FLAVONOIDS IN SOME AQUEOUS-Ethanolic EXTRACTS FROM HERBA HYPERICI AND THEIR ANTIMICROBIAL ACTIVITY

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Abstract. In recent years, herbal products containing St. John’s wort extracts are among the most consumed worldwide. This medicinal plant has a long history of medical use even in Albania. Inhabitants in the regions of Korça and Pogradec quite frequently prepare herbal products using dried flowers, tops, and roots of the plant Hypericum perforatum L. Alcoholic and oily extracts are the most commonly used. Our study aims to evaluate the influence of alcohol percentage on phytochemical components extraction, especially on flavonoids. The presence of flavonoids and hypericin was demonstrated using thin layer chromatography. The extracts antimicrobial activity was tested against Staphylococcus aureus, applying the disc diffusion technique on nutrient agar-broth. Using 50µl/disc of the extracts prepared with 70 and 80% ethanol the observed inhibition zones were similar, having a diameter of 7 mm. The inhibition zone formed with the same quantity of the oily extract was 8 mm.

Key words. Hypericum perforatum L., extracts, flavonoids, TLC, extracts antimicrobial activity.

Introduction. Hypericum perforatum L., St. John’s wort, is presently one of the most studied, as well as consumed, medicinal plants worldwide. St. John’s wort is a member of genus Hypericum, of which there are known more than 400 species. The plant has been used for centuries as a folk remedy to heal burns and wounds, as well as for the treatment of anxiety and bacterial infections. Hypericum has a complex and diverse chemical composition [1-3]. Extracts from dried flowering tops are available as pharmacy products etc. According to European Pharmacopoeia 8th edition, standardization of the dried extracts obtained from H. perforatum is based on the quantification of total hypericins, flavonoids and hyperforin. The plant has undergone extensive laboratory and clinical testing [4-6]. Hyphenated instrumental techniques have been applied for separation and structure verification, enabling characterization of H. perforatum constituents. The coupling of LC with spectroscopic techniques such as MS or NMR has been reported in numerous applications [6-8]. Recent studies outline that authorized preparations in the European market, containing purified extracts, revealed considerable differences in the phytochemical composition, depending on the production mode [9]. There is a traditional use of Hypericum extracts in Albanian folk-medicine, due to the anti-inflammatory, antimicrobial and antidepressant effect [10]. Hydroalcoholic and oily extracts of the dried flowering tops are frequently used by residents of Korça and Pogradec regions. Extracts usually find use for external application in burns, wounds, eczema; they are also orally used for the treatment of mood disorders. Our study aims to compare flavonoids presence in extracts produced by maceration, using extractive solvents containing different percentages of ethanol, as well as their antimicrobial activity.

Material and methods. Plant material. Flowering tops of H. perforatum L. were collected in the area of Lliza, in the outskirts of Tirana, in May 2013. The plant material was dried in a shady place. Standardization was performed as described by BP 2009. Loss on drying was 7%, determined on the powdered drug in an oven at 105°C, for 2 hours. The drug identity and purity were conducted using the pharmaceutical monograph for St. John’s Wort. Quantitative evaluation of the total hypericins was carried out according to the spectrophotometric method given by BP 2009, measuring the absorbance of the test solution at 590 nm and calculating the content of hypericins using the specific absorbance of hypericin [11]. Dried plant material contained 0.1% (w/w) total hypericins, expressed as hypericin. Preparation of aqueous-ethanolic extracts. In accordance with the folk-medicine procedure used, extraction was carried out using dried flowers, buds, and roots of the plant. Hypericum perforatum L. Anhydrous formic acid: water: ethyl acetate (6:9:90). The plant material mentioned above was covered having with refined sunflower oil and the dish with the mixture was exposed to sunlight. The mixture was reshuffled frequently. Extraction and fermentation continued for six weeks, and then the herbal material was pressed out. The oily extract thus obtained had a bright red color (Figure 1).

Identification of flavonoids and hypericin by thin layer chromatography. TLC is considered a powerful technique to screen plant extracts for the presence of phenolic compounds [11, 12]. The experimental conditions for the TLC are described in the following. Reference standards used, hyperoside and rutin, were Sigma-Aldrich products. Layer: silica gel F254 plate, Merck (10 x 20 cm, thickness 0.25 cm). Visualization: examination in UV light 254 nm; sprayed with the reagent boric acid 3% : oxalic acid 10% (15:5) and then heated at 120°C for 10 minutes (DAB 9).

Method used for the determination of extracts antimicrobial activity. Sample preparation: 50 µl liquid extract of each sample was applied on paper discs, and then the items were left for 1 hour into a sterile cabinet, to remove the ethanol.

Procedure. 1 ml Staphylococcus aureus bacterial suspension, at a concentration of 10 cfu/ml, was added over the Petri dishes containing 15 ml nutrient broth-agar, to get a uniform microbial growth. The absorbent paper discs impregnated with the extracts were placed on the Petri dishes surface. The plate was left to stand at 4°C for 2 hours. Incubation was performed at 35°C for 24 hours. Paper discs with only 50 µl solvent were used as a negative control, whereas the paper discs containing 10 µg gentamicin base as a positive control. The diameters of inhibiting zones were measured with a precision of 0.1 mm and the mean values reported.

Result and Discussion. Figure 2 shows the chromatograms obtained using sample solutions M5, M3 and M2, examined in ultraviolet light at 254 nm. Close to the start line was a yellow band due to flavonoid rutine. Comparing the color intensity and the sizes of separated zones (eight well distinguished bands of yellow color), the presence of flavonoids was similar on chromatograms M1 and M2 with hypericin (RF 0.39) as the main component. On the chromatogram M3 (corresponding to the sample prepared with ethanol 50%) four slightly colored zones were visible and only traces of hyperoside were present in it. In the lower part of the upper third zone were present two grey colored constituents that, based on the schematic chromatogram given by BP 2009 (the same mobile phase), correspond to pseudohypericin (naphthodianthrone) and hypericin (RF 0.84). Although in the same chromatographic conditions, on the chromatogram M3, there were no yellow zones visible in the range from the start line to the elongated zone of light brown color. Figure 3. According to EMA considerations on Hypericum Oil obtained by sunlight maceration, breakthrough products of hypericin and other lipophilic components such as phloroglucinol derivatives are present in it. It seems, that in the experimental conditions, they cannot be separated and thus migrate together. Pertaining to some other papers on the topic, it is not transparent whether the authors determined hypericin or the artifacts of it, by using only spectrophotometric method [9]. The results of extracts antimicrobial activity against Staphylococcus aureus are given in Figure 4. Gentamicin was used as a positive control. All the inhibition zones having a diameter of 15 mm. Inhibition zones obtained by the action of extracts M5 and M4 were relatively narrow, with similar sizes of 7 mm. A higher quantity of extracts of cotton and extracts (marked as M4, M2 and M3) stored in dark glass bottles. The ratio between plant material and solvent was 1:9. Preparation of oily extract. “Hyperici oleum” was prepared using sunlight maceration method, as described by folk-medicine, in the same ratio 1:9. The plant material mentioned above was covered having with refined sunflower oil and the dish with the mixture was exposed to sunlight. The mixture was reshuffled frequently. Extraction and fermentation continued for six weeks, and then the herbal material was pressed out. The oily extract thus obtained had a bright red color (Figure 1).
for each paper disc would have been more appropriate, because the ratio 1:9 herbal substance: extracting solvent leads to diluted extracts. According to some approved standards the recommended ratio is 1:5 (Bradley 2006). Oily extract M1 had also antimicrobial activity, showing well defined inhibition zones of 8 mm. By using sunlight maceration method described above, lipophilic breakdown products of hypericin with antimicrobial activity are present in oily extracts. They give the red color [13, 14]. Even phloroglucinol derivatives found frequently in the lipophilic fraction have demonstrated antibacterial activities [15]. It seems that Hypericum oil composition differs considerably from aqueous-ethanolic extracts. As reported by different researchers, the main compounds responsible for antimicrobial action of Hypericum extracts are hypericin, flavonoids and especially hyperforin (phloroglucinol derivative) [16-19]. Generally, the extracts were found to be more active against Gram-positive bacteria than Gram-negative bacteria.

Conclusion. TLC chromatograms of Hypericum extracts, prepared by using ethanol 80% and 70% as extractive liquids, showed the presence of more than eight yellow bands corresponding to the flavonoid compounds, whereas only four of them were present in the extract produced with ethanol 50%. Hypericum oil extract obtained by using sunlight maceration method differs from aqueous-ethanolic extracts in terms of the constituents. No yellow bands were observed on the corresponding chromatogram, but in the upper part of it a light brown elongated zone was distinguished. Lipophilic components present in the extract were not separated in the experimental conditions. Using the mixture of anhydrous formic acid : water : ethyl acetate (6:9:90) as a mobile phase hypericin and pseudohypericin were identified in the samples M1 and M2, but not in the sample M3 that represents the extract for which ethanol 50% was used. Hydroalcoholic extracts as well as the oily extract have shown to possess antimicrobial activity against Staphylococcus aureus. The inhibition zones for extracts prepared with ethanol 80% and 70% and the ones of the oily extract differ only slightly.

References.