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Short Communication

THE EFFECTS OF WILD THYME (*THYMUS SERPYLLUM* L.) ESSENTIAL OIL COMPONENTS AGAINST OCHRATOXIN-PRODUCING *ASPERGILLI*

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The aim of this study was to determine the effects of the essential oil of *Thymus serpyllum* L. and of its components thymol and total phenols (total phenolic content, TPC) extracted from the plant on the growth and mycotoxin production of *Aspergillus ochraceus*, *A. carbonarius*, and *A. niger*. Minimal inhibitory concentration (MIC) determined for the essential oil and thymol, and selected concentration of the TPC extract inhibited fungal growth and ochratoxin A biosynthesis by more than 60 %, depending on the conditions and duration of incubation with the fungi. Essential oil showed the strongest inhibitory effect which may have been related to the synergistic or cumulative effects of its components.

KEY WORDS: *antifungal activity,* Aspergillus sp.*, ochratoxin A, thymol, total phenols*

Moulds from the genus *Aspergillus* often spoil food products and produce mycotoxins. In terms of food safety, one of the most important is ochratoxin A (OTA). In nature it is produced by *Aspergillus ochraceus, A. carbonarius*, *Penicillium verrucosum*, and to a lesser extent by *A. niger*. *A. ochraceus* is responsible for OTA in coffee, rice, beverages, while *A. carbonarius* and *A. niger* are responsible for OTA in grapes, raisins, and wine (1, 2). Moulds and/or mycotoxins in food can endanger human health and pose a huge burden on economy. In line with green technologies, current trends in food preservation disfavour synthetic additives and welcome new methods of safe food production. These include prevention of prevention of food contamination with mycotoxins in the field and during storage, and systematic monitoring and control.

Over the last few years essential oils have attracted significant interest due to their antimicrobial activity, including activity against a wide range of fungi. These natural products could safely be used as alternative and desirable replacements of chemical fungicides. Recent publications (3, 4) rank the essential oil of *Thymus serpyllum* L. among the most potent against fungi. This activity has mainly been attributed to its components thymol and carvacrol.

The aim of this study was to investigate the antiochratoxigenic and antifungal effect of wild thyme (*Thymus serpyllum* L.) essential oil, thymol, and total phenols extracted from the wild thyme on four ochratoxigenic*Aspergillus* species.

MATERIALS AND METHODS

OTA-producing strains *A. ochraceus* NRRL 3174, *A. ochraceus* 318, *A. carbonarius* 328, and *A. niger* 388, were obtained from the Collection of Microorganisms of the Laboratory of General Microbiology and Food Microbiology, Faculty of Food Technology and Biotechnology, University of Zagreb (Zagreb, Croatia). They were stored on potato dextrose agar (PDA) slants (Biolife, Italy) at 4 °C. To prepare inoculum, the moulds were first incubated on PDA slants at 25 °C for 10 to 14 days and then added two 5 mL portions of Triton X-100 (5 mg L^{-1}) (Sigma-Aldrich, USA) in sterile water to adjust the concentration of spores in suspension to about 10^6 spores per mililitre, as determined with a Thoma counting chamber.

The essential oil (EO) of wild thyme (*Thymus serpyllum* L.), purchased from Kemika (Croatia), was dissolved in 96 % (by volume) ethanol (Kemika, Croatia) to the final concentration of 100 μL mL^{-1}. Thymol was purchased from Sigma (Sigma-Aldrich, USA) and used in the final concentration of 4 mg mL^{-1}, while TPC was obtained by extraction procedure and used in the final concentration of 0.293 mg mL^{-1}. Single concentration of TPC (0.293 mg mL^{-1}) was chosen and used here as mean concentration of TPC which was usually obtained from different herbs collected form Dalmatian area (data to be published). For the extraction 1 g of air-dried herb (flowered tops and stalks, collected in central Dalmatia, Croatia) was added 40 mL of 30 % ethanol. The mixture was placed in a water bath at 70 °C for 30 min, filtered through Whatman No. 4 paper, and the filtrate adjusted to 50 mL by adding 30 % ethanol. Total phenolic content was determined using the Folin-Ciocalteau colorimetric method according to Kulišić et al. (5). Calibration was done against gallic acid as the reference standard, and the results are expressed as gallic acid equivalents (GAE).

The minimum inhibitory concentrations (MIC) of EO and thymol against the *Aspergillus* species were determined by microdilution, following the NLCLS procedure (6). The medium used for serial dilution of EO and oil components was RPMI-1640 (Sigma-Aldrich, USA). Spore suspension was prepared in a 0.1 % solution of Tween 80 in sterile water, and the final concentration was 10^4 spores per mililitre. Fifty microlitres of spore suspension per well were added on sterile 96-well microtitre plates already containing 50 μL of serially diluted stock solutions of essential oils or thymol in 96 % ethanol. The plates were placed on a shaker for 1 min and incubated at (25±1) °C for 48 h. MIC was determined as the lowest concentration of EO or thymol which resulted in 100 % inhibition of visible mould growth. The obtained MICs were the starting point for further testing of the inhibitory activity. Growth inhibition of mould colonies was determined by supplementing PDA with EO and thymol at corresponding MICs, or TPC at 0.293 mg mL^{-1}(Table 1).

Control samples were not treated with antifungal agents. After two hours of incubation, two microlitres per conidial suspension of *A. ochraceus* NRRL 3174, *A. ochraceus* 318, *A. carbonarius* 328, and *A. niger* 388 were transferred to Petri dishes at the concentration of 10^6 conidia per millilitre and kept incubated at 25 °C. To establish growth inhibition we measured radial growth every four days over 28 days. Each experiment was repeated at least twice.

OTA production was measured after 14 and 21 days of mould incubation in YES broth (yeast extract 2 %, sucrose 20 %, distilled water up to 1 L) to which antifungal agents were added in concentrations shown in Table 1. Fifty millilitres of the broth was sterilised in Erlenmeyer flasks (250 mL) at 121 °C for 20 min. Broth pH was adjusted to 5.5±0.2 by adding HCl (c=1 mol L^{-1}) or NaOH (c=1 mol L^{-1}). Each broth was inoculated by adding 1 mL of spore suspension containing 10^6 spores. Control samples were incubated together with the test samples at 28 °C for 21 days. After incubation, OTA was extracted from the suspension as follows: the suspension (50 mL) was first added the same volume of chloroform, and the mixture was shaken at 50 cycles per minute for 15 min. The procedure was repeated twice and pooled extracts dried over anhydrous sodium sulphate and concentrated in a rotary evaporator to a volume of approximately 5 mL. OTA from the concentrated extract was purified using a 40 mmx400 mm preparative chromatographic column filled with silica gel (7). Its concentration was determined at 650 nm in a Sunrise microplate reader (Tecan, USA) using the ELISA test kit Veratox® 8610 (Neogen, USA) following the manufacturer's instructions.

The antifungal and antiochratoxigenic activity of the tested compounds were expressed as mean value of inhibition of colony growth ± standard deviation. For statistical analysis we used Student's t-test (SAS software, version 6.1, SAS Institute, USA) and set the level of significance at 95 % ($p<0.05$).

RESULTS AND DISCUSSION

Table 1 shows the MICs for EO and thymol obtained with ochratoxigenic *Aspergillus* strains. The two strains of *A. ochraceus* showed higher susceptibility to the antifungal activity of EO than *A. carbonarius* 328 and *A. niger* 388. The most resilient to EO was *A. niger* 388.

Table 1 *Minimum inhibitory concentration (MIC) of wild thyme (*Thymus serpyllum *L.) essential oil (EO) and thymol*

Strain	MIC	
	EO / µL mL^{-1}	Thymol / mg mL^{-1}
A. ochraceus NRRL 3174	0.625	0.0156
A. ochraceus 318	0.625	0.0156
A. carbonarius 328	1.25	0.125
A. niger 388	2.50	0.0078

Figure 1 shows that during the first 16 days of treatment inhibition of fungal growth was significant, as determined by the reduction of radial growth, but kept dropping from that point on until it was negligible on day 28. At the end of experiment, there was no inhibitory effect of EO, thymol, and TPC on colony growth, as compared to control.

The strongest antifungal activity was observed for EO against *A. carbonarius* 328 (Figure 1), but none of the agents did completely inhibit mould growth. Soliman and Badeaa (8) reported a complete inhibition of *A. flavus*, *A. parasiticus*, and *A. ochraceus* by thyme essential oil at levels of 250 µg g^{-1} and 500 µg g^{-1}. In their work, the inhibitory effect of essential oils or their components on mould growth was proportional to their concentration in the medium. They suggested that essential oils may act by attacking the cell wall and forcing the cytoplasm to withdraw into the hyphae, which ultimately kills the mycelium. The inhibitory mechanism may also include the effects of EO components on enzymatic reactions involved in cell wall synthesis. This interference may directly affect fungal growth and morphogenesis (9, 10). Rasooli and Owlia (11) observed irreversible damage of the cell wall (degenerative changes), cytoplasmic membrane (irregular, dissociated from the cell wall, invaginated), and nuclear membrane (folding) of *Aspergillus parasiticus* in the presence of thyme oils. The

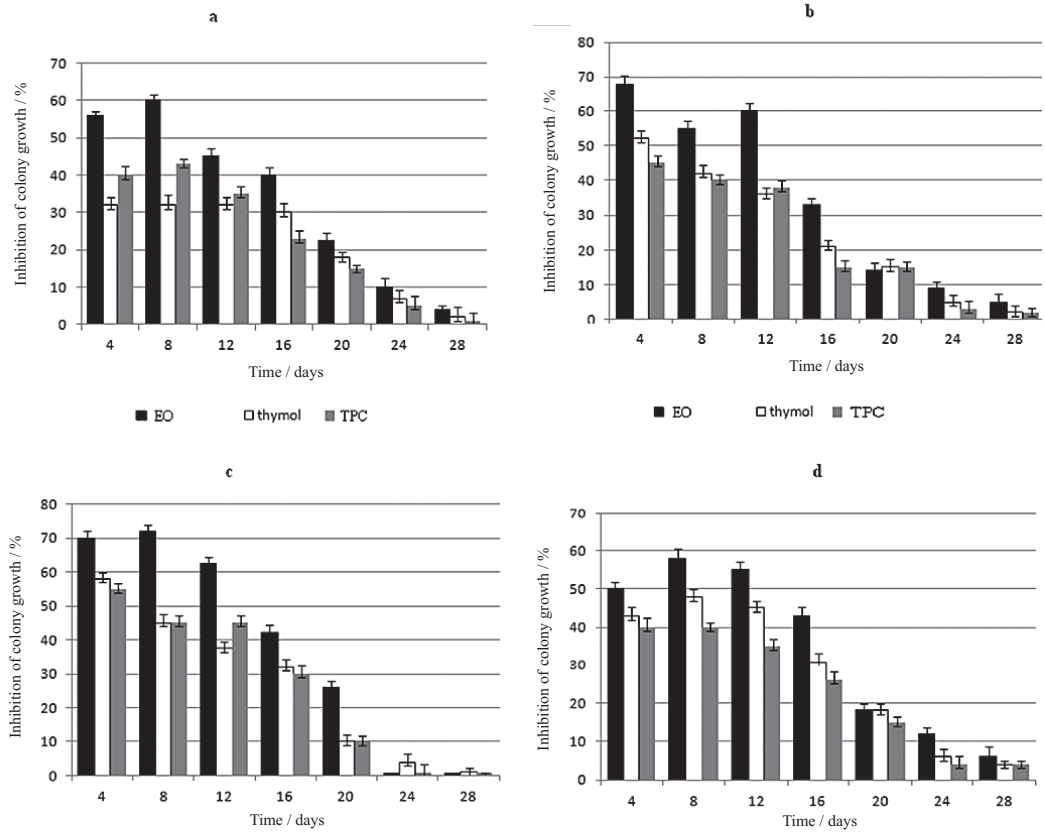

Figure 1 *Inhibition of colony growth of* A. ochraceus *NRLL 3174 (a);* A. ochraceus *318 (b);* A. carbonarius *328 (c) and* A. niger *388 (d) by wild thyme (EO), thymol and total phenols (TPC).*
Values presented here and in Figure 2 are means ± SD ($p<0.05$). Control serves as baseline (0 % inhibition).

antioxidant, antibacterial, and antifungal activities of thyme (5, 12) are related to its phenolic compounds, particularly thymol and *p*-cymene, which mostly abound in the leaves (13). Thymol is known to act against the *Asperigillus* species (14). This fact could further increase quality value of the plant due to its role as antioxidant agent. Considering the MICs obtained in our study it had the strongest growth inhibitory effect on *A. carbonarius* and the weakest effect against *A. ochraceus* NRRL 3174.

TPC added at concentration of 0.293 mg mL^{-1} showed the most potent antifungal activity against *A. carbonarius,* followed by *A. ochraceus* and *A. niger* (Figure 1). Our results confirm that thymol and TPC, the main components in EO of wild thyme, possess antifungal activity. The mechanism of antioxidantive action regarding the inhibition of mycelial growth of toxigenic fungi is not clear. Many studies have described antioxidative effects of antifungal compounds, their relation to protoplasmic poisoning, disruption of microbial cell wall, and precipitation of cell proteins (10, 15). The activity of these compounds has been attributed to the presence of an aromatic nucleus and an OH group which are known to form hydrogen bonds within active sites of enzymes (10, 16, 17). The EO showed more inhibitory activity than thymol and TPC alone. Assuming that their concentrations in EO are much lower than MICs obtained in our study, this activity implies an additive combination or even a synergism of the two components in EO, which has been supported by the findings of Pina-Vazk et al. (12) and Lee et al. (13).

Figure 2 shows the effects of EO, thymol, and TPC on OTA production on days 14 and 21 of incubation. Addition of thyme essential oil inhibited significantly OTA production by both *A. ochraceus* species ($p<0.05$, 74 % and 78 %, respectively). In *A. carbonarius* this inhibition was nearly 90 % and in *A. niger* about 68 %. Thymol and TPC showed similar OTA inhibition in all fungi, ranging from 58 % to 77 %. Our results are in agreement with data published by Pereira et al. (18), who showed that wild thyme EO reduced OTA production by between 75 % and 100 %.

As expected, the antifungal and antiochratoxigenic activities of EO, thymol, and TPC decreased with time (Figures 1 and 2). These results are in agreement with data obtained by Basílico and Basílico (16), who showed a slight inhibition of mould growth by EO over time.

Our results suggest that thyme essential oil and its phenolic components could be used against fungi growing on stored food as an effective alternative to chemical fungicides. Aerosol preparations of essential oil or its components could be sprayed over stored crops and as their concentration of active and volatile compounds is relatively low, could be used for coating plastic bags used for packing fruit and vegetables.

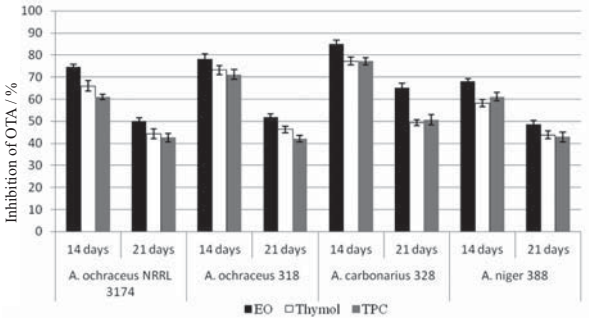

Figure 2 *Inhibition of OTA biosynthesis in* A. ochraceus *NRLL 3174;* A. ochraceus *318;* A. carbonarius *328 and* A. niger *388 by wild thyme (EO), thymol and total phenols (TPC).*

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Sažetak

MAJČINA DUŠICA (*THYMUS SERPYLLUM* L.) I NJEZINE KOMPONENTE PROTIV OKRATOKSIKOTVORNIH VRSTA *ASPERGILLUSA*

Cilj ovog rada bio je odrediti utjecaj eteričnog ulja majčine dušice (*Thymus serpyllum*), uljne komponente timola i ukupnih fenola ekstrahiranih iz biljke, na rast plijesni i sintezu mikotoksina. U odabranim uvjetima minimalna inhibitorna koncentracija (MIC) eteričnog ulja i timola, koja je određena u ovom radu, i koncentracija ukupnih fenola (TPC) inhibirale su više od 60 % rasta odabranih plijesni i biosinteze okratoksina A. Njihova inhibitorna učinkovitost ovisila je o uvjetima i trajanju inkubacije s plijesnima. U odnosu na timol i ekstrakt eterično ulje pokazalo je snažniji inhibitorni utjecaj, koji je uvjetovan sinergističkim ili kumulativnim djelovanjem uljnih komponenata.

KLJUČNE RIJEČI: *antifungalna aktivnost, eterično ulje, okratoksin A, timol, ukupni fenoli*

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