LEK. SIROV.	God. XXXIII	Broj 33	Str. 111 – 118	Beograd 2013.
LEK. SIROV.	Vol. XXXIII	No. 33	PP. 111 – 118	Belgrade 2013.

Prikaz doktorske teze – Ph.D. Thesis prewiev ***Rukopis prihvaćen:*** *27.11.2013.*
UDC: 633.881/.883

UTICAJ USLOVA GAJENJA NA MORFOLOŠKE I HEMIJSKE OSOBINE I BIOLOŠKE EFEKTE EKSTRAKATA *Arnica montana* L.

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Arnica montana L. (Asteraceae) je višegodišnja planinska biljka čije se cvetne glavice (*Arnicae flos*) uglavnom koriste za spoljašnju upotrebu kod modrica i uganuća u fitofarmaceutskim preparatima kao što su masti, kreme i gelovi. Do pre par godina svetsko tržište pomenute sirovine je zadovoljavano isključivo iz spontanog resursa, ali su biljne populacije, zbog prekomernog sakupljanja, znatno degradirane, zbog čega je u većini evropskih zemalja stavljena zabrana za njeno dalje sakupljanje iz prirode. Obzirom da tražnja za ovom sirovinom na tržištu ne opada, kultivacija arnike se nameće kao jedino održivo rešenje zadovoljenja potreba farmaceutske industrije.

Budući da *A. montana* (narodni naziv – arnika) nije autohtona biljna vrsta za područje Srbije, iako se spontano javlja u planinskim oblastima nekih susednih zemalja i Balkanskog poluostrva uopšte, cilj ovih istraživanja je bio da se iznađe tehnološko rešenje njivske proizvodnje na osnovu ispitivanja uticaja vremena zasnivanja, primene đubrenja i tipa sadnica. U istraživanju su pored cvetne glavice, kao glavne droge, posmatrani i podzemni organi arnike: rizom i koren, kao sporedne sirovine. Prva faza eksperimentalnog rada obuhvatila je trogodišnji trofaktorijalni poljski ogled načina proizvodnje arnike, pri čemu su se kao faktori ispitivali: vreme zasnivanja useva (prolećna i jesenja sadnja), đubrenje (stajnjak i NPK) i način zasnivanja useva (iz semena i klonskom propagacijom – deobom bokora). U drugoj fazi eksperimenta, izvršena je karakterizacija sirovine u smislu njene brze identifikacije, hromatografskim tehnikama identifikovani su prisutni sekundarni metaboliti i određen njihov sadržaj u ispitivanim uzorcima i testirani su biološki efekti odabranih ekstrakata.

Ispitivanja su vršena na ogledu postavljenom 2008. godine na lokalitetu Kaluđerskih bara (1008 m n.v.), na planini Tara, u krugu rasadnika Nacionalnog Parka „Tara“. Obzirom na to da arnika prvu vegetaciju provodi u fazi rozete sva merenja morfoloških parametara, prinosa i hemijskih karakterizacija sirovina

vršena su tokom 2009. i 2010. godine. Na ogledu su praćeni sledeći parameri: prečnik rozete, visina cvetnog izdanka, broj cvetnih izdanaka, broj cvetnih glavica, prečnik cvetne glavice, broj sekundarnih rozeta. Određeni su sledeći prinosi: prinos cvetne glavice, prinos rizoma, prinos korena i prinos etarskog ulja (rizoma i korena). U okviru identifikacije biljne sirovine izvršena je poredbena mikroskopska analiza i određen hemijski profil tankoslojnom hromatografijom droge *Arnicae flos* u odnosu na moguće falsifikate: cvetne glavice biljaka *Calendula officinalis* i *Doronicum columnae*. U cilju lokalizacije i određivanja mehanizama ekskrecije etarskog ulja u rizomu i korenu arnike, izvršena je: mikroskopska analiza ultra-tankih preseka pod svetlosnim mikroskopom, ispitivana je fluorescencija i urađeno više različitih histohemijskih bojenih testova, pripremljeni su i analizirani preseci skenirajućom i transmisionom elektronskom mikroskopijom. Hemijska karakterizacija cvetnih glavica arnike obuhvatila je kvalitativnu i kvantitativnz analizu sekundarnih metabolita iz grupe seskviteprenskih laktona i fenolnih jedinjenja (fenolne kiseline i flavonoidi). U okviru hemijske karakterizacije etarskog ulja rizoma i korena izvršena je identifikacija i kvantifikacija glavnih komponenti.

Od svih varijanti faktora indukovanih na ogledu, varijanta prolećne sadnje sadnica iz vegetativne propagacije se pokazala kao nepoželjna. Kod ove varijante došlo je do cvetanja velikog broja biljaka odmah nakon sadnje, što je uslovilo iscrpljivanje potencijala biljke na formiranje cvetonosne stabljike, cveta i ploda. Ova pojava je, kako je pokazano u narednoj godini, bila posledica uznapredovale generativne faze razvića biljaka u trenutku rasađivanja. Kao posledica slabog ukorenjavanja biljke ove varijante su u velikoj meri uginule, a preživele biljke su se slabije razvile. Vrednosti dobijene sa ove varijante zastupljene su u statističkim analizama, ali nisu uzimane u obzir za poređenje sa literaturnim podacima. Faktor vremena sadnje je bio statistički značajan za sve merene parametre osim za prinos korena u 2009. i prinos rizoma u 2010. godini. Takođe je variranje faktora vrste sadnica imalo statistički značajan uticaj na sve merene parametre, osim na prinos rizoma i korena u 2009. godini i prinos rizoma i prečnik cvetne glavice u 2010. godini. Faktor vrste đubrenja je imao statistički značajan uticaj na variranje vrednosti gotovo svih morfoloških parametara, ali su statistički značajne faktorske interakcije u većini slučajeva prikrivale doprinos unetih hraniva, tako da se diskusija o značajnosti tretmana đubrenja u gajenju arnike morala rasčlaniti na pojedinačne varijante vrste sadnica i vremena sadnje.

Vrednosti prečnika rozete su se kretale od 12,4 – 23,4 cm (14,5 – 28,2 cm) u drugoj (odnosno trećoj) vegetaciji. Visina cvetnih izdanaka se kretala od 20,5 – 34,1 cm (25,5 – 41,7 cm), dok su se prosečne vrednosti broja cvetnih izdanaka kretale od 1,5 – 5,0 (2,4 – 12,6). Broj cvetnih glavica po biljci je varirao od 7,2 – 16,3 (8,4 – 38,9), prečnik cvetnih glavica se kretao od 6,0 – 7,5 cm (6,4 – 9,1 cm), a broj sekundarnih rozeta od 5,7 – 18,3 (25,3 – 35,4). Prinos cvetnih glavica se

kretao od 59,8 – 143,5 kg/ha (116,2 – 258,7 kg/ha), prinos rizoma od 106,3 – 373,7 kg/ha (475,9 – 897,5 kg/ha), a prinos korena od 194,3 – 426,4 kg/ha (420,9 – 615,0 kg/ha). Sadržaj etarskog ulja se u rizomu kretao od 4,0 – 4,8% (2,1 – 3,1%), dok je u korenu bio od 1,1 – 3,2% (1,7 – 2,4%).

Mikroskopskim snimanjem samlevenog biljnog materijala pod različitim uveličanjima ustanovljena je jednostavna procedura identifikacije cveta arnike u odnosu na dva pretpostavljena falsifikata. Tako, pored ostalih karakterističnih detalja, cvetne glavice arnike i *D. columnae* sadrže vidljive i lako prepoznatljive papuse, za razliku od *C. officinalis*, dok se po debljini papusi arnike (69±16 μm) mogu razlikovati od papusa *D. columnae* (30±7 μm). Analizom hromatograma dobijenih tankoslojnom hromatografijom pouzdano se može identifikovati cvetna glavica nevena koji se od ostale dve vrste razlikuje po prisustvu zone izražene narandžaste fluorescencije u donjoj polovini hromatograma na Rf ~ 0,4. Ova zona po položaju odgovara rutinu u smeši standarda, a odsutna je u uzorcima cvetnih glavica *A. montana* i *D. columnae*. U uzorku *A. montana* jasno se uočava još jedna zona narandžaste fluorescencije na Rf ~ 0,65 koja je potpuno odsutna u uzorku C. officinalis i jedva primetna u uzorku D. columnae. Ova zona po položaju i fluorescenciji odgovara standardu izokvercitrina.

Seskviterpenski laktoni prisutni u cvetnoj glavici arnike su identifikovani i određen je njihov ukupni sadržaj izražen kao dihidrohelenalin tiglat (DHHT), koji se u 2009. godini kretao od 7,9 – 13,2 mg/g, a u 2010. godini od 4,6 – 13,9 mg/g. U obe posmatrane godine biljna sadržaj seskviterpenskih laktona u drogi *Arnicae flos* je bio veći od minimuma (4 mg/g) koji propisuje Evropska farmakopeja 6.0. U uzorcima cvetne glavice arnike identifikovano je pet fenolnih jedinjenja i određen sadržaj jedne fenolne kiseline (hlorogenska kiselina) i dva dominantna flavonoida (kvercetin-3-O-glukozida i kemferol-3-O-glukozida). Sadržaj hlorogenske kiseline se kretao od 3,1 – 6,0 mg/g (1,9 – 6,57 mg/g) u drugoj (odnosno trećoj) vegetaciji, kvercetin-3-O-glukozida od 8,4 – 13,9 mg/g (7,8 – 12,5 mg/g), a kemferol-3-O-glukozida od 2,1 – 4,7 mg/g (2,1 – 4,5 mg/g). U hemijskom profilu etarskog ulja rizoma i korena dominantno (ca. 80%) su bili zastupljeni derivati timola i to prvenstveno 2,5-dimetoksi-p-cimen (28,9 – 40,7%) i timol metil etar (9,6 – 27,2%).

U ispitivanju antioksidativne aktivnosti ekstrakata cvetnih glavica arnike veću sposobnost neutralizacije DPPH radikala imao je ekstrakt sa maksimalnom vrednošću sadržaja ukupnih seskviterpenskih laktona u odnosu na ekstrakt sa minimalnom vrednošću, dok su razlike u antioksidativnoj aktivnosti esktrakata sa maksimalnom i minimalnom vrednošću sume sadržaja tri kvantifikovana fenolna jedinjenja bile neznatne. Testirani ekstrakti ispoljili su i antimikrobnu aktivnost, gde su se minimalne vrednosti za inhibiciju rasta devet sojeva bakterija i jednog kvasca kretale od 2 – 15 μl/ml. Antimikrobna aktivnost etarskih ulja je bila selektivna, gde su MIC vrednosti za određene mikroorganizme (*Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Pseudomonas aeruginosa,*

Micrococcus flavus, Listeria monocytogenes i *Salmonella enteritidis*) bile izuzetno male (5 µl/ml), dok je kvasac *Candida albicans* bio visoko rezistentan na sva testirana etarska ulja (MIC koncentracija kretala se i do 83 µl/ml).

Plantažnim gajenjem arnike u agroekološkim uslovima Srbije može se dobiti do 300 kg/ha kvalitetne sirovine *Arnicae flos* i oko 1000 kg/ha podzemnih organa iz kojih se može destilacijom dobiti oko 30 l etarskog ulja bogatog aromatičnim jedinjenjima. Kao najbolja varijanta gajenja izdvojila se kombinacija jesenje sadnje sadnicama proizvedenim iz semena u bloku mineralnog đubrenja, dok se kao najnepovoljnija varijanta pokazala kombinacija zasnivanja plantaže na proleće sadnicama dobijenim vegetativnom propagacijom.

Ključne reči: arnika, lekovito bilje, seskviterpenski laktoni, fenolna jedinjenja, etarsko ulje, mikroskopska analiza, histohemija, antioksidativna aktivnost, antimikrobna aktivnost.

Naučna oblast: *Biotehnologija*
Uža naučna oblast: *Ratarstvo i povrtarstvo*

INFLUENCE OF GROWING CONDITIONS ON MORPHOLOGICAL AND CHEMICAL PROPERTIES AND BIOLOGICAL EFFECTS OF EXTRACTS OF *Arnica montana* L.

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SUMMARY

Arnica (*Arnica montana* L., Asteraceae) is a perennial mountain plant whose flower heads (*Arnicae flos*) are mainly used for topical treatment of bruises and sprains in phytopharmaceutical preparations such as ointments, creams and gels. Until few years ago, the world market demand for this raw material was almost exclusively covered from the spontaneous resources, but plant populations are, due to over-collecting, significantly degraded, what caused prohibition of its collection from the wild in most of European countries. Since the demand for this raw material on the market is constant rise, cultivation arnica is imposed as the only sustainable solution to meet the needs of the pharmaceutical industry.

Since the *A. montana* plant species is not native to the region of Serbia, although it occurs spontaneously in some mountain areas of neighboring countries and the Balkans in general, the aim of this study was to find a suitable technology solution of field cultivation based on the examination of induced various ecological factor effects. In this research, beside observations connected with flower heads, as the main drug, underground parts of arnica: rhizome and roots, were also studied as side products. The first phase of the experimental work included a three-year three-factorial field experiment in different modes of production, where as the factors examined: time of plantation establishment (spring and autumn planting), fertilization (manure and NPK) and methods of propagation (from seed and clonal propagation - division of the tuft). In the second phase of the experiment, characterization of the raw material was carried out in the terms of its rapid identification, qualitative evaluations based on the content of secondary metabolites and testing of biological effects of extracts obtained.

Tests were performed on the field experiment conducted in 2008 at locality of Kaludjerske Bare (1008 m) on mountain Tara, in area of Nursery production department of the National Park "Tara". Since arnica its first vegetation remains in the rosette phenophase, all measurements of morphological parameters, yields and chemical characterizations of raw materials was carried out in 2009 and 2010 year. Following parameters were examined at the trial: rosette diameter, height of flower shoots, number of flower shoots, number of flower heads per plant, flower head diameter and number of secondary rosettes. Yields were observed for the flower

heads, rhizomes, roots and essential oils (in rhizomes and roots). Identification of the raw material *Arnicae flos* against possible forgeries, *Calendulae flos* and flower heads of *Doronicum columnae*, was performed by comparative microscopic imaging and thin layer chromatography. We examined the localization and mechanisms of excretion of essential oil in rhizome and roots by recording of ultra-thin sections under the light microscope, by examining the fluorescence after histochemical staining, and by recording with scanning and transmission electron microscopy. Chemical characterization of flower heads included quantification and qualification of secondary metabolites from the group of sesquiterpene lactones and phenolic compounds (phenolic acids and flavonoids). In the frame chemical characterization of essential oils of the rhizomes and roots, quantification and identification of key components were done.

From all factor combinations induced in an experiment, variant of spring planting of seedlings from vegetative propagation is shown to be highly undesirable. In this variant large number plants flowered immediately after planting, causing depletion of plant potential on forming flower shoots, flower heads and seeds. This phenomenon, as shown in the following year, was the consequence of an advanced generative development stage of plants at the time of planting. As a result of poor rooting, plants these variants were withered in a large scale, while survived plants were small and weak. The values obtained from these variants are represented in statistical analysis, but were not considered for comparison with literature data. Factor of planting time was statistically significant for all measured parameters except for root, in 2009, and rhizome yield in 2010 year. Similarly, the variation of type of propagation factor had a statistically significant effect on all the measured parameters, except for the root and rhizome yield in 2009, and rhizome yield and the diameter of the flower head in 2010. Type of fertilizer factor had a significant influence on variations of almost all morphological parameters, but it also had statistically significant interactions with other two factors in most cases, which dissembled nutrient contribution in plant development, therefore the discussion on the significance of fertilization treatments in growing arnica had to be divided on individual observations within other two factor combinations.

Rosette diameter values ranged from 12.4 - 23.4 cm (14.5 - 28.2 cm) in the second (third) vegetation. Height of flower shoots ranged from 20.5 - 34.1 cm (25.5 - 41.7 cm), while the average value of the number of flowering shoots ranged from 1.5 - 5.0 (2.4 - 12.6). Number of flower heads per plant ranged from 7.2 - 16.3 (8.4 - 38.9), diameter of flower heads ranged from 6.0 - 7.5 cm (6.4 - 9.1 cm), and the number of secondary rosette of 5.7 - 18.3 (25.3 - 35.4). Flower heads yield ranged from 59.8 - 143.5 kg/ha (116.2 - 258.7 kg/ha), rhizome yield from 106.3 - 373.7 kg/ha (475.9 - 897.5 kg/ha), and root yield from 194.3 - 426.4 kg/ha (420.9 - 615.0

kg/ha). The content of essential oil in the rhizome ranged from 4.0 - 4.8% (2.1 - 3.1%), while in the roots ranged from 1.1 to 3.2% (1.7 - 2.4%).

Through microscopic imaging of grounded plant material a simple identification procedure of arnica flower heads against two presumed forgeries has been established. Among to other typical details, arnica flower heads and *D. columnae* contain visible and easily recognizable pappi, unlike C. officinalis. Also pappi of arnica are much thicker (69±16 μm) than pappi of *D. columnae* (30±7 μm). Furthermore, thin-layer chromatography can reliably distinguish *C. officinalis* from the other two species, since the extract of this plant in the lower half of the chromatogram (Rf ~ 0.4) has zone with strong orange florescence, which corresponds to location of rutin in the standard mixture, while is absent in *A. montana* and *D. columnae*. In the sample of *A. montana* another zone with orange fluorescence was clearly visible at Rf ~ 0.65, which was absent in sample of *C. officinalis* and barely visible in *D. columnae*. This zone by its position and fluoresnece responds to isoquercitrin standard.

Sesquiterpene lactones present in arnica flower were identified and quantified in its overall content, expressed as dihydrohelenalin tiglate (DHHT), which ranged, in 2009, from 7.9 – 13.2 mg/g and, in 2010, from 4.6 - 13.9 mg/g. Considering both years, the herbal drug *Arnicae flos* meet the quality criteria of minimum total sesquiterpene lactones content (4 mg/g) prescribed by the European Pharmacopoeia 6.0. In the flower head samples five phenolic compounds has been identified, out of which content of one phenolic acid (chlorogenic acid) and two dominant flavonoids (quercetin-3-O-glucoside and kaempherol-3-O-glucoside) has been estimated. Chlorogenic acid content ranged from 3.1 - 6.0 mg/g (1.9 - 6.6 mg/g) in the second (third) vegetation, quercetin-3-O-glucoside from 8.4 - 13.9 mg/g (7.8 - 12.5 mg/g) and kaempherol-3-O-glucoside from 2.1 - 4.7 mg / g (2.1 - 4.5 mg/g). In chemical profiles of the essential oils of rhizomes and roots dominant components were thymol derivates (ca. 80%), mainly 2,5-dimethoxy-p-cymene (28.9 - 40.7%) and thymol methyl ether (9.6 - 27.2%).

In examined antioxidant activity of extracts of arnica flower heads greater ability to neutralize DPPH radicals had extract with maximum content of total sesquiterpene lactones compared with extract with minimum content, while differences in the antioxidant activities of extracts with maximum and minimum value of the sum of the three quantified phenolic compounds were negligible. Tested extracts exhibited also antimicrobial activity, where minimum inhibitory concentrations (MICs) of nine strains of bacteria and one yeast ranged from 2-15 μl/ml. The antimicrobial activity of the essential oil was selective, where the MIC values for certain microorganisms (*Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus flavus, Listeria monocytogenes* and *Salmonella enteritidis*) were very small (5 μl/ml), but the yeast

Candida albicans was highly resistant to all tested essential oils (MIC concentrations ranged up to 83 µl/ml).

From cultivation of arnica in agro-ecological conditions of Serbia can be obtained up to 300 kg/ha of quality raw material *Arnicae flos* and up to 1000 kg/ha of underground organs, from which can be extracted, by distillation, about 30 l of essential oils rich in aromatic compounds. As the best variant proved to be combination of autumn planting of seedlings produced from seeds in a block of mineral fertilizer, while the least favorable option has been shown combination of spring established variants where seedlings are obtained from vegetative propagation.

Key words: arnica, herbs, medicinal plants, yield, sesquiterpene lactones, DHHT, flavonoids, essential oils, antioxidant activity, antimicrobial activity.

Scientific field: *Biotechnology*
Scientific discipline: *Field and Vegetable Crops*