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Effects of thyme and rosemary essential oils on the growth of two aflatoxigenic *Aspergillus flavus* strains

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Summary

In this study, antifungal effects of thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) essential oils (EOs) on two aflatoxigenic *Aspergillus flavus* strains (MAM-200682 and MAM-2006113) previously isolated from hazelnut were investigated. Five different concentration of the EOs (500, 250, 125, 62 and 31 µL/mL) were prepared in methanol. Minimal inhibitory concentration (MIC) values of the thyme and rosemary EOs against two cultures were determined as 31 and 125 µL/mL, respectively. 250 µL/mL of thyme EO on *A. flavus* MAM-200682, and 125 µL/mL of thyme EO and 500 µL/mL of rosemary EO on *A. flavus* MAM-2006113 had fungicidal effect. Fungal growth was almost completely inhibited after 90 min application of thyme EO concentrations (250 and 125 µL/mL). Rosemary EO caused slight growth inhibition. Thyme EO has the most fungistatic and fungicidal effect against aflatoxigenic *A. flavus* strains. These results indicated that the thyme and rosemary EOs could be used as natural antimicrobial agents against aflatoxigenic *A. flavus* in the food preservation practices.

Introduction

Aspergillus flavus, A. parasiticus, A. fumigatus, A. candidus, A. niger, A. ochraceus, A. tamar and *A. nomius* are responsible for off flavour formation and production of allergenic compounds and mycotoxins, which lead to both important qualitative and economic losses of foodstuffs and are important risk factors for human and animal health (HEPERKAN, 2005). *A. flavus*, a dominant mycotoxin-producing storage fungus, is commonly controlled by synthetic chemicals; however, most of fungicides of this group create several side effects in the forms of carcinogenicity, teratogenicity and residual toxicity. These fungi are mainly present in peanut, nuts and their products, dried fruits and figs, spices and maize (ASAN, 2004).

Spice essential oils (EOs) and extracts have been used for a wide variety of purposes for many thousands of years (HAMMER et al., 1999). It was reported that the EOs delay or inhibit the growth of food-borne pathogen fungi (MARINO et al., 2001; KUMAR et al., 2007). The preservation of foodstuffs by natural nontoxic substances is of great concern both to the consumer and food scientists (OZKAN et al., 2003; SAGDIC et al., 2009). EOs have been evaluated an alternative protective matters (MISHRA and DUBEY, 1994; SAGDIC et al., 2009). Several researchers reported fungistatic or fungicidal effects of thyme and rosemary (NGUEFACK et al., 2004; ATANDA et al., 2007).

Additionally the fungal growth inhibition in several studies, mycotoxin production could be prevented using some plant EOs (VELLUTI et al., 2003). Most of the previous studies showed that hazelnut was available for fungal growth and important mycotoxigenic fungi such as *Aspergillus* and *Penicillium* were isolated (SIMSEK et al., 2002; ALUC and ALUC, 2003). In this research, antifungal effects of thyme and rosemary EOs on the growth of two *A. flavus* strains isolated from hazelnut and the EOs compositions of the thyme and rosemary were investigated.

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Material and methods

Solvent (methanol), peptone, Tween-80, the basal medium (Potato Dextrose Agar, PDA) were obtained from Merck, Germany.

EOs of thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) were purchased from Sifam Baharat (Izmir-Turkey).

GC-MS analysis of essential oil (EO)

The chemical composition of thyme and rosemary EOs were characterized by GS/MS (SAGDIC et al., 2009). The composition of the volatile constituents of the material was established by GC-MS/Quadrupole detector analyses, using a Shimadzu QP 5050 system, fitted with an FFAP (50 m×0.32 mm (i.d.), film thickness: 0.25 µm) capillary column. Detector and injector temperature were set at 230 °C. The temperature program for FFAP column was from 120 °C (1 min) to 230 °C at a rate of 6 °C min^{-1} and then held at 200 °C for 35 min. Helium was used as a carrier gas at a flow of 14 psi (Split 1:10), and injection volume of each sample was 1 µL. The identification of the components was based on comparison of their mass spectra with those of Wiley and Nist, Tutore Libraries. The ionisation energy was set at 70 eV.

Preparation of the EO concentrations

Five EO concentrations (500, 250, 125, 62 and 31 µL/mL in absolute methanol) of the thyme and rosemary were prepared using methanol as a diluting agent (RASOOLI and ABYANEH, 2004). Absolute methanol without the EOs was used as a control in antifungal assays.

Antifungal assays: Determination of MIC and MFC values

Two aflatoxigenic *Aspergillus flavus* MAM-200682 and MAM-2006113 strains isolated from hazelnut in previous study were used in this research. They were obtained from Mould Culture Collection (MRC) of TUBITAK (The Scientific and Technological Research Council of Turkey). Stock cultures were maintained on PDA slants at 30 °C for 7 days (PAWAR and THAKER, 2006; SHARMA and TRIPATHI, 2008). Spores were harvested from slant surface by a sterile lop needle. Washing and preparing were made with sterile yeast extract sucrose (YES) broth. The spore suspension was adjusted to approximately 10^5-10^6 spores/mL in 5 mL of YES broth tubes by haemocytometer (SOKOVIC et al., 2002). The initial spore counts were confirmed using pour plate method, and control 1 and 2 (with and without absolute methanol, respectively) were prepared.

Minimal inhibitory concentration (MIC) and Minimal fungicidal concentration (MFC) tests were performed by using broth dilution method (RASOOLI and ABYANEH, 2004; KLARIC et al., 2007). The method of kinetic assay was carried out as described by RANA et al. (1997) and RASOOLI et al. (2006).

Fifty microliters from five EO concentrations were added in 5 mL sterile YES broth tubes containing 10^6 spores/mL and these solutions were gently mixed. The tubes were incubated at 30 °C for 7 days. After the incubation period, the tubes were controlled for either

mycelia growth or turbidity and sediments. The pH values of MIC tubes were measured by using an inoLab-pH meter (inoLab, Weilheim, Germany) after MIC assay. Every measurement was made three times and the average value was reported.

MFC test was used to determine if the inhibition was reversible or permanent. The inoculations of 1 mL from the tubes in which inhibition was seen were subcultured with two-fold dilutions using pour plate technique and incubated at 30 °C for 48 hours. MFC of EO is observed at concentration level at which *A. flavus* can not grow on the plates. Additionally, the inhibition percentages of the spices added were calculated according to the following formula:

$$\% \text{ Inhibition} = \frac{A.\ flavus \text{ count in control group} - A.\ flavus \text{ count in EO treatment group}}{A.\ flavus \text{ count in control group}}$$

Kinetics of inhibiting of colony formation

Spores of *A. flavus* were obtained from a 7-day-old culture. The slants were scraped gently using a sterile needle, washed with 7 mL of sterile physiological saline solution (0.85 %) with Tween-80 (0.1 % w/v). This suspension was collected in sterile tubes. The viable spore counts were controlled according to pour plate method after determining of spore numbers approximately by haemocytometer. Five milliliter of the spore suspensions including 10^5-10^6 spores/mL were transferred to sterile tubes. Fifty microliters of EO concentrations at MFC levels were added to the tubes. These tubes were mixed and incubated at 30 °C in 30 min intervals for 300 min. One milliliter of the reaction mixtures was taken and diluted until six-fold. Two parallel inoculations were made and the plates were incubated for 48 h at 30 °C. After the incubation period, fungal colonies were counted and calculated.

Tab. 1: Compositions of thyme and rosemary EOs (%)

No	Compounds	Thyme	Rosemary
1	Thymol	26.87	-
2	*p*-Cymene	19.77	-
3	γ-Terpinene	17.52	-
4	L-Linalool	6.38	0.86
5	α-Terpinolene	5.18	-
6	α-Terpineol	-	2.35
7	α-Terpinene	-	1.69
8	β-Myrcene	3.21	3.16
9	Caryophyllene	2.52	-
10	Endo-borneol	2.21	-
11	Camphene	1.83	6.90
12	Cyclohexene	1.80	-
13	3-Cyclohexen-1-ol	1.69	1.03
14	Phenol	1.31	0.77
15	1-Octen-3-ol	1.23	-
16	Camphene bicyclo heptane	0.97	-
17	1,8-Cineole	-	56.24
18	Δ^3-Carene	-	11.68
19	Camphor	-	4.35
20	1-Borneol	-	4.31
21	Endobornyl acetate	-	1.35
22	*trans*-Caryophyllene	-	0.67
23	α-Pinene	-	0.53
	Total	95.99	95.89

- : not detected.

Results and discussion

The results of analysis of thyme and rosemary EOs are shown in Tab. 1. GC-MS analyses of the EOs were done, and fourteen different components of thyme and rosemary EOs were characterized. According to our results the common main constituents of thyme EO were thymol (26.87 %), *p*-cymene (19.77 %) and γ-terpinene (17.52 %), the common main constituents of rosemary EO were 1,8-cineole (56.24 %) and Δ^3-carene (11.68 %). The main components present in EOs of thyme and rosemary were determined to be thymol (26.87 %) and 1,8-cineole (56.24 %), respectively (Tab. 1).

The compositions of the EOs of the *Thymus* species and *Rosmarinus officinalis* have also been studied (KARAMAN et al., 2001; HUDAIB et al., 2002). These results are in accordance with the finding of LEE et al. (2005) who demonstrated that major components of *T. vulgaris* were thymol, linalool and α-terpineol. Also, LOZIENE et al. (2007) described that linalool, thymol and α-terpineol were the main constituents in different *Thymus* species. Additionally, TOUAFEK et al. (2004) and WANG et al. (2008) reported that main component of the rosemary EO was 1,8-cineole.

FARAG et al. (1989) concluded that the antimicrobial effect of essential oils was mainly due to the most abundant components. The composition of essential oils in plant samples varies significantly because of different species and chemotypes (TANTAOUI-ELARAKI et al., 1993), geographical origin (PERRY et al., 1999), season (SENATORE, 1996), extraction procedure, time of the harvest and the plant part (SCANEBERG and KHAN, 2002) collected.

The differences and similarities between the MIC results in this study and those in the previous studies could have been due to the differences in the compositions of EO. For example, thymol content in *T. vulgaris* was determined to be 52.89 % (SUHR and NIELSEN, 2003) and 27.5 % (AMVAM et al., 1998) and 1,8-cineole content in rosemary was found to be ~30 % (ZAOUALI and BOUSSAID, 2008). Differences between the results could be attributed to the differences between the geographical origins, extraction procedures, harvest seasons and the plant parts collected.

Thymol in thyme (FARAG et al., 1989) and 1,8-cineole in rosemary (GIANNI et al., 2005) as the major compounds are responsible for antimicrobial effects.

The results of experimental design in this research are presented in Tab. 2 and 3, Fig. 1. Two aflatoxigenic *A. flavus* strains (MAM-200682 and MAM-2006113) grew in all of the control tubes. The pure forms of thyme and rosemary EOs had fungistatic effect on both of two strains.

The data on MIC is presented in Tab. 2. MIC values for both of two cultures were determined as 31 µL/mL concentration of thyme EO and 125 µL/mL concentration of rosemary EO. Thyme EO exhibited the strongest fungistatic activity against two *A. flavus* strains.

RASOOLI and ABYANEH (2004) determined that at 125 µL/mL concentration of thyme EO was evaluated as MIC value against *A. parasiticus*. This concentration was higher than our findings. It was reported that rosemary EO had very limited effects compared to thyme EO (GUYNO et al., 2003; PAWAR and THAKER, 2006). SUHR and NIELSEN (2003) reported that thyme EO proved overall more growth inhibitor than rosemary against rye bread fungi. These findings are similar to our results. In the other researches, 200 mg/mL and 500 µg/mL levels of thyme EO were evaluated as MIC value (SOLIMAN and BADEAA, 2002; KUMAR et al., 2007).

The results of this examination by broth dilution method revealed that thyme EO was the best antifungal agent than the other EOs. Some of EO concentrations evaluated as fungistatic effect had also fungicidal effect (Tab. 2). Irreversible inhibitions of *A. flavus* MAM-200682 and *A. flavus* MAM-2006113 for 2nd day obtained from thyme EO at 250 and 125 µL/mL were determined as MFC values, respectively.

Tab. 2: MIC/MFC values of thyme and rosemary EOs against aflatoxigenic *A. flavus* strains (n=3).

A. flavus strains	Types of EOs	EO concentrations in absolute methanol (µL/mL)				
		500	250	125	62	31
MAM-200682	Thyme	+/+	+/+	+/-	+/-	+/-
	Rosemary	+/-	+/-	+/-	-/-	-/-
MAM-2006113	Thyme	+/+	+/+	+/+	+/-	+/-
	Rosemary	+/+	+/-	+/-	-/-	-/-

+ : Growth positive, -: Growth negative

Tab. 3: pH values in MIC (+) and (-) broth media (n=3)

A. flavus strains	Controls	Types of EOs	EO concentrations in absolute methanol (µL/mL)				
			500	250	125	62	31
MAM-200682	6.14	Thyme	6.74	6.78	6.80	6.80	6.78
		Rosemary	6.73	6.71	6.63	6.51	6.35
MAM-2006113	6.25	Thyme	6.74	6.70	6.73	6.70	6.68
		Rosemary	6.60	6.65	6.67	6.34	6.20

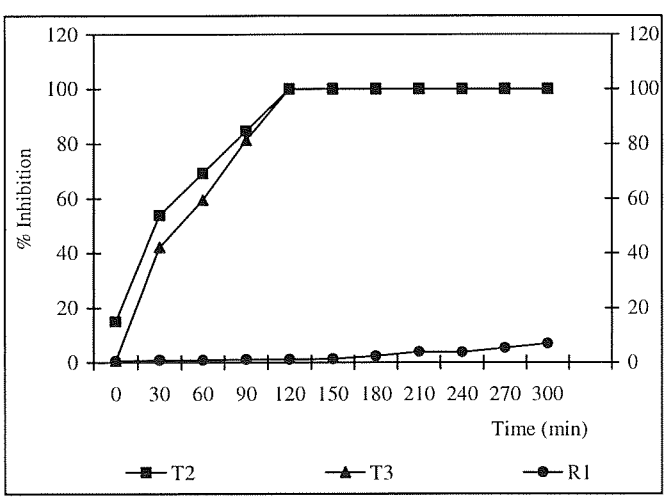

Fig. 1: The percentage inhibition during 300 min (n=3)
T2: Thyme at 250 µL/mL concentration against *A. flavus* MAM-200682,
T3: Thyme at 125 µL/mL concentration against *A. flavus* MAM-2006113,
R1: Rosemary at 500 µL/mL concentration against *A. flavus* MAM-2006113.

The results of MFC are in good agreement with the findings of RASOOLI and ABYANEH (2004). They reported that *Thymus eriocalyx* and *T. x-porlock* EOs had fungicidal activity at 250 µL/mL EO concentration. MFC values of thyme EO in present study were higher than those of the results such as 500 mg/mL and 1000 mg/mL in other studies (NGUEFACK et al., 2004; GACHKAR et al., 2007). Rosemary EO at only 500 µL/mL concentrations had the lethal effect on *A. flavus* MAM-2006113. No study has appeared on the fungicidal effect of *Rosmarinus officinalis* EO. On the other hand, GACHKAR et al. (2007) reported bactericidal activity of the herb EO.

The pH was measured in all of the MIC tubes after 6th day of incubation (Tab. 3). The average pH value in the tubes for inhibition was 6.71 and the value was higher than that of the controls (6.2). KARAPINAR (1985) determined that the 2 % concentration of thyme delayed mold growth at pH 6.6-6.7. LOPEZ-MALO et al. (2005) indicated that natural antimicrobial agent (carvacrol, thymol, vanillin, eugenol, and citral) addition in combination with pH reduction might result in an interesting and promising food preservation approach.
The growth of two *A. flavus* strains was completely inhibited after 90 min in the presence of thyme EO at MFC levels (Fig. 1). In a previous study on fungicidal kinetics of thyme and rosemary was found the 90-100 % lethal effects with in 2 hours and the more than 50 % the death with in 15 and 30 min (RASOOLI and ABYANEH, 2004). In this study, lethal effects of thyme oil were observed against *A. flavus* strains at 90th min. *A. flavus* MAM-2006113 in rosemary EO (500 µL/mL) remained 93.06 % at the end of 300 min. As a result of kinetic activity study, thyme EO at 250 and 125 µL/mL concentrations completely inhibited the growth of *A. flavus* strains. The inhibitory effect of spice EOs is mainly due to their main components. KLARIC et al. (2007) reported that thymol, the main antimicrobial component of thyme, has been demonstrated to have a high antimicrobial and anti-aflatoxigenic effects due to the presence of phenolic-OH group present in thymol.

Conclusions

This study demonstrated that thyme EO had the most fungistatic and fungicidal affect on both two *A. flavus* strains. Mycotoxin production might be prevented if mycotoxigenic strains could be inhibited by using EOs. So, they could be added to nuts and their products to protect from fungal contamination. Additionally, these results indicated that the thyme and rosemary EOs could be used as natural antimicrobial agents against aflatoxigenic *A. flavus* in the food preservation practices. However, given the strong aromatic flavour of these EOs, they should be added in lower amounts so as not to negatively affect the organoleptic qualities of the food. The results in present study could be performed on stored hazelnuts in the model systems.

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