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RESEARCH ARTICLE

The effect of Propolis on inhibition of *Aspergillus parasiticus* growth, aflatoxin production and expression of aflatoxin biosynthesis pathway genes

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Abstract

Background and purpose Aflatoxins are one of the most important mycotoxins, which have been classified as Group I carcinogenic compounds by the International Agency for Research on Cancer. This investigation aimed to examine the effect of Propolis on inhibition of the *Aspergillus parasiticus* growth, aflatoxin production and expression of aflatoxin biosynthesis pathway genes.

Materials and methods A standard strain of *Aspergillus parasiticus* (ATCC 15517) was used to perform antifungal susceptibility test, using a microdilution method in accordance with the CLSI M38-A2 guidelines. The aflatoxin concentrations in the control and treated media were determined by HPLC. Also, the quantitative changes in the level of *nor-1*, *ver-1* and *omtA* genes expression in aflatoxin biosynthetic pathway were analyzed using Real-Time PCR method.

Results The results showed that the minimum inhibitory concentrations (MIC) of propolis was 100 μg/ml. The results showed that total levels of aflatoxin decreased from 386.1 ppm to 3.01 ppm at 50 μg/ml of propolis. In addition, quantitative real-time PCR analysis showed that the level of *nor-1, ver-1* and *omtA* genes expression was significantly decreased after treatment with propolis extract.

Conclusions The findings reveal that propolis extract, have a significant inhibitory effect on important genes for aflatoxin biosynthesis pathway in aflatoxin production.

Keywords Aflatoxin · *Aspergillus parasiticus* · Gene expression · Propolis

Introduction

Aflatoxins (AFs) are one of the most dangerous and known mycotoxins have characteristic of mutagenic, carcinogenic, teratogenic, hepatotoxic, estrogenic, immunosuppressive and histopathological effects for mammals [1–3]. These secondary metabolites, produced by different strains of *Aspergillus* species, under certain conditions in different foods cause various harmful diseases such as: depression, growth retardation, abdominal pain, diarrhea, immuno suppression, kidney damage, edema, hepatic carcinoma and ultimately death in human [4–6]. Four major types of aflatoxins are B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) that can cause food and agricultural commodities contamination [7, 8]. Among them, AFB_1 is the most potent natural carcinogenic compounds in mammals [9–11]. Therefore, the International Agency for Research on Cancer (IARC), AFB_1 classifies it as a group I human carcinogen [12]. Therefore, contamination of crops with aflatoxins has become of international public health concern.

Biochemical pathways and genetic regulation of aflatoxin biosynthesis in *A. parasiticus* and *A. flavus* are encoded by more than 25 genes in a 75-kb cluster. The genes *nor-1, ver-1* and *omtA* encode significant enzymes in aflatoxin biosynthesis pathway [13, 14].

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The first aflatoxin biosynthesis gene cloned was *nor-1* in *A. parasiticus* [13, 15]. The *nor-1* gene encodes a reductase/dehydrogenase enzyme, which is involved in the conversion of norsoloronic acid to averantin in aflatoxin biosynthesis. and this reaction is reversible depending on NADPH or NADH [13, 16]. The *ver-1* gene is involved in the conversion of versicolorin A to demethylsterigmatocystin by a ketoreductase enzyme [13, 17]. The *omtA* gene by encodes O-methyltransferase II in the next step of aflatoxin biosynthesis is involved in the conversion of sterigmatocystin to O-methylsterigmatocystin and dihydrosterigmatocystin to dihydro-O-methylsterigmatocystin [13, 18]. Therefore, mutation in these genes could be a suitable target for deletion of this toxin [19].

Given that contamination of food and agricultural products with mycotoxins threatens the quality of food and human health. Thus, identification of methods for eliminating and reducing mycotoxins in order to countering with production of aflatoxin and Increase the level of food safety has drawn the attention of many experts during the past years. So far from different physical, chemical and biological methods have been utilized to remove or reduce aflatoxins produced in different foods and crops. But the results concerning physical and chemical methods due to need special equipment, expensive materials and high costs have not been very satisfactory [4, 20, 21]. Today the attention of researchers has been drawn to the use of biological methods, such as: genetic engineering, natural biopreservatives and natural sources with lower toxicity for eukaryotic systems [4, 22–25]. Among the natural bioactive substances whose antimicrobial effects have been proven, in traditional medicine for centuries, propolis produced by honey bees [26]. Propolis is a natural product derived from plant resins collected by honeybees for building and preserving their hives. More than 300 different compounds of polyphenols, aldehyde phenolics, monoterpenes, amino acids, steroids and other inorganic compounds have been found in the propolis structure [27]. It has been shown that the mechanism of action of these substances through the inhibition of free radicals has strong antioxidant activity. It has also shown to inhibits the process of lipid peroxidation in the in vitro and in vivo environment [28]. Therefore, this study aimed to examine the effect of extract of propolis on inhibition of growth, aflatoxin production and expression of aflatoxin biosynthesis pathway genes in *A. parasiticus*.

Materials and methods

Preparation of samples extracts

Propolis used in this study was collected from beehives (Apis mellifera L.) from different areas of Kurdistan Province, Iran. In this study, extract of propolis was prepared according to the method used in Koc et al. study [29]. At first, 7 g of crude propolis was dissolved in 93 ml of 80% ethanol. Then, it was incubated at 50° C with shaking at 40 rpm for 72 h. The obtained aqueous ethanol extract was filtered using Whatman No. 1 (Maidstone, UK) filter paper and concentrated at 50° C. To evaporate the ethanol from the solution, Rotary (Heidolph, Germany) was used at 100 rpm and 50° C. The purified extract was kept at -20° C for 1 day and then transferred to the freeze-dryer (LTE) for lyophilization. After 24 h, the extract was dried, pulverized and stored in a closed container wrapped in aluminum foil at -20° C until next use.

Fungal strain and susceptibility test

In this study, *Aspergillus parasiticus* standard strain American type culture collection 15517 (ATCC15517) was cultured on Sabouraud Dextrose Agar medium (Merck, Germany), and was incubated at 30° C for 3 days. For evaluation determination of the minimum inhibitory concentration (MIC) of the samples was performed using a microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 methods [30]. Fungal spores from culture were suspended in distilled water. Then, their optical density was adjusted to a transmittance of 78%-82% at 530 nm wavelengths using a spectrophotometer. The final suspension was made by 1:50 dilution of the stock suspension with the RPMI 1640 medium (Sigma Chemical Co.), which resulted in a colony count of 0.4×10^4 to 5×10^4 cfu/ml. propolis solutions were prepared at the concentrations of (800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 μg/ml), and then dispensed into a 96-well microdilution plate. The next step, 100 μl of fungal suspension was added to the plates. Negative (only RPMI 1640 medium) and positive (RPMI 1640 medium and fungal spores) controls were also run alongside each experiment. The plates were incubated for 2 days at 37° C. Each experiment was repeated three times.

Detection of aflatoxin production by HPLC

The amount of aflatoxin produced by *A. parasiticus* in the presence of sub-MIC concentrations of extract was measured using high-performance liquid chromatography (HPLC). For this purpose, 5 ml of the spore suspension with a final density of 0.4×10^4 to 5×10^4 CFU/ml was added to 5 ml Potato Dextrose Broth (PDB) medium in the presence of sub-MIC values of propolis. PDB plates along with positive control (PDB with spore suspension but without extract) and negative control (PDB without spore suspension), were incubated at 35 °C for 7 days. then the contents of each well were centrifuged at 3500 rpm for 5 min. Subsequently, AFs concentration in the culture medium was detected using HPLC (Scanning Fluorescence

Table 1 Primers used in Real-Time PCR

Gene	Primer name	Sequence
nor-1	Forward *nor-1*	5'- ACGGATCACTTAGCCAGCAC-3'
	Reverse *nor-1*	5'- AATGAGACCTCGTCCGATGC-3'
ver-1	Forward *ver-1*	5'-CGGAGCCAAAGTCGTGGTTA-3'
	Reverse *ver-1*	5'- ATCGGCCTGGATTGCGATAG-3'
omtA	Forward *omtA*	5'- ACGGACCTAGTCCGACATCA-3'
	Reverse *omtA*	5'- CTCGAAAATGAAGCGCTGGG-3'
β-actin	Forward *β-actin*	5'-ACGGTATTGTTTCCAACTGGGACG-3'
	Reverse *β-actin*	5'- TGGAGCTTCGGTCAACAAAACTGG-3'

Detector Water 474) as follows: 6 ml from each supernatant was dissolved in 34 ml of phosphate buffer saline (PBS) and passed through the glass-fiber filter paper (GFFP). Then the diluted extract of 500 µl of methanol (MeOH-HPLC) was added to the column and the solution was collected in a vial. This process was stopped for 1 min and 750 µL of MeOH-HPLC was added and the resulting solution was collected in the same vial. Afterwards, 1750 µL of H2O-HPLC was added to the vial and mixed using Vortex. The column was then washed with 20 ml of PBS and finally, 200 ml of the extract was injected into HPLC. The amounts of AFs in the samples were measured at wavelength of 365 nm by comparison of the under-curved area with the authentic standards (λem = 435, λex = 365) [5].

RNA preparation and real-time PCR

In this study, quantitative changes in structural genes (*nor-1, ver-1 and omtA*) expression were measured using Real-Time PCR. For fungal RNA extraction, *A. parasiticus* was cultured in RPMI 1640 medium in the presence of propolis at the sub-MIC concentrations for 3 days at 35 °C. An RNX-Plus kit (Sinaclon Company, Iran) was used to extract RNA from the fungal samples according to the manufacturer's instructions. The concentrations and purity of the extracted RNAs were determined by a spectrophotometer (Biophotometer, Eppendorf, Germany). The cDNA was synthesized from mRNA using random primers and reverse transcriptase (BioFACT, South Korea). The cDNA of target genes was quantified through Real-time quantitative PCR analysis using ABI StepOnePlus (Applied Biosystems Device). Amplification of beta-actin was performed as an internal control. Quantitative Real-Time PCR was performed using 5x evagreen qPCR mix (Solis BioDyne, Estonia). The primer sets (Table 1) in a two-step PCR conditions were used with an initial incubation at 95 °C for 15 min, 45 cycles of 94 °C for 15 s, 58 °C for 20 s and 72 °C for 30 s. The relative expression of genes was analyzed using REST software (2008 V2.0.7, Corbett Research, Sydney, Australia).

Results

Effect of propolis on *A. parasiticus* growth

The results of this study showed that the minimum inhibitory concentrations (MICs)values of propolis which inhibited *A. parasiticus* growth was 100 µg/ml. According on the results obtained at this step, 50 µg/ml of propolis was determined as sub-MIC concentrations and used for next steps.

Effects of propolis on aflatoxin production

The amount of aflatoxin produced by *A. parasiticus* exposed to propolis extract was measured by HPLC method (Table 2). According to the results, in the positive control, all types of AFs, including AFB_1, AFG_1, AFB_2, and AFG_2 were detected, with a total level of 386.1 ppm; whereas no aflatoxin was detected in the negative control. in the presence of 50 µg/ml propolis, only AFB_1 was detected and the total amount of aflatoxins produced was 3.01 ppm.

Effect of propolis on the expression of aflatoxin biosynthetic pathway genes

The results of the *nor-1, ver-1* and *omtA* genes expression by real-time PCR showed that the expression level of all genes in the presence of 50 µg/ml of propolis was significantly decreased. (Fig. 1; Table 3).

Table 2 The effect of propolis on aflatoxin production

Growth condition	AF_{total}	AFG_2	AFG_1	AFB_2	AFB_1 (ppm)
Negative control	0.00	0.00	0.00	0.00	0.00
Positive control	386.1	5.70	136.65	6.30	237.45
propolis (50 µg/ml)	3.01	ND	ND	ND	3.01

AF: aflatoxin, ND: not detectable

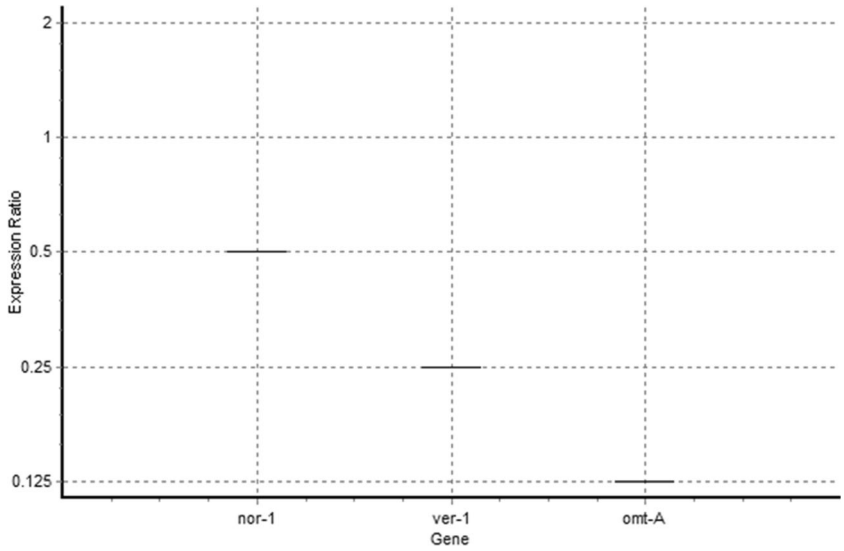

Fig. 1 Comparison of *nor-1, ver-1* and *omtA* genes expression in the presence of propolis extract

Discussion

In the present study, it was shown that propolis extract had the ability to inhibit the growth of *A. parasiticus*, reduce the *nor-1, ver-1* and *omtA* genes expression and the production of aflatoxin. Aflatoxicosis is an important food-borne disease it has always been one of the major health threatening diseases in different countries of the worldwide. Many countries reported that aflatoxicosis, as a food-borne disease, causes poisoning, sickness, and death in humans and animals following the consumption of aflatoxin-contaminated foods [31]. For example: studies on the Qidong area of China showed that the consumption of aflatoxin contaminated foodstuff is the main cause of liver cancer in this area [32]. Therefore, the lethal effects of aflatoxins caused the serious measures by governments were taken to elimination or reduction aflatoxin from foodstuff. Today one of the new methods of controlling food pathogens is the use of compounds derived from natural products [33, 34]. In recent years, extensive research on the exploring bioactive compounds with inhibitory effect on toxigenic fungal growth and reduction aflatoxin production [5, 35]. In the present study, the antifungal and anti-toxin activity of propolis extract on *A. parasiticus* as a natural bioactive substance with respect to reduction in expression of aflatoxin biosynthesis pathway genes was evaluated. There is clear evidence that fungal growth and aflatoxin biosynthesis are linked and may share regulatory elements [36]. Based on the results in this study, we found that the higher the extract concentration, the lower the mycelium production, and the highest fungal growth inhibition by propolis was, in the concentration 100 μg/ml. This result is in accordance with the results of other similar studies reported from Iran and around the world [37, 38]. Maria Belen Agüero et al. (2011) studied the effect of propolis extract from different regions of Argentina on dermatophytes and yeast strains, and showed that the minimum inhibitory concentration was between 125 and 31.25 μg/ml [39]. This finding is consistent with that of the present study. Other studies have shown that in the concentration 125 μg/ml of propolis extract could inhibit the growth of *Aspergillus niger*, *Microsporum canis*, *Trichophyton rubrum* and *Epidermophyton floccosum* [40, 41]. which is in line with the finding of the present study. Other findings of this study showed that the reduction and inhibition aflatoxin production in *A. parasiticus* was affected by 50 μg/ml concentration of propolis extract could be due to a significant reduction in the expression of tested the genes for aflatoxin biosynthesis pathway including; *nor-1, ver-1* and *omtA*. *omtA* compared to the control, it had the most significant effect and reduction at mRNA level; this results are consistent with the results of studies conducted by Jahanshiri et al. [42], Yoshinar et al. [43], Jahanshiri et al. [14] and Yahyaraeyat et al. [36]. which showed that reduced expression of aflatoxin biosynthesis pathway genes could inhibit aflatoxin production in *A. parasiticus*.

Table 3 *nor-1*, *ver-1* and *omtA* genes expression in the presence of 50 ug/ml propolis extract by Real-Time PCR

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
β-actin	REF	1.0	1.000				
nor-1	TRG	1.0	0.500	0.500–0.500	0.500–0.500	0.000	DOWN
ver-1	TRG	1.0	0.250	0.250–0.250	0.250–0.250	0.000	DOWN
omtA	TRG	1.0	0.125	0.125–0.125	0.125–0.125	0.000	DOWN

REF: reference gene, TRG: target gene

Conclusions

According to the results of the present study, propolis extract, have inhibitory effects on the growth, aflatoxin production and the expression of aflatoxin biosynthesis pathway genes in *A. parasiticus*. Therefore, propolis may be consider successfully as a potential candidate for controlling aflatoxin contamination on food and crops.

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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