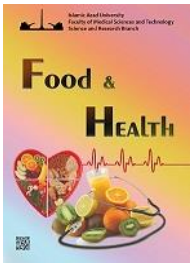

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Antimicrobial effect of carvacrol on *Aspergillus flavus* and reduce expression of *aflR* gene in the aflatoxin biosynthetic pathway

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ABSTRACT

Carvacrol was found in essential oils of thyme, oregano, wild bergamot, and some other plants. Although, a wide range of bioactivities such as anticancer, antioxidant, and antimicrobial has been identified for carvacrol, however, among all therapeutic properties it possesses a potent antimicrobial activity. The present study investigates the inhibitory effect of carvacrol as an active compound against the growth of *Aspergillus flavus*, besides its effect on the expression of aflatoxin-related (*aflR*) gene. Twelve fungal samples of *A. flavus* were used and the antimicrobial activity of carvacrol was tested against them using the minimum inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) according to the broth microdilution procedure. The expression of the aflatoxin regulatory (*aflR*) gene was examined by Reverse Transcriptase polymerase chain reaction (RT-PCR) technique. The results of MIC and MFC tests showed that carvacrol at 0.8 µg/ml and 3.5 µg/ml concentrations displayed antimicrobial activities on *A. flavus*, respectively. The RT-PCR result indicated that the expression level of *aflR* gene had decreased to 33% in the presence of carvacrol compared to 67% in the absence of the mentioned active compound. Together the results demonstrated that carvacrol not only exhibited antimicrobial activity against *A. flavus* but also reduced its gene expression level.

1. Introduction

Studies of the past decade have presented that fungal contamination has been dramatically increased in agricultural products (1-4). Although several antifungal synthetic drugs are currently available, however, the increasing consumer concern about chemical additives has led to their replacement by the natural compound in food industries (5). Recently there has been growing attention in the study of plant ingredients because of their accessibility and fewer side effects as compared to the existing synthetic antifungal agents (6-8). Medicinal plants are found to have a broad range of therapeutic properties such as anti-inflammatory, antimutagenic, and antimicrobial activities (7-10). Plants derived compound can control or even inhibit the synthesis of DNA and RNA in fungal cells by inducing modifications similar to the influences of antibiotic action (8, 11). Previous findings confirmed the antimicrobial activity and inhibitory action of plants` essential oils (EOs) or their corresponding components which comprise a high amount of these substances (12-14). Among active compounds separated from plants, carvacrol, is proved to have a wide range of biological activities including antioxidant, anti-inflammatory, anti-parasitic, and antimicrobial (9, 10, 15). Carvacrol is the main constituent of the EOs of many plants such as thyme and oregano with several reports have indicated its potent antimicrobial effect against some fungus (1, 8, 9). Fungi is one of the microorganisms involved in food spoilage especially for preserved food (4, 16, 17). The fungal toxin causes agricultural product corruption and endangers human health (16, 18). The most important fungus cause food contamination are *Aspergillus ochraceus*, *Penicillium digitatum* and *Aspergillus flavus* (5). Among them, *Aspergillus flavus* is the most frequent contaminant and subsequent aflatoxin production in food and its derivatives (19). Aflatoxins (AFs) are a group of associated fungal secondary metabolite produced by *Aspergillus flavus* and are extremely

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toxic (8, 9, 16, 20). It has been reported that aflatoxin has 18 types, however, only four types namely aflatoxin B_1, B_2, G_1, and G_2 are usually found in food (9). Among them, AFB_1 was considered the most toxic and was classified as a human carcinogen (9, 19, 21). Aflatoxicosis is the disease caused by aflatoxin consumption and many studies have stated that it can cause liver cancer (22-24). Food contamination by aflatoxin is a major concern in food industries due to significant economic losses for consumers and manufacturers (16). A standard method to inhibit aflatoxin growth is the use of food preservatives. In recent years, there has been a growing interest to use plants' active compounds instead of artificial food preservatives. In this context, carvacrol is accepted as a safe food preservative by the FDA (US Food and Drug Administration) (9). Therefore, the aim of this study was to examine the antimicrobial activity of carvacrol on *Aspergillus flavus* growth as well as to evaluate its effect on the expression of the aflatoxin production regulatory (*aflR*) gene.

2. Material and methods

2.1. Aspergillus flavus collection and preparation

A total of 12 isolates of *Aspergillus flavus* were obtained from the Mycology laboratory, Science and Research Branch, Islamic Azad University, Tehran, Iran. Each isolate of *Aspergillus flavus* (*A. flavus*) was sub-cultured on potato dextrose agar (PDA) to confirm purity and viability. Isolates were grown on PDA slants at 30 °C for 7 days to make sure acceptable sporulation (25). Each inoculum was suspended and collected by adding up sterile water to the PDA slants. The suspension density was determined using a spectrophotometer and then modified to a concentration of 1×10^6 spores/mL, equivalent to 80 % transmittance at 530 nm wavelength (26). The agar formulation used was RPMI 1640 medium combined with 2% glucose plus 1.5% agar then buffered to pH 7.0 with 0.17 M morpholine propane sulfonic acid (MOPS) buffer (26).

2.2. Aflatoxin production in broth

Seven-day-old broth cultures of the fungal isolates were continuously filtered and then centrifuged for 30 min at 4°C to gain a supernatant followed by filtration to remove any contamination. Then the culture filtrate was examined to produce aflatoxin.

2.3. Antifungal agent

Carvacrol was purchased from Sigma. Then stock solutions were aliquoted and kept at –70°C until use.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from a 100 mg of *A. flavus* which transferred to the 1.5 microtube containing RNeasy Plant Mini RNA Kit. The integrity and purity of the isolated RNA were verified by agarose gel electrophoresis. The RNAs concentrations were calculated by measuring the absorbance at 260 and 280 nm using a spectrophotometer. Complementary DNA (cDNA) was produced applying the Superscript II Reverse transcriptase kit according to the manufacture's instruction.

2.5. Determination of minimal inhibitory concentration (MIC)

For MIC determination we followed NCCLS (National Committee for Clinical Laboratory Standards) approved method using standard broth microdilution (27, 28). Sterile microtiter 96-plates containing flat-bottomed wells were used. The stock conidial suspension (106 spores/ml) was diluted to a final inoculum concentration range of 0.3×10^4 to 5×10^4 CFU/ml and distributed into the microdilution wells. Each well contained 100 µL of RPMI 1640 as a culture medium, 100 µL of serial dilutions of the carvacrol as the antifungal drug, and the spore suspension (0.3×10^4 to 5×10^4 CFU/ml) together with distilled water to adjust the final volume to 3 mL. Two wells holding RPMI 1640 and distilled water in common with spore suspension were utilized as a positive control to provide growth controls and two other wells containing RPMI 1640 and distilled water together with a nontoxigenic fungal extract were used as a negative control to provide sterility. The final concentrations of carvacrol were serially diluted from 0.8 to 0.2 µg/mL and added into the microdilution wells, then microdilution plates were sealed and incubated at 35°C for 72 hours. To determine the growth of microorganisms, the optical density (OD) of each 96-well was measured by a microplate spectrophotometer set at 540 nm wavelength, and the MIC was identified as the lowest carvacrol concentration causing at least 50% growth failure compared with controls (8).

2.6. Determination of minimal fungicidal concentration (MFC)

After MIC determination, the 96-plates were shaken for ten seconds; next, a 50 µL was removed from each well indicating no fungal growth and seeded on the PDA and incubated at 35°C for one week. To determine MFC, the number of colonies forming units (CFU) per plate was counted using a colony counter. The MFC was identified as the lowest drug concentration from which less than 3 colonies (equal to 99% death rate of microorganism) were formed on the agar plate or the lowest drug concentration of carvacrol at which ≥ 99.99% of the initial inoculum was killed (10).

2.7. Evaluation of expression of aflR gene by RT-PCR method

The effect of carvacrol on the expression of *A. flavus* aflatoxin synthesis gene (*aflR*) was defined using the RT-PCR method (29). The *A. flavus* was grown with or without the inhibitory concentration of carvacrol (0.02%) in10 mL of PDB at 25◦C for 7 days. RT-PCR was done with primers 1 and 2 ('5-TATCTCCCCCCGGGCATCTCCCGG-3' and '5-CCGTCAGACAGCCACTGGACACGG-3), respectively. Comparative gene expression was defined by the relative

critical threshold method using a 7500 Fast Real-Time PCR system (Applied Biosystems). Data were standardized to the β-tubulin as endogenous control and the level of *aflR* gene expression between samples with or without the carvacrol was concluded. Experiments were performed in replicates.

2.8. Statistical Analysis

Data analysis was performed using SPSS version 22.0. The data of MIC, MFC, and RT-PCR were statistically analyzed by two-tailed t-test and one-way ANOVA using the GraphPad Prism software package. The variations between different groups of the fungal isolates were examined using Duncan's test and values considered significant when $p<0.05$.

3. Results and discussion

The MIC value of carvacrol against *A. flavus* showed the highest antimicrobial effect of concentration at 0.8 µg/ml in group F1 while were in lower concentration at 0.4 µg/ml for samples F9 and F11 (Fig. 1).

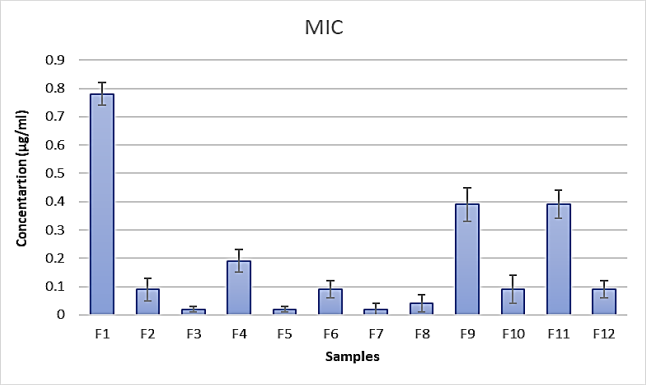

Fig. 1. The results of MIC experiment.

The results of MFC experiment demonstrated that carvacrol`s concentration was at 3 µg/ml (for sample F1) and in less than 1µg/ml concentration (for sample F9 and F11) which display significant antimicrobial activity against *A. flavus* (Fig. 2).

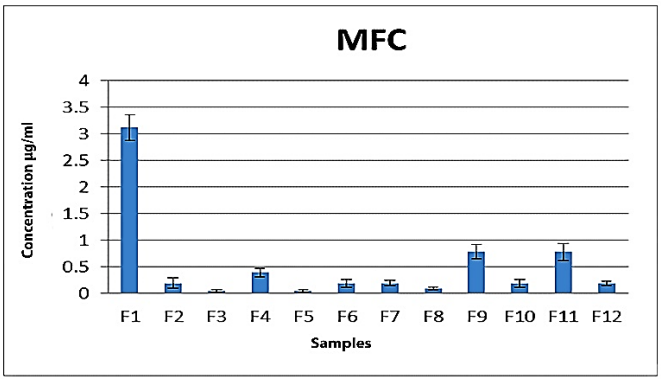

Fig.2. The results of MFC experiment.

For samples F1, F9 and F11, the MFC determined for carvacrol was almost three times of their MIC value. The results of MIC and MFC indicated a significant difference with the control group ($p<0.05$). Both MIC and MFC experiments were done in replicates and the results showed consistency. The results in Fig. 3 demonstrated the expression of aflatoxin *aflR* gene on *A. flavus* without and with the inhibitory effect of carvacrol. The mean expression level of *aflR* gene, was significantly lower in the presence of carvacrol than that of the non-carvacrol (33% and 67%), respectively.

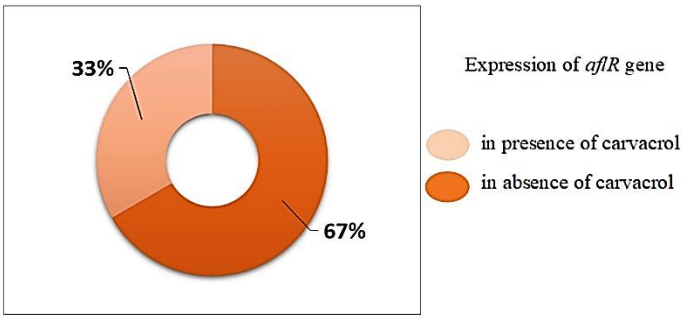

Fig. 3 The expression of *aflR* gene in *Aspergillus flavus* with and without the inhibitory effect of carvacrol.

Fig. 4 indicated the RT-PCR products on gel electrophoresis. Ten µL of each amplified product was loaded into all wells on 2% agarose gel. The wells 2, 3, and 4 showed the 100 bp ladder, positive and negative control respectively. The wells 5 to 16 were used for RT-PCR products as S1, S2, S4, and S9 indicated the non-expression of *aflR* gene in presence of carvacrol, S3, S5, S6, S7, S8, and S10 showed expression of *aflR* gene in absence of carvacrol. Table 1; show the results of MIC and MFC tests (µg/ml). This table indicates the high antimicrobial effect of carvacrol on *A. flavus* growth.

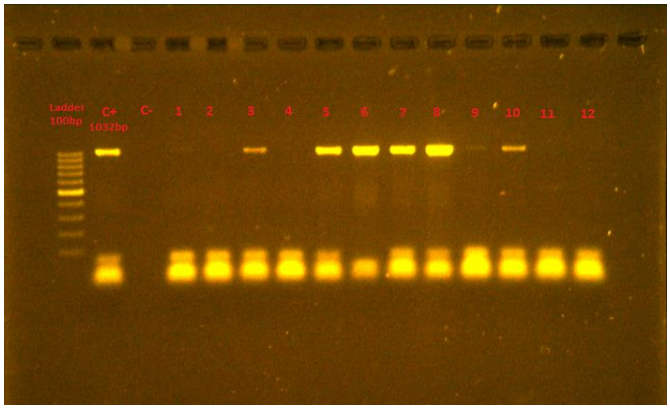

Fig.4. The RT-PCR products on gel electrophoresis. The wells S1, S2, S4 and S9 indicated the non-expression of *aFlR* gene in presence of carvacrol while wells S3, S5, S6, S7, S8 and S10 showed expression of *aFlR* gene in absence of carvacrol.

In the present study, we evaluate the inhibitory effect of carvacrol against the growth of *A. flavus*, as well as its inhibitory effect on expression of aflatoxin-related (*aflR*) gene.

The results of MIC and MFC demonstrated that carvacrol has both preventive activities to inhibit *A. flavus* growth and reduce the aflatoxin related gene expression. Growing evidence has revealed the antifungal and antimicrobial effect of some plant active compounds as a source of natural preservatives in food (1, 15, 30). These results open a bright window into the food industry to extend the shelf life of canned food, as well as satisfy many consumers who looking for food without artificial preservatives. Fungi is the main problem at all stages of the food processing chain due to its ability to grow in severe conditions. Aspergillus is general contamination of agricultural products and a major threat to processed food as a result of its mycotoxin production (19, 31, 32). Aflatoxins are the serious threats to human health, either by direct consumption of poisoned food or by aflatoxins in derived food such as milk (33). Recent investigations identified the antimicrobial and antifungal activities of several plant ingredients. Carvacrol was found to be significantly protective against *Aspergillus flavus* growth (7, 10, 15). Razzaghi-Abyaneh et al. (34) have investigated the essential oil of some pharmaceutical plants to test the extract inhibitory activity on fungus growth and subsequent aflatoxin contamination. Among medicinal plants tested, *Thymus vulgari* and *Citrus aurantifolia* were observed to prevent both aflatoxin production and *A. parasiticus* growth. The same results also observed that carvacrol can prevent *Aspergillus flavus* growth in various doses, however, the most effective dose concentration was found at 0.015 µg/mL. Furthermore, a study by Fani-Makki et al. (35) examined the effect of three plant extracts including, *Thymus vulgaris*, *Silybum marianum*, and *Aloe vera* on *Aspergillus flavus* growth along with aflatoxin B_1 production. They conducted MIC and MFC approaches to evaluate the antimicrobial effect of the mentioned active compound. Their results were in accordance with the present study that showed the positive antimicrobial effect of carvacrol. Abbas Zadeh et al. (36) have examined the antifungal activity of carvacrol, thymol, menthol, and eugenol against various fungal isolates. Among tested compounds, the most efficacy was exhibited by carvacrol. They also concluded that these plant components might be considered as a substitute for synthetic fungicides in the food industry. The results gained on the biological properties of some EOs showed better antimicrobial activity of carvacrol. This was supported by several researches, for example, Nguefack et al. (37) found that essential oil of thyme (carvacrol is the main contributor to EO of the thymus) reduced 81% of the *Aspergillus flavus* growth. Moreover, it has been demonstrated that potent antimicrobial activity might be related to EOs comprising a high proportion of thymol (*Thymus vulgaris*). It is probably due to thymol components that may interfere with cell wall enzymes of the fungus (37). Zambonelli et al. (38) have reported that thymol was associated with cell damage by changes in normal conditions of the cell such as enhancing the number of vacuoles of the cytoplasm and accumulation of lipids precipitations in the plasma membrane. In another study by Rasooli et al. (39) was showed the permanent destruction to cell wall and cell organelles such as mitochondria which treated by EOs of *Thymus vulgaris*.

4. Conclusions

To sum up, this study demonstrates the potential of carvacrol as a natural preserver against *Aspergillus flavus* growth and aflatoxin production in food contamination and foodborne diseases ($p<0.05$). Furthermore, carvacrol down-regulated the aflatoxin *aflR* gene in *Aspergillus flavus*. It would be of interest to evaluate aflatoxin production by the HPLC method. Moreover, studies using other plant-derived active compounds are proposed to test their inhibitory effect on aflatoxin production.

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