LC-DAD-MS-Assisted Quantification of Marker Compounds in *Hypericum perforatum* L. (St. John’s Wort) and its Antioxidant Activity

Ilkay ERDOGAN ORHAN1,*, Murat KARTAL2,3

1Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, 06330 Ankara, TURKEY, 2Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, 06100 Ankara, TURKEY, 3Bezmialem Vakif University, Faculty of Pharmacy, and Center of Phytotherapy Education, Research and Practice, Department of Pharmacognosy, 34093 Istanbul, TURKEY

*Correspondence: E-mail: iorhan@gazi.edu.tr; Tel: +90.312.202 31 86

**Hypericum perforatum** L. (St. John’s Wort) is a reputed plant with a long service to humankind. In the current study, antioxidant activity of the methanol extract of the aerial parts of *H. perforatum* growing in Turkey along with hyperoside and hyperforin was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, metal-chelation, and ferric-reducing antioxidant power (FRAP) assays. The major components including chlorogenic acid, the flavonoid derivatives; rutin, hyperoside, quercitrin, quercetin, and baicarpin, the naphthodianthrons; pseudohypericin and hypericin, and the phloroglucinol derivatives; hyperforin and adhyperforin were quantified in the extract by LC-DAD-MS. Hypericin (16 ± 0.08 µg/g) and hiperforin (1164 ± 0.02 µg/g) contents in *H. perforatum* were found to be in accordance with the amounts required by the European Pharmacopeia. The extract and hyperoside exerted a remarkable antioxidant activity in DPPH radical scavenging and FRAP assays, whereas they did not have metal-chelation capacity.

**Key words:** Antioxidant activity, *Hypericum perforatum*, LC-DAD-MS, Phenolic compounds, Hypericin, Hyperoside
INTRODUCTION

Hypericum perforatum L. (Hypericaceae), commonly known as “St. John's Wort, Klamath weed, and goat weed”, is a perennial herb distributed predominantly in the temperate regions of the world (1), while it is known as “sarı kantaron, binbirdelik otu, kan otu, koyunkıran” in Turkish. The Hypericum genus is represented by 89 species in the flora of Turkey (2) and among them; H. perforatum, known by several local names such as “sarı kantaron, binbirdelik otu, kılc otu, kan otu, mayası otu”, shows a wide distribution throughout the country (3). H. perforatum was described as a remedy since the middle ages to the present day. It has been one of the best studied medicinal plants throughout the world and its chemical constituents are well-characterized. The phytopharmaceuticals based on standardized extracts obtained from the flowering tops of this plant have been approved to be effective against mild to moderate depression (4,5). The bioactive compounds found in H. perforatum are naphthodianthron derivatives; hypericin and pseudohypericin, acylated phloroglucinol derivatives; hyperforin and adhyperforin, as well as several flavonoid derivatives such as quercetin, quercitrin, hyperoside, rutin, kaempferol, biapigenin, and amentoflavon. Among its constituents, hyperforin has been reported to exert antidepressant, antibiotic, and antitumoral activities (6). Besides, adhyperforin has been also stated to contribute to the antidepressant effect of the plant (7).

H. perforatum and some other species of the genus are also economically important, and used as edible, medicinal, fodder, fuel, dye, etc. The herb and the fruits are consumed as a tea substitute (8,9). Besides, the plant has been recorded to have traditional utilizations internally and externally against several disorders such as wounds, burns, cuts, hemorrhoids, gastric spasm, insomnia, and muscular pain (3,10). In our ongoing research on Hypericum perforatum from Turkey (11,12), we have now aimed to determine antioxidant capacity and to identify the individual characteristic compounds of the methanol extract obtained from H. perforatum growing in Turkey in order to provide additional scientific evidence.

EXPERIMENTAL

Plant material
The sample of H. perforatum was collected from the vicinity of Eskişehir province in June, 2007 and identified by Prof. Dr. Hayri Duman from Department of Biology, Faculty of Art and Science, Gazi University, Ankara, Turkey. The voucher specimen (AEF 23971) is preserved at the Herbarium of Faculty of Pharmacy, Ankara University, Ankara, Turkey.

Preparation of the extract and standards for LC-DAD-MS analysis
The aerial parts of H. perforatum (1.05 g) were extracted with 100 mL of methanol (MeOH) for 5 h on a magnetic stirrer. After filtrating the methanol phase, it was evaporated in vacuo at 40°C and the residue obtained was dissolved completed up to 25 mL in a volumetric flask. The obtained solution was filtered through a cartridge type sample filtration unit prior to LC analysis.

Chemicals used in LC-DAD-MS analysis
Standards of rutin trihydrate (SR04-072-D), hyperoside (SR04-093-A), quercitrin (Bu04-015-A) and hyperforin (SY04-047-A) were kindly provided by Dr. Willmar Schwabe Pharmaceuticals (Germany). Quercetin hydrate (34120) was purchased from Serva Chemical Co. (NY, USA), while hypericin (H9252), pseudohypericin (H9416), I3,II8-biapigenin (73962), adhyperforin (APH-20012), and chlorogenic acid (C-3878) were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany). Chromatographic grade-double distilled water, HPLC-grade acetonitrile (Merck; 1.00030), and analytical grade formic acid 98% (Merck; 263) were employed in LC-DAD-MS analyses.

LC-DAD-MS apparatus and chromatographic conditions
Analyses were performed using an Agilent Technologies 1200 series high pressure liquid chromatography (HPLC), including a binary pump, vacuum degasser, autosampler, diode array detector, and coupled to an Agilent Technologies 1200 series Model VL single quadrupole mass spectrometer (MS) equipped with an multimode ionization interface. Nitrogen drying gas was generated using a Claind LC-MS 1 model nitrogen generator. Chromatographic separations were achieved using Eclipse XDB-C18 column (15 cm × 4.6 mm, 5 µm) at room temperature. A mobile phase consisted of two eluents; (solution A) acetonitrile and (solution B) 40 mM formic acid in water. All solvents were filtered through a 0.45 µm Milipore filter prior to use and degassed in an ultrasonic bath. Separation of the compounds was carried out with gradient elution profile. A linear gradient program was applied with a slight modification of Brolis et al.’s method (13). The flow rate of 1.0 mL/min and an injection volume of 10 µL were applied. Quantification was measured at 270 nm using photo-diode array detector (DAD). The chromatographic run time was 60 min, while the column void volume was 1.60 min. Retention times (min) for chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, biapigenin, pseudohypericin, hypericin, hyperforin, and adhyperforin were 9.31, 17.45, 18.01, 18.38, 21.07, 29.28, 34.77, 37.70, 39.05, 51.82, and 54.37, respectively. Quantitative analysis parameters of the analysis is given in Table 1.

The system was controlled and data analysis was performed with Agilent ChemStation. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas. The LC-MS instrumentation described here utilizes a quadrupole MS system operating in selective ion mode (SIM) mode to achieve the requisite detection sensitivity. Operating in SIM mode precludes the ability to simultaneously detect and identify non-target analytes. The API-ES process was used for mass spectral measurements. The positive-ion mass spectra of chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, biapigenin, pseudohypericin, hypericin, hyperforin, and adhyperforin were recorded in the total-ion monitoring mode using a series of fragmentor potentials to establish their fragmentation patterns. The mass spectrum (MS) consisted of the protonated molecular ion [M+H]+ at m/z 355 for chlorogenic acid, m/z 303 for quercetin, m/z 611 for rutin, m/z 465 for hyperoside, m/z 449 for quercitrin, m/z 539 for biapigenin, m/z 537 for hyperforin, m/z 551 for adhyperforin, m/z 521 for pseudohypericin, and m/z 505 for hypericin. The fragmentor was set at 20 V for all compounds to observe the pseudomolecular ion. Spray chamber parameters were as follows: 5.0 L/min drying gas, 325°C drying gas temperature, 200°C vaporizer temperature, 60 psig. nebulizer pressure, and 2000 V capillary voltage. The compounds were identified by LC-DAD-MS analysis by

| Table 1. Quantitative analysis parameters for the calibration data for and the recovery analysis of the standards via LC-DAD-MS (each value is mean of three experiments) |
|----------------------------------|---|---|
| Retention time (min) | 7.928 | 12.616 |
| Linearity range (ppm) | 0.05-1500 | 0.05-100 |
| Slope (mAu/ppm) | 16.861 | 22.914 |
| Intercept (mAu) | -53.304 | -6.372 |
| Correlation coefficient | 0.999 | 0.999 |
| Limit Of Detection (pg/µL) | 0.019 | 0.002 |
| Limit Of Quantification (pg/µL) | 0.056 | 0.005 |
| Within-day precision (RSD %) | 4.586 | 4.520 |
| Between-day precision (RSD %) | 5.152 | 4.452 |
comparing the retention time of the peaks in the extract with those of the authentic reference samples. Purity of the peaks was checked by DAD. UV spectra of the peaks were compared with those of the authentic reference samples.

**Antioxidant activity assays**

**DPPH radical scavenging assay**

The hydrogen atom or electron donation capacity of the samples was computed from the bleaching property of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The stable DPPH radical scavenging activity of the samples was determined by the method of Blois (14). The samples (2700 µL) dissolved in methanol were mixed with 300 µL of DPPH solution (1.5 × 10⁻⁴ M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (Dayton, NJ, USA). The results were compared to that of gallic acid employed as the reference. Inhibition of DPPH in percent (I%) was calculated as given below:

\[ I% = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100, \]

where \( A_{\text{blank}} \) is the absorbance of the control reaction (containing all reagents except the test sample), and \( A_{\text{sample}} \) is the absorbance of the extracts/reference. Experiments were run in triplicate and the results were conveyed as average values with S.E.M. (Standard error mean).

**Fe²⁺-ferrozine test system for metal-chelating**

The ferrous ion-chelating effect of the test samples by Fe²⁺-ferrozine test system was estimated by the method of Chua et al. (15). Accordingly, 740 µL of ethanol and 200 µL of the samples dissolved in methanol were incubated with 2 mM FeCl₂ solution. The reaction was initiated by the addition of 40 µL of 5 mM ferrozine solution into the mixture, shaken vigorously, and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of ferrozine-Fe²⁺ complex formation was calculated as follows:

\[ I\% = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100. \]

The control contained only FeCl₂ and ferrozine.

Analyses were run in triplicate and expressed as average values with S.E.M. Ethylenediaminetetraacetic acid (EDTA) was employed as the reference in this assay.

**Ferric-reducing antioxidant power (FRAP)**

FRAP of the samples was tested using the assay of Oyaizu (16) based on the chemical reaction of Fe(III) ⇒ Fe(II). Different concentrations of the samples dissolved in methanol were added into 2500 µL of phosphate buffer (pH 6.6) and 2500 µL of potassium ferricyanide \([K₃Fe(CN)₆]\) (1%, w/v). Later, the mixture was incubated at 50°C for 20 min and then 2500 µL of trichloroacetic acid (10%) was added. After the mixture was shaken vigorously, this solution was mixed with 2500 µL of distilled water and FeCl₃ (100 µL, 0.1%, w/v). After 30 min incubation, absorbance was read at 700 nm using a Unico 4802 UV-visible double beam spectrophotometer (Dayton, NJ, USA). Analyses were achieved in triplicate. Increase in absorbance of the reaction indicated increase in reducing power of the extracts. Chlorogenic acid was the reference in this assay.

**Statistical analysis**

Data obtained from the experiments are presented as the mean ± the standard error.

**RESULTS AND DISCUSSION**

The methanol extract prepared from the aerial parts of *H. perforatum* was analyzed for its phytochemical content in terms of chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, biapigenin, pseudohypericin, hypericin, hyperforin, and adhyperforin. As tabulated in Table 2, the most abundant flavonoid derivative was found to be rutin (1124 ± 0.09 µg/g), while hyperforin was another compound existed in major quantity in the extract (1164 ± 0.02 µg/g). On the other hand, the extract as well as hyperforin and hyperoside were tested for their antioxidant activity by three methods. They (except for hyperforin) displayed high antioxidant activity in DPPH radical scavenging activity assay (Fig. 1) comparable to that of gallic acid used
The radical scavenging activity of the samples was determined by the method of Blois (14). The stable DPPH control contained only FeCl₂ and ferrozine. A blank is the absorbance of the control reaction mixture. The inhibition of DPPH in percent (I%) was calculated as given below:

\[ I\% = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100 \]

where \( A_{\text{blank}} \) is the absorbance of the reaction mixture and \( A_{\text{sample}} \) is the absorbance of the reference. Inhibition of DPPH in percent (%I) was calculated as given below:

\[ I\% = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100 \]

The ferrous ion-chelating effect of the test samples dissolved in methanol were checked by DAD. UV spectra of the peaks were compared with those of the authentic reference samples. Purity of the peaks was compared by comparing the retention time of the peaks in the extract with those of the authentic reference samples.

The methanol extract prepared from the aerial parts of Hypericum perforatum was analyzed for its phytochemical content in terms of bioactive compounds. Bioactive compounds were quantified by LC-DAD-MS. The results were presented as the mean ± the standard error of the mean (S.E.M.) calculated as follows:

\[ \text{Amount} = \left( \frac{\text{peak area}}{\text{peak area of the reference compound}} \right) \times \text{concentration of the reference compound} \]

The St. John’s Wort extracts are classified under the registered medicines in Germany, where psychovegetative disorders, moderate depression, nervous disturbances, and anxiety are listed in the monograph of the German Turku Journal of Pharmacology and Toxicology. The St. John’s Wort extracts are classified under the registered medicines in Germany, where psychovegetative disorders, moderate depression, nervous disturbances, and anxiety are listed in the monograph of the German Turku Journal of Pharmacology and Toxicology.

Table 2. Amounts of the phenolic compounds in the aerial parts of H. perforatum analyzed by LC-DAD-MS

| Compounds analyzed | Amounta (µg/g ± S.E.M.b) |
|--------------------|--------------------------|
| Chlorogenic acid   | 374 ± 0.02               |
| Rutin              | 1124 ± 0.09              |
| Hyperoside         | 805 ± 0.02               |
| Quercitin          | 144 ± 0.01               |
| Quercitrin         | 39 ± 0.01                |
| Biapigenin         | 189 ± 0.03               |
| Pseudohypericin    | 14 ± 0.01                |
| Hypericin          | 16 ± 0.08                |
| Hyperforin         | 1164 ± 0.02              |
| Adhyperforin       | 156 ± 0.08               |

aData are expressed as micrograms of each individual phenolic compound per gram of the methanol extract; bStandard error mean (n=3)

Figure 1. DPPH radical scavenging activity (Inhibition % ± S.E.M.) of the MeOH extract of H. perforatum (HP) and the compounds (hyperforin and hyperoside). Concentrations are given µg/mL. For hyperforin and hyperoside, the concentrations are indicated in parentheses as the reference compound and moderate activity in FRAP assay (Fig. 2). Nevertheless, none of them exerted metal-chelation capacity in this test.
Commission E as indications of the aqueous and alcoholic Hypericum extracts for the internal use (17). The European Pharmacopeia (Eur. Ph.) monograph on *H. perforatum* requires standardization for the extract considering hypericin and hyperforin contents (18). In our study, we showed that hypericin and hyperforin contents of the plant material of *H. perforatum* were in accordance with the criteria of Eur. Ph. The preparations of St. John's Wort, sold in European countries usually contain dry hydroalcoholic extracts, prepared either with 60% (w/w) ethanol or 80% methanol from the aerial parts of the plant (17). The extracts include a variety characteristic compounds belonging to six foremost chemical classes; naphthodianthrones, phloroglucinols, flavonol glycosides, biflavones, proanthocyanidins, and phenylpropanes. Consequently, a number of analytical studies have been performed on *H. perforatum* samples from different countries in order to determine flavonoid, phenolic acid, naphthodianthrone, and phloroglucinol contents using various methods (19-23) in which the results varied more or less as compared to ours. Although a few studies have been done to examine hypericin content of *H. perforatum* growing in Turkey, such a detailed analytical study has not been reported on the plant of Turkish origin up to date. In another study similar to ours, quantities of hypericin (440-2820 µg/g), chlorogenic acid (0-1860 µg/g), rutin (0-8770 µg/g), hyperoside (5410-22280 µg/g), quercitrin (1640-3980 µg/g), and quercetin (1010-1760 µg/g) were reported in the aerial parts of *H. perforatum* collected from the northern region of Turkey, which seems to be quite different from our results. This might be due to the fact that several factors such as genetic variation, environmental conditions, altitude, collection time, climate, and drying techniques may influence hypericin variation as underlined by some researchers (24-26), whereas altitude variation was concluded to have no effect on hypericin content of *H. perforatum* samples collected at varying...
altitudes (125, 155, 300, 400, 500, 650, 700, 940, 1000, 1010, 1070, and 1100 m) from Bursa province (27).

Antioxidant activity of the ethanol extract from *H. perforatum* has been evaluated according to several *in vitro* and *in vivo* methods and the *H. perforatum* extract containing rutin, hyperoside, isoquercitrin, avicularin, quercitrin, and quercetin was revealed with high metal-chelating effect (28), which is in disagreement with the relevant data obtained in the present work. Since we showed that hyperoside and hyperforin did not possess metal-chelating capacity, some other components might be considered to contribute to metal-chelating effect. In consistent with our data, Silva et al. concluded that high DPPH radical scavenging activity of *H. perforatum* was stated to result from its flavonoids along with caffeoyl quinic acid and hypericin and hyperforin did not contribute to antioxidant potential of the plant (29).

Accordingly, we also earlier reported that the ethyl acetate, MeOH, and water extracts of *H. perforatum* growing in Turkey did not possess metal-chelation capacity (30).

As illustrated in Figures 1 and 2, hyperoside displayed approximately the same level of antioxidant activity as the extract *per se* in the assays applied, flavonoids, hyperoside in particular, might be the major contributors to antioxidant capacity of the extract.

**CONCLUSION**

Our findings from the current study point out to the fact that *H. perforatum* growing in Turkey has a rich polyphenol and phloroglucinol content and meets the standardization criteria required by Eur. Ph., which might be used in preparation of phytopharmaceuticals/nutraceuticals. Preliminarily, it can be suggested that hyperoside seems to be more associated with the antioxidant activity of *H. perforatum* according to the experimental models studied herein. We herein describe the first detailed analytical study evaluating *H. perforatum* from Turkey for its afore-mentioned compounds.

**REFERENCES**

1. Robson NKB, *Hypericum* botany. In: *Hypericum: The genus Hypericum* (Ernst E, Ed), Taylor and Francis; New York, 1-22, 2003.
2. Davis PH, Cullen J, *Hypericaceae* In: “Flora of Turkey and the East Aegean Islands” (Davis PH, Ed), Vol.10, Edinburgh University Press, 96-103, 1984.
3. Gurhan G, Ezer N, Halk arasında hemoroi tedavisinde kullanılan bitkiler, J Pharm Fac Hacettepe Univ 24, 37-55, 2004.
4. Di Carlo G, Borrelli F, Ernst E, Izzo AA, St. John’s Wort: Prozac from the plant kingdom, Trends Pharmacol Sci 22, 292-297, 2001.
5. Bilia AR, Gallori S, Vincieri FF, St. John’s wort and depression, efficacy, safety, tolerability-An update, Life Sci 70, 3077-3096, 2002.
6. Beerhues L, Hyperforin, Phytochemistry 67, 2201-2207, 2006.
7. Lensen AG, Hansen SH, Nielsen EO, Adhyperforin as a contributor to the effect of *Hypericum perforatum* L. in biochemical models of antidepressant activity, Life Sci 67, 1593-1605, 2001.
8. Samant SS, Palni LMS, Diversity, distribution and indigenous uses of essential oil yielding medicinal plants of Indian Himalayan region, J Med Arom Plant Sci 22, 671-684, 2000.
9. Brown T, Guide to Wild Edible and Medicinal Plants (Field Guide), Berkley Publishing Group, New York, 1985.
10. Altun ML, Sever Yılmaz B, Orhan IE, Saltan Çitoğlu G, Assessment of cholinesterase and tyrosinase inhibitory and antioxidant effects of *Hypericum perforatum* (St. John’s wort), Ind Crops Prod 43, 87-92, 2013.
11. Orhan IE, Kartal M, Gülpinar AR, Cos P, Matheussen A, Maes L, Tasdemir D, Assessment of antimicrobial and antiprotozoal activity of the olive oil macerate samples of *Hypericum perforatum* and their LC-DAD-MS analyses, Food Chem 138, 870-875, 2013.
12. Jarić S, Popović Z, Macukanovic-Jocic M, Djurdjevic L, Mijatovic M, Karadzic B, Mitrovic M, Pavlovic P, An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia), J Ethnopharmacol 111, 160-175, 2007.
13. Brolis M, Gabetta B, Fuzzati N, Pace R, Panzeri F, Peterlongo F, Identification by high pressure liquid chromatography-diod
array detection-mass spectrometry and quantification by high pressure liquid chromatography-UV absorbance detection of active constituents of *Hypericum perforatum*, J Chrom A 825, 9-16, 1998.

14. Blois MS, Antioxidant determinations by the use of a stable free radical, Nature 181, 1199-1200, 1958.

15. Chua MT, Tung YT, Chang ST, Antioxidant activities of ethanolic extracts from the twigs of *Cinnamomum osmophleum*, Biorec Technol 99, 1918-1925, 2008.

16. Oyaizu M, Studies on products of browning reactions-antioxidative activities of products of browning reaction prepared from glucosamine, Jap J Nutr 44, 307-315, 1986.

17. Erdelmeier CAJ, Hoerr R, *Hypericum perforatum* - St. John's wort, chemical, pharmacological, and clinical aspects, In: “Studies in Natural Product Chemistry” (Atta-ur-Rahman, Ed), Vol. 22, Elsevier Science B.V., The Netherlands, pp. 643-716, 2000.

18. European Pharmacopoeia, Council of Europe, 4th Edition, Strausbourg, 2008.

19. Southwell IA, Bourke CA, Seasonal variation in hypericin content of *Hypericum perforatum* L. (St. John’s Wort), Phytochemistry 56, 437-441, 2001.

20. Kosuth J, Kopperdaková J, Tolenov A, Hohtola A, Cellarova E, The content of hypericins and phloroglucinols in *Hypericum perforatum* L. seedlings at early stage of development, Plant Sci 165, 515-521, 2003.

21. Skerget M, Kotnik P, Hadolin M, Hras AR, Simonic M, Knez Z, Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities, Food Chem 89, 191-198, 2005.

22. Williams FB, Sander LC, Wise SA, Girard J, Development and evaluation of methods for determination of naphthodianthrone and flavonoids in St. John’s Wort, J Chrom A 1115, 93-102, 2006.

23. Bagdonaite E, Janulis V, Ivanauskas L, Labokas J, *Ex situ* studies on chemical and morphological variability of *Hypericum perforatum* L. in Lithuania, Biologica 53, 63-70, 2007.

24. Upton R, Graff A, Williamson E, Bunting D, Gatherum DM, Walker EB, Butterweck V, Lieflünder U, Nahrstedt A, Winterhoff H, Cott J, St. John’s Wort Monograph, In: American Herbal Pharmacopoeia and Therapeutic Compendium, Herbal Gram, 40, 1-32, 1997.

25. Büter B, Orlacchio C, Soldati A, Berger K, Significance of genetic and environmental aspects in the field cultivation of *Hypericum perforatum*, Planta Med 64, 431-437, 1998.

26. Zobayed S, Saxena PK, Production of St. John’s Wort plants under controlled environment for maximizing biomass and secondary metabolites, In vitro Cell Dev Biol Plant 40, 108-114, 2004.

27. Kaçar O, Azkan N, Determination of effect of different altitudes on hypericin amount of St. John’s wort (*Hypericum perforatum* L.) populations in the natural flora of Bursa, J Agric Fac Uludag Univ 19, 77-89, 2005.

28. Zhou Y, Li Y, Wei D, Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. *in vitro*, J Agric Food Chem 52, 5032-5039, 2004.

29. Silva BA, Ferreres F, Malva JO, Dias ACP, Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts, Food Chem 90, 157-167, 2005.

30. Altun ML, Sever Yılmaz B, Orhan IE, Saltan Çitoğlu, Assessment of cholinesterase and tyrosinase inhibitory and antioxidant effects of *Hypericum perforatum* L. (St. John’s wort), Ind Crops Prods 43, 87-92.