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Chemical components and antibacterial activities of essential oil of wild, *in vitro* and acclimatised plants of *Ziziphora tenuior* L.

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Abstract

Ziziphora tenuior is widely used in traditional medicine for the treatment of various diseases. Therefore, medicinal plants such as this require continuous investigation to obtain the beneficial compounds they might possess. The essential oils of wild, *in vitro* and acclimatised *Z. tenuior* were identified by GC/MS analysis. The compounds in the essential oils were determined by co-injection of the sample with a solution containing the homologous series as standard of C9 to C22 *n*-alkanes. The activity of the essential oils was evaluated against five bacterial strains by disc diffusion, and the MIC was determined based on a micro-well plate (96 well) dilution method. To obtain the MBC, 10 µL was taken from each well and inoculated on Müller-Hinton agar. Seventeen volatile compounds were determined, the main one being pulegone (45.02%-46.43%), followed by bicyclo[3.1.1]hept-3-en-2-one,4,6,6-trimethyl- (12.96%-14.68%) and bicyclo[2.2.1]heptan-2-ol,1,5,5-trimethyl- (11.62%-13.18%). Two compounds; copaene and β-cubebene, were only isolated from the essential oil of *in vitro* plants. The chemical compositions and their percentages differed based on the source of plants. The results obtained demonstrated the influence of plant tissue culture media on the resulting essential oils. The essential oil of all plants showed various antimicrobial activities against *Escherichia coli, Klebsiella pneumoniae, Salmonella Typhi* and *Staphylococcus aureus* with diameters of inhibition zone ranging between 8 and 42 mm, while none was observed on *Pseudomonas aeruginosa*. The largest inhibition zone (42 mm) was observed from the essential oil of *in vitro* plants (100%) against *Staph. aureus.* The MIC ranged between 0.25 to 2 while MBC between 0.5 to 8 µg/mL. It seems that plant tissue culture will be a useful method to modify the chemical composition and their concentration of *Z. tenuior* essential oil, like pulegone and *n*-hexadecanoic acid. The different chemical compositions are likely to be the result of genetic differences and/or the effect of some plant growth regulators.

Introduction

Since ancient times, plants have been the sources of medicinal compounds which played a dominant role in the maintenance of humans' health (Kirbag *et al.*, 2009). Many plant species among the flora of Syria play an important role in traditional medicine. *Ziziphora* spp. (Lamiaceae) are among the plants commonly used in folk medicine in the Kalamoon Mountains areas of Syria for cough, stomach ache and dysentery. *Ziziphora* spp. are also used as aperitif, carminative and antiseptic treatments of various diseases (Ozturk and Ercisli, 2007). One of the species, *Z. tenuior*, has been used to treat fever and dysentery (Talebi *et al.*, 2012), diarrhoea, gut inflammation, cough (Safa *et al.*, 2012), expectorant, bladder stone and painful menstruation (Naghibi *et al.*, 2005), while *Z. clinopodioides* is widely used in Iranian traditional medicine for the treatment of common cold, gastrointestinal disorders and inflammations (Naghibi *et al.*, 2005). Sarac and Ugur have isolated the essential oils from some species of Lamiaceae, and shown that *Z. tenuior* was effective against bacteria and fungi (Sarac and Ugur, 2009). In another study, the *Z. tenuior* extract has shown antibacterial effect against *Klebsiella pneumoniae* and *Staphylococcus aureus*, and could be used as food preservative and human health maintenance (Ghasemi Pirbalouti, 2012; Mahboubi *et al.*, 2012). The chemical compounds in the essential oil of *Z. tenuior* were identified by Mahboubi *et al.* (2012), and the main components are α-terpineole (16.2%),

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thymol (10.4%), *p*-cymene (6.1%), geranylacetate (5.2%), 1,8-cineole (5.1%), geraniol (4.4%), linalool (4.1%) and carvacrol (2.5%). The antimicrobial effects of *Z. tenuior* extracts were also evaluated on *Escherichia coli* PTCC 1330 and *Staph. aureus* PTCC 1337 which revealed that the methanolic extracts were quite effective at 2,000 µg/mL (Yazdi *et al.*, 2013). *Z. tenuior* is also a good source of pulegone (71.2 to 85.3%) which has been widely used in food and drug industries. Pirbalouti (2013) also showed that pulegone, limonene and thymol were among the main constituents of *Z. tenuior* essential oil and that the chemical composition would differ based on the environmental conditions on which the species is planted. Delnavazi et al (2014) assessed four species; *Z. tenuior*, *Scutellaria orientalis, Eremostachys laciniata* and *Phlomis herba-venti* using GC-MS, and revealed that the plants' essential oil contained 1,8-cineol (19.6%), germacrene D (16.5%), and linalool (10.2%) (Delnavazi *et al.*, 2014). In another study, Ganjali *et al.* (2014) evaluated the effect of vermicompost fertiliser on the chemical composition quality and quantity of *Z. tenuior* essential oil, and they noticed the increase of pulegone from 69.9% (pre-fertilisation) to 81.3% (post-fertilization), and limonene from 4.8% to 6.7%. However, cyclohexane decreased from 4.1% to 1.3%. Ganjali and Harati (2014) also studied the effect of warm and dry climate (weather conditions of Kahnooj, Iran) on the quality of *Z. tenuior* essential oil compounds, and found pulegone (82.6%) and limonene (6.8%).

Nowadays, researchers often turn to folk medicines all the while looking for new leads to develop better drugs against microbial infections (Parekh and Chanda, 2007). Many folk medicines have been scientifically corroborated based on their traditional uses (Anyinam, 1995). Nevertheless, there is a continuous and urgent need to discover more new sources of medicinal plants to obtain beneficial compounds with health inducing and/or maintaining properties. Therefore, the aims of the present work were to determine the chemical components of *Z. tenuior* essential oils obtained from different sources (wild, *in vitro* and acclimatised), and compare these components' antibacterial activity against selected foodborne pathogens.

Material and methods

Plant materials and extraction of essential oil

Wild samples of *Z. tenuior* were collected from Assal Al-ward in the Kalamoon Mountains, Syria. The plant materials were authenticated by Dr. Imad Alkadi, an expert on plant diversity at Damascus University. The voucher specimens of the plants were deposited in the Department of Plant Biology, Faculty of Sciences. The *in vitro* cultured plants previously prepared (Dakah *et al.*, 2014) and the acclimatised ones were collected from the plant tissue culture laboratory in the Department of Plant Biology, Faculty of Science. The wild plant materials were washed with clean water to remove soil, debris and impurities. The plant parts were air-dried and grounded into powdered form. For the production of oil, air-dried plant materials (100 g) were hydrodistilled for 3 h using a Clevenger apparatus. The oil was dried over anhydrous Na_2SO_4 and kept in a sealed vial at 4°C.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was performed using a Variane 3400 equipped with a HB-5MS column (30 × 0.25 mm internal diameter, film thickness 0.25 µm, and with helium as a carrier gas) with analytic conditions: the injector and detector temperatures were held at 260°C. The oven temperature was first set to 60°C for 4 min, and increased to 200°C for 8 min. The final temperature was 260°C and held for 7.5 min. The SFE samples (1 µL) were injected using split mode with a split ratio of 1:40, and the carrier gas was helium with split flow of 1mL/min. The ionisation energy was 69.922 eV with a scan time of 1 sec and mass range of 35-450 amu.

Identification of components

The retention indices (RI) for all the compounds of essential oils were determined by co-injection of the sample with a solution containing the homologous series as standard of C9 to C22 *n*-alkanes. The identification of components was based on the comparison of their mass spectra with those found in the literature, and using the NIST 2.1.0 and WILEY mass spectral database.

Test bacterial strains

The test bacterial strains were obtained from the Atomic Energy Commission in Syria (AECS) which included Gram-positive (*Staph. aureus*) and Gram-negative (*S.* Typhi*, E. coli, K. pneumoniae, P. aeruginosa*.

Preparation of microbial suspension

A 24 h culture of each bacterium was prepared in suspension prior to the tests. To prepare the bacterial suspensions, the bacterial culture stocks were inoculated onto fresh Nutrient Agar and incubated for 24 h at 37°C. Following incubation, the bacterial

colonies were flooded with 1 mL physiologic liquid (NaCl 9 g/L) and the density of each test bacterial suspension was adjusted to 0.5 McFarland standard (≈10^8 CFU/mL).

Antibacterial assay

Disc diffusion

The antibacterial activity of *Z. tenuior* essential oils were determined by the disc diffusion method. Firstly, the Müller-Hinton agar was spread with bacterial suspension (300 µL). Sterile blank discs (6 mm diameter) were impregnated with 10 µL of essential oil at three concentrations (25, 50, 100%). Next, the impregnated discs were applied to the bacterial surface with equal distance from each other. The inoculated plates were incubated at 37°C for 24 h, and the results of antibacterial activity were recorded by measuring the diameter (mm) of inhibition zone surrounding the discs. All experiments were repeated three times.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of *Z. tenuior* essential oils against bacterial strains was determined based on a micro-well plate (96 well) dilution method (Swanson *et al.*, 1992; Shahla, 2012). The plates were prepared by distributing into each well 95 µL Müller-Hinton broth and 5 µL bacterial suspension. Next, 100 µL essential oil (8 µg/mL) was added into the first well. Then, serial dilutions were prepared by transferring 100 µL from the first well to the next. The final well contained 100 µL Müller-Hinton broth without oil, and served as negative control. The plates were then placed in a shaker at 300 rpm, and incubated at 37°C for 24 h. Microbial growth was determined by absorbance at 600 nm. The lowest concentration inhibiting growth was regarded as the MIC of the essential oil (Sahin, 2003). To confirm MIC and to find MBC, 10 µL was taken from each well and inoculated on Müller-Hinton agar. The results were taken depending on the presence or absence of bacterial growth. The minimum concentration preventing visible growth of the bacteria is taken as the MBC (Cosentino *et al.*, 1999). Each bacterial strain was tested in triplicates.

Results

Components of essential oils

The chemical compositions of essential oils of *Z. tenuior* which were identified by GC/MS analysis are presented in Table 1. In total, 17 volatile compounds were identified with pulegone being the predominant, followed by bicyclo[3.1.1]hept-3-en-2-one,4,6,6-trimethyl- and bicyclo[2.2.1]heptan-2-ol,1,5,5-trimethyl-. The chemical compositions and their percentages differed based on their different sources. The results revealed the influence of plant tissue culture media on the resulting essential oils.

Table 1. Chemical compositions of essential oils of wild, *in vitro* and acclimatised plants of *Ziziphora tenuior*

Compounds	%			
	RI	Wild	Acclimatised	In vitro
Bicyclo[2.2.1]heptan-2-ol,1,5,5-trimethyl-	1150	13.18	13.10	11.62
Menthone	1156	1.99	1.99	2.56
4-isopropyl-3-methoxymethylene-1,1-dimethyl-cyclohexane	1162	0.96	0.97	-
Isomenthol	1166	3.79	3.77	3.93
1,3,4-trimethyl-3-cyclohexenyl-1-carboxaldehyde	1178	1.94	1.89	1.56
Cyclohexanol,1-methyl-4-(1-methylethyl)-	1191	1.16	1.12	1.09
5-isopropenyl-2-methylcyclopent-1-enecarboxaldehyde	1212	1.07	1.06	-
3,7,7-trimethyl-1-penta-1,3-dienyl-2-oxabicyclo[3.2.0]hept-3-ene	1220	3.58	3.56	2.68
Pulegone	1245	45.02	45.27	46.43
3-cyclohexen-1-one,2-isopropyl-5-methyl-	1257	2.39	2.38	1.99
2-acetyl-4-methyl-1,3-cyclopentanedione	1267	2.46	2.45	1.91
Isomenthyl acetate	1297	1.72	1.72	1.22
Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	1345	14.60	14.68	12.96
Copaene	1378	-	-	0.97
β-caryophyllene	1422	1.50	1.51	2.71
β-cubebene	1484	-	-	0.97
Caryophyllene oxide	1587	1.83	1.86	1.72
Hexahydrofarnesyl acetone	1846	0.98	0.96	1.47
n-hexadecanoic acid	1965	1.83	1.71	4.22

Table 2. Antibacterial activities of essential oils of wild, *in vitro* and acclimatised plants of *Ziziphora tenuior*

Tested bacteria	Source of plants, concentration of essential oils (%) and diameter of inhibition zone (mm)								
	25%			50%			100%		
	Wild	In vitro	Acclimatised	Wild	In vitro	Acclimatised	Wild	In vitro	Acclimatised
Staphylococcus aureus	9.0 ± 1.1	30.1 ± 0.6	15.4 ± 0.7	14.0 ± 0.5	36.4 ± 0.9	18.3 ± 0.8	15.6 ± 0.3	42.0 ± 0.5	18.3 ± 0.8
Salmonella Typhi	9.6 ± 0.6	10.8 ± 0.7	8.9 ± 0.4	12.6 ± 0.8	16.0 ± 0.0	12.4 ± 0.3	17.0 ± 0.5	18.3 ± 0.8	14.6 ± 0.6
Escherichia coli	11.0 ± 1.0	19.6 ± 0.8	14.9 ± 0.6	16.7 ± 0.9	21.0 ± 0.5	17.6 ± 0.6	18.6 ± 0.8	21.4 ± 0.8	20.3 ± 0.3
Klebsiella pneumoniae	12.0 ± 0.5	13.3 ± 1.2	8.0 ± 0.0	14.8 ± 0.3	17.6 ± 1.2	9.7 ± 0.6	14.5 ± 1.2	19.2 ± 0.6	10.6 ± 0.8
Pseudomonas aeruginosa	na	na	na	na	na	na	na	na	na

na: not active. Data are mean ± standard deviation.

We noticed an increase in pulegone from 45.02 to 46.43%, and *n*-hexadecanoic acid from 1.83 to 4.22%. However, bicyclo[2.2.1]heptan-2-ol,1,5,5-trimethyl- decreased from 13.18 to 11.62%. Two compounds were absent from the essential oil of *in vitro* plants: 4-isopropyl-3-methoxymethylene-1,1-dimethyl-cyclohexane and 5-isopropenyl-2-methylcyclopent-1-enecarboxaldehyde. Two other compounds were absent from wild and acclimatised plants; copaene and β-cubebene.

Antibacterial activity

The essential oil of all plant sources showed various antimicrobial activities against the tested bacteria with diameters of inhibition zone ranging between 8 and 42 mm (Table 2). The largest zone of inhibition (42 mm) was observed from essential oil (100%) of *Z. tenuior* against *Staph. aureus.* It can also be seen that the antimicrobial activity of essential oil of *in vitro Z. tenuior* was high as compared to wild and acclimatised plants. It is clear that there was a difference in effect according to the species of bacteria, where the essential oil (100%) of *Z. tenuior* was active against *Staph. aureus* (42 mm) more than E. coli (21.4 mm), K. pneumoniae (19.2 mm) and S. Typhi (18.3 mm). Also, the antibacterial activity of essential oil (100%) of acclimatised *Z. tenuior* against *Staph. aureus* (18.3 mm) and E. coli (20.3 mm) were better than wild *Z. tenuior*. On the contrary, the antibacterial activity of essential oil of wild *Z. tenuior* against *S.* Typhi (17 mm) and *K. pneumoniae* (14.5 mm) were superior then acclimatised *Z. tenuior*. Similar trend was also observed in 25 and 50% concentration treatments. Figure 1 shows the antibacterial activity of essential oil at 100% concentration.

Determination of MIC

As shown in Table 3. *P. aeruginosa* exhibited resistance to essential oil of all plant sources and concentrations tested in the present work. *Staph aureus* was the most sensitive strain to essential oil of *in vitro Z. tenuior* (MIC = 0.25 µg/mL). The second sensitive bacterium was *E. coli* (MIC = 0.5 µg/mL) followed by *S.* Typhi and *K. pneumoniae* (MIC = 1 µg/mL). We also noticed that *in vitro Z. tenuior* was more effective than wild and acclimatised *Z. tenuior*. At 1 µg/mL concentration, the essential oil of wild and acclimatised *Z. tenuior* completely inhibited all bacteria except for *S.* Typhi and *K. pneumoniae*. At 2, 4 and 8 µg/mL concentrations of all plant sources, all bacterial strains were inhibited.

Determination of MBC

The results from Table 3 show that the essential oil of all plant sources killed the tested bacteria (99.9%) except for *P. aeruginosa*. The MBC values varied among the tested bacteria and depended on the source of plants. In general, the essential oils from *in vitro Z. tenuior* were more effective than wild and acclimatised *Z. tenuior*. For *in vitro Z. tenuior*, the MBC was 0.5 µg/mL for *Staph. aureus*, 1 µg/mL for *E. coli* and *K. pneumoniae*, 2 µg/mL for *S.* Typhi. At 8 µg/mL, all tested bacteria were killed except for *P. aeruginosa*. *S.* Typhi and *K. pneumoniae* managed to survive at 4 µg/mL of acclimatised *Z. tenuior* essential oils, while did not grow when other sources of *Z. tenuior* essential oils were used.

Discussion

Components of essential oils

Pulegone was the main compound isolated from essential oils of *Z. tenuior*. This result agrees with many other studies that showed *Ziziphora* spp. (*Z.*

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of essential oils of wild, *in vitro* and acclimatised plants of *Ziziphora tenuior*

Tested bacteria	Source of plants	MIC µg/mL							MBC µg/mL						
		0	0.25	0.5	1	2	4	8	0	0.25	0.5	1	2	4	8
Staphylococcus aureus	Wild	+	+	-	-	-	-	-	+	+	+	+	-	-	-
	In vitro	+	-	-	-	-	-	-	+	+	-	-	-	-	-
	Acclimatised	+	+	+	-	-	-	-	+	+	+	+	-	-	-
Salmonella Typhi	Wild	+	+	+	+	-	-	-	+	+	+	+	+	-	-
	In vitro	+	+	+	-	-	-	-	+	+	+	+	-	-	-
	Acclimatised	+	+	+	-	-	-	-	+	+	+	+	+	+	-
Escherichia coli	Wild	+	+	+	-	-	-	-	+	+	+	+	+	-	-
	In vitro	+	+	-	-	-	-	-	+	+	+	-	-	-	-
	Acclimatised	+	+	+	-	-	-	-	+	+	+	+	-	-	-
Klebsiella pneumoniae	Wild	+	+	+	-	-	-	-	+	+	+	+	-	-	-
	In vitro	+	+	+	-	-	-	-	+	+	+	-	-	-	-
	Acclimatised	+	+	+	+	-	-	-	+	+	+	+	+	+	-
Pseudomonas aeruginosa	Wild	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	In vitro	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Acclimatised	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+: bacterial growth.

taurica, Z. vychodceviana, Z. persica) are rich in pulegone (Sezik and Tümen, 1990; Dembistikii *et al.*, 1995; Nezhadali and Zarrabi, 2010). Sezik *et al.* (1991) also reported that pulegone was the major constituent in essential oil of *Z. tenuior* (Sezik and Tumen, 1990). *Z. clinopodioides* and *Z. pamiroalaica* have also been found to be rich in pulegone (Meral *et al.*, 2002; Salehi *et al.*, 2005; Sonboli *et al.*, 2006; Verdian-rizi, 2008; Si-lei *et al.*, 2010; Shahla, 2012). Comparing among the different sources for essential oils, the percentage of some compounds like pulegone and *n*-hexadecanoic acid increased, while bicyclo[2.2.1]heptan-2-ol,1,5,5-trimethyl- and bicyclo[3.1.1]hept-3-en-2-one,4,6,6-trimethyl- decreased. Two compounds (copaene and β-cubebene) were present and others (4-Isopropyl-3-methoxymethylene-1,1-dimethyl-cyclohexane) was absent. This might suggest that the tissue culture media and plant growth regulators (auxin and cytokinin) could have affected the compound production since these factors also affected the gene expression. So, it could be said that the essential oils' components and concentrations change according to the environmental conditions. These results agree with Feizbakhsh *et al.* (2014) which showed that some growth hormones like 1-naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) significantly influenced the concentrations and compositions of the essential oil of *Sambucus ebulus*. Medina-Holguın *et al.* (2007) also showed that the concentration of two compounds: thymol and piperitone from the essential oil of *Anemopsis californica* increased by increasing irrigation. Abdelmajeed *et al.* (2013) also reported that the environmental factors such as temperature, relative humidity, irradiation, photoperiod, wind, soil properties, fertilisation and harvest time impact the composition and quality of essential oils.

Antibacterial activity

It appears that the bacteria were sensitive to the essential oils, and the effectiveness of *in vitro Z. tenuior* clearly outperformed the others. The inefficiency of the essential oils against *P. aeruginosa* is in accordance with another study which showed that the *Z. clinopodioides* essential oil had no effect on *P. aeruginosa* (Anzabi *et al.*, 2013). Additionally, Sarac and Ugur (2009) have also demonstrated that the essential oils of *Z. tenuior* and other Lamiaceae members were very effective against Gram-positive and Gram-negative bacteria, except *P. aeruginosa* ATCC 27853 and *P. fluorescens* MU 87. However, Rabah *et al.* (2013) showed antibacterial activity with inhibition zone of 37 mm of essential oil *Z. hispanica* against *P. aeruginosa* (Rabah *et al.*, 2013). Nevertheless, this could be due to the difference in *Ziziphora* species used. In general, Gram-negative bacteria are more resistant than Gram-positive bacteria which could be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (Adwa and Abu-Hasan, 1998). Our results are also consistent with Mahboubi *et al.* (2012) who studied the inhibition of *Z. tenuior* oil against *K. pneumoniae* (11.8 mm in diameter). However, our result showed higher inhibition (8 to

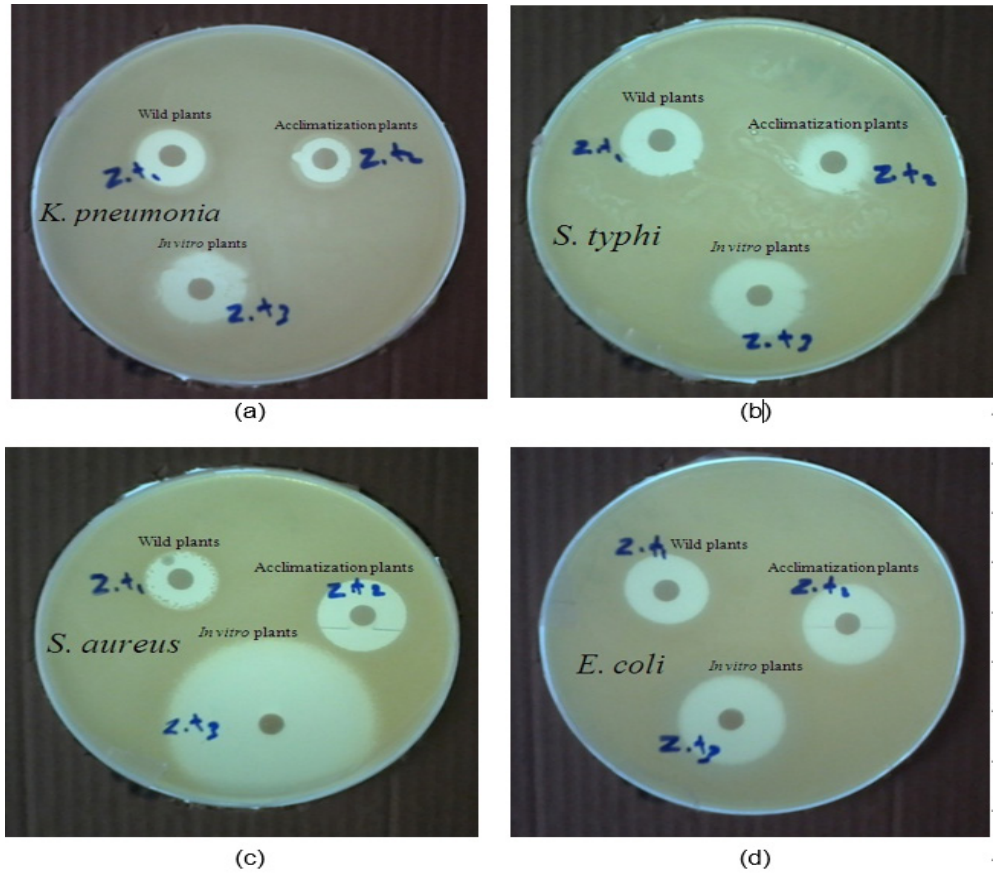

Figure 1. Antibacterial activities of essential oils 100% (10 µL) of wild, in vitro and acclimatised plants of *Ziziphora tenuior* against some bacteria. (a) *Klebsiella pneumoniae*, (b) *Salmonella* Typhi, (c) *Staphylococcus aureus*, and (d) *Escherichia coli*.

19 mm in diameter). The overall antibacterial activity of *Z. tenuior* essential oil could well be associated to the relatively high pulegone content as reported by many researchers (Meral *et al.*, 2002; Bakkali and Averbeck, 2008; Beikmohammadi, 2011; Rabah *et al.*, 2013). Pulegone from the essential oil of *Mieromeria thymfolia* also showed antibacterial activity against 28 bacterial species (Kalodjera *et al.*, 1994). The presence of copaene in the essential oils of *in vitro Z. tenuior* might also affect the bacterial inhibition as compare to wild and acclimatised *Z. tenuior*. Lin *et al.* (2012) has confirmed that copaene was considered a powerful antimicrobial agent against *Staph. aureus* and *E. coli*. The MIC and MBC values obtained showed that the essential oil of *in vitro Z. tenuior* were superior to the wild and acclimatised *Z. tenuior*, and this could well be due to pulegone and the presence of copaene and β-cubebene.

Conclusion

It seems that plant tissue culture will be a useful method to modify the chemical composition and concentration of *Z. tenuior* essential oil. The different chemical compositions are likely to be the result of genetic differences and/or the effect of some plant growth regulators like Kin and NAA. It was showed that the essential oil of *Z. tenuior* exhibited antimicrobial activity against Gram-positive (*Staph. aureus*) and Gram-negative (*S.* Typhi, *E. coli, K. pneumoniae*). However, no activity was observed against the Gram-negative *P. aeruginosa*.

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