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Chemical Composition And Antifungal Activity Of Essential Oil Of Satureja Calamintha Spp. Nepeta (L.) Briq Against Some Toxinogenous Mold

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ABSTRACT

The resistance of the toxigenic mycoflora to chemical treatments based on synthetic fungicides guides us in the search for more effective and safer alternatives. In this context, the aim of this study was to evaluate the chemical and antifungal properties of the essential oil of S. calamintha nepeta against three fungal strains namely: A. flavus, A. parasiticus, and A. ochraceus. Indeed, the oil obtained by hydrodistillation had a yield of 1,36 %. The chemical composition performed by GC-MS identified 110 compounds, dominated by oxygenated monoterpenes. Piperitenone oxide, trans piperitenone oxide, caryophyllene oxide, 3-methyldiphenyl ether, (E) -caryophyllene, gensmin, germacrene D, (Z) -jasmone, trans-calamenene, γ-gurjunene, and pulegone are the main constituents of this oil. The antifungal potency was studied by the poisoned food method in order to determine the percentage inhibition of mycelial growth and the microdillution technique for the minimum inhibitory, fungistatic and fungicidal concentrations. Overall, all tested molds were inhibited with 1/100 and 1/250 (v/v) concentrations after seven days of incubation. The minimum inhibitory and fungicidal concentrations of EO were in the order of 0.666-2.666 µl/ml and 2.666-5.333 µl/ml respectively. It can be concluded that the essential oil of S. calamintha nepeta can be exploited in the food industry as natural fungicides against storage fungi to protect food from spoilage caused by these microorganisms.

Keywords: Essential oil, Aspergillus, antifungal activity, Penicillium, Satureja.

1. INTRODUCTION

Food contamination by toxigenic molds is a serious concern that is receiving increased attention from consumers, governments and producers due to the real danger to human and animal health, in particular, in countries tropical and subtropical where unscientific harvesting, collecting, transporting and storage practices promote mold growth and the secretion of highly toxic substances, generally referred to as "secondary metabolites" or "mycotoxins" [1,2].

These are toxic substances produced naturally by certain molds in a random fashion on the plant in the open field or during storage. Environmental conditions such as temperature, humidity and the nature of the substrate play a major role in the production of different types of mycotoxins, which can contaminate different foodstuffs [3]. Aflatoxins and ochratoxin A are among the most toxic mycotoxins and produced by certain strains of Aspergillus and penicillium. The range of harmful effects of mycotoxins is very wide: they have carcinogenic, mutagenic, immunomodulatory, estrogenic, necrotizing, neurotoxic, nephrotoxic, hepatotoxic and hematotoxic effects [4-8]. In addition, this dangerous contamination causes enormous economic losses. In fact, the Food and Agriculture Organization of the United Nations (FAO) estimates that 25% of the world's foodstuffs are contaminated by mycotoxins [9].

The use of chemicals is currently the most widely used technique for controlling harmful molds, due to their efficacy, as well as their easy and convenient applications. However, their use remains a very delicate task because of their very high cost and their harmful side effects on consumers on the one hand, and the emergence of strains resistant to certain common antifungals on the other hand [10-13].

These various difficulties aroused our interest in the search for other fungitoxic substances of natural origin which could constitute an alternative solution to chemicals. Medicinal and aromatic plants constitute a very important natural wealth, the valuation of which requires a perfect knowledge of the properties to be valued. The medicinal properties of medicinal plants depend on the presence of various bioactive agents belonging to different chemical classes [14]. Among the natural substances extracted from aromatic plants, there are essential oils (EOs) which are defined as fragrant products, generally of complex composition, obtained from a botanically defined raw material, either by entrainment with water vapor either by dry distillation or by a suitable mechanical process without heating [15].

The geographical location and the diversity of the bioclimatic stages of Algeria, offer a rich and diverse vegetation, mainly concerning medicinal aromatic plants which grow spontaneously, some of them give natural substances with proven antifungal properties. From this vegetation, we have chosen Satureja calamintha ssp. Nepeta (L.) Briq. (Syn: Calamintha nepeta) belonging to the lamiaceae family. It is a small perennial and annual plant 40 to 80 cm tall with a minty scent. The stems are soft and hairy, bear opposite leaves, lanceolate and pointed at their tips. The flowers are clearly visible from July to October, white to purple in color [16]. Several works has been done on the essential oil of this plant regarding its antifungal, antibacterial, anti-inflammatory, and antioxidant activity [17-27]. Moreover, thanks to its smell it is used in various culinary recipes [16].

The objective of this work is to highlight the characterization of the chemical composition of the EO of S. calamintha nepeta, and to study its antifungal power against three fungal strains synthesizing mycotoxins in order to develop a herbal formulation to reduce or inhibit the growth of toxigenic molds in foods

2. MATERIELS AND METHODS

2.1. Plant material

The plant species was collected in September 2019 from the mountains of the Skikda region located in northeastern Algeria. First, the plant was cleaned thoroughly with tap water and sterile distilled water to remove dust and other inert materials. The plant parts were dried in the open air, protected from light and moisture for three weeks, and then stored until use.

2.2. Fungal strains

To evaluate the antifungal activity of our essential oil, we used a collection of three molds belonging to the genus Aspergillus: A. flavus, A. parasiticus, and A. ochraceus. These molds were isolated by the dilution method, from the traditional food marketed in Bechar city located in Southwest Algeria. The microorganisms were then identified by the "Single Spore" technique. The choice of these molds was based on their degree of toxicity and their ability to synthesize mycotoxins.

2.3. Extraction of essential oil

The essential oil extraction was carried out by steam distillation (hydrodistillation) by a clevenger type apparatus [28]. By putting the dried plant material (100 g) in direct contact with 1 liter of distilled water in a flask surmounted by a column of 60 cm in length, then brought to the boil. Heating is maintained for four hours. The column is connected to a condenser which allows the accumulation of water vapor charged with droplets of essential oil. The traces of water in the essential oil obtained were removed by a micro syringe. To avoid their degradation, due to the action of air or light, we stored the essential oil at 4 °C in the dark until use. The yield of essential oil is defined as the ratio between the mass of essential oil obtained and the mass of the plant material to be treated. The yield is calculated by the following formula:

$$\text{Oil } \% \left(\frac{v}{w}\right) = \frac{\text{weight of oil extracted in (g)}}{\text{weight of the treated sample in (g)}} \text{ x } 100$$

2.4. Chromatographic analysis

GC-MS analysis was performed using the Hewlett-Packar 6890 gas chromatograph (Palo Alto, CA, USA) interfaced with an HP 5973 mass spectrometry detector (Palo Alto, CA, USA). The settings were as follows: The injector temperature was 280 °C and the volume of essential oil injected in split mode (50: 1) was 0.2 µl. The column was of the HP-5 MS type (5 % diphenyl and 95% dimethyl lpolysyloxane; diameter: 30 m × 0.25 mm; film thickness of 0.25 µm). The initial oven temperature was maintained at 60 °C (0-8min), then it's increased by 2 °C/min to 250 °C (8-113min), the carrier gas was helium with a flow rate of 0.5 ml/min. The column was operated in electronic ionization mode at 70 eV with a transfer temperature maintained at 270 °C, while the temperature of the quadrupole and ion source was maintained at 230 °C. Mass spectrometry was obtained with a scanning range of 40-550 m/z with a solvent delay of 3 min. A series of alkanes (C_8-C_{29}) was injected under the same chromatographic conditions described for the sample. The identification of the compounds was carried out by comparing their retention indices with those of the alkane series, by referring to compounds known in the literature, and by comparing their mass spectra with those stored on the NIST and Wiley computer libraries [29].

2.5. Antifungal activity

Antifungal activity was assessed by the poisoned food method to determine the level of inhibition and in liquid medium to determine the minimum inhibitory concentrations (MIC), minimum fungistatic concentrations (MFCs), and minimum fungicidal concentrations (MFC).

2.6. Poisoned Food Method

The technique used was that reported by Satrani et al [21] and Remmal et al [30] with some minor modifications. Due to the immiscibility of the essential oil with water and therefore with culture media, the emulsification was carried out using dimethyl sulfoxide (DMSO) in order to disperse the compounds and improve their contact with the germs tested. Dilutions were prepared at 1/10, 1/25, 1/50, 1/100, 1/200, 1/300 and 1/500 in DMSO. In test tubes each containing 13.5 ml of sterilized PDA (20 min at 121 °C), acidified with 25% lactic acid and cooled to 45 °C, 1,5 ml of each of the dilutions of so as to obtain the final concentrations of 1/100, 1/250, 1/500, 1/1000, 1/2000, 1/3000 and 1/5000 (v/v). The tubes were then shaken well before pouring them into Petri dishes (9 cm in diameter) with enough care to avoid trapping air bubbles. Controls, containing the culture medium and DMSO alone, were also prepared. Seeding was done by depositing 6 mm diameter discs taken from the periphery of the 48 hour young cultures and aseptically inoculated in the center of the Petri dishes. Each test was repeated three times, and the plates were incubated for 7 days at 28 °C in the dark, with the colony diameter measured each day. The fungal toxicity of the essential oil was expressed as a percentage inhibition of mycelial growth according to the following formula described by Philippe et al [31]:

$$\text{Inhibition rate } \% = \frac{\text{Dt} - \text{Di}}{\text{Dt}} \times 100$$

Where Dt = mean diameter of the control fungal colony; Di = mean diameter of the fungal colony treated.

2.7. Microdillution method

The determination of the minimum inhibitory concentration (MIC), the minimum fungistatic concentration (MFCs) and the minimum fungicidal concentration (MFC) of this EO was carried out with the microdilution method according to the CLSI M38-A3 protocol [32]. Briefly, the essential oil was dissolved in DMSO, in order to obtain a final concentration of the stock solution of EO of 16 µl/ml. a volume of 100 µl of PDP (Potato Dextrose Broth) was placed in the wells of a 96-well microplate. Afterwards, a volume of 100 µl of the prepared mother solution was transferred into the first well, in order to that a half (½) dilution series was prepared in the culture medium. Then, a volume of 50 µl of fungal inoculum (10^6 CFU/ml) is homogenized in each well. The final concentrations of the range thus generated are between 5.33 and 0.08 µl/ml. Wells containing Amphotericin B (ATB) and Fungazol are used as positive controls, and those containing DMSO are used as negative control. The plate was incubated for 72 hours at 28 °C. Fungal growth was determined by adding 30 µl of an aqueous solution of triphenyl tetrazolium chloride (TTC). In addition, the MFCs was defined as the lowest concentration of essential oil that can remove 99.9% of the fungal inoculum. For this, 10 µl of the broth was taken from each well without visible growth and inoculated in PDA for 7 days at 28 °C. Resumption of growth was considered MFC, while resumption of fungal growth was defined as MFCs.

2.8. Statistical analysis

All experiments were spotted three times. Ms Excel 2007 was used to express the values as the mean ± deviation.

3. RESULTS

3.1. Yield

The chemical composition of an essential oil is very complex and subject to many variables. Knowing exactly the constituents of an essential oil is fundamental, both to check its quality, to explain its properties and to predict its potential toxicity. The essential oil obtained forms a group of highly volatile substances, with a mild aromatic minty odor, and pale yellow in color, with an average yield of 1.39 %.

3.2. Composition of essential oil

Chromatographic analysis of the chemical composition allowed us to identify 110 compounds representing 95.677 % of the total oil of C. nepeta. Among the compounds identified, piperitenone oxide is the major compound with a content of 26.062 %. The latter is followed by trans piperitenone oxide (9.351 %), caryophyllene oxide (4.26 %), 3-methyldiphenyl ether (3.86 %), (E)-caryophyllene (3.83 %), gensmin (2.86 %), germacrene D (2.85 %), (Z)-jasmone (2.70 %) and trans-calamenene (2.19 %), γ-gurjunene (2.14 %), pulegone (2.06 %). Other compounds are identified but in relatively low percentages such as cis-cadina-1(6), 4-diene (1.98 %), D-limonene (1.96 %), E-(β)-famesene (1.80 %), viridiflorol (1.74 %), and thymol (1.56 %).

Table 1. Chemical composition of the essential oil of Satureja calamintha nepeta from the Skikda region (North-Eastern Algeria).

N	Compound	KI	RT	Concentration (%)
1	Ethyl 2-methylbutanoate	842	06.16	0.020
2	Tricyclene	925	09.82	0.042
3	α-Pinene	932	10.22	0.629
4	Camphene	949	11.32	0.263
5	Sabinene	972	12.77	0.213
6	β-Pïnene	978	13.11	0.737
7	1-Octen-3-ol	982	13.41	0.292
8	β-Myrcene	990	13.88	0.268
9	Octan-3-ol	1000	14.5	0.058
10	α-Phellandrene	1005	14.87	0.012
11	Pseudo limonene	1007	15.05	0.027
12	α-Terpinene	1018	15.79	0.255
13	Para-cymene	1026	16.41	0.164
14	**D-limonene**	**1030**	**16.71**	**1.962**
15	1,8-Cineole	1034	16.95	0.134
16	(z)-β-Ocimene	1037	17.2	0.130
17	γ-Terpinene	1059	18.78	0.428
18	cis- Sabinene hydrate	1073	19.79	0.161

19	Terpinolene	1085	20.71	0.155
20	p-Cymene	1092	21.21	0.051
21	Linalool	1100	21.77	0.105
22	1-Octen-3-yl acetate	1111	22.61	0.779
23	cis p-Menth-2-en-1-ol	1127	23.77	0.040
24	α-Campholenal	1130	23.99	0.027
25	Dihydro linalool	1137	24.52	0.299
26	trans-Pinocarveol	1142	24.88	0.024
27	trans p-Menth-2-en-1-ol	1145	25.1	0.039
28	Camphor	1151	25.49	0.019
29	Menthone	1159	26.11	0.067
30	Lavandulol	1168	26.73	0.134
31	Isomenthol	1173	27.15	0.198
32	2-methyl isoborneol	1176	27.34	1.067
33	trans-isopulegone	1180	27.6	0.057
34	Terpinen-4-ol	1183	27.89	0.718
35	para-Cymen-8-ol	1192	28.48	0.065
36	α-Terpineol	1199	29	0.316
37	Methyl chavicol (estragol)	1201	29.19	0.080
38	trans-Piperitol	1213	30	0.024
39	α-Methylcinnamaldehyde	1217	30.28	0.422
40	Coahuilensol methyl ether	1221	30.58	0.684
41	Sobornyl formate	1230	31.24	0.065
42	(Z)-3-Hexanyl 3- methylbutyrate	1239	31.87	0.141
43	**Pulegone**	1243	32.14	**2.069**
44	Cumin aldehyde	1248	32.45	0.219
45	trans-Piperitone oxide	1264	3362	**9.351**
46	cis-Carvone oxide	1266	3375	0.143
47	2-Hydroxy-3-isopropyl-6- menthyl-2 cyclohexen-1-one	1268	33.9	0.121
48	α-Terpinen-7-al	1281	34.82	0.090
49	Bornylacelate	1287	35.18	0.497
50	Dihydroedulan I	1294	35.67	0.526
51	2-Undecanone	1296	35.86	0.118
52	2-Hydroxypiperitone	1300	36.09	0.325
53	**Thymol**	1304	36.41	**1.560**
54	Carvacrol	1313	36.99	0.361
55	p-vinyl-Guaiacol	1319	37.41	0.035
56	6-Methoxy-1,2-dihydronaphthalene	1329	38.05	0.111
57	trans-Carvyl acetate	1336	38.48	0.015
58	Piperitenone	1343	38.96	0.297
59	**Piperitenone oxide**	1377	41.24	**26.062**

60	β-Bourbonene	1383	41.64	0.591
61	β-Elemene	1390	42.07	0.386
62	cis,trans-Nepetalactone	1401	42.83	1.052
63	**(Z)-jasmone**	1403	42.93	**2.708**
64	α-Gurjunene	1405	43.07	0.210
65	β-Lonol	1408	43.22	0.249
66	**Gensmin**	1415	43.72	**2.862**
67	**(E)-Caryophyllene**	1420	44.02	**3.838**
68	β-Cedrene	1424	44.24	0.087
69	β-Copaen	1429	44.56	0.087
70	cis-Muurola-3,5-diene	1445	45.55	0.175
71	**(E)-β-Famesene**	1455	46.17	**1.803**
72	Allo-Aromadendrene	1458	46.39	0.151
73	**Cis-Cadina-1(6),4-diene**	1462	46.66	**1.981**
74	α-Acoradiene	1465	46.82	0.079
75	**γ-Gurjunene**	1475	47.46	**2.141**
76	**Germacrene D**	1481	47.85	**2.856**
77	Aristolochene	1485	48.09	0.026
78	Phenethyl 3-methylbutanoate	1489	48.3	0.214
79	σ-Silenene	1491	48.45	0.039
80	cis-β-Guaiene	1494	45.66	0.222
81	α-Muurolene	1497	48.85	0.063
82	γ-Amorphene	1501	49.08	0.046
83	(Z)-α-Bisabolene	1508	49.46	0.200
84	σ-Amorphene	1512	49.73	0.293
85	**trans-Calamenene**	1521	50.25	**2.190**
86	α-Cadinene	1536	51.14	0.396
87	α-Calacorene	1441	51.45	0.059
88	Muurol-5-en-4-α-ol	1557	52.41	0.278
89	β-Calacorene	1562	52.69	0.027
90	**3-Methyldiphenyl ether**	1576	53.3	**3.868**
91	Spathulenol	1581	53.83	1.313
92	**Caryophyllene oxide**	1585	54.06	**4.296**
93	β-Copaen-4-α-ol	1588	54.19	0.310
94	**Viridiflorol**	1596	54.7	**1.749**
95	Ledol	1604	55.18	0.144
96	Humulene epoxide II	1611	55.54	0.762
97	1,10-di-epi-cubenol	1616	55.85	1.537
98	β-cedrene epoxide	1623	56.21	0.077
99	1-epi-cubenol	1628	56.52	0.081
100	Muurola-4,10(14)-dien-1-β-ol	1633	56.8	0.231
101	α-epi-Cadinol	1643	57.37	0.324

102	3-isi-thujospanone	1649	57.69	0.386
103	A-Cadinol	1658	58.17	1.187
104	trans-Calamenen-10-ol	1667	56.68	0.103
105	Khusinol	1680	59.41	0.156
106	cis-14-nor-Muurol-5-en-4-one	1686	59.79	0.580
107	Amorpha-4,9-dien-2-ol	1692	60.12	0.773
108	Calamenen-10-ol <10-nor->	1708	61	0.117
109	6,10,14-Trimethylpentadecan-2-one	1843	68.05	0.111
110	(5E,9E) Farnesylacetone	1911	71.49	0.022
	Total identified (%)			**95.677**
	Hydrocarbon monoterpenes (%)			**5.467**
	Oxygenated monoterpenes (%)			**47.510**
	Hydrocarbon sesquiterpenes (%)			**16.946**
	Oxygenated sesquiterpenes (%)			**13.687**
	Other compounds (%)			**12.067**

In fact, the monoterpene fraction is larger and represents about 52.977 % of the overall chemical composition of the oil, of which the oxygenated monoterpenes and the hydrocarbon monoterpenes have 47.510 % and 5.467 % respectively of this fraction. The sesquiterpene fraction is smaller, representing 30.633 % of the overall chemical composition of the oil, and is shared between the hydrocarbon sesquiterpenes (16.946 %), and the oxygenated sesquiterpenes (13.687 %). In addition, oxygenated monoterpenes are distributed among ketones (38.511 %), alcohols (4.950 %), esters (2.393 %), ethers (0.898 %), and aldehydes (0.758 %). While, the oxygenated sesquiterpenes are distributed between alcohols (15 compounds) and ethers (3 compounds), the contents of which were 8.552 % and 5.135 % respectively (Table 2).

Chemical classes		KI	Number of compounds	Majority compounds
Hydrocarbon monoterpenes		925 ; 932 ; 949 ; 972 ; 978 ; 990 ; 1005 ; 1007 ; 1018 ; 1026 ; 1030 ; 1037 ; 1059 ; 1073 ; 1085 ; 1092	16 (5.467 %)	D-limonene
Hydrocarbon sesquiterpenes		1383 ; 1390 ; 1405 ; 1420 ; 1424 ; 1429 ; 1445 ; 1455 ; 1458 ; 1462 ; 1465 ; 1475 ; 1481 ; 1485 ; 1491 ; 1494 ; 1497 ; 1501 ; 1508 ; 1512 ; 1521 ; 1536 ; 1541 ; 1562 ; 1151 ; 1159 ; 1180 ; 1243 ; 1264 ; 1266 ; 1268 ; 1300 ; 1343 ; 1377	24 (16.946 %)	(E)-Caryophyllene Germacrene D γ-Gurjunene
Oxygenated monoterpenes	Ketones	1151 ; 1159 ; 1180 ; 1243 ; 1264 ; 1266 ; 1268 ; 1300 ; 1343 ; 1377	10 (38.511 %)	Piperitenone oxide Trans-piperitone oxide Pulegone
	Alcohols	1100 ; 1127 ; 1137 ; 1142 ; 1145 ; 1168 ; 1173 ; 1176 ; 1183 ; 1192 ; 1199 ; 1213 ; 1304 ; 1313	14 (4.950 %)	Thymol
	Esters	1111 ; 1230 ; 1287 ; 1401	4 (2.393 %)	cis, trans-Nepetalactone
	Ethers	1034 ; 1201 ; 1221	3 (0.898 %)	Coahuilensol methyl ether
	Adehydes	1130 ; 1217 ; 1248 ; 1281	4 (0.758 %)	α-Methylcinnamaldehyde
Oxygenated sesquiterpenes	Alcohols	1408 ; 1557 ; 1581 ; 1588 ; 1596 ; 1604 ; 1616 ; 1628 ; 1633 ; 1643 ; 1658 ; 1667 ; 1680 ; 1692 ; 1708	4 (0.758 %)	Viridiflorol 1,10-di-epi-cubenol
	Ethers	1585 ; 1611 ; 1623	4 (0.758 %)	Caryophyllene oxide
Other compounds	Ethers	1294 ; 1329 ; 1576	3 (5.135 %)	3-Methyldiphenyl ether
	Ketones	1296 ; 1403 ; 1649 ; 1843 ; 1911 ; 1686	3 (4.505 %)	(Z)-jasmone
	Alcohols	982 ; 1000 ; 1319 ; 1415	6 (3.925 %)	Gensmine

	Esters	842 ; 1239 ; 1336 ; 1489	4 (3.247 %)	Phenethyl 3-methylbutanoate

Table 2. Main constituents of the different chemical classes of essential oil of Satureja calamintha nepeta from the Skikda region (north-eastern Algeria).

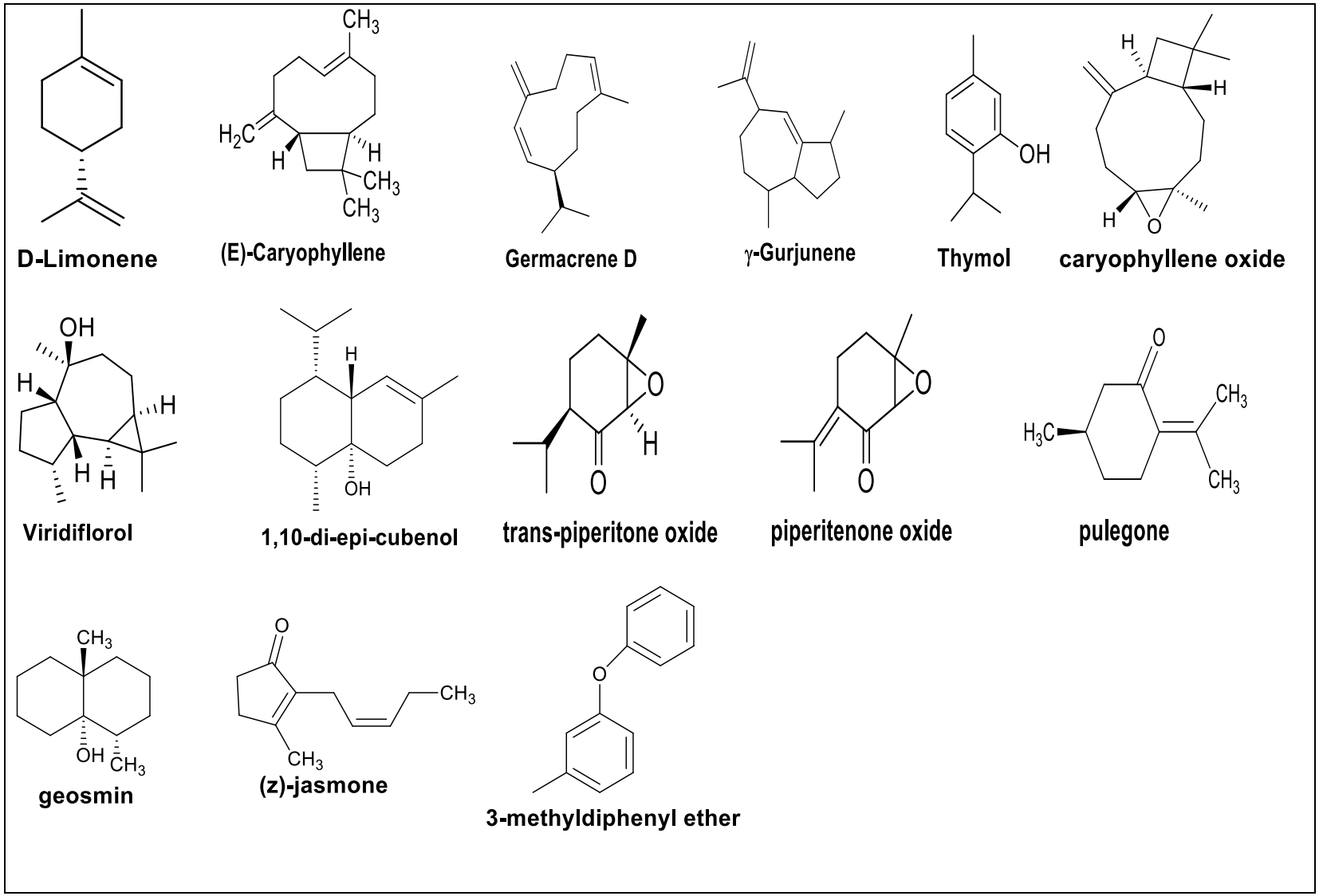

Figure 1. Chemical structures of the major compounds.

3.3. Antifungal activity

Faced with the problems linked to the resistance of various microorganisms to synthetic antibiotics, much work has been done on the antimicrobial power of natural products such as the essential oils of certain aromatic plants. In this study, the antifungal potency of C. nepeta EO was investigated against three pathogenic fungal strains. This power was evaluated by determining the inhibition rate (Table 3), MIC, MFCs, and the MFC (Table 4,5).

Table 3. Mycelial growth inhibition rate (%) of the essential oil of S. Calamintha nepeta on three toxinogenic molds after seven days of incubation.

	Inhibition of mycelial growth (%)		
Concentration (v/v)	**A. flavus**	**A. parasiticus**	**A. ochraceus**
1/5000	67.52±0.74	53.90±1.88	58.53±1.21
1/3000	78.20±1.28	68.72±0.71	62.60±1.86
1/2000	84.18±0.74	77.77±1.23	67.07±1.21
1/1000	100.00±0.00	95.88±0.71	74.79±1.40
1/500	100.00±0.00	100.00±0.00	92.68±1.21
1/250	100.00±0.00	100.00±0.00	100.00±0.00
1/100	100.00±0.00	100.00±0.00	100.00±0.00

The results obtained showed that this oil had significant antifungal activity against all the molds tested. Complete inhibition of mycelial growth was observed at a concentration of 1/100 and 1/250 (v/v) for all fungi tested. However, A. flavus was shown to be the most sensitive, it was completely inhibited from the 1/1000 (v/v) concentration. Thus, the concentration of 1/500 (v/v) was sufficient to stop the growth of A. parasiticus. Whereas, the strain that showed some remarkable resistance is A. ochraceus which resisted up to the oil concentration 1/250 (v/v) (Table 3).

The minimum inhibitory and fungicidal concentrations of EO were in the order of 0.666-2.666 µl/ml and 2.666-5.333 µl/ml, respectively. Indeed, this inhibitory activity is more marked on A. flavus by completely inhibiting its growth from an MIC = 0.666 µl/ml, while the MIC for A. partasiticus corresponded to 1.333 µl/ml. While A. ochraceus was the most resistant strain with a MIC = 2.666 µl/ml (Table 4,5).

Table 4. The sensitivity of different strains studied to essential oil and classic antifungals by the microdilution method

	Inhibition of mycelial growth (%)		
Concentration (µl/ml)	**A. flavus**	**A. parasiticus**	**A. ochraceus**
0,083	+ + +	+ + +	+ + +
0,166	+ + +	+ +	+ +
0,333	+ +	+ +	+ +
0,666	-	+	+
1,333	-	-	+

2,666	-	-	-
5,333	-	-	-
ATB	+	+	+
Fungazol	-	-	-
DMSO	+	+	+

- Inhibition; + low growth; + + average growth; + + + very strong growth

Furthermore, the fungitoxic nature shows that the fungal strains tested are all sensitive to this oil. The latter shows a fungicidal activity MFC = 2.666 µl/ml, and fungistatic MFCs = 0.666 µl/ml for A. flavus. Whereas, for A. parasiticus, the oil showed a fungicidal activity CMF = 2.666 µl/ml and a fungistatic effect MFCs = 1.333 µl/ml. While, the oil studied showed a fungicidal activity MFC = 5.333 µl/ml, and fungistatic MFCs = 2.666 µl/ml for A. ochraceus, which shows that at least double the MIC is required to obtain the fungicidal effect on these two fungal species (Table 4,5). It can be concluded that the essential oil of S. calamintha nepeta can be exploited in the food industry as natural fungicides against storage fungi to protect food from spoilage caused by these microorganisms.

Table 5. Summary of antifungal parameters of the effects of C. nepeta EO on the growth of strains studied by the microdilution method

Fungal strain	MIC	MFCs	MFC
A. flavus	0,666	0,666	2,666
A. parasiticus	1,333	1,333	2,666
A. ochraceus	2,666	2,666	5,333

In addition, this study allowed us to make an interesting comparison of the inhibitory activity of the growth of germs with commercial fungicides on the one hand, and our essential oil on the other hand. The results obtained are shown to be very effective, since all the strains studied for this study showed almost total resistance to fungazol. In contrast, all strains have some resistance to ATB (Table 4). This finding leads us to conclude that this EO can be used as an antifungal alternative for toxinogenic molds resistant to commercial antifungals.

4. DISCUSSION

The yield found in our study is appreciable and can be profitable on an industrial scale. The comparison of yield obtained in our study with those reported in the literature clearly shows that C. nepeta from the Skikda region is richer in essential oil. Indeed, this rate is similar to that found by Kerbouche et al [25] (1.4 %), Labiod et al [27] (1.48 %), and Bouzidi et al [33] (1.3 %) for different populations of C. nepeta from Algeria. In addition, our yield is higher than that found by Cozzolino et al [34] (0.6 %), and Velasco-Negueruela et al [35] (0.3 %). On the other hand, it is lower compared to that by Souleles et al [36] (2 %). These variations in content may be due to several factors including the degree of maturity, the interaction with the environment (type of climate, soil), the time of harvest, and the method of extraction [37].

Concerning the chemical composition, this work complements and enriches the studies carried out on the essential oils of S. calamintha nepeta. In Algeria, the chemical composition of C. nepeta EOs has been reported previously, and it differs from region to region. Studies carried out in different

regions of Algeria have shown a clearly observed chemical variability depending on the origin of the samples. The study carried out by Kerbouche et al [27], shows that the EO of C. nepeta collected in the Blida region is mainly composed of oxygenated monoterpenes (97.4 %) including pulegone (39.5 %), neo-menthol (33 %) and I somenthone (19.6 %) were the main components of this oil. The same result was reported by Bouzidi et al [33] where the EO of C. nepeta, collected in the region of Bouhanifia near Mascara, was dominated by pulegone (73.54 %) associated with isomenthone (7.89 %), cis-peperitone oxide (2.28 %), limonene (1.37 %) and trans-isopulegone (1.09 %). Contrary to these results, Labiod et al [27] declared that the EO of C. nepeta from the Jijel region had menthone (26.46 %) as the major component, associated with piperitone oxide (22.26 %) and pulegone (14.04 %). While in Morocco, completely different results were recorded compared to those of Algerian studies. The study carried out by Satrani et al [21] reports that Moroccan oil contained ρ-cymene (20.9 %), γ-terpinene (18.7 %), and thymol (34.94 %) as predominant products.

In Europe, a study carried out by Marongiu et al [38] on essential oils obtained by hydrodistillation (HD) and by supercritical fluid (SFE) from C. nepeta species collected in Portugal as well as in the region of Sardinia (Italy), show that EOs were characterized by high contents of pulegone (64.4 - 39.9 %), piperitone (6.4 - 7.7 %) and piperitenone oxide (2.5 - 19.1 %), with the presence of other minority compounds such as limonene (4,8 - 2,8 %), menthone (2.8 - 2.5 %) and I somenthone (1.9 - 2.0 %). The Portuguese EO chemical composition appeared to be quite different from the Italian variety.

Another study carried out by Riela et al [39] on EO obtained by a solvent free extraction assisted by microwave (SFME) and by hydrodistillation (HD) from C. nepeta collected in Sicily (Italy), the main compounds were pulegone (25.2 % and 21.4 %, respectively, in oils obtained by SFME and HD), piperitone (13.1 %, SFME and 6.4 %, HD), piperite-none (12.3 %, SFME and 16.4 %, HD), menthone (11.6%, SFME and 19.8%, HD) and I somenthone (12.0 %, SFME and 2.1 %, HD). In addition, Flamini et al [40] reported the chemical composition of the essential oil of C. nepeta collected in Tuscany (Italy). The main constituent was pulegone (about 50 %), with the presence of other minor compounds: menthone (9.4 %), limonene (7.0 %), menthol (4.6 %), piperitenone oxide (4.6 %) and piperitone oxide (3.9 %). Similar results have been described by Panizzi et al [41] whose pulegone was the major constituent of EO in the region of Tuscany in Italy. In France, Rossi et al [42] reported pulegone (49 %) as the main compound followed by menthone (21,5 %), I somenthone (8.3 %) and limonene (3.5 %) of the EO of C. nepeta collected in Corsica. The same results were reported in the work carried out by Couladis and Tzakou [43] on the EO of Korinthos in Greece. In addition, several experiments carried out in several countries around the world have reported results similar to that of our study, where the oxides of piperitone and piperitone were the major components of EOs [36, 44 - 46].

An important chemical variability of EOs from Calamintha nepeta has been shown by two studies carried out by Baldovini et al [47] and Negro et al [48] on species collected respectively in Ajaccio (France) and in Apulia in the south-eastern Italy. The results of Baldovini et al [47] on 40 species showed that 16 were characterized by the predominance of menthone (43.4 %) associated with pulegone (18.9 %), with piperitone II oxide (8.3 %) and limonene (5.2 %), and 11 were characterized by high contents of piperitone II oxide (30.5 %) and piperitone oxide (12.5 %). While 13 species were characterized by a strong predominance of pulegone (55.6 %) with the presence of regular amounts of limonene (6 %). However, the results of Negro et al [48] on 46 species show that 10 were characterized by the predominance of piperitone oxide (49 %) associated with piperitone oxide (14 %), D-limonene

(8 %) and thymol (4 %), and 2 were strongly characterized by the presence of piperitone oxide (39 % and 48 % respectively. While, 9 species were characterized by the strong presence of piperitone (21.4 %) and menthone (22.53 %), while 25 species were characterized by a strong predominance of pulegone (42 %).

In conclusion, we can see a very important chemical polymorphism, of which most of the studies reported in the literature, three types of oils can be distinguished. The first type is characterized by pulegone as the main component, associated with a range of compounds: menthone, menthol and its isomers, piperite, piperitone and oxides of piperite. The second type is characterized by the predominance of piperitone oxide and/or piperitone oxide. Finally, as a third type, an atypical sample is distinguished by the presence of carvone and 1,8-cineole as main components [49]. Our results reinforce previous data on the variability of essential oils, which may be due to many factors: climate, geographic area, seasons, soil conditions, period of cultivation and extraction technique.

Furthermore, all the results obtained in the present study show that the essential oil of C. nepeta exhibited significant antifungal activity against the fungi tested. However, numerous studies have shown that the antifungal activities of EOs in aromatic plants are linked to their chemical composition and in particular to the major compounds. Thus, the nature of the chemical structures which constitute it, and their proportions play a determining role. However, it is likely that this activity will also depend on the presence of minority compounds which can interact directly, or synergistically or antagonistically, to create a mixture with antifungal activity [50, 51].

In addition, several studies have shown the antifungal activity of this oil. Labiod et al [27], by studying the antifungal activity of EO of Calamintha nepeta from the Jijel region located in eastern Algeria, showed that the minimum dose used to totally inhibit the growth of some post-pathogenic fungal strains harvest (Fusarium and Aspergillus spp) summer 2 µl/ml. The results demonstrated an excellent antifungal property of this oil. The authors pointed out that this antifungal action can be attributed to the high content of menthone, piperitone oxide and pulegone in this oil, which agrees with our results.

Another study carried out in Egypt, on this oil which showed remarkable antifungal activity against a frequent contaminant of many foodstuffs, namely A. niger [52]. In addition, Marongiu et al [38], during a study carried out on two samples of C. nepeta from Italy and Portugal, reported that Italian oil was more active than Portuguese oil, showing a Significant antifungal activity against certain strains of Aspergillus (A. flavus, A. fumigatus and A. niger), with minimum inhibitory concentration values ranging from 0.32 up to 1.25 µl/ml. The authors concluded that the higher antifungal activity of Italian oil could be associated with the contribution of pulegone, and suggested its use for therapeutic purposes, in particular in the treatment of certain aspergillosis. In addition, the antifungal activities of C. nepeta of the Italian vegetation coming more precisely from the region of Pisa carried out against certain pathogenic fungi, namely: Fusarium moniliforme, Botrytis cinerea, A. niger and Pyricularia oryzae. The oil has shown a broad spectrum of antifungal action with inhibition diameters ranging from 10.33 up to 14.67 mm. The authors suggested that the major constituents of this oil (pulegone and piperitenone oxide) were responsible for its antimicrobial activities [19].

The antifungal potency of Italian EO has also been proven by Panizzi el al [41], against two fungi (Saccharomyces cerevisiae and C. albicans). The oil was very rich in pulegone, piperitone and

piperite with their oxides, and showed good potency against the microorganisms tested (MIC = 2 µg/ml). According to the authors, the activity of this oil could be mainly due to its major constituents.

Still in the same context, the EO of C. nepeta from Montenegro proved to be hyper potent on several microorganisms, and in particular A. niger (diameter of the zone of inhibition = 40 mm) responsible for the production of some toxic metabolites [22] This was confirmed by another study carried out on C. nepeta collected in Serbia, and which included the same test microorganisms previously [52]. In both experiments, the essential oils had pulegone, piperitone and piperitone with their oxides as the major compounds. In addition, a study carried out on the EO of C. nepeta collected in Italy with the aim of knowing its antifungal capacities against two fungi namely Microsporum canis and Microsporum gypseum responsible for human cutaneous mycosis disseminated by domestic animals. The authors reported very remarkable fungistatic and fungicidal minimum concentrations. This oil is characterized by pulegone, piperitenone and piperitenone oxide as major compounds [53].

This essential oil antifungal property of C. nepeta can be attributed to its complex chemical makeup. On one side, the oxygenated monoterpenes that go into its composition are generally more active than the hydrocarbon monoterpenes which on the other hand, are known for their low antifungal power [54]. On the other hand, sesquiterpenes can have strong antifungal potency, sometimes even greater than that of monoterpenes [55]. The mode of action responsible for this growth reduction until complete inhibition, components known for their strong antifungal power are hydrophobic. This property facilitates their penetration between membrane phospholipids of fungal cells, which leads to the leakage of the contents of vital cells, the impairment of fungal enzyme systems consequently disrupting cellular functions [56]. It is then concluded that the antifungal activity of this EO is due to the presence of both sesquiterpenes and monoterpenes and to the synergy between its components. Therefore, more in-depth studies can be undertaken for the development of natural products based on essential oil to exploit its antifungal properties in the prevention, and treatment of certain fungal infections, and to combat molds resistant to conventional antifungals.

Conflict of interest statement

We declare that there is no conflict of interest

References

[1] Domijan AM, Peraica M., Miletic-Medved M, Lucic A, Fuchs R. Two different clean-up procedures for liquid chromatographic determination of ochratoxin A in urine. J Chromatogr B 2003; **798**(2): 317-321.

[2] Dinis AMP, Lino CM, Pena AS. Ochratoxin A in nephropathic patients from two cities of central zone in Portugal. J Pharmaceut Biomed Anal 2007; **44**(2): 553-557.

[3] Daou R, Joubrane K, Maroun RG, Khabbaz LR, Ismail Z, El Khoury A. Mycotoxins: Factors influencing production and control strategies. AIMS Agric Food 2021; **6**(1): 416-447.

[4] Reed KM, Mendoza KM, Abrahante JE, Coulombe RA. Comparative Response of the Hepatic Transcriptomes of Domesticated and Wild Turkey to Aflatoxin B_1. Toxins 2018; **10**(1): 1-24.

[5] Pierron A, Alassane-Kpembi I, Oswald IP. Impact of mycotoxin on immune response and consequences for pig health. Animal Nutr 2016; **2**(2): 63-68.

[6] Ladeira C, Frazzol C, Orisakwe OE. Engaging One Health for Non-Communicable Diseases in Africa: Perspective for Mycotoxins. Front Pub Hea 2017; **5**(266): 1-15.

[7] Dai Y, Huang K, Zhang B, Zhu L, Xu W. Aflatoxin B1-induced epigenetic alterations: An overview. Food Chem Toxicol 2017; **109**(1): 683-689.

[8] Zhu L, Zhang B, Dai Y, Li H, Xu W. A Review: Epigenetic Mechanism in Ochratoxin A Toxicity Studies. Toxins 2017; **9**(4): 1-11.

[9] Food and Agriculture Organization, Basic facts on the world cereal situation. Food Outlook, 5/6. Rome. 1996.

[10] Liu C, Zhao C, Pan HH, Kang J, Yu XT, Wang HQ, Li BM, Xie YZ, Chen RY. Chemical Constituents from Inonotus obliquus and Their Biological Activities. J Nat Prod 2014; **77**(1): 35-41.

[11] Balakumar S, Rajan S, Thirunalasundari T, Jeeva S. Antifungal activity of Aegle marmelos (L.) Correa (Rutaceae) leaf extract on dermatophytes. Asian Pac J Trop Biomed 2011; **1**(4): 309-312.

[12] Shoemaker M, Hamilton B, Dairkee SH, Cohen I, Campbell MJ. In vitro anticancer activity of twelve Chinese medicinal herbs. Phytother Res 2005; **19**(7): 649-651.

[13] Pezzuto JM. Plant-derived anticancer agents. Biochem Pharmacol 1997; **53**(2): 121-133.

[14] Maihebiau P. La nouvelle aromathérapie. Biochimie aromatique et influence psychosensorielle des odeurs. Lausanne. 1994

[15] International Organization for Standardization, ISO 9235:2013. Aromatic natural raw materials–Vocabulary. 2014.

[16] Božović M, Ragno R. Calamintha nepeta (L.) Savi and its Main Essential Oil Constituent Pulegone: Biological Activities and Chemistry. Molecules 2017; **22**(2): 1-50.

[17] Ristorcelli D, Tomi F, Casanova J. Essential Oils of Calamintha nepeta subsp. nepeta and subsp. glandulosa from Corsica (France). J Essent Oil Res 1996; **8**(4): 363-366.

[18] Fraternale D, Giamperi L, Ricci D, Manuta A. Composition of Essential Oil as a Taxonomic Marker for Calamintha nepeta (L.) Savi ssp. Nepeta. J Essent Oil Res 1998; **10**(5): 568-570.

[19] Flamini G, Cioni PL, Puleio R, Morelli IPanizzi L. Antimicrobial activity of the essential oil of Calamintha nepeta and its constituent pulegone against bacteria and fungi. Phytother Res 1999; **13**(4): 349-351.

[20] Baldovini N, Ristorcelli D, Tomi F, Casanova J. Infraspecific variability of the essential oil of Calamintha nepeta from Corsica (France). Flavour Fragt J 2000; **15**(1): 50-54.

[21] Satrani B, Farah A, Fechtal M, Talbi M, Blaghen M, Chaouch A. Composition chimique et activité antimicrobienne des huiles essentielles de Satureja calamintha et Satureja alpina du Maroc. Ann Fals Exp Chim 2001; **94**(956): 241-250.

[22] Kitic D, Jovanovic T, Ristic M, Palic R, Stojanovic G. Chemical Composition and Antimicrobial Activity of the Essential Oil of Calamintha nepeta (L.) Savi ssp. glandulosa (Req.) P.W. Ball from Montenegro. J Essent Oil Res 2002; **14**(2): 150-152.

[23] Kürkçüoglu M, Iscan G, Ozek T, Baser KHC, Alan S. Composition and Antimicrobial Activity of the Essential Oils of Calamintha betulifolia Boiss. et Bal.. J Essent Oil Res 2007; **19**(3): 285-287.

[24] Singh PP, Jha S, Irchhaiya R. Antidiabetic and antioxidant activity of hydroxycinnamic acids from Calamintha Officinalis Moench. Med Chem Res 2012; **21**(8): 1717-1721.

[25] Kerbouche L, Hazzit M, Baaliouamer AJ. Essential Oil of Satureja calamintha subsp. nepeta (L.) Briq from Algeria: Analysis, Antimicrobial and Antioxidant Activities. Bio Act Prod Nat 2013; **3**(4): 266-272.

[26] L. Cherrat, L. Espina, M. Bakkali, R. Pagán, A. Laglaoui. Chemical composition, antioxidant and antimicrobial properties of Mentha pulegium, Lavandula stoechas and Satureja calamintha Scheele essential oils and an evaluation of their bactericidal effect in combined processes. Innov Food Sci Emerg Technol 2014; **22**: 221-229.

[27] Labiod R, Aouadi S, Bouhaddouda N. Chemical composition and antifungal activity of essential oil from Satureja Calamintha nepeta against phytopathogens fungi. Inter J Pharm Pharmaceutic Sci 2015; **7**(7): 208-211.

[28] Clevenger JF. Apparatus for the determination of volatile oil. J Am Pharm Assoc 1928; **17**(4): 346-349.

[29] Adams RP. Identification of essential oil components by gas chromatography/quadrupole mass spectrometry. Allured Publishing Corporation, Carol Stream. 2001.

[30] Remmal A, Bouchikhi T, Rhayour K, Ettayebi M, Tantaoui-Elaraki A, Improved Method for the Determination of Antimicrobial Activity of Essential Oils in Agar Medium. J Essen Oil Res 1993; **5**(2): 179-184.

[31] S. Philippe, F. Souaïbou, A. Guy, D.T. Sébastien, Y. Boniface, A. Paulin, Y. Issaka, S. Dominique. Chemical Composition and Antifungal activity of Essential oil of Fresh leaves of Ocimum gratissimum from Benin against six Mycotoxigenic Fungi isolated from traditional cheese wagashi. Res J Bio Sci 2012; **1**(4): 22-27.

[32] Clinical and Laboratory Standards Institute (CLSI). M27-A3: Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard—3rd. Wayne, PA, USA, 2008.

[33] Bouzidi N, Mederbal K, Bouhadi D. Chemical composition of the essential oil of Satureja calamintha subsp. Nepeta of west Algerian. Mor J Chem 2018; **6**(2): 213-217.

[34] Cozzolino F, Fellous R, Vernin G, Parkanyi C. GC/MS Analysis of the Volatile Constituents of Calamintha nepeta (L.) Savi ssp. nepeta from Southeastern France. J Essent Oil Res 2000; **12**(4): 481-486.

[35] Velasco-Negueruela A, Pérez-Alonso MJ, Esteban JL, Vallejo MCG, Zygadlo JA, Guzmán CA, Ariza-Espinar L. Essential Oils of Calamintha nepeta (L.) Savi and Mentha off. suaveolens Ehrh., Grown in Cordoba, Argentina. J Essent Oil Res 1996; **8**(1): 81-84.

[36] Souleles C, Argyriadou N, Philinos S. Constituents of the essential oil of Calamintha nepeta. J Nat Prod 1987; **50**(3): 510-522.

[37] Botton B, Bertron A, Fevere M, Gauthier S, Guph D, Larpent JP, Reymond P, Sanglier JJ, Vaysser Y, Veau S, Moisissures utiles et nuisibles importance industrielle .Ed : Masson collection biotechnologies. 1990.

[38] Marongiu B, Piras A, Porcedda S, Falconieri D, Maxia A, Gonçalves MJ, Cavaleiro C, Salgueiro L. Chemical composition and biological assays of essential oils of Calamintha nepeta (L.) Savi subsp. nepeta (Lamiaceae). Nat Prod Res 2010; **24**(18): 1734-1742.

[39] Riela S, Bruno M, Formisano C, Rigano D, Rosselli S, Saladino ML, Senatore F. Effects of solvent-free microwave extraction on thechemical composition of essential oil of Calamintha nepeta (L.) Savi compared with the conventional production method. J Sep Sci 2008; **31**(6-7): 1110-1117.

[40] Flamini G, Cioni PL, Puleio R, Morelli I, Panizzi L. Antimicrobial Activity of the essential oil of Calamintha nepeta and its Constituent pulegone against bacteria and fungi. Phytother Res 1999; **13**(4): 349-351.

[41] Panizzi L, Flamini G, Cioni PL, Morelli I. Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae. J Ethno 1993; **39**(3): 167-170.

[42] Rossi PG, Berti L, Panighi J, Luciani A, Maury J, Muselli A, Serra DDR, Gonny M, Bolla JM. Antibacterial Action of Essential Oils from Corsica. J Essent Oil Res 2007; **19**(2): 176-182.

[43] Couladis M, Tzakou O. Essential Oil of Calamintha nepeta subsp. glandulosa from Greece. J Essent Oil Res 2001; **13**(1): 11-12.

[44] Kirimer N, Baser KHC, Özek T, Kürkçüoglu M. Composition of the Essential Oil of Calamintha nepeta subsp. Glandulosa. J Essent Oil Res 1992; **4**(2) 189-190.

[45] Fraternale D, Giamperi L, Ricci D, Manunta A. Composition of Essential Oil as a Taxonomic Marker for Calamintha nepeta (L.) Savi ssp. nepeta. J Essent Oil Res 1998; **10**(5): 568-570.

[46] Mastelic J, Milos M, Kustrak D, Radonic A. The Essential Oil and Glycosidically Bound Volatile Compounds of Calamintha nepeta (L.) Savi. Croa Chem Acta 1998; **71**(1): 147-154.

[47] Baldovini N, Ristorcelli D, Tomi F, Casanova J. Infraspecific variability of the essential oil of Calamintha nepeta from Corsica (France). Flavour Fragrance J 2000; **15**(1): 50-54.

[48] Negro C, Notarnicola S, De Bellis L, Miceli A. Intraspecific variability of the essentialoil of Calamintha nepeta subsp. nepeta from Southern Italy (Apulia). Nat Prod Res 2013; **27**(4-5): 331-339.

[49] S. Burt. Essential oils: their antibacterial properties and potential applications in foods —a review. Int J Food Microbiol 2004; **94**(3): 223-253.

[50] Garzoli S, Pirolli A, Vavala E, Di Sotto A, Sartorelli G, Božovi M, Angiolella L, Mazzanti G, Pepi F, Ragno R. Multidisciplinary Approach to Determine the Optimal Time and Period for Extracting the Essential Oil from Mentha suaveolens Ehrh. Molecules 2015; **20**(6) : 9640-9655.

[51] Monforte MT, Tzakou O, Nostro A, Zimbalatti V, Galati EM. Chemical Composition and Biological Activities of Calamintha officinalis Moench Essential Oil. J Med Food 2011; **14**(3): 297-303.

[52] Kitic D, Stojanovic G, Palic R, Randjelovic V. Chemical Composition and Microbial Activity of the Essential Oil of Calamintha nepeta (L.) Savi ssp. Nepeta var. subisodonda (Borb.) Hayek from Serbia. J Essent Oil Res 2005; **17**(6): 701-703.

[53] Perrucci S, Mancianti F, Cioni PL, Flamini G, Morelli I, Macchioni G. In Vitro Antifungal Activity of Essential Oils Against some Isolates of Microsporum canis and Microsporum gypseum. Planta Med 1994; **60**(2): 184-187.

[54] Kalemba D, Kunicka A. Antibacterial and antifungal properties of essential oils. Med Chem 2003; **10**(10): 813-829.

[55] Chang HT, Cheng YH, Wu CL, Chang ST, Chang TT, Su YC. Antifungal activity of essential oil and its constituents from Calocedrus macrolepis var. formosana Florin leaf against plant pathogenic fungi. Bioresource Technol 2008; **99**(14): 6266-6270.

[56] Sharma N, Tripathi A. Effects of Citrus sinensis (L.) Osbeck epicarp essential oil on growth and morphogenesis of Aspergillus niger (L.) Van Tieghem. Microbiol. Res 2008; **163**(3): 337-344.