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The effects of *Heracleum platytaenium Boiss* essential oil on the growth of ochratoxigenic *Penicillium verrucosum* (D-99756) isolated from Kashar Cheese

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Summary

The antifungal effects of essential oil isolated from *Heracleum platytaenium* Boiss on the growth of ochratoxigenic *Penicillium verrucosum* (D-99756) isolated from Kashar cheese were investigated. Minimal inhibitory concentration (MIC) and Minimal lethal concentration (MLC) tests were performed by using broth dilution method. Controls were prepared with three groups; with pure essential oils (Eo) (positive control), methanol and without Eos. The serial doubling dilution of oil was prepared in methanol with concentrations ranging from 500 to 31µL/mL. Applied incubation periods were 25 ºC for 10 days for MIC and 48 hours at the same temperature for MLC. After the incubation period, the tubes were controlled for either mycelia growth or turbidity and sediments. The maximal inhibitory dilution (the minimal inhibitory concentration), exhibiting any growth, was regarded as MIC. Tubes containing *Penicillium verrucosum* which did not display any growth were subcultured to determine if the inhibition was reversible or permanent. The Eo dilution, which is the not visible growth of typical *P. verrucosum* colonies on the plates, was accepted as lethal effect. MLC was the lowest concentration of antifungal to completely inhibit fungal growth. The MIC and MFC values were determined as 31 and 125 µL/mL, respectively.

Introduction

The presence and growth of fungi in food may cause spoilage and result in a reduction in quality and quantity. Growth of commonly occurring filamentous fungi in foods may result in production of toxins known as mycototoxins, which can cause a variety of ill effects which has a range of biological activities including acute toxicity, teratogenicity, mutagenicity and carcinogenicity in humans, beginning from allergic responses to immunosuppression and cancer. The most important mycotoxins are aflatoxins, ochratoxin-A, fumonisins, trichothecenes and zearalenone. Ochratoxin-A produced by *Aspergillus ochraceus*, *A. niger*, *A. carbonarius*, *Penicillium verrucosum* and *P. viridicatum* (Pitt, 1987; Abarca et al., 1994; Varga et al., 1996) is probably carcinogenic and may cause urinary tract cancer and kidney disease (Krogh et al., 1974; Pitt, 2000). Penicillium species are frequent contaminants of different food products and known as producers of a variety of secondary metabolites (Frisvad and Fitenborg, 1983). *P. verucosum* is primarily encountered on cereals and cheeses (Larsen et al., 2001).

Various species of Heracleum have been reported to possess antibacterial and antifungal properties (Ciesla et al., 2008). *Heracleum* sp. is a member of *Umbelliferae* family whose essential oils (Eos) have antibacterial and antifungal effects (İşcan et al., 2004). The fruits and the leaves of this genus are used as flavouring agent and spice for food and also antiseptic, carminative, digestive and analgesic effect (Firuzi et al., 2010). Chemical composition of the Eos of some *Heracleum* species was investigated by some researches. The major compounds of different *Heracleum* species were reported as octyl acetate by Işcan et al. (2004), 1-octanol, octylbutyrate, octanol by Janssen et al. (1986) and Kürkçüoğlu et al. (1995) and myristicin, E-anethole, octyl isobutanoate, hexyl butanoate, octyl acetate and elemicin by Firuzi et al. (2010).

Materials and Methods

Materials

The essential oil of the plant *Heracleum platytaenium* Boiss was obtained from Biological Science Department, Faculty of Science and Literature in Samsun-Turkey.

Preparation of the essential oil dilutions

The serial doubling dilution of each oil (Pinto et al., 2007) was prepared in methanol (Rasooli and Abyaneh, 2004) with concentrations ranging from 500 to 31 µL mL^{-1}.

Fungal strain and preparation of spore suspension

The fungal strain used in this study was *Penicillium verrucosum* Dierck (D-99756), obtained from Faculty of Food Engineering in Istanbul Technical University, Istanbul-Turkey. The strain was cultured from frozen stocks and maintained on Malt Extracted Agar (MEA, 2% malt, 0.1% peptone, 1.5% agar) slants at 25 ºC in the dark for 7-10 days. The strain was cultivated on MEA slants at 25 ºC in the dark for 7-10 days (Cabanas et al., 2009). Spores were harvested by adding 10 ml of sterile saline solution (NaCl, 0.85%) containing Tween 80 (0.1% v/v). The spore suspension was adjusted to approximately 10^6 spores mL^{-1} using haemocytometer (Elias et al., 2006). Test organism was enumerated in growing broth media by serial dilution method (Özkan et al., 2010). The viability and inoculums size of the strains were checked using quantitive colony counts. The plates were incubated for 72 h at 25 ºC in the dark (Tavares et al., 2008).

Growth media and chemicals

Yeast Extract Sucrose (YES) agar, YES broth, methanol, Tween-80, Sodium chlorid (NaCl) were obtained from Merck, Germany. YES culture media was used to determine inhibitory concentrations and YES agar was used to determine if the inhibition was reversible or permanent (Bragulat et al., 2001). 5 mL sterile YES broth tubes containing 10^6 spores mL^{-1} were dispensed into sterile tubes using for broth dilution method. Petri dishes (90 mm) were filled with 15-20 mL of YES agar for fungicidal effect. Controls with and without methanol (negative controls) and using pure Eos (positive controls) incubated under the same conditions.

Antifungal activity of essential oils with macrodilution method

A macrodilution broth method was used to determine the minimal inhibitory concentrations (MIC) and minimal lethal concentrations

(MLC) (Lass-Flörl et al., 2003; Tavares et al., 2008). Fifty micro liters from four Eos dilutions at various concentrations (500, 250, 125, 62, 31, µL mL^{-1}) were added in 5 mL sterile YES broth tubes containing 10^6 spores mL^{-1} and these solutions mixed gently. The tubes were incubated at 25 ºC for 7-10 days on an incubator shaker as to evenly disperse the oils throughout the broth in tubes. The fungal growth was indicated by the turbidity. The highest dilution (lowest concentration), showing no visible growth compared with Eo-free controls, was regarded as MIC. The oil dilutions supplying inhibitory effect were tested for lethal effect as well. The inoculations of 1 mL for pour plate and 30 µL for three point inoculation methods from the tubes were subcultured on YES agar plates and than incubated at 25 ºC for 72 hours. The essential oil dilution, which is not visible growth of typical *P. verrucosum* colonies on the plates, was accepted as lethal effect. The MLC was defined as the lowest Eo concentration at which 99% of the inoculum was killed. All the experiments were performed in duplicate and three independent experiments were run with concordant results.

Results

The results of experimental design in this research are presented in Tab. 1, Fig. 1 and Fig. 2a - 2b.

The mycelial growth was visible in negative control tube (Fig. 1b) while any fungal growth wasn't seen in positive control tubes (Fig. 1a). The highest dilution concentration (31 µL mL^{-1}) was MIC value (Fig. 1c). The growth of *P. verrucosum* was inhibited 100% at MIC value.

Heracleum species has just been investigated for anticandidal activity by Naeini et al. (2009), İşcan et al. (2003 and 2004) and Kuljanabhagavad et al. (2010). The researches reported that different *Heracleum* spp. had notable anticandidal effect. In this study, it was determined that the mycelial formation of *P. verrucosum* could be prevented using *H. platytaenium* essential oil.

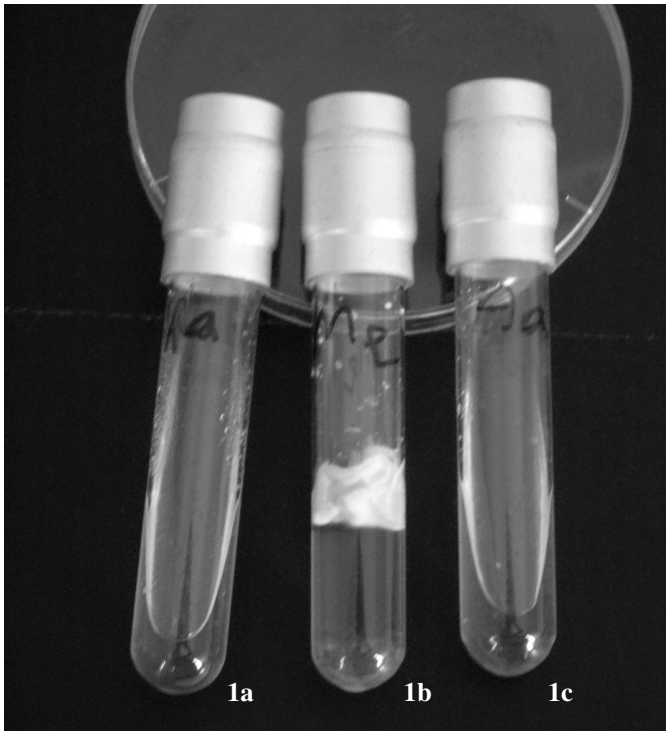

Fig. 1: The inhibitory effects of *H. platytaenium* Eo dilutions on *P. verrucosum* after 9 days of incubation at 25°C. The left tube represents complete inhibition at pure form of Eo, the right tube represents complete inhibition at 31 µL mL^{-1} and the middle tube represents negative control tube.

The lethal effect was assayed using two methods. The results both were the same. Oil required for MLC value is more than MIC. The inoculations subcultured on YES agar plates could be killed at 125 µL mL^{-1} oil dilutions (Fig. 2a - 2b).

Tab. 1: MIC/MFC values of *Heracleum platytaenium* Eo against ochratoxigenic *P. verrucosum* strain.

	The inhibitory and lethal effects of *H. platytaenium* Eo dilutions (µL mL^{-1}) on *P. verrucosum.*						
	Positive controls	Negative controls	500	250	125	62	31
MIC	+	-	+	+	+	+	+
MLC	+	-	+	+	+	-	-

+: inhibition positive -: inhibition negative

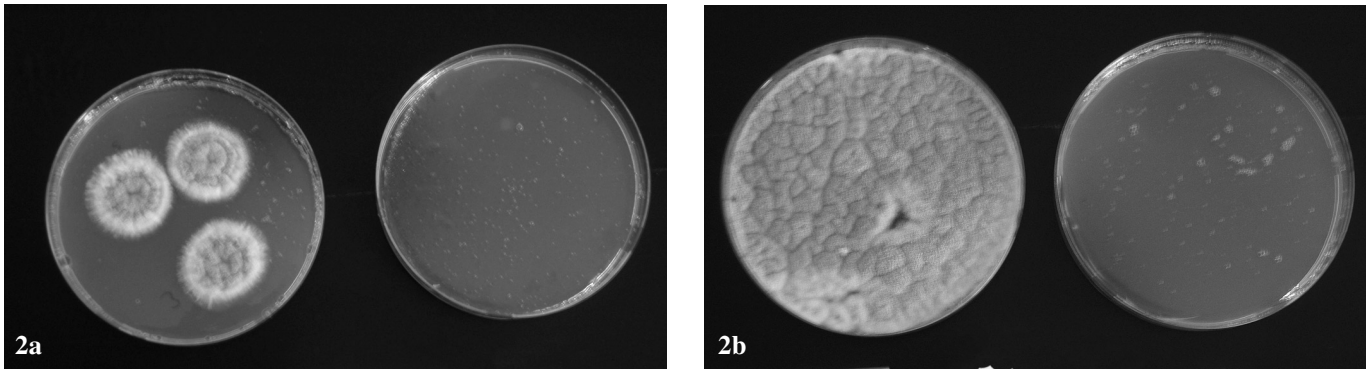

Fig. 2: MFC results after 72 hours of incubation at 25 °C using three point inoculation (Fig. 2a) and pour plate mehods (Fig. 2b). The left petri dishes for non-additive and the right petri dishes for additive with *H. platytaenium* Eo dilutions represent subcultured inoculations from MIC tubes.

Discussion

In this study, it was demonstrated that *H. platytaenium* had inhibitory and toxic effects against *P. verrucosum*. In general, the cytotoxic activity of Eos is mostly due to the presence of aldehydes, alcohols, methylene dioxy compounds and phenols (KULJANABHAGAVAD et al., 2010). FIRUZI et al. (2010) reported that *Heracleum* species had the cytotoxic activity mostly due to the presence of myristicin, elemicin and (*E*)-anethole. *Heracleum* species has just been investigated for anticandidal activity by NAEINI et al. (2009), İŞCAN et al. (2003 and 2004) and KULJANABHAGAVAD et al. (2010). This survey is the first report that the inhibitory effect of *H. platytaenium* against ochratoxigenic *P. verrucosum.*

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