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Investigation of the effects of carvacrol and nanocarvacrol on the expression of aflatoxin biosynthesis pathway genes in Aspergillus parasiticus using RT-qPCR

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A B S T R A C T

Introduction: Carvacrol is an important medicinal compound from the marjoram plant, whose antimicrobial properties on pathogenic and toxin-producing fungi have received little attention so far. Access to medicinal plants with nanocomposites provides the possibility of improving the properties and increasing the efficiency of medicinal plants in preventing the production of fungal toxins such as aflatoxin, given the limited availability of antifungal drugs and their resistance in the food and pharmaceutical industries. This study aimed to evaluate the effect of the antifungal properties of carvacrol and nanocarvacrol on the expression of aflatoxin biosynthesis genes, aflS, aflQ, aflD, and aflR, of Aspergillus parasiticus using RT-qPCR.

Materials & Methods: The standard strain of toxin-producing Aspergillus parasiticus (PTCC 5018) was used. SDA (Saboro Dextrose Agar) medium was used to evaluate the minimum inhibitory concentration (MIC). PDB (Potato Dextrose Agar) medium was used to perform HPLC and aflatoxin production. Dynamic light scattering (DLS) and Zeta analysis were used to examine the size and shape of particles with a scanning electron microscope to confirm the carvacrol nanoemulsion. To measure the expression of aflR, aflS, aflQ, and aflD genes, RNA extraction was performed using the guanidine isothiocyanate method. For cDNA synthesis, primers for afl genes and the 18S rRNA internal control gene were designed, and after cDNA synthesis, the RNA of the samples was examined using the RT-qPCR technique.

Results: In the presence of carvacrol and nanocarvacrol, the highest inhibition of fungal growth was observed at concentrations of 0.97 and 97 µg/µl, respectively. Using HPLC, a significant reduction in toxin production was determined by nanocarvacrol compared to carvacrol. The results of RT-qPCR also proved that nanocarvacrol inhibited aflatoxin-producing genes more than carvacrol at the molecular level.

Conclusion: The results showed that nanocarvacrol can inhibit fungal growth more than carvacrol and significantly reduced the expression of genes involved in aflatoxin biosynthesis in Aspergillus parasiticus.

Keywords: Nanocarvacrol, Aspergillus parasiticus, Gene expression, Aflatoxin biosynthesis, Zeta analysis

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Introduction

Human fungal diseases are recognized as a major phenomenon of the 21st century. The use of corticosteroids and antimicrobial drugs in clinical medicine has increased the frequency of fungal diseases. The administration of antimicrobial agents and the use of medical devices in patients with severe immune system conditions have made these patients particularly susceptible to fungal diseases (1). Today, a large number of antifungal drugs are produced in the world. However, the main problem is drug resistance to these treatments. Unfortunately, there is still a long way to go in the fight against aflatoxin. For this reason, finding new treatments or substances that have antifungal effects is always of interest to researchers in various fields (2).

Fungi of the genus Aspergillus are widely distributed in the environment in the form of conidia. These fungi are part of the natural flora of water and soil and cause widespread pollution. Aspergillus is relatively heat-resistant and has high adaptability. Aflatoxin is a natural fungal toxin that originates from species such as Aspergillus flavus and Aspergillus parasiticus. These toxins can be carcinogenic if ingested through food (3). There are different types of aflatoxins, such as aflatoxin B1 and B2 or aflatoxin G1 and G2. The importance of aflatoxin toxin studies is because humans are exposed to the risks of the toxin by consuming foods contaminated with Aspergillus fungi. Since it is not easy to prevent the growth of fungi in foods, it is difficult to prevent fungal diseases in humans and animals (4).

Most of the genes associated with aflatoxin are grouped into 75 regions of the genome. The nor-1, ver-1, omt-A, aflR, aflS, aflQ, and aflD genes are required for aflatoxin production. aflR plays an important role in regulating aflatoxin production in Aspergillus flavus and Aspergillus parasiticus. The aflR protein contains the GAL4 type, which is transcriptionally involved in the biosynthetic pathway of many structural genes such as ver-1 and nor-1 as an activator (30, 5). The genus Origanum belongs to the Lamiaceae family and is considered an evergreen perennial aromatic medicinal plant. Carvacrol is known as an important phenolic compound found in the origanum plant. Carvacrol is known as a potent antioxidant, anti-oxidative stress, and anti-inflammatory agent. The neuroprotective, hepatoprotective, and renal effects of these polyphenolic substances have also been identified in various disease models. The protective role of carvacrol, the main compound of the oregano plant, on various biological systems can be investigated in a model of disease caused by Aspergillus parasiticus aflatoxin (6, 31, 32). Currently, the biosynthesis of nanoparticles is available as a simple, biocompatible, cheap, and safe method to replace chemical and physical methods (7, 33). Several physical and chemical methods have been investigated to produce metal nanoparticles on a commercial scale to achieve biological and non-biological goals (8, 34).

Nanocarriers can protect plant compounds from environmental factors such as light, heat, and moisture and increase their stability. They can also increase the absorption and availability of active compounds of medicinal plants in the body. By using nanocarriers, the drug can be precisely directed to the desired area in the body and prevent possible side effects. Nanotechnology allows for precise control of the release rate of active compounds from nanocarriers, thereby increasing drug efficacy. Many herbal compounds have poor absorption due to their low water solubility. Nanotechnology can increase the solubility of these compounds by creating nanoparticles, and by increasing stability, absorption, targeting, and

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release control, the overall efficacy of herbal medicines is increased (9).

In 2019, a study by Karim et al. investigated the industrial importance of mountain basil essential oil with nano-coated chemical characteristics against fungi contaminating herbal raw materials and secreting aflatoxin B1. Basil nanocoated with chitosan showed increased antifungal and anti-aflatoxigenic potential compared to basil without nano. The nanocoated basil plant reduced ergosterol content. This study recommended the use of nano-basil to increase the shelf life of stored herbal raw materials (9). In 2018, a study was conducted to investigate the role of carvacrol and thymol nanoemulsion, which have a wide range of biological effects, including antispasmodic, anti-inflammatory, and anticancer. Carvacrol and thymol also have strong odor, taste, and antioxidant effects. Therefore, the biological effects, along with their favorable toxicity, make thymol and carvacrol an additive to prevent microbial spoilage of foods and potent antimicrobial agents against antibiotic-resistant bacteria (10).

The aim of this study was to investigate the antifungal properties of carvacrol and nanocarvacrol on the fungus Aspergillus parasiticus. Nanotechnology using nanoparticles allows for the improvement of the properties and increased efficacy of medicinal plants. By creating nanocarriers for plant compounds, this technology allows for the targeting of drugs, increased stability, and controlled release in the body. In this way, the effectiveness and efficiency of herbal medicines in pharmaceutical and medical applications increase, and the effect of these compounds on the expression of aflatoxin biosynthesis genes, aflS, aflQ, aflD, and aflR, was determined using the RT-qPCR method.

Materials and methods

Preparation of fungal strains, culture medium, carvacrol, nanocarvacrol

A standard strain of toxin-producing Aspergillus parasiticus (PTCC 5018) was used. This strain produces aflatoxins B1, B2, G1, and G2. Saborodextrose agar (SDA) medium was purchased from Merck, Germany, and for fungal cultivation, Aspergillus parasiticus samples were cultured in Sabouraud dextrose agar (SDA) medium and kept in an incubator at 30°C. Carvacrol is used industrially with 100% purity. Carvacrol, the main component of marjoram essential oil, was prepared from Sigma, UK. The compound was diluted with dimethyl sulfoxide (DMSO) solution from Merck, Germany, and after preparing a liquid suspension, it was added to the liquid fungal culture medium at different concentrations (11).

To synthesize the carvacrol nanoemulsion, first 0.2 ml of Sigma carvacrol (UK) and 0.8 ml of Tween 80 surfactant (Merck, Germany) were mixed together and stirred at 250 rpm for ten minutes. Then 2.33 ml of deionized water was added to it, and the resulting mixture was homogenized again at 250 rpm for ten minutes. Then the resulting emulsion was subjected to ultrasonication for half an hour at a power of 60 kHz. To confirm the carvacrol nanoemulsion, DLS (dynamic light scattering) experiments and Zeta analysis were used to examine the size and shape of particles with a scanning electron microscope (12, 25, 35).

Evaluation of the antifungal susceptibility of carvacrol and nanocarvacrol

Based on the CLSI reference protocol, the microplate dilution method and 96-well plate were used to determine the MIC of the nanoemulsion containing carvacrol and pure carvacrol. The concentration of the pure compound was 100%, and a concentration of 0.01 mg/ml was used for nanocarvacrol. In short,

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first, a seven-day culture of the fungus was prepared in a specific Sabouraud dextrose agar medium at a temperature of 28°C. In order to determine the MIC, first 100 µl of RPMI1640 medium was poured into each well of a 96-well plate, then 100 µl of the base concentration of the tested compounds was poured into the first well of the 96-well plate, and from that, twofold serial dilutions (from 5000 to 9.7 ppm for carvacrol and from 500 to 0.97 ppm for nanocarvacrol) were prepared up to well 10. All experiments were performed in triplicate (13, 26).

Performing RT-qPCR on Aspergillus parasiticus

Initially, to purify RNA from fungal samples, the Sinaclone Gene kit was used according to the manufacturer's instructions. For cDNA synthesis, the Sinaclone Kit (Cat. No.: RT5201) was used.

Briefly, 1 µl of primer was added to the microtube containing RNA that had undergone the DNA contamination removal step and was heated at 70°C for 5 min and then immediately placed on ice. Next, 4 µl of 5x buffer, 2 µl of dNTP mix (10 mM), and 20 units of ribonuclease inhibitor were added and incubated at 37°C for 5 min. Then, 200 units of reverse transcriptase enzyme were added and incubated at 42°C for 1 h. Finally, to stop the reaction, the samples were placed at 70°C for 10 min. The BIORAD RT-PCR kit was used to perform RT-qPCR. Total RNA obtained from fungi was used directly in this kit. The contents of this kit include a reverse transcriptase enzyme and a PCR master mix (including a mixture of dNTPs, a Taq DNA polymerase enzyme bound to an antibody, fluorescein, SYBR Green, and a stabilizer). According to the PCR program for this kit in LightCycler devices, first all the materials were mixed together in appropriate concentrations with total RNA extracted from the mushroom in a 0.2 ml tube and placed at 50°C for 10 minutes to make cDNA from total RNA, and after confirming that the melting temperature was a single band and also obtaining a CT value of less than 20, cDNA dilution cycles were performed to determine the reaction efficiency and standard curve (14, 27).

For normalization, small amounts of mRNA in mushroom samples were grown in tubes. In order to amplify the 18S rRNA and aflQ, aflR, aflC, and aflD genes, Karaj Technology Probe primers were used (Table 2).

Analysis of CT results was performed using the Livak or CT method according to the following formula. The number obtained indicated an increase or decrease in expression depending on the distance from the one-order number.

$$FolaChange = \frac{\left(E_{t\,\mathrm{arg}et}\right)^{\Delta Ct_{t\,\mathrm{arg}et}\,(control-sample)}}{\left(E_{ref}\right)^{\Delta Ct_{ref}\,(control-sample)}}$$

In this formula, E is the reaction efficiency, which is considered to be 2 in the optimal case. Target is equivalent to functional genes, and ref is the reference gene under study. The ratio also shows the order of gene expression changes. Statistical analysis was performed using one-way ANOVA. ($P < 0.05$) was considered significant (15, 27).

Table 1. Names and sequences of primers used for RT-qPCR test.

Gene Name	Primer (5′→3′)	Direction	Product Size (bp)	Melting Temperature (°C)
alfR	CCTTTCTCACTACTCGGGTTT	F	21	59
	GCAGGTAATCAATAATGTCGC	R	21	57
alfC	TGCATGGCGATGTGGTAGTT	F	20	58

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	GTAAAGGCCGCGGAجAGAAAG	R	19	59
alfQ	GCACCAACAATTCGGCTCTG	F	20	60
	TGTGGAAGGGTGAAGATGC	R	20	60
alfD	ATGCTCCCGTCCTACTGTTT	F	20	58
	—	—	—	56
	ATGTTGGTGATGGTGCTGAT	R	20	—
18S rRNA	GCTCTTTTGGGTCTCGTAATTGG	F	23	93
	CGCTATTGGAGCRGGAATTACC	R	21	82

The diluted primers were stored in a -20°C freezer, and the used primers in a 4°C refrigerator. In the PCR reaction, the primers bind on both sides of the target sequence and enable the polymerase activity of the DNA polymerase enzyme to begin. Therefore, the first characteristic of the primer is its correct sequence for the desired location for amplification (16, 28).

The temperature and time program implemented for RT-qPCR was obtained according to Table (2):

Table 2. RT-qPCR temperature conditions for gene expression.

Stage	Temperature (°C)	Time	Cycles
Initial Denaturation	94	30 s	
Annealing	60	30 s	35
Extension	72	30 s	
Final Extension	72	10 min	1
Hold	4	—	

Results

Evaluation of the antifungal properties of carvacrol and nano carvacrol

The results obtained showed that carvacrol and nanocarvacrol have a very effective and favorable inhibitory effect on various types of Aspergillus parasiticus fungi.

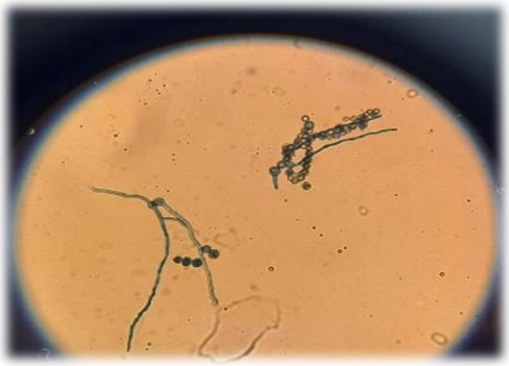

Figure 1. Microscopic view of the fungus Aspergillus parasiticus under the influence of carvacrol at 100x magnification.

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Figure 1. Microscopic view of the fungus Aspergillus parasiticus under the influence of nanocarvacrol with magnification of 100.

B- Results of the minimum growth inhibition test (MIC): Aspergillus parasiticus fungus was kept in a 30°C incubator under the influence of carvacrol and nanocarvacrol in SDA medium for two days, and after this period, macroscopic examination of the microplates showed that with increasing concentrations of carvacrol and nanocarvacrol, fungal growth also decreased. The concentration of the pure compound was 100%, and a concentration of 0.01 mg/ml was used for nanocarvacrol. After seven days of mushroom cultivation in a specific Sabouraud dextrose agar medium at 28°C, the tested compounds were poured onto the first well of a 96-well plate. At the end, the last well in which no growth was observed was considered the MIC. All experiments were performed in triplicate. Comparison of microplates with positive control demonstrated the ability of nano-carvacrol to inhibit fungal growth, and after performing several MICs, the lowest growth inhibition (MIC) was observed at 97 ppm of carvacrol and nano-carvacrol at a concentration of 0.97 ppm.

Determination of the characteristics of carvacrol nanoemulsion

According to the results, the type of surface charge is negative, which depends on the emulsifier, and its surface charge is 16.5 mV, which is acceptable on a scale. SEM images showed the spherical morphology of particles and an average particle size of about 80 nm. Comparison of microplates with positive control proved the ability of nanocarvacrol to inhibit fungal growth, and after performing several MIC tests, the lowest growth inhibition (MIC) was observed at 97 ppm of carvacrol and nanocarvacrol at a concentration of 0.97 ppm. DLS analysis was used to determine the size of the synthesized nanoemulsion. According to the results, the sample has a size of 88.5 nm, which is close to the ideal value according to the results announced in numerous articles (Table 3). Also, to determine the type of surface charge and the degree of stability, Zeta analysis was performed on the sample. According to the result, the type of surface charge is negative, which depends on the emulsifier, and its surface charge is 16.5 mV, which is acceptable (Table 4). SEM images showed the spherical morphology of particles and an average particle size of about 80 nm (Figure 3).

Table 3. Determination of the size of synthesized nanoemulsion by DLS analysis method.

Maximum Number	S.P. Surface Ratio	Total Mean	Mean	Mode
1	1	5.85 nm	8.16 nm	7.87 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1	5.85 nm	8.16 nm	7.87 nm

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Table 4. Determination of surface charge type and stability, zeta analysis.

Maximum Number	S.P. Surface Ratio	Total Mean	Mean	Mode
1	1	5.85 nm	8.16 nm	7.87 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1	5.85 nm	8.16 nm	7.87 nm

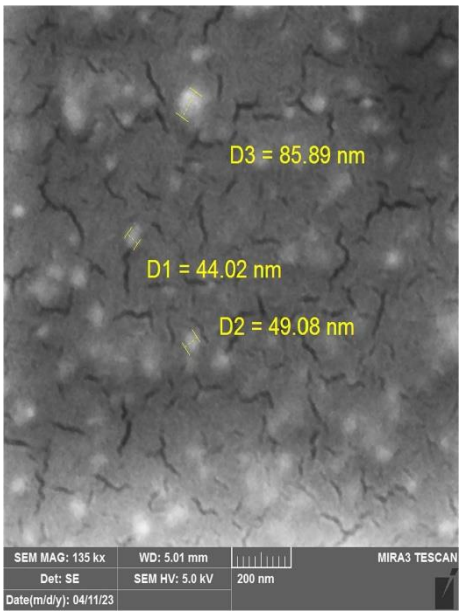

Figure 4. Electron microscope image of particles.

Results of expression of aflR, aflD, aflQ, aflC genes producing aflatoxin after and before the effect of carvacrol and nanocarvacrol by RT-qPCR

According to the results obtained by the RT-qPCR method and according to the obtained graphs, the black graph in the figure shows the upward and increasing expression of each of the studied aflatoxin genes alone and in pure culture, and the downward gray graphs show the decreasing expression of aflatoxin genes in the presence of carvacrol and nanocarvacrol. The obtained results also confirm the greater and stronger decreasing effect of nanocarvacrol on the expression of aflatoxin genes aflR, aflD, aflQ, and aflC (Figures 4 and 5).

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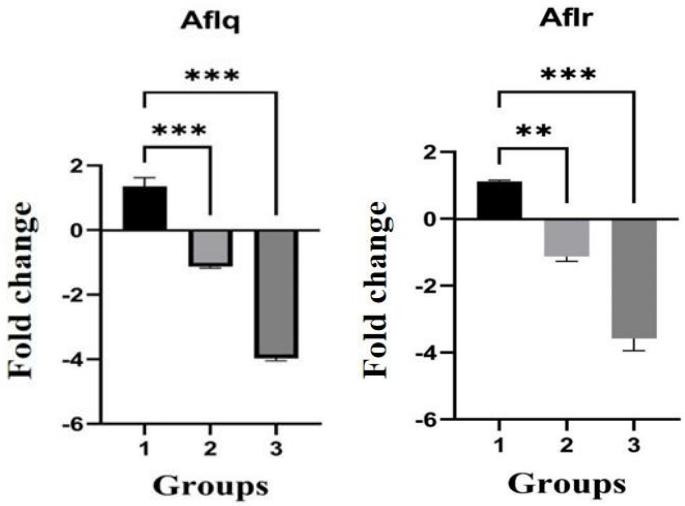

Figure 4. Amplification graphs of R and Q afl genes in Aspergillus parasiticus containing carvacrol and nanocarvacrol and control sample.

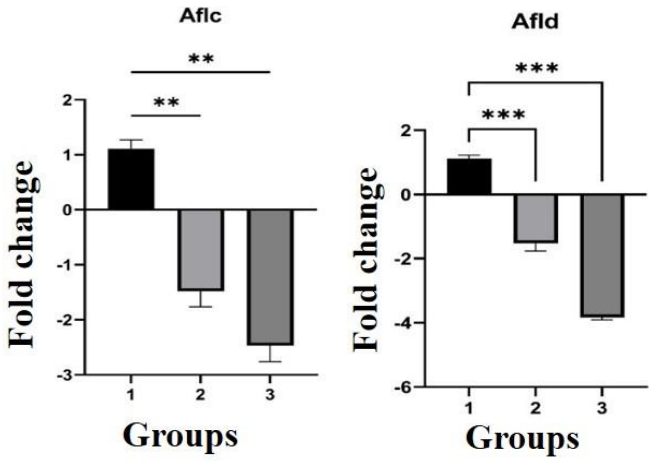

Figure 5. Amplification graphs of D and C afl genes in Aspergillus parasiticus containing carvacrol and nanocarvacrol and control sample.

Analysis of RT-qPCR results of aflR, aflD, aflQ, aflC genes

The threshold cycle, or CT, of the expression of aflR, aflD, aflQ, and aflC genes in the presence of carvacrol and nanocarvacrol was performed on the positive control sample of Aspergillus parasiticus (Figure 6). In this graph, the expression of aflR, aflD, aflQ, and aflC genes was examined using the CT analysis method compared to the positive control. According to the threshold cycle graph or CT graph, the results of gene analysis were obtained. According to the above graph, the lower the expression of the tested genes, the higher the level of the CT curve of the genes will be compared to the graph of the internal control gene 18S rRNA, which confirms the lower level of expression of the genes in the above study, considering that the CT graph of the experiment has an increasing curve and a higher level (Figure 6).

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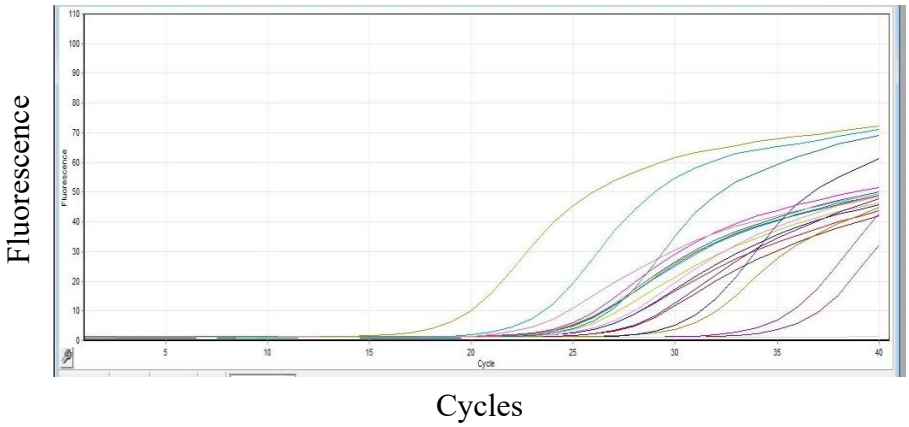

Figure 6. CT analysis curve diagram examining the expression of aflR, aflD, aflQ, aflC genes.

Standard melting curve diagram

It is used to check the non-specificity of the fluorescence emitted from the amplified sample. Since the fluorescent dye Cybergreen is able to bind non-specifically to all double-stranded DNA and emit light (Figure 6), this radiation becomes positive for all replicated strands. The longer the DNA length, the higher the melting temperature. In the absence of contamination, the melting temperature curve is correct in that all peaks of a gene should be at the same temperature, but the height of the peaks does not need to be the same, which indicates the correctness of the melting temperature diagram. According to the standard melting temperature curve of the RT-qPCR reaction, the melting temperature curves of the aflR, aflD, aflQ, and aflC genes match correctly (Figure 7) (16, 38).

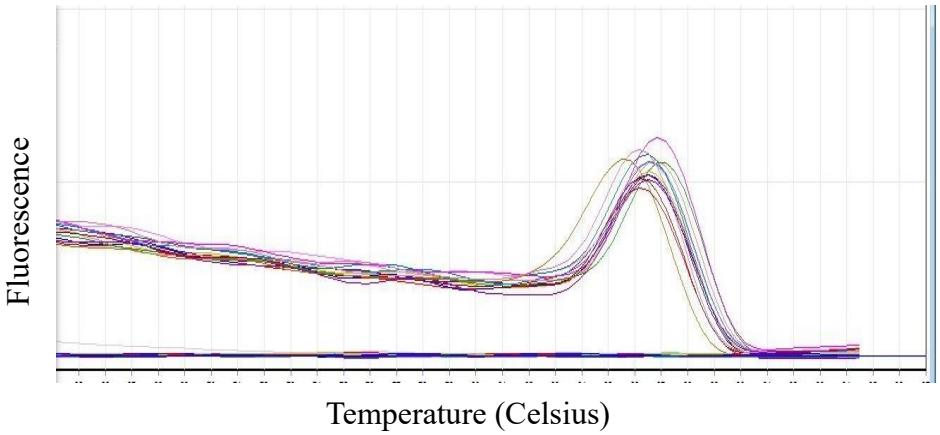

Figure 7. Melting temperature curve diagram of aflR, aflD, aflQ, aflC genes.

Discussion

Aflatoxin is a large group of mycotoxins. Today, widespread contamination of agricultural products and animal feed with aflatoxin is considered a threat to human and animal health. Aflatoxin can cause acute or chronic poisoning in humans and animals after consumption of contaminated food products such as wheat, barley, cereals, cottonseed, and peanuts. Aflatoxins are secondary metabolites produced by a group of fungi, including Aspergillus, Penicillium, Fusarium, and Alternaria (17, 36, 37). Since fungi of the genus Aspergillus are widely distributed in the environment, they produce aflatoxin when exposed to suitable

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growth conditions, including temperature, humidity, and an appropriate environment in the fields or during storage and warehousing of agricultural products, and thus enter the human and animal food chains.

In recent years, studies on plant extracts and compounds have shown remarkable effects in cases such as reducing microbial growth and affecting microorganisms in not producing toxins. Among the medicinal plants whose antimicrobial and antifungal effects have been proven is the marjoram plant. According to the results of this study, carvacrol, as an important compound of the marjoram plant, has shown the ability to inhibit the growth of the fungus A. parasiticus and also prevent the production of various aflatoxins. Phenolic compounds in this plant, along with the alcoholic extract, contain succinic acid, gluconic acid, glucose, and rhamnose, and its aqueous extract contains malic and oxalic acids (17). Easy biopreparation of nanoparticles with control of size and shape is of great importance in the presented methods. There are many plants that have the ability to make nanoparticles and use them in such a valuable and expensive industry. Considering that the medicinal compound carvacrol from the marjoram plant has the ability to synthesize nanoparticles and also that the use of this plant for the biological reduction of ions has not been reported so far, the results of this study clearly show the good function of this plant for the first time (18).

Previous studies have shown that the synthesis of nanoparticles using parsley flower extract was a biocompatible method. The obtained nanoparticles were examined by spectrophotometry, X-ray diffraction, and transmission electron microscopy. They reported that the size of the triangular particles was about 200 nm and the spherical particles were smaller and about 8-10 nm (19). However, in the present study, the carvacrol nanoparticles were produced with a size of about 80 nm and in an almost spherical shape.

Recent research has shown that combinations of Juniperus procera fruit extract with silver nanoparticles are effective against Aspergillus fumigatus, Fusarium, and Chlamydosporum. The combination of silver nanoparticles with J. procera fruit extract effectively inhibited the production of the mycotoxins gliotoxin and nivalenol compared to the extract alone. This is consistent with the fact that in the present study, the production of aflatoxin was reduced more in the presence of nanocarvacrol than in pure carvacrol alone (40).

In 2021, Bafghi et al., in their study of the effect of plant essential oils along with silver and selenium nanoparticles on the growth of Aspergillus and Candida, showed that the MIC level was significantly reduced using nanomaterials. Comparison of the results showed that even these nanoparticles have a better effect than the antifungal drug group in some cases. Therefore, the use of biosynthesized nanoparticles is more convenient and cost-effective due to the increased drug resistance of opportunistic and pathogenic microorganisms compared to the targeted drugs applied. In the present study, nanocarvacrol also proved its antifungal effects on the fungus Aspergillus parasiticus.

Similar results have been obtained from various studies, which show that the compounds obtained from this plant contained 49% carvacrol. The results also indicated the antioxidant properties of the essential oil. The antioxidant power and antimicrobial properties of the essential oil were affected by the presence of phenolic compounds in the essential oil. In the present study, carvacrol was used in terms of antimicrobial properties on Aspergillus parasiticus under the influence of phenolic substances (19).

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In another study in 2023 by Moghadasi et al., the effect of aloe vera, carvacrol, thymol, and nanocarvacrol on the growth and production of aflatoxin B1 in Aspergillus parasiticus and Aspergillus flavus was investigated. In this study, carvacrol and nano-carvacrol caused a very significant reduction in both mycelial growth and aflatoxin production. Thus, the results of the MIC of nano-carvacrol are similar to the present study (20). The aflR gene is a positive regulator gene in aflatoxin biosynthesis and requires aflR alleles in the first step, when neurosulonic acid is formed (41). This gene is required for the transcription of most structural genes in Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nidulans. In the present study, considering the expression level of afl genes and the observation of a decrease in gene expression both in the presence of carvacrol and in the presence of nanocarvacrol, where a decrease in aflatoxin gene expression was more pronounced, we conclude that there is a clear agreement (20).

In a study conducted on the effects of growth and aflatoxin production in Aspergillus niger using tea extract, it was shown that the reduction of aflatoxin B1 was affected by the extract. In addition, genomic sequence analysis showed that the fungus is considered safe due to the absence of pathogenic genes and gene clusters for the synthesis of mycotoxins. These results indicate that the RAF106 gene of Aspergillus niger induced by tea extract has promising potential for commercial application in the pharmaceutical and food industries for AFB1 detoxification. In the present study, using RT-q PCR and downregulation of toxin-producing genes using carvacrol, there is agreement (42).

In a study conducted by Aljelehawy et al. in 2023, the antifungal activity of carvacrol and nanocarvacrol, as well as anticancer activity and inhibition of the growth of fungi, viruses, and bacteria, was evaluated. The aim was to investigate the extent to which the antifungal activity of carvacrol depends on the test method used. In this study, a toxin assay was not performed, but the effect of carvacrol and nanocarvacrol was performed. Thus, loading of carvacrol by lipid and polymer nanocarriers showed effective therapeutic activity in vivo and in vitro. According to the present study, the use of carvacrol nanoparticles was seen to have a significant effect on reducing the growth of Aspergillus parasiticus (44). In another study, the effect of Chinese bell pepper and black pepper on reducing the expression of aflQ, aflD, aflC, and aflR genes of aflatoxin Aspergillus parasiticus was investigated. According to the results obtained, a decrease in the expression of the above genes was evident in the presence of extracts of these plants and is consistent with the present study (22).

In another study, it was shown that the use of plant compounds along with nanoparticles had a significant effect on reducing aflatoxin production. Accordingly, different ratios of plant compounds (citral (C), geraniol (G), and terpineol (T)) were prepared and encapsulated inside nanochitosan. This study recommended its use as a plant antifungal agent for managing the growth of fungal contamination and AFB1 in agricultural food products (23).

Under the influence of concentrations of 9.7 ppm carvacrol and 9.7 ppm nanocarvacrol and within a period of seven days, the morphology of the fungus Aspergillus parasiticus changes. These changes are in the form of reduced mycelium production, reduced conidial heads, reduced conidia, and a change in colony color from green to white. The results of this study showed the inhibitory effects of carvacrol and nanocarvacrol from oregano essential oil on the expression of aflQ, aflD, aflC, and aflR genes encoding aflatoxin in the fungus Aspergillus parasiticus, which was reduced by the RT-qPCR technique.

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It is noteworthy that the importance of this study compared to similar works carried out; the impact and importance of nanotechnology and the use of nanoparticles together with carvacrol as an antifungal herbal medicine and its greater effectiveness over carvacrol to prevent and reduce the expression of aflatoxin-producing genes in the fungus Aspergillus parasiticus; and, in general, the importance of using nanoparticles together with antifungal herbal medicines.

The present study clearly shows that nanocarvacrol has a significant advantage in inhibiting the growth of Aspergillus parasiticus and the expression of key genes of the aflatoxin biosynthesis pathway compared to its free form. This advantage is mainly due to the unique characteristics of the nanoemulsion form (approximately 80 nm), suitable negative zeta potential (-16.5 mV), and spherical morphology, which leads to increased contact surface area, improved permeability through the fungal cell wall, and controlled release of the active ingredient. From a mechanism of action perspective, nanocarvacrol causes fungal cell death by disrupting the integrity of the cell membrane, inducing oxidative stress through the production of reactive oxygen species (ROS), and inhibiting cell signaling pathways. At the molecular level, a significant decrease in the expression of the master regulator gene aflR (more than 8-fold) indicates the direct targeting of this nanoformulation at the top of the regulatory cascade of the aflatoxin biosynthesis pathway. This cascade effect leads to a significant reduction in the expression of structural genes aflD, aflQ, and aflC, which are involved in key steps in neurosulfuric acid conversion and methylation, respectively. This multi-point inhibition pattern is of great importance because it minimizes the possibility of resistance development in the fungus. Comparison of these findings with previous studies, including Moghadasi et al. (2023), who reported a decrease in mycelial growth and aflatoxin B1 production, and Buitimea-Cantua et al. (2020), who showed inhibition of aflR and aflD gene expression by phenolic compounds, is in full agreement. Furthermore, the findings of this study are in line with recent work on carvacrol nanoformulations, including the study of Rostami et al. (2025), who showed effective protection of pistachios during storage, and the study of Alizadeh et al. (2025), who reported enhanced antifungal activity through nanoencapsulation in chitosan. Therefore, the use of nanotechnology not only increases the antifungal potency of carvacrol by up to 100-fold based on quantitative comparisons but also reduces the potential for side effects by reducing the consumption of the active ingredient and providing more specific targeting. These features make nanocarvacrol a promising candidate for application in the food and agricultural industries as a natural and safe alternative to conventional chemical compounds. However, further studies at the proteomic and metabolomic levels are recommended to better understand the molecular mechanisms, as well as in vivo studies on real food products to evaluate the practical efficacy of this nanoformulation.

Conclusion

In general, the results of the present study proved the antifungal effect of nanoparticles containing carvacrol with appropriate physicochemical properties and increased drug stability on Aspergillus parasiticus. All concentrations of nanocarvacrol used had an inhibitory effect on fungal growth. Also, in the present study, the minimum lethal concentration of nanocarvacrol was higher than that of carvacrol on fungal aflatoxin toxins, which may indicate that the active ingredients of nanoparticles with carvacrol are more effective and can be a promising antifungal agent with high antifungal effects and low side effects. Nanocarvacrol shows promise as a natural preservative in food industries, a

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solution to antifungal resistance due to its multi-point mechanism, and a model for enhancing the efficacy of herbal medicines through nanotechnology. Finally, it can be concluded that nanocarvacrol is a promising candidate for the development of new-generation antifungal agents with high efficacy and low side effects, which has great potential for application in integrated management strategies for fungal pests and diseases.

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CRediT authorship contribution statement

Conceptualization, Methodology, Validation, Formal Analysis, Investigation: SM, Resources, Software, Data Curation: DN Writing–Original Draft Preparation, Writing–Review & Editing, Visualization, Supervision, Project Administration: AK, AS.

Conflict of interest

The authors declare no conflict of interest.

Ethical considerations

As this study did not involve the use of human or animal subjects, obtaining an ethics approval code was not applicable. The authors avoided data fabrication, falsification, plagiarism, and misconduct.

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