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Zataria multiflora Boiss.: A review study on chemical composition, anti-fungal and anti-mycotoxin activities, and ultrastructural changes

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ABSTRACT

Introduction: *Zataria multiflora* is a valuable medicinal plant from Lamiaceae family with various pharmacological and therapeutic properties. In this article we reviewed the various aspects of *Z. multiflora* properties including botanical characteristics, chemical composition, anti-fungal and anti-mycotoxin activity and fungal ultrastructural changes.

Methods: Google Scholar, PubMed, EBSCO, Directory of open access journals (DOAJ), EMBASE, and Web of Science were searched using the keywords *Z. multiflora* and pathogenic and toxigenic fungi.

Results: The essential oil (EO) of *Z. multiflora* is frequently used in pharmaceutical industries. Thymol and carvacrol are the most important active components of *Z. multiflora* that are used for the treatment of a wide variety of diseases such as candidiasis and dermatophytosis. Aflatoxin production inhibitory effect of *Z. multiflora* EO was at the transcription level and this herb may cause reduction in aflatoxin biosynthesis. Ultrastructural changes showed that the main sites of action of EO were the plasma membrane and cell wall of fungi.

Conclusion: *Zataria multiflora* has the potential to be considered as a new natural drug for the treatment of some fungal infections. Morphological and structural changes may be one of the mechanisms involved in growth inhibition of the fungi and reducing aflatoxin production by *Z. multiflora* EO.

Implication for health policy/practice/research/medical education:
Zataria multiflora can be used effectively for the most clinically relevant opportunistic fungal infections. Cautious about the inhibitory effects of *Z. multiflora* against pathogenic and toxigenic fungi is very important.

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Introduction

The emergence of opportunistic fungal infections, especially in immunocompromised individuals, highlights the need to elucidate new therapeutic options, especially because the fungi involved usually have remarkable morphological plasticity and express genes involved in the mechanisms of resistance to antifungal agents (1). In fact, indiscriminate use and the small number of available antifungal agents have promoted the development of resistant strains, especially in immunocompromised individuals (2). This fact justifies the development of new therapies for use in clinical practice. Among these new therapies, natural products stand out; they are considered sources of bioactive molecules with potential therapeutic applications in medicine (3).

In search of phytochemicals with potential antimicrobial activity in the past few years, attentions have been directed on *Zataria multiflora* quite reasonably. From the vegetable families, Lamiaceae is one of the biggest families, with worldwide distribution, which has 200 genuses and 2000-5000 species of aromatic bush and short shrubs producing terpenes and other types of compounds that are stored in epidermal gland of leafs, stalks and generative organs (4). *Zataria multiflora* Boiss. (Avishan-e-Shirazi in Persian and Saatar or Zaatar in the old Iranian medical books) is a thyme-like plant, which is a member of this family

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and is the aborigine of Iran, Afghanistan and Pakistan (5). *Zataria multiflora* can be recognized by the orbicular, densely gland-dotted, ovate leaves and the dense white, hairy, round buds on the leaf axils. It is an aromatic shrub that reaches 60-90 cm in height. Mature branches are woody and leafless whereas young branches are white with dense glandular, spreading, pilose indumentums. Leaves (5-10 × 5-10 mm^2) are orbicular ovate to orbicular. Flowering stems are usually un-branched, sometimes having short lateral branches. Flowers are white, sub-sessile, very small and often male sterile (6). *Zataria multiflora* (aerial parts) is not only a popular condimental plant but is also used in traditional folk remedies for its anti-microbial, analgesic, carminative, anthelmintic and anti-diarrheal properties (7). Modern pharmacological studies have shown that *Z. multiflora* possesses wide ranging biological properties including anti-nociceptive, anti-microbial, spasmolytic and anti-inflammatory effects (5,8-10). Currently, some pharmaceutical forms of this plant, such as syrups, oral drops, soft capsules and vaginal creams are sold as treatment remedies for various diseases. To outline the extensive uses of *Z. multiflora* in healthcare and medicine and to provide a probable scope for future research, several pharmacological and clinical studies on this plant and its active components are provided in this review article.

Methods

Google Scholar, PubMed, EBSCO, Directory of open access journals (DOAJ), EMBASE, and Web of Science were searched using the keywords *Z. multiflora* and pathogenic and toxigenic fungi.

Chemical composition of *Zataria multiflora*

Various extraction techniques like distillation, effleurage, CO_2 extraction, expression and solvent extraction are applied for essential oil (EO) extraction from various plants. But conventional methods that are usually used to extract EO oil from plants, are the distillation methods like steam-distillation, hydro-distillation and water-steam-distillation method.

Quantitatively, the most abundant components in hydrodistilled *Z. multiflora* EOs are oxygenated monoterpenes (approximately 70%), followed (in order) by monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated sesquiterpenes (10,11). Two studies from Pakistan identified carvacrol as the main constituent of the oil (12,13). To date, a large number of studies have focused on *Z. multiflora* EO: some reported carvacrol as the main compound, but others reported thymol, the isomer of carvacrol, as the main compound. Saei-Dehkordi et al (14) collected *Z. multiflora* from five different areas of Iran and analyzed its EOs. According to the GC–MS data, the main EO constituents remained similar between plants from different geographical regions, but their relative quantities differed among plants from different regions. Thymol was the most abundant compound among all constituents in all samples (14). In a study conducted by Shokri et al (15), *Z. multiflora* main components were carvacrol (61%) and thymol (25%). Additionally, Saleem et al (16) showed that thymol was the main constituent of the fresh plant (73.21%), while carvacrol was the primary constituent in the dried plant (62.87%). It is clear that geographical variation, cultivar differences, stage of plant growth, preparation process and other factors may influence the EO composition both quantitatively and qualitatively. According to previous studies, the EOs of *Z. multiflora* contain significant amounts of thymol and carvacrol, which are well-known anti-fungal agents (17-19). *p*-Cymene is the other main component in *Z. multiflora* EO. Zatariol, zataroside A (glycoside and non-volatile), zataroside B (glycoside and non-volatile), multiflotriol and multiflorol have been reported as new derivatives of *p*-cymene isolated from *Z. multiflora* (20,21). Linalool, caryophyllene, γ-terpinene and borneol are some of the other main components in the EOs (22).

Zataria multiflora also contains other compounds belonging to different classes of natural products, including alkanes such as n-nonacosane (C_{29}), n-hentriacontane (C_{31}), n-dotriacontane (C_{32}), n-tritriacontane (C_{33}) and n-pentatriacontane (C_{35}); fatty acids such as behenic acid (C_{22}), lignoceric acid (C_{24}), cerotic acid (C_{26}) and montanic acid (C_{28}) (23); phytosterols such as β-sitosterol and stigmasterol; triterpenes such as betulin, betulinic acid and oleanolic acid (24); and hydroxycinnamic acids such as rosmarinic acid (25). Moreover, flavonoids such as apigenin, luteolin and 6-hydroxyluteolin are also among the phytochemicals reported from *Z. multiflora* (22). This plant also contains small amounts of tannins, resins and saponins while lacking alkaloids (26).

Pharmacological and therapeutic activities of *Zataria multiflora*

1. Anti-yeast activity of *Zataria multiflora*

Various studies have shown that many species of yeasts, especially *Candida* and *Malassezia* species, have developed resistance to standard antifungal drugs. Nowadays traditional medicine and the use of herbal medicines to treat yeast infections are important because herbal medicines have fewer side effects and are less likely to develop drug resistance compared with chemical ones (27,28). Medicinal plants have been shown to eliminate the drug resistant yeasts and can be beneficial for their therapeutic effects (29). Zarei-Mahmoudabadi et al evaluated anti-*Candida* activity of three extracts of *Z. multiflora* (aqueous, ethanolic and methanolic) against 14 isolates of *Candida*. Aqueous extract of *Z. multiflora* showed no activity against *Candida* species. The ethanolic and methanolic extracts showed remarkable activities against *Candida* species. The minimum inhibitory concentration (MIC) for both extract was between 50 and 150 mg/L. The lowest MIC was for seven isolates of *C. albicans* (125 mg/L). Others MICs were respectively *C. glabrata* (126 mg/L), *C. parapsilosis* (125 mg/L) and *C. tropicalis* (131 mg/L). Totally, the MIC of ethanolic extract for 14 isolates of *Candida* was 127 mg/L. Both *C. albicans* and

C. parapsilosis were more susceptible than other species. The isolates of *C. parapsilosis* (64 mg/L) were more susceptible to methanolic extract of *Z. multiflora*, followed by *C. glabrata* (66 mg/L), *C. albicans* (76 mg/L) and *C. tropicalis* (76 mg/L). In addition, the MIC of methanolic extract for tested *Candida* was 70 mg/L. In that study methanolic extract showed more activities than ethanolic extract against 14 isolates of *Candida* (30). In Moghim et al (31) study, the obtained MIC of *Z. multiflora* extract was 0.13 mg/mL for *C. albicans*, which was lower than the effect on *C. albicans* obtained for this extract in the study of Zarei Mahmoudabadi et al (30). In another study by Fuladi et al (32), the MIC of *Z. multiflora* methanol and ethanol extracts were estimated to be 0.079 and 0.125 mg/mL, respectively. The contrasting results could be due to the different species of *Z. multiflora*, plant chemical compounds and/or methodologies. Katiraei et al (33) compared the MIC of EOs of *Z. multiflora*, *Geranium* and *Artemisia* with regard to the growth of *C. albicans* isolates resistant to azole drugs. They showed that the MIC levels of EOs of the plants were statistically significant from those of azoles. In that study, the obtained MIC of *Z. multiflora* for *C. albicans* was 0.18 mg/mL. In a study conducted by Esfandiary et al (34), a set of *C. glabrata* (29 strains), *C. krusei* (3 stains) and *C. patapsilosis* (2 strains) were studied. The results revealed that 33 isolates were resistant (MIC = 64 μg/mL), 4 isolates were susceptible (MIC ≤ 8 μg/mL) and 7 isolates had dose-dependent susceptibility (MIC = 16-32 μg/mL) to fluconazole, respectively. With regard to fluconazole, high resistance rate was observed in *C. glabrata* and *C. krusei*. However, ciclopirox olamine was found to inhibit the growth of all non-*albicans Candida* species (MIC ≤8 μg/mL). Also, favorable anti-fungal activity against *non-albicans Candida* species was obtained by *Z. multiflora* despite having a wide range of MICs (34.9-139.5 mg/mL). Mohammadi-Pourfard and Kavoosi (35) showed that EO from *Z. multiflora* significantly ($P<0.01$) inhibited the growth of *C. albicans*. MIC for *C. albicans* was 2.8 ± 0.8 mg/mL of EO. At concentration > 5 mg/mL, EO significantly ($P<0.01$) reduced the growth of *C. albicans* by 100%. However, the aqueous extract did not show any such activity at any concentration used. In addition, Zomorodian et al (36) determined the MICs and minimum fungicidal concentrations (MFCs) of the EO from *Z. multiflora* against *Candida* and *Trichosporon* species, showing strong anti-yeast activities with MIC values ranging from 0.007 to 0.5 μg/mL. This finding is similar to that of the study by Mahboubi et al (37) who reported strong anti-*Candida* activity of *Z. multiflora* EO with high thymol and carvacrol concentrations.

In a randomized clinical trial conducted by Khosravi et al (38), the application of 0.1% *Z. multiflora* cream in patients with acute vaginal candidiasis decreased vulvar pruritus in 80.9% of patients, vaginal pruritus in 65.5% of patients, vaginal burning in 73.9% of patients, urinary burning in 100% of patients, painful intercourse in 92.6% of patients, and vaginal secretion in 90% of patients. In addition, 0.1% *Z. multiflora* cream reduced erythema and satellite vulvar lesions in 100% of patients, vulvar edema in 100% of patients, vaginal edema in 83.3% of patients, vulvo-vaginal excoriation and fissures in 92% of patients, and white, sticky vaginal secretions in 86.2% of patients. After treatment with 0.1% *Z. multiflora* cream a laboratory using standard methods reported negative mycologic results on microscopic evaluation for 90% of patients; negative mycologic cultures in 86.7% of patients; and negative mycologic results on microscopy and culture combined for 93.3% of patients (38). Abou Fazeli et al (39) demonstrated the activity of the EO against *C. albicans*. They also suggested *Z. multiflora* oil-containing vaginal suppositories as a successful replacement for current drugs for the treatment of vaginitis caused by *C. albicans* (39). In a comparative study, a 7-day therapy with *Z. multiflora* as an intravaginal cream was more effective than clotrimazole vaginal cream in the treatment of Candidal vaginitis (40). An open-label, randomized and controlled study with two parallel treatment groups was conducted to evaluate the efficacy of a miconazole 2% gel compared with a *Z. multiflora* 0.1% gel applied four times daily for two weeks in the treatment of *Candida*-associated denture stomatitis. The results indicated that the *Z. multiflora* gel reduced the surface erythema of the palate more efficiently than miconazole gel but did not reduce the colony count on the denture surface as efficiently as miconazole (41). In another study, the physicochemical properties and stability of creams containing different concentrations (1%–3%) of *Z. multiflora* were evaluated and suggested as a successful replacement in the treatment of *C. albicans* induced vaginitis. In a previous study by Khosravi et al (43), intraperitoneally administration of 64 mg/kg of the EO in mice with systemic candidiasis had the highest efficacy in reducing *C. albicans* and produced 39.5, 21.8, 141.5, 174 and 501-fold reductions in mean colony forming units per 0.1 gram in *Candida* infections of the liver, spleen, lungs, brain and kidneys, respectively, as compared to itraconazole. Avaei et al (44) demonstrated that *Saccharomyces cerevisiae* (MIC = 200 μg/mL and MFC = 1600 μg/mL) was more resistant than *C. utilis* to *Z. multiflora* EO.

Naeini et al (45) showed that *Malassezia* species were susceptible to *Z. multiflora* EO ranging from 10 to > 50 mm (mean value: 28.1 mm). The highest inhibitory effect was recorded with *Malassezia obtusa*, followed by *M. furfur* and *M. globosa*. In another study conducted by Naeini et al (46), the EO from *Z. multiflora* presented anti-*Malassezia* activity against the tested yeasts. All *Malassezia* species were very susceptible to *Z. multiflora* EO, with MICs ranging from 0.015 to 0.06% (v/v), and approximately 52.4% of the strains had a MIC value of 0.015% (v/v). The mean MIC values of the EO against *M. sympodialis*, *M. furfur* and *M. pachydermatis* were 0.03, 0.024 and 0.02%, respectively. Khosravi et al (47) showed that *Z. multiflora* EO had the best anti-fungal activity against various *Malassezia* species isolated from dogs with atopic dermatitis, with MIC values ranging from 30 to 80 μg/mL. *M. nana* isolate was the most susceptible one (30

μg/mL), while *M. sloofiae* isolates showed the highest MIC value (80 μg/mL) (P<0.05).

2. Anti-dermatophyte activity of *Zataria multiflora*

Dermatophytes are the major cause of superficial mycoses, and remain a public health problem. They have the ability to invade keratinized tissues and cause dermatophytosis, the most common human contagious fungal disease (48). In recent years, the incidence of dermatophytosis has increased considerably, especially among individuals with impaired immunity, as well as in pediatric and geriatric populations (49). Some of these infections are still difficult to resolve completely, and remissions and relapses are often observed. The poor availability of antifungals and the increasing number of treatment failures have motivated current searches for therapeutic alternatives which can work effectively as potential anti-dermatophyte agents. Khosravi et al (50) showed that EO obtained from *Z. multiflora* possessed anti-fungal activity against a wide number of clinical isolates of various dermatophytes, with MIC ranging from 0.25 to 4 mg/mL. However, variation of susceptibility from one species to another was evident. The MIC values were 0.5 mg/mL for *Microsporum canis* and *M. gypseum*, 1.5 mg/mL for *Trichophyton mentagrophytes* and *Epidermophyton floccosum* and 2 mg/mL for *T. rubrum*. The MFCs recorded for plant oil tested ranged from 0.5 to 8 mg/mL. In a study conducted by Effatpanah et al (51), total (100%) fungal growth inhibition was observed from *Z. multiflora* EO at concentration of > 8 mg/mL for *T. mentagrophytes*, *T. rubrum* and *E. floccosum* (P < 0.01). In another study, the minimum inhibitory activity of *Z. multiflora* methanolic extract against various dermatophytes was found to be approximately 0.5% (w/v) (42).

3. Anti-filamentous fungi activity of *Zataria multiflora*

Literature studies indicated that there are many reports on non-dermatophytic filamentous fungi causing fungal infections in human and animals and food spoilage (18,52). Gandomi Nasr-Abadi et al (53) found that all concentrations of EO from *Z. multiflora* had a significant effect on the growth and sporulation of *Aspergillus flavus*. Also, they reported the levels of MIC and MFC 400 and 1000 ppm, respectively. In a study conducted by Mohammadi-Pourfard and Kavoosi (35), the EO from *Z. multiflora* at all tested concentrations significantly (P < 0.01) inhibited the growth of *A. niger*. MIC for *A. niger* was 2.2 ± 0.5 mg/mL of EO. At concentration > 5 mg/mL, EO significantly (P < 0.01) reduced the growth of *A. niger* by 100%. In a study by Shokri et al (18), the EO from *Z. multiflora* showed the inhibitory effects on four tested fungi including *A. flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium verticillioides* at all concentrations (500-2000 ppm). It completely inhibited four fungi at 2000 ppm. At 1000 ppm concentration, *Z. multiflora* EO significantly decreased the growth of *Aspergillus* species compared with the control, whereas it caused complete growth inhibition of *F. verticillioides* (P < 0.05). Therefore, *F. verticillioides* had a higher susceptibility than *Aspergillus* species against the EO tested. In addition, Nasseri et al (54) showed that the EO from *Z. multiflora* has anti-fungal activity; the lowest inhibition (75%) was observed in *A. niger*, while the highest inhibition (95.3%) was observed in *Alternaria solani*. The MICs for *A. solani*, *Rhizoctonia solani*, *Rhizopus stolonifer*, *A. flavus, A. ochraceus* and *A. niger* were 200, 200, 200, 300, 300 and 200 ppm, respectively. In addition, the MFCs for *A. solani*, *R. solani*, *R .stolonifer, A. niger* and *A. ochraceus* were 600, 400, 300, 900 and 700 ppm, respectively, and none of the tested concentrations were fatal for *A. flavus. A. solani* and *R. solani* showed a strong susceptibility to *Z. multiflora* EO at all concentrations. The fungal pathogens studied were classified according to their susceptibility to the EO in the following order: *A. solani* > *R. stolonifer* > *R. stolonifer* > *A. ochraceus* > *A. niger* > *A. flavus*. Sharif Rohani et al (55) showed that dosage of 800 ppm of *Z. multiflora* EO for *Fusarium solani* had obvious inhibitory rate. In a study by Amini et al (56), *Z. multiflora* EO was very effective on the four studied pathogenic fungi including *Pythium aphanidermatum, R. solani*, *Fusarium graminearum* and *Sclerotinia sclerotiorum* with growth inhibition average of 100% at 200 μL/L concentration. Nevertheless, MIC and MFC of the EO were variable depending to species of fungi. *P. aphanidermatum* and *S. sclerotiorum* were the most susceptible and most resistant to the studied EO with average growth inhibition 89.54% and 75.35%, respectively. Khosravi et al (57) determined the anti-fungal assay of EO from *Z. multiflora* against *Saprolegnia parasitica* isolated from fish eggs. The infected fish eggs were treated with EO oil at concentrations of 1, 5, 10, 25, 50, and 100 ppm daily. The MIC of *Z. multiflora* EO against *S. parasitica* was 0.9 ppm. *Z. multiflora* at concentrations of 25, 50, and 100 ppm had significant differences in comparison with negative control (P<0.05). The most hatching rates were recorded with *Z. multiflora* (11%) EO. *Z. multiflora* was effective for the treatment of *S. parasitica*-infected rainbow trout eggs in aquaculture environment (57).

4.Inhibition of mycotoxin production by *Zataria multiflora*

Fungi are significant spoilage microorganisms of foodstuffs during the storage, rendering them unfit for human consumption by retarding their nutritive value and sometimes by producing mycotoxins (58). Mycotoxins are polyketide secondary metabolites produced by the important food and feed contaminating species *Aspergillus*, *Penicillium* and *Fusarium* and are known as potent carcinogens for a wide variety of animal species, including humans (59). EOs and their phenolic compounds have been used as natural inhibitors of fungal growth and mycotoxin production to preserve foods and feeds in some countries during recent decades. Many of the spices and herbal EO which have been tested have an antagonistic effect against aflatoxigenic *Aspergillus* strains (60). Previous studies demonstrated that thymus EO, which mainly consists of thymol, has been shown to inhibit both the growth and aflatoxin production in *A.*

flavus and *A. parasiticus* (61,62). Yahyaraeyat et al (63) determined the effects of *Z. multiflora* EO on growth, aflatoxin production and transcription of aflatoxin biosynthesis pathway genes of *A. parasiticus*. The results showed that mycelial dry weight and aflatoxin production reduce in the presence of *Z. multiflora* EO (100 ppm) on day 5 of growth. It was found that the expression of *nor-1*, *ver-1*, *omt-A* and *aflR* genes was correlated with the ability of fungus to produce aflatoxins on day 5 in yeast extract sucrose medium. RT-PCR showed that in the presence of *Z. multiflora* EO (100 ppm), *nor-1, ver-1* and *omtA* genes expression was reduced. It was suggested that toxin production inhibitory effects of *Z. multiflora* EO on day 5 may be at the transcription level and this herb may cause reduction in aflatoxin biosynthesis pathway genes activity (63). Also, Gandomi et al (64) investigated the effect of *Z. multiflora* EO against growth, spore production and aflatoxin formation by *A. flavus* ATCC 15546. EO effectively inhibited radial growth and spore production on potato dextrose agar (PDA) in a dose-dependent manner. At 200 ppm, the radial growth and sporulation reduced by 79.4% and 92.5%, respectively. The growth was completely prevented at EO ≥400 ppm on PDA, and MFC of the oil was estimated at 1000 ppm. The EO also significantly suppressed mycelial growth and aflatoxin synthesis in broth medium at all concentrations tested ($P<0.05$). In that study, *Z. multiflora* EO had a significant inhibitory effect on aflatoxin formation, which was reduced by 31% and 99.4% at 50 and 150 ppm, respectively. In a similar study conducted by Farag et al (65), *Z. multiflora* EO was effective at concentrations ≥100 ppm in reducing aflatoxin formation by 50%. It has been suggested that the regulation of aflatoxin synthesis and conidiogenesis may be interlinked, since the loss of aflatoxigenic capabilities in the nonaflatoxigenic variant strains of *A. parasiticus* was correlated with alterations in the conidial morphology (66). Furthermore, it has been shown that lysis of the mycelia and spores of the toxigenic fungi are one of the characteristics of aflatoxin deactivation process (67).

It was shown by several investigators that chemical compounds of the EOs may cause reduction or stimulation in the toxin production and the anti-toxigenic effects of the EOs are not necessarily related to their anti-fungal activity. Values for growth inhibition were calculated as 0.79 and 0.86mM for carvacrol and thymol, while for AFB_1 and AFG_1, it was reported as 0.50 and 0.06mM for carvacrol and 0.69 and 0.55mM for thymol (68). It was also shown by Wright et al (69) that aflatoxin production by the fungus was reduced by *n*-decyl aldehyde and hexanal, but was stimulated by octanal. Their results indicated that all three volatile compounds reduced radial growth but only *n*-decyl aldehyde significantly inhibits aflatoxin biosynthesis in *A. parasiticus*. Difference in anti-fungal and aflatoxin inhibition efficacy of *Thymus* and *Zataria* EOs in different studies may be attributable to the EO compositions. Two major components of *Thyme* and *Zataria* are thymol and carvacrol. *Thyme* contains higher thymol than *Zataria* and *Zataria* contains more carvacrol. The other factors are also important for causing these differences such as differences in culture media used, culture conditions, temperature, pH and durations of culture.

There are little reports on the effect of *Z. multiflora* in inhibiting the other mycotoxins. In this regard, Vazquez et al (70) reported that phenolic compounds, i.e., thymol and eugenol, inhibited growth and citrinin production by *Penicillium citrinum* in some Galician cheeses. In another study by Noori et al (71), the EO from *Z. multiflora* significantly ($P<0.05$) suppressed citrinin formation by *P. citrinum* in mozzarella cheese at all concentrations, as citrinin accumulation was reduced by 30% and 87% at 50 and 1000 ppm, respectively.

Ultrastructural changes of fungi treated with *Zataria multiflora*

1. Scanning electron microscopy

The effect of *Z. multiflora* EO on morphology of *A. flavus* observed by Scanning electron microscopy (SEM) is illustrated in Figure 1. Hyphae grown on PDA without EO showed swollen and turgid feature with smooth and uniform surface. In presence of 50 ppm EO, hyphae lost their turgidity and uniformity to some extent and these modifications increased at higher concentrations of EO. Furthermore, 100 ppm EO induced the shrinkage of the hyphae and formation of pits along the mycelia. The severity of damage increased with 200 ppm EO and the hyphae were frequently collapsed and their destruction was often evident (72).

2. Light and transmission electron microscopy

Semi-thin sections of *A. flavus* grown in broth medium containing different concentrations of *Z. multiflora*

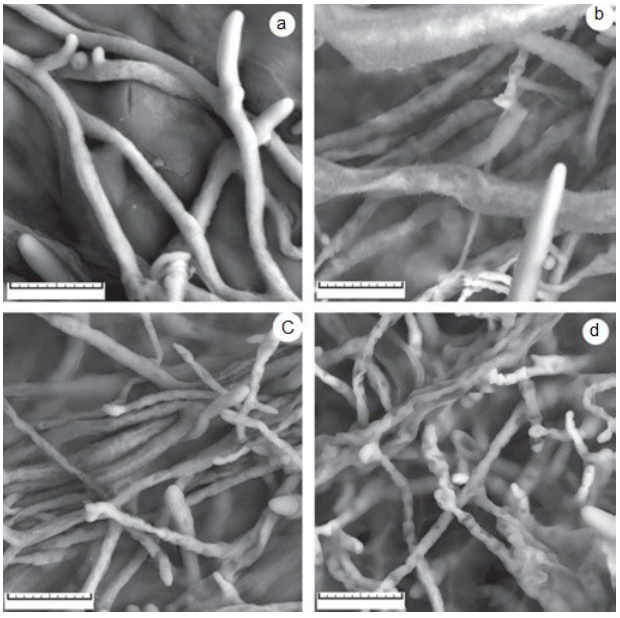

Figure 1. Scanning electron micrographs ×2000, *Aspergillus flavus* mycelia: (a) untreated; (b) treated with 50 ppm *Zataria multiflora* essential oil (EO); (c) treated with 100 ppm *Z. multiflora* EO; (d) treated with 200 ppm *Z. multiflora* EO (72).

EO are illustrated in Figure 2. Longitudinal sections of untreated culture displayed normal mycelia that had a steady arrangement with continuous and homogenous cytoplasm (Figure 2a). Vacuolization of the cytoplasm was the major effect induced in the presence of 50 ppm EO, which led to the cell swelling (Figure 2b). However, mycelia treated with 100 ppm EO revealed greater damage characterized with detachment of the cell membrane from the cell wall that was collapsed and herniated in different intervals along the longitudinal sections resulting in the deformation of mycelia (Figure 2c). In presence of 150 ppm EO, cell membrane was fully disconnected from the cell wall and mycelia obtained an electron-lucent appearance due to cytoplasm shedding from the cell (Figure 2d). Moreover, swelling and deformation of the mycelia were the other changes observed (Figure 2d). The concentration of 200 ppm EO effectively inhibited the germination of the fungal conidia and no mycelia formed at this concentration (Figure 2e). Some of the conidia had a normal shape but the others showed changes ranging from the vacuolization to cell membrane detachment and deformation (Figure 2e). With increasing EO concentration to 400 ppm, more severe damages including depletion of the cytoplasm and frequent degradation of the cell wall were observed (Figure 2f). Furthermore, deformation and destruction of conidia were often evident (Figure 2f) (72). Examination of ultrathin sections under transmission electron microscopy (TEM) resulted in more obvious findings. Untreated hypha was enclosed by the cell wall that was integrated and intact. The plasma membrane was fully attached to the cell wall along the hypha and was smooth and unwrinkled. Cytoplasm was homogeneous and dense and intercellular septum was intact and healthy. At concentration of 50 ppm of *Z. multiflora* EO, the cell wall lost its integrity and uniformity to some extent. The cell membrane in some areas was detached from the cell wall and invaginated into the cytoplasm. The loss of cytoplasm density and vacuolization were the other changes. At concentration of 100 ppm of *Z. multiflora* EO, the plasma membrane was completely separated from the cell wall and vacuolization of the cytoplasm was more evident. The presence of 150 ppm of EO increased the severity of injury characterized by the depletion of cytoplasm content, and hypha lost its normal shape and was collapsed. In addition, detachment and fragmentation of the plasma membrane and formation of the lomasome, small membrane-bound vesicles, beneath the cell wall, were seen and membrane fragments were spread over the cytoplasm. At concentration of 200 ppm EO, no evident change was observed in the cell wall, whereas cell membrane was completely destroyed and cytoplasm was released from the cell. With increase in oil concentration to 400 ppm, complete destruction of the plasma membrane and cell wall was evident and hypha was totally free of cytoplasm (73).

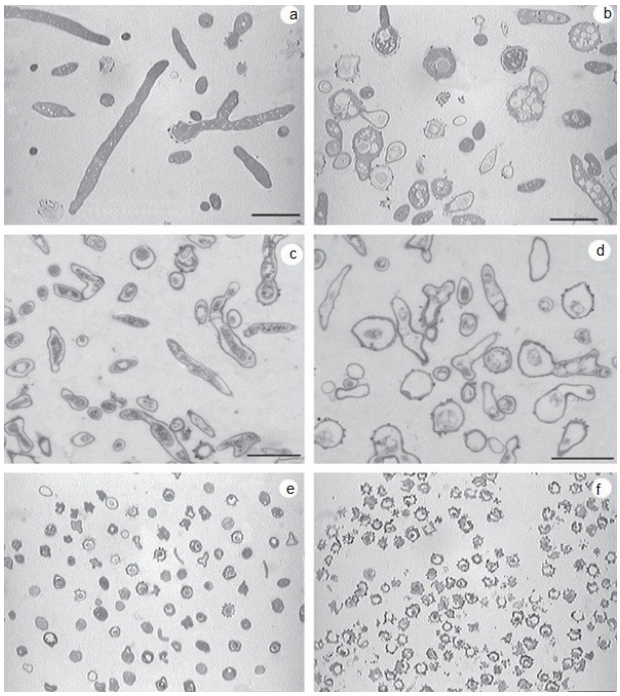

Figure 2. Semi-thin sections × 1000, *Aspergillus flavus* grown in broth medium: (a) untreated; (b) treated with 50 ppm *Zataria multiflora* essential oil (EO); (c) treated with 100 ppm *Z. multiflora* EO; (d) treated with 150 ppm *Z. multiflora* EO; (e) treated with 200 ppm *Z. multiflora* EO; (f) treated with 400 ppm *Z. multiflora* EO.

Conclusion

In summary, the microscopic examinations showed that *Z. multiflora* EO suppressed the size of the colony as well as sporulation of fungi. Mycelia treated with EO showed morphological alterations ranging from loss of turgidity and uniformity of mycelia at low concentrations of EO to evident destruction of the hyphae at higher concentration of EO. The major change at level as low as 50 ppm of EO was limited to vacuolization of cytoplasm resulting in cell swelling, while at higher concentrations, detachment of the cell membrane from the cell wall, deformation of mycelia and shedding the cytoplasm from the cell were the main alterations. These damages were well documented by TEM, which showed that the main sites of action of EO were the plasma membrane and cell wall. In conclusion, morphological and structural changes observed in this study may be one of the mechanisms involved in growth inhibition of the fungi and reducing aflatoxin production.

Authors' contributions

All authors contributed to the conception of the study, confirmed the final version of the article and approved all aspects of the study.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission and redundancy) were completely observed by authors.

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