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The inhibitory effect of oregano extract on the growth of *Aspergillus* spp. and on sterigmatocystin biosynthesis

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ABSTRACT

The aim of this study was to investigate the effect of oregano extract on the growth of *Aspergillus* species (*Aspergillus niger*, *Aspergillus carbonarius*, and *Aspergillus wentii*) isolated from food and biosynthesis of sterigmatocystin (STC) by *Aspergillus versicolor*. Antifungal determinations were conducted using the agar plate method. The effect of oregano extract on the biosynthesis of STC was determined in a Yeast Extract Sucrose (YES) broth. The STC content and mycelial growth of *A. versicolor* were determined on the 7th, 14th, and 21st day with all applied concentrations of oregano extract and control sample. The composition of oregano extract was determined by gas chromatography–mass spectrometry (GC–MS) and 21 different components were identified. The major components were carvacrol (34.20%) and carvone (18.05%). At 2.5 mL/100 mL concentration, the oregano extract completely inhibited the growth of *A. wentii*, while the growth of *A. carbonarius* and *A. niger* was reduced by 95.6% and 45.6%, respectively. Significant reductions in STC biosynthesis during 21 days of incubation were observed for extract concentrations higher than 0.06 mL/100 mL. The obtained results showed that the tested oregano extract could be used as a food preservative to prevent food-borne fungal infections and mycotoxin production.

1. Introduction

Aspergillus species are frequent contaminants of medium (a_w range from 0.75 to 0.90) and low moisture food ($a_w < 0.75$). Metabolic activity of these microorganisms causes food spoilage and enormous economic loss. Besides that, toxin producing species biosynthesize toxic secondary metabolites-mycotoxins: aflatoxins (AF) (*Aspergillus flavus, Aspergillus parasiticus*), ochratoxin A (OA) (*Aspergillus ochraceus, Aspergillus carbonarius, Aspergillus niger, Aspergillus melleus, Aspergillus ostianus, Aspergillus sulphurues*), naphto-γ-piron, malphormin (*A. niger*), nidulotoxin, sterigmatocystin (STC) (*Aspergillus versicolor, Aspergillus ustus, Aspergillus rugulosus, Aspergillus biploaris, Aspergillus aurantio-brunens, Aspergillus quadrilineatus*), emodin, ventilacton (*Aspergillus wentii*) etc. (Frisvad & Thrane, 2004). Intake of fungal toxins through food consumption provokes intoxications in animals and humans, so called mycotoxicoses which can occur on a large-scale (Diaz, 2005). Mycotoxicoses are manifested in the form of acute and chronic toxicity, cytotoxicity, hepatotoxicity, neurotoxicity, teratogenicity and mutagenicity (Diaz, 2005). On the cellular level, some mycotoxins react with nucleic acids and inhibit the biosynthesis of proteins and DNA and RNA macromolecules. Others affect the structure and functions of biological membranes or impair the energy metabolism (Diaz, 2005; Mayer, Engelhart, Kolk, & Blome, 2008).

Several strategies have been used in controlling the fungal growth and mycotoxin biosynthesis in food products. Changes in consumers' preferences toward more natural products than synthetic food additives, and reduction of salt and sugar in foods for dietary reasons, stimulate the use of spices and/or aromatic plants, which are low in sodium and calories (Dadalioglu & Evrendilek, 2004). Consequently, in recent years, large number of studies have targeted the usage of essential oils, extracts and oleoresins extracted from spices and aromatic herbs as alternative food preservatives (Benkeblia, 2004; Burt, 2004; Chutia, Deka Bhuyan, Pathak, Sarma, & Boruah, 2009; Sağdiç, 2003; Soliman & Badeaa, 2002).

Oregano is a widely used spice in the food industry. It is mainly used for its aromatic properties with a primary role to enhance the taste and aroma of foods. Due to high content of oleanolic, ursolic, caffeic, rosemarinic, lithospermic acids, flavonoids, hydroquinones,

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tannins, and phenolic glycosides, oregano has been shown to exhibit antioxidative and antimicrobial activity.

Many studies showed the inhibitory activity of oregano extracts and essential oils against the growth of many bacteria (Aligiannis, Kalpoutzakis, Mitaku, & Chinou, 2001; Bagamboula, Uyttendaele, & Debevere, 2004; Celikel & Kavas, 2008; Dadalioglu & Evrendilek, 2004; Nostro et al., 2007; Özcan & Erkmen, 2001; Vági, Simándi, Suhajda, & Héthelyi, 2005; Valero & Francés, 2006) and fungi (Baratta, Dorman, Deas, Biondi, & Ruberto, 1998; Bouchura, Achouri, Hassani, & Hmamouchi, 2003; Daouk, Dagher, & Sattout, 1995; Gumus, Sukuru Demirci, Sagdic, & Arici, 2010; Kocić-Tanackov, Dimić, Tanackov, & Tuco, 2011; Özcan & Erkmen, 2001; Paster, Menasherov, Ravid, & Juven, 1995; Tantaoui-Elaraki Beraoud, 1994; Vági et al., 2005; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2007; Wogiatzi, Gougoulias, Papachatzis, Vagelas, & Chouliaras, 2009). However, there is a limited number of studies related to their effect to mycotoxin production (Basilico & Basilico, 1999; Salmeron, Jordano, & Pozo, 1990; Velluty, Sanchis, Ramos, Egido, & Marin, 2003; Velluty, Sanchis, Ramos, Turon, & Marin, 2004).

The purpose of this study was to investigate the effect of oregano extract on the growth of *Aspergillus* species (*A. niger, A. carbonarius*, and *A. wentii*), isolated from food and determination of STC biosynthesis by *A. versicolor*.

2. Materials and methods

2.1. Materials

For the antifungal activity testing, a commercially available, food grade oregano extract was provided by ETOL (Celje, Slovenia).

Investigated fungal species (*A. niger* van Tieghem, *A. carbonarius* (Bainier) Thom, *A. wentii* Wehmer and *A. versicolor* (Vuill.) Tiraboschi) were isolated from cakes and fresh ready-for-use salads (prepared with different kinds of vegetables) bought in a supermarket.

2.2. Chemicals

The reagents and mycological media: sodium nitrate p.a. ($NaNO_3$), dipotassium phosphate p.a. (K_2HPO_4), potassium chloride p.a. (KCl), magnesium sulphate heptahydrate p.a. ($MgSO_4 \cdot 7H_2O$), ferrous sulphate heptahydrate p.a. ($FeSO_4 \cdot 7H_2O$), formic acid p.a. (HCOOH), ammonium acetate p.a. (CH_3COONH_4), ammonium formate p.a. ($HCOONH_4$), Tween 80 p.a., yeast extract (for microbilogy), sucrose (for microbiology), agar (for microbiology), Dihloran 18% Glicerol Agar (DG18) and Potato Dextrose Agar (PDA) were purchased from Merck, Darmstadt. Acetonitrile (HPLC gradient grade) was purchased from J.T. Baker, Decenter. Sterigmatocystin (STC) reference standard was purchased from Sigma Aldrich, Steinheim, Germany.

2.3. Mycological analyses

2.3.1. Isolation and identification of Aspergillus species from food

Samples of cakes and fresh ready-for-use salads (prepared with different kinds of vegetables) were bought in a supermarket and analyzed straight after. Isolation of *Aspergillus* species (*A. niger*, *A. carbonarius*, *A. wentii*, and *A. versicolor)* from cakes and fresh ready-for-use salads was performed on Dichloran 18% Glycerol Agar (DG18). Under aseptic conditions 20 g of cakes or fresh salads was homogenized in 180 mL of sterile peptone water (0.1 g of peptone/100 mL of distilled water). After this, samples were shaken for 10 min at 200 rpm (Unimax 1010, Heidolph, Germany). One mililiter of the obtained stock solution was transferred into a Petri plate (θ 9 cm), in which medium was poured and samples were incubated for 7 days at 25 ± 2 °C. In order to obtain pure cultures and perform the identification, colonies that were suspected to belong to the *Aspergillus* genera (according to the macro-morphological characteristics) were re-inoculated to the Czapek Yeast Autolysate Agar, CYA ($NaNO_3$ 3 g; K_2HPO_4 1 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; yeast extract 5 g; sucrose 30 g; agar 20 g; distilled water 1000 mL). After this, samples were incubated for 7 days at 25 ± 2 °C.

Obtained pure cultures of *Aspergillus* genera were identified according to the keys for determination (colony diameter, color and texture; microscopic characteristics − hyphae and conidiophore appearance, size and shape of vesicles, metulae, phialides, and conidia) described by Klich (2002) and Samson, Hoekstra, and Frisvard (2004).

Isolated and identified *Aspergillus* species were kept on Potato Dextrose Agar (PDA) at 4 °C as a part of the collection of the Laboratory for Food Microbiology at the Faculty of Technology, University of Novi Sad, Serbia.

2.3.2. Determination of the oregano extract effect on the Aspergillus species growth

Effect of the oregano extract on the *A. niger, A. carbonarius*, and *A. wentii* growth was determined according to the modified agar plate method described by Matamoros-León, Argaiz, and López-Malo (1999).

2.3.2.1. Preparation of media. Potato Dextrose Agar (PDA) was used a medium for antifungal investigations. PDA was divided into equal volumes (150 mL), poured into Erlenmeyer (250 mL) flasks and autoclaved at 121 °C for 15 min after which it was cooled to 45 °C. The extracts were added to the PDA to achieve the following concentrations: 0, 0.35, 0.7, 1.5, and 2.5 mL/100 mL. PDA containing different concentration of oregano extract was poured in sterile Petri plate (∅ 9 cm), 12 mL into each plate.

2.3.2.2. Preparation of fungal spore suspension. Seven-day fungal cultures grown on PDA were used for preparation of the fungal spore suspension tests. The spores were harvested with sterile loop in10 mL of medium which contained 0.5 mL/100 mL Tween 80 and 0.2 g/100 mL agar in sterile distilled water and aseptically transferred into sterile test tubes. The spore suspensions were adjusted with the same solution to give a final spore concentration of 10^6 spores/mL using the haemocytometer.

2.3.2.3. Inoculation and incubation. For each extract concentration and fungal species, including the controls, plates were centrally inoculated by spotting the 1 μL of a spore suspension (10^3 spores/mL) in the middle of the plate using an inoculation needle to give a circular inoculum of approximately 2 mm in diameter (one inoculum per plate). The control sample was inoculated by spotting the 1 μL of sterile distilled water. After inoculation, the Petri plates were closed with a parafilm and incubatied at 25 ± 2 °C.

The inhibitory effect of oregano extract on fungal growth was evaluated by a daily measurement of the diameter of the radial colony growth during 14 days and was calculated by equation: $I\ (\%) = (C - T)/C \times 100$, where I is inhibition (%), C is colony diameter on the control plate (cm) and T is colony diameter on the test plate (cm) (Pandey, Tripathi, Tripathi, & Dixit, 1982).

For determination of minimal inhibitory concentration (MIC) or minimal fungicidal concentration (MFC) the parafilm was removed from the Petri plates (so oxygen could enable development of fungal spores) in which no colony growth was observed after 14 days, and the plates were further incubated for 16 days (30 days in total) at 25 ± 2 °C. In Petri plates in which fungal growth was