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.

Keywords

Ziziphora tenuior
Assal Al-Ward
Antibacterial
Pulegone

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%),
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$_2$SO$_4$ and kept in a sealed vial at 4°C.

Gas chromatography-mass spectrometry (GC-MS) analysis

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

<table>
<thead>
<tr>
<th>Tested bacteria</th>
<th>Source of plants</th>
<th>MIC µg/mL</th>
<th>MBC µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Wild</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Acclimatised</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella Typhi</em></td>
<td>Wild</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Acclimatised</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>In vitro</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Acclimatised</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>Wild</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Acclimatised</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>In vitro</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Acclimatised</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+: bacterial growth.

*Z. 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. pamoioalaica* have also been found to be rich in pulegone (Meral et al., 2002; Salehi et al., 2005; Sonboli et al., 2006; Verdiyan-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 (coapaene 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 Feizbaksh 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-Holguin 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
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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### References


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