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Chemical analyses, antioxidant and antifungal effects of oregano and thyme essential oils alone or in combination against selected *Fusarium* species

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Abstract

Essential oils (EOs) extracted from aromatic plants are interesting natural products due to their large therapeutic potential and benefits as natural preservatives. In the present work, the chemical composition, antioxidant, and antifungal effects of the essential oils of *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme), alone or in combination, were investigated. The chemical analysis was performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) techniques. The antioxidant activity was investigated using a DPPH radical scavenging activity assay. The *in vitro* antifungal activity was evaluated by disc diffusion, agar dilution, and spore germination inhibition methods against *Fusarium* species isolated from dry rot of potato tubers, whereas the combined effect of the two essential oils was evaluated by the checkerboard technique. GC and GC-MS analyses resulted in the identification of 37 and 41 components in *T. vulgaris* and *O. vulgare* EOs, representing 80.17% and 93.00% of the total oils, respectively. The main compound of thyme oil was thymol (46.97%) and that of oregano oil was carvacrol (59.03%). The DPPH scavenging assay showed a high antioxidant capacity for both essential oils, alone or in combination. The antifungal activity revealed that *O. vulgare* and *T. vulgaris* oils exhibited a great potential antifungal activity with minimal inhibitory concentration (MIC) values of 0.078 - 0.156 µL/mL and 0.156 - 0.313 µL/mL, respectively. The fractional inhibitory concentration index (FICI) of combined applications of the tested oils ranged from 0.375 to 0.500, suggesting synergistic interactions. Moreover, the oils were found to be effective against the spore germination of all tested *Fusarium* species. *In vivo* experiments against *Fusarium oxysporum* with wounded potato tubers supported these results. The findings indicated that both essential oils, alone or in combination, possess antioxidant and antifungal properties and can therefore be used as a potential source of bioactive molecules for preventing lipid peroxidation and fungal contamination of food.

Introduction

Essential oils, also known as volatile oils, are concentrated volatile hydrophobic liquids extracted from aromatic plants and mainly include terpenes, terpenoids, and aromatic and aliphatic constituents, all having low molecular weights. These compounds are of growing interest in the food industry due to their benefits as natural preservatives (Prakash *et al.,* 2015). Several of them are known antioxidants (Teixeira *et al.,* 2017; Aziz and Karboune, 2018); they have been proposed to replace synthetic antioxidants used in the food industry, such as butylated hydroxyltoluene (BHT) and butylated hydroxylanisole (BHA), which have been shown to carry potential health risks and toxicity (Öğretmen and Inanan, 2014). Additionally, essential oils are considered as new agents that exhibit strong antifungal activities against various storage fungi (Ferdes and Ungureanu, 2012); they have a relatively safe status, are biodegradable and nontoxic, and enjoy wide consumer acceptance (Yen *et al.,* 2015; Periasamy *et al.,* 2016).

Potato *Fusarium* dry rot is one of the most important potato diseases worldwide, and among the most dangerous fungal diseases that contaminate members of the Solanaceae family (Al-Mughrabi *et al.,* 2010). The disease causes significant losses in both quality and quantity of seed tubers. *Fusarium* spp. produce a range of toxic metabolites called mycotoxins, endangering human and animal health (Marin *et al.,* 2013; Kocić-Tanackov and Dimić, 2013). Alimentary intake of these metabolites in

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animals and humans causes intoxication (mycotoxicosis) that is manifested in the form of acute and chronic toxicity, including cytotoxicity, hepatotoxicity, neurotoxicity, teratogenicity, mutagenicity, and carcinogenicity (Kocić-Tanackov and Dimić, 2013). The principal mean to control fungal growth and therefore the production of mycotoxins is based on the application of fungicides (Becher *et al.,* 2010; Chowdhary *et al.,* 2013). However, the use of synthetic chemical compounds has now been discouraged due to their possible adverse health effects (Hoppin *et al.,* 2017; Mie *et al.,* 2018). For this reason, natural substances, such as essential oils, have been considered as reservoirs of new therapeutic and biopesticide agents (Garcia and Copetti, 2019; Ishaq *et al.,* 2019). Numerous scientific reports have described their strong ability to suppress the growth of *Fusarium* species (Nazzaro, 2017; Perczak *et al.,* 2019).

Essential oils extracted from oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.) have achieved greater importance in the fragrance, pharmaceutical, food, and flavour industries (Teixeira *et al.,* 2013; Prasanth Reddy *et al.,* 2014). In the present work, the essential oils extracted from Algerian oregano and thyme were screened (alone and in combination) for the first time to detect their potential antioxidant and antifungal activities against *Fusarium* spp. that cause potato dry rot. *In vivo* bioassays were also performed to evaluate the fungicidal potency against *F. oxysporum*. Furthermore, chemical analysis of the two essential oils was carried out using gas chromatography (GC) and mass spectroscopy (GC-MS) techniques.

Materials and methods

Plant materials

The aerial parts of thyme and oregano were collected in 2018 in the north east of Algeria. The plant identification was confirmed in the botanical department of the High National School of Agronomy (ENSA), Algiers, Algeria. After transport to the laboratory, the samples were thoroughly rinsed and shade-dried. The dry plants were then ground and stored for further use.

Essential oil isolation

The shade-dried and finely powdered plant leaves were hydrodistilled for 3 h using a modified Clevenger-type apparatus. The oil was extracted from distilled water with diethyl ether, dried over anhydrous sodium sulphate, filtered and the solvent was removed at room temperature under reduced pressure on a rotary evaporator, thus yielding the oil. The resulting oil was stored in the dark at 4°C until analysis.

Chromatographic analysis

Both essential oils were subjected to analysis by using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The GC analysis was performed on a model 6890 N gas chromatograph (Agilent Technologies, Sta. Clara, CA), equipped with a flame ionisation detector (FID) and split ratio of 1:50 using a fused silica capillary column, HP5-MS (30 m × 250 µm i.d., 0.25 µm film thickness). The injector and detector temperature for each analysis were held at 220 and 280°C, respectively. The carrier gas was helium at a flow rate of 1 mL/min. The gas chromatography-mass spectrometry (GC-MS) was carried out on an Agilent model 5975 mass spectrometry (Agilent Technologies, Sta. Clara, CA) operating in the ionising energy mode at 70 eV, combined with the GC described earlier. The temperature of the column was programmed from 60 to 240°C at a rate of 4°C/min. The injector and ion source temperatures were the same as mentioned earlier. Scanning was performed from m/z 50 - 550 at 1.9 scan/s. The retention indices (RI) were determined in relation to homologous series of *n*-alkanes (C_8 - C_{22}) under similar operating conditions. Identification of the compounds was made by visual interpretation, comparing their retention indices and mass spectra with data published in the literature (Adams, 2007) and by matching their recorded mass spectra with reference spectra in the computer library (NIST MS library, Version 2.0). Quantification was computed as the percentage contribution of each compound to the total amount present.

Antioxidant activity

DPPH radical scavenging activity assay

The method of Braca *et al.* (2002) was used for the determination of the scavenging activity of DPPH free radical. α-tocopherol and BHT served as standards. The inhibition of the DPPH free radical in percent (I%) was calculated based on control reading, which contained equal volumes of DPPH solution and methanol without any test compound using Eq. 1:

$$\% \text{ inhibition} = [(A_C - A_S)/A_C] \times 100 \qquad \text{(Eq. 1)}$$

where, A_C = absorbance of control reaction, and A_S = absorbance of the sample. The essential oil concentration providing 50% inhibition (IC_{50}) was calculated from the graph of scavenging effect percentage

against essential oil concentration.

Determination of antioxidant combination Index (CI)

To evaluate a possible synergistic antioxidant activity between the essential oils tested, an isobologram analysis based on the median effect index IC50 was used (Bounimi and Chebli, 2017):

$$CI = (D)1/(Dx)1+(D)2/(Dx)2 \quad \text{(Eq. 2)}$$

where, (D)1 and (D)2 = IC_{50} values of two essential oils in combination; (Dx)1 and (Dx)2 = IC_{50} values of two active essential oils individually. The antioxidant interaction was interpreted based on CI values as follows: CI < 1: synergistic; CI = 1: additive; CI > 1: antagonistic.

Antifungal activity

Fungal material

The fungal pathogens *Fusarium* spp. used in this experiment were isolated from infected potato tubers that showed typical dry rot symptoms. As reported by Rapilly (1968), the identification of the fungi was performed according to the macro- and micro-morphological characteristics, as described by Toussoun and Nelson (1968), Booth (1971), Domsch *et al.* (1980), Nelson *et al.* (1983), and Pitt and Hocking (1999). The identification was confirmed at the Pasteur Institute in Algiers. Conidia were isolated from agar media using sterile 0.85% saline containing 0.1% Tween-80 (v/v), and the inoculum size was assessed using a chamber cell counting (haemocytometer) and adjusted to approximately 10^7 spores/mL for each strain.

In vitro experiment

Disk diffusion assay

The method of Rota *et al.* (2004) was followed with slight modifications. Sterile blank filter discs (6 mm diameter, Whatman Paper No 3) containing 3 µL of each pure essential oil were applied onto the surface of potato dextrose agar (Difco Laboratories), which was previously seeded by spreading 1 mL of overnight fresh inoculum suspension (10^7 spores/mL) of each fungal strain. All Petri dishes were stored in the dark at 4°C for one hour to enable diffusion of the essential oil and were incubated for up to 72 h at 25°C. Discs without oil were used as a control.

The antifungal activity was evaluated by measuring the diameters of the inhibition zones including the disc diameter (6 mm). Clear inhibition zones around the discs indicate the presence of antifungal activity. The values shown are the means of tests performed in triplicate.

Agar dilution method

The minimum inhibitory concentration (MIC) was assessed by the agar dilution method (Fandohan *et al.,* 2004) with slight modifications. The agar medium (potato dextrose agar) was supplemented with various concentrations of essential oils, ranging from (0.039 to 5 µL/mL), which were immediately mixed and poured into Petri dishes. Tween-20 (0.1%) was used to dissolve each essential oil in the medium. The plates were spot inoculated with 1 µL of strain suspension that contained 10^7 conidia/mL and were then incubated for up to 72 h at 25°C.

The MIC was determined as the lowest EO concentration showing complete growth inhibition of the tested fungal strains under a binocular microscope compared with the negative control (agar medium without essential oil).

Minimal fungicidal concentration (MFC) was assessed by re-inoculation of the treated inoculums onto sterile PDA. The lowest concentrations of the tested EOs giving no visible growth after re-inoculation were regarded as MFCs.

Effect of oregano and thyme oils by checkerboard assay

The antifungal effect of the combination of the two EOs against *Fusarium* spp. was evaluated using the checkerboard method, followed by the FIC index calculation as described by Turgis *et al.* (2012). Serial two-fold dilutions corresponding to the MIC, 1/2 MIC, 1/4 MIC, and 1/8 MIC of each essential oil were prepared, and all the oregano oil dilutions were then mixed with the same proportions of thyme oil. The MICs of the essential oils in combination were determined by the dilution method. The fractional inhibitory concentration index (FICI) was calculated using Eq. 3:

$$FICI = FICa + FICb \quad \text{(Eq. 3)}$$

where, FICa = MIC of EO_a in combination / MIC of EO_a alone, and FICb = MIC of EO_b in combination / MIC of EO_b alone. The results were interpreted according to the FIC indices as follows: FIC $\leq$ 0.5 synergy; $\leq$ 1.0 additive; $\leq$ 2.0 indifference; > 2.0 antagonistic. All experiments were performed in triplicate (Pillai *et al.,* 2005).

Spore germination assay

The antifungal activities of the plants were also evaluated by inhibition of spore germination using the slide technique (Paul *et al.,* 1993), with

some modifications. Each essential oil of oregano and thyme was dissolved with 0.1% Tween-20 to obtain 0.019, 0.039, 0.078 and 0.156 µL/mL concentrations of the oils. Aliquots of 25 µL of the essential oil solutions at different concentrations were mixed with 25 µL of 5% dextrose solution and 50 µL of the strain spore suspension (approximately 10^7 conidia/mL) in a cavity slide; these were then incubated in a moist chamber at 25°C for 24 h. After incubation, the slides were fixed in lactophenol cotton blue and examined under a light microscope to record the percentage of spore germination. The spores that generated germ tubes were enumerated, and percentage of spore germination was calculated in comparison with the control assay. A spore was considered germinated when the length of its germinal tube reached one-half of the spore diameter (Paul *et al.,* 1993). The germination rate was measured for approximately 100 spores per treatment. Each treatment was replicated three times, and the experiment was repeated twice. The control (0.01% Tween-20 without EOs) was tested separately for spore germination of the tested *Fusarium* spp.

In vivo experiment with wounded potato tubers

F. oxysporum was selected for the *in vivo* study based on frequency of isolation and the *in vitro* results. This assay was carried out following the method of Feng *et al.* (2011) with some modifications. Fresh unspoiled potato tubers were purchased from a local market in Algiers. The potatoes were chosen based on the absence of physical defects, their uniform size, and being devoid of injuries and infections. After disinfection in 2% sodium hypochlorite for 3 min, the potatoes were washed with autoclaved tap water, dried on sterile filter paper and were wounded with a sterile puncher to make one uniform 4 mm deep by 4 mm wide wound on their peel at the equatorial region. Aliquots of 20 µL of different dilutions of oregano and thyme EOs alone (prepared at the highest MIC value obtained against *F. oxysporum*) or in combination (prepared using a checkerboard process described above) were pipetted onto the wounds. After 1 h, 10 µL of conidial suspension (10^7 conidia/mL) of *F. oxysporum* was added into each wound. The tubers inoculated with the pathogens without essential oil were considered as control. The treated potatoes were stored at 25°C. Following 2 w of incubation, each tuber was cut through the wound sites using a clean, flame-sterilised knife, and the width and depth of the rotted tissue was measured. As reported by Peters *et al.* (2008), rots were assumed to be conical for the purposes of analysis. Therefore, the volume of each rot was calculated using Eq. 4:

$$\text{Volume} = \pi r^2 (h/3) \qquad \text{(Eq. 4)}$$

where, r = half the width of the rot, and h = depth of the rot. Each treatment was replicated three times with 20 tubers per replicate, and the entire experiment was repeated twice.

Statistical analysis

All experiments were carried out in triplicate. The data were expressed as means ± standard deviation (SD). Differences were evaluated by one-way analysis of variance (ANOVA) and Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Chemical composition of EOs

The essential oils of the aerial parts of thyme and oregano were obtained with a yield of 0.85 and 1.97% (w/w), respectively, based on the dry weight of the plants. The chemical constituents of the essential oils identified by GC and GC-MS are listed in Table 1. As can be seen, 37 and 41 components were identified in the EOs of thyme and oregano, representing 80.17% and 93.00% of total oil, respectively. The major components of thyme oil were thymol (46.97%), linalool (3.94%), *p*-cymene (3.32%), and carvacrol (3.20%). The oregano oil predominantly contained carvacrol (59.03%), *p*-cymene (12.32%), γ-terpinene (4.63%), thymol (2.42%), and ß-caryophyllene (2.21%). The analysis indicated that the thyme and oregano used in the present work belonged to the "thymol chemotype" and "carvacrol chemotype", respectively. Both essential oils were characterised by high percentage of monoterpene fractions, especially oxygenated monoterpenes, which was the most abundant compound class.

Antioxidant activity

The potential antioxidant activity of oregano and thyme essential oils and their mixture was determined based on the scavenging activity of the stable free radical DPPH. As shown in Table 2, thyme essential oil exhibited stronger antioxidant potential than oregano essential oil, and the oregano/thyme essential oil combination displayed a synergistic antioxidant interaction. The examined samples possessed stronger antioxidant effects than standard BHT.

Table 1. Composition of oregano and thyme essential oils as assessed using GC and GC-MS analyses.

Peak No.	[a]RT	[b]RI	[c]Compound	Relative content (%)	
				Oregano oil	Thyme oil
1	5.28	922	Tricyclene	n.d.	0.11
2	5.63	927	α-Thujene	0.64	0.58
3	5.89	934	α-Pinene	0.52	2.12
4	6.12	948	camphene	0.10	0.27
5	6.94	954	Verbenene	n.d.	0.29
6	7.06	973	Sabinene	0.12	n.d.
7	7.21	976	β-Pinene	0.19	1.18
8	7.30	982	1-Octen-3-ol	0.48	n.d.
9	7.58	987	Octanone-3	0.17	0.15
10	7.76	994	β-Myrcene	1.15	0.27
11	7.92	998	α-Phellandrene	0.18	0.12
12	8.03	1010	δ-3-Carene	0.11	n.d.
13	8.19	1020	α-Terpinene	2.51	1. 20
14	8.24	1026	*p*-Cymene	**12.32**	**3.32**
15	8.28	1029	Limonene	0.29	0.31
16	8.36	1032	β-Phellandrene	0.17	n.d.
17	8.83	1035	1,8-cineole	0.10	2.12
18	9.48	1047	Trans-β-Ocimene	0.41	0.17
19	10.20	1058	γ-Terpinene	**4.63**	0.81
20	10.86	1072	Cis-Sabinene-	0.26	1.01
21	10.93	1084	hydrate	n.d.	0.15
22	11.69	1087	Camphenilone	0.18	0.47
23	12.25	1093	Terpinolene	1.36	**3.94**
24	12.98	1100	Linalool	0.41	n.d.
25	13.28	1120	Sabinene hydrate	n.d.	0.08
26	13.70	1139	Campholenal	0.16	1.38
27	14.28	1158	Camphor	0.11	1.76
28	14.44	1167	Borneol	0.28	1.8
29	14.63	1178	Terpinen-4-ol	n.d.	1.20
30	14.88	1185	*p*-Cymen-8-ol	0.13	1.21
31	15.42	1223	α-Terpineol	0.21	0.16
32	15.70	1237	Thymol methyl-ether	0.11	n.d.
33	16.21	1265	Carvone	0.15	0.2
34	16.76	1290	Bornyl acetate	**2.42**	**46.97**
35	18.26	1300	Thymol	**59.03**	**3.20**
36	20.08	1356	Carvacrol	**2.21**	0.13
37	21.62	1393	β-Caryophyllene	0.10	n.d.
38	21.97	1441	α-Humulene	0.16	0.16
39	22.73	1498	Germacrene D	0.12	0.28
40	23.54	1510	α-Terpineol	0.07	0.14
41	24.17	1559	γ-Cadinene	0.15	n.d.
42	24.63	1562	δ-Cadinene	0.29	0.37
43	25.49	1579	α-Bisabolene	0.55	2.04
44	25.64	1586	Spathulenol	0.12	0.22
45	26.21	1637	Caryophyllene oxide	0.15	0.28
46	27.76	1656	Viridiflorol	0.11	n.d.
			β-Eudesmol		
			δ-Muurolene		
			γ-Cadinene		
Monoterpenes hydrocarbons				24.19%	12.38%
Oxygenated monoterpenes				64.13%	64.02%
Sesquiterpenes hydrocarbons				2.92%	0.71%
Oxygenated sesquiterpenes				1.11%	2.91%
Others				0.65%	0.15%
Total oils				**93.00%**	**80.17%**

[a]time retention; [b]retention index; [c]compounds are listed in order of their elution from an HP column using the homologous series of *n*-alkanes. n.d. = not detected.

Table 2. The *in vitro* antioxidant activities of oregano and thyme essential oils (individual and in combination) and BHT in DPPH radical scavenging test.

Sample	DPPH (IC_{50} in µg/mL)	CI
Oregano oil	745.90 ± 8.12	-
Thyme oil	616.90 ± 5.11	-
Oregano/thyme oils	305.00 ± 2.00	0.89
BHT	> 1	-

CI = antioxidant combination index.

In vitro antifungal efficacy

Isolated and identified fungi

Six species were identified, and their frequency varied from 4.38% to 38.75%. *F. oxysporum* was isolated from the greatest number of samples with a high frequency (38.75%), followed by *F. culmorum* (24.85%), *F. moniliforme* (16.75%), *F. equiseti* (13.75%), *F. avenaceum* (8.66%), and *F. solani* (4.38%). The relative density reached 33.33% for *F. oxysporum* and dropped to 5.64% for *F. solani.*

Single antifungal effect

The *in vitro* antifungal activity assessed by the disc diffusion assay was estimated by the diameter of the inhibition zone (mm). As shown in Table 3, both essential oils were shown to be effective against all *Fusarium* species, and the diameters of growth inhibition ranged from 23.77 ± 1.17 mm to 51.50 ± 0.65 mm for oregano essential oil, and from 20.43 ± 0.79 to 45.13 ± 2.13 mm for thyme essential oil. *F. moniliforme* was the most sensitive species, exhibiting the highest diameters of inhibition zone (51.50 ± 0.65 and 45.13 ± 2.13 mm for oregano and thyme essential oils, respectively), whereas *F. oxysporum* was the most resistant to the oils, showing a clear zone of inhibition. The efficiency of oregano and thyme essential oils estimated by the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) are shown in Table 3. Oregano essential oil showed an excellent inhibitory effect against all tested species, with MIC values ranging from 0.156 to 0.078 µL/mL. It could inhibit the development of four species (*F. culmorum*, *F. equiseti*, *F. avenaceum*, and *F. moniliforme*) at a MIC value of 0.078 µL/mL.

The essential oil of thyme had the same efficiency against different strains, but its MIC values were two-fold lower than those of oregano, indicating the stronger antifungal effect of oregano essential oil. Regarding the MFC values of the essential oils, the MFC values of the thyme essential oil were almost similar to their MIC values and were 2-fold higher in the case of oregano essential oil (Table 3). In fact, the MFC values of thyme and oregano essential oils ranged from 0.313 to 0.156 µL/mL. Thus, it can be concluded that both essential oils exhibited a fungicidal effect.

Table 3. Determination of antifungal activity, Fractional Inhibitory Concentration Index, and antifungal effect of the investigated essential oils against the tested *Fusarium* species.

Fusarium species	Oregano oil				Thyme oil				Combined oil	
	Θ inhibition zone (mm)		[b]MIC µL/mL	[c]MFC µL/mL	Θ inhibition zone (mm)		[b]MIC µL/mL	[c]MFC µL/mL	[d]FICI	[e]Effect
	[a]C	Treated			[a]C	Treated				
F. oxysporum	-	23.77 ± 1.17	0.156	0.313	-	20.43 ± 0.79	0.313	0.313	0.499	S
F. culmorum	-	35.43 ± 1.54	0.078	0.313	-	30.93 ± 1.13	0.156	0.313	0.500	S
F. solani	-	27.33 ± 0.69	0.313	0.313	-	24.53 ± 0.24	0.313	0.313	0.499	S
F. equiseti	-	38.80 ± 0.33	0.156	0.156	-	36.50 ± 0.30	0.156	0.156	0.500	S
F. avenaceum	-	33.67 ± 0.50	0.078	0.313	-	31.40 ± 0.37	0.156	0.313	0.375	S
F. moniliforme	-	51.50 ± 0.65	0.078	0.156	-	45.13 ± 2.13	0.156	0.156	0.375	S

Θ = diameter of the inhibition zone (mm); [a]control (the disc without oils); [b]Minimal Inhibitory Concentration; [c]Minimal Fungicidal Concentration; [d]Fractional Inhibitory Concentration Index; [e]synergistic effect; (-) = no inhibition zone. Values are means ± standard deviation (SD) of three experiments (n = 3).

Combined antifungal effect

Interactions between the EOs in combination were assessed by the checkerboard technique, which was used to investigate the effects of various combinations of thyme and oregano essential oil fractions. Combining these oils together caused a significant decrease in the MIC of each oil against the tested *Fusarium* species as compared to the individual MIC values. For example, the MIC of oregano essential oil alone against *F. oxysporum* was 0.156 µL/mL, but was reduced to 0.078 µL/mL in the presence of thyme essential oil. Similarly, the MIC of thyme essential oil alone was 0.313 µL/mL, but was reduced to 0.039 µL/mL when it was combined with oregano essential oil. The FIC index calculated from these results was approximately 0.5, which indicated a synergistic effect of the target essential oils in combination.

Regarding the other tested species, the results demonstrated that all four combinations generated by the checkerboard assay displayed FIC index values ranging from 0.375 to 0.5 (Table 3), thus indicating the synergistic effect of the essential oils.

Effect of oregano and thyme essential oils on spore germination

The results obtained for the essential oils of the spore germination assay for each of the test fungi are shown in Figure 1. The control (Tween-20, 0.1% v/v) did not inhibit the spore germination of any of the tested species. There was a significant inhibition of fungal spore germination at different essential oil concentrations, and the inhibitory effects were enhanced with increasing concentrations of each essential oil alone or in combination (Figure 1). A 100% inhibition of all tested fungal spore germinations was observed at 0.078 µL/mL of the combined essential oil.

In vivo antifungal efficacy against Fusarium oxysporum

The *in vivo* antifungal experiments were performed using the wounded potato tuber technique. Following incubation at 25°C, the tubers in the control group developed a rot volume equal to 1.711 ± 0.054 cm3. In the group treated with essential oils, a very significant reduction ($p < 0.05$) in the rot volume was found as compared to that of the control group (Figure 2).

In addition, an inverse relationship between the concentration of the essential oils and the rot volume was observed, indicating that growth inhibition due to the essential oils was dose-dependent against the target strain. In general, oregano essential oil was shown to be slightly more active than thyme essential oil. The effect of combining the essential oils was tested based on the checkerboard method. It was clearly shown that the same concentrations in combination had more inhibitory activity than the individual concentrations. The analysis of fractional inhibitory concentration obviously revealed a highly synergistic interaction against *F. oxysporum* (FIC index ≈ 0.5).

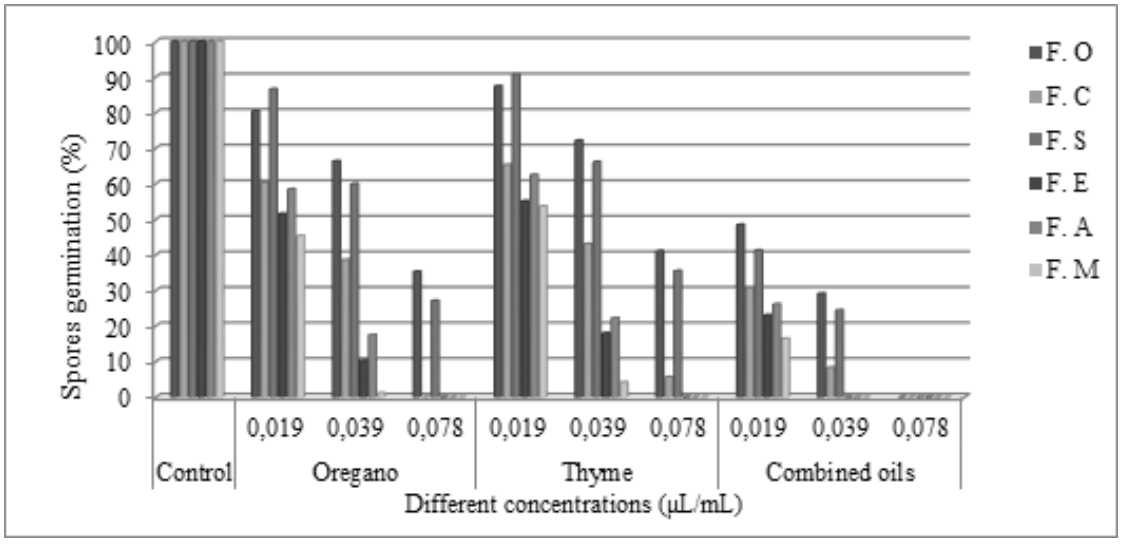

Figure 1. Effects of different concentrations of oregano and thyme essential oils alone and in combination on the inhibition of spore germination of targeted *Fusarium* species. FO = *F. oxysporum*; FC = *F. culmorum*; FS = *F. solani*; FE = *F. equiseti*; FA = *F. avenaceum*; FM = *F. moniliforme*.

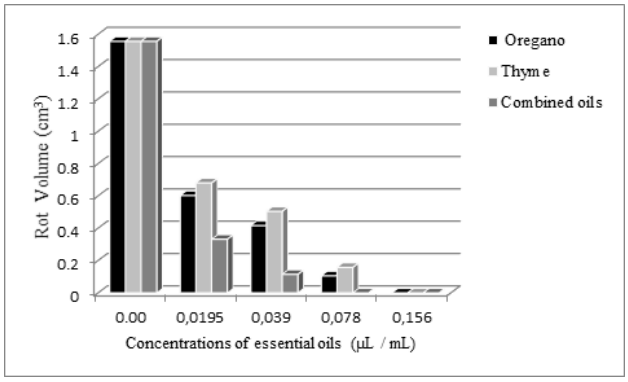

Figure 2. Antifungal effect of tested oils against *F. oxysporum* rot.

Discussion

Many reports have shown that essential oils derived from aromatic plants can be considered as non-phytotoxic, easily biodegradable, and have prominent biological activities (Dhifi *et al.,* 2016; Ayaz *et al.,* 2017). Our results demonstrated that the tested oils were characterised by a high percentage of monoterpene fractions, amounting to 88.52% and 76.40% in the thyme and oregano essential oils, respectively, with oxygenated monoterpenes (OMT) being the most important fraction. These results are in accordance with previous reports that have shown OMTs as predominant components with a similar chemotype (Moghtader, 2012; Miladi *et al.,* 2013; Stupar *et al.,* 2014). However, Nguefack *et al.* (2012) found that thyme essential oil contained almost as much monoterpene hydrocarbon as OMT, and thymol was not the primary component. Similarly, Khosravi *et al.* (2011) revealed that oregano belonged to the chemotype linalool, while Bharti *et al.* (2013) reported that the major compound of oregano essential oil collected from India was carvacrol (86.50%). The different essential oil compositions found in different studies can be due to physiological variation, genetic factors, and evolution, as well as to the time of harvest, species, season, environmental factors, the technique employed for plant drying, the part of plant studied and the oil extraction method (Lee and Ding, 2016; Tsasi *et al.,* 2017; Sampaio and Da Costa, 2018).

To evaluate the antioxidant activity, the DPPH radical scavenging assay was employed. This assay is widely used to measure the ability of compounds to act as free radical scavengers or hydrogen donors (Pyrzynska and Pękal, 2013; Han *et al.,* 2017). The results obtained in the present work are consistent with previous studies. Several studies have reported the high radical scavenging activity of essential oils extracted from *Origanum* and *Thymus* species (Gavaric *et al.,* 2015; Khadir *et al.,* 2016). The antioxidant activity detected in oregano and thyme essential oils may be due to their major compounds, carvacrol and thymol, respectively. These compounds can act alone or together with other minority compounds that could act synergistically (Gavaric *et al.,* 2015; Bounimi and Chebli, 2017). Our work was focused on the antifungal activity effect of oregano and thyme essential oils against *Fusarium* species. For this, six isolates known for their high ability to produce rot in potato tubers were selected. *F. oxysporum* was found to be the most isolated species; this result is in accordance with that obtained by Gachango *et al.* (2012). Nevertheless, Aydin and Inal (2018) observed that *F. coeruleum* was the more prevalent strain among the *Fusarium* species isolated from dry rot potato tubers.

Our results showed that both oils had high antifungal activity against all targeted species and that they were also effective in inhibiting spore germination of the tested species, with the highest efficacy displayed by a combination of the essential oils. Several previous reports have also found an antifungal activity of essential oils, including that of thyme and oregano, against different *Fusarium* species (Sumalan *et al.,* 2013; Stevic *et al.,* 2014; Gao *et al.,* 2016). The present work confirms previous findings regarding the antifungal activity of essential oils on spore germination (Costa *et al.,* 2015; López-Meneses *et al.,* 2015; Sharma *et al.,* 2017). However, the MIC and MFC results indicated that thyme essential oil was slightly less efficient in inhibiting mycelial growth as compared to oregano essential oil.

Based on GC and GC-MS analysis, the antifungal activity observed could be related to their high content of the OMT. These observations confirmed the strongest antimicrobial activity of OMT, especially carvacrol and thymol, as reported by several authors (Stevic *et al.,* 2014; Memar *et al.,* 2017).

Regarding the combined antifungal effects, the oregano and thyme essential oil combinations exhibited a synergistic effect against all the tested *Fusarium* species and caused a significant decrease in the MICs as compared to their individual MIC values. This could mainly be due to interactions between the major and/or minor components of the two combined essential oils. In the literature, an increase in the antifungal activity of combined essential oils has been explained by the combination of the major compounds, such as carvacrol and thymol, in a mixture of oregano and thyme essential oils

(Stevic *et al.,* 2014). Moreover, it has also been suggested that the antifungal activity of the main components may be modulated by the minor components, consequently contributing to the final interactive effect of the combination (Ouedrhiri *et al.,* 2017; Nikkhah *et al.,* 2017). Based on previous studies, it was supposed that the interaction between aldehydes, alcohols, esters, and phenolic components (thymol and carvacrol) and their precursors (*p*-cymene and γ-terpinene) leads to an increase in the inhibitory effect against *Fusarium* species. The increase of antifungal activity via the synergistic effect between different components of essential oils would be an advantage in pre- and post-harvest protection, because pathogens cannot easily acquire resistance to the multiple components contained in two or more essential oils (Stevic *et al.,* 2014).

Conclusion

The present work clearly demonstrated that oregano and thyme EOs in combination exhibited both synergistic antioxidant and antifungal activities. Thymol and carvacrol were the likely bioactive compounds responsible for the biological activities and the synergistic interaction observed. These findings suggest that the mixture of oregano and thyme EO at suitably low concentrations should find a practical application in the control of *Fusarium* dry rot in potatoes. Furthermore, these natural biological compounds may successfully replace synthetic and chemical antimicrobial and antioxidant agents and provide an alternative method of preservation.

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References

Adams, R. P. 2007. Identification of essential oil components by gas chromatography/mass spectrometry. 4th ed. United States: Allured Publishing Corporation.

Al-Mughrabi, K. I. 2010. Biological control of *Fusarium* dry rot and other potato diseases using *Pseudomonas fluorescens* and *Enterobacter cloacae*. Biological Control 53(3): 280-284.

Ayaz, M., Sadiq, A., Junaid, M., Ullah, F., Subhan, F. and Ahmed, J. 2017. Neuroprotective and anti-aging potentials of essential oils from aromatic and medicinal plants. Frontiers in Aging Neuroscience 9: article ID 168.

Aydin, M. H. and Inal, B. 2018. Comparative susceptibility of some commercial potato cultivars to *Fusarium sambucinum* and *F. solani* isolates causing tubers dry rot. Applied Ecology and Environmental Research 16(4): 4879-4892.

Aziz M. and Karboune S. 2018. Natural antimicrobial/antioxidant agents in meat and poultry products as well as fruits and vegetables: a review. Critical Reviews in Food Science and Nutrition 58(3): 486-511.

Becher, R., Hettwer, U., Karlovsky, P., Deising, H. B. and Wirsel, S. G. 2010. Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. Phytopathology 100(5): 444-453.

Bharti, V., Vasudeva, N. and Duhan, J. S. 2013. Bacteriostatic and fungistatic activities of *Origanum vulgare* extract and volatile oil and interaction studies in combination with antibiotics and antifungal agents against food poisoning pathogens. International Food Research Journal 20(3): 1453-1458.

Booth, C. 1971. The genus *Fusarium*. United Kingdom: Commonwealth Mycological Institute.

Bounimi, S. and Chebli, B. 2017. Synergistic antioxidant activity of three essential oils of Lamiacea family from Morocco. Applied Journal of Environmental Engineering Science 3(2): 195-200.

Braca, A., Sortino, C., Politi, M., Morelli, I. and Mendez, J. 2002. Antioxidant activity of flavonoids from *Licania licaniaeflora*. Journal of Ethnopharmacology 79(3): 379-381.

Chowdhary, A., Kathuria, S., Xu, J. and Meis, J. F. 2013. Emergence of azole-resistant *Aspergillus fumigatus* strains due to agricultural azole use creates an increasing threat to human health. PLoS Pathogens 9(10): article ID e1003633.

Costa, L. C. B., Pinto, J. E. B. P., Bertolucci, S. K. V., Costa, J. C. B., Alves, P. B. and Niculau, E. S. 2015. *In vitro* antifungal activity of *Ocimum selloi* essential oil and methylchavicol against phytopathogenic fungi. Revista Ciencia Agronomica 46(2): 428-435.

Dhifi, W., Bellili, S., Jazi, S., Bahloul, N. and Mnif, W. 2016. Essential oils: chemical characterization and investigation of some biological activities: a critical review. Medicines 3(4): article ID 25.

Domsch, K. H., Gams, W. and Anderson, T. H. 1980. Compendium of soil fungi. London: Academic Press.

Fandohan, P., Gbenou, J. D., Gnonlonfin, B., Hell, K., Marasas, W. F. and Wingfield, M. J. 2004.

Effect of essential oils in the growth of *Fusarium verticillioides* and fumonisin contamination in corn. Journal of Agricultural and Food Chemistry 52(22): 6824-6829.

Feng, W., Chen, J., Zheng, X. and Liu, Q. 2011. Thyme oil to control *Alternaria alternata in vitro* and *in vivo* as fumigant and contact treatments. Food Control 22(1): 78-81.

Ferdes, M. and Ungureanu, C. 2012. Antimicrobial activity of essential oils against four food-borne fungal strains. UPB Scientific Bulletin, Series B: Chemistry and Materials Science 74(2): 87-98.

Gachango, E., Hanson, L. E., Rojas, A., Hao, J. J. and Kirk, W. W. 2012. *Fusarium spp*. causing dry rot of seed potato tubers in Michigan and their sensitivity to fungicides. Plant Disease 96: 1767-1774.

Gao, T., Zhou, H., Zhou, W., Hu, L., Chen, J. and Shi, Z. 2016. The fungicidal activity of thymol against *Fusarium graminearum* via inducing lipid peroxidation and disrupting ergosterol biosynthesis. Molecules 21(6): article ID 770.

Garcia, M. V. and Copetti, M. V. 2019. Alternative methods for mould spoilage control in bread and bakery products. International Food Research Journal 26(3): 737-749.

Gavaric, N., Mozina, S. S., Kladar, N. and Bozin, B. 2015. Chemical profile, antioxidant and antibacterial activity of thyme and oregano essential oils, thymol and carvacrol and their possible synergism. Journal of Essential Oil-Bearing Plants 18(4): 1013-1021.

Han, F., Ma, G.-G., Yang, M., Yan, L., Xiong, W., Shu, J.-C., … and Xu, H.L. 2017. Chemical composition and antioxidant activities of essential oils from different parts of the oregano. Journal of Zhejiang University Science B 18(1): 79-84.

Hoppin, J. A., Umbach, D. M., Long, S., London, S. J., Henneberger, P. K., Blair, A., … and Sandler, D. P. 2017. Pesticides are associated with allergic and non-allergic wheeze among male farmers. Environmental Health Perspectives 125(4): 535-543.

Ishaq, A., Syed, Q. A., Khan, M. I. and Zia, M. A. 2019. Characterising and optimising antioxidant and antimicrobial properties of clove extracts against food-borne pathogenic bacteria. International Food Research Journal 26(4): 1165-1172.

Khadir, A., Sobeh, M., Gad, H. A., Benbelaid, F., Bendahou, M., Peixoto, H., … and Wink, M. 2016. Chemical composition and biological activity of the essential oil from *Thymus lanceolatus*. Zeitschrift fur Naturforschung C, Journal of Biosciences 71(5-6): 155-63.

Khosravi, A. R., Shokri, H., Kermani, S., Dakhili, M., Madani, M. and Parsa, S. 2011. Antifungal properties of *Artemisia sieberi* and *Origanum vulgare* essential oils against *Candida glabrata* isolates obtained from patients with vulvovaginal candidiasis. Journal de Mycologie Médicale 21(2): 93-99.

Kocić-Tanackov, S. D. and Dimić, G. R. 2013. Antifungal activity of essential oils in the control of food-borne fungi growth and mycotoxin biosynthesis in food. In Méndez-Vilas, A. (ed). Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education. Spain: Formatex Research Centre.

Lee, Y. L. and Ding, P. 2016. Production of essential oil in plants: ontogeny, secretion structures and seasonal variations. Pertanika Journal of Scholarly Research Reviews 2(1): 1-10.

López-Meneses, A. K., Plascencia-Jatomea, M., Lizardi-Mendoza, J., Rosas-Burgos, E. C., Luque-Alcaraz, A. G. and Cortez-Rocha, M. O. 2015. Antifungal and antimycotoxigenic activity of essential oils from *Eucalyptus globulus, Thymus capitatus* and *Schinus molle*. Food Science and Technology 35(4): 664-671.

Marin, S., Ramos, A. J., Cano-Sancho, G. and Sanchis, V. 2013. Mycotoxins: occurrence, toxicology, and exposure assessment. Food and Chemical Toxicology 60: 218-237.

Memar, M. Y., Raei, P., Alizadeh, N., Akbari Aghdam, M. and Kafil, H. S. 2017. Carvacrol and thymol: strong antimicrobial agents against resistant isolates. Reviews in Medical Microbiology 28(2): 63-68.

Mie, A., Rudén, C. and Grandjean, P. 2018. Safety of safety evaluation of pesticides: developmental neurotoxicity of chlorpyrifos and chlorpyrifos-methyl. Environmental Health 17: article ID 77.

Miladi, H., Slama, R., Mil, D., Zouari, S., Bakhrouf, A. and Ammar, E. 2013. Essential oil of *Thymus vulgaris* L. and *Rosmarinus officinalis* L.: gas chromatography-mass spectrometry analysis, cytotoxicity and antioxidant properties and antibacterial activities against foodborne pathogens. Natural Science 5(6): 729-739.

Moghtader, M. 2012. Antifungal effects of the essential oil from *Thymus vulgaris* L. and comparison with synthetic thymol on *Aspergillus niger*. Journal of Yeast and Fungal Research 3(6): 83-88.

Nazzaro, F., Fratianni, F., Coppola, R. and Feo, V. 2017. Essential oils and antifungal activity. Pharmaceuticals 10(4): article ID 86.

Nelson, P. E., Tousson, T. A. and Marasas, W. F. O. 1983. *Fusarium* species. An illustrated manual for identification. United States: Pennsylvania State University Press.

Nguefack, J., Tamgue, O., Lekagne Dongmo, J. B., Dakole, C. D., Leth, V., Vismer, H. F., ... and Nkengfack, A. E. 2012. Synergistic action between fractions of essential oils from *Cymbopogon citratus*, *Ocimum gratissimum* and *Thymus vulgaris* against *Penicillium expansum*. Food Control 23(2): 377-383.

Nikkhah, M., Hashemi, M., Habibi Najafi, M. B. and Farhoosh, R. 2017. Synergistic effects of some essential oils against fungal spoilage on pear fruit. International Journal of Food Microbiology 257: 285-294.

Öğretmen, F. and İnanan, B. E. 2014. Effect of butylated hydroxytoluene (BHT) on the cryopreservation of common carp (*Cyprinus carpio*) spermatozoa. Animal Reproduction Science 151(3-4): 269-274.

Ouedrhiri, W., Mounyr, B., Harki, E. H., Moja, S. and Greche, H. 2017. Synergistic antimicrobial activity of two binary combinations of marjoram, lavender, and wild thyme essential oils. International Journal of Food Properties 20(12): 3149-3158.

Paul, G. C., Kent, C. A. and Thomas, C. R. 1993. Viability testing and characterization of germination of fungal spores by automatic image analysis. Biotechnology and Bioengineering 42(1): 11- 23.

Perczak, A., Gwiazdowska, D., Marchwińska, K., Juś, K., Gwiazdowski, R. and Waśkiewicz, A. 2019. Antifungal activity of selected essential oils against *Fusarium culmorum* and *F. graminearum* and their secondary metabolites in wheat seeds. Archives of Microbiology 201(8): 1085-1097.

Periasamy, V. S., Athinarayanan, J. and Alshatwi, A. A. 2016. Anticancer activity of an ultrasonic nanoemulsion formulation of *Nigella sativa* L. essential oil on human breast cancer cells. Ultrasonics Sonochemistry 31: 449-455.

Peters, J. C., Lees, A. K., Cullen, D. W., Sullivan, L., Strout, G. P. and Cunnington, A. C. 2008. Characterization of *Fusarium* spp. responsible for causing dry rot of potato in Great Britain. Plant Pathology 57(2): 262-271.

Pillai, S. K., Moellering, R. C. and Eliopoulos, G. M. 2005. Antimicrobial combinations. In Lorian, V. (ed). Antibiotics in Laboratory Medicine (5th ed), p. 365-440. United States: Lippincott Williams and Wilkins.

Pitt, J. I. and Hocking, A. D. 1999. Fungi and feed spoilage. 2nd ed. Gaithersburg: Aspen Publication.

Prakash, B., Kedia, A., Mishra, P. K. and Dubey, N. K. 2015. Plant essential oils as food preservatives to control moulds, mycotoxin contamination and oxidative deterioration of agri-food commodities - potentials and challenges. Food Control 47: 381–391.

Prasanth Reddy, V., Ravi Vital, K., Varsha, P. V. and Satyam, S. 2014. Review on *Thymus vulgaris*: traditional uses and pharmacological properties. Medicinal and Aromatic Plants 3(3): article ID 164.

Pyrzynska, K. and Pękal, A. 2013. Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples. Analytical Methods 5(17): 4288-4295.

Rapilly, F. 1968. Les techniques de mycologie en pathologie végétale. France: Institut National de la Recherche Agronomique.

Rota, M. C., Carramiñana, J.J., Burillo, J. and Herrera, A. 2004. *In vitro* antimicrobial activity of essential oils from aromatic plants against selected foodborne pathogens. Journal of Food Protection 67(6): 1252-1256.

Sampaio, B. L. and Da Costa, F. B. 2018. Influence of abiotic environmental factors on the main constituents of the volatile oils of *Tithonia diversifolia*. Revista Brasileira de Farmacognosia 28(2): 135-144.

Sharma, A., Rajendran, S., Srivastava, A., Sharma, S. and Kundu, B. 2017. Antifungal activities of selected essential oils against *Fusarium oxysporum* f. sp. *lycopersici* 1322, with emphasis on *Syzygium aromaticum* essential oil. Journal of Bioscience and Bioengineering 123(3): 308-313.

Stevic, T., Beric, T., Savikin, K., Sokovic, M., Godevac, D., Dinkic, I. and Stankovic, S. 2014. Antifungal activity of selected essential oil against fungi isolated from medicinal plant. Industrial Crops and Products 55: 116-122.

Stupar, M., Grbić, M. L., Džamić, A., Unković, N., Ristić, M., Jelikić, A. and Vukojević J. 2014. Antifungal activity of selected essential oils and biocide benzalkonium chloride against the fungi isolated from cultural heritage objects. South African Journal of Botany 93: 118-124.

Sumalan, R. M., Alexa, E. and Poiana, M.-A. 2013. Assessment of inhibitory potential of essential oils on natural mycoflora and *Fusarium* mycotoxins production in wheat. Chemistry Central Journal 7: article ID 32.

Teixeira, B., Marques, A., Ramos, C., Serrano, C., Matos, O., Neng, N. R., ... and Nunes, M. L. 2013. Chemical composition and bioactivity of different oregano (*Origanum vulgare*) extracts and essential oil. Journal of the Science of Food and Agriculture 93(11): 2707-2714.

Teixeira, T. S., Vale, R. C., Almeida, R. R., Ferreira, T. P. S. and Guimarães, L. G. L. 2017. Antioxidant potential and its correlation with the contents of phenolic compounds and flavonoids of methanolic extracts from different medicinal plants. Revista Virtual de Quimica 9(4): 1546-1559.

Toussoun, T. A. and Nelson, P. E. 1968. A pictorial guide to the identification of *Fusarium* species according to the taxonomic system of Snyder and Hansen. United States: Pennsylvania State University Press.

Tsasi, G., Mailis, T., Daskalaki, A., Sakadani, E., Razis, P., Samaras, Y. and Skaltsa, H. 2017. The effect of harvesting on the composition of essential oils from five varieties of *Ocimum basilicum* L. cultivated in the island of Kefalonia, Greece. Plants 6(3): article ID 41.

Turgis, M., Vu, K. D., Dupont, C. and Lacroix, M. 2012. Combined antimicrobial effect of essential oils and bacteriocins against foodborne pathogens and food spoilage bacteria. Food Research International 48(2): 696-702.

Yen, H. F., Hsieh, C. T., Hsieh, T. J., Chang, F. R. and Wang, C. K. 2015. *In vitro* anti-diabetic effect and chemical component analysis of 29 essential oils products. Journal of Food and Drug Analysis 23(1): 124-129.