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Full Length Research Paper

Antifungal properties of *Allium ursinum* L. ethanol extract

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Allicin analysis of *Allium ursinum* L. flower and leaf ethanol extracts by LC/MS and *in vitro* germination and growth inhibition effects on *Aspergillus niger, Botrytis cinerea, Botrytis paeoniae, Fusarium oxysporum* f.sp. *tulipae, Penicillium gladioli*, and *Sclerotinia sclerotiorum* were performed. The antifungal activity of the flower extract was stronger than that of the leaf extract, and this was correlated with a higher allicin content.

Key words: *Allium ursinum,* allicin, antifungal, flower extract, leaf extract.

INTRODUCTION

Allium species have been used in traditional medicine for many centuries, with more than 300 *Allium* species described (Brullo et al., 2003, 2009; Ledezma and Apitz-Castro, 2006). The Romanian ethnobotanical data record 32 among wild and cultivated species of *Allium* L. (Ciocârlan, 2009). One of these is *Allium ursinum* L. ("ramson", "wild garlic") a wild-growing species found in Europe and Northern Asia forests (Schmitt et al., 2005).

In recent years, the potential health benefits of ramson have been attributed mainly to the sulfur-containing compounds (Schmitt et al., 2005). Several biological activities of *A. ursinum* plants, such as antioxidative (Stajner et al., 2008), cytostatic (Sobolewska et al., 2006), and antimicrobial (Ivanova et al., 2009; Sobolewska et al., 2006), were reported.

In the present study we investigated the *in vitro* antifungal properties of *A. ursinum* flower and leaf extracts on some phytopathogenic fungi. Because allicin is an important antifungal compound from *Allium* plant extracts, the second aim was to perform a quantitative allicin analysis from *A. ursinum* extracts.

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MATERIALS AND METHODS

Plant material

A. ursinum aerial parts have been collected shortly after the blooming period, from the Botanical Garden of Babes-Bolyai University, Cluj-Napoca (Romania). The plant was identified at the Department of Botany, by Dr. Marcel Pârvu and a voucher specimen (CL 659750) was deposited at the Babes-Bolyai University Herbarium.

Extraction

Fragments of 0.5 - 1 cm of fresh flowers and respectively fresh leaves of *A. ursinum* were harvested at the same time from plants

grown in the same place, and samples were extracted with 50% ethanol (Merck, Bucuresti, Romania) in the Mycology Laboratory of Babes-Bolyai University, Cluj-Napoca, Romania, by modified Squibb's repercolation method. Briefly, three successive applications of the same menstruum were repercolated to the plant material. In each percolator plant material (150 g in the first, 90 g in the second, 60 g in the third percolator) was moistened with the menstruum, macerated for two days and then percolated at a rate of about 4 - 6 drops per minute for each 100 g of crude material. From each percolator the first percolated fractions were reserved and the next fractions were poured upon the next percolator. Then reserved fractions (60 ml from the first, 90 ml from the second and 150 ml from the third) were mixed and the final extract of flower and leaf were 1:1 (w:v) respectively (Pârvu et al., 2010a).

Quantitative analysis of allicin

The analysis of allicin (reference standard from Allicin International LTD, Rye, UK) from the flower and the leaf extracts was carried out using a newly developed liquid chromatography coupled with mass spectrometry detection (LC/MS). Briefly, an Agilent 1100 series high performance liquid chromatography (HPLC) system was used (Agilent Technologies, Darmstadt, Germany), coupled with an Agilent Ion Trap SL mass spectrometer equipped with an electrospray ion source. The chromatographic separation of allicin was made using a Synergi Polar column, 100 mm x 2.0 mm i.d., 4 µm (Phenomenex, SUA). The mobile phase consisted in 100% aqueous ammonium acetate 1 mM, isocratic elution, flow 0.6 ml/min. A 1 mM aqueous silver nitrate solution was post-column added using a mixing tee with a flow rate of 10 µl/min. The mass spectrometer operated in positive multiple reactions monitoring (MRM) mode, using nitrogen as nebulising and dry gas. The nebuliser was set at 60 psi, the dry gas flow was 12 L/min at 350°C temperature. The mass spectrometer was set to record the transition m/z (449+451)> m/z (269; 271; 287; 289), which is specific to allicin-silver adduct. The retention time of allicin in the above described conditions was 0.9 min.

Preparation of fungal colonies

Aspergillus niger Tiegh. isolated from *Allium cepa* L. bulbs, *Botrytis cinerea* Pers. isolated from Rose Kordes'Perfecta flowers, *Botrytis paeoniae* Oudem. isolated from *Paeonia officinalis* L. flowers, *Fusarium oxysporum* f. sp. *tulipae* W.C. Snyder and H.N. Hansen isolated from *Tulipa gesneriana* L. flowers, *Penicillium gladioli* Machacek isolated from *Gladiolus* x *hybridus* C. Morr. corms, *Sclerotinia sclerotiorum* (Lib.) de Bary isolated from *Daucus carota* L. roots were included in this study.

Fungal cultures were obtained from the collection of the Mycology Laboratory, Babes-Bolyai University Cluj-Napoca, and were grown in Petri dishes containing Czapek-agar medium (BD Difco, Budapest, Hungary), following inoculation into the central point and incubation at 22°C for 5 days.

Assay of antifungal activity

The agar-dilution assay was used to determine the *in vitro* antifungal activity of *A. ursinum* flower and leaf extracts, expressed as minimum inhibitory concentration (MIC). Ethanol was used as a solvent control (C). The antimycotic drug fluconazole (2 mg ml-1) (Krka, Novo Mesto, Slovenia) was the positive control.

The diameter of fungal growth was measured and expressed as percentage (P) of mycelial growth inhibition of four replicates using the formula: $P = (C-T) \times 100/C$, where C is the diameter of the control colony (nutritive medium and 50% EtOH) and T is the diameter of the treated colony (Nidiry and Babu, 2005).

Statistical analysis

The results for each group were expressed as mean ± SEM. Data were evaluated by analysis of variance (ANOVA). Statistical differences were considered significant at the $p<0.05$ level. The correlation analysis was performed by the Pearson test.

RESULTS

Quantitative analysis of allicin

Allicin analysis of *A. ursinum* extracts by LC/MS determined 1.946 mg allicin/ml flower extract (Figure 1) and 0.028 mg allicin/ml leaf extract (Figure 2).

Antifungal activity

The results of the antifungal assays of *A. ursinum* flower and leaf extracts are presented in Table 1. *A. ursinum* flower extract MIC was 100 µl/ml for *A. niger,* 60 µl/ml for *B. cinerea*, 70 µl/ml for *B. paeoniae*, 140 µl/ml for *F. oxysporum* f. sp. *tulipae,* 90 µl/ml for *P. gladioli*, and 60 µl/ml for *S. sclerotiorum*. *A. ursinum* leaf extract MIC was 120 µl/ml for *A. niger,* 80 µl/ml for *B. cinerea*, 100 µl/ml for *B. paeoniae*, 160 µl/ml for *F. oxysporum* f. sp. *tulipae,* 120 µl/ml for *P. gladioli*, and 80 µl/ml for *S. sclerotiorum*. The inhibitory effect of *A. ursinum* flower extract was stronger than that of the leaf extract for all concentrations and for all tested fungi ($p<0.001$). The antifungal effect of the *A. ursinum* flower and leaf extracts positively correlated with the allicin content ($r = 0.92$). Fluconazole MIC was 300 µl/ml for *A. niger,* 120 µl/ml for *B. cinerea* and *B. paeoniae*, 100 µl/ml for *F. oxysporum* f. sp. *tulipae,* 300 µl/ml for *P. gladioli*, and 80 µl/ml for *S. sclerotiorum* (Table 1). Compared on a weight basis, the antifungal properties of *A. ursinum* flower and leaf extracts were stronger than that of fluconazole ($p<0.01$) against all tested fungi.

DISCUSSION

The development of strategies to control fungal infections may be an effective mean for therapeutic interventions. This study has established that *A. ursinum* flower extract possesses a more potent antifungal activity against *A. niger, B. cinerea, B. paeoniae, F. oxysporum* f.sp. *tulipae, P. gladioli*, and *S. sclerotiorum,* than *A. ursinum* leaf extract. LC/MS analysis has shown that *A. ursinum* flower extract contains a higher amount of allicin than the leaf extract.

The differences of MIC values obtained from *A. ursinum* flower and leaf extracts for each tested fungi, are in accordance with other studies which showed that

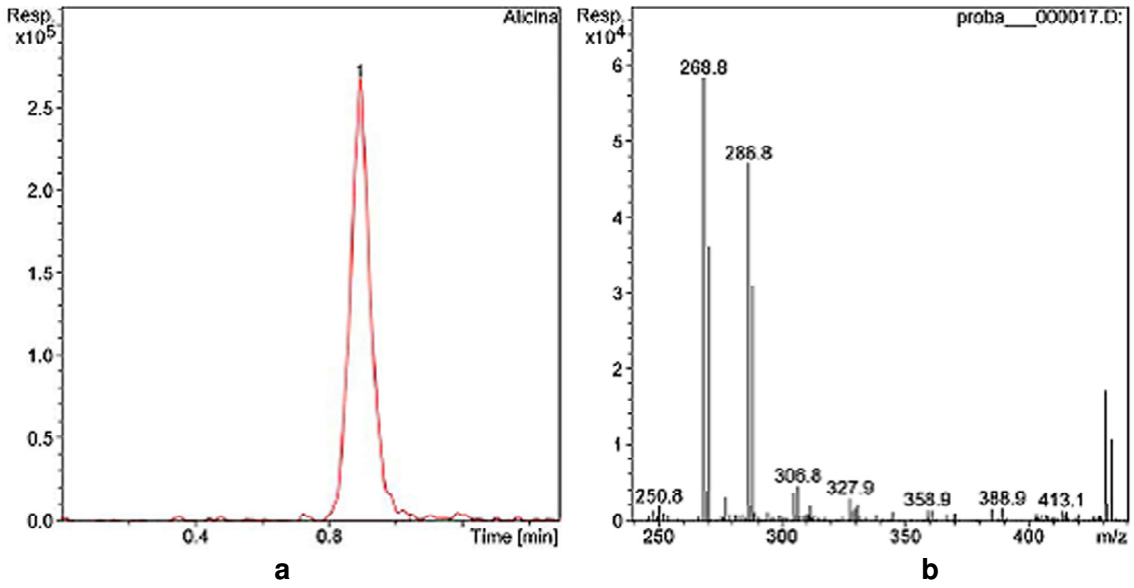

Figure 1. The chromatogram (a) and mass spectrum (b) of allicin from *A. ursinum* flower extract.

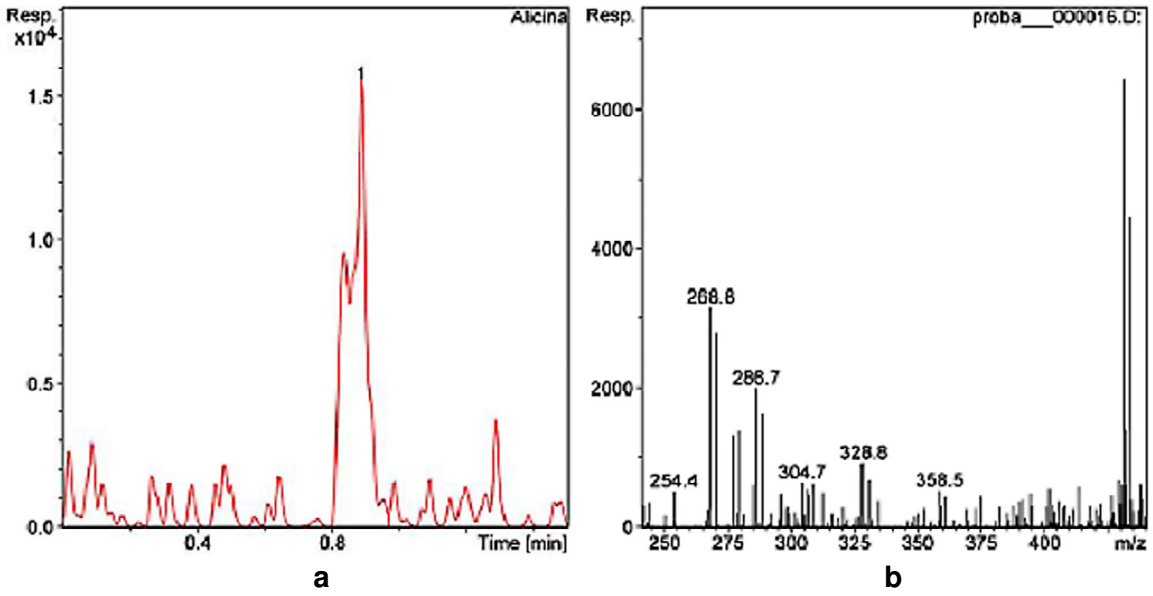

Figure 2. The chromatogram (a) and mass spectrum (b) of allicin from *A. ursinum* leaf extract.

antibacterial and antifungal effects of *Allium* plant extracts depend on the pathogenic species and on the type of plant extract (Abubakar, 2009; Mahmoudabadi and Nasery, 2009; Pârvu et al., 2009, 2010a).

The quality and quantity of the biologically active compounds from *Allium* species significantly depend on the species, the plant organ and the harvest time. In *A. ursinum* plant extracts, alliin, isoalliin (Schmitt et al., 2005; Fritsch and Keusgen, 2006), methiin (Fritsch and Keusgen, 2006), flavonoid glycosides (Wu et al., 2009), saponins (Sobolewska et al., 2006), polyphenolic compounds (Pârvu et al., 2010b), volatile oil (Godevac et al., 2008) and other secondary metabolites (Ivanova et al., 2009) were determined. Alliin dominates in the widely used “garlic-type”, which includes wild leek (*A. obliquum*) and sand leek (*A. scorodoprasum*). Alliin and isoalliin rarely co-dominate, being only found in the cultivated Chinese leek (*A. tuberosum*). A mix of almost equal amounts of methiin, alliin and isoalliin is present in *A. ursinum* (Fritsch and Keusgen, 2006). Our previous

Table 1. Antifungal activity of *Allium ursinum* extracts on *in vitro* germination and growth of some phytopathogenic fungi.

Fungal species	Flower extract of *Allium ursinum* (µl/ml)	Colony [a] diameter (mm)	P [a] (%)	Leaf extract Of *Allium ursinum* (µl/ml)	Colony [b] diameter (mm)	P [b] (%)	Fluconazole (µl/ml)	Colony [c] diameter (mm)	P [c] (%)
Aspergillus niger	C	22	0	C	22	0	C	22	0
	20	20	9.09 ± 0.02	40	20	9.09 ± 0.05	100	11.66	47 ± 0.45
	40	15	45.45 ± 0.8	60	15	31.82 ± 0.4	200	7.66	65.18 ± 0.62
	60	8	72.72 ± 0.57	80	7	63.64 ± 0.87	250	4.33	80.31 ± 0.75
	80	2	86.36 ± 0.82	100	2	90.91 ± 0.91	300	0	100 ± 0.91
	100	0	100 ± 1.02	120	0	100 ± 0.7			
Botrytis cinerea	C	65	0	C	65	0	C	65	0
	10	60	7.69 ± 0.1	20	58	11.67 ± 0.2	20	40.33	37.95 ± 0.32
	20	50	36.92 ± 0.25	40	32	48.33 ± 0.31	60	20	69.23 ± 0.52
	30	26	70.76 ± 0.78	60	12	73.33 ± 0.54	100	5.33	91.80 ± 0.92
	50	5	92.30 ± 0.89	70	6	91.67 ± 0.71	120	0	100 ± 0.87
	60	0	100 ± 0.9	80	0	100 ± 0.80			
Botrytis paeoniae	C	60	0	C	60	0	C	60	0
	20	53	11.67 ± 0.11	40	52	13.33 ± 0.11	20	50	16.66 ± 0.14
	40	31	48.33 ± 0.32	60	23	61.67 ± 0.42	60	24	60 ± 0.48
	50	16	73.33 ± 0.46	80	3	95.00 ± 0.71	100	5	91.66 ± 0.87
	60	5	91.67 ± 0.93	100	0	100 ± 0.81	120	0	100 ± 0.92
	70	0	100 ± 0.57						
Fusarium oxysporum f.sp. *tulipae*	C	32	0	C	32	0	C	32	0
	40	30	9.37 ± 0.09	60	25	21.88 ± 0.13	20	20	37.50 ± 0.22
	60	22	25 ± 0.21	80	17	46.88 ± 0.22	60	8	75 ± 0.63
	80	14	40.62 ± 0.33	100	12	62.50 ± 0.51	80	2	93.75 ± 0.8
	100	8	50 ± 0.51	120	7	78.13 ± 0.81	100	0	100 ± 0.75
	120	3	62.5 ± 0.57	160	0	100 ± 0.66			
	140	0	100 ± 1.02						

Table 1. Contd.

Penicillium gladioli	C	15	0	C	15	0	C	15	0
	20	14	20 ± 0.14	20	14	6.67 ± 0.08	100	11	26.66 ± 0.19
	40	10	40 ± 0.21	40	11	26.67 ± 0.12	160	10	26.66 ± 0.2
	60	6	53.33 ± 0.38	60	9	40.00 ± 0.42	200	8	46.66 ± 0.31
	80	2	73.33 ± 0.44	80	5	66.67± 0.31	250	5	66.66 0.74
	90	0	100 ± 0.85	100	3	80.00 ± 0.61	300	0	100 ± 0.08
				120	0	100 ± 0.74			
Sclerotinia sclerotio-rum	C	64	0	C	64	0	C	64	0
	10	60	6.25 ± 0.07	20	58	9.38 ± 0.08	20	30	53.12
	20	46	28.13 ± 0.21	40	32	50.00 ± 0.42	40	15	76.56
	30	22	65.63 ± 0.42	50	20	68.75 ± 0.31	60	5	92.18
	40	12	81.25 ± 0.51	60	12	81.25 ± 0.61	80	0	100
	50	4	93.75 ± 0.63	70	5	92.19 ± 0.42			
	60	0	100 ± 0.72	80	0	100 ± 0.67			

Legend: [a] = The effect of *A. ursinum* flower extract; [b] = the effect of *A. ursinum* leaf extract; [c] = the effect of Fluconazole; C = control (50% aq. EtOH); P = mycelial growth inhibition - results are the mean ± SEM of 4 experiments. The same doses of *A. ursinum* flower and leaf extracts were tested against all fungal species.

analysis of 19 polyphenolic compounds (Pârvu et al., 2010b) showed large qualitative and quantitative differences among five Allium species (*A. obliquum*, *A. senescens* subsp. montanum, *A. schoenoprasum* subsp. schoenoprasum, *A. fistulosum*, and *A. ursinum*). Experiments that studied the antioxidative properties of *A. ursinum* bulb, leaf and stalk extracts, demonstrated that the leaf extract has the highest effect (Stajner et al., 2008). In our case, *A. ursinum* flower extract has proven to be a more effective fungal inhibitor than the leaf extract.

In *A. ursinum* leaves and bulbs, the highest amount of volatile precursors was found in March and April, shortly before flowering time (Schmitt et al., 2005). In order to compare *A. ursinum* flower and leaf extracts, our plants were harvested shortly after blooming. Allicin has an efficient action against many fungal species, such as *Aspergillus flavus*, *A. niger*, *Candida albicans*, *Fusarium laceratum*, *Microsporum canis*, *Mucor racemosus*, *Penicillium* spp., *Rhizopus nigricans*, *Saccharomyces* spp., *Trichophyton granulosum* (Josling, 2003), *F. oxysporum* (Ogita et al., 2006). The stronger inhibitory activity of the *A. ursinum* flower extract, compared to the leaf extract, may be attributed to the higher content of allicin, and may explain the positive correlation between the antifungal activity and allicin content.

In conclusion, three main original results regarding the antifungal effect of *A. ursinum* flower and leaf extracts against some phytopathogenic fungi have been reported in the present study: (i) the flower extract has a stronger inhibitory activity than that of the leaf extract; (ii) *A. ursinum* flower extract has a high allicin content; (iii) the antifungal activity is positively correlated to the allicin content. These data may be helpful in the development of new natural antifungal products.

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