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BRINE SHRIMP LETHALITY BIOASSAY OF SELECTED *CENTAUREA* L. SPECIES (ASTERACEAE)

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Abstract — Ether extracts of 15 *Centaurea* L. species (Asteraceae) methanol extracts of 12 species, and cnicin isolated from *C. derventana* were tested for general bioactivity using the brine shrimp lethality test. Cnicin showed the most potent activity with LC50 0.2. Also, ether extract of *C. splendens* showed significant activity with LC50 7.3, as did methanol extract of *C. arenaria* with LC50 12.4.

Key words: *Centaurea*, bioactivity, cnicin, cytotoxicity, brine shrimp lethality bioassay

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INTRODUCTION

Centaurea L. is a large genus of the family Asteraceae, comprising about 500 species. Most species are predominantly distributed around the Mediterranean area and in West Asia (Mabberley, 1997). Various biological tests have been used in investigation of different extracts of *Centaurea* spp. (Monya et al., 1968; Llombart and Perez-Minguez, 1969; Ivorra et al., 1988; Robels et al., 1997; Roy et al., 1995; Güven et al., 2005). It has been shown that a number of *Centaurea* species possess biologically active compounds. Several species are used in traditional medicine. Thus, *C. cyanus* L. is an astringent, diuretic, emmenagog, and antiseptic, in addition to which it is used to treat collyrium, fever, and tumors (eye) (Johnson, 2003; Tucakov, 1978; Sarker et al., 2001; Valles et al., 1996). *Centaurea jacea* L. is known as a diuretic and antidiabetic that is also used to treat fever (Johnson, 2003; Valles et al., 1996). *Centaurea montana* L. is an astringent, cyanogenetic, diuretic, emmenagog, pectoral, stimulant, and tonic that is also used to treat collyrium and fever. Finally, *C. salonitana* Vis. is used to treat tumors (Johnson, 2003).

MATERIALS AND METHODS

Plant material

Plant material (Table 1) of *Centaurea* spp. was collected from native habitats in Serbia and Montenegro. Voucher specimens with accession numbers are deposited in the Herbarium of the Institute of Botany, Faculty of Biology, University of Belgrade.

Sample preparation

Sample preparation was performed as described previously (Meyer et al., 1982; Mc Laughlin, 1991). Table 2 shows the percent of dry weight and fresh weight yields of extracts of the investigated species. Ether extracts were prepared by mixing 50 ml of Et_2O with 5 g of dried leaves, methanol extracts by mixing 50 ml of MeOH with 5 g of fresh leaves. Mixtures were extracted continuously in an ultrasonic bath for 30 min and then were kept for 24 h in the dark at room temperature. After filtration, dry residues from extracts were obtained by evaporation in a rotary evaporator (t = 40°C). Solutions with starting concentration of 1 mg/ml of methanol extracts, ether extracts, and cnicin were prepared by dissolving 10 mg of the given extracts and cnicin in 10 ml of methanol in volumetric flasks. Appropriate amounts of solutions (10, 100, and 1000 µl, for 10, 100, and 1000 µg/ml, respectively) were transferred to 1.5-cm disks of filter paper (Schleicher and Schuell, no. 589[1], Ø70 mm). The disks were dried in air, placed in 2-dram vials, and then dried further *in vacuo* for an hour. Control disks were prepared

Table 1. Investigated *Centaurea* species.

Species	Locality	Voucher specimens, accession numbers
Centaurea affinis Friv.	Lake Vlasina, Serbia	CAf491998, CAf06072001
C. arenaria Bieb. ex Willd.	Belgrade, Serbia	CAr18062001
C. atropurpurea Waldst. & Kit.	Zlot, Serbia	CAt27062000
C. chrysolepis Vis.	Niševac, Serbia	CCh13071999
C. cyanus L.	Mt. Fruška Gora, Serbia	CCy28052001
C. grisebachii (Nyman) Form.	Zabel, Razgojna, Serbia	CGr25082001
C. incompta Vis.	Mt. Orjen, Montenegro	CIn02081999E
C. jacea L.	Belgrade, Serbia	CJ26072001
C. jacea L.	Mt. Zlatibor, Serbia	CJ2061998
C. montana L.	Derventa Canyon, Perućac, Serbia	CMo13062001
C. nervosa Willd.	Mt. Kučki Kom, Montenegro	CNr12072001
C. rupestris L.	Vranje, Serbia	CR071998
C. salonitana Vis.	Niševac, Serbia	CS05091998, CS18072001
C. scabiosa L.	Belgrade, Serbia	CSc24052001
C. scabiosa L.	Premeća, Serbia	CSc1972000lf
C. solstitialis L.	Belgrade, Serbia	CSo23052001
C. splendens L.	Mt. Orjen, Montenegro	CSpl3062001
C. stoebe L.	Svrljig, Serbia	CSt18072001
C. stoebe L.	Maglič, Serbia	CSt17072000
C. triniifolia Heuff.	Zlot, Serbia	CTrin27062000
C. triumfetti All.	Mt. Midžor, Serbia	CTri13062001
C. triumfetti All.	Godulja Canyon, Serbia	CTri15062000

Table 2. Percent of dry and fresh yield of extracts of investigated *Centaurea* spp. (np): extract not prepared.

Centaurea species	Yield of extract (%)	
	Fresh weight (methanolic)	Dry weight (aetheric)
C. splendens L.	3.13	2.26
C. nervosa Willd.	np	0.62
C. rupestris L.	np	5.03
C. montana L.	np	0.89
C. atropurpurea Waldst. & Kit.	np	0.64
C. chrysolepis Vis.	7.86	0.91
C. incompta Vis.	np	1.87
C. cyanus L.	1.71	2.06
C. triniifolia Heuff.	np	4.96
C. jacea L.	4.21	1.14
C. stoebe L.	np	2.61
C. triumfetti All.	1.14	0.73
C. salonitana Vis.	4.27	3.77
C. arenaria Bieb. ex Willd.	4.17	1.66
C. scabiosa L.	5.22	3.63
C. solstitialis L.	3.67	np
C. affinis Friv.	9.51	0.54
C. grisebachii (Nyman) Form.	5.35	np

Table 3. Cytotoxic activity of methanol and ether extracts of *Centaurea* spp. and cnicin. Potassium dichromate (50 μg/ml) was used as a positive control. (*): confidence interval 95%; (nt): not tested.

Centaurea species	Percent deaths at 24 h							
	Methanol extract				Ether extract			
	10 μg/ml	100 μg/ml	1000 μg/ml	LC_{50}* μg/ml	10 μg/ml	100 μg/ml	1000 μg/ml	LC_{50}* μg/ml
C. splendens L.	17	26.6	97	114.6	58.3	75.2	100	7.3
C. nervosa Willd.	nt	nt	nt	nt	55.2	56.9	100	13.8
C. rupestris L.	nt	nt	nt	nt	41.6	56.9	100	27.8
C. montana L.	nt	nt	nt	nt	33	44	100	47.2
C. atropurpurea Waldst. & Kit.	nt	nt	nt	nt	27	32.2	100	73.1
C. chrysolepis Vis.	0	7	96.8	277.7	18.8	48.8	100	86.9
C. incompta Vis.	0	4	100	232.8	20.8	24.7	100	100.2
C. cyanus L.	23	67	100	37.9	22.2	22.2	100	102.7
C. triniifolia Heuff.	nt	nt	nt	nt	16.6	24.7	100	110.2
C. jacea L.	27	40	100	61.2	16	24.7	100	111.6
C. stoebe L.	nt	nt	nt	nt	16.6	21.5	100	118
C. triumfetti All.	4	11	90	266.5	11	11	100	166.6
C. salonitana Vis.	14	27	100	111.2	4.4	4.4	93.5	275.4
C. arenaria Bieb. ex Willd.	54	64	100	12.4	0	0	100	319.5
C. scabiosa L.	0	30	100	142.1	0	0	18.2	>1000
C. solstitialis L.	30	34	100	64.8	nt	nt	nt	nt
C. affinis Friv.	4.1	7	100	218.5	nt	nt	nt	nt
C. grisebachii (Nyman) Form.	24	33.3	70	219.1	nt	nt	nt	nt
Cnicin	80	94	96	0.2	80	94	96	0.2

using potassium dichromate (VI). Five replicates were prepared for each dose level.

Brine shrimp cytotoxicity assay

The test was performed as described in Meyer et al. (1982) and McLaughlin (1991). Each extract and cnicin isolated earlier from *C. derventana* (Tešević et al., 1998) were tested at concentrations of 10, 100, and 1000 μg/ml. Brine shrimp eggs (*Artemia salina* Leach) were purchased in a pet shop in Belgrade and hatched in artificial sea water (solution of NaCl 3.8%) at room temperature. After 48 h, the larvae (nauplii) were collected. A suspension of 10 nauplii in artificial sea water was added to each sample vial to a volume of 5 ml and the sample vials incubated for 24 h at room temperature. After this period, the number of dead nauplii in each sample vial was counted using a binocular microscope (MBS 9, USSR, 4.8 x). Pure methanol was used as a positive control. Finney's statistical method of probit analysis (Finney, 1971) was used to calculate the concentration of the extract or cnicin that would kill 50% of brine shrimps within 24 h of exposure, i.e., the LC_{50} with 95% confidence intervals. The extracts were considered as bioactive when LC_{50} was 1000 μg/ml or less. The value of LC_{50} was determined using the LdP Line® program (Bakr, 2007).

RESULTS AND DISCUSSION

As shown in Table 3, methanol extracts from all investigated species were very active. The methanol extract of *C. arenaria* showed very significant activity (LC_{50} = 12.4 μg/ml). The lowest activity was found with *C. chrysolepis* (LC_{50} = 277.7 μg/ml). The ether extract of *C. splendens* was the most active (LC_{50} = 7.3 μg/ml). The lowest activity was recorded for the ether extract of *C. scabiosa* (LC_{50} = 2605.9 μg/ml, i.e., >1000 ppm). The sesquiterpene lactone

cnicin showed significantly higher activity than any extract of the investigated *Centaurea* species (LC_{50} = 0.2 μg/ml). *Centaurea splendens* is already known as a rich source of cnicin (Janaćković et al., 2003). It can be presumed that the high content of cnicin in *C. splendens* is in direct relation to strong activity of the ether extract of this species. Most of the investigated species contain cnicin (Nowak et al., 1984; Gousiadou and Skaltsa, 2003) and other potentially active compounds like lignans, flavones, etc. (Janaćković et al., 2004). From results of the brine shrimp lethality bioassay used in this work, it can be concluded that this test is useful in determining the biological activity of *Centaurea* species. Since many of these species are used in traditional medicine, their extracts should be subject to further investigation for isolation and identification of biologically active compounds.

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БИОТЕСТ *ARTEMIA SALINA* КОД ОДАБРАНИХ ВРСТА РОДА *CENTAUREA* L. (ASTERACEAE)

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У раду је испитивана биолошка активност метанолних и етарских екстраката 18 врста рода *Centaurea* помоћу Биотеста *Artemia salina.* Врсте овог рода представљају значајне природне изворе биолошки активних секундарних метаболита. Такође, одређена је и биолошка активност (токсичност) сесквитерпенског лактона кницина, чисте супстанце, издвојене из *C. derventana*. Екстракти су добијени екстракцијом 5 g листова у етру и метанолу, кницин је изолован из *C. derventana,* према Tešević et. al. (1998).

Узорци и тест су припремљени и спроведени према Meyer et al. (1982) и McLaughlin (1991). Као контрола коришћен је калијум дихромат (VI). Утврђено је да метанолни и етарски екстракти свих испитиваних врста показују значајну биолошку активност. Највећу активност испитиваних метанолних екстраката показао је екстракт врсте *C. arenaria* LC_{50} 12.4 μg/ml, док је најмању активност показала врста *C. chrysolepis*, чија вредност LC_{50} износи 277.7 μg/ml. Највећу активност етарских екстраката показао је екстракт врсте *C. splendens*, чија вредност LC_{50} износи 7.3 μg/ml, а најмању активност показала је врста *C. scabiosa* чија вредност LC_{50} износи 2605.9 μg/ml, тј. >1000. Сесквитерпенски лактон кницин је показао највећу биолошку активност (LC_{50} 0.2 μg/ml). Резултати биотеста сони рачић (*Artemia salina* Leach) показују значајну активност свих испитиваних екстраката анализираних врста рода *Centaurea*. Наши резултати, литературни подаци, као и употреба врста рода *Centaurea* у етномедицини, наводе на закључак да је истраживање врста овог рода са аспекта биолошке активности како укупног екстракта, тако и појединачних компоненти, веома значајно.

Поред осталог, на основу резултата може се закључити да биотест сони рачић представља једноставан, статистички прихватљив, брз и поуздан метод за детекцију компоненти виших биљака које могу испољавати читав спектар фармаколошких активности.