Chemical Composition and Antifungal Activity of Essential Oil of Zataria Multiflora

Vida Rahimi; MSc¹, Seyedhossein Hekmatimoghaddam; PhD², Ali Jebali; PhD³, Elham Khalili Sadrabad; PhD³⁵, Ali heydari; MSc¹⁴ & Fateme Akrami Mohajeri; PhD⁴⁵

¹ Department of Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
² Department of Advanced Medical Sciences and Technologies, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
³ Department of Laboratory Sciences, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
⁴ Zoonotic Diseases Research Center, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
⁵ Department of Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

ABSTRACT

Background: Aspergillus flavus is a toxic contaminant in foods, which can induce mutagenic, teratogenic, and carcinogenic effects. In last decades, synthetic fungicides have been used for inhibition of fungal growth in plants. The public attention was also attracted to contamination of food chain by these chemicals. Therefore, in the current study, we decided to use Zataria multiflora (ZM) essential oil to inhibit the Aspergillus flavus growth. Method: The essential oil from ZM was obtained by hydro-distillation and analyzed by GC/MS. The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of ZM essential oil were determined at different concentrations (0-1000 ppm). Results: In this study, Carvacrol (33.45%) and Thymol (34.44%) were the most frequent compounds of the ZM essential oil. The minimum inhibitory and fungicidal concentrations were 100 and 400 ppm, respectively. Conclusions: The presence of phenolic compounds such as Thymol and Carvacrol, as the major components of ZM essential oil inhibits the Aspergillus flavus growth. Furthermore, this essential oil has a very strong anti-bacterial effect. Considering these results, it seems that ZM essential oil is potentially an appropriate natural alternative for controlling Aspergillus flavus fungus.

Keywords: Aspergillus flavus; Chemical composition; Zataria multiflora; Antifungal activity

Introduction

Aflatoxins are produced by three strains of Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nomius (Allameh et al., 2011). The Aspergillus flavus can contaminate crops such as cotton seed, corn, pistachio, and peanuts. Pre- and post-harvest as well as the biological and environmental factors are important in mycotoxin contaminations (Nikan and Ghamari, 2015). The secondary metabolism of Aspergillus flavus, aflatoxin consists of the toxic contaminants in...
foods that can induce mutagenic, teratogenic, and carcinogenic effects. Despite of health problems, contamination of crops with aflatoxin has direct and indirect economics effects. The regulatory guide lines of Food and Drug Administration (FDA) recommend that the standard level of aflatoxin in food products is 20 ppb (Bhatnagar-Mathur et al., 2015). Moreover, the International Agency for Research on Cancer (IARC) has classified aflatoxin as a class I carcinogenic agents. In last decades, synthetic fungicides have been produced for inhibition of fungal growth in plants. However, the development of resistant strain and adverse environmental effects of these chemicals have drawn the attentions to substitute them by bioactive compounds such as essential oils (Ferreira et al., 2013). Many researchers investigated the anti-fungal activity of herbal extracts to inhibit mycotoxin production (Ferreira et al., 2013).

Zataria multiflora (ZM) is a thyme-like plant in Laminaceae family, which grows in Iran, Pakistan, and Afghanistan. ZM has been used in traditional medicine as a herbal drug for treatment of different diseases (Jafari et al., 2015). ZM essential oil is rich in phenolic compounds including carvacrol, thymol, and gamma-terpinene (Azizkhani et al., 2013). The antibacterial, antifungal, and antiviral activities of ZM essential oil were proved in many studies (Jafari et al., 2015, Mohammadi et al., 2015). In the present study the effects of ZM essential oil as the anti-fungal plants were investigated.

Materials and Methods

The ZM was prepared from the local market and the herbarium confirmation process was performed by the Academic Center for Education, Culture, and Research (ACECR) of the University of Tehran. We dried the purchased ZM and obtained its essential oil by hydro-distillation method using a Clevenger. Then, the extracted oil was dried over anhydrous sodium sulfate followed by filtration. Later, it was stored in 4°C until injection to GC/MS apparatus (Saei-Dehkordi et al., 2010).

The components of the essential oils were estimated by Gas Chromatography-Mass Spectrometry device model (GC Hewlett-Packard 6890N) equipped with a HP-5MS (0.25x30) column and a Hewlett-Packard 5973N mass spectrophotometer. For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 ml min−1. Injector and mass transfer line temperatures were set at 250 °C and 300 °C, respectively. The oven temperature was programmed from 50 °C to 200 °C at 8 °C min−1 and then held isothermally for 20 min and finally raised to 300 °C at 10 °C min−1. Diluted samples (1/100 v/v, in methanol) of 0.2 μl were manually injected in the split less mode. Identification of compounds of the essential oil was based on GC retention time on VF-5 capillary column, computer matching of mass spectra with standards. The type of components entering the mass spectrometry was determined by the library study of Wiley and the ISIRI No 5192 (Saei-Dehkordi et al., 2010)

Evaluation of the anti-fungal effects of essential oil of ZM: The antifungal activity of oil was determined by disc diffusion method. The sterile potato dextrose agar (PDA) containing 0, 50, 75, 100, 200, 300, and 400 ppm essential oil of ZM was prepared. A 5mm Whatman No.1 disk paper was inoculated with 10μl of a suspension of 10⁶ spore/mL and placed in the center of the plate. The plates were incubated for 9 days under 26°C. The diameter of the colony was measured daily in control group (lacking essential oils). The plate was completely covered with the fungi. In order to determine the minimal inhibitory concentration (MIC) or minimal fungicidal concentration (MFC), the disk of the plates with no fungal growth was transferred to a medium without essential oil. After that, discs with no growth were transferred to PDA plates without essential oil and incubated for 48 hours to determine the fungi static or fungicidal effect. The lowest concentration that inhibited the growth of the fungus was considered as MIC and the lowest concentration of essential oil with no observed growth in plate (which killed the test fungus) was taken as the minimum fungicidal
concentration (MFC). All treatments were carried out in triplicate (Akrami et al., 2015).

Data Analysis: The tests were performed in randomized design with three repetitions and SPSS version 18 was used for statistical analysis of data. In addition, the inhibition of radial mycelia growth was examined using analysis of variance (ANOVA) and the mean scores were compared. It should be noted that the statistical difference was significant at the level of $P < 0.05$.

Results

The yield of ZM essential oil used in the current study was measured as 1.51% (v/w). The chemical compositions of the essential oil of ZM were identified using GC-MS analysis (Table 1).

Thymol (34.44%), Carvacrol (33.45%), and Parasymn (15.62%) were identified as the principle components in essential oil.

In order to analyze the effect of ZM essential oil on the growth of Aspergillus flavus, the fungus was cultured on a sterile PDA medium. In this experiment, the media containing 0, 50, 75, 100, 200, 300, and 400 ppm of the ZM essential oil were cultured for 9 days using a 5mm disk. The results are presented in Table 2. As shown, the plates containing ≤ 100 ppm essential oil did not show any fungal growth after 9 days. The disks without any growth were transferred to the PDA medium to determine the MIC and the MFC. The results are shown in Table 2. The concentration of MIC and MFC were 100 and 400 ppm, respectively.

<table>
<thead>
<tr>
<th>Type of compound</th>
<th>RTa</th>
<th>KIb</th>
<th>Percentage</th>
<th>Type of compound</th>
<th>RT</th>
<th>KI</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpin-4-ol</td>
<td>99.13</td>
<td>1200</td>
<td>1.11</td>
<td>γ-Pyronene</td>
<td>7.62</td>
<td>915</td>
<td>0.33</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>44.14</td>
<td>1222</td>
<td>0.93</td>
<td>α-Propylphenol</td>
<td>8.57</td>
<td>954</td>
<td>0.25</td>
</tr>
<tr>
<td>Thymyl methyl ether</td>
<td>14.93</td>
<td>1248</td>
<td>0.50</td>
<td>1,3-Cyclopentadiene</td>
<td>8.69</td>
<td>959</td>
<td>0.18</td>
</tr>
<tr>
<td>Carvacrol methyl ether</td>
<td>15.13</td>
<td>1258</td>
<td>0.85</td>
<td>α-Terpinolene</td>
<td>8.98</td>
<td>971</td>
<td>0.28</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>15.93</td>
<td>1299</td>
<td>0/18</td>
<td>1,3-Cyclohexadiene</td>
<td>9.33</td>
<td>986</td>
<td>0.52</td>
</tr>
<tr>
<td>Thymol</td>
<td>16.76</td>
<td>1345</td>
<td>34.44</td>
<td>Myrcene</td>
<td>9.55</td>
<td>995</td>
<td>1.65</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>16.98</td>
<td>1357</td>
<td>33.45</td>
<td>α-Phellandrene</td>
<td>9.89</td>
<td>1010</td>
<td>0.45</td>
</tr>
<tr>
<td>Thymol acetate</td>
<td>17.27</td>
<td>1373</td>
<td>0.86</td>
<td>α-Terpinolene</td>
<td>10.17</td>
<td>1022</td>
<td>1.12</td>
</tr>
<tr>
<td>Carvacryl acetate</td>
<td>17.65</td>
<td>1393</td>
<td>0.95</td>
<td>p-Cymene</td>
<td>10.46</td>
<td>1035</td>
<td>15.62</td>
</tr>
<tr>
<td>Isoeucalyphyllene</td>
<td>18.20</td>
<td>1426</td>
<td>0.64</td>
<td>γ-Terpinene</td>
<td>11.17</td>
<td>1067</td>
<td>1.80</td>
</tr>
<tr>
<td>Alloaromadendrene</td>
<td>19.74</td>
<td>1516</td>
<td>1.23</td>
<td>α-Terpinolene</td>
<td>12.29</td>
<td>1118</td>
<td>1.85</td>
</tr>
<tr>
<td>Acetophenone</td>
<td>21.54</td>
<td>1615</td>
<td>0.53</td>
<td>E,E-Alloocimene</td>
<td>13.02</td>
<td>1153</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*: Retention Time; b: Kovats Index

Table 2. The effect of Zataria multiflora Essential oil on radial growth by Aspergillus flavus on PDA

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>4th</th>
<th>5th</th>
<th>7th</th>
<th>9th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30 ± 1</td>
<td>43 ± 1.7</td>
<td>63 ± 2.6</td>
<td>all over the plate</td>
</tr>
<tr>
<td>50</td>
<td>8 ± 0.6</td>
<td>11.5 ± 2.1</td>
<td>20 ± 1.5</td>
<td>32 ± 1.5</td>
</tr>
<tr>
<td>75</td>
<td>Inhibition</td>
<td>8 ± 0.6</td>
<td>12 ± 1.5</td>
<td>28 ± 1.7</td>
</tr>
<tr>
<td>100</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>200</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>300</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>400</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
</tbody>
</table>

Discussion

ZM is a popular and medicinal plant in Iran. During the recent years, due to its remarkable antimicrobial activity in food industry, the plant has received high attention. The current study determined the chemical composition and
antifungal properties of ZM essential oil. ZM essential oil is a yellow liquid with a favorable odor and hot flavor. Thymol is a phenolic compound and the most effective component in the essential oil. Another important component of the essential oil is Carvacrol, which can be solved easily in the alcohol and organic solvents. These materials are generally stored in young leaves during the growth of the plant (Nabavi et al., 2015). According to the literature, frequencies of the essential oils range from 0.8 to 2.6 percent in different parts of the ZM. This is consistent with the results of the present study (1.51%) (Nabavi et al., 2015). The most frequent components of the ZM essential oil are Thymol and Carvacrol. In accordance with these results, (Hudaib et al., 2002) Thymol, Carvacrol, and Parasyms were introduced as the major components of the essential oil of ZM with 5.08, 14.4, and 10.6 percent. In another study (Imelouane et al., 2009), it was found that Carvacrol (71.12%) was the most frequent component in the ZM. Moreover, a study (Safari et al., 2015) reported a similar result and measured the percentage of the Carvacrol as 60.3 percent. This confirms the results obtained by the present study. In another study, Goodarzi et al. reported Carvacrol as the most effective component of ZM. Most compounds found in the essential oils of ZM include anti-oxidant, anti-bacterial, anti-inflammatory, and analgesic (Goudarzi et al., 2006). The anti-bacterial activity of ZM essential oils is attributed to existence of Thymol and Carvacrol. In this regard, Lambert et al. stated that a proper combination of Thymol and Carvacrol may prevent the growth of microorganisms more effectively than other herbal essential oils (Lambert et al., 2001). This inhibitory effect occurs due to the damage to the cellular membrane resulted from influences on homeostatic pH and inorganic ion balance. Viuda-Martos et al. found that the essential oil of ZM inhibits the growth of mycelium of Aspergillus flavus fungus by denaturing the enzyme proteins (Viuda-Martos et al., 2007). This inhibitory effect is attributed to the compounds such as Thymol, Carvacrol, and Eeugenol. Furthermore, Centeno et al. showed that the Thymol, Carvacrol, and Borneol compounds prevented the mycelium of Aspergillus flavus fungus from germinating by granulation of the cytoplasm contents and destroying the cytoplasm membrane (Centeno et al., 2010).

The MIC is defined as a dose of an anti-microbial agent that may inhibit the in vitro growth of fungi or bacteria. Moreover, the MFC means the minimum anti-microbial agent that destroys the fungi or bacteria. In the present study, the MIC and MFC concentrations were 100 ppm and 400 ppm for the Aspergillus flavus fungus, respectively. Our results are consistent with the results of Fani-Makki et al. which reported the MIC and MFC of the Zataria for Aspergillus flavus as 200 and 400ppm, respectively (Fani-Makki et al., 2015). In another study, Omid-Beigi et al. indicated that the ZM essential oil with the concentration of 350 μL/mL in a liquid medium prevented the growth of Aspergillus flavus with the inhibition rate of 100 percent (Omidbeygi et al., 2007). Nikan et al. revealed that the concentrations of 0.06, 0.1, and 0.14 mg/mL of ZM essential oils were effective in prevention of the growth of Aspergillus flavus (Nikan and Ghamari, 2015). In addition, regarding the effects of the ZM essential oil on other microorganisms, Mohajerfar et al. reported the MIC of the ZM essential oils for Listeria monocytogenes as 400 ppm (Mohajerfar et al., 2012). However, the lysozyme enzyme could not inhibit the growth of the bacteria even at 1000 ppm. In another study, Akhond-Zadeh Basti et al. found that the MIC of the ZM essential oils for the Vibrio parahaemolyticus bacteria was 100 ppm. (Mohajerfar et al., 2012).

Conclusions
The existence of phenolic compounds in the ZM such as Thymol and Carvacrol as the major components may control the Aspergillus flavus effectively. In the current study, the Carvacrol and Thymol were found to be the most prevalent components of the ZM essential oils with frequencies of 33.45 and 34.44 percent, respectively. We found strong anti-bacterial effects for the essential oils of ZM. Therefore, use of essential oils is very advantageous. So, the
essential oils may be used as natural preservatives for most foods.

Acknowledgement
This paper is a part of the thesis for MSc from the Food Hygiene and Safety department of the School of Public Health of Yazd Shahid Sadoughi University of Medical Sciences. The authors acknowledge the cooperation and assistance of the laboratory department of Food Hygiene and Safety Faculty of the School of Public Health.

Authors’ Contributions
Akrami, Hekmatimoghaddam, Jebali and Khalili conceived and designed the experiments. Rahimi and heydari carried out the experiment. All authors contributed to the final version of the manuscript.

Conflict of interest
The authors declare no conflict of interests.

References
Akrami F, et al. 2015. Antioxidant and antimicrobial active paper based on Zataria (Zataria multiflora) and two cumin cultivars (Cuminum cimumin). Food science and technology. 60 (2): 929-933.


Mohajerfar T, et al. 2012. Determination of minimum inhibitory concentration (MIC) of Zataria Multiflora boiss. essential oil and
Activity of essential oil of Zataria Multiflora


