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Full Length Research Paper

Assaying the variation in secondary metabolites of St. John's wort for its better use as an antibiotic

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The present study is aimed at investigating the effects of variation in secondary metabolites of St. John's wort for its better use as an antibiotic. The seasonal dynamics investigation of St. John's wort secondary metabolites was carried out on annual, biennial and triennial wild-growing plants of the suburban localities, and on the indigenous perennial plants of the mountainous localities. The effects of variation in secondary metabolites of the plant material were monitored using a complex antimicrobial preparation imanin. As plant secondary metabolites, imanin was isolated from flowers and leaves of St. John's wort by aqueous-alkaline extraction. The quality of imanin contained in St. John's wort was determined by FTIR and HPLC methods. The imanin extracts were tested for antimicrobial activity against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus diphteriae*, *Bacillus tetani*, *Clostridium histolyticus*, *Bacillus mesentericus* and *Bacillus mycoides*. The quantitative effects of temperature and light intensity on imanin accumulations in St. John's wort were examined depending on the sampling periods and location. The results of antimicrobial activity and quantitative effects of climatic conditions were correlated with vegetation phases of hypericum plants.

Key words: Imanin, St. John's wort, antimicrobial activity, hyperforin, plant vegetation.

INTRODUCTION

St. John's wort (*Hypericum perforatum* L.) is a perennial herbaceous weed with a spindly and very developed branchy root. The stalk is upright, very robust, angular and branchy (Figure 1). St. John's wort is a medicinal herb widely spread in Europe, Asia and North Africa. In the region of Southeast Europe (Balkan), four sub-species of *H. perforatum* L.: *Vulgare*, *Latifolium*, *Veronese* and *Angustifolium* (Serbian academy, 1972) have been found. In the flora of Serbia 19 species of this genus have been found (Kojic et al., 1998). The habitat of St. John's wort includes both lowland and mountainous terrains. The conditions necessary for growth and survival of St. John's wort are soils moderately rich in nutrients, temperate and fairly light habitats. It usually grows on dry hills, in oak woods, insolated meadows, in the shrubbery, near the roads, rocky areas, clearings and fire sites, barren and uncultivated land and around swamps. It blossoms during the whole summer (from May to September), but mostly in July. The harvest time is in May and June. It propagates by seeds. The top half of the blooming herb (*Hyperici herba*) is sampled. The drug has a characteristic scent, and astringent, bitter and aromatic flavor. It is wide-spread in many taxonomic forms. In principle, we can distinguish between male (*Centaurium umbellatum*) and female (*H. perforatum*) centauriums (Kojic et al., 1998). The medicinal parts of the blooming plant are flowers and leaves. The production of plant secondary metabolites (a historical perspective) was detailed in literature (Bourgaud et al., 2001).

As a medicinal plant, St. John's wort shows a wide range of active effects (Kelet et al., 2001; Reichling et al., 2001; Borchardt et al., 2008), and has a long-standing application in traditional medicine. When its bioactive components, such as hypericin, hyperforin, quercetin, and the proper essential oil were isolated, it found its application in modern medicine as well. It is used as an antiseptic, sedative, analgesic, diuretic, antidepressant, anti-inflammatory, antibiotic, antitumoral, antiangiogenic

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Figure 1. Profile of St. John's wort and distinctions between female (*Hypericum perforatum*) and male (*Centaurium umbellatum*) centauriums.

(Bauer et al., 2001; Sanchez-Mateo et al., 2002; Schwarz et al., 2003; Roz and Rehavi, 2004). It can be used both externally and internally. The internal use refers to herbal tea used for alleviating gastric spasms, the elimination of pulmonary secretion, as a cure for kidney, liver, gall, spleen, and urinary bladder diseases, a chronic cough, asthma, blood coagulation, gastric acidity, gastritis, and intestinal parasites, irregular and profuse menstruations, a vaginal discharge, etc. (Jancic, 1990). It is especially recommended for uncontrolled bedwetting. Mixed with other medicinal herbs, it is an excellent means for treating a chronic cough and asthma. Recent studies have revealed the effects in treating viral and retroviral infections (Reichling et al., 2001), or cancer treatments (Dona et al., 2004; Guedes and Eriksson, 2005). Externally, St. John's wort is used as an oil balm for burns, cuts, insect bites, hemorrhoids, and for healing wounds (Jancic, 1990). In cosmetics and medicine it serves as a skin care and protection agent (massage, dry and scaly skin care, protection against sun and radiation, acne, boils, burns). The application of St. John's wort's oil is not limited with respect to duration because there are no side effects, so it can be used continually for long periods of time (Glisic et al., 2006). St. John's wort has a number of problematic interactions with many drugs (Goldstein et al., 2006). It has been reported to interact with amphetamines, asthma inhalants, decongestants, diet pills, narcotics, tryptophan and tyrosine (amino acids), as well as with antidepressant medications and certain foods. There is also a risk when combining it with various medications. The effect may be pharmacokinetic by altering the absorption or metabolism, and may be pharmacodynamic, by changing the final effect of the drug. St. John's wort, an antidepressant herbal remedy, may pharmacodynamically interact with specific serotonin reuptake inhibitors, causing a serotonin syndrome. St. John's wort also causes serious pharmacokinetic interactions by activating the cytochrome, dangerously decreasing blood levels of cyclosporin, warfarin, and theophylline, and reducing the efficacy of contraceptive pills and AIDS therapy.

The multiple effects of St. John's wort are due to its complex chemical composition (American Herbal Ph., 1997). It contains essential oil comprised of kadinen and other sesquiterpenes, esters of isovaleric acid and some azulene. It contains resins, iron, anthocyans, red pigment hypericin, carotene and flavonoid heterosides (hyperosid, rutosid, quercitrosid and others giving it the yellow color), choline, vitamin C, traces of alkaloids and mixed tannin, where catechin is prevalent. Active substances participating in the composition of the plant are: hypericin, pseudohypericin, isohypericin (in the umbel), hyperosid, quercetin, rutin, quercitrin, biapigenin, amenthoflavon, catechin, epicatechin, chlorogenic acid, caffeic acid, hyperforin, carotenoids, sterols and essential oil (Tatsis et al., 2007).

Interestingly, in the mid-20th century, imanin and novoimanin were introduced into medical practice as antibiotics (Maksyutina and Koget, 1973). Imanin was isolated by water-alkali solution extraction of St. John's wort (Derbenceva, 1961) and represents a complex antibacterial preparation with the components of polyphenolic nature, mostly from dianthron and flavonoid groups. Apart from hydrolysis products and resinous matters, hyperforin was isolated and characterized as its main active ingredient. Hyperforin and hypericin are two of the most important components from the standpoint of pharmacological activity of St. John's wort (Figure 2). Hypericin is a derivative of naphthodiantrone. Hyperforin has bicyclo-(3,3,1)-nonan structure and typically occurs only in St. John's wort.

In literature, there are numerous references on the investigations of the complex composition of St. John's wort (Chandrasekera et al., 2005; Smelcerovic et al., 2006a; Williams et al., 2006). Nearly all components and techniques of their separation have been defined, as well as the content of hypericin, flavonoid, essential oil and other components in specific parts of the plant (Smelcerovic et al., 2006b). Phytochemical analysis and genetic characterization of *Hypericum* species from various localities in Serbia are known (Smelcerovic et al., 2006b, c). However, the change of antibiotic properties of

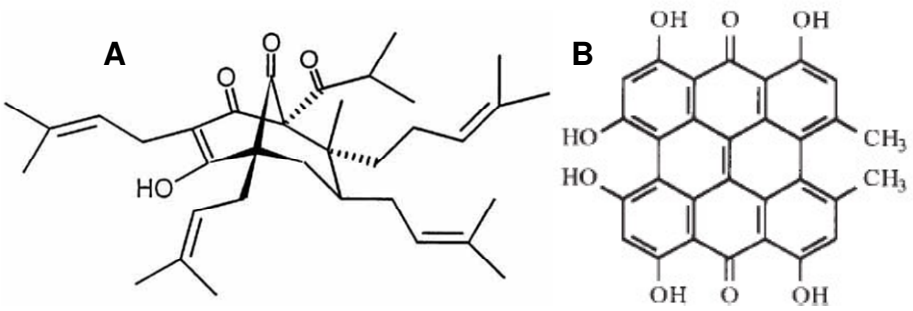

Figure 2. Structure of (A) Hyperforin (phloroglucinol) and (B) Hypericin (naphtodianthrone).

St. John's wort as a function of the biological cycle of the plant has been partly investigated in Europe (Felkova and Stranski, 1957). Thus, Czech authors have investigated the antimicrobial properties of alcoholic extracts of St. John's wort during the whole period of the generative development of the plant. Likewise, Russian authors have developed St. John's wort and an imanin active matter extraction method (Drobotko et al., 1958), and then investigated the dynamics of antibiotic activity properties in St. John's wort (Omelcuk-Miakusko and Fastovskaja, 1968). Additionally, the quantitative effects of temperature and light intensity on hyperforin, hypericin and pseudohypericin accumulations in *H. perforatum* have been examined (Odabas et al., 2009). A new model to estimate hyperforin, hypericin and pseudohypericin accumulations in greenhouse-grown *H. perforatum* plants as affected by temperature and light intensity was also developed (Odabas et al., 2009). It is well know that a chemical profile of plants and the accumulation level of a special metabolite in plant tissues can be influenced by several environmental factors such as temperature (Couceiro et al., 2006), light quality (Upadhyaya et al., 1994) and light intensity (Yamamaura et al., 1989). In this sense, the determination of optimum temperatures and light intensities for chemical accumulation, as well as the plant growth and development, is an important topic in obtaining the increased concentration of phytochemicals (Abreu and Mazzafera, 2005; Zobayed et al., 2006). Also, the effects of the water and temperature stress on the content of active constituents, as well as different effects of light and nitrogen on production of hypericins and leaf glands in *H. perforatum* were investigated (Briskin and Gawienowski, 2001).

However, there is no data on the dynamics investigations of antimicrobial properties of St. John's wort active secondary metabolites from water-alkali solutions in different vegetation periods, especially with respect to the plant material from the Balkan localities. Therefore, the interest of this work has been monitoring and investigation of seasonal dynamics of collecting St. John's wort in Serbia (Leskovac area) from the aspect of maximal exploitation of antimicrobial properties of the plant's components. For this reason the present study is aimed at investigating the effects of variation in secondary metabolites of the St. John's wort for its better use as an antibiotic. In the sense, the quantitative effects of temperature and light intensity on secondary metabolites accumulations in St. John's wort were examined depending on the sampling periods and location. These investigations have practical importance for the determination of rational deadlines for the collection of plant material, i.e. St. John's wort harvesting, as well as its use in the pharmaceutical-cosmetic industry or for medicinal purposes. For the determination of antimicrobial properties of St. John's wort in the plant material collected during the season, the method of the extraction of active substances from St. John's wort was used with the aim of obtaining imanin. Imanin, as a complex antibacterial preparation, is from the practical point of view far more suitable for monitoring the total St. John's wort activity than individual active ingredients. On one hand, this is due to a less complicated technique of isolation and storage, mainly to the high instability of its active components in pure state. Hyperforin, the main bioactive component of imanin, is hard to isolate due to high lipophility and limited stability. In addition, the longer retention of imanin antimicrobial properties compared to its components enables a better correlation of the activities and the dynamics of St. John's wort biological cycle. A comparative study of such investigations would lead to a better understanding of the influence of the geographic location, climate conditions and natural habitat on the process of development and conservation of the medicinal plant material. In the present study, the *Staphylococcus* test (*Staphylococcus aureus* breed 209) has been chosen to monitor the antimicrobial activity of St. John's wort secondary metabolites.

MATERIALS AND METHODS

Plant and locality

The first investigated Balkan's plant St. John's wort (*H. perforatum* L.) was registered in the General regional herbarium of Balkan Peninsula (int. sign BEO), the Natural museum, Belgrade, Serbia (registration number 8632, date 16/08/1936). Specimen of the plant from the location of "Vlasina lake pl. Plana" was identified by P.

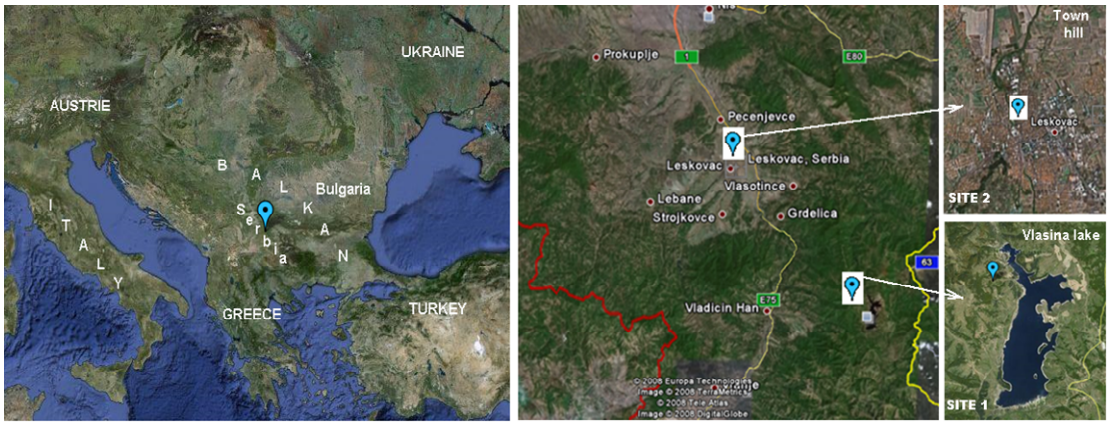

Figure 3. Satellite Balkan map and study locations of the tested St. John's wort: Vlasina lake (Site 1) and Leskovac town-hill (Site 2) (Southeast Serbia, coordinates 42.7°N and 22.3°E) [Europa Technologies Image (2008), Terra Metrica, Tele Atlas, (http://en.wikipedia.org)].

Cernjavski (Serbian academy, 1972). The second investigated plant St. John's wort (*C. umbellatum*) at the foothill of the Leskovac Hisar-hill (Serbia, Balkan), on an uncared-for, uncultivated lot, converted into grass-land with wild plants, is not registered in the major regional herbarium. The Balkan map with the study location and sampling points are shown in Figure 3.

Isolation of imanin

Carefully sampled plants from the corresponding locality were left to dry naturally on air for 20 days, in a drafty place away from the direct sun (at temperature 20 ± 2°C). The part of the plant (leaves and flowers) were cut up into small pieces and spread over a clean fabric and dried for another 10 days in a warm room with good exchange of air. The samples of St. John's wort (10 g air-dried plant material, with moisture content of 4.6%) were mechanically ground with a laboratory mill to obtain a homogeneous drug powder. Imanin isolation was carried out inside 10 min by boiling with 0.5% solution of NaOH and 1:10 g/g solution to drug ratio. After cooling, 10% HCl was added to the solution (a weak acid reaction), whereby a precipitate is formed. The water layer was carefully decanted, and the precipitate was separated by centrifuging. The solution can also be filtered, since the imanin does not apparently decrease. The precipitate was flushed by water to eliminate the traces of acid, and dried in a thin layer at the temperature of 40°C, protected from the sun. The precipitate was ground in a colloid grinder. The powdery substance obtained, dark brown in color, is imanin. The imanin yield amounts 7 to 10% of the weight of the air dried plant. Imanin is stored in airtight dark container, at the temperature of 6°C (± 0.5), to preserve its activity for a longer period.

Characterization of imanin

The quality of St. John's wort and imanin composition in the alkaline extracts were determined by the HPLC method under the following conditions: apparatus Hewlett Packard 1100 (binary pumps Agilent 1200; DAD spectrophotometric detector; Agilent HPLC software); column Lichrosorb RP-C18 (5 µm, 250 x 4 mm); eluent methanol:acetone (80:20); flow rate 1.0 cm^3/min; sample volume 20 µl; detection 590 nm; at room temperature. The identification of hyperforine and hypericine were determined using standard substances. The quality of the product (dry extracts of St. John's wort) was determined by FTIR method. The FTIR spectra as an average of 40 scans were recorded at room (298K) temperature on a BOMEM MB-100 FTIR spectrometer (Hartmann and Braun, Canada), equipped with a standard DTGS/KBr detector in the range 4000 - 400 cm^{-1} with a resolution of 2 cm^{-1}. In the region, all spectra were baseline corrected and area normalized. A fourier self-deconvolution based on the Griffiths/Pariente method was applied to enhance the resolution in a spectral region of 4000 - 400 cm^{-1}. A gamma factor of 12 corresponding to a peak width of 24 cm^{-1} was used. Deconvoluted spectra were smoothed by the 30-point Savitzky-Golay filter method. For a sample preparation the KBr pastille method was used. Fine pulverized, water-free samples (cca. 1 mg) were mixed with potassium bromide (150 mg, Merck) stored at 80°C for 6 h, and then pressed at 1000 MPa to obtain a transparent pellet. The reference measurement was performed with pure KBr. The dryness of the pastille was controlled using the Win-Bomem Easy software, by the band at cca. 1640 cm^{-1}, which is associated with the deformation vibrations of the O-H bond from water molecules.

Antimicrobial activity

Staphylococcus test

For seasonal dynamics investigation of metabolites antimicrobial activity, the *Staphylococcus* test has been chosen. The imanin antimicrobial activity against *Staphylococcus* was carried out on broth, by a dilution method, whereby the bacteria were inoculated at 200,000 microorganisms per 1 cm^3 of the medium. The investigation of the activity was carried out on liquid media, by the method of serial growth. As a test sample, an 18 h culture of Staphylococcus (*S. aureus*, breed 209) was used. Various concentrations of imanin tested were applied onto the medium with peptonic broth, where the test-culture was also introduced (calculated 200,000 bacteria per 1 cm^3 of the medium). The accessory was thermostatted 18 h at 37°C. The antibacterial titer of the preparation was considered its dilution, whereby the broth remained completely clear. Every experiment was repeated three times.

Disc diffusion method

For testing the antimicrobial activity of imanin against *S. aureus*, *Streptococcus agalactiae*, *Bacillus diphteriae*, *Bacillus tetani*,

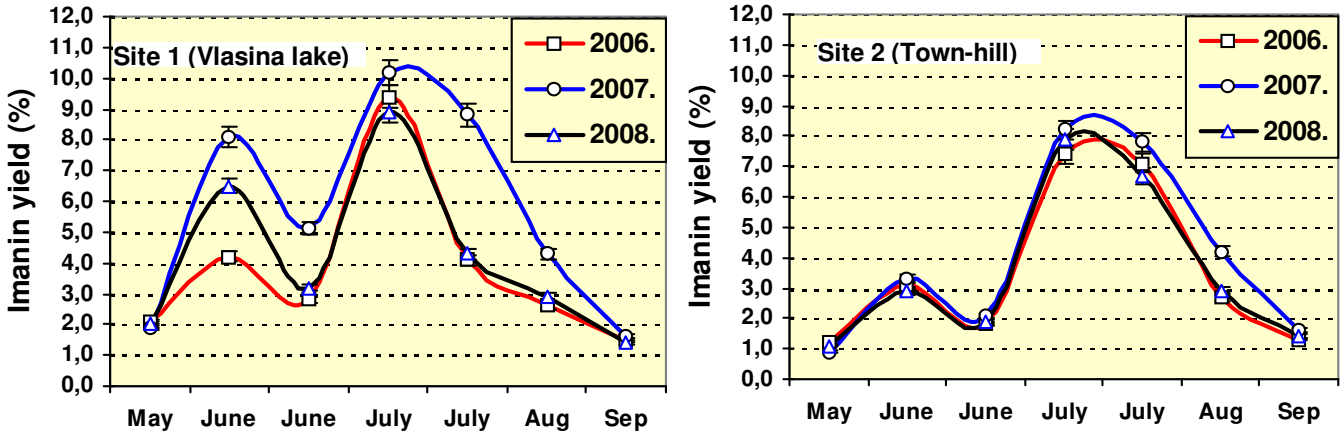

Figure 4. The imanin yield amounts (% of the air-dried plant weight) dependent of sampling periods and location: Site 1 (Vlasina lake) and Site 2 (town-hill area).

Clostridium histolyticus, *Bacillus mesentericus*, *Bacillus mycoides* a diffusion method with a paper disc (diameter: 6 mm, Biolife Italiana SRL - Viale, Italy) was used. Nutritive media Antibiotica-Agar No. 1 (Merck, Darmstadt, Germany) was used for the diffusion method. 10 µl of the selected samples of different concentrations were applied onto the paper discs by micropipette. After soaking, the paper discs were put into Petri dishes on nutrient agar medium inoculated with selected test-microorganisms. Petri dishes were placed in thermostat and incubated for 18 h at 37°C. The diameter of the inhibition zone, as the measure of an antimicrobial activity, was measured.

Metabolites accumulation

To determine the quantitative effects of environmental conditions on St. John's wort metabolites accumulation the literature model was applied (Odabas et al., 2009). The prediction of the contents of metabolites was conducted with the independent variables: temperature and light intensity. The relationships between the temperatures and light intensity and phytochemical (PC) accumulations were formulized by equations (Odabas et al., 2009) for: hyperforin content (Equation 1), for hypericin content (Equation 2) and for pseudohypericin content (Equation 3), where PC – phyto-chemicals, T - temperature (°C) and L - light intensity (µmolm^{-2}s^{-1}):

$$PC_{hyperforin} = (88.85) + (-\ 2.27 \times T) + (-0.06 \times L) + (7.86E\text{-}6 \times L^2) + [1.3E\text{-}3 \times (T \times L)] \quad (1)$$

$$PC_{hypericin} = (-\ 0.63) + (0.066 \times T) + (1.14E\text{-}3 \times L) + (-\ 2.4E\text{-}7 \times L^2) + [-\ 2.2E\text{-}5 \times (T \times L)] \quad (2)$$

$$PC_{pseudohypericin} = (-\ 0.65) + (0.076 \times T) + (1.18E\text{-}3 \times L) + (-\ 2.3E\text{-}7 \times L^2) + [-\ 2.12E\text{-}5 \times (T \times L)] \quad (3)$$

Statistical analysis

The results were statistically analyzed using Microcal^{TM}Origin software version 9.1. Statistical significance result was tested by independent *t*-test and one-way analysis of variance (ANOVA), at the 0.05 level. The multiple regression analysis was carried out until the least sum of the square was obtained.

RESULTS

Plant sampling and dynamics of imanin formation

For the investigation of the dynamics of the biological cycle and the analysis of antimicrobial properties of the St. John's wort from the Balkan region, the wild plant material was gathered during the years 2006, 2007, and 2008. St. John's wort was sampled in various vegetation periods: spring sprouts (mid May), budding (beginning of June), the first flowers (mid June), a full flowering season (end of June and beginning of July), fructification beginning (end of July), pod ripening (August), and haulm withering (September). In order to enable the comparison according to the habitat, the sampling was carried out on two localities (Figure 3): south upland - site 1 (meadow near the Vlasina lake) and west suburban area - site 2 (at the foothill of Leskovac town, on an uncared-for, uncultivated lot, converted into grass-land with wild plants). St. John's wort grows on these terrains every year. From each lot, 5 bushes were chosen, cut 10 to 20 cm below the plant top (depending on the plant height), left to dry on air, protected from the rain fall and direct sunlight. Under laboratory conditions, these samples were used to produce imanin according to the procedure described in the experimental part, and after that tested for the accumulation and antimicrobial activity. The dynamics of imanin production are shown in Figure 4. The imanin yield amount is ranged from 8 to 10% of the weight of the air-dried plant, dependent of location and sampling periods.

Extract testing

The identification of standardized imanin, isolated from St. John's wort extracts, with active and non-active solid state substances in various quantitative ratios, was carried out by use of standard physicochemical methods,

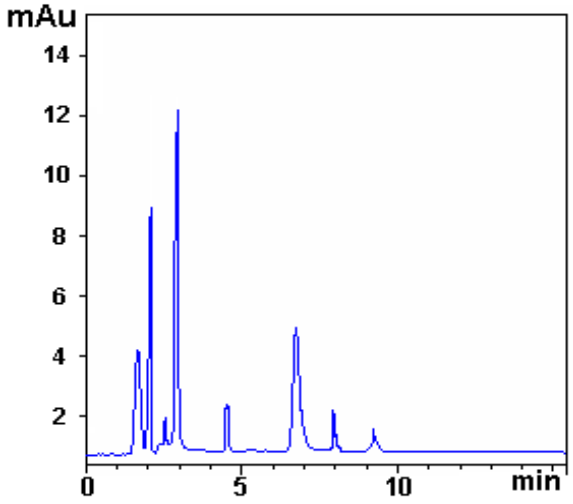

Figure 5. HPLC chromatogram of St. John's wort dry extract - imanin (identified as hyperoside Rt 1.8 min, isoquercitrin, pseudohypericin, hyperforin Rt 2.9 min, quercitrin, hypericin Rt 6.8 min, quercetin and catehin).

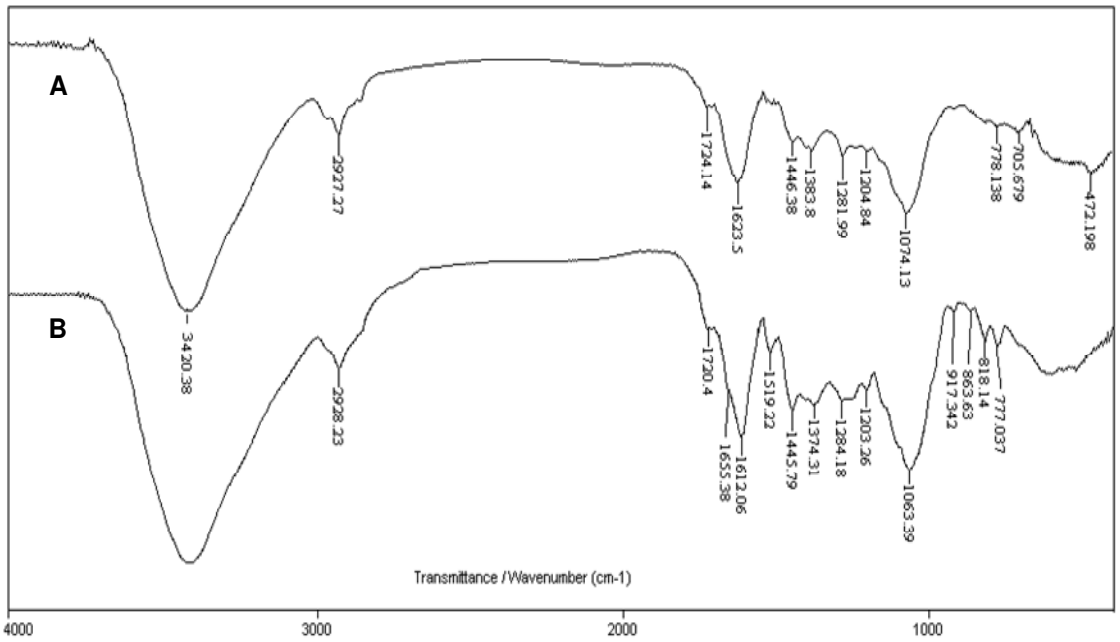

Figure 6. FTIR spectra of St. John's wort dry extract - imanin from *Hypericum perforatum* (A) and *Centaurium umbellatum* (B).

FT-IR spectroscopy and HPLC chromatography. The matter has no characteristic melting point. It is poorly soluble in neutral water, somewhat better soluble in alcohol, ether, acetone and glycerol. It is completely soluble in 0.1 M aqueous solution of NaOH (pH 9.0) when heated. A corresponding HPLC chromatograph of the isolated imanin is shown in Figure 5. The chromatographic separation was performed on a C18 column. The identification of the major known constituents present in St. John's wort extracts (hyperforin and hypericin) was determined using the standard substances.

The imanin fraction was generally analyzed by FTIR spectroscopy to determine the major known constituents present in St. John's wort extract. FTIR spectra of the isolated imanin are shown in Figure 6. The extract

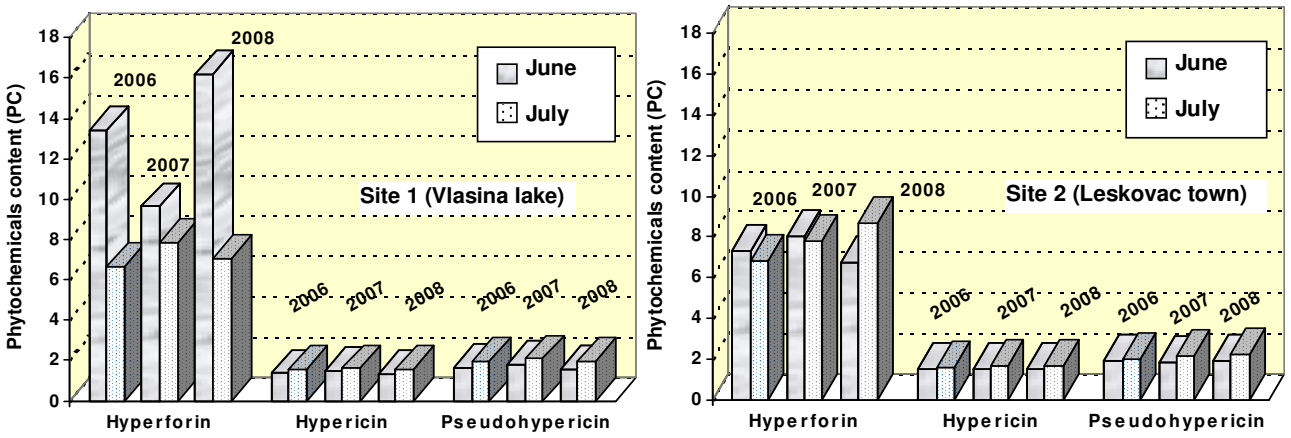

Figure 7. Estimated contents and quantitative effects of some environmental conditions (average monthly temperature and light intensity) on metabolites accumulations (PC mg/10 g plant) in St. John's wort of the sampling periods from two localities (Sites 1 and 2).

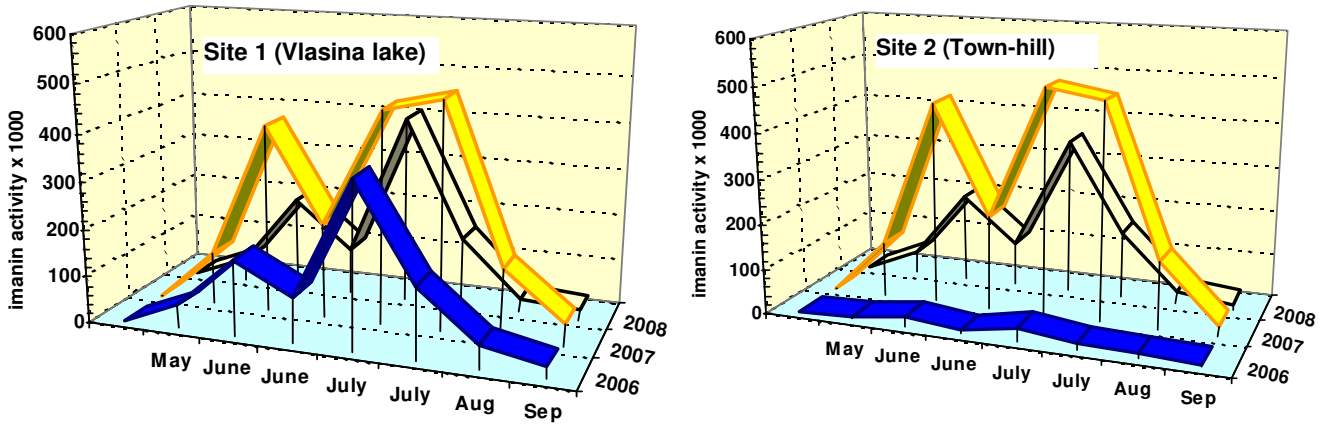

Figure 8. Diagram of mean activity in relation to the vegetation phases of imanin obtained from St. John's wort, harvested in the years 2006 - 2008 on mountainous Site 1 and suburban Site 2.

contains a group of bioactive compounds with noteworthy pharmacological activities. The FTIR spectroscopy analysis showed that the extract contains several investigated constituents obtained and identified by the HPLC method. The presence and identification of major imanin constituents (hyperforin and hypericin) was confirmed by FTIR data (KBr, cm^{-1}): 3420 (OH), 3350 (OH), 3050 (Ar CH), 2930 (CH), 1725 (C=O), 1655 (C=C), 1620, 1612, 1520, 1070 (C-O).

Climate effects on metabolites accumulations

St. John's wort plants in growth under different environmental conditions of the sampling periods (2006 - 2008 years), from two localities (Sites 1 and 2, Figure 3) were examined in the present study in order to determine the effect of different temperatures on the contents of some bioactive compounds (hypeforin, hypericin and pseudohypericin). The relationship between the content of some phytochemicals (hyperforin, hypericin and pseudohypericin) in the plant material (calculated by Equations 1 - 3), and environmental conditions (average monthly temperature and light intensity, Table 1) during the sampling periods of 2006 - 2008 years from two localities is shown in Figure 7.

Studied antimicrobial activity

The antibiotic properties of the St. John's wort plants from two different localities were investigated in the present work by *Staphylococcus* test. For a more detailed analysis of the total St. John's wort activity as being dependent on the harvesting season and locality, the obtained results are presented by diagrams given in Figure 8. It can be seen that in all cases the antimicrobial activity on imanin reaches two maximum values during

the harvesting season. The first maximum refers to the budding period, when the plant is preparing to bloom. The first, milder decrease of the activity is a characteristic for the period of the initial flowering, while the second, sharp drop of the activity appears in the fructification and pod ripening phases. This drop of the activity can be explained by higher accumulation of hyperforin and similar compounds in the fruit (seed) in the fructification phase, and not in the flowers. Similarly, the maximum activity value is reached in the period of mass flowering when hyperforin is accumulated in the flowers.

Apart from testing the antimicrobial activity of St. John's wort metabolites against the Staphylococci culture (*S. aureus* strain 209), an additional investigation was carried out for testing the sensitivity of some gram-positive microorganisms to the isolated imanin. The imanin extracts were tested for antimicrobial activity against *S. aureus*, *S. agalactiae*, *Bacillus diphteriae*, *B. tetani*, *C. histolyticus*, *B. mesentericus* and *Bacillus mycoides* by disc diffusion method. The results from the antimicrobial disc diffusion assay were shown in Figure 9. The antimicrobial activity was assessed qualitatively and quantitatively by the presence or absence of inhibition zones. The imanin extract of St. John's wort showed great antimicrobial activities against all bacteria tested.

The data indicated that gram-positive *Staphylococcus* was the most sensitive strain tested to imanin, with the greatest inhibition zone of 25 mm. The obtained testing values show that Staphylococci and Streptococci are almost equally susceptible to imanin. A considerable susceptibility to imanin was also manifested by the *Bacillus diphteriae*. The microorganism was also found to be more sensitive, with the inhibition zone of 21 mm. The *Bacillus mycoides* and *C. histolyticus* were also found to be sensitive with the inhibition zones of 18 and 16 mm, respectively. The imanin extract also showed a significant activity against tested *B. mesentericus*, with the inhibition zone of 12 mm. The *B. tetani* was least sensitive to imanin, with the inhibition zone of 9 mm. The corresponding results of the susceptibility of some microorganisms to imanin, obtained by *Staphylococcus* test are given in Table 6.

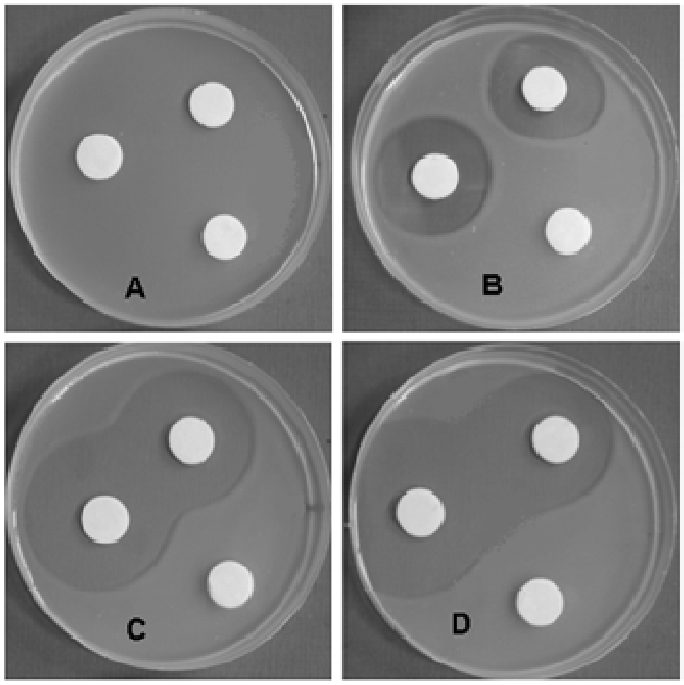

Figure 9. Antimicrobial activity of aqueous-alkaline solution (A) and imanin extract against some gram-positive microorganisms: *Clostridium histolyticus* (B), *Bacillus diphteriae* (C), and *Staphylococcus aureus* (D).

DISCUSSION

The main function of plant secondary metabolites is thought to be the adaptation of plants to their environment (Kliebenstein, 2004). By interacting with the ecosystems, these natural compounds largely contribute to plant fitness, example, protection of plants from pathogens by phytoalexins and/or phytoanticipins (Çirak et al., 2005) or preventing the serious leaf damage from the light by several UV absorbing compounds (Bourgaud et al.,2001). Thus, environmental factors such as light, temperature, CO_2 availability, soil conditions etc. have a prominent effect on the secondary metabolism resulting in the extreme variability in the phytochemical contents of wild/cultivated plants and the products derived from them (Kirakosyan et al., 2003). Hence, a description of the effect of different environmental factors on the secondary metabolism seems to be the first step in optimizing the production methodology of consistent medicinal plant materials in terms of a chemical profile.

Climate conditions and location of St. John's wort

Vlasina Lake (Site 1, Figure 3) is surrounded by mountains exceeding 1800 m above sea level.

The climate is sub-mountain with short, fresh and dry summers and cold winters. Favorable climatic conditions (cold and snowy winters, temperately warm summers) and natural resources are the basic characteristics of the first site. Extremely attractive characteristics of the region are: rich water resources of high water quality, clean air, unpolluted soil, the area abounding in flora and fauna with plants and animal kinds of the rarest and endemic character. The plateau is surrounded by three mountains. It is overgrown with dense vegetation including birch trees. Most of the time, it is anchored along the shores. Flora and fauna of the lake are rich and include several endemic species. The surroundings of the lake are the mixture of meadows and high-altitude forests, especially birch, beech, pine and juniper (the former two indigenous,

and the latter chiefly introduced by afforestation of the western shore). The indigenous tree of downy birch and yellow beech (characteristic for its ever-yellow leaf color) stand out among the species of the trees. Sundew is the only carnivorous plant in Serbia and is unique for the Vlasina region. By the decision of the Government of Serbia in 2006, the Vlasina region is protected as the first category nature of special interest. The total protected area is 12741 ha out of which 9.6 are on the 1st level of protection, 4354 on the 2nd level and 8377 on the 3rd level of protection.

The town of Leskovac (Site 2, Figure 3) is surrounded by five mountains. Four rivers flow through the Leskovac depression. Leskovac and its surroundings have a temperate continental climate, with extremely warm summers and moderately cold winters and two transitional periods - spring and autumn. During the summer period, Leskovac has 44 sunny days on average, and 29 days of snowfall in winter. July is the hottest month, and January is the coldest. The number of windy days in Leskovac is small, and the prevailing wind is a weak north wind. The variety of climates in the wider Leskovac area is conditioned by its geographic position, topography, altitude, vegetation and other factors, but three climate districts could be distinguished: valley-hill, transitive and mountain districts. This variety is further intensified by the air current from the region south of the mountain Kukavica and north-east from the mountain Selicevica. The valley region includes the valleys of the three rivers and their tributaries. The temperate continental climate reaches this region, but is modified by the influence of the surrounding mountains. The transitive district includes the terrains at the altitudes of between 500 and 1200 m. It is characterized by long, cold winters with heavy snowfalls. The summers are coldish and short with chilly nights and hot days, with more or less frequent precipitations. The total yearly precipitation amounts about 1080 mm.

In the present work, the dynamics investigation of the St. John's wort antimicrobial properties was carried out on annual, biennial and triennial wild-growing plants from the suburban localities, and on the indigenous perennial plants from the mountainous localities. Carefully sampled plants from the corresponding locality were left to dry naturally on air for 20 days, in a drafty place away from direct sun (at the temperature of 20 ± 2°C). The part of the plant (leaves and flowers) were cut up into small pieces and spread over a clean fabric and dried for another 10 days in a warm room with good exchange of air. For the storage of dried plants glass jars or cartons were used.

Climate effects on metabolites accumulations

To determine the effect of different temperatures and light intensities on secondary metabolites contents St. John's wort plants in growth under different environmental conditions of the sampling periods (2006 - 2008 years), from two localities (Sites 1 and 2), were examined in the present work. Exchanging temperatures from 25 to 40°C, and light intensities from 923 to 1780 $\mu molm^{-2}s^{-1}$ (Table 1) resulted in a modification in some metabolites as hyperforin, hypericin and pseudohypericin contents. Using Equations 1 - 3 and the values for the average temperature and light intensity (Table 1), throughout St. John's wort growth period, the content of each compound were estimated (Figure 7). The obtained results revealed that most of the variations in the phytochemical levels could be explained by temperature and light intensity and changes in the parameters affecting significantly the contents of hyperforin, hypericin and pseudohypericin in the plant material.

According to the results (Figure 7), hyperforin contents during June were generally 20 - 40% lower in the town area (Site 2) than in the lake area (Site 1). Pseudohypericin and hypericin contents in the same period were similar in both locations. Also, hyperforin contents in the town area (Site 2) were similar in both months. The fact is that hyperforin contents on the Vlasina lake location were lower at the environment temperatures of 31 - 35°C (in July) than at 24 - 27°C (in June, Site 1, Figure 7). Pseudohypericin and hypericin contents on the same location (Site 1) were slightly higher in July. It can be assumed that the changes in secondary metabolite levels may be due to the biochemical pathway of a given metabolite that could be stimulated by stress factors like high temperatures. This assumption is consistent with the allegations of the literature by other authors (Zobayed et al., 2005). Similarly, the total phytochemicals yield per plant was the highest in plants grown under 25°C, then followed temperature above 30°C. In the present study it can be concluded that the best temperature for highest hyperforin content per plant is 25 - 30°C.

Recently, several studies reported that change in the light intensity can significantly alter the secondary metabolite concentrations in *H. perforatum* (Briskin and Gawienowski, 2001). In the present study and based on experimental results (Table 1 and Figure 7), we observed that increases in light intensities resulted in the increase on the contents of phytochemicals. The presented findings have indicated that temperature and light are the major environmental factors affecting plant physiology, especially the photosynthesis and development. The physiological changes in plants, in response to different stress factors, may stimulate the secondary metabolite production for the restoration of the defensive systems. The changes in secondary metabolite contents of the plants observed in the present study, under higher temperatures and light intensities, may be attributed to those possible physiological changes (Odabas et al., 2009). It is also possible that available carbon in plant tissues may be used unusually for the biosynthesis of the secondary metabolites rather than the plant growth under

Table 1. Data on the environmental conditions (average monthly temperature and light intensity) of the sampling periods of 2006 - 2008 year, from two sampled localities.

Site 1			
Year	**Month**	T_{AV} (°C ± 0.5)	L_{AV} (µmolm^{-2}s^{-1} ± 2.5)
2006	June	25	923
	July	31	1515
2007	June	27	1105
	July	35	1730
2008	June	24	809
	July	32	1625
Site 2			
2006	June	31	1520
	July	37	1710
2007	June	32	1610
	July	38	1730
2008	June	31	1540
	July	40	1780

Site 1: Vlasina Lake (area of 16 sq. km, latitude 42°42′, longitude 22°20′, altitude 1213 m, *T* max 31.6°C, *T* min - 31.5°C, rainfall 800 mm/m^2, snowfall 204 cm, average annual temperature 5.7°C, sunny 60 days, rainy 96 days, snow 56 days).
Site 2: Leskovac town (area of 1025 sq. km, latitude 42°52′, longitude 21°57′, altitude 228 m, *T* max 42°C, *T* min - 30.3°C, rainfall 628 mm/m^2, snowfall 124 cm, average annual temperature 11.3°C, sunny 44 days, rainy 115 days, snow 29 days).

stressful conditions. Similarly, some authors (Briskin et al., 2001) reported that the increase of the light intensity illuminating from 106 to 402 µmolm^{-2}s^{-1} resulted in a continuous increase on the level of leaf hypericins. Each 70 to 100 µmolm^{-2}s^{-1} increase in light intensity yielded about a 1.2 - 1.5-fold increase in the leaf total hypericin level. It was concluded that the high photosynthetic activity under high light intensity resulted on the increased amount of carbon assimilation and enhanced the hypericin concentration in leaf tissues. Thus, Mosaleeyanon et al. (2005) reported that cultivating *H. perforatum* plants under 100, 300 and 600 µmolm^{-2}s^{-1} photosynthetic photon flux in combination with different CO_2 levels resulted in a significant increase on plant biomass and accumulation of hypericin, pseudohypericin and hyperforin. Authors attributed the increases in biomass and secondary metabolite production to the increase of net photosynthetic rate. The above findings were also confirmed by an open field experiment in which shading lowered the content of hypericin (Hevia et al., 2002).

Studied antimicrobial activity

It is well known from literature that hyperforin, the main ingredient of imanin, shows antimicrobial properties upon more than 40 varieties of microorganisms, typically upon *S. aureus*, *Bacillus cereus*, *Bacillus subtilis* and *Nocardia gardene* (Jayasuriya et al., 1991; Rocha et al., 1995; Trifunovic et al., 1998; Schempp et al., 1999). Isolated from St. John's wort, imanin is also very effective against gram-positive bacteria (MIC 0.1-1.0 µg/ml) (Reichling et al., 2001). It is very successful in inhibiting the growth of *Staphylococci* when diluted to 1:250,000 (Bakyrel et al., 2001). The antibacterial active substances of imanin isolated from St. John's wort flowers only have the activity of up to 1:1,000,000. The antimicrobial activity has been confirmed repeatedly upon multi-resistant bacterium *S. aureus* with MIC values of 1.0 µg/ml. Therefore, the *Staphylococcus* test (*S. aureus* breed 209) has been chosen to monitor the antimicrobial activity of St. John's wort secondary metabolites.

The antibiotic properties of the plant from two different localities were investigated in the present work. One of the investigated plants was sampled up in the first crop (in the course of the year 2006.), when St. John's wort first appeared on an uncared-for lot on site 2 (Figure 3) as a wild growing plant. On this site the male centaurium (*C. umbellatum*) typically prevailed. Taking into consideration the literature data (Kojic et al., 1998), blooming of the first crop was expected to be scarce and short-lived. As a result of this study, the extracts of *C.*

Table 2. Antimicrobial activity of imanin from St. John's wort from two localities against *Staphylococcus aureus* (year 2006).

Vegetation phase	Harvesting date	Imanin activity			
		Site 1	Site 2	SD	SE
Spring sprouts	May 14th	1 : 50,000	1 : 5,000	329.15	35.50
Budding	June 7th	1 : 160,000	1 : 20,000	458.25	264.57
First flowers	June 13th	1 : 100,000	1 : 8,000	360.56	208.16
Full flowering	July 1st	1 : 350,000	1 : 30,000	826.13	476.96
Fructification beginning	July 14th	1 : 120,000	1 : 15,000	389.42	224.82
Pod ripening	Aug 25th	1 : 50,000	1 : 10,000	251.66	145.29
Haulm withering	Sept 14th	1 : 25,000	1 : 5,000	100.11	57.73

N = 7; Mean = 11,625; Variance = 9.3982E7; Two population t-Test: t = 3.3903; p = 0.0044; One-Way ANOVA: F = 1.1274E-4; p = 0.9998 at the 0.05 level.

Table 3. Antimicrobial activity of imanin from St. John's wort from two localities against *Staphylococcus aureus* (year 2007).

Vegetation phase	Harvesting date	Imanin activity			
		Site 1	Site 2	SD	SE
Spring sprouts	May 16th	1 : 110,000	1 : 120,000	870.07	502.34
Budding	June 8th	1 : 400,000	1 : 450,000	1750.23	1010.50
First flowers	June 15th	1 : 200,000	1 : 210,000	1000.01	577.35
Full flowering	July 1st	1 : 450,000	1 : 500,000	2551.47	1473.09
Fructification beginning	July 17th	1 : 480,000	1 : 480,000	1497.77	864.74
Pod ripening	Aug 24th	1 : 150,000	1 : 150,000	1075.09	620.71
Haulm withering	Sept 15th	1 : 50,000	1 : 25,000	611.01	352.76

N = 7; Mean = 24,875; Variance = 4.2356E10; Two population t-Test: t = 3.3240; p = 0.0051; One-Way ANOVA: F = 1.0948E-4; p = 0.9918 at the 0.05 level.

umbellatum were found to be antimicrobially effective against gram-positive bacteria, with a special activity towards methicillin-restistant strains of *S. aureus* (MIC values: 1.2 to 2.6 mg herb/ml). Hyperforin exhibited an excellent effect against methicillin-resistant strains of *S. aureus* with a MIC value of 1.0 µg/ml. The antimicrobial activity results of the first crop of the plant are given in Table 2. Imanin obtained from the sample gathered from the first year crop (2006.) practically showed a very low activity. Its maximum antibacterial effect was manifested when diluted 1:30,000. It is well known that the activity of imanin against *S. aureus* is 1:25,000 to 1:500,000.

On site 1 (Figure 3), St. John's wort grew as an indigenous plant in earlier years. On this site the female centaurium (*Hypericum perforatum*) typically dominated. During the first harvest (in the year 2006) the blooming was abundant but the changeable meteorological conditions did not allow a longer duration of this vegetative phase. However, the antimicrobial activity results for the obtained imanin were slightly better (Table 2). In the second year of harvesting the plant material (year 2007.), St. John's wort bloomed abundantly and produced seed on both investigation sites. This was favored by temperately warm climate and temperately sunny habitats. The flowering periods of the biennial St. John's wort were found to be longer, and it was possible to make two samplings in this vegetation phase. Under favorable weather conditions the flowering is known to last as long as two months, and from the moment of pods formation to their mass ripening it takes about 6 weeks. The data on the antimicrobial activity of the second St. John's wort crop, according to vegetation phases are given in Table 3.

When grown in a nursery, St. John's wort, as a perennial herb, can sometimes live up to 10 years. However, most often, it begins to wither after three years of growth. For comparison purposes, in this work, the third harvest of St. John's wort (in 2008.) was also investtigated. The results of the antimicrobial activity investigations per vegetation phases are presented in Table 4. In view of less grown vegetation of the three-year-old plant the imanin activity results were expected to be lower than those of the previous year on Site 2. Still, the antimicrobial activity of the three-years-old St. John's wort had the same character and dynamics as those of the two-year-old plant. Although the values were slightly lower, the activity variations had passed through the same vegetation phases as during the firs t two years.

Table 4. Antimicrobial activity of imanin from St. John's wort from two localities against *Staphylococcus aureus* (year 2008).

Vegetation phase	Harvesting date	Imanin activity			
		Site 1	Site 2	SD	SE
Spring sprouts	May 15th	1 : 50,000	1 : 50,000	638.74	285.65
Budding	June 10th	1 : 200,000	1 : 200,000	1204.16	538.51
First flowers	June 16th	1 : 100,000	1 : 100,000	673.05	300.99
Full flowering	July 1st	1 : 400,000	1 : 350,000	1316.43	588.72
Fructification beginning	July 13th	1 : 150,000	1 : 150,000	952.36	425.91
Pod ripening	Aug 23rd	1 : 30,000	1 : 30,000	460.43	205.91
Haulm withering	Sept 17th	1 : 20,000	1 : 15,000	476.44	213.07

N = 7; Mean = 111,875; Variance = 1.4071E10; Two population t-Test: t = 2.6674; p = 0.0183; One-Way ANOVA: F = 1.4238; p = 0.2355 at the 0.05 level.

Table 5. Comparative study of antimicrobial activity of the first and second mowing of St. John's wort against *Staphylococcus aureus*.

Mowing	Mowing date	Vegetation phase	Imanin activity
First	June 10th	budding	1 : 200,000
Second	August 16th	full flowering	1 : 450,000

The activity of St. John's wort from Site 1 showed very similar values (Table 4).

One should bear in mind that all these results were influenced by the area of St. John's wort's growth, meteorological conditions, temperatures that were different each year, and a geographic location of the habitat of the plant. Typically, the biennial St. John's wort from the habitat on site 2 (in the year 2007.) showed the highest antimicrobial activity in all vegetation phases, most probably due to favorable meteorological conditions, advantageous humidity in the spring period and extremely hot climate during the summer. However, it is interesting that the habitat on Site 1, in spite of long-standing wild growth, has conserved the tendency of two-phase increase of antimicrobial substances content both in budding and full flowering phases.

Having in mind the two obvious phases of accumulation of active substances during one vegetation season (clearly visible maximums in Figures 4 and 8), further investigations were directed towards the examining of the individual activity of the first and second mowing in the same year. The second harvest of St. John's wort was obtained after the interruption of the third vegetation phase of the first crop (beginning of the flowering), by mowing the plant and the repeated growth on the same locality. To that end, on one part of the lot on site 2, in the third year of harvesting St. John's wort (in the year 2008.) and after taking samples in the first three vegetation phases (spring sprout, budding and beginning of flowering), the whole crop of St. John's wort was mown. In six weeks' time a new generation of St. John's wort grew in the place of the mown plant, and flowered again. In this case, the aim of the investigations was the possibility of multiple cultivations and harvesting of St. John's wort during a single season, and the maximum utilization of pharmaceutically active substances.

By the end of the flowering phase and the beginning of fructification of aftermath, the plants were mown and subjected to antimicrobial activity testing. The data obtained are given in Table 5. Other vegetation phases were not tested from this aspect because, in accordance with the results obtained (Tables 2 - 4), a low yield of active substances was expected and even a negative effect of collecting antibiotic substances in these vegetation phases. Table 5 shows that St. John's wort from the second mowing has a significantly greater antimicrobial activity. This leads to the conclusion that in this period, from the first to the second mowing, the active recollection of antimicrobial substances by the plant was observed. The total antimicrobial activity from the two mowings (Table 5) was higher than the total of the corresponding activities per analyzed vegetation phases from the single mowing (Table 4). This result indicates a possibility of better utilization of the plant material from the economic point of view, confirming the expectations and justifying the aim of this investigation. The tested plants had preserved a high activity after the second mowing in the course of several months. Based on these results we can come to the conclusion that when growing St. John's wort, in order to achieve maximal utilization of this plant as an industrial culture, the first mowing should be carried out in the period of budding, and the second in the period of full flowering. In that way, the maximum values of antimicrobial substance in St. John's wort can be obtained.

The interesting fact is that in the third year (2008), on

Table 6. Susceptibility of some microorganisms to imanin. Comparison between the literature (Trifunovic et al., 1998; Chandrasekera et al., 2005) and experimental values of imanin bacteriostatic activity.

Microorganism	Reference literature values of imanin bacteriostatic activity (dilution)	Experimental values of imanin bacteriostatic activity (dilution ± SD)
Staphylococcus aureus	1 : 25,000 - 1 : 500,000	1 : 500,000 ± 2430
Streptococcus	1 : 10,000 - 1 : 100,000	1 : 450,000 ± 1690
Bacillus diphteriae	1 : 25,000 - 1 : 250,000	1 : 200,000 ± 1220
Bacillus tetani	1 : 33,000	1 : 50,000 ± 620
Clostridium histolyticus	1 : 50,000 - 1 : 100,000	1 : 100,000 ± 940
Bacillus mesentericus	1 : 100,000	1 : 80,000 ± 730
Bacillus mycoides	1 : 100,000	1 : 150,000 ± 1190

the wild growing suburban lot in town surroundings (Site 2), some degenerative phenomena were recorded. These were manifested by the decrease of the bush height, less flowers, smaller flowers and pods in some bushes. Contrary to that, indigenous St. John's wort growing on the mountainous area near the lake (Site 1), though generally it gave a lower yield of antimicrobial substances, still preserved the dynamics of its biological cycle. This is probably due to the more favorable climate and the healthier and cleaner environment where the habitat has existed for a number of years.

Conclusion

Assaying the variation in secondary metabolites of St. John's wort for its better use as an antibiotic was performed in this study. For the investigations of the biological cycle dynamics and the analysis of antimicrobial properties the wild plant material was gathered during the years 2006 - 2008. The plant was sampled and analyzed in various vegetation periods. The effects of variation in secondary metabolites of the plant material were monitored using a complex antimicrobial preparation. As the plant secondary metabolites, imanin was isolated from the flowers and leaves of St. John's wort by the aqueous-alkaline extraction. The quality of imanin was determined by FTIR and HPLC methods. From this study, the main function of the plant secondary metabolites is thought to be the adaptation of plants to their environment. The data presented here suggest that the imanin yield amount is ranged from 8 to 10% of the weight of the air-dried plant, dependent of the location and sampling periods. Our results suggested that the temperature and light are important environmental factors to optimize the phytochemical production in St. John's wort plants grown under natural conditions. The higher levels of these factors (temperatures from 25 to 40°C, and light intensities from 923 to 1780 $\mu molm^{-2}s^{-1}$) can significantly change the hyperforin, hypericin and pseudohypericin contents. According to our results, hyperforin contents during June were generally 20 - 40% lower in the town area than in the lake area. In the present study it can be concluded that the best temperature for highest hyperforin content per plant is 25 - 30°C.

The present study has consistently demonstrated the effectiveness of St. John's wort secondary metabolites and antimicrobial activity against all bacteria tested: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus diphteriae*, *Bacillus tetani*, *Clostridium histolyticus*, *Bacillus mesentericus* and *Bacillus mycoides*. The antibiotic properties of imanin were investigated by *Staphylococcus* test. It can be concluded that the imanin antimicrobial activity reaches two maximum values during the harvesting season. The first maximum refers to the budding period, when the plant is preparing to bloom. The second maximum activity is reached in the period of mass flowering when hyperforin is accumulated in the flowers.The decrease of the activities is a characteristic for the period of the initial flowering and fructification. This drop of the activity can be explained by higher accumulation of hyperforin and similar compounds in the seed. The fact that the use of plants as medicine is dependent of the accumulation of secondary metabolites opens a new horizon, which in turn varies according to the season. Harvesting the plant at a specific season will give higher antimicrobial activity and thus will be more beneficial as an antibiotic. The relationships between environmental conditions, the accumulation of phytochemicals and antimicrobial properties of St. John's wort in the present study scientifically validates the use of the plant secondary metabolites in traditional medicine.

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