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Research Article

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

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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* AfS35 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 ($p<0.001$) 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. multifora Boiss* is promising to eradicate *A. fumigatus* infections.

Keywords: *Aspergillus fumigatus* AfS35, *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 1×10^6 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 *Z. Multiflora* Boiss were prepared in normal saline added to the fungal suspension in 96-well plates, and incubated for 24hr.

Quantitative real-time PCR

The total RNAs were obtained using RNeasy Mini Kit (Qiagen, Hilden, Germany) 1×10^7 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 ($2^{-\Delta\Delta CT}$) 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

Primer	Sequence: 5'-3'	Product (bp)	Ann. (°C)	Ref.
TUBB-F	CAR GCY GGT CAR*TGY*GGT AAC CA	195	60	(Ries *et al*., 2017)
TUBB-R	CAR GCY GGK CAR TGY GGB AAC CA			
*rpdA*F	AAATGGTACCATCCAACGACTGTTTACCATCCAACGACT	220	61	(Bauer *et al*., 2019)
*rpdA*R	TTTATAATACATATGTGAGAAAGGTCGTGTATGTG			
*crzA*F	GCTCTTGGTGATAGCGACC	190	60	(de Castro *et al*., 2014)
*crzA*R	GGCAAAGAGCTATGCAGAC			

*indicate intron positions

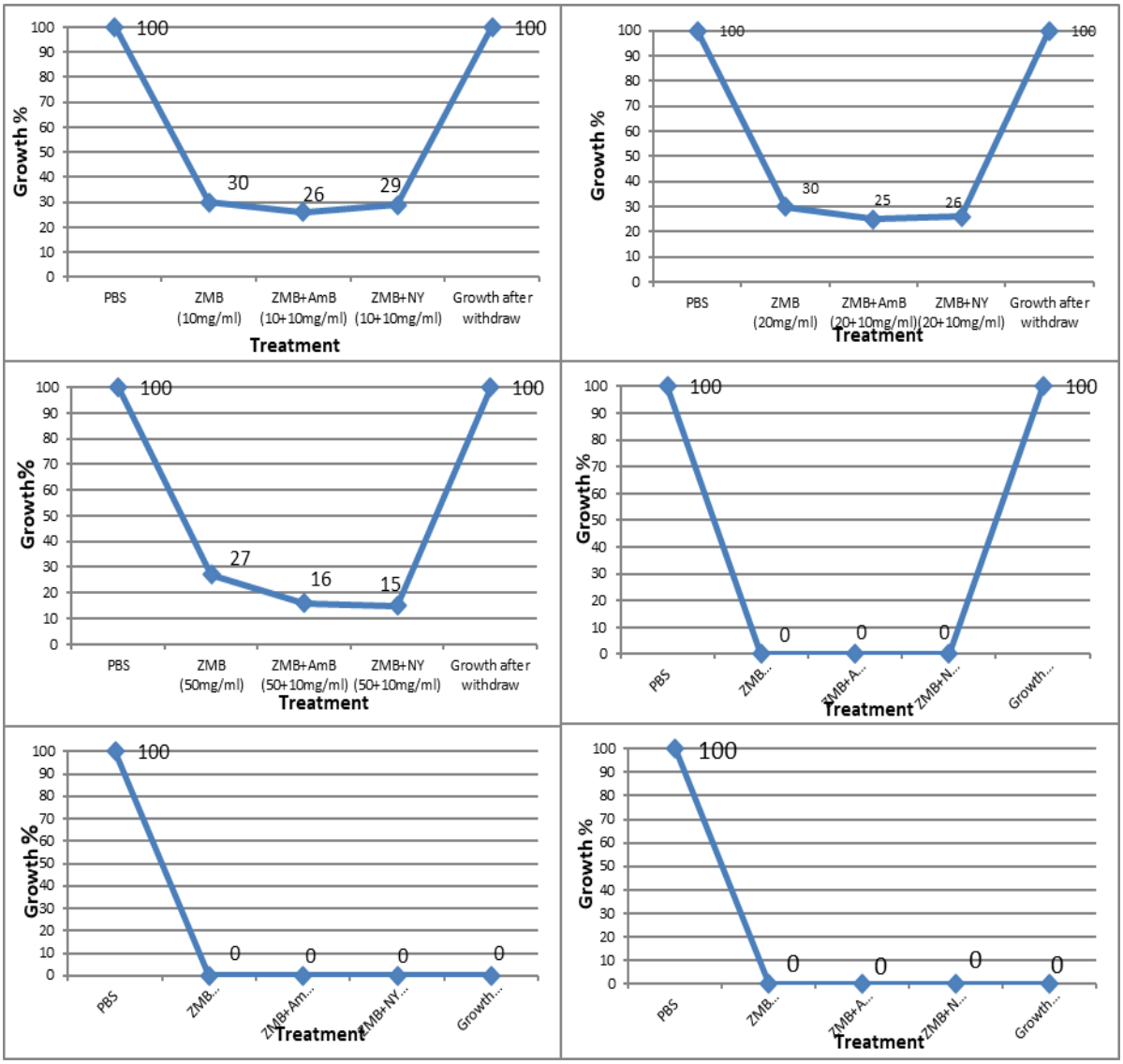

Fig. 1. *Growth inhibition of Z. multiflora Boiss (ZMB) at concentrations ≥100mg/mL which significantly differed (p<0.0001) from control (PBS); significant synergistic antifungal effect of 10mg/mL of each AmB and nystatin (NY) plus 50mg/mL of ZMB was revealed against A. fumigatus.*

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

Concentration of extract	Viability %
10 mg/ml	95
20 mg/ml	95
50 mg/ml	85
100 mg/ml	75
150 mg/ml	75
300 mg/ml	50
400 mg/ml	30
500 mg/ml	25

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

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

*Significant differences; **non-Significant differences

medicine. In this study, *Z. Multiflora Boiss* ethanolic 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 10mg/mL of each AmB and nystatin plus 50mg/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. multifora Boiss* is promising to eradicate *A. fumigatus* infections.

Conflict of interest

The authors declare that they have no conflict of interest.

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.

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