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Full Length Research Paper

Anti-*Aspergillus* properties of different extracts from selected plants

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In vitro **antifungal activity of methanol, acetone and ethyl-acetate extracts from** ***Equisetum telmateia*** **Ehrh.,** ***Allium flavum*** **L.,** ***Sedum acre*** **L.,** ***Sideritis montana*** **L.,** ***Marubium peregrinum*** **L. and** ***Xeranthemum anuum*** **L. grown in Serbia, were investigated by microdilution method. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) have been determined. Testing was conducted against four** ***Aspergillus*** **species -** ***A. flavus, A. fumigatus, A. niger*** **and** ***A. restrictus*****. All statistical analyses were performed using SPSS package. The tested extracts showed significant antifungal activity against** ***A. restrictus*** **and moderate activity against other** ***Aspergillus*** **spp. The best results showed ethyl acetate extract of** ***S. montana*****,** ***X. anuum*** **and** ***S. acre*****.**

Key words: Antifungal activity, plant extracts, *Aspergillus* spp.

INTRODUCTION

Fungi are significant destroyers of foodstuffs during storage, rendering them unfit for human consumption by retarding their nutritive value and sometimes by producing mycotoxins. Some *Aspergillus* species are xerophilic fungi, which are responsible for many cases of food and feed contamination all over the world (Nobuo and Tadao, 2000; Kumar et al., 2007; Samapundo et al., 2007). Antimicrobial preservatives inhibit the growth of microbes such as fungi. Using the synthetic chemicals to inhibit the fungal growth in/on foods leads to negative consumer reaction due to different ecological and medical problems - residual toxicity, carcinogenicity, teratogenicity, hormonal imbalance, spermatotoxicity, etc. (Omura et al., 1995; Pandey, 2003). Due to that, attention goes more and more toward natural alternatives (Rasooli and Owlia, 2005). Different crude extracts of spices, herbs and other plant materials, rich in polyphenolics, are becoming increasingly important in food industries because of their antifungal, antiaflatoxigenic and antioxidant activity (Kumar et al., 2007).

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Out of five families, we chose six plants that have not been tested in this direction: *Equisetum telmateia* Ehrh., *Allium flavum* L., *Sedum acre* L., *Sideritis montana* L., *Marubium peregrinum* L. and *Xeranthemum anuum.*

Great horsetail (*E. telmateia*, fam. Equisetaceae) is a herbaceous perennial plant used in traditional medicine for treating acne, rheumatism, diuretic, expectorant, kidney stones, strengthen hair, skin and nails, mouth infections, chronic eczema, strain and as antifungal (Uzun et al., 2004). Most of *Allium* plants are known in traditional medicine and used in different purposes (Griffiths et al., 2002) while for wild *A. flavum* L. there are no available data. The results of newer investigations show high antioxidant activites (Štajner et al., 1998; (Štajner and Szцllфsi Varga, 2003). Goldmoss stonecrop (*S. acre* L. - Crassulaceae) is a perennial herbaceous plant, which is widely used in traditional medicine for treating ulcers, infected wounds and hypotension (Leporatti and Ivancheva, 2003). *S. montana* L. (Lamiaceae) is the annual species which has antispasmodic, carminative (Tabanca et al., 2001) and antimicrobial properties (Karanika et al., 2001; Kursat and Erecevit, 2009). Horehound, *M. peregrinum* L. (Lamiaceae) is a perennial plant whose medicinal substances show antihypertensive (El Bardai et al.,

2004) and antispasmodic activities (Rigano et al., 2009). The literature data indicate that the *X. annuum,* annual herb, is used in traditional medicine as a source of active substances (Vogl-Lukasser and Vogl, 2004; Spiridonov, 2008).

The aim of this study is to evaluate acetone, ethyl acetate and methanol extracts of aerial parts of six Serbian plant species (*E. telmateia, A. flavum*, *S. acre*, *S. montana*, *M. peregrinum* and *X. anuum*), for their anti-aspergillus properties, *in vitro*, using microdilution method.

MATERIALS AND METHODS

Chemicals

Organic solvents and sodium hydrogen carbonate were purchased from ‘’Zorka pharma“ Šabac, Serbia. Gallic acid, rutin hydrate, chlorogenic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. Folin-Ciocalteu phenol reagent and aluminium chloride hexahydrate were purchased from Fluka Chemie AG, Buchs, Switzerland. Nutrient liquid medium, sabouraud dextrose broths were from Torlak, Belgrade. Antimycotic, fluconazole, was obtained from Pfizer Inc., USA. All other solvents and chemicals were of analytical grade.

Plant material

Aerial flowering parts of *A. flavum* (Goč Mt., central Serbia, July 2010), *S. montana* (Stara Planina Mt., eastern Serbia, July 2008) *M. peregrinum* (Prokuplje, southeast Serbia, August 2009), *E. telmateia* (Kragujevac, Central Serbia, July 2009), *S. acre* (Trgoviste, south Serbia, June 2010) and *X. annuum* (Vranje, south Serbia, August 2009) were collected from natural populations. The voucher specimen was confirmed and deposited in Herbarium at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air-dried in darkness at room temperature (20°C). Dried plant parts were cut up and stored in tight-seal dark containers until needed.

Preparation of plant extracts

Prepared plant material (10 g) was transferred to dark-coloured flasks, mixed with 200 ml of solvent and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40°C using Rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C (Stanković et al., 2011).

Antifungal assay

Test microorganisms

Antimicrobial activity of acetone, ethyl acetate and methanol extracts were tested against four species *Aspergillus - A. fumigatus* PMFKG-F23*, A. Flavus* PMFKG-F24*, A. restrictus* PMFKG-F25 and *A. niger* PMFKG-F26. The microorganisms were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation

Initial suspensions of fungal spores were prepared by gentle stripping of spore from slopes with growing aspergilli. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland’s standard (Andrews, 2005). The resulting suspensions were 1:1000 diluted in sterile 0.85% saline.

Microdilution method

Antifungal activity was tested by determining the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) using microdilution method (CLSI, 2003). The 96-well plates were prepared by dispensing 100 L of Sabouraud dextrose broth into each well. A 100 L from the stock solution of tested compound (concentration of 80 mg/ml) was added into the first row of the plate. Then, twofold, serial dilutions were performed by using a multichannel pipette. The obtained concentration range was from 40 to 0.156 mg/ml. A 10 L of diluted suspension of spores was added to each well to give a final concentration of 5×10^3 CFU/cm^3. The inoculated plates were incubated at 28°C for 72 h. MIC values of the tested substances were determined as the lowest concentration that visibly inhibited mycelia growth.

Minimum fungicidal concentration (MFC) was determined by plating 10 L of samples from wells, where no mycelia growth was recorded, on nutrient agar medium. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum fungicidal concentration.

Fluconazole dissolved in nutrient liquid medium, was used as a positive control. The tested compounds were dissolved in DMSO and then diluted into nutrient liquid medium to achieve a concentration of 10% DMSO. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganism. It was observed that 10% DMSO did not inhibit the growth of microorganism. Also, in the experiment, the concentration of DMSO was additionally decreased because of the twofold serial dilution assay (the working concentration was 5% and lower). Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Statistical analysis

All statistical analyses were performed using SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 17, 2008). Mean differences were established by Student’s *t*-test. Data were analyzed using one-way analysis of variance (ANOVA). In all cases P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The results of *in vitro* testing antifungal activities of acetone, ethyl acetate and methanol extracts of *E. telmateia, A. flavum*, *S. acre*, *S. montana*, *M. peregrinum* and *X. anuum* are shown in Table 1. For comparison, MIC and MFC values for fluconazole are also listed in Table 1. The solvent (10% DMSO) did not inhibit the growth of the tested microorganisms.

Antifungal activities of tested extracts of selected plants were evaluated by determining MICs and MFCs in relation to the four *Aspergillus* species. MICs and MMCs values were in range from 0.625 mg/ml to 40 mg/ml. The intensity of antimicrobial action varied depending on the

Table 1. Antifungal activities of acetone, ethyl acetate and methanol extracts of *E. telmateia*, *A. flavu*, *S. acre*, *S. montana*, *M. peregrinum* and *X. anuum* against four *Aspergillus* spp.

Plant species	Type of extract	mg/ml	*Aspergillus restrictus*	*Aspergillus fumigatus*	*Aspergillus flavus*	*Aspergillus niger*
Sideritis montana	Acetone extract	MIC	2.5	2.5	10	10
		MFC	2.5	20	20	20
	Ethyl acetate extract	MIC	1.25	1.25	2.5	10
		MFC	2.5	10	10	20
	Methanol extract	MIC	5	20	10	1.25
		MFC	10	20	20	5
Xeranthemum anuum	Acetone extract	MIC	1.25	10	1.25	20
		MFC	20	20	20	40
	Ethyl acetate extract	MIC	0.625	10	10	1.25
		MFC	1.25	20	20	5
	Methanol extract	MIC	0.625	20	20	20
		MFC	1.25	20	20	20
Equisetum telmateia	Acetone extract	MIC	10	2.5	20	20
		MFC	10	20	20	20
	Ethyl acetate extract	MIC	1.25	2.5	10	20
		MFC	10	20	20	20
	Methanol extract	MIC	10	20	20	10
		MFC	10	20	20	10
Marrubium peregrinum	Acetone extract	MIC	10	20	40	10
		MFC	10	20	40	20
	Ethyl acetate extract	MIC	1.25	10	20	20
		MFC	1.25	20	20	20
	Methanol extract	MIC	10	20	10	10
		MFC	10	20	20	20
Allium flavum	Acetone extract	MIC	10	5	10	10
		MFC	10	5	10	20
	Ethyl acetate extract	MIC	/	5	5	/
		MFC	/	5	5	/
	Methanol extract	MIC	2.5	10	10	10
		MFC	5	10	10	10
Sedum acre	Acetone extract	MIC	5	2.5	10	10
		MFC	10	2.5	10	20
	Ethyl acetate extract	MIC	1.25	2.5	10	10
		MFC	1.25	5	10	20
	Methanol extract	MIC	5	10	10	20
		MFC	10	10	10	20
Fluconazole		MIC	0.5	0.5	1	0.5
		MFC	2	1	1	1

MIC, minimum inhibitory concentration; MMC, minimum microbiocidal concentration; / , not tested.

tested species of *Aspergillus*, the tested species of plants and on the type of the extracts.

In general, the tested extracts demonstrated selective to moderate antifungal activity. They showed more potent

inhibitory effects on the growth of *A. restrictus* than to other tested fungi ($p < 0.05$). Statistically significant difference in activity between the extracts was observed in ethyl acetate extract ($p < 0.05$). From all the plants, *S. montana*, *X. anuum* and *S. acre* showed the highest activity while *M. peregrinum* and *E. maximum* showed the lowest ($p < 0.05$).

The tested extracts showed high antifungal activity against *A. restrictus*. MICs values were in range from 0.625 to 1.25 mg/ml for *X. anuum* (all the 3 exracts) and 1.25 to 5 mg/ml for *S. montana* and *S. acre* (all the 3 exracts). Ethyl acetate extract of *S. montana* showed significant effect against *A. fumigatus* (MIC value is 1.25 mg/ml). Acetone and ethyl acetate extracts of *S. acre* and *E. telmateia*, and acetone extract of *S. montana* showed some weaker activity related to the presented results (MIC value is 2.5 mg/ml). Against *A. Flavus*, acetone extract of *X. anuum* showed significant action (MIC value is mg/ml) while ethyl acetate extract of *S. montana* was weaker (MIC value is 2.5 mg/ml). The methanol extract of *S. montana* and ethyl acetate extract of *X. anuum* had the most significant influence against *A. niger* (MIC value is 1.25 mg/ml).

Antifungal activity of the extracts from the tested plants has been little investigated. Petroleum ether extract of *E. telmateia* showed the action on *Candida albicans* (Uzun et al., 2004), while hydro-alcoholic extract of *E. telmateia* showed lower action from the controle on *Aspergilus niger* ATCC 16404 and *Candida albicans* (Milovanović et al., 2007). The methanol extracts of *S. acre* exhibited antifungal effect on *C. albicans* ATCC 10231 and *C. krusei* ATCC 6258 (Tosun et al., 2006). Methanol extract of *S. montana* inhibited the growth of *C. albicans* (Kursat and Erecevit, 2009), while seed extracts of *S. montana* with vitamins or flavonoids were showing antifungal activity against *C. albicans, C. glabrata, Trichophyton* sp and *Epidermophyton* sp better than control (Emre et al., 2011). For antifungal action of *A. flavum* L., *M. peregrinum* L. and *X. anuum* there are no available data.

This research of antifungal activity of plants *E. telmateia, A. flavum*, *S. acre*, *S. montana*, *M. peregrinum* and *X. anuum* is the first of that kind on *Aspergillus* spp. Achieved results indicate that plant extracts from these plants could be used as natural sources of preservative substances with high importance in food industry aganist some *Aspergillus* species.

Conclusions

The results indicate that tested extracts of selected plants showed different degree of antifungal activity in relation to the tested species. Extracts demonstrated more potent inhibitory effects on the growth of *A. restrictus* than on other tested fungi. The best results showed ethyl acetate extract of *S. montana*, *X. anuum* and *S. acre*. All of this suggests the possible use of the plant extracts as potential botanical antifungal agents in ecofriendly control of post harvest biodeterioration of food commodities from storage fungi.

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