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Original scientific paper

DIFFERENT EXTRACTS AND ISOLATED SUBSTANCES FROM THE ROOT OF *Onosma visianii* Clem. AS ANTIFUNGAL AGENTS

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ABSTRACT. The aim of this study was to determine the antifungal activity of extracts and isolated substances from the root of *Onosma visianii* Clem. (Boraginaceae) by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The results show that antifungal activity depends on species of microorganism and the type of substances or extracts. All tested substances showed selective and moderate antifungal activity. MIC and MFC values for the extracts ranged from <0.015 to 15 mg/ml, while those for substances ranged from 0.04 to 5 mg/ml. Among all tested extracts, petroleum ether and methylene chloride in proportion 1:1 (against *Penicillium expansum*) and acetone extract (against *Saccharomyces boulardii*) showed the strongest antifungal activity. Among the isolated substances, the most significant was the effect of 5,8-O-dimethyl deoxyshikonin against *P. expansum*. The results of this study show that both extracts, and the isolated substances can be potentially used in pharmacology, biotechnology, or agriculture.

Key words: *O. visianii*, antifungal activity, naphthoquinones, 5,8-O-dimethyl deoxyshikonin

INTRODUCTION

When microorganisms are exposed to antibiotics for a long period of time, due to the treatment of certain infections, they develop resistance to these agents (WORLD HEALTH ORGANIZATION (WHO), 2019, 2020). Today, many plants are used as a source of bioactive compounds that can be used as auxiliary substances against microorganisms. Nowadays, both plant extracts and isolated substances that can be used to control the growth and development of bacteria or fungi are being tested (RADOJEVIĆ *et al.*, 2013; GRUJIĆ *et al.*, 2014; MLADENOVIĆ *et al.*, 2016; MURUZOVIĆ *et al.*, 2016; RADOJEVIĆ *et al.*, 2021), and this trend of investigation is increasing. Due to the large number of medicinal plants and substances isolated from them,

they represent an alternative to synthetic antibiotics to which microorganisms develop resistance (VEERESHAM, 2012).

Onosma visianii Clem. is a plant species from the Boraginaceae family. The species is widely distributed in the areas of the Balkan Peninsula and Southeastern Europe. *Onosma* sp. is traditionally used in medicine for the treatment of infections of the respiratory tract, digestive system, urinary tract, etc. (DI GIORGIO *et al.,* 2008).

The first report on the chemical composition of *O. visianii* was presented a long time ago, in 1971. The results indicated a rich chemical composition, including the presence of shikonin (SHCHERBANOVSKII, 1971). Phytochemical studies of the roots of *Onosma* plants indicated the presence of shikonin and alkannin derivatives, as compounds that exhibited different biological potentials (NAZ *et al.,* 2006; WANG *et al.,* 2015). Alkannins and shikonins belong to the group of lipophilic red pigments. Except in genus *Onosma* they are found on the surface of the roots of species belonging to the genera *Alkanna*, *Lithospermum*, *Echium, Anchusa* and *Cynoglossum* (al from Boraginaceae family) (PAPAGEORGIOUA *et al.,* 2006).

The biological properties of shikonin and its derivatives have been studied by many researchers. ANDÚJAR *et al*. (2013) presented a review of the literature published between 2002 and 2013 on the antifungal activity of shikonin and its derivatives. The authors also pointed out that shikonin has many biological potentials such as antioxidant, anti-inflammatory, antithrombotic, antimicrobial activity etc. These findings are of great importance as they have increased the interest of scientists in their studies. The use of alkannin, shikonin, and related derivatives in therapeutic areas, especially in cancer chemotherapy, is under development. Naphthoquinones isolated from *O. visianii* showed antibacterial and cytotoxic activities (VUKIC *et al.,* 2017). Shikonin family naphthoquinones, isolated from the root extract of *O. visianii*, could be potential candidates for use in agriculture (for the crop protection) (SUT *et al.,* 2017). *Onosma* species have compounds with a large variety of biological effects including cholinesterase and tyrosinase inhibitory activities with antioxidant activity, antibacterial, antitumor (KRETSCHMER *et al.,* 2012, YILDIRIM *et al.,* 2013), hypoglycemic (KUMAR *et al.,* 2010), parasiticidal and antifungal activities (AHMAD *et al.,* 2009).

However, there is very limited research data regarding the antifungal activity of *O. visianii*. Therefore, this study attempted to investigate the antifungal properties of petroleum ether and methylene chloride (1:1), chloroform, ethyl acetate, acetone and methanol extracts, as well as isolated compounds (deoxyshikonin, isobutyrylshikonin, α-methyl butyrylshikonin, acetylshikonin, β-hydroxy isovalerylshikonin, 5,8-O-dimethyl isobutyrylshikonin, 5,8-O-dimethyl deoxyshikonin) obtained from the roots of *O. visianii.* The results of this study reveal the potential of this plant species for use in pharmacology, biotechnology, or agriculture.

MATERIALS AND METHODS

Chemicals

The extracts and isolated substances were dissolved in DMSO and then diluted in a liquid nutrient medium (Sabouraud dextrose broth; Torlak, Belgrade), to achieve a 10% concentration. Antimycotics fluconazole (Pfizer Inc., USA), ketoconazole (Sigma-Aldrich, USA), amphotericin B (Sigma-Aldrich, USA) and itraconazole **(**Actavis Ltd**.,** UK**)** were dissolved in the same nutrient liquid medium. Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (New Jersey, USA). All other solvents and chemicals were of analytical grade.

Plant material, extraction method and compound isolation

The roots of *O. visianii* were collected in June 2015 in Rumija mountain (southern Montenegro, altitude 650 m, 42º06'10'' N, 19º11'37'' E). The herbarium specimen was deposited at the Department of Botany, Faculty of Biology, University of Belgrade, Serbia.

The extraction method involved dried roots (135 g) of *O. visianii* where the plant was pulverized and extracted at room temperature in different solvents: mixture of petroleum ether and methylene chloride (1:1), chloroform, ethyl acetate, acetone, and methanol. The prepared extracts were evaporated under reduced pressure to obtain the concentrated extract as a reddish brown semi-viscous residue.

The general procedure for isolation of the compound implied a petroleum ether methylene chloride extract subjected to column chromatography on silica gel. Twenty-nine fractions were analyzed on TLC plates. After TLC analysis, compounds ***1*** and ***2*** were obtained from fractions F1-11, compounds ***2*** and ***3*** from F12-15, compounds ***3*** and ***4*** from F16-20, compound ***5*** from F21-23, and compounds ***6*** and ***7*** from F24-29. To achieve high purity, all isolated compounds were subjected to semi-preparative HPLC on a Zorbak Eclipse KSDB C18 reversed phase column with isocratic elution using a mixture of water and methanol (40:60). All roots extracts and isolated compounds were obtained according to the methods of VUKIC *et al*. (2017).

Antifungal assay

Test microorganisms

The antifungal activity of five types of extracts and seven isolated substances was tested against ten fungal species – four yeasts (*Rhodotorula mucilaginosa*, *Saccharomyces boulardii, Candida albicans*, and *Candida albicans* ATCC 10231) and six filamentous fungi (*Penicillium expansum, Penicillium chrysogenum, Mucor mucedo* ATCC 52568, *Trichoderma viride* ATCC 13233, *Aspergillus flavus* ATCC 9170, and *Aspergillus niger* ATCC 16404). The microorganisms originate from a collection of the Microbiology Laboratory of the Faculty of Science, University of Kragujevac.

Suspension preparation

Yeast suspensions were prepared by the direct colony method (ANDREWS, 2005). Turbidity of the initial suspension was adjusted by comparison with the 0.5-McFarland standard (approximately 10^6 CFU/ml), and diluted 1:100 in sterile 0.85% saline. The fungal spores suspension was prepared by carefully stripping the spores from the nutrient agar slants and rinsed with sterile 0.85% saline, used for spectrophotometric determination of turbidity at 530 nm, and then further diluted to approximately 10^6 CFU/mL according to the procedure recommended by NATIONAL COMMITEE FOR CLINICAL LABORATORY STANDARDS and PFALLER (1998).

Microdilution method

Minimum inhibitory concentration (MIC) and minimum fungicide concentration (MFC) were determined by the broth microdilution method using 96-well microtiter plates (SARKER *et al.*, 2007). The 96-well plates were prepared by adding 100 μL of Sabouraud dextrose broth into each well. 100 μL of stock solution of extracts or isolated compounds were added to the first row of the plate. Then, twofold serial dilutions were performed using a multichannel pipette. The obtained concentration range was between 15 mg/mL and 0.015 mg/ml for the extracts and between 5 mg/mL and 0.005 mg/mL for the isolated substances. To each well, a 10 μL of the diluted yeast suspension was added. Finally, 10 μL of resazurin solution was added to each well inoculated with yeast, as an indicator of yeast growth. No indicator is added for

filamentous fungi. The inoculated plates were incubated at 28°C for 48 h (yeast) or 72 h (filamentous fungi). The MIC was defined as the lowest concentration of a tested substance that prevents a color change of the resazurin from blue to pink. For filamentous fungi, MIC values of the tested substance were determined as the lowest concentration that visibly inhibited mycelial growth. The minimum fungicidal concentration (MFC) was determined by plating 10 μL of the samples from the wells, in which no color change of the resazurin or no mycelial growth was detected onto a nutrient agar medium. At the end of the incubation period, the lowest concentration with no growth (no colony) was defined as the minimum fungicidal concentration.

Antimycotics, Fluconazole, Ketoconazole, Aamphotericin B, and Itraconazole dissolved in a nutrient liquid medium, were used as a positive control. The tested compounds were dissolved in DMSO and then diluted in a liquid nutrient medium to achieve a concentration of 10% DMSO. A solvent control test was performed to investigate the effect of 10% DMSO on the growth of the tested fungi. It was found that 10% DMSO did not inhibit the growth of the tested fungi. Each test included growth control and sterility control and was performed in duplicate.

RESULTS AND DISCUSSION

The *in vitro* antifungal activity of five extracts and seven isolated substances from the root of *O. visianii* against four yeast strains and six filamentous fungi are presented in Tables 1, 2, and 3.

Petroleum ether and methylene chloride (1:1) and acetone extract showed the best effect (Tab. 1), with an effective concentration below 1 mg/mL. The exception was *A. niger* ATCC 16404, which was resistant to the effect of the tested extracts and isolated substances (Tab. 1 and 2). The weakest effect was shown by the methanolic extract, except on *P. expansum*.

Among the isolated substances (Tab. 2), substance ***7*** (5,8-O-dimethyl deoxyshikonin) showed the most significant effect. The best was on yeasts and tested species of the genus *Penicillium* (MIC and MFC from 0.04 mg/mL to 1.25 mg/mL). In general, the tested yeasts (with the exception of *R. mucilaginosa*) showed higher sensitivity to the extracts (with the exception of methanol extract) than to the tested compounds (with the earlier mentioned exception of 5,8-O-dimethyl deoxyshikonin). Among the filamentous fungi, *A. niger* ATCC 16404 and *A. flavus* ATCC 9170 showed the highest resistance (results close to, or above 1 mg/mL).

When comparing the effect of the tested extracts with the positive controls (Tab. 3), it can be noted that the extracts, with the exception of the methanol extract, showed an effect on the yeasts *S. boulardii* and *C. albicans* in the same range as fluconazole (MIC or MFC). The MIC values of the petroleum ether and methylene chloride (1:1) and methanol extracts were in the same range as Amphotericin B for *P. expansum*, while the ethyl acetate extract was in the same range as Ketoconazole for the same fungus. *Mucor mucedo* showed sensitivity to most of the tested extracts in the range of Ketoconazole, while the acetone extract showed an effect in the range of Fluconazole on *A. flavus* ATCC 9170. The isolated compounds showed an effect on the tested fungi that was close to the values of the positive controls.

Substances ***1*** (deoxyshikonin), ***2*** (isobutyrylshikonin), and ***3*** (α-methyl butyrylshikonin) showed the effect on *S. boulardii* in the range of Fluconazole. Substances ***2*** (isobutyrylshikonin), ***3*** (α-methyl butyrylshikonin), and ***4*** (acetylshikonin) showed the effect on *P. expansum* in the range of Ketoconazole, while substance ***7*** (5,8-O- dimethyl deoxyshikonin) showed better effect than Ketoconazole. Substance 3 (α-methyl butyrylshikonin) showed an effect similar to that of Ketoconazole on *P. chrysogenum*.

Table 1. Antifungal activity of different extracts from the root *O. visianii*.

Type of extracts	**Petroleum ether and methylene chloride (1:1)**		**Chloroform**		**Ethyl acetate**		**Acetone**		**Methanol**	
Species	**MIC[1]**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
R. mucilaginosa	1.88	15	1.88	15	1.88	15	3.75	15	7.5	>15
S. boulardii	0.015	0.47	0.12	0.94	0.12	0.94	0.12	0.94	7.5	7.5
C. albicans	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.94	7.5	7.5
C. albicans ATCC 10231	0.12	0.23	0.23	0.47	0.23	0.47	0.47	0.94	3.75	7.5
P. expansum	<0.015	0.47	0.47	0.47	0.06	0.47	0.47	0.47	<0.015	0.47
P. chrysogenum	0.47	1.88	0.94	1.88	0.94	1.88	1.88	1.88	3.75	7.5
M. mucedo ATCC 52568	0.23	0.23	0.23	7.5	0.23	3.75	0.23	0.47	3.75	15
T. viride ATCC 13233	1.88	1.88	0.94	3.75	0.47	1.88	0.94	3.75	15	15
A. flavus ATCC 9170	3.75	3.75	1.88	1.88	1.88	3.75	0.94	0.94	>15	>15
A. niger ATCC 16404	1.88	7.5	>15	>15	>15	>15	>15	>15	>15	>15

[1] MIC and MFC values are given as mg/mL.

Table 2. Antifungal activity of isolated substances from the root *O. visianii*.

Type of substances	***1*** Deoxyshikonin		***2*** Isobutyrylshikonin		***3*** α-methyl butyrylshikonin		***4*** Acetylshikonin		***5*** β-hydroxy isovalerylshikonin		***6*** 5,8-O-dimethyl isobutyrylshikonin		***7*** 5,8-O-dimethyl deoxyshikonin	
Species	**MIC[1]**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
R. mucilaginosa	2.5	5	2.5	5	2.5	>5	2.5	5	2.5	5	2.5	5	5	5
S. boulardii	0.08	2.5	0.04	2.5	0.08	0.63	2.5	5	1.25	2.5	1.25	2.5	0.16	1.25
C. albicans	5	5	5	5	2.5	2.5	2.5	2.5	1.25	2.5	1.25	2.5	0.63	1.25
C. albicans ATCC 10231	2.5	2.5	2.5	2.5	0.63	5	2.5	2.5	2.5	2.5	2.5	2.5	0.63	1.25
P. expansum	1.25	1.25	0.16	0.16	0.16	0.16	0.16	0.16	1.25	1.25	0.31	0.31	0.04	0.04
P. chrysogenum	1.25	1.25	1.25	2.5	0.63	2.5	0.63	0.63	0.63	2.5	0.63	2.5	1.25	1.25
M. mucedo ATCC 52568	5	5	2.5	2.5	2.5	2.5	5	>5	5	>5	5	5	>5	>5
T. viride ATCC 13233	1.25	5	0.63	5	2.5	2.5	5	5	5	5	5	5	2.5	2.5
A. flavus ATCC 9170	1.25	1.25	2.5	2.5	5	5	5	5	2.5	2.5	5	5	2.5	2.5
A. niger ATCC 16404	>5	>5	>5	>5	>5	>5	5	>5	>5	>5	>5	>5	5	>5

[1]MIC and MFC values are given as mg/mL.

Table 3. Antifungal activity of antimycotics.

Positive controls	**Amphotericin B**		**Itraconazole**		**Fluconazole**		**Ketoconazole**	
Species	**MIC[1]**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
R. mucilaginosa	0.004	0.004	0.002	0.008	0.063	1	<0.9[2]	<0.9[2]
S. boulardiii	<0.1[2]	<0.1[2]	–	–	0.031	1	<0.9[2]	3.91[2]
C. albicans	0.001	0.002	0.002	0.002	0.063	1	–	–
C. albicans ATCC 10231	0.001	0.002	0.002	0.002	0.031	1	<0.9[2]	1.95[2]
P. expansum	0.004	–	–	–	–	–	0.125	–
P. chrysogenum	0.2[2]	0.39[2]	0.001	–	1000[2]	1000[2]	62.5[2]	125[2]
M. mucedo ATCC 52568	<0.1[2]	0.39[2]	–	–	250[2]	250[2]	1.95[2]	7.81[2]
T. viride ATCC 13233	0.78[2]	1.56[2]	0.008	–	500[2]	1000[2]	62.5[2]	125[2]
A. flavus ATCC 9170	0.39[2]	0.78[2]	0.001	0.001	500[2]	500[2]	1.95[2]	1.95[2]
A. niger ATCC 16404	0.2[2]	0.39[2]	0.002	0.002	1000[2]	1000[2]	125[2]	250[2]

[1]MIC and MFC values are given as mg/mL; [2]MIC and MFC values are given as μg/mL; "–" means not determined.

In this research, the antifungal activity of extracts and isolated substances from the roots of *O. visianii* was studied for the first time. The antibacterial activity of the same extracts and isolated substances was studied by VUKIC et al. (2017). According to them the naphthoquinones with bioactive properties have activity against selected resistant bacterial strains, and cytotoxic activity against tumor cell lines. Compounds ***3*** (α-methylbutyrylshikonin) and ***4*** (acetylshikonin) showed good activities against all tested gram-positive and gram-negative bacterial species. In our study, among all of the isolated substances, the most significant effect was observed for the substance ***7*** (5,8-O-dimethyl deoxyshikonin), which showed the best effect on the tested yeasts and the tested *Penicillium* species (MIC and MFC between 0.04 and 1.25 mg/ml).

Some authors studied the antifungal activity of other species from the genus *Onosma*. OZGEN *et al*. (2003) showed that *O. argentatum* has no antifungal activity. According to AHMAD *et al*. (2009), the methanolic extract of *O. griffithii* showed moderate antifungal activity against *A. flavus* (55%) and *Fusarium solani* (40%), while the chloroform fraction showed good antifungal activity against *A. flavus* (59%) and *F. solani* (60%). The remaining fractions tested (n-hexane, ethyl acetate, n-butanol, aqueous extracts) did not show antifungal activity against the mentioned fungal strains. The results of our study indicate that the antifungal activity of the tested extracts and substances from the roots of *O. visianii* depends on the type of extracts and substances, as well as on the fungal species. The difference in the effect of the extracts lies in the secondary metabolites obtained from the tested plant with different solvents.

CONCLUSION

The present work provided scientific information on naphthoquinones which belong to the shikonin group. The mentioned lipophilic substances isolated from the roots of *O. visianii* are potential antifungal agents because our research has exhibited their moderate antifungal activity. This fact promotes isolation of substances with potential bioactive properties. Our study indicates the necessity of further research on the potential use of naphthoquinones from *O. visianii* as antifungal agents, especially against *Penicillium* species.

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