Antifungal effects of Zataria multiflora Boissss (Shirazi Thyme) extract against Aspergillus fumigatus Afs35 genes: crza and rpda

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How to Cite

Abstract
Aspergillus fumigatus causes a fatal infection particularly among immunocompromised individuals. Nowadays herbal and medicinal essential oils play a vital role as an alternative safe medication. Zataria multiflora Boiss essential oils have antifungal activities mainly on the expression of some gene required for fungal survival. The main objective of the study was evaluation of antifungal effects of Z. multiflora Boiss extract against A. fumigatus AIS35 strain. The strain was obtained from department of biology, Baghdad University. Various concentrations (10-300mg/mL) of Z. multiflora Boiss extract were prepared and the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using micro-broth dilution method. The expression of crza and rpda genes was evaluated using the quantitative real-time PCR (RT-qPCR) technique. The concentrations ≥100mg/mL of extract inhibited the fungal growth which was non-toxic to normal cell line (MIC=100mg/mL and MFC=150mg/mL). The combination of 50mg/mL of extract plus each of amphotericin B (10mg/ml) and nystatin (10mg/mL) significantly decreased the MIC level (p<0.001). The concentration of 10mg/mL of the extract decreased the expression of crza and rpda genes 2.4 (p<0.001) fold and 3.6 (p<0.0001) fold, respectively. The same concentration of amphotericin B non-significantly decreased the crza and rpda genes expression by 0.6 (p=0.224) and 1.1 (p=0.033) fold, respectively. The study concluded that Z. multiflora Boiss extract exerted antifungal effect against A. fumigatus by inhibiting the cell wall biosynthesis (down-regulation of crza), sustainability and virulence expression (rpda). The combination therapy with Z. multiflora Boiss is promising to eradicate A. fumigatus infections.

Keywords: Aspergillus fumigatus AIS35, crza, extract, rpda, Zataria multiflora Boiss

INTRODUCTION: Aspergillus fumigatus (A. fumigatus) causes fatal systemic and pulmonary infections reaching more than 90% among those immunocompromised individuals mostly through escape (using glucan and chitin) and counteract the immune response (Van De Veerdonk et al., 2017; Resendiz Sharpe et al., 2018; Latgé and Chamilos, 2019). In A. fumigatus, the crza gene contributes in cell wall biosynthesis via regulation of the expression of specific chitin synthase genes. Additionally, the rpda gene is responsible for the expression of virulence genes and sustainability in axenic conditions. Treatment of severe or systemic A. fumigatus infections include antifungal drugs such as azoles, polyenes and echinocandins, which target ergosterol biosynthesis and cell wall (CW) polysaccharide glucan (Paulussen et al., 2017). However, some drawbacks following application of chemotherapies such as side effects, costs and poor specificity have led to consideration of alternative approaches in addition to accurate diagnosis (Rieber et al., 2016). Herbal medicines are
considered as non-toxic agents, which contain various fractions to be applied against microorganisms. *Zataria multiflora* Boiss (commonly called Shirazi thyme) is a potential herbal drug which has exhibited antifungal, antibacterial and antiparasitic properties (Moazeni et al., 2014; Al-Abodi et al., 2019; Golkar et al., 2020). The present study aimed to assess antifungal effects of *Z. Multiflora* Boiss against *A. fumigatus* phenotypically and on gene expression of *crzA* and *rpdA* genes

**MATERIALS AND METHODS**

**Strain and culture**

*A. fumigatus* strain was obtained from department of biology, Baghdad University and was subcultured onto the *Aspergillus* minimal and complete media at 37°C for an overnight and next a suspension of fungus was prepared (Ries et al., 2017; Takahashi et al., 2017).

**MTT assay**

The cytotoxic effect of *Z. Multiflora* Boiss extract was investigated using HEL 12469 normal human embryo lung cells by the MTT assay as previously described (Lammel et al., 2013; Okoro et al., 2019).

**Preparation of extract**

The herbal extract was prepared using maceration method (Keikhaie et al., 2018). After collecting the ground part and leaves and drying, 50 gr of it was added to 500mL of 70% ethanol for 24h and with shaking. Dimethyl sulfoxide solvent (5% DMSO) was used to prepare various extract concentrations. Then, fungal suspension was obtained in a number of 1×10⁶ CFU/mL. The AmB (Sigma Aldrich) and Nystatin (Sigma Aldrich) were used as the control. Concentrations of 10, 20, 50, 100, 150 and 300mg/mL of AmB and Z. multioflora Boiss included 100 and 150mg/mL, respectively (Fig.1).

**Quantitative real-time PCR**

The total RNAs were obtained using RNeasy Mini Kit (Qiagen, Hilden, Germany) 1 × 10⁷ conidia culture onto the CM (37°C for 16h) and treated with DNase for removing the DNA according to the protocol provided by the manufacturer. The cDNA was synthesized using 2mg of RNA using the high-capacity cDNA reverse transcription kit (Thermo Scientific). RT-qPCR was performed using a StepOne Plus real-time PCR system (Thermo Scientific) employing Sybr green PCR master mix purchased from this company. The biological replicates were conducted in triplicate and separately. The β-tubulin gene was used as the control and the threshold method (2^ΔΔCT) was conducted for expression analyses. The primers sequences of *crzA* and *rpdA* genes have been described previously (Ries et al., 2017; de Castro et al., 2014; Bauer et al., 2019) (Table 1). A 10mg/mL concentration of each AmB and *Z. multiflora* Boiss extract was applied for gene expression.

**RESULTS**

**Cytotoxicity of *Z. multiflora* Boiss extract**

The results of the MTT assay revealed that, the extract is safe at concentrations 10-150mg/ml for HEL 12469 normal human embryo lung cells while exerting their cytotoxicity at concentrations of 300, 400, 500 mg/ml (Table 2).

**Fungal growth inhibition**

There was no growth inhibition at 10-50mg/mL concentrations of *Z. multiflora* Boiss extract using naked eye, but the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Z. multiflora* Boiss included 100 and 150mg/mL, respectively (Fig.1).

**Gene expression**

The concentration of 10mg/mL of the extract decreased the expression of *crzA* and *rpdA* genes 2.4 (p<0.001) and 3.6 (p<0.0001) fold, respectively. The same concentration of AmB decreased the *crzA* and *rpdA* genes expression by 0.6 (p=0.224) and 1.1 (p=0.033) fold, respectively. Additionally, the same dosage of Nystatin decreased the *crzA* and *rpdA* genes expression by 0.43 (p=0.424) and 0.8 (p=0.123) fold, respectively (Table 3).

### Table 1. Primer pairs used for *crzA* and *rpdA* gene expression

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence: 5’-3’</th>
<th>Product (bp)</th>
<th>Ann. (°C)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUBB-F</td>
<td>CAR GGY GGT CAR<em>TGY</em>GTT AAT CA</td>
<td>195</td>
<td>60</td>
<td>(Ries et al., 2017)</td>
</tr>
<tr>
<td>TUBB-R</td>
<td>CAR GGY GGT CAR GAT GBT AAT CA</td>
<td>220</td>
<td>61</td>
<td>(Bauer et al., 2019)</td>
</tr>
<tr>
<td>rpdAF</td>
<td>AATGGTTACCTCCAACGACTTACATCCAACGACT</td>
<td>190</td>
<td>60</td>
<td>(de Castro et al., 2014)</td>
</tr>
<tr>
<td>rpdAR</td>
<td>TTATTATATATATATGAGAAAGCTGTCGTATGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crzAF</td>
<td>GCTCTTGGTATAGCCACC</td>
<td>190</td>
<td>60</td>
<td>(de Castro et al., 2014)</td>
</tr>
<tr>
<td>crzAR</td>
<td>GCCAAAGAGCTAGCGAAC</td>
<td>190</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

*indicate intron positions
DISCUSSION

Herbal medicines have advantages in human health by acting as anti-oxidant, antimicrobial and anti-inflammatory effects (Al-Abodi et al., 2019). Moreover, the compounds contain low toxicity against normal cells (Table 2). Fractions from herbal medicines exert an antifungal effect on cell membrane permeability and cell wall (hydrophobicity and ergosterol production). Noticeably, various solvents leave a different series of compounds which affect the antifungal trait of a herb. *Z. Multiflora* Boiss has demonstrated substantial antimicrobial and antiparasitic effects from previous studies (Moazeni et al., 2014; Akrami et al., 2015; Moradi et al., 2016; Al-Abodi et al., 2019; Golkar et al., 2020). Thymol and carvacrol are major compounds in this herbal

Table 2. Cytotoxicity of *Z. multiflora* Boiss extract on HEL 12469 normal human embryo lung cells

<table>
<thead>
<tr>
<th>Concentration of extract</th>
<th>Viability %</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/ml</td>
<td>95</td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>95</td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>85</td>
</tr>
<tr>
<td>100 mg/ml</td>
<td>75</td>
</tr>
<tr>
<td>150 mg/ml</td>
<td>75</td>
</tr>
<tr>
<td>300 mg/ml</td>
<td>50</td>
</tr>
<tr>
<td>400 mg/ml</td>
<td>30</td>
</tr>
<tr>
<td>500 mg/ml</td>
<td>25</td>
</tr>
</tbody>
</table>
medicine. In this study, Z. Multiflora Boiss ethanol extract exhibited an antifungal effect against A. fumigatus through inhibiting cell wall biosynthesis, fungal sustainability and virulence. We observed that Z. Multiflora Boiss MIC and MFC included 100 and 150mg/mL, respectively (Fig. 1). Furthermore, Z. Multiflora Boiss at concentrations ≥100mg/mL significantly exerted an antifungal effect (p<0.0001) compared to the control (PBS) (Fig. 1). More importantly, significant synergistic antifungal effect of 10µg/mL of each AmB and nystatin plus 50µg/mL of Z. Multiflora Boiss was revealed against A. fumigatus (Fig. 1). There is no previous study regarding the assessment of the antifungal effect of Z. Multiflora Boiss against A. fumigatus. In a study by Hoda et al. (2020), the hexane extract of Myristica fragrans exhibited an inhibitory effect against A. fumigatus via decrease in the melanin and ergosterol production and hydrophobicity of the cell. Herein present study, the Z. Multiflora Boiss decreased the expression of crzA and rpdA genes significantly (Table 3), which have roles in cell wall biosynthesis and fungal survival. Therefore, this herbal medicine exerts its effect on different pathways in the A. fumigatus. However, other possible mechanisms are needed to be verified. Considering the nontoxicity and wide availability of Z. Multiflora Boiss, the application of the related compounds (thymol and carvacrol) as formulations of antifungal agents seems beneficial.

### Conclusion

The present study concluded that Z. Multiflora Boiss extract exerted antifungal effect against A. fumigatus by inhibiting the cell wall biosynthesis (down-regulation of crzA), sustainability and virulence expression (rpdA). The combination of antifungal drugs with Z. Multiflora Boiss is promising to eradicate A. fumigatus infections.

### Conflict of interest

The authors declare that they have no conflict of interest.

### Table 3. Gene expression of crzA and rpdA gene after treatment with extract, Amphotericin B and Nystatin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Folding without treatment</th>
<th>Folding with treatment</th>
<th>Difference in folding</th>
<th>Sig. Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10mg/ml of Zataria multiflora Boiss extract</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crzA</td>
<td>3.8</td>
<td>1.4</td>
<td>2.4</td>
<td>0.001*</td>
</tr>
<tr>
<td>rpdA</td>
<td>4.9</td>
<td>1.3</td>
<td>3.6</td>
<td>0.001*</td>
</tr>
<tr>
<td><strong>10mg/ml of Amphotericin B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crzA</td>
<td>2.8</td>
<td>2.2</td>
<td>0.6</td>
<td>0.224**</td>
</tr>
<tr>
<td>rpdA</td>
<td>3.7</td>
<td>2.6</td>
<td>1.1</td>
<td>0.033**</td>
</tr>
<tr>
<td><strong>10mg/ml of Nystatin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>crzA</td>
<td>2.5</td>
<td>1.07</td>
<td>0.43</td>
<td>0.424**</td>
</tr>
<tr>
<td>rpdA</td>
<td>2.2</td>
<td>1.4</td>
<td>0.8</td>
<td>0.123**</td>
</tr>
</tbody>
</table>

*Significant differences; **non-Significant differences

### Ethical approval

The Research related to human use has complied with all the relevant national regulations, and institutional policies and is in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors’ institutional review board or equivalent committee. Project no. M201002 was approved at 7th October 2020.

### REFERENCES