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## EFFECT OF *ORIGANUM HERACLEOTICUM* L. ESSENTIAL OIL ON FOOD-BORNE *PENICILLIUM* *AURANTIOWISEUM* AND *PENICILLIUM* *CHRYSOGENUM* ISOLATES

**ABSTRACT:** Molds are ubiquitously distributed in nature and their spores can be found in the atmosphere even at high altitudes. The difficulty of controlling these undesirable molds, as well as the growing interest of the consumers in natural products, have been forcing the industry to find new alternatives for food preservation. The modern trends in nutrition suggest the limitation of synthetic food additives or substitution with natural ones. Aromatic herbs are probably the most important source of natural antimicrobial agents. *Origanum heracleoticum* L. essential oil has been known as an interesting source of antimicrobial compounds to be applied in food preservation. In this work, we have investigated the effect of essential oil obtained from *O. heracleoticum* on growth of six isolates of *Penicillium aurantiowiseum* and four isolates of *Penicillium chrysogenum* isolated from meat plant for traditional Petrovačka sausage (Petrovska klobasa) production.

The findings reveal that the essential oil of *O. heracleoticum* provides inhibition of all of fungal isolates tested. *O. heracleoticum* L. essential oil exhibited higher antifungal activity against the isolates of *P. chrysogenum* than the isolates of *P. aurantiowiseum*. *O. heracleoticum* essential oil showed a MIC value ranging from 25 to 100 µL/mL. The fungi cultivated in the medium with higher concentration of essential oil showed certain morphological changes. The alterations included lack of sporulation and loss of pigmentation.

**KEY WORDS:** antifungal activity, essential oil, *Origanum heracleoticum*

## INTRODUCTION

Molds are ubiquitously distributed in nature and their spores can be found in the atmosphere even at high altitudes (Carmona et al., 2008). Mold contamination is often associated with unpleasant appearance, odor and changes in taste and nutritional value of foods (Papagian et al., 2007).

Some molds are able to produce mycotoxins, which apart from the toxic effects, frequently have degenerative, toxinogenic or carcinogenic effects (Miličević et al., 2010).

The difficulty of controlling these undesirable fungi, as well as the growing interest of the consumers in natural products, have been forcing the industry to find new alternatives for food preservation. One of the possibilities is the usage of essential oils as antifungal additives. Many naturally occurring compounds found in plants, herbs, and spices have been shown to possess antimicrobial functions and serve as a source of antimicrobial agents against food borne pathogens (Škrinjar and Nemet, 2009; Škrinjar et al., 2009). Their systematic screening may result in the discovery of novel effective antimicrobial compounds.

Among the aromatic plant species from *Lamiaceae* (*Labiatae*) family, genus *Origanum* occupies a special position. Essential oils from genus *Origanum* are known to exhibit antimicrobial activities against bacteria and fungi (Skandamis et al., 2001; Džamić et al., 2008). This is basically due to their major components, carvacrol and thymol (Govariss et al., 2010) which have potential to be used as food preservatives (Burt, 2004). Biological activity of essential oils depends on their chemical composition, which is determined by genotype and influenced by environmental and agronomic conditions (Burt, 2004).

Chemical analysis of the oregano (*O. heracleoticum* L.) essential oil revealed the presence of several ingredients, most of which have important antioxidant activity (Tsimogiannis et al., 2006), and antimicrobial properties (De Martino et al., 2009). Carvacrol and thymol, two main phenolic compounds that constitute about 78–85% of oregano oil, are principally responsible for the antimicrobial activity of the oil (Kokkini et al., 1997; Govariss et al., 2010). In addition, other minor constituents, such as monoterpene hydrocarbons  $\gamma$ -terpinene and p-cymene, also contribute to antimicrobial activity of the oil (Burt, 2004).

Thymol and carvacrol do not exhibit adverse effects on human health, and are proved to cause neither significant nor marginal toxic effects at cellular level. Also, the concentrations at which they exhibit antimicrobial activity do not reach the possible genotoxic level (Stamati et al., 1999; Burt, 2004). One limitation on the use of essential oils or their constituents in foods is their herbal aroma; in fact carvacrol and thymol are permitted food flavorings in the U.S. and Europe (CFSA, 2006).

The aim of this study was to investigate antifungal effects of various concentrations of essential oil *O. heracleoticum* on the six isolates of *Penicillium aurantiogriseum* and four isolates of *Penicillium chrysogenum* isolated from meat plant for traditional Petrovacka sausage (Petrovská klobása) production.

## MATERIALS AND METHODS

**Plant Material:** Aerial parts of *O. heracleoticum* L. (*O. vulgare* L. ssp. *hirtum*) were collected during blooming stage (August 2009) from the locality Kamendol near Smederevo, Serbia. The plant material was dried under laboratory conditions (20-25 °C). Institute of Medicinal Plant Research Dr. Josif Pančić identified the plants, and voucher specimens were stored in the herbarium of the Institute of Medicinal Plant Research Dr. Josif Pančić.

**Isolation of essential oil:** The essential oils were isolated from dried plant material by hydro-distillation according to the standard procedure reported in the Sixth European Pharmacopoeia (European pharmacopoeia, 2008). Distillation was performed using Clevenger type apparatus for 2.5 hours. The resulting essential oil was dried over anhydrous sodium sulfate and stored at 4°C. The oil solution (1%) in ethanol was used for chromatographic analysis.

**Chemical analysis of the essential oil:** Gas chromatography-mass spectrometry (GC-MS) analyses were carried out using Agilent 5975C Series GC-MSD system (7890A GC and 5975C inert MSD), equipped with a HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.50 µm). 1 µl of diluted essential oil sample was injected in split mode (50:1), and inlet temperature was held at 250 °C. Helium was used as carrier gas in constant flow mode at 1 ml/min. The oven temperature was programmed as follows: 70 °C increased to 180 °C (2 °C/min) without holding, and then to 200 °C (4 °C/min) which was held for 10 min. Ion source was operated at 70 eV, and mass spectra were acquired in scan mode in the 50-550 m/z range.

**Identification of the essential oil components:** Essential oil components were identified by comparing their retention indices and mass spectra with those published by Adams (2007) and with Wiley and NIST/NBS mass spectral libraries. Experimental values of retention indices were calculated using calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1., DTRA/NIST, 2002).

**Microorganisms:** The antifungal activity of essential oil was evaluated using six isolates of *Penicillium aurantiogriseum* and four isolates of *Penicillium chrysogenum* isolated from Petrovačka sausage (Petrovská klobása) processing unit. Test cultures belonged to the culture collection from the Laboratory for Food Microbiology, Faculty of Technology in Novi Sad. The cultures were maintained on Sabouraud maltose agar (SMA) slants and were stored at +4 °C. The fungal isolates used in the experiments are shown in Table 1.

**Antifungal assay:** Evaluation of the effectiveness of oregano essential oil against ten isolates of *P. aurantiogriseum* and *P. chrysogenum* was carried out *in vitro* by disc-diffusion method. The suspension of the fungal isolates was prepared from 7-day-old cultures. Spores were taken by adding 10 ml of sodium chloride solution containing 0.5 % tween 80 onto slant, scraped with sterile loop and aseptically transferred into sterile test tubes. Determination of total mold count per 1ml of suspension was performed using the standard Koch's method. Final concentration of spore suspension was approximately  $1 \times 10^8$  spores/ml. One ml of suspension was inoculated in sterile Petri dishes

Tab. 1 – *P. aurantiogriseum* and *P. chrysogenum* isolated from traditional Petrovačka sausage (Petrovská klobása) processing unit

Species	Sign	Origin
<i>P. aurantiogriseum</i>	PS-1	Air in the ripening chamber
<i>P. aurantiogriseum</i>	PS-2	Air in the ripening chamber
<i>P. aurantiogriseum</i>	PS-3	Air in the ripening chamber
<i>P. aurantiogriseum</i>	PS-4	Pepper
<i>P. aurantiogriseum</i>	PS-5	Swab of sausage casing
<i>P. aurantiogriseum</i>	PS-6	Air in the ripening chamber
<i>P. chrysogenum</i>	PS-7	Air in the ripening chamber
<i>P. chrysogenum</i>	PS-8	Swab of sausage casing
<i>P. chrysogenum</i>	PS-9	Swab of sausage casing
<i>P. chrysogenum</i>	PS-10	Air in the ripening chamber

poured with about 15 mL of Sabouraud maltose agar (SMA) tempered at 47 °C. Essential oil was diluted in dimethylsulphoxide (DMSO) to the test concentrations of 250, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56 µl/mL. The filter paper discs (7 mm in diameter) were impregnated with 10 µl of the oil dilution in the concentration range from 250 to 1.56 µl/mL and placed on the inoculated agar. Negative controls were prepared using the same solvents to dissolve the essential oil – dimethylsulphoxide (DMSO). The plates were incubated at 25 °C for seven days. At the end of the incubation period, the minimal inhibitory concentration (MIC) was the lowest essential oil concentration showing growth inhibition zones with diameter equal to or greater than 10 mm. Diameters of the inhibition zones were measured in millimeters. All analyses were performed in duplicate, and the mean values with the standard deviations (SD) are reported.

## RESULTS AND DISCUSSION

It is well known that both environmental and genetic factors have effects on the observed variations among *O. heracleoticum* accessions with high accuracy (Jerković et al., 2001). Because of this, yield and chemical composition of essential oil can vary among the populations of the same species from different localities.

From the collected plant material of *O. heracleoticum* L. total of 2.05% (v/w) of essential oil has been isolated by the process of hydro-distillation. The oil was intensively yellow, with characteristically strong and pleasant odor. The results of chemical analysis of *O. heracleoticum* essential oil are presented in Table 2.

Tab. 2 – Chemical constituents of the essential oil of *O. heracleoticum* L. [%]

No.	Constituents	*KIL	%	No.	Constituents	*KIL	%
<b>Monoterpenes hydrocarbons</b>				18	Thymol	1289	7.94
1	$\alpha$ -Thujene	924	0.20	19	Carvacrol	1298	69.00
2	$\alpha$ -Pinene	932	0.65	20	Carvacrol acetate	1370	0.18
3	Camphene	946	0.19	$\Sigma$			<b>79.21</b>
4	$\beta$ -Pinene	974	0.30	<b>Sesquiterpenes hydrocarbons</b>			
6	$\beta$ -Myrcene	988	0.60	21	$\beta$ -Caryophyllene	1417	1.52
7	$\alpha$ -Phellandrene	1002	0.11	22	Aromadendrene	1439	0.06
8	$\Delta^3$ -Carene	1008	0.05	23	$\alpha$ -Humulene	1452	0.23
9	$\alpha$ -Terpinene	1014	0.99	24	$\beta$ -Bisabolene	1505	1.01
10	p-Cymene	1020	10.50	25	$\delta$ -Cadinene	1522	0.15
11	$\beta$ -Phellandrene	1025	0.11	$\Sigma$			<b>2.97</b>
12	$\gamma$ -Terpinene	1054	2.86	<b>Oxygenated sesquiterpenes</b>			
$\Sigma$			<b>16.56</b>	26	Caryophyllene oxide	1582	0.51
<b>Oxygenated monoterpenes</b>				$\Sigma$			<b>0.51</b>
13	Terpinolene	1086	0.14	<b>Other</b>			
14	Linalool	1095	0.21	5	1-octen-3-ol	974	0.07
15	Borneol	1165	0.31	$\Sigma$			<b>0.07</b>
16	Terpinen-4-ol	1174	0.73	$\Sigma$			<b>92.86</b>
17	Carvacrol methyl ether	1241	0.70				

\*KIL: literature values (Adams, 2007)

Twenty six components (92.86 %) were identified as constituents of this essential oil by GC/MS analyses. The major components were carvacrol (69.00%), p-cymene (10.50%), thymol (7.94%) and  $\gamma$ -terpinene (2.86%). Except  $\beta$ -caryophyllene (1.53%) and  $\beta$ -bisabolene (1.01%), the amount of all remaining oil components was less than 1%.

Aromatic alcohol carvacrol was also dominant compound in *O. heracleoticum* oil analyzed by other authors (Džamić et al., 2008; Govaris et al., 2010).

The predominant group of compounds in the oil were monoterpenes (95.77 %), with significantly more oxidized compounds (79.21 %) than hydrocarbons (16.56 %). Sesquiterpenes were present in low percentage in the oil (3.48 %).

Results for antifungal activity of *O. heracleoticum* L. essential oil obtained by the disc diffusion method are shown in Table 3. The results show that the essential oil of *O. heracleoticum* provides inhibition of all of the fungal isolates tested. *O. heracleoticum* L. essential oil exhibited higher antifungal activity against isolates of *P. chrysogenum* than isolates of *P. aurantiogriseum*. Isolate of *P. chrysogenum* (PS-9) shows complete inhibition (Figure 1) at the highest tested concentration (250  $\mu$ L/mL) of *O. heracleoticum* L. essential

oil. The oil also exhibited high antifungal activity against other tested isolates of *P. chrysogenum*. The zones of inhibition tested isolates of *P. chrysogenum* were in the range from 54.0 mm (PS-7) to 87.6 mm (PS-10). For the same concentration of *O. heracleoticum* L. essential oil isolates of *P. aurantiogriseum* achieved lower inhibition zones ranging from 16.2 mm (PS-4) to 38.0 mm (PS-2) (Table 3). At the highest tested concentration (250  $\mu\text{L/mL}$ ) most susceptible to the effects of essential oil was isolate PS-2 ( $38.0 \pm 3.11$ ) (Table 3, Figure 2, a, b). Essential oil did not have any influence on the tested fungal isolates at concentration lower than 25  $\mu\text{L/mL}$ .

Tab. 3 – Effect of various concentrations of essential oil of *Origanum heracleoticum* L. on the growth of fungal isolates

Isolate sign	Concentration ( $\mu\text{L mL}^{-1}$ )			
	250	100	50	25
PS-1	$26.4^* \pm 0.28^{**}$	$15.2 \pm 4.24$	$9.7 \pm 0.14$	$7.87 \pm 0.25$
PS-2	$38.0 \pm 3.11$	$13.6 \pm 0.84$	$10.2 \pm 1.09$	$7.75 \pm 0.27$
PS-3	$19.2 \pm 0.84$	$15.4 \pm 2.82$	$9.3 \pm 1.27$	-
PS-4	$16.2 \pm 1.69$	$13.4 \pm 2.26$	$8.2 \pm 0.28$	-
PS-5	$22.1 \pm 4.10$	$19.6 \pm 0.28$	$8.6 \pm 0.54$	-
PS-6	$31.8 \pm 1.09$	$24.4 \pm 0.81$	$8.0 \pm 0.0$	$7.87 \pm 0.25$
PS-7	$54.0 \pm 4.24$	$22.4 \pm 4.80$	$12.4 \pm 3.67$	$8.3 \pm 0.14$
PS-8	$67.8 \pm 2.26$	$19.2 \pm 1.41$	$10.8 \pm 0.84$	$8.7 \pm 0.42$
PS-9	c.i	c.i	$12.4 \pm 0.28$	$11.0 \pm 1.41$
PS-10	$87.6 \pm 1.15$	$52.0 \pm 7.07$	$12.8 \pm 0.84$	$9.5 \pm 1.64$

\* diameter of inhibition zone (mm) including disc diameter of 7mm

\*\* standard deviation (SD)

c.i. – complete inhibition

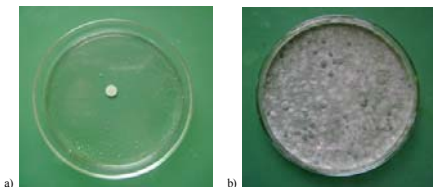


Fig. 1 – a) Complete inhibition of *P. chrysogenum* (PS-9) by *Origanum heracleoticum* L. essential oil (250  $\mu\text{L/mL}$ ) obtained by disc-diffusion method; b) Blank sample

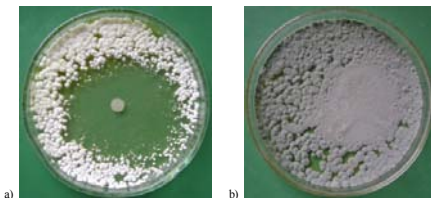


Fig. 2 – a) Diameter of inhibition zone for *Origanum heracleoticum* L. essential oil (250  $\mu\text{L}/\text{mL}$ ) against *P. aurantiogriseum* (PS-2) obtained by disc-diffusion method; b) Blank sample

Minimal inhibitory concentration (MIC) was the lowest essential oil concentration showing growth inhibition zones with diameter equal to or greater than 10 mm. *O. heracleoticum* essential oil showed MIC value of 50  $\mu\text{L}/\text{mL}$  against isolates of *P. chrysogenum*, with the exception of PS-9 isolates which showed MIC value of 25  $\mu\text{L}/\text{mL}$ .

MIC value of essential oil against isolates of *P. aurantiogriseum* was higher. Essential oil showed MIC value of 100  $\mu\text{L}/\text{mL}$ , against tested isolates of *P. aurantiogriseum*, with the exception of PS-2 isolates that showed MIC value of 50  $\mu\text{L}/\text{mL}$  (Figure 3). These results are in agreement with the findings of Carmo et al. (2008). They found the MIC values of *O. vulgare* essential oil ranging from 20 to 80  $\mu\text{L}/\text{mL}$  against *Aspergillus* species. Obtained results are in contrast with data published by Džamić et al. (2008) who reported lower MIC values. Their results concerning the essential oil of *O. heracleoticum* obtained by broth microdilution assay exhibited fungicidal characteristics with MIC and MFC of 0.1–1  $\mu\text{L}/\text{mL}$ .

This disparity in results could be related to a great extent to the volatility of the essential oil in an open air system when using the solid medium diffusion technique (Duarte et al., 2005). However, it has been established that the antifungal activity of different essential oils ranges from narrow to wide spectrum depending on the assayed essential oil, its concentration and fungal target (Burt and Reinders, 2003).

The applied disc diffusion method can be used only for preliminary screening of antimicrobial substances, since the easily volatile components of essential oils evaporate over a period of incubation together with the solvent, while poorly dissolved components do not pass through the medium (Griffin and Markham, 2000).

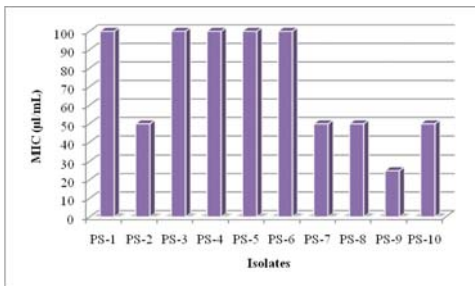


Fig. 3 – The MIC values ( $\mu\text{L/mL}$ ) of the of *Origanum heracleoticum* L. essential oil against isolates of *P. aurantiogriseum* and *P. chrysogenum* by the disc – diffusion method

The fungi cultivated in the medium with concentration of essential oil higher than  $50 \mu\text{L/mL}$  showed certain morphological changes. The alterations included lack of sporulation and loss of pigmentation (Figure 2. a, b). Previous studies have reported that essential oils are able to cause morphological changes in *Aspergillus* species including lack of sporulation, loss of pigmentation, aberrant development of conidiophores (flattened and squashed), and distortion of hyphae (De Billerberk et al., 2001; Rasooli and Abyaneh, 2004; Sharma and Tripath, 2008). These findings suggest that the mode of antifungal activity of essential oils could include an attack on the cell wall and retraction of the cytoplasm in the hyphae ultimately resulting in the death of the mycelium.

Velluti et al. (2003) suggested that the antimicrobial activity of the essential oil depends on the chemical structure of their components. Carvacrol and thymol, phenolic compounds known as major constituents of *Origanum* essential oil, have their antimicrobial property attributed to the presence of an aromatic group that is known to be reactive and to form hydrogen bonds with active sites of target enzymes (Souza et al., 2007). However, it is also suggested that the effectiveness of complete essential oils is higher than the activity of each separated compound (Miloš et al., 2000). Based on all our results, essential oil of *O. heracleoticum* L. may be used against molds, but at higher concentration.



## CONCLUSION

Results obtained by disk diffusion method showed that the essential oil of *O. heracleoticum* provides inhibition of all tested fungi isolates. *O. heracleoticum* L. essential oil exhibits higher antifungal activity against isolates of *P. chrysogenum* than isolates of *P. aurantiogriseum*. At the highest concentration tested (250 µL/mL), *O. heracleoticum* L. essential oil shows complete inhibition of the *P. chrysogenum* (PS-9) isolate. The oil also exhibited high antifungal activity against other tested isolates of *P. chrysogenum*. The inhibition zones of tested *P. chrysogenum* isolates were in the range from 54.0 mm (PS-7) to 87.6 mm (PS-10). For the same oil concentration used, *P. aurantiogriseum* isolates were less inhibited, with inhibition zones ranging from 16.2 mm (PS-4) to 38.0 mm (PS-2). For concentration lower than 25 µl/mL, essential oil has not influenced any of the tested fungal isolates. The fungi cultivated in the medium with concentration of essential oil higher than 50 µl/mL showed certain morphological changes. The alterations included lack of sporulation and loss of pigmentation.

Future research will be focused on determination of MIC and MFC using more precise broth microdilution method and examination of the influence of *O. heracleoticum* oil on the production of mycotoxins.

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УТИЦАЈ ЕСЕНЦИЈАЛНОГ УЉА *ORIGANUM HERACLEOTICUM L.*  
НА ИЗОЛАТЕ *PENICILLIUM AURANTIOWRISEUM* И *PENICILLIUM*  
*CHRYSOGENUM* ПОПЕКЉОМ ИЗ ХРАНЕ

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Резиме

Плесни су широко распрострањене у природи и њихове споре се могу наћи у атмосфери чак и на врло великим висинама. Тешкоће у контроли ових непожељних микроорганизама, као и све већа потражња за природним производима, приморавају индустрију да пронађе нове алтернативе у конзервисању хране. Модерни трендови предлажу ограничену примену синтетичких адитива и њихову замену природним. Ароматично биље је вероватно најважнији извор природних антимикробних агенаса. Есенцијално уље *Origanum heracleoticum L.* је познато као интересантан извор антимикробних компонента у погледу примене у конзервисању хране. У овом раду испитивали смо утицај есенцијалног уља добијеног из *O. heracleoticum* на раст шест изолата *Penicillium aurantiowriseum* и четири изолата *Penicillium chrysogenum*, изолованих у погону за производњу традиционалне Петровачке кобасице (*Petrovska klobasa*).

Резултати су показали да есенцијално уље *O. heracleoticum* инхибира све испитане изолате. Јачи антифунгални ефекат уље је показало према изолатима *P. chrysogenum* него према изолатима *P. aurantiowriseum*. Минимална инхибиторна концентрација уља кретала се у опсегу од 25 до 100 µl/ml. Плесни гајене на подлози са вишом концентрацијом есенцијалног уља показују одређене морфолошке промене. Те промене укључују смањену спорулацију и одсуство пигментације.