Antifungal activity of Oregano (*Origanum vulgare* L.) extract on the growth of *Fusarium* and *Penicillium* species isolated from food

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Abstract

The effect of the oregano extract (*Origanum vulgare* L.) on the growth of *Fusarium* and *Penicillium* species isolated from cakes and ready-for-use fresh salads from different kinds of vegetables was investigated. The contents of the active component of extract were identified by GC-MS and they include: carvacrol (34.2%), carvone (18.5%), *p*-cimene (8.05%), thymol (3.74%). The oregano extract showed the ability to reduce mould growth at all applied concentrations. Stronger inhibitory effect on the growth of *Penicillium* species, contrary to *Fusarium*, was determined. At extract concentration of 2.50 mL/100 mL, growth of *P. aurantiogriseum, P. glabrum* and *P. brevicompactum* was completely inhibited during 14 days of incubation. At the same concentration, growth of *Fusarium proliferatum* was inhibited by 81.71%, *F. oxysporum* by 85.84%, *F. verticillioides* by 86.50%, *P. chrysogenum* by 86.2% and *F. subglutinans* by 88.85%.

Keywords: Oregano extract; antifungal activity; *Fusarium* spp.; *Penicillium* spp.

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Moulds comprise a large group of microorganisms, which are frequent contaminants, and causes of spoilage in many food commodities. *Fusarium* species are field moulds, which require higher substrate moisture (20–21%) and lower temperatures for growth and commonly contaminate plants on fields. They are frequent contaminants of cereals (corn, barley, wheat, oats, rye, rice, etc.), cereal products (flour, bread, cakes, etc.), fruits and vegetables [1–4]. *Penicillium* species are storage moulds with lower requirements regarding substrate moisture (13–18%), but higher temperature requirements and are frequently isolated from stored products [5–8]. Enormous economic damages take place as a consequence of food deterioration caused by metabolic activity of microorganisms [7]. In addition, species possessing the genetic base for mycotoxin production can biosynthesize zearalenone, trichothecenes, fumonisins, monilioformin, fusarin C, etc. (*Fusarium* spp.), ochratoxin A, penicillic acid, verrucosidin, patulin, nephrotoxic glycopeptides, roquefortine C, chrysogine, penitrem A, etc. (*Penicillium* spp.) [9–11]. The consumption of food contaminated with mycotoxins has been associated with various diseases in humans, livestock and domestic animals. They have been recognized as causes of cytotoxicity, hepatotoxicity, teratogenicity, mutagenicity, neurotoxicity, etc. [9,11].

Several strategies have been used in controlling the fungal growth and mycotoxin biosynthesis in food products. However, today consumers increasingly demand that their food is minimally processed without synthetic preservatives and additives, because of their possible harmful effects on human health. The problem that the food industry now faces is how to produce food that will satisfy these criteria and also be safe to use. It is known that certain types of spice plants have antimicrobial properties. Therefore, essential oils, extracts, oleoresins and their main components extracted from natural herbs as antimicrobial agents attracted attention as one of the possible solutions for controlling the fungal growth and mycotoxin biosynthesis in food that are not toxic in contrast to chemical additives [12–17].

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

The objective of this work was to study the antifungal potential of commercial oregano (*Origanum vulgare* L.) extract, intended for usage in food, against some *Fusarium* and *Penicillium* species isolated from cakes and ready-for-use fresh salads from different kinds of vegetables.

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MATERIALS AND METHODS

Oregano extract

For antifungal activity testing, a commercially available food grade oregano (*Origanum vulgare* L.) extract was provided from ETOL "Tovarna arom in eteričnih olj d.d.", Celje, Slovenia.

Determination of oregano extract composition

The composition of extracts was determined by gas chromatography–mass spectrometry (GC–MS) analyses. The GC–MS analysis was carried out on a Varian T2100 GC–MS instrument equipped with data processor. A fused silica capillary column VF-5MS (30 m×0.25 mm i.d., 0.25 μm film thickness, Varian) was used for the separation of the sample components. The carrier gas ultra pure helium was passed through moisture and oxygen traps with constant flow rate of 0.62 cm^3/min. The following temperature program was used: injector temperature 230 °C, initial temperature 40 °C (held 5 min), temperature increase 5 °C/min to 200 °C and held at this temperature for 25 min. The mass spectrometer was operated in the electron ionization mode. The data acquisition was carried out in the scan mode (range 50–550 *m*/*z*). The injection volume was 1 μl. The compounds were identified by matching the mass spectra with NIST Mass Spectra Library stored in the GC–MS database.

Fungal strains

As testing microorganisms, the following fungal strains from the genera *Fusarium* and *Penicillium* were used: *F. oxysporum* Schlecht., *F. proliferatum* (Matsushima) Nirenberg, *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas and *F. verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* Sheld.), *P. aurantiogriseum* Dierckx, *P. brevicompactum* Dierckx, *P. glabrum* (Wehmer) Westling, *P. chrysogenum* Thom. The fungal cultures were isolated from cakes and ready-for--use fresh salads from different kinds of vegetables and identified according to determination keys described by Leslie and Summerell [3], Lević [4], Samson and Frisvard [6], and Samson *et al*. [18]. The isolated cultures were maintained on Potato Dextrose Agar (PDA) (Merck, Darmstadt) 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.

Efficacy of oregano extract on the growth of *Fusarium* and *Penicillium* species

The agar plate method was applied in testing of antifungal activity of oregano extract. The basic medium for antifungal tests was PDA (Merck, Darmstadt). The medium was divided into equal volumes (150 mL), poured into Erlenmeyer (250 mL) flasks and autoclaved at 121 °C for 15 min. Concentrations of 0, 0.35, 0.70, 1.50 and 2.50 mL/100 ml were tested. The extracts were added to the medium after cooling to 45 °C. The culture medium was poured into sterile Petri dishes (∅ 9 cm), 12 mL into each plate.

Seven-day fungal cultures grown on PDA were used to prepare fungal spore suspension tests. Suspensions of funal spores were prepared in a medium containing 0.5% Tween 80 and 0.2% agar in distilled water and adjusted to 10^6 spores/mL using a haemocytometer. For each extract dose and fungi species, including the controls, plates were centrally inoculated with 1 μl of a spore suspension (10^3 spores/mL) using an inoculation needle. After inoculation, the Petri plates were closed with a parafilm.

The efficacy of the treatment was evaluated by daily measurement of the diameter of the radial colony growth during 14 days of incubation at 25±2 °C. The parafilms were removed from the Petri dishes without fungal growth after 14 days, and they were further incubated up to 30^{th} day. If there were no observable growth during this period, fungal spores were transferred using a wet cotton swab to the PDA medium free from extract and were incubated for 5 days at 25±2 °C to confirm the fungicidal effect (MFC).

The inhibitory effect of the oregano extract was calculated following the formula:

$$I\,(\%) = 100(C - T)/C$$

where I is inhibition, C is colony diameter on the control plate and T is colony diameter on the test plate [19].

Monitoring of macroscopic and microscopic changes in moulds

Changes in macroscopic and microscopic features of moulds were observed and compared to the controls. The macroscopic and microscopic features were observed using a stereoscopic binocular microscope (Technival 2, Carl Zeiss) and microscope (Aristoplan, Leitz), respectively.

Statistical analysis

Determination of the effect of oregano extract on the growth of *Fusarium* and *Penicillium* species was carried out in 3 series and 2 replications. Microsoft Statistica® 4.5 (Microsoft, USA) was used to calculate means and standard deviations. Significant differences between the colony growth inhibition values assessed on the fourteenth day of the testing were determined by Duncan's multiple range test ($p < 0.05$), following one-way ANOVA procedure.

RESULTS AND DISCUSSION

Extract composition

The constituents of the oregano extract are shown in Table 1. A total of 20 components were identified, with major components being carvacrol (34.20%), carvone (18.05%), *p*-cymene (8.05%), thymol (3.74%) limonene (3.36%), γ-terpinene (2.35%).

The majority of researchers named carvacrol and thymol as the main compounds associated with the antimicrobial activity of oregano [20–23]. *p*-Cymene and γ-terpinene were evaluated as weaker antimicrobial agents when compared to carvacrol and thymol, although they are their bioprecursors [24,25].

Effect of oregano extract on the growth of *Fusarium* and *Penicillium* species

The oregano extract at the tested concentrations showed a capacity to reduce or inhibit the growth of *Fusarium* and *Penicillium* species. The inhibitory effect of the extract increased proportionally with concentration and was also affected by treatment duration. Growth rate and colony growth inhibition of tested moulds in the presence of oregano extract are presented in Figures 1–8. Table 2 presents the results of mould inhibition assessed on fourteenth day of exposure to oregano extract.

During 14 days of incubation, the lowest concentration of oregano extract significantly inhibited the growth of *F. proliferatum* (24.06%), *F. oxysporum* (23.22%) and *F. subglutinans* (22.30%) whereas its effect was negligible to *P. glabrum* (3.07%) and *P. chrysogenum* (5.99%). At 0.70 mL/100 mL extract concentration the inhibition of fungal growth ranged from 10.27% (*P. glabrum*) to 54.47% (*P. aurantiogriseum*). The extract concentration of 1.50 mL/100 mL exhibited the highest inhibitory effect against the growth of *F. subglutinans* (76.45%), *P. aurantiogriseum* (76.43%), *F. verticillioides* (72.61%) and *F. proliferatum* (72.21%). At this concentration, the inhibitory activity against other tested moulds ranged from 53.74% (*P. glabrum*) to 62.86% (*P. brevicompactum*). At 2.50 mL/100 mL, the growth of *P. aurantiogriseum*, *P. glabrum* and *P. brevicompactum* was totally inhibited after 14 days of incubation. The colony growth was reduced by 86.2% in *P. chrysogenum,* 81.71% in *F. proliferatum,* 85.84% in *F. oxysporum*, 86.50% in *F. verticillioides* and 88.85% in *F. subglutinans* (Figures 1–8, Table 2).

Increasing concentrations of oregano extract (0.70, 1.50 and 2.50 mL/100 mL) caused an absence or delay in spore germination and showed different inhibitory effects.

At extract concentration of 0.70 mL/100 mL, spore germination in *P. aurantiogriseum* was delayed for one day in relation to the control whereas it was unaffected

Table 1. Chemical composition of Oregano extract

Peak	Compound	Percentage[a]	*RT*[b] / min	Identification method[c]
1	α-Thujene	0.23	10.259	GC–MS[c]
2	α-Pinene	0.28	10.512	GC–MS
3	Camphene	0.31	11.123	GC–MS
4	Myrcene	0.31	12.656	GC–MS
5	α-Phellandrene	0.97	13.221	GC–MS
6	α-Terpinene	0.20	13.601	GC–MS
7	*p*-Cymene	8.05	13.895	GC–MS
8	Limonene	3.36	14.034	GC–MS
9	γ-Terpinene	2.35	15.042	GC–MS
10	Linalol	1.51	16.521	GC–MS
11	Borneole	0.22	18.840	GC–MS
12	4-Terpineole	0.05	19.048	GC–MS
13	Dill ether	1.69	19.184	GC–MS
14	α-Terpineole	0.29	19.558	GC–MS
15	Dihydrocarvone	0.24	19.751	GC–MS
16	Carvone	18.05	20.934	GC–MS
17	Thymol	3.74	22.537	GC–MS
18	Carvacrol	34.20	22.766	GC–MS
19	Triacetin	22.91	23.619	GC–MS
20	β-Caryophyllene	0.90	25.658	GC–MS
21	Other compounds	0.14	–	GC–MS

[a]Relative area percentage; [b]retention time on VF-5MS column; [c]gas chromatography–mass spectrometry

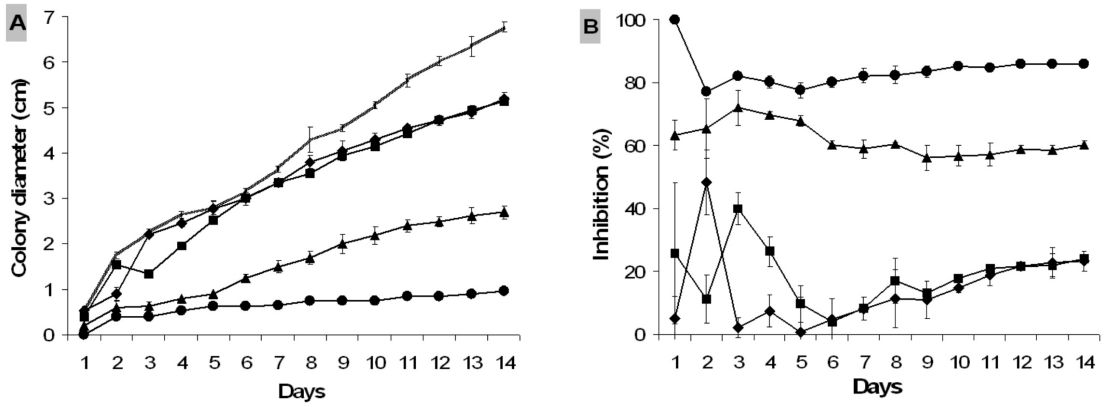

Figure 1. Growth rate (A) and colony growth inhibition (B) of F. oxysporum; control (▩), 0.35 (◆), 0.70 (■), 1.50 (▲) and 2.50 mL/100 mL (●).

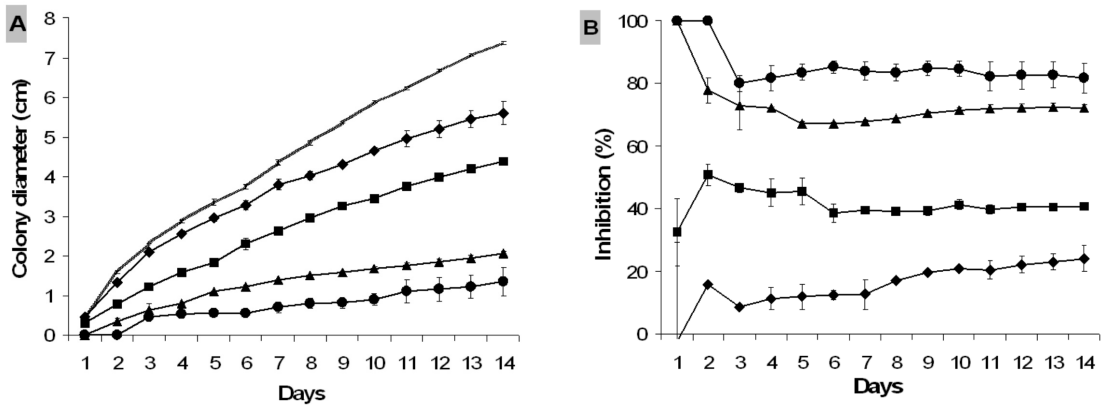

Figure 2. Growth rate (A) and colony growth inhibition (B) of F. proliferatum; control (▩), 0.35 (◆), 0.70 (■), 1.50 (▲) and 2.50 mL/100 mL (●).

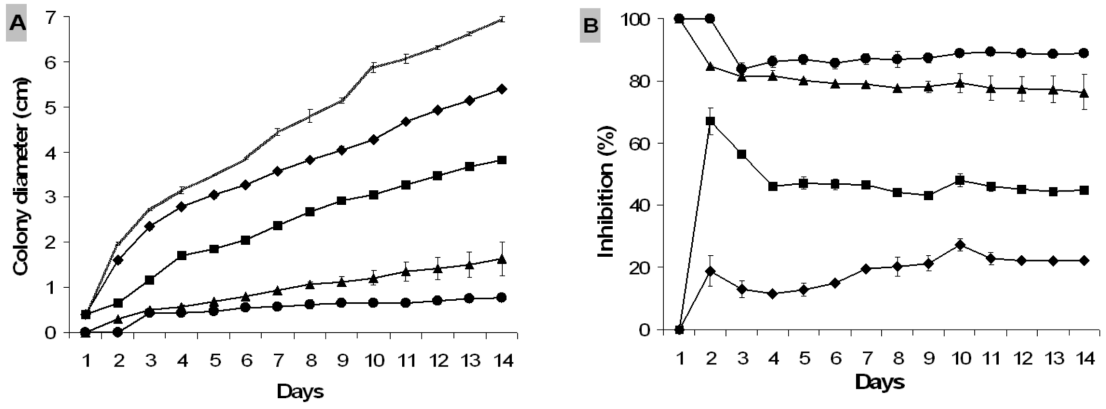

Figure 3. Growth rate (A) and colony growth inhibition (B) of F. subglutinans; control (▩), 0.35 (◆), 0.70 (■), 1.50 (▲) and 2.50 mL/100 mL (●).

in other assayed species. At 1.50 mL/100 mL concentration, the colony growth of *P. aurantiogriseum* was delayed for 3 days, *P. brevicompactum* for 2 days, *P. glabrum*, *F. proliferatum* and *F. subglutinans* for one day. During 30 days of incubation at 2.50 mL/100 mL extract concentration, the growth of *P. aurantiogriseum* was not observed. This concentration delayed the growth of *P. brevicompactum* for 23 days and *P. glabrum* for 17 days. The growth of *P. chrysogenum*, *F. proliferatum*, *F. subglutinans* was observed on the third day and *F. oxysporum* after 2 days of incubation. This extract concentration was MFC for *P. aurantiogriseum* and MIC for P. *brevicompactum* and *P. glabrum*.

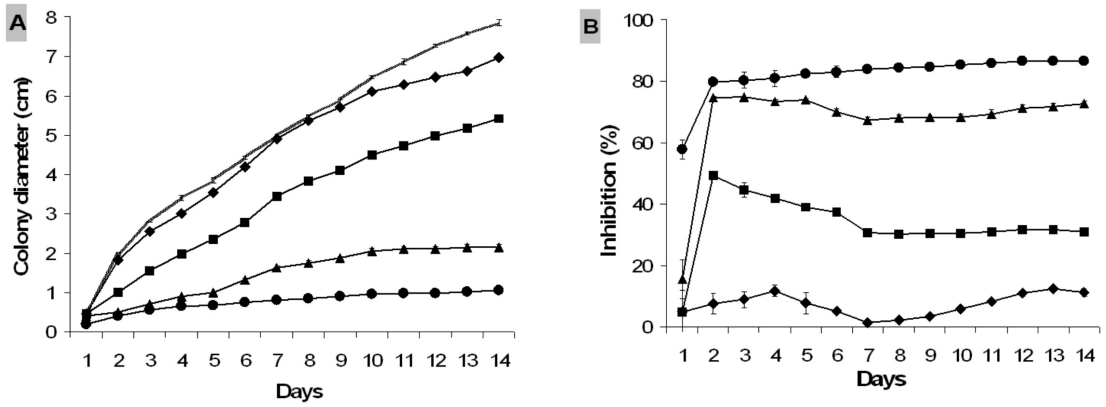

Figure 4. Growth rate (A) and colony growth inhibition (B) of F. verticillioides; control (▪), 0.35 (◆), 0.70 (■), 1.50 (▲) and 2.50 mL/100 mL (●).

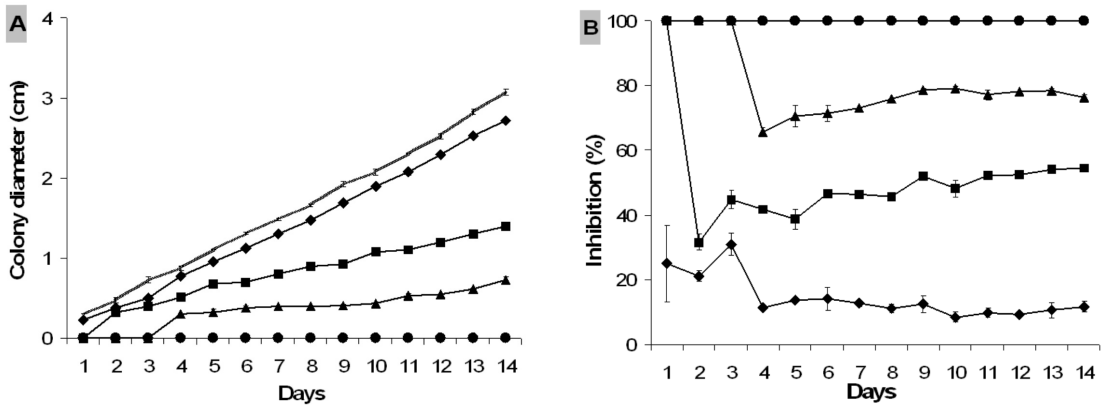

Figure 5. Growth rate (A) and colony growth inhibition (B) of P. aurantiogriseum; control (▪), 0.35 (◆), 0.70 (■), 1.50 (▲) and 2.50 mL/100 mL (●).

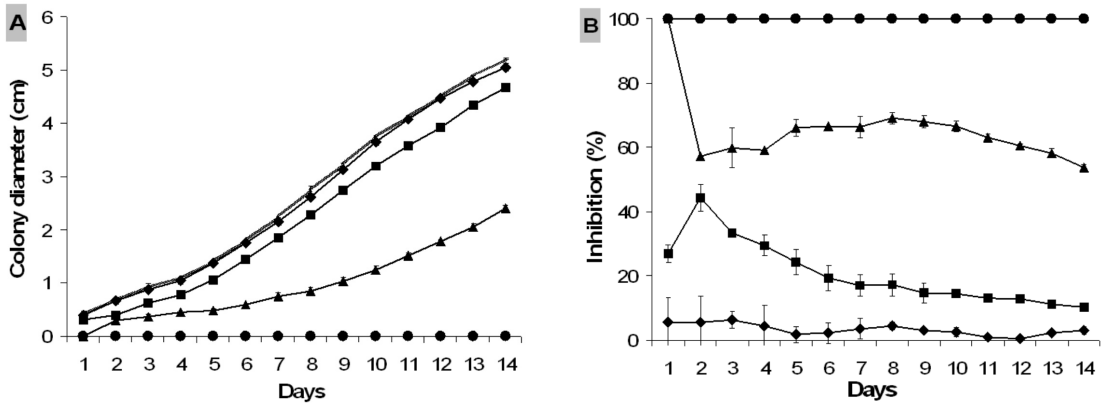

Figure 6. Growth rate (A) and colony growth inhibition (B) of P. glabrum; control (▪), 0.35 (◆), 0.70 (■), 1.50 (▲) and 2.50 mL/100 mL (●).

Colony growth reduction induced by increased Oregano extract concentrations was more evident in *Penicillium* species than in *Fusarium* species, which implies their higher sensitivity (Figures 1–8).

Besides the colony growth reduction effect, the tested oregano extract caused changes in fungal macro and micromorphology. At higher concentrations (1.50 and 2.50 mL/100 mL), the formation of uncharacteristic colonies with centre elevation, granular structure and diminished conidiation (*P. brevicompactum, P. chrysogenum, F. oxysporum*) occurred. Moreover, hyphae deformations with frequent occurrence of fragmentations and thickenings or bending, deviations of reproductive organs (occurrence of irregular vesicle frequently without

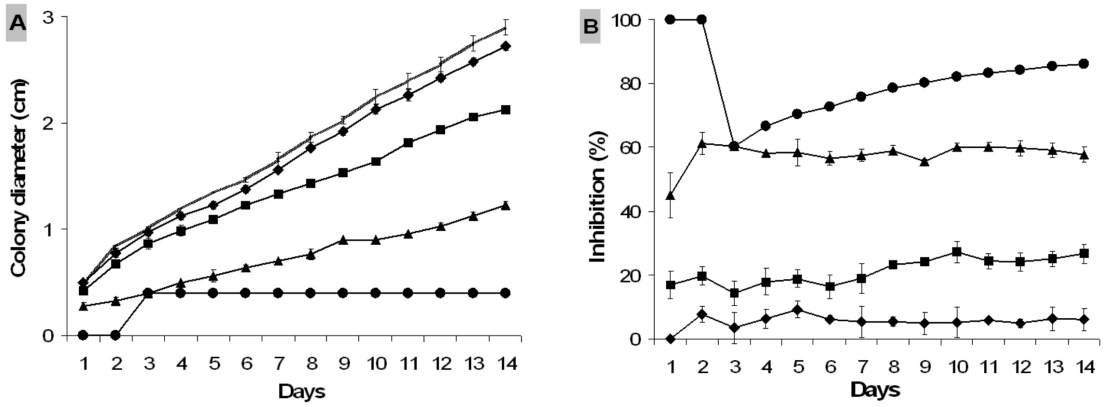

Figure 7. Growth rate (A) and colony growth inhibition (B) of P. chrysogenum; control (▪), 0.35 (◆), 0.70 (■), 1.50 (▲) and 2.50 mL/100 mL (●).

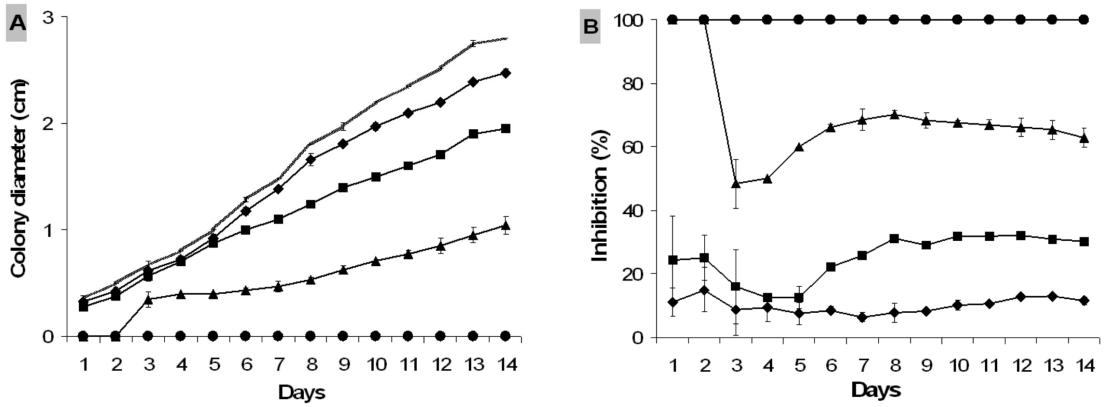

Figure 8. Growth rate (A) and colony growth inhibition (B) of P. brevicompactum; control (▪), 0.35 (◆), 0.70 (■), 1.50 (▲) and 2.50 mL/100 mL (●).

Table 2. Colony growth inhibition (%) of tested moulds after 14 days of incubation. Values followed by the same small letter (a–d) within the same column are not the significantly different (p > 0.05) according to Duncan's multiple rang test. Values followed by the same big letter (A–E) within the same row are not the significantly different (p > 0.05) according to Duncan's multiple rang test

Moulds	Concentration of oregano extract, mL/100 mL			
	0.35	0.70	1.50	2.50
F. oxysporum	$23.22\pm3.29^{ab,C}$	$23.98\pm0.15^{ab,C}$	$60.16\pm1.46^{c,C}$	$85.84\pm0.61^{d,B}$
F. proliferatum	$24.06\pm4.20^{a,C}$	$40.68\pm0.76^{b,E}$	$72.21\pm0.83^{c,A}$	$81.71\pm4.71^{d,C}$
F. subglutinans	$22.30\pm0.79^{a,C}$	$44.96\pm0.05^{b,F}$	$76.45\pm5.63^{c,A}$	$88.85\pm0.40^{d,B}$
F. verticillioides	$11.14\pm1.25^{a,A}$	$30.89\pm1.07^{b,D}$	$72.61\pm1.15^{c,A}$	$86.50\pm0.96^{d,B}$
P. aurantiogriseum	$11.70\pm1.71^{a,A}$	$54.47\pm0.52^{b,A}$	$76.43\pm0.88^{c,A}$	$100.00\pm0.00^{d,A}$
P. glabrum	$3.07\pm1.09^{a,B}$	$10.27\pm0.92^{b,B}$	$53.74\pm0.96^{c,B}$	$100.00\pm0.00^{d,A}$
P. chrysogenum	$5.99\pm3.51^{a,AB}$	$26.69\pm3.01^{b,C}$	$57.73\pm2.25^{c,BC}$	$86.20\pm0.34^{d,B}$
P. brevicompactum	$11.61\pm1.26^{a,A}$	$30.36\pm0.00^{b,D}$	$62.86\pm3.03^{c,C}$	$100.00\pm0.00^{d,A}$

metulae and phialides, enlarged metulae, deformations of monophialides) and absence of reproductive organs (*P. aurantiogriseum, P. brevicompactum, P. chrysogenum*) were observed at the same concentrations.

Numerous studies of other researchers have also demonstrated considerable antifungal activity of dried oregano, oregano extracts and its essential oils. Akgula and Kivanc [26] found that, among ten tested spices, only oregano exerted antifungal activity against nine tested fungi. The addition of ground oregano and thyme into the growth medium reduced the production of aflatoxins by *Aspergillus parasiticus* [27]. Oregano extract at 2 g/100g completely inhibited the growth of *A. parasiticus* during 10 days of incubation at 30 °C [28]. Essential oil of oregano was described as a strong inhibitor of fungal growth and mycotoxin biosynthesis. In a

growth medium that contained 0.10 mL/100 mL oregano essential oil, the growth of *A. parasiticus* and aflatoxin production was completely inhibited [29]. Paster *et al*. [30] demonstrated the antifungal activity of oregano essential oil at concentration of 2.0 and 2.50 µL/L on mycelium and spores of *A. niger, A. flavus* and *A. ochraceus*. Basilico and Basilico [31] reported that 1000 ppm of oregano essential oils inhibited the growth of *A. ochraceus* and ochratoxin A production in YES broth during 21 days. Essential oils of Greek oregano at the applied concentration of 4 µL significantly inhibited the growth of *Pythium* spp., *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *lycopersici* and *Sclerotina sclerotiorum* [32]. Etheric oil of oregano showed particularly strong inhibition to the growth of *Fusarium proliferatum, F. graminearum* and biosynthesis of fumonisin B1, zearalenone and deoxynivalenol [33,34]. Baratta *et al*. [35], Bouchra *et al*. [36] and Vuida-Martos *et al*. [37] implied that essential oil of oregano possesses stronger antifungal activity against *A. niger* and *A. flavus* in comparison to those of rosemary, sage, thyme and clove. Gumus *et al*. [38] reported that oregano essential oil completely inhibited the mycelial growth of *Aspergillus fumigatus* and *Paecilomyces variotii* at 0.25 g/100 g during 10 days of incubation.

Antifungal activities of tested oregano extract depend on content of major and minor components. Thymol and carvacrol in concentration of 0.0025 g/100 g and 0.05 g/100 g at pH 5.5 completely inhibited the growth of *A. flavus*, *A. niger, Geotrichum candidum, Mucor* spp*., P. roqueforti* and *Penicillium* spp. on PDA agar. The volatile oil *Origanum syriacum* that contain carvacrol and thymol as the major components completely inhibited the mycelial growth of *A. niger*, *Penicillium* spp., and *F. oxysporum* at concentration of 0.1 µL/mL in YES broth [39].

At cellular level, these compounds are able to inhibit enzymes, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins [40,41]. Their possible consequence is damage to membrane integrity, which could affect pH homeostasis and equilibrium of inorganic ions [41,42].

CONCLUSION

The oregano extract at concentration of 2.50 mL/100 mL was found to efficiently reduce and inhibit the growth of assayed moulds. This concentration was MFC for *P. aurantiogriseum* and MIC for P*. brevicompactum* and *P. glabrum*. The growth of *P. chrysogenum* and *Fusarium* species was significantly reduced (81.69–88.84%). The extract exerted stronger effect on the *Penicillium* species. Among *Penicillium* species, *P. aurantiogriseum* was the most sensitive to the extract whereas *P. chrysogenum* was the least sensitive. *F. subglutinans* showed higher sensitivity to the extract activity in relation to other *Fusarium* species whereas *F. proliferatum* had the lowest sensitivity.

The obtained results suggest that the oregano extract is suitable as antifungal agent in food applications. However, additional research is necessary to assess more in detail the practical application of the tested concentrations in the real food system (*e.g*., bakery products).

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IZVOD

ANTIFUNGALNA AKTIVNOST EKSTRAKTA ORIGANA (*Origanum vulgare* L.) NA RAST *Fusarium* I *Penicillium* VRSTA IZOLOVANIH IZ HRANE

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(Naučni rad)

U radu je prikazan uticaj ekstrakta origana (*Origanum vulgare* L.) na rast *Fusarium* i *Penicillium* vrsta izolovanih iz kolača i salata od svežeg povrća. Antifungalna ispitivanja su izvedena metodom agar ploča. Sastav aktivnih komponenti ekstrakta je određen GC–MS metodom i kao glavne komponente ustanovljene su: karvakrol (34,2%), karvon (18,5%) i *p*-cimen (8,05%). Ispitivani ekstrakt je na svim primenjenim koncentracijama pokazao sposobnost redukcije rasta plesni. Jači inhibitorni efekat je utvrđen na rast *Penicillium* vrsta. Pri koncentraciji od 2,50 mL/ /100 mL ekstrakta rast *P. aurantiogriseum*, *P. glabrum* i *P. brevicompactum* je kompletno inhibiran tokom 14 dana inkubiranja. Pri istoj koncentraciji rast *F. proliferatum* je inhibiran za 81,71%, *F. oxysporum* za 85,84%, *F. verticillioides* za 86,50%, *P. chrysogenum* za 86,2% i *F. subglutinans* za 88,85%. Ispitivani začinski ekstrakt origana je pored ograničavanja rasta kolonija plesni uzrokovao i promene u makro- i mikromorfologiji. Pri višim koncentracijama (1,50 i 2,50 mL/100 mL) došlo je do formiranja nekarakterističnih, kolonija sa izdignutim centrom, zrnaste strukture i smanjenom konidijacijom (*P. brevicompactum, P. chrysogenum, F. oxysporum*). Takođe, na ovim koncentracijama su u mikroskopskom preparatu uočene deformacije hifa (sa čestom fragmentacijom i zadebljanjima, ugibanje) i reproduktivnih organa (vezikule nepravilnog oblika, često bez metula i fijalida, pojava proširenih metula, deformacija monofijalida) ili bez reprouktivnih organa (*P. aurantiogriseum, P. brevicompactum i P. chrysogenum*). Rezultati pokazuju da bi ispitivani ekstrat origana mogao imati zaštitni efekat u kontroli rasta plesni u hrani tokom čuvanja ili čak produžiti rok trajnosti hrane.

Ključne reči: Ekstrakt origana • Antifungalni uticaj • *Fusarium* spp. • *Penicillium* spp.