ANTIMICROBIAL ACTIVITY OF Hypericum annulatum MORIS AND Hypericum elegans STEPHAN EX WILLD. ESSENTIAL OILS FROM SERBIA

The in vitro antimicrobial activity of Hypericum annulatum and Hypericum elegans essential oils was evaluated against a panel of standardized bacteria and fungi using broth microdilution assay. Both essential oils showed antimicrobial activity against all the tested microorganisms. Hypericum annulatum essential oil showed better antibacterial than antifungal activity, being more effective against Pseudomonas aeruginosa and Escherichia coli while H. elegans essential oil showed no significant difference between antibacterial and antifungal activity. Antimicrobial testing of α-pinene, β-pinene and β-myrcene compounds was also performed. All the compounds were active against all the tested microorganisms, however, based on the MIC, MBC and MFC values, none of these compounds could be thought of as the main bearer of the oils’ antimicrobial activity. This is the first report regarding the antimicrobial activity of the essential oils of the two Hypericum species.

Keywords: Hypericum annulatum, Hypericum elegans, essential oil, antimicrobial activity.

Hypericum L. (Hypericaceae) is a genus represented by more than 400 species, widespread in warm-temperate areas throughout the world, as well as on the Balkan Peninsula [1]. Plants of the genus Hypericum have traditionally been used as highly esteemed medicinal plants [2]. Among these, H. perforatum is one of the best chemically investigated plant species. The volatile profiles of the other related Hypericum species, including those originating from southeastern Serbia, have been investigated [3-9].

The extracts, as well as the essential oil of Hypericum species have been shown to possess significant antiviral, wound healing, antioxidant and antimicrobial activities [4,7,10-15]. Some of these actions were attributed to the presence of phloroglucinols (antibacterial, wound healing, antimalarial and antidepressant activity), naphthodianthrones (antiviral and antidepressant activity), tannins (antiviral, antimicrobial and antioxidant activity), flavonoids (antifungal, antioxidant and antidepressant activity) and volatile oils (antimicrobial activity) [16-19]. Hypericum annulatum Moris (syn.: Hypericum atomarium Boiss. subsp. degenii (Bormm.) Hayek and Hypericum degenii Bornm.) is no exception in this respect. This species’ natural habitats are slightly inclined, shady and hilly slopes shielded from strong wind. In Serbia this plant grows mostly on siliceous geological substratum, but it also inhabits limestone habitats. Hypericum annulatum subsp. annulatum is used in folk medicine for the treatment of gastric and liver disorders [20]. Recent investigations on several benzophenone glycosides and xanthones isolated from aerial parts of H. annulatum indicate their potential chemoprotective/radioprotective properties as well as their protective effect against some toxic agents [21,22]. A new isocoumarin from H. annulatum, annulatomin, exhibited a modest growth-inhibitory activity in vitro against human chronic myeloid leukemia [23]. A prenylated phloroglucinol, hyperatomarin, also a secondary metabolite of H. annulatum, has been shown to possess significant antibiotic activity against Gram-positive bacteria [24]. The very scarce literature
data on *H. elegans* includes mostly its non-volatile chemistry [17,25,26], with no reports on its biological activities. This rare species, in Serbia inhabits xerothermic habitats, dry meadows, pastures, rocky terrains, sandy soils and rocks [27].

Therefore, the aim of this study was to investigate the antimicrobial potential of *H. annulatum* and *H. elegans* essential oils, as well as some of their main constituents.

**EXPERIMENTAL**

**Plant material**

Aerial parts of *H. annulatum* in the flowering and beginning of fructiferous phase were collected in the valley of the river Pčinja (near Trgovište, southeastern Serbia) in July 2008. Voucher specimens were deposited in the Herbarium of Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade (BEOU), under the acquisition number 16269. Aerial parts of *H. elegans* in the flowering phase were collected in the region of the Lalinačka Slatina Salt Marsh (Svrljig, near Niš, southeastern Serbia) in July 2008. Voucher specimens were deposited in the Herbarium of the Faculty of Science and Mathematics, University of Niš, under the acquisition number 200812.

**Essential oil isolation**

Fresh aerial parts, of *H. annulatum* (400 g) and *H. elegans* (300 g), were subjected to hydrodistillation for 2.5 h using an original Clevenger-type apparatus and yielded 0.05 and 0.08% (w/w) of pale yellow essential oils, respectively. The obtained oils were separated, dried over anhydrous magnesium sulfate and stored under appropriate conditions before they were tested.

**Chemicals and reagents**

α-pinene, β-pinene and β-myrcene standards were obtained from Sigma Aldrich (St. Louis, USA). Diethyl ether was purchased from Carlo Erba (Italy) while anhydrous magnesium sulfate was purchased from Merck (Darmstadt, Germany). All chemicals and reagents were of analytical grade purity.

**Broth microdilution assay**

The in vitro antimicrobial activity of essential oils and commercial standards - α-pinene, β-pinene and β-myrcene was tested against a panel of laboratory control strains belonging to the American Type Culture Collection Maryland, USA (except one, belonging to National Collection of Type Cultures, UK, see below). Antibacterial activity was evaluated against two Gram-positive (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538) and three Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NCTC 6017). The antifungal activity was tested against two organisms *Aspergillus niger* ATCC 16404 and *Candida albicans* ATCC 10231.

The minimal inhibitory concentration (MIC) of the samples against tested bacteria and fungus/yeast was determined by using a broth microdilution method according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) [28]. The inocula of the tested strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Dimethyl sulfoxide (10%, v/v aqueous solution) was used to dissolve and to dilute samples. A stock concentration of all tested samples was prepared at 100 mg/ml. A serial double dilution of the samples was prepared in 96 multi-well microtitre plates, using the method of Sarker et al. [29]. Two rows in each plate were used as controls. One row was used as a positive control and contained a broad-spectrum antibiotic (doxycycline in a serial dilution of 200-0.05 μg/ml) to determine the sensitivity of Gram-negative and Gram-positive bacteria and an antifungal (nystatin in a serial dilution of 50-0.02 μg/ml) to determine the sensitivity of fungi. The other row contained the solvent as negative control. The minimal bactericidal/fungicidal concentration (MBC/MFC) was determined by spreading the content of each well of the microtitre plate in which colour change occurred on sterile nutrient agar plates (prepared according to the manufacturer’s instructions) set in Petri dishes. These plates were incubated at 37 °C for 24 h for bacteria or at 28 °C for 48 h for fungi. The MBC/MFC was evaluated as the lowest concentration of essential oil at which 99.9% of inoculated microorganisms were killed. Tests were carried out in triplicate.

**RESULTS AND DISCUSSION**

The results obtained in the broth microdilution assay are presented in Tables 1 and 2. The antimicrobial activities of *H. annulatum* and *H. elegans* essential oils were evaluated against five bacterial strains, one mold and one yeast species. The results indicated that the MIC values against tested organisms ranged between 3.13 and 12.50 mg/ml, while the MBC and MFC values were 50.00 mg/ml and higher, for both of tested oils. The assayed samples were less effective than the antibiotic/antimycotic used as reference standard (Table 1).
Essential oil of *H. annulatum* showed antimicrobial activity against all tested strains. No significant difference in the activity was observed against Gram-positive and Gram-negative bacterial strains. However, the oil showed better antibacterial activity than antifungal activity (Table 1). The results indicated that the most susceptible microorganisms were *P. aeruginosa* and *E. coli* (MIC = 3.13 mg/ml, MBC = 50.00 mg/ml, for both bacterial strains). The oil was equally effective against *S. aureus* with MIC = 3.13 mg/ml, however, the bactericidal concentration in this case was higher than 50.00 mg/ml (Table 1). The most resistant bacterial strain was *S. abony* (MIC = 12.50 mg/ml, MBC = 50.00 mg/ml). The antifungal activity of the oil was evenly manifested against both tested strains *C. albicans* and *A. niger* (MIC = 3.13 mg/ml, MBC = 50.00 mg/ml). These results represent the first data on the antimicrobial activity of *H. annulatum* essential oil.

Hypericum *elegans* essential oil showed antimicrobial activity against all tested strains with no significant differences between antibacterial and antifungal activity. Based on the MIC values, the most susceptible strains were *E. coli* and *S. aureus* (3.13 mg/ml, for both the strains). Regarding the MBC/MFC values, the oil showed the best effect against *P. aeruginosa* (MBC = 25.00 mg/ml, Table 1). The most resistant microorganism was *B. subtilis* (MIC = 12.50 mg/ml, MBC > 50.00 mg/ml). The oil showed antifungal activity against both the tested fungal strains, and was found to be more effective against the pathogenic yeast *C. albicans* (MIC = 3.13 mg/ml, MBC = 50.00 mg/ml).

Our previous studies revealed the chemical composition of *H. annulatum* and *H. elegans* essential oils [8,9]. Some of the components, identified in the essential oils of the two species, were: α-pinene, β-pinene and β-myrcene. Therefore, their antimicrobial activity was also tested in this study. The obtained results showed moderate to strong activity against all tested microorganisms (Table 2). α-Pinene, the major component of *H. annulatum* essential oil and the second most abundant one in *H. elegans* essential oil, exhibited the highest activity against *S. abony* and *C. albicans*, whereas the most resistant was fungus *A. niger*. β-Pinene was most effective against *S. abony* and *C. albicans* strains (exhibiting strong antimicrobial activity) and the most resistant was *P. aeruginosa*. The most susceptible to β-myrcene activity were *S. aureus* and *C. albicans* strains (exhibiting strong antimicrobial activity) and the most resistant was *P. aeruginosa*. The most susceptible to β-myrcene activity were *S. aureus* and *C. albicans* strains. Neither of the components could be thought of as the main bearer of the antimicrobial activity of the essential oil of the two *Hypericum* species (regardless of their percentage in oil), hence the results suggest a synergistic and/or antagonistic activity of oil components.

### Table 1. Minimal inhibitory (MIC) and minimal bactericidal/fungicidal concentrations (MBC/MFC) of *H. annulatum* and *H. elegans* essential oils; n.t. – not tested

<table>
<thead>
<tr>
<th>Microorganism</th>
<th><em>H. elegans</em> (mg/ml)</th>
<th><em>H. annulatum</em> (mg/ml)</th>
<th>Doxycycline (µg/ml)</th>
<th>Nystatin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC/MFC</td>
<td>MIC</td>
<td>MBC/MFC</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>12.50</td>
<td>&gt;50.00</td>
<td>6.25</td>
<td>50.00</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>3.13</td>
<td>50.00</td>
<td>3.13</td>
<td>&gt;50.00</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>3.13</td>
<td>50.00</td>
<td>3.13</td>
<td>50.00</td>
</tr>
<tr>
<td><em>S. abony</em></td>
<td>12.50</td>
<td>50.00</td>
<td>12.50</td>
<td>&gt;50.00</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>6.25</td>
<td>25.00</td>
<td>3.13</td>
<td>50.00</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>3.13</td>
<td>50.00</td>
<td>25.00</td>
<td>&gt;50.00</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>6.25</td>
<td>50.00</td>
<td>25.00</td>
<td>&gt;50.00</td>
</tr>
</tbody>
</table>

### Table 2. Minimal inhibitory (MIC) and minimal bactericidal/fungicidal concentrations (MBC/MFC) of selected constituents of *H. annulatum* and *H. elegans* essential oils

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>α-Pinene (mg/ml)</th>
<th>β-Pinene (mg/ml)</th>
<th>β-Myrcene (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC/MFC</td>
<td>MIC</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>6.25</td>
<td>50.00</td>
<td>6.25</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>1.56</td>
<td>&gt;50.00</td>
<td>0.78</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>12.50</td>
<td>&gt;50.00</td>
<td>6.25</td>
</tr>
<tr>
<td><em>S. abony</em></td>
<td>1.56</td>
<td>25.00</td>
<td>0.39</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>12.50</td>
<td>&gt;50.00</td>
<td>12.50</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>1.56</td>
<td>6.25</td>
<td>0.20</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>12.50</td>
<td>&gt;50.00</td>
<td>3.13</td>
</tr>
</tbody>
</table>
CONCLUSIONS

From the earlier stated data, it can be concluded that *H. annulatum* and *H. elegans* essential oils may be used against bacterial and fungal infections in traditional and modern medicine. To the best of our knowledge, this is the first report on the antimicrobial activity of the mentioned essential oils.

Acknowledgments

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REFERENCES


AMIKROBNA AKTIVNOST ETARSKIH ULJA BILJNIH VRSTA Hypericum annulatum MORIS I Hypericum elegans STEPHAN EX WILLD. POREKLOM IZ SRBIJE

Antimikrobna aktivnost etarskih ulja biljnih vrsta Hypericum annulatum i Hypericum elegans određena je na setu standardnih bakterija i gljivica mikrodilucionom metodom, in vitro. Oba etarska ulja pokazala su antimikrobnu aktivnost na sve testirane mikroorganizme. Etarsko ulje izolovano iz H. annulatum pokazalo je bolju antibakterijsku od antifungalne aktivnosti, tako što je efikasnije delovalo na Pseudomonas aeruginosa i Escherichia coli (u odnosu na testirane gljivice), dok etarsko ulje H. elegans nije pokazalo značajnu razliku između antibakterijske i antifungalne aktivnosti. Takođe, ispitana je i antimikrobna aktivnost sledećih komponenti etarskih ulja: α-pinena, β-pinena i β-mircena. Sve komponente pokazale su antimikrobnu aktivnost, mada se na osnovu MIC, MBC i MFC vrednosti ni jedna od njih ne može smatrati glavnim nosiocem aktivnosti koju pokazuju ispitivana ulja. Ovo su prvi podaci o antimikrobnoj aktivnosti etarskih ulja izolovanih iz dve gore pomenute Hypericum vrste.

Ključne reči: Hypericum annulatum, Hypericum elegans, etarsko ulje, antimikrobna aktivnost.