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PYRETHRUM *(TANACETUM CINERARIIFOLIUM)* FROM THE NORTHERN ADRIATIC AS A POTENTIAL SOURCE OF NATURAL INSECTICIDE

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ABSTRACT

Pyrethrum Tanacetum cinerariifolium *(Trevir.) Schultz-Bip., a species native to the Eastern Adriatic coastal mountains and islands, is a plant widely used in the production of natural insecticides, pyrethrins. The biosynthetic potential of Pyrethrum from two different locations in the Northern Adriatic for pyrethrin production was determined. In all the samples obtained, all 6 pyrethrins were detected, as measured by HPLC. The highest pyrethrin content was detected in the flower heads, which contained on average 1.2% pyrethrins of dry weight. The pyrethrin content of flower heads from the Northern Adriatic populations is comparable with the content levels in conventional production of Pyrethrum, but not as high as the content levels for highly productive Pyrethrum clones from countries currently producing Pyrethrum.*

Key words: Pyrethrum, *Tanacetum cinerariifolium,* pyrethrins, HPLC, Northern Adriatic

PIRETRO *(TANACETUM CINERARIIFOLIUM)* DEL NORD ADRIATICO COME FONTE POTENZIALE DI INSETTICIDI NATURALI

SINTESI

Il piretro, Tanacetum cinerariifolium *(Trevir.) Schultz-Bip., è una specie nativa delle montagne a ridosso dell'Adriatico orientale e delle isole, ampiamente usato nella produzione di insetticidi naturali quali le piretrine. È stato pertanto determinato il potenziale biosintetico, per la produzione di piretrine, del piretro proveniente da due zone del Nord Adriatico. In tutti i campioni analizzati, con l'ausilio dell'HPLC, sono state trovate tutte e sei le piretrine. Il contenuto di piretrine maggiore è stato riscontrato nei capolini, che contengono in media 1,2% di piretrine (peso secco del campione). Il contenuto di piretrine dei capolini raccolti nelle zone del Nord Adriatico è paragonabile ai livelli ottenuti tramite produzione tradizionale di piretrine, ma non raggiunge i livelli ricavati dagli altamente produttivi cloni di piretro, utilizzati nei paesi che attualmente producono piretrine.*

Parole chiave: piretro, *Tanacetum cinerariifolium,* piretrine, HPLC, Nord Adriatico

INTRODUCTION

Pyrethrum *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip., previously classified within the genus *Chrysanthemum*, and with a still commonly used (Obukosia *et al.*, 2005) synonym *Chrysanthemum cinerariaefolium* Vis., Asteraceae (Figs. 1a, b), is a plant widely used for natural insecticide production (Hitmi *et al.*, 2000). It is the only agronomically important source of pyrethrins, identified also in other members of the genus *Tanacetum* and other genera of the same family, such as *Calendula*, *Chysanthemum*, *Tagetes* and others (Hitmi *et al.* 2000).

The term "pyrethrum" refers to the plant, flower head or flower extract, with the active insecticidal components of pyrethrum, being known as "pyrethrins" (Morris *et al.*, 2006). Pyrethrins are a combination of six monoterpene esters (Keskitalo, 1999; Gspan *et al.*, 2004). Pyrethrin I, cinerin I, and jasmolin I are collectively referred to as "pyrethrins I", whereas pyrethrin II, cinerin II, and jasmolin II are collectively referred to as "pyrethrins II". The typical extract contains pyrethrins, cinerins and jasmolins in the proportions 10:3:1 (Crombie, 1995), with the ratio of pyrethrins I to pyrethrins II (PyI/PyII) being typically around 1.0, although it can vary between 0.5 and 3.5 (Bhat, 1995). The activity of pyrethrins is a consequence of the esters mixture, depending on the ratio of PyI/PyII (Bhat, 1995). Pyrethrins affect the nervous system of insects, blocking nerve junctions and the action of voltage-sensitive sodium channels (Sonderlund, 1995). The advantages of pyrethrins include effectiveness at low dosage, on a wide range of household and public health insects, rapid insecticidal action – knock-down and killing effects, repellency, less toxicity than other insecticides to mammals, and other homeotherms, rapid degradation on exposure to light and air, and the lack of bioaccumulation in food chains and ground water (Cohran, 1995; Jovetić & de Gooijer, 1995). To avoid instability in light and air, they are formulated with antioxidants, stabilizers and synergists (Jovetić & de Gooijer, 1995; Hitmi *et al.* 2000).

The Pyrethrum plant is a long-stemmed perennial plant, 45 cm to 60 cm in height, that blooms from spring to summer and that can be harvested for up to five years. The Pyrethrum flower head (Figs. 1b, c, d), the main source of pyrethrins, is a compound inflorescence consisting of two floret types: disc florets with yellow corollas in the centre of the head and ray florets, with white corollas from the head's outer rim (Bhat, 1995). Pyrethrins mostly accumulate in achenes (94%) located under the flower's receptaculum (Brewer, 1973) and in minor quantities in the disc florets, ray florets and in the receptacles (Fig. 1d) (Head, 1966). They accumulate in two types of secretory tissue in the system of secretory channels inside the achene wall (Fig. 1e) and in cells of the oil glands on the achene surfaces (Figs. 1e, f) and the leaves (Pal & Dhar, 1985). The flower heads are first cut and allowed to dry in the field. Pyrethrins from the dried flower heads are extracted with petroleum-based solvents in order to produce a dark oleoresin, which is then refined into a coloured extract without wax. Pyrethrin content depends on several factors: genotype, flower maturity, harvesting interval, climate, drying method (Zieg *et al.*, 1983) and storage conditions (Morris *et al.*, 2006). The highest reported pyrethrins content in selected high producing clones from Australia was 2.4% (Morris *et al.*, 2006), in Kenya 2.0% (Ikahu *et al.*, 1994) and India 2.5% (Ravishankar *et al.*, 1989; Rajasekaran *et al.*, 1993) of flower dry weight. It was also reported that selected Pyrethrum varieties contain up to 3.0% pyrethrins of flower dry weight (Hitmi *et al.*, 2000).

Pyrethrum is a species native to the area of the Eastern Adriatic coastal mountains and islands of the former SFR Yugoslavia (*i.e.* Slovenia, Croatia, Bosnia and Herzegovina, Montenegro) and Albania, although it has been cultivated and locally naturalized in other parts of Europe (Heywood, 2004). The cultivation of Pyrethrum for the production of insecticide pyrethrins started in the middle of the19th century in the regions of the native species, which was part of the Austro-Hungarian Monarchy at that time. At the beginning of the 20th century, it was cultivated on more than 2,000 ha of coastal regions of the Eastern Adriatic, in Dalmatia and the islands (Kathe *et al.*, 1993). These regions dominated in the cultivation of Pyrethrum for insecticide pyrethrins until World War I, when the plant was introduced to Japan. Later, other countries such as Kenya, Tanzania, India, Tasmania (Australia), and the USA became producers of pyrethrins. It is also cultivated in Europe, in Austria, Germany, France, Hungary, Italy, Spain and Russia (Keskitalo, 1999).

As an insecticide, pyrethrins are healthier and constitute an environmentally aware and efficient method of insect control (Jovetić & de Gooijer, 1995). Currently, pyrethrins are used mainly for protecting foodstuffs or for storage in the dark, for anti-lice shampoo and indoor sprays, and they are also approved for use on organic farms (Hitmi *et al.*, 2000). The world production of natural pyrethrins lags behind the demand from the global market (Hitmi *et al.*, 2000). Therefore data about the pyrethrin content of Pyrethrum from their native area could represent a basis for further studies of this agronomically important plant species.

The main purpose of our work was to determine the biosynthetic potential of native species from the area of the Adriatic coastal mountains and islands. The pyrethrins content of flower heads of Adriatic origin collected on the island of Cres, Croatia and in the Botanical Garden of Ljubljana, Slovenia, was determined. In addition, we determined the pyrethrins content in different plant parts, at different developmental stages of flowers, as well as in leaves, stems and roots.

Fig. 1: Pyrethrum *Tanacetum cinerariifolium* ***(Trevir.) Schultz Bip. from both locations. (a, b) flowering Pyrethrum plant; (c) capitulum with ¾-open disc florets reached maximum pyrethin content; (d) vertical cross section of flower head; (e) longitudinal cross section through disc florets ovary (200x); (f) disc florets with oil glands on the surfaces of the ovary wall.***
Legend: o-df = open disc florets, c-df = closed disc florets, rf = white rye florets, df = yellow disc florets, r = capitulum's flattened axis, sc = secretory channels, og = oil glands. Secretory channels and oil glands are the site of pyrethrins accumulation.
Sl. 1: Bolhač *Tanacetum cinerariifolium* ***(Trevir.) Schultz Bip. z obeh lokacij. (a, b) bolhač v polnem cvetju; (c) cvetne glavice dosegajo maksimalne vsebnosti piretrinov takrat, ko je odprtih ¾ cevastih cvetov; (d) vzdolžno prerezana cvetna glavica bolhača; (e) plodnica cevastega cveta prečno (200x); (f) cevasti cvetovi z oljnimi žlezami na steni plodnice.***
Legenda: o-df = odprti cevasti cvetovi, c-df = zaprti cevasti cvetovi, rf = jezičasti cvetovi, df = cevasti cvetovi, r = cvetišče, sc = sekretorni kanali, og = oljne žleze na površini plodnice. Sekretorni kanali in oljne žleze so mesto akumulacije piretrinov.

MATERIAL AND METHODS

Plant material

Flower heads, leaves, stems and roots of Pyrethrum, *Tanacetum cinerariifolium* (Trevir.) Schultz-Bip. (Figs. 1a, b), of Adriatic origin, cultivated in the Botanical Garden of Ljubljana (Latitude 46°3'19N, Longitude 14°30'52E, Altitude 281 m), and years ago cultivated, wild grown on the island of Cres (Latitude 44°57'41N, Longitude 14°24'28E, Altitude 10 m), Croatia, were used for analysis. Pyrethrum flowers were collected in the middle of June in the years 1999 and 2000 in the Botanical Garden of Ljubljana and at the beginning of June 2000 on the island of Cres. Plant material, flowers and various plant parts were cold stored in the fridge (5 °C) for a few days or in the deep freeze (-80 °C) for a longer period until extraction procedures were performed.

Extraction and analysis of pyrethrins

For the quantitative and qualitative determination of pyrethrins in various plant parts, high-pressure liquid chromatography (HPLC) was used (Gspan *et al.*, 2004). Briefly, pyrethrins were extracted with petroleum ether (40-60 °C) from plant material, crushed in a mortar with silica sand and anhydrous sodium sulphate, evaporated to dryness, re-dissolved in CH_3CN, filtered through a 0.22 µm mesh filter and analysed using a Waters HPLC system with a diode array (PDA) detector. Separations were performed on a Nova Pack C18 column (Waters, 150 x 3.9 mm), using a gradient of solvent CH_3CN and Milli Q H_2O with a flow rate of 1.4 ml/min. Absorbance was monitored at 225 nm. Pyrethrins in the sample were identified on the basis of retention time and characteristic absorption spectra. The standards used (Figure 2a) were pyrethrins I (cinerin I, pyrethrin I, and jasmolin I), and pyrethrins II (cinerin II, pyrethrin II and jasmolin II) (pyrethrins technical mixture, PESTANAL, Riedel-de-Haën).

Statistics

The Student's t-test was used for evaluating levels of statistical significance (*P*) between samples of each pyrethrin from flowers of different origin.

RESULTS AND DISCUSSION

Biosynthetic potential of native Pyrethrum

The pyrethrins content in flower heads of native species from the area of the Adriatic coastal mountains and islands was studied (Fig. 1). In all flower samples

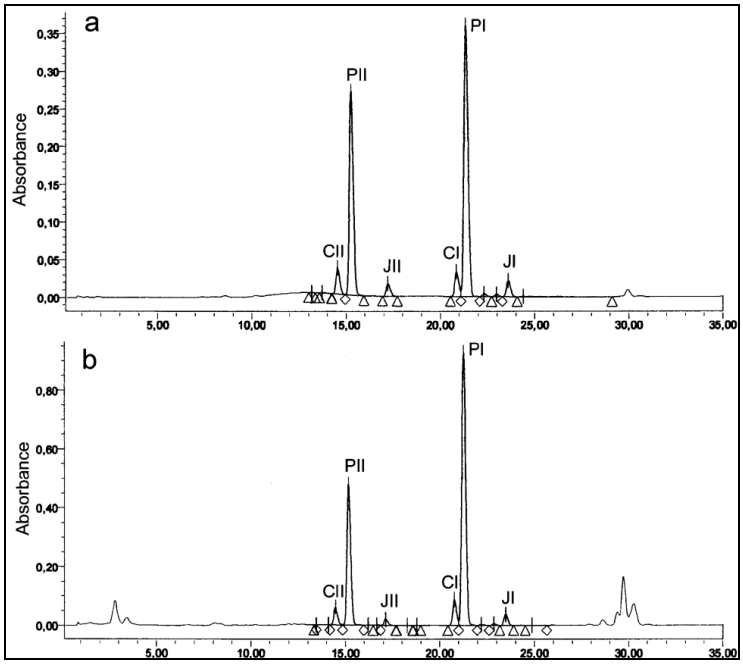

Fig. 2: (a) HPLC chromatogram of standard pyrethrin mixture; (b) HPLC chromatogram of pyrethrum flower head from the Ljubljana Botanical Garden.
Legend: CII = cynerin II, PII = pyrethrin II, JII = jasmolin II, CI = cynerin I, PI = pyrethrin I, JI = jasmolin I.
Sl. 2: (a) HPLC kromatogram standardne mešanice piretrinov; (b) HPLC kromatografska analiza piretrinov iz cvetnih glavic bolhača, nabranega v Botaničnem vrtu v Ljubljani.
Legenda: CII = cinerin II, PII = piretrin II, JII = jasmolin II, CI = cinerin I, PI = piretrin I, JI = jasmolin I.

Tab. 1: Pyrethrin content in various plant parts of Pyrethrum, collected in the Botanical Garden of Ljubljana and on the island of Cres: flower heads, leaves, stems, roots, in different parts of flower heads, open florets, closed florets and the receptaculums with the remaining parts of flower heads. Average (n = 3–5) contents of pyrethrins and standard deviations (SD) are shown. DW = dry weight.
Tab. 1: Vsebnost piretrinov v posameznih delih bolhača, nabranega v Botaničnem vrtu v Ljubljani in na otoku Cresu: cvetnih glavicah, listih, steblu, koreninah, v posameznih delih cvetne glavice, odprtih in zaprtih cvetovih ter razširjeni osi socvetja s preostanki cvetne glavice. Prikazana je povprečna vsebnost posameznih piretrinov (n = 3–5) in standardna deviacija (SD). DW = suha masa.

Sample	Pyrethrins (mg/g dry weight)										
	Cinerin II	Pyrethrin II	Jasmolin II	Cinerin I	Pyrethrin I	Jasmolin I	Pyrethrins II	Pyrethrins I	PyI/ Py II	Pyrethrins	Pyrethrins (% DW)
Flower heads* (botanical garden)	0.97	6.65	0.19	0.42	4.06	0.12	7.81	4.59	0.59	12.40	**1.2**
SD	0.16	0.99	0.03	0.05	0.77	0.03	1.16	0.84		0.63	
Flower heads* (Cres)	0.47	4.01	0.15	0.49	6.09	0.20	4.62	6.79	1.45	11.41	**1.1**
SD	0.04	0.27	0.02	0.03	0.50	0.01	0.32	0.54		0.63	
Open disc and ray florets**	0.67	5.62	0.20	0.82	9.12	0.32	6.49	10.25	1.58	16.75	**1.7**
SD	0.04	0.38	0.02	0.03	0.65	0.02	0.43	0.70		1.13	
Closed disc florets**	0.48	4.67	0.14	0.66	9.24	0.25	5.29	10.16	1.92	15.45	**1.5**
SD	0.02	0.16	0.00	0.05	0.31	0.01	0.18	0.37		0.20	
Receptaculums**	0.16	1.45	0.05	0.20	2.49	0.07	1.66	2.76	1.66	4.42	**0.4**
SD	0.02	0.10	0.00	0.01	0.08	0.00	0.12	0.09		0.22	
Leaves	0.03	0.46	0.01	0.05	1.48	0.04	0.49	1.57	3.18	2.06	**0.2**
SD	0.01	0.20	0.00	0.01	0.26	0.01	0.21	0.27		0.45	
Steam	0.002	0.02	0.001	0.01	0.11	0.002	0.03	0.12	4.65	0.15	**0.01**
SD	0.001	0.01	0.000	0.00	0.02	0.001	0.01	0.03		0.03	
Roots	0.012	0.08	0.002	0.02	0.17	0.003	0.10	0.19	1.92	0.28	**0.03**
SD	0.005	0.03	0.001	0.00	0.00	0.00	0.04	0.01		0.04	

* with 3/4-opened disc florets
** isolated from flower heads

obtained, all 6 pyrethrins were detected (Fig. 3). The highest pyrethrin content was detected in the flower heads, with ¾-open disc florets (Fig. 1c), which contained on average 1.2% (Fig. 3), maximally 1.4%, and minimally 1.0% pyrethrins of dry weight (data not shown). The chromatogram of the flower heads (Fig. 2b) is almost identical to the chromatogram for the pyrethrins standard (Fig. 2a). Although the content of the flower heads did not reach that of the highly productive pyrethrum clones from present-day production countries (Ravishankar *et al.*, 1989; Rajasekaran *et al.*, 1993; Ikahu *et al.*, 1994; MacDonald, 1995; Hitmi *et al.*, 2000; Morris *et al.*, 2006), it is comparable with pyrethrins from conventional production in Kenya, Australia and India (Head, 1966; Keskitalo, 1999), with 0.1% to 1.8% pyrethrins of flower dry weight. Thus the Adriatic origin (Heywood *et al.*, 2004), as a source of pyrethrum genetic variability, could still be of interest for a breeding program to select of high-producing clones.

Biosynthesis of pyrethrins in various plant parts

Different parts of the same flower head, separated into three parts – receptaculum, closed disc florets and open disc florets with ray florets – did not contain the same quantity of pyrethrins. The highest pyrethrins content was analysed in completely open and most mature disc florets with ray florets, which contained 1.7% pyrethrins of dry weight, less in closed disc florets, which contained 1.5% pyrethrins of dry weight, and least in the remaining part of the flower, the receptaculum, which contained only 0.4% pyrethrins of dry weight (Tab. 1). Leaves, stems and roots contained less pyrethrins than flower heads in both locations (Tab. 1). Leaves contained an average of 0.2%, steam an average of 0.01% and roots an average of 0.03% pyrethrins of dry weight and are not suitable for pyrethrins production. Besides the lower content of pyrethrins in plant parts other than the flower, they also contained a higher PyI/PyII ratio. The PyI/PyII ratio of the flower heads of pyrethrum was always lower than the ratio of other plant parts (Tab. 1). This confirms previous observations (Head, 1966).

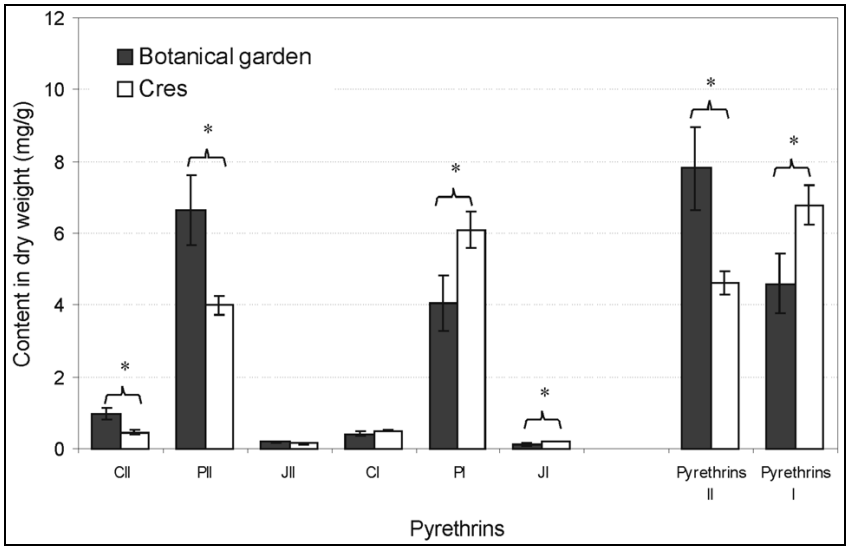

Fig. 3: The effect of location conditions on content levels of individual pyrethrins, pyrethrins I and II and total pyrethrins in flower heads of pyrethrum collected in the Botanical Garden of Ljubljana and on the island of Cres. Average (n = 5) contents of pyrethrins, standard deviations (SD) and statistically significant differences (t-test, ∗ denotes $P < 0.05$) are shown between samples from two locations. Legend: see Figure 2.
Sl. 3: Vpliv različnih rastiščnih razmer na vsebnost posameznih piretrinov, piretrinov I in II ter celokupnih piretrinov, v cvetnih glavicah bolhača, nabranega v Botaničnem vrtu v Ljubljani in na otoku Cresu. Prikazana je povprečna vsebnost posameznih piretrinov (n = 5), standardna deviacija (SD) in statistično značilne razlike (t-test, ∗ označuje $P < 0,05$) med vzorci obeh rastišč. Legenda: glej Sliko 2.

Biosynthesis of pyrethrins in plants cultivated in regions with different climate conditions

Differences between plants cultivated under the different climate conditions of the island of Cres, Croatia, and the Botanical Garden in Ljubljana, had only a slight and insignificant influence on the total pyrethrin content in the flower heads (Fig. 3). Flower heads from island of Cres contained an average of 1.2% and flower heads from the Botanical Garden in Ljubljana contained an average of 1.1% pyrethrins of flower dry weight.

Differences between plants cultivated in different climate conditions of the island of Cres, Croatia, and the Botanical Garden in Ljubljana, however, did significantly influence the PyI/PyII ratio (Fig. 3), which is important for the insecticidal activity of pyrethrins and therefore affects the quality of the Pyrethrins extract (Jovetić & de Gooijer, 1995). Pyrethrins II were shown to have rapid knock-down ability and pyrethrins I slow killing effect (Jovetić & de Gooijer, 1995). The PyI/PyII ratio in flower heads from the island of Cres were noticeably higher (1.4) than the PyI/PyII ratio in flower heads from the Botanical Garden of Ljubljana (0.6) (Fig. 3). Ratios lower than 1 were observed in four of five samples from the Botanical Garden of Ljubljana, while in the remaining one this ratio increased to a level comparable to that observed in flower heads from Cres (data not shown). Beside the difference in environmental conditions, variations in PyI/PyII ratio may be caused by other factors, not examined in our study, such as slightly different stages of maturity at the time of flower sampling (Pattenden, 1970), slightly different management of flower samples or different cultivation conditions during plant growth on each site.

In conclusion, these data indicate that differences in location and climate conditions did not influence the total pyrethrin content in the flower heads, but did influence the PyI/PyII ratio. The pyrethrin content of flower heads from native species of the Northern Adriatic is comparable with content levels in the conventional production of pyrethrins, but did not reach the content levels of highly productive Pyrethrum clones from countries currently producing pyrethrins. Thus the Adriatic origin, as a source of Pyrethrum genetic variability, could be of potential interest in creating a breeding program for the selection of high-producing clones.

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SEVERNOJADRANSKI BOLHAČ *(TANACETUM CINERARIIFOLIUM)* KOT POTENCIALNI VIR NARAVNIH INSEKTICIDOV

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POVZETEK

Bolhač Tanacetum cinerariifolium *(Trevir.) Schultz-Bip. je najpomembnejši vir naravnih insekticidov piretrinov. Prispevek opisuje možnost pridobivanja piretrinov iz bolhača, s primarnih rastišč na kraških otokih in pobočjih priobalnega pasu Jadranskega morja nekdanje Jugoslavije in Albanije, nabranega na otoku Cresu in v Botaničnem vrtu v Ljubljani. V vseh vzorcih, analiziranih s tekočinsko kromatografijo visoke ločljivosti (HPLC), smo zaznali vseh 6 piretrinov. Največ piretrinov smo zaznali v cvetnih glavicah, ki so vsebovale povprečno 1.2% piretrinov v suhi masi vzorca. Razlike v rastiščnih razmerah obeh lokacij so malo in neznačilno vplivale na celokupno vsebnost piretrinov. Vsebnosti piretrinov v bolhaču, ki izvira iz naravnih rastišč severnega Jadrana, so primerljive z vsebnostjo piretrinov bolhača iz konvencionalne proizvodnje piretrinov, ki pa ne dosegajo vsebnosti visoko-produktivnih klonov bolhača, o katerih poročajo nekatere države sedanje proizvajalke piretrinov. Primarna rastišča priobalnega pasu Jadranskega morja, ki so vir genetske pestrosti bolhača, so zato še vedno zanimiva za programe žlahtnjenja in selekcijo visoko-produktivnih klonov.*

Ključne besede: bolhač, *Tanacetum cinerariifolium*, piretrini, HPLC, severni Jadran

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