and collecting data at a m/z range of 45–300. Compounds were tentatively identified by comparing their mass spectra with those contained in the NIST08 library (USA). Results were expressed as spectrum % and the molecules were successively classified in main chemical groups.

Motility assay. *Panagrolaimus rigidus* adults were suspended in M199 medium and, then, approximately 100 larvae in 100 μL of suspension were added to each well of a 96-well microplate, followed by immediate addition of 100 μL of the essential oil dilutions. Microplates were stored in incubator at 20°C. After 1, 3, 5, 12 and 24 h, nematodes were removed from each well and mobile and immobile roundworms were counted using an optical microscope at 40x magnification. Nematodes were considered paralyzed or died when presenting with straight body and absence of any motility after manipulation stimulus. M199 solution with 0.05% DMSO, and 50, 25, 5, 0.5, 0.05 and 0.005% methanol were used as controls. Experiments were carried out in triplicate for each essential oil concentration and controls, and results are expressed as LD_{50} (mg/mL).

Results and Discussion

Analysis by GC-MS of the essential oil obtained by hydrodistillation of the aerial parts of *Nepeta nuda* ssp. *pubescens* allowed the identification of 49 compounds, representing 100% of the total oil composition. The most represented chemical classes included monoterpene hydrocarbons (18.58%), aldehydes/ketones (13.86%) and oxygenated monoterpenes (9.83%) (Tab. 1). The essential oil was obtained in a yield of 0.2% (w/w), based on the dry weight of the sample. The most abundant (> 5%) components were pinane (12.89%), 1-ethyl-1H-pyrrole (12.67%), 1-cycloethyl-1-(2-methylenecyclohexyl)ethanol (10.37%), 3-methyl-2-cyclohexen-1-one (9.17%) and 2,3-dimethyl-3-hexanol (5.88%) (Table 1). Among oxygenated monoterpenes, two nepetalactones were identified, *i.e.* (*E,Z*)-nepetalactone (2.24%) and (*Z,E*)-nepetalactone (0.31%) (Tab. 1).

The essential oil composition of *N. nuda* ssp. *pubescens* investigated in this study is different from that reported for other subspecies of *N. nuda*. In fact, the essential oil from *N. nuda* L. ssp. *albiflora* (Boiss.) Gams. growing wild in Lebanon was characterized by higher contents of three nepetalactones: (*Z,E*)-nepetalactone (0.8-3.6%), (*E,Z*)-nepetalactone (4.4-8.0%) and (*Z,Z*)-nepetalactone (0.5-2.9%) (Mancini et al. 2009). However, these compounds were not detected in the essential oil from *N. nuda* ssp. *albiflora* collected in Turkey, characterized by higher amount of spathulenol (7.35%) compared with our *N. nuda* ssp. *pubescens* samples (2.92%) (Alim et al., 2009). Similarly, nepetalctones were not found in *N. nuda* L. ssp. *nuda* essential oil from Turkey, where higher levels of caryophyllene oxide (21.8%), spathulenol (13.8%) and *allo*-aromadendrene (9.0%) were measured (Kökdil et al., 1998).

The steam distillation of *N. curviflora* aerial parts yielded 0.4% (w/w on dry weight basis) essential oil. GC-MS analysis identified 41 constituents, representing 100% of the total oil composition (Tab.

2). The most represented chemical classes were oxygenated sesquiterpenes (41.65%) and oxygenated monoterpenes (24.73%) (Tab. 2). The major components (> 5%) were 2-isopropyl-5-methyl-3-cyclohexen-1-one (12.51%), (-)-spathulenol (11.73%), *cis*-*Z*-α-bisabolene epoxide (8.07%), widdrol (7.0%), (*E,Z*)-5,7-dodecadiene (6.93%), dihydronepetalactone (5.57%) and 4-propyl-cyclohexene (5.43%) (Tab. 2).

In a previous report, the most abundant compounds in *N. curviflora* essential oil were β-caryophyllene (41.6%), caryophyllene oxide (9.5%), (*E*)-β-farnesene (6.2%) and (*Z*)-β-farnesene (4.8%) (Mancini et al., 2009). Noteworthy, lower amounts of dihydronepetalactone were previously reported in essential oils from *Nepeta persica* Boiss. (3.5%), *N. nuda* ssp. *albiflora* (0.2%) and *Nepeta cataria* L. (0.08%) (Kökdil et al., 1996; Javidnia et al., 2002; Dmitrović et al., 2015). Therefore, the composition of essential oil of *N. curviflora* is quite different from the ones previously reported in the literature (Senatore et al., 2005; Mancini et al., 2009; Al-Qudah 2016). However, it should be noted that chemical polymorphism is characteristic of this species, and the oil composition depends on variety, growing site, climatic conditions, balsamic period, time of collection and analytical method (Formisano et al., 2011).

A number of biological properties have been ascribed to *Nepeta* species. In particular, phytotoxic, antibacterial, antiviral and antioxidant activities have been reported in *N. nuda* ssp. *albiflora*, *N. nuda* ssp. *nuda* and *N. curviflora*. (Alim et al., 2009; Mancini et al., 2009; Gormez et al., 2013; Todorov et al., 2015). As regards nematicidal activity of *Nepeta* species, it was only reported for *Nepeta cataria* L. Both solvent (methanol, ethanol and water) extracts and essential oil from aerial parts of this plant species exhibited anthelmintic effects on *Meloidogyne chitwoodi*, *Meloidogyne incognita* and *Haemonchus contortus* (Bandh et al., 2011; Pavaraj et al., 2012; Alam et al., 2015; Faria et al., 2016). Our results demonstrated that the essential oils of *N. curviflora* and *N. nuda* ssp. *pubescens* are endowed with nematicidal activity as well. In particular, the essential oil of *N. curviflora* was more active than the *N. nuda* ssp. *pubescens* one against *Panagrolaimus rigidus*. According to the motility assay, LD_{50} was 0.5 mg/mL and 2.5 mg/mL 24 h after treatment with *N.*

curviflora and *N. nuda* ssp. *pubescens* essential oil, respectively (Fig. 1). *P. rigidus*, a free-living bacteriovorus nematode, represents a model organism for research on novel anthelmintics. Indeed, this nematode has been widely used in basic research on anthelmintic pharmacology of human and agricultural parasites, as well as at understanding the mechanisms of resistance to anthelmintics. Several characteristics make *P. rigidus* a reliable experimental model, such as rapid life cycle, easy growth and manipulation in laboratory conditions, knowledge of its genome and phylogenetic proximity to other nematodes.

The different *in vitro* nematicidal activity of the two *Nepeta* species can be due to the diverse essential oil composition. In particular, a number of reports highlighted the anthelmintic potential of terpenes. The nematicidal activity of these compounds against *Meloidogyne incognita* was found to decrease in the order carvone > pulegone > *trans*-anethole > geraniol > eugenol > carvacrol > thymol > terpinen-4-ol, with EC_{50} values (24 h) calculated in the range of 115-392 µg/mL (Ntalli et al., 2010). Similar results and EC_{50} values were reported against *M. javanica* (Oka et al., 2000). The highest mortality (100%) on this nematode species was induced by carvacrol, geraniol and thymol (0.5 mg/mL, 72 h incubation) (Andrés et al., 2012). Carvacrol, thymol, nerolidol, *α*-terpinene, geraniol, citronellol, farnesol, limonene, pseudoionone and eugenol also caused 50% mortality at 50 *µ*g/mL on the model nematode *Caenorhabditis elegans* (Abdel-Rahman et al., 2013). Noteworthy, the contribution of each terpene constituent to the overall activity of an essential oil seems to be due to a rather complicated pattern of interactions, since they may act together synergistically or antagonistically. Some terpene pairs exhibiting synergistic activity on *M. incognita* paralysis in decreasing order were: *trans*-anethole/geraniol > *trans*-anethole/eugenol > carvacrol/eugenol > geraniol/carvacrol (Ntalli et al., 2011). Therefore, it is very important to understand the synergy and antagonism interactions among individual components of a nematicidal essential oil. In this view, the presence of nematicidal terpenes in the *N. curviflora* essential oil, *i.e.* pulegone, carvacrol, eugenol and thymol, could contribute to explain its higher activity against *P. rigidus* motility, compared with *N. nuda* ssp. *pubescens* essential oil.

In conclusion, the present study revealed that essential oil of *N. curviflora* exhibits promising nematicidal activity, in addition to the previously reported phytotoxic effects. On the whole, these results highlight the possibility of using this plant product as biopesticide in soil treatment to control phytopathogenic nematodes and weeds.

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Table 1. Essential oils of *Nepeta nuda* L. ssp. *pubescens*

RT (min)	%	Compound
14.50	0.15	1-Octen-3-one
16.67	0.11	Cytronellol hydrate
20.18	0.58	Linalool oxide *trans*
20.96	1.48	Furfural
23.18	1.32	Benzaldehyde
24.72	0.42	Linalool
25.23	0.11	Cyclopentanol 1-(methylenecyclopropyl)-
25.59	0.26	2-Cyclopentene-1,4-dione
28.66	1.57	2-Methoxy-5-methylphenol
30.9	1.74	Azulene
32.09	0.50	Methyl salicylate
32.65	0.11	Phorone
33.52	0.43	β-Damascenone
33.95	0.91	Fenchone
34.14	1.84	Hexanoic acid
34.78	2.11	Benzyl alcohol
35.27	0.71	Cyclohexane ethenyl-
35.62	3.02	Phenylethyl alcohol
35.72	2.51	Bicyclo[10,1,0]tridec-1-ene
36.69	1.39	Methyl ethyl cyclopentene
36.85	1.16	Ethanone 1-(1H-pyrrol-2-yl)-
37.02	0.31	(*Z*,*E*)-Nepetalactone
37.54	0.49	(+)-3-Carene 10-(acetylmethyl)-

37.59	1.20	(-)-Isosativene
37.83	0.17	α-Santalol
38.06	3.95	2,6 octadiene 2,6-dimethyl-
38.43	2.24	(*E*,*Z*)-Nepetalactone
38.56	1.25	Ethanone 1-(2-methyl-1-cyclopenten-1-yl)-
38.64	0.61	Octanoic acid
38.76	1.04	6-Methyl-3,5-heptadiene-2-one
38.95	9.17	2-Cyclohexen-1-one 3-methyl-
39.25	5.88	3-Hexanol 2,3-dimethyl-
39.52	10.37	1-Cyclohexyl-1-(2-methylenecyclohexyl)ethanol
39.86	2.92	Spathulenol
39.95	1.54	Cyclopentanecarboxylic acid 3-methylene-1,7,7-trimethylbicyclo[2,2,1]hept-2-yl ester
40.2	12.67	1H-Pyrrole 1-ethyl-
40.37	2.03	Isopulegol
40.53	1.03	2,3-Dehydro-1,8-cineole
40.85	12.89	Pinane
41.10	0.27	Thymol
41.55	1.26	Artemiseole
41.65	3.69	Eicosanoic acid
41.99	0.18	1,7-Octadien-3-one 2-methyl-6-methylene-
42.15	0.23	Cariophyllene oxide
42.32	0.79	α-Caryophylladienol
43.39	0.52	Alloaromadendrene oxide-(1)
43.93	0.13	1-Cyclohexene-1-butanal α-2,6,6-tetramethyl-

44.10	0.49	Indole
44.76	0.28	Benzoic acid 2,6-dimethoxy- methyl ester
Grouped compounds		
Monoterpene hydrocarbons	18.58	
Oxygenated monoterpenes	9.83	
Sesquiterpene hydrocarbons	1.37	
Oxygenated sesquiterpenes	4.46	
Aliphatic hydrocarbons	4.61	
Aldehydes/ketones	13.86	
Fatty acids and esters	8.46	
Others	38.8	
Total	100	

Table 2. Essential oils of *Nepeta curviflora* Boi

RT (min)	%	Compound
15.14	0.28	2-Methoxy-5-methylphenol
29.91	0.39	Cyclohexanecarboxylic acid 3-phenylpropyl ester
33.14	1.77	Pulegone
34.14	1.04	Hexanoic acid
35.60	0.90	Phenylethyl alcohol
36.70	5.43	Cyclohexene 4-propyl-
36.74	0.70	Bicyclo[2,2,1]heptane 2-methyl-
36.78	6.93	5,7-Dodecadiene, (E,Z)-
36.97	0.98	Cyclohexene, 3-(2-methylpropyl)-
37.34	1.68	Caryophyllene oxide
37.79	2.72	Germacrene
38.56	0.58	Ethanone 1-(1,3-dimethyl-3-cyclohexen-1-yl)-
38.64	1.86	Octanoic acid
38.76	1.73	Ledol
38.97	12.51	3-Cyclohexen-1-one 2-isopropyl-5-methyl-
39.43	1.44	2-nonenoic acid methyl ester
39.54	5.57	Dihydronepetalactone -
39.73	0.68	Dehydrocurdione
39.85	11.73	(-)-Spathulenol
40.21	3.15	γ-Cadinene
40.34	1.20	Eugenol
40.68	2.76	Carvacrol
41.09	0.93	p-Thymol

41.40	0.54	Isoaromadendrene epoxide
41.54	7.00	Widdrol
41.77	0.48	3-Octyne
42.25	0.37	Alloaromadendrene oxide
42.34	4.84	caryophylla-3(4),8-dien-5-ol
42.55	1.01	Phenol 2,4-bis(1,1-dimethylethyl)-
42.87	8.07	*cis*-*Z*-α-Bisabolene epoxide
43.01	3.50	α-Selinene
43.40	3.59	*trans*-*Z*-α-Bisabolene epoxide
43.61	0.54	α-pinene oxide
43.93	0.65	L-Fenchone
44.25	0.27	Patchoulane
44.76	0.38	N-(1-Cyclohexen-1-yl)piperidine
45.05	0.43	Isolongifolol
46.39	0.22	Thujopsene
46.55	0.63	(-)-Neoclovene-(I) dihydro-
48.17	0.31	2,6-Dimethylbicyclo[3,2,1]octane
49.17	0.23	Caryophyllene-(II)

Grouped compounds

Monoterpene hydrocarbons	0.31
Oxygenated monoterpenes	24.73
Sesquiterpene hydrocarbons	9.73
Oxygenated sesquiterpenes	41.65
Aliphatic hydrocarbons	14.52

Fatty acids and esters	4.73
Others	4.35
Total	100

Figure 1

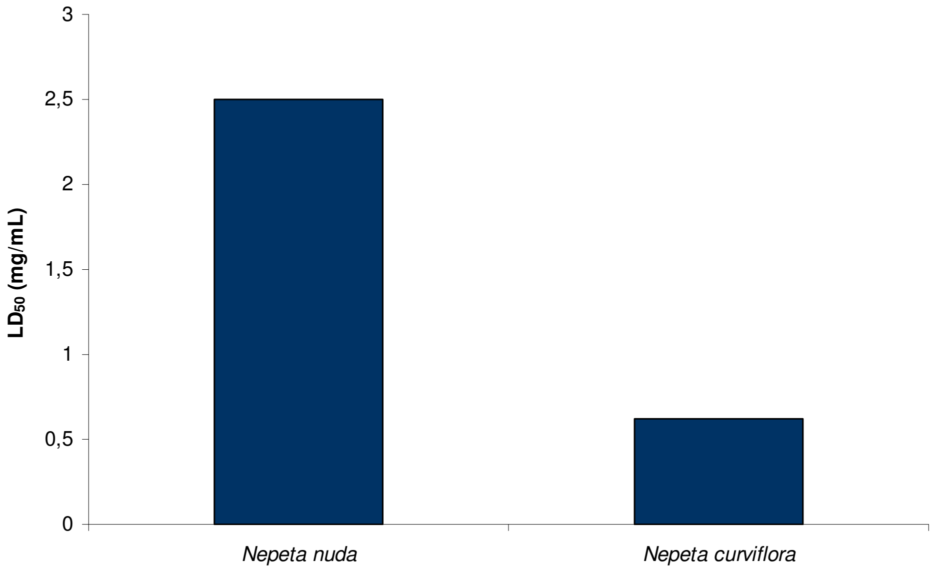

Figure 1. *In vitro* nematicidal activity of essential oils obtained from *Nepeta nuda* L. ssp. *pubescens* and *Nepeta curviflora* Boiss on *Panagrolaimus rigidus* adults.

Regular articles

Chemical characterization and nematicidal activity of the essential oil of *Nepeta nuda* L. ssp. *pubescens* and *Nepeta curviflora* Boiss. from Lebanon

Abstract

The chemical characterization and the nematicidal activity of the essential oils from *Nepeta nuda* L. ssp. *pubescens* and *Nepeta curviflora* Boiss. growing wild in Lebanon are reported. A comparative study was carried out as, to the best of our knowledge, no information is available on *Nepeta nuda* L. ssp. *pubescens*. In addition, both *Nepeta* species were collected in the same geographical area in order to rule out the environmental factors influencing essential oil composition and bioactivity. The most abundant (> 5%) components of *N. nuda* ssp. *pubescens* essential oil were pinane (12.89%), 1-ethyl-1H-pyrrole (12.67%), 1-cycloethyl-1-(2-methylenecyclohexyl)ethanol (10.37%), 3-methyl-2-cyclohexen-1-one (9.17%) and 2,3-dimethyl-3-hexanol (5.88%). Among oxygenated monoterpenes, two nepetalactones were identified, *i.e.* (*E,Z*)-nepetalactone (2.24%) and (*Z,E*)-nepetalactone (0.31%). The major constituents (> 5 %) of *N. curviflora* essential oil were 2-isopropyl-5-methyl-3-cyclohexen-1-one (12.51%), (-)-spathulenol (11.73%), *cis*-*Z*-α-bisabolene epoxide (8.07%), widdrol (7.0 %), (*E,Z*)-5,7-dodecadiene (6.93%), dihydronepetalactone (5.57%) and 4-propyl-cyclohexene (5.43%). The essential oil of *N. curviflora* was more active than the *N. nuda* ssp. *pubescens* one against the nematode *Panagrolaimus rigidus*. According to the motility assay, LD_{50} was 0.5 mg/mL and 2.5 mg/mL 24 h after treatment with *N. curviflora* and *N. nuda* ssp. *pubescens* essential oil, respectively.

Keywords: aromatic plants, nematodes, isoprenoids, biopesticides, anthelmintics, Lebanese flora.

Introduction

The genus *Nepeta* (Lamiaceae) consists of around 300 herbaceous, perennial, rarely annual species. This genus is distributed over a wide area including Europe, Asia, Africa and the Middle East (Formisano et al., 2011). Some of these species are widely used in traditional medicine and are endowed with a number of pharmacological properties, usually attributed to their essential oils (Sharma et al. 2013). The main bioactive compounds are a variety of secondary metabolites, including monoterpenes (iridoids and their glycosides), diterpenes, triterpenes as well as phenols.

Nepeta nuda L. is one of the most common species of the genus *Nepeta*. It is widespread from central and southeast Europe to Russia and towards southwest and central Asia. The essential oil composition of *N. nuda* was studied in a number of investigations (De Pooter et al. 1987; Alim et al. 2009; Turkey et al., 2011; Bozari et al., 2013; Gormez et al., 2013).

Nematodes are parasites of humans, vertebrates, insects and plants. In particular, plant diseases caused by nematodes threaten the world agriculture because of their severity and economic burden. In the developing countries, crop production losses attributable to nematodes are estimated around 15%, compared with around 9% in developed countries. Not least, chemical control of nematodes is troublesome after the ban of soil fumigants such as methyl bromide, and, in addition, non-volatile nematicides, organophosphates and carbamates, can represent environmental and human threats due to their toxicological and ecotoxicological risks. Therefore, environmentally friendly and low-cost alternatives to chemical control measures for phytoparasitic nematodes are urgently needed. In this context, essential oils from plants could be attractive nematicide compound sources, possibly safe for non-target organisms.

As part of a continuing investigation on essential oils of *Nepeta* species growing in Lebanon, in the present study, we report, for the first time, the chemical characterization and the nematicidal activity of the essential oil from *N. nuda* ssp. *pubescens*. As, to the best of our knowledge, no information is available on this plant species, we used *Nepeta curviflora* Boiss. as reference species (Senatore et al., 2005; Mancini et al., 2009; Al-Qudah 2016), in order to compare these two *Nepeta* species growing wild in Lebanon. In this comparative study, both *Nepeta* species were collected in the same geographical area, in order to rule out the environmental factors influencing essential oil composition and bioactivity.

Materials and methods

Plant material. The dried parts of *Nepeta nuda* ssp. *pubescens* (flowering tops, seeds and leaves) were collected in April 2005 in a mountain area (Laklouk, Mount Lebanon, 1780 m above sea level) near to Tannourine, Lebanon (average temperature 8.4 ± 0.8 °C and 1105 mm of rain in 2005; GPS coordinates: 34°8'19"N 35°52'20"E). The dried parts of *Nepeta curviflora* (flowering tops, seeds, and leaves) were collected in April 2005 in Lebanon, near to the St. Antonio of Qozhaya convent, Ehden region at 664 m above sea level (average temperature 13.07 ± 1.3 °C and 940 mm of rain in 2005; GPS coordinates 34°16'59"N 35°56'50"E).

Essential oil production. Essential oils were obtained by steam distillation (250 mL water) from a sample (5 g) of air-dried aerial parts of the plant. The collected aqueous phase (150 mL) containing the essential oil was extracted with diethyl ether (40 mL × 5). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to obtain 12 mg (0.2%) of a light yellow oil from *N. nuda* and 19 mg (0.4%) from *N. curviflora.* The extracted EO samples were stored in sealed vials at low temperature (4 °C) before gas chromatography coupled with mass spectrometry (GC-MS) analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis. Oil compositions were analysed by SPME/GC-MS, as reported by Scaglia et al. (2011) with some modifications. A manual SPME device and divinylbenzene (DVB)/Carboxen/polydimethylsiloxane (PDMS) 50-30 µm fiber - Supelco, Bellefonte, PA, USA) was used. The compounds were adsorbed from the gas-phases by exposing the fiber, preconditioned for 3 h at 250 °C as suggested by the Supplier, to the oils on 20 ml vials. The fibers were incubated at 40 °C for 60 min under agitation condition (speed of 500 rpm with on time of 5 s and off time of 2 s).

Oils analysis was performed using an Agilent 5975C Series GC/MSD and FID as detector. Volatiles were separated using a polar capillary column Zebron-WAX (Zebron, Phenomenex, USA) of 30 m x 0.25 mm (ID) and a film thickness of 0.25 µm. Carrier gas was helium at a flow rate of 1 mL min^{-1}. Molecules were desorbed exposing the SPME in the GC injection port in splitless condition for 600 seconds at 250 °C. The temperature program was isothermal for 3 min at 40 °C, then three different temperature ramps were done to raise 115 °C, 150 °C and 220 °C at a rate of 3 °C min^{-1}, 5 °C min^{-1} and 7 °C min^{-1} respectively. Finally an isothermal at 220 °C for 15 min was done. The transfer line to the mass spectrometer was maintained at 150 °C. The mass spectra were obtained by electronic impact at 70 eV, a multiplier voltage of 1294 V and collecting data at a *m/z* range of 45–300. The retention indices were determined in relation to a homologous series of *n*-alkanes (C7-C30) under the same operation conditions. Compounds were tentatively identified by comparing their mass spectra with those contained in the NIST08 library (USA) and peak retention indices (RI) relative to *n*-alkanes. From the raw spectra (Table SI-1 and Table S-2), the typical constituents of the essential oils (Babushok et al., 2001; Dobravalskyte et al., 2013; Zebib et al., 2015) have been selected and considered. Therefore, concentrations of these typical components have been recalculated as percent of the sum of raw spectra of essential oil constituents.

Motility assay. *Panagrolaimus rigidus* adults were suspended in M199 medium and, then, approximately 100 larvae in 100 µL of suspension were added to each well of a 96-well microplate, followed by immediate addition of 100 µL of the essential oil dilutions. Microplates were stored in incubator at 20°C. After 1, 3, 5, 12 and 24 h, nematodes were removed from each well and mobile and immobile roundworms were counted using an optical microscope at 40x magnification. Nematodes were considered paralyzed or died when presenting with straight body and absence of any motility after manipulation stimulus. M199 solution with 0.05% DMSO, and 50, 25, 5, 0.5, 0.05 and 0.005% methanol were used as controls. Experiments were carried out in triplicate for each essential oil concentration and controls, and results are expressed as LD_{50} (mg/mL).

Statistical analysis. Statistical analysis was performed using OriginPro® software (version 8.0). All experiments were carried out in triplicate and results are expressed as mean ± standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA) and comparison among means was determined according to Fisher's least significant difference (LSD) test. Differences were considered significant at $P < 0.05$ and represented by different letters.

Results and Discussion

Essential oil composition of *N. nuda* ssp. *pubescens*

The essential oil was obtained in a yield of 0.2% (w/w), based on the dry weight of the sample. Among the molecules recognised with the GC MS analysis (Table SI 1), 49 have been identified as typical of essential oils and considered for the successive discussion (Table 1) (Babushok et al., 2001; Dobravalskyte et al., 2013; Zebib et al., 2015; Yermakov et al., 2010)

The most represented chemical classes included monoterpene hydrocarbons (18.58 ± 0.70% oil composition), aldehydes/ketones (13.86 ± 0.20% oil composition) and oxygenated monoterpenes (9.83 ± 0.20% oil composition) (Tab. 1). The most abundant (> 5%) components were pinene (12.89 ± 0.80%), 1-ethyl-1H-pyrrole (12.67 ± 0.40%), 1-cycloethyl-1-(2-methylenecyclohexyl)ethanol (10.37 ± 0,50%), 3-methyl-2-cyclohexen-1-one (9.17 ± 0.60%) and 2,3-dimethyl-3-hexanol (5.88 ± 0.50%) (Table 1). Among oxygenated monoterpenes, two nepetalactones were identified, *i.e.* (*E,Z*)-nepetalactone (2.24 ± 0.20%) and (*Z,E*)-nepetalactone (0.31 ± 0.00%) (Tab. 1).

The essential oil composition of *N. nuda* ssp. *pubescens* investigated in this study is different from that reported for other subspecies of *N. nuda*. In fact, the essential oil from *N. nuda* L. ssp. *albiflora* (Boiss.) Gams. growing wild in Lebanon was characterized by higher contents of three nepetalactones: (*Z,E*)-nepetalactone (0.8-3.6%), (*E,Z*)-nepetalactone (4.4-8.0%) and (*Z,Z*)-nepetalactone (0.5-2.9%) (Mancini et al. 2009). However, these compounds were not detected in the essential oil from *N. nuda* ssp. *albiflora* collected in Turkey, characterized by higher amount of spathulenol (7.35%) compared with our *N. nuda* ssp. *pubescens* samples (2.92%) (Alim et al., 2009). Similarly, nepetalctones were not found in *N. nuda* L. ssp. *nuda* essential oil from Turkey, where higher levels of caryophyllene oxide (21.8%), spathulenol (13.8%) and *allo*-aromadendrene (9.0%) were measured (Kökdil et al., 1998).

Essential oil composition of *N. curviflora*

The steam distillation of *N. curviflora* aerial parts yielded (0.4% w/w) essential oil. GC-MS analysis identified 41 constituents (Tab. 1). The most represented chemical classes were oxygenated sesquiterpenes (41.65 ± 0.70%) and oxygenated monoterpenes (24.73 ± 0.30%) (Tab. 1). The major components (> 5%) were 2-isopropyl-5-methyl-3-cyclohexen-1-one (12.51 ± 0.60%), (-)-spathulenol (11.73 ± 0.60%), *cis*-*Z*-α-bisabolene epoxide (8.07 ± 0.20%), widdrol (7.0 ± 0.30%), (*E,Z*)-5,7-dodecadiene (6.93 ± 0.60%), dihydronepetalactone (5.57 ± 0.50%) and 4-propyl-cyclohexene (5.43 ± 0.20%) (Tab. 1).

In a previous report, the most abundant compounds in *N. curviflora* essential oil were β-caryophyllene (41.6%), caryophyllene oxide (9.5%), (*E*)-β-farnesene (6.2%) and (*Z*)-β-farnesene (4.8%) (Mancini et al., 2009). Noteworthy, lower amounts of dihydronepetalactone were previously reported in essential oils from *Nepeta persica* Boiss. (3.5%), *N. nuda* ssp. *albiflora* (0.2%) and *Nepeta cataria* L. (0.08%) (Kökdil et al., 1996; Javidnia et al., 2002; Dmitrović et al., 2015). Therefore, the composition of essential oil of *N. curviflora* is quite different from the ones previously reported in the literature (Senatore et al., 2005; Mancini et al., 2009; Al-Qudah 2016).

Effects of geographical origin on essential oil composition

A number of factors could affect essential oil composition, including genetic traits, soil, climatic conditions, environmental constraints, harvesting as well as extraction and analytical methods. Not least, a high level of chemical polymorphism is characteristic of *Nepeta* species (Formisano et al., 2011). Indeed, *N. nuda* ssp. *pubescens* and *N. curviflora* collected from two different areas in

Lebanon showed different essential oil composition, probably due to diverse climates and environments. As previously introduced, due to the lack of information available on *N. nuda* ssp. *pubescens*, we compared our results with those reported on other subspecies of *N. nuda*. Essential oil from *N. nuda* ssp. *albiflora* harvested in Lebanon was devoid of monoterpene hydrocarbons and richer in sesquiterpenes, both hydrocarbons and oxygenated derivatives (Mancini et al., 2009), compared with *N. nuda* ssp. *pubescens* essential oil (Tab. 2). Similarly, higher levels of oxygenated sesquiterpenes were detected in essential oil from *N. nuda* ssp. *albiflora* collected in Turkey (Alim et al. 2009) than in *N. nuda* ssp. *pubescens* essential oil (Tab. 2). Finally, in *N. nuda* ssp. *nuda*, a much higher sesquiterpene fraction was measured (Tab. 2) (Kökdil et al., 1998), thus pointing out that geographical origin represents a relevant constraint in essential oil composition.

As regards *N. curviflora*, essential oil the same species harvested in Lebanon and Jordan was characterized by much higher levels of sesquiterpene hydrocarbons (Senatore et al., 2005; Mancini et al., 2009; Al-Qudah 2016), in comparison with our *N. curviflora* essential oil (Tab. 2).

Nematicidal activity

A number of biological properties have been ascribed to *Nepeta* species. In particular, phytotoxic, antibacterial, antiviral and antioxidant activities have been reported in *N. nuda* ssp. *albiflora*, *N. nuda* ssp. *nuda* and *N. curviflora*. (Alim et al., 2009; Mancini et al., 2009; Gormez et al., 2013; Todorov et al., 2015). As regards nematicidal activity of *Nepeta* species, it was only reported for *Nepeta cataria* L. Both solvent (methanol, ethanol and water) extracts and essential oil from aerial parts of this plant species exhibited anthelmintic effects on *Meloidogyne chitwoodi*, *Meloidogyne incognita* and *Haemonchus contortus* (Bandh et al., 2011; Pavaraj et al., 2012; Alam et al., 2015; Faria et al., 2016). Our results demonstrated that the essential oils of *N. curviflora* and *N. nuda* ssp. *pubescens* are endowed with nematicidal activity as well. In particular, the essential oil of *N. curviflora* was more active than the *N. nuda* ssp. *pubescens* one against *Panagrolaimus rigidus*. According to the motility assay, LD_{50} was 0.6 ± 0.1 mg/mL and 2.5 ± 0.3 mg/mL 24 h after treatment with *N. curviflora* and *N. nuda* ssp. *pubescens* essential oil, respectively ($P < 0.05$) (Fig. 1). *P. rigidus*, a free-living bacterivorous nematode, represents a model organism for research on novel anthelmintics. Indeed, this nematode has been widely used in basic research on anthelmintic pharmacology of human and agricultural parasites, as well as at understanding the mechanisms of resistance to anthelmintics. Several characteristics make *P. rigidus* a reliable experimental model, such as rapid life cycle, easy growth and manipulation in laboratory conditions, knowledge of its genome and phylogenetic proximity to other nematodes.

The different *in vitro* nematicidal activity of the two *Nepeta* species can be due to the diverse essential oil composition. In particular, a number of reports highlighted the anthelmintic potential of terpenes. The nematicidal activity of these compounds against *Meloidogyne incognita* was found to decrease in the order carvone > pulegone > *trans*-anethole > geraniol > eugenol > carvacrol > thymol > terpinen-4-ol, with EC_{50} values (24 h) calculated in the range of 115-392 µg/mL (Ntalli et al., 2010). Similar results and EC_{50} values were reported against *M. javanica* (Oka et al., 2000). The highest mortality (100%) on this nematode species was induced by carvacrol, geraniol and thymol (0.5 mg/mL, 72 h incubation) (Andrés et al., 2012). Carvacrol, thymol, nerolidol, *α*-terpinene, geraniol, citronellol, farnesol, limonene, pseudoionone and eugenol also caused 50% mortality at 50 *µ*g/mL on the model nematode *Caenorhabditis elegans* (Abdel-Rahman et al., 2013). Noteworthy, the contribution of each terpene constituent to the overall activity of an essential oil seems to be due to a rather complicated pattern of interactions, since they may act together synergistically or antagonistically. Some terpene pairs exhibiting synergistic activity on *M. incognita* paralysis in decreasing order were: *trans*-anethole/geraniol > *trans*-anethole/eugenol > carvacrol/eugenol > geraniol/carvacrol (Ntalli et al., 2011). Therefore, it is very important to understand the synergy and antagonism interactions among individual components of a nematicidal essential oil. In this view, the presence of nematicidal terpenes in *N. curviflora* essential oil, *i.e.* pulegone, carvacrol, eugenol and thymol, could contribute to explain its higher activity against *P. rigidus* motility, compared with *N. nuda* ssp. *pubescens* essential oil.

Conclusion

The present study confirmed that geographical origin of plants represents a major factor influencing essential oil composition, as well as environmental variables due to annual rainfall and temperature. Noteworthy, difference in nematicidal activity of the two essential oils could be attributed to their diverse chemical composition. The presence of nematicidal terpenes in the *N. curviflora* essential oil, *i.e*. pulegone, carvacrol, eugenol and thymol could contribute to explain the observed biological activity. Overall, these results revealed that essential oil of *N. curviflora* exhibits promising nematicidal activity, in addition to the previously reported phytotoxic effects, thus highlighting the possibility of using this plant product as biopesticide/bioherbicide in soil treatment to control phytopathogenic nematodes and weeds.

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Table 1. Essential oils of *Nepeta nuda* L. ssp. *pubescens* and *Nepeta curviflora* Boiss.

RI^a	Compound	*Nepeta nuda* L. ssp. *pubescens*	*Nepeta curviflora* Boiss.
1224.2	1-Octen-3-one	0.15±0.10	-
1300.13	2-Methoxy-5-methylphenol	-	0.28±0.00
1300.9	Cytronellol hydrate	0.11±0.00	-
1400.5	Linalool oxide *trans*	0.58±0.00	-
1401.1	Furfural	1.48±0.10	-
1500.1	Benzaldehyde	1.32±0.20	-
1500.8	Linalool	0.42±0.00	-
1501.4	Cyclopentanol 1-(methylenecyclopropyl)-	0.11±0.00	-
1502.0	2-Cyclopentene-1,4-dione	0.26±0.10	-
1601.0	2-Methoxy-5-methylphenol	1.57±0.20	-
1605.42	Cyclohexanecarboxylic acid 3-phenylpropyl ester	-	0.39±0.20
1700.2	Azulene	1.74±0.10	-
1701.3	Methyl salicylate	0.50±0.10	-
1703.2	Phorone	0.11±0.00	-
1713.44	Pulegone	-	1.77±0.30
1800.1	β-Damascenone	0.43±0.10	-
1800.3	Fenchone	0.91±0.20	-
1800.5	Hexanoic acid	1.84±0.30a	1.04±0.10a
1801.4	Benzyl alcohol	2.11±0.20	-
1803.7	Cyclohexane ethenyl-	0.71±0.10	-
1812.9	Phenylethyl alcohol	-	0.9±0.00
1813.7	Phenylethyl alcohol	3.02±0.50	-
1835.5	Bicyclo[10,1,0]tridec-1-ene	2.51±0.20	-
1900.8	Methyl ethyl cyclopentene	1.39±0.10	-
1900.82	Cyclohexene 4-propyl-	-	5.43±0.20
1900.89	Bicyclo[2,2,1]heptane 2-methyl-	-	0.7±0.00
1900.96	5,7-Dodecadiene, (E,Z)-	-	6.93±0.60
1901.1	Ethanone 1-(1H-pyrrol-2-yl)-	1.16±0.20	-
1901.4	Cyclohexene, 3-(2-methylpropyl)-	-	0.98±0.20
1901.6	(*Z,E*)-Nepetalactone	0.31±0.00	-
1903.28	Caryophyllene oxide	-	1.68±0.10
1906.4	(+)-3-Carene 10-(acetylmethyl)-	0.49±0.00	-
1908.1	(-)-Isosativene	1.20±0.10	-
1987.09	Germacrene	-	2.72±0.20
2000.0	α-Santalol	0.17±0.00	-
2000.2	2,6 Octadiene 2,6-dimethyl-	3.95±0.00	-
2000.6	(*E,Z*)-Nepetalactone	2.24±0.20	-
200.76	Ethanone 1-(1,3-dimethyl-3-cyclohexen-1-yl)-	-	0.58±0.00
2000.8	Ethanone 1-(2-methyl-1-cyclopenten-1-yl)-	1.25±0.10	-
2000.9	Octanoic acid	0.61±0.00a	1.86±0.30b
2001.2	6-Methyl-3,5-heptadiene-2-one	1.04±0.20	-
2001.22	Ledol	-	1.73±0.10
2001.9	2-Cyclohexen-1-one 3-methyl-	9.17±0.60	-
2002.04	3-Cyclohexen-1-one 2-isopropyl-5-methyl-	-	12.51±0.60

2005.0	3-Hexanol 2,3-dimethyl-	5.88±0.50	-
2015.11	2-Nonenoic acid methyl ester	-	
2100	Dihydronepetalactone -	-	5.57±0.50
2100.14	Dehydrocurdione	-	0.68±0.10
2100.4	1-Cyclohexyl-1-(2-methylenecyclohexyl)ethanol	10.37±0.50a	1.44±0.00b
2100.3	(-)-Spathulenol	2.92±0.20a	11.73±0.60b
2100.4	Cyclopentanecarboxylic acid 3-methylene-1,7,7-trimethylbicyclo[2,2,1]hept-2-yl ester	1.54±0.20	-
2100.78	γ-Cadinene	-	3.15±0.30
2100.8	1H-Pyrrole 1-ethyl-	12.67±0.40	-
2101.2	Isopulegol	2.03±0.10	-
2101.1	Eugenol	-	1.2±0.00
2101.8	2,3-Dehydro-1,8-cineole	1.03±0.00	-
2102.93	Carvacrol	-	2.76±0.00
2106.0	Pinene	12.89±0.80	-
2200.0	Thymol	0.27±0.10a	0.93±0.2a
2200.3	Isoaromadendrene epoxide	-	0.54±0.00
2200.5	Artemiseole	1.26±0.10	-
2200.51	Widdrol	-	7.0±0.30
2200.7	Eicosanoic acid	3.69±0.20	-
2200.98	3-Octyne	-	0.48±0.10
2201.9	1,7-Octadien-3-one 2-methyl-6-methylene-	0.18±0.00	-
2203.2	Cariophyllene oxide	0.23±0.20	-
2205.1	Alloaromadendrene oxide	-	0.37±0.00
2207.7	α-Caryophylladienol	0.79±0.20	-
2208.88	Caryophylla-3(4),8-dien-5-ol	-	4.84±0.30
2300.05	Phenol 2,4-bis(1,1-dimethylethyl)-	-	1.01±0.00
2300.42	*cis*-*Z*-α-Bisabolene epoxide	-	8.07±0.20
2300.67	α-Selinene	-	3.5±0.10
2302.3	Alloaromadendrene oxide-(1)	0.52±0.10	-
2302.34	*trans*-*Z*-α-Bisabolene epoxide	-	3.59±0.3
2306.19	α-Pinene oxide	-	0.54±0.00
2400.06	L-Fenchone	-	0.65±0.20
2400.1	1-Cyclohexene-1-butanal α-2,6,6-tetramethyl-	0.13±0.00	-
2400.1	Indole	0.49±0.20	-
2400.22	Patchoulane	-	0.27±0.00
2400.6	Benzoic acid 2,6-dimethoxy- methyl ester	0.28±0.10	-
2400.61	N-(1-Cyclohexen-1-yl)piperidine	-	0.38±0.10
2400.96	Isolongifolol	-	0.43±0.00
2500.02	Thujopsene	-	0.22±0.20
2500.14	(-)-Neoclovene-(I) dihydro-	-	0.63±0.200
2600.11	2,6-Dimethylbicyclo[3,2,1]octane	-	0.31±0.10
2601.5	Caryophyllene-(II)	-	0.23±0.20
Grouped compounds			
Monoterpene hydrocarbons		18.58±0.70b	0.31±0.10a
Oxygenated monoterpenes		9.83±0.20a	24.73±0.30b
Sesquiterpene		1.37±0.20a	9.73±0.20b

hydrocarbons		
Oxygenated sesquiterpenes	4.46±0.10a	41.65±0.70b
Aliphatic hydrocarbons	4.61±0.30a	14.52±0.50b
Aldehydes/ketones	13.86±0.20	-
Fatty acids and esters	8.46±0.30b	4.73±0.20a
Others	38.8±0.60b	4.35±0.30a
Total	100	100

[a] RIs are retention indices calculated using a polar column (ZebronWax). Data are mean ± standard deviation of three replicates and values with different letters within the same line are statistically different ($P < 0.05$).

Table 2. Main constituents and geographical origin of essential oils from *Nepeta nuda* subspecies and *Nepeta curviflora*

***Nepeta* species**	**Geographical origin**	**Main essential oil constituents**	**References**
N. nuda ssp. *albiflora*	Turkey	*trans*-Caryophyllene (23.9%), isopulegone (12.6%), *cis*-sabinol (10.1%), *β*-pinene (10.0%)	Alim et al., 2009
N. nuda ssp. *albiflora*	Lebanon	Oxygenated monoterpenes (17.5-27.3 %) sesquiterpene hydrocarbons (22.7-38.4%) oxygenated sesquiterpenes (15.6-22.5%)	Mancini et al., 2009
N. nuda ssp. *nuda*	Turkey	Sesquiterpene fraction (81.9%)	Kökdil et al., 1998
N. curviflora	Lebanon	Sesquiterpene hydrocarbons (62.5%)	Senatore et al., 2005
N. curviflora	Lebanon	Sesquiterpene hydrocarbons (61.2%)	Mancini et al., 2009
N. curviflora	Jordan	Sesquiterpene hydrocarbons (55.27%)	Al-Qudah 2016

Figure 1

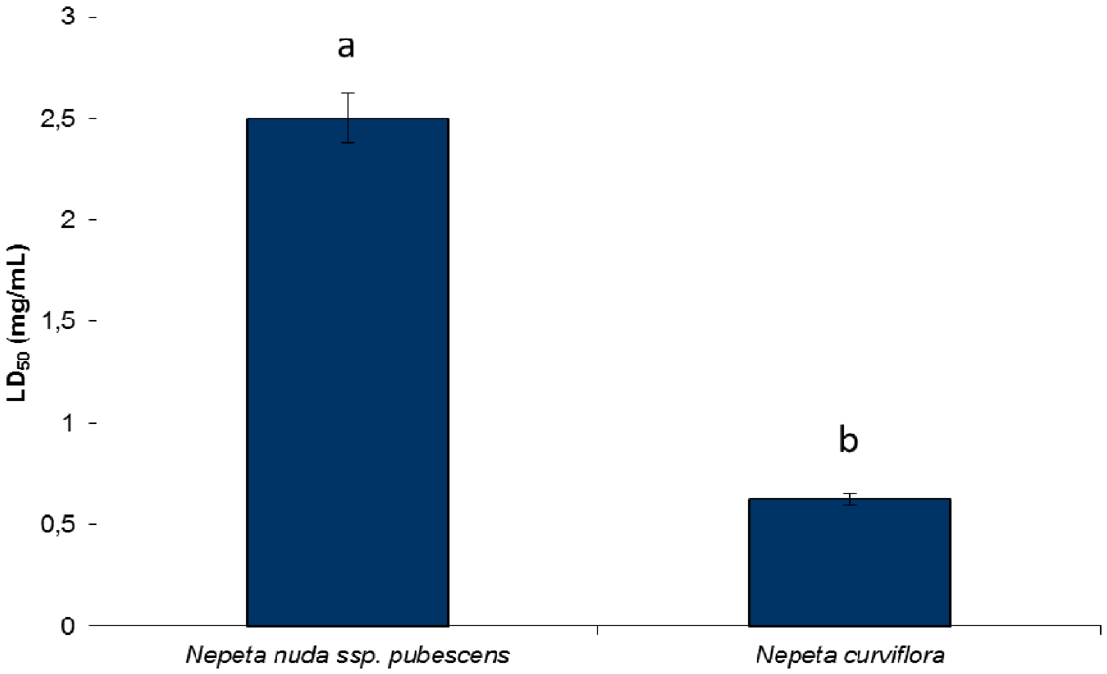

Figure 1. *In vitro* nematicidal activity of essential oils obtained from *Nepeta nuda* L. ssp. *pubescens* and *Nepeta curviflora* Boiss. on *Panagrolaimus rigidus* adults. Results are mean of three replicates; bars indicate standard deviation; bars carrying different letters are significantly different at $P < 0.05$ (Fisher's LSD test).