ORIGINAL ARTICLE

PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *HYPERICUM HUMIFUSUM* L. (*HYPERICACEAE*)

ANCA TOIU[1], LAURIAN VLASE[2], CRISTINA MANUELA DRĂGOI[3*], DAN VODNAR[4], ILIOARA ONIGA[1]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, 8, V. Babes Street, Cluj-Napoca, Romania*
[2]*Department of Pharmaceutical Technology and Biopharmaceutics, Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy, 8, V. Babes Street, Cluj-Napoca, Romania*
[3]*Department of Biochemistry, Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, 6, Traian Vuia Street, sector 2, Bucharest, Romania*
[4]*Department of Food Science, Faculty of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine, 3-5, Manăştur Street, Cluj-Napoca, Romania*

**corresponding author: manuela.dragoi@gmail.com*

Manuscript received: January 2016

Abstract

The study focused on the chemical composition, antioxidant and antibacterial evaluation of *Hypericum humifusum* aerial parts. Total phenolic content (TPC), total flavonoid content (TFC) and total hypericins (TH) were determined by spectrophotometric methods, and the identification and quantitation of polyphenolic compounds by LC/UV/MS. Ethanolic extracts were the richest in total phenols (8.85%), flavonoids (4.52%) and total hypericins (0.12%). Gentisic, caffeic and chlorogenic acids, hyperoside, isoquercitrin, rutin, quercitrin, and quercetin were identified and quantified by HPLC/UV/MS. The antioxidant potential determined by DPPH assay showed a better antioxidant activity for *H. humifusum* ethanolic extract and a positive correlation between the antioxidant properties, TPC and TFC. Antimicrobial activity by dilution assays, minimal inhibitory concentration and minimal bactericidal concentration were assessed. *H. humifusum* aerial parts represent an important alternative source of natural antioxidants and antimicrobials.

Rezumat

Obiectivul studiului a constat în determinarea compoziţiei chimice şi evaluarea activităţii antioxidante şi antibacteriene pentru părţile aeriene ale speciei *Hypericum humifusum*. Conţinutul de polifenoli totali (TPC), flavonoide totale (TFC) şi hipericine totale (TH) a fost determinat printr-o metodă spectrofotometrică, iar pentru analiza şi identificarea compuşilor polifenolici a fost utilizată o metodă LC/UV/MS. Extractele etanolice au fost mai bogate în polifenoli totali (8,85%), flavonoide totale (4.52%) şi hipericine totale (0,12%). Acidul gentizic, acidul cafeic, acidul clorogenic, hiperozida, izoquercitrozida, rutozida, quercitrozida şi quercetolul au fost identificaţi prin HPLC/UV/MS în toate probele. Potenţialul antioxidant determinat prin tehnica DPPH a arătat efecte mai bune pentru extractul etanolic de *H. humifusum* şi o corelaţie pozitivă între conţinutul în polifenoli, flavonoide şi efectul antioxidant. Acţiunea antimicrobiană a fost evaluată prin tehnica microdiluţiilor, fiind determinate concentraţia minimă inhibitorie şi concentraţia bactericidă. Părţile aeriene ale speciei *H. humifusum* reprezintă o sursă alternativă importantă de compuşi naturali cu efect antioxidant şi antibacterian.

Keywords: *Hypericum humifusum* L., HPLC/MS, polyphenols, antioxidant, antimicrobial.

Introduction

Hypericum species (*Hypericaceae*), widely distributed in temperate, tropical and mountainous regions, have been used in folk and modern medicine for many years due to their various therapeutic properties: antidepressant, analgesic, antiviral, antioxidant, antimicrobial, anti-inflammatory [9]. Phytochemical investigations showed the presence of naphtodianthrones, flavonoids, tannins, essential oils, acylphloroglucinols in *Hypericum sp.* [3, 9]. Numerous phenolic antioxidants have demonstrated scavenging radical activities and are considered promising bioactive compounds for free radical pathologies related with chronic diseases (atherosclerosis, neurodegenerative disorders, cerebral and cardiac ischemia, and rheumatic disorders). The major substances with antioxidant properties are considered the flavonoids, whereas essential oils of *Hypericum sp.* have notable antimicrobial activity [5, 19].

The most recognized species of this genus, *H. perforatum* is widely used for the treatment of depressive disorders due to its content in bioactive compounds such as hypericins, hyperforins and flavonoids [9, 10]. Comprehensive analysis of the published research on the phytochemistry and biological effects showed that other species have similar potential as *H. perforatum*, with considerable amounts of active metabolites [3].

However, phytochemical investigations on other Romanian *Hypericum species* are scarce and little is

known about their biological activities. Thus, investigation and quantification of the main bioactive compounds in extracts should be carried out, in order to correlate the plant constituency with potential activities. Therefore, this study was initiated in order to evaluate total phenolic content, total flavonoid content, total hypericins and the antioxidant and antibacterial activity of *H. humifusum* aerial parts extracts for the first time.

Materials and Methods

Materials

Plant material: the plants were harvested from wild populations from Cluj County on July 2013 at full flowering stage. A voucher specimen of the studied plants was stored in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania, with the accession number HH-54.

Extraction procedure: the air-dried natural product was reduced to a powder and extracted with different solvents. The methanolic extract was obtained using 0.5 g vegetal product and 50mL methanol for 30 min on a water bath at 60ºC [14]; the ethanolic extract was prepared from 10 g natural product and 100 g ethanol 50% at room temperature [18].

Total phenolic content (TPC) for both extracts was assessed by Folin-Ciocalteu method, with some modifications [13]. The absorbance was measured at 760 nm with a JASCO UV-VIS spectrophotometer. Different concentrations of gallic acid were used in order to obtain the standard curve, and TPC was expressed as mg gallic acid/g dry material plant (mg GAE/g plant material).

Total flavonoid content (TFC) was determined and expressed as rutin equivalents (mg RE/g plant material), using the method described in the Romanian Pharmacopoeia (X^{th} Edition) [18] and the absorbance was measured at 430 nm.

The content in total hypericins (TH) was determined by a spectrophotometric method, and was expressed in hypericin (mg hypericin/g plant material) [17].

HPLC analysis of polyphenols

The identification and quantitation of polyphenolic compounds was assessed using an Agilent 1100 HPLC Series system equipped with UV detector, degasser, binary gradient pump, column thermostat, autosampler. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). The analysis was achieved in previously described conditions and 18 standards were employed: caftaric, gentisic, caffeic, chlorogenic, ferulic, *p*-coumaric, sinapic acids, hyperoside, rutin, isoquercitrin, myricetin, fisetin, quercitrin, quercetin, patuletin, luteolin, kaempferol, apigenin. The detection and quantification of polyphenols was performed in UV assisted by mass-spectrometry (MS) detection. Calibration curves in the 0.5 - 50 µg/mL range with good linearity ($R^2 > 0.999$) for a five point plot were used [1, 2, 16].

DPPH Radical Scavenging Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was used to evaluate the antioxidant capacity, by bleaching of purple methanolic solution of the stable radical. The measure of antioxidant effect is the disappearance of the DPPH absorbtion by the action of antioxidants. 20 µL of diluted extracts were added to 980 µL DPPH solution (100 µM). After 30 min incubation period, the decrease in absorbance was measured at 517 nm, using a UV-VIS JASCO V-530 spectrophotometer. Both hydrophilic and lipophilic synthetic antioxidants, quercetin and butylated hydroxytoluene (BHT) were used as standards. The percentage inhibition of the DPPH radical after adding individual samples was calculated using the following equation:

$$I = 100(A_c-A_s)/A_c,$$

where I - DPPH inhibition (%), A_c - absorbance of control sample, A_s - absorbance of the tested sample. The Antioxidant activity was also expressed as inhibitory concentration IC_{50}, defined as the concentration of the sample required to cause a 50% decrease in initial DPPH radical absorbance. IC_{50} values in DPPH assay were calculated graphically. All experiments were performed in triplicate [1, 15].

Antibacterial activity

Microorganisms and culture conditions

For the bioassay six bacterial strains were used, three Gram positive: *Staphylococcus aureus* (ATCC 49444), *Listeria monocytogenes* (ATCC 19114), *Bacillus cereus* (ATCC 11778) and three Gram negative: *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028) and *Escherichia coli* (ATCC 25922). All microorganisms were obtained from Food Biotechnology Laboratory, Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine Cluj Napoca, Romania. The bacteria were cultured on Müller-Hinton Agar and cultures were stored at 4°C and subcultured once a month [8].

Microdilution method

In order to evaluate the antimicrobial activity, a modified microdilution technique was used. Bacterial species were cultured overnight in Tryptic Soy Broth (TSB) medium at 37°C. The bacterial cell suspensions were adjusted with sterile saline to a concentration of approximately 2.5 x 10^5 colony forming units CFU/mL (final volume of 100 µL per well). The inoculum was stored at 4°C. Determinations of MIC were performed by a serial dilution technique using 96-well microtiter plates. Into each well containing 100 µL of TSB, different extract dilutions and 10 µL of inoculum were added. The microplates were incubated for 24 - 48 h (37°C). The MIC of the samples was detected following the addition of 20 µL (0.2 mg/mL) of resazurin solution

to each well. The plates were incubated again for 2 h (37°C). A change from blue to pink indicates reduction of resazurin, thus bacterial growth. The MIC was defined as the lowest drug concentration that prevented this colour change. The minimum bactericidal concentrations (MBC) were determined by serial subcultivation of a 2 µL into microtiter plates containing 100 µL of broth per well and further incubation for 48 h at 37°C. The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculum. Streptomycin was used as positive control for bacterial growth, and a 10% ethanol solution in water was used as negative control [8].

Statistical analysis

In all cases, analyses were performed in triplicate. Data are presented as mean ±standard deviation (SD). Statistical analysis was carried out using Excel software package.

Results and Discussion

Polyphenolic compounds analysis

Considering that the type and polarity of solvent and extraction method can influence the amount of bioactive compounds and the composition of plant extracts [6], ethanol/water and methanol/water mixtures were used in order to evaluate the polyphenolic compounds and hypericins from *H. humifusum* aerial parts.

We observed that the total phenolic content, total flavonoid content and total hypericins determined in extracts varied due to different extraction solvent and method employed, with higher amounts in ethanolic extract. The content of total polyphenols (81.11 - 88.57 mg GAE/g plant material), flavonoids (37.24 - 45.21 mg RE/g plant material) and total hypericins (1.21 - 1.24 mg hypericin/g plant material) are presented in Table I.

Table I

TPC, TFC and TH in *H. humifusum* aerial parts extracts (± SD)

Extract	TPC (mg GAE/g)	TFC (mg RE/g)	TH (mg hypericin/g)
Methanolic extract (ME)	81.11 ± 1.23	37.24 ± 0.89	1.21 ± 0.07
Ethanolic extract (EE)	88.57 ± 1.59	45.21 ± 1.08	1.24 ± 0.15

Previous work on *Hypericum sp*. has shown that they represent good sources of phenolic compounds. The present TPC values for the *H. humifusum* aerial parts are mainly in correlation with earlier published data on *H. perforatum* and other investigated species: *H. perforatum* (64.4 - 91.6 mg GAE/g plant material), *H. olympicum* (73.58 mg GAE/g plant material), *H. barbatum* (57.68 mg GAE/g plant material) [3, 4, 11]. Taking into account the content in hypericins, our results are in accordance with quality standards required by Eur Ph. (*H. perforatum* contains not less than 0.08% of total hypericins).Thus *H. humifusum* can be considered as a valuable alternative with good content in polyphenols and hypericins.

The specific compounds from *H. humifusum* aerial parts, mostly responsible for the wide range of biological activities, have been identified and quantified by an optimised LC/UV/MS method using 18 polyphenolic compounds as standards (phenolic acids, flavonoid glycosides and aglycones). By comparing retention times, UV and MS data with those of the reference standards [1, 7], eight polyphenolic compounds were identified and quantified in both extracts: three cinnamic acid derivatives (gentisic acid, caffeic acid, chlorogenic acid), four flavonoid glycosides (hyperoside, isoquercitrin, rutin, quercitrin) and one flavone (quercetin).

The amounts of the identified polyphenols in *H. humifusum* aerial parts ethanolic extract are presented in Table II, and HPLC chromatogram of *H. humifusum* aerial parts is presented in Figure 1.

Table II

Quantitative determinations of polyphenols by HPLC (mg/100) in *H. humifusum* ethanolic extract

Compound	R_T ± SD	Concentration (mg/100g)
Gentisic acid	3.69 ± 0.03	< 0.02
Caffeic acid	5.60 ± 0.04	< 0.02
Chlorogenic acid	6.43 ± 0.05	< 0.02
Hyperoside	19.32 ± 0.12	229.83 ± 5.42
Isoquercitrin	20.29 ± 0.10	106.82 ± 4.01
Rutin	20.76 ± 0.15	1.4 ± 0.09
Quercitrin	23.64 ± 0.13	27.08 ± 2.64
Quercetin	27.55 ± 0.15	6.90 ± 0.18

Note: NF - not found, below the limit of detection. Values are the mean ± SD (n = 3).

Quantitative determinations by LC/UV/MS revealed that hyperoside and isoquercitrin are the main compounds found in both extracts, with higher amounts in *H. humifusum* aerial parts ethanolic extract (229.83 mg/100g, and respectively 106.825 mg/100g). Other flavonoid glycosides, rutin and quercitrin were identified and quantified in methanolic and ethanolic extracts in small quantities (1.1 - 1.4 mg/ 100g, and respectively 25.78 - 27.08 mg/100g).

H. humifusum extracts contain three phenolic acids (gentisic acid, caffeic acid, chlorogenic acid) and one free aglycone (quercetol). The results show that aerial parts of *H. humifusum* are rich in polyphenols, which are widely known as good antioxidants and antimicrobial agents [1, 7].

This is the first report (as far as the authors are aware) of the polyphenolic and hypericin content of *H. humifusum* aerial parts.

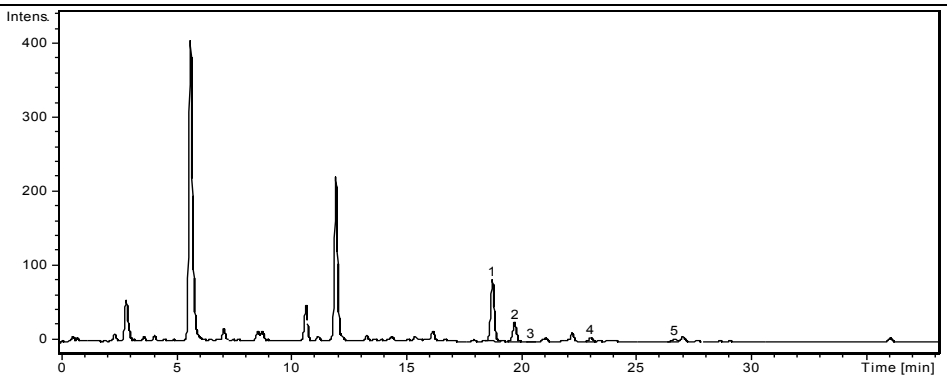

Figure 1.
HPLC chromatogram of *H. humifusum* ethanolic extract

Antioxidant activity assay

In order to evaluate the antioxidant potential of *H. humifusum* aerial parts extracts and synthetic antioxidants (quercetin and BHT), the stable free radical DPPH was used. Both extracts of *H. humifusum* were able to reduce DPPH radical with different degrees of scavenging activity. Higher bleaching effect reflected a better antioxidant effect, thus a lower IC_{50} value. The obtained results for the evaluation of the antioxidant activity using the DPPH bleaching assay are presented in Table III.

Table III
Antioxidant activity measured by DPPH method

Sample	IC_{50} (µg/mL)
H. humifusum ME	19.75 ± 5.32
H. humifusum EE	18.51 ± 4.94
Quercetin	5.59 ± 0.13
BHT	15.88 ± 1.06

Note: Values are the mean ± SD (n = 3).

The positive control, quercetin, was the strongest antioxidant, with a IC_{50} value of 5.59 µg/mL. The examined extracts showed lower DPPH scavenging activity than the reference compounds, quercetin and BHT. The highest radical scavenging activity was determined for *H. humifusum* ethanolic extract (18.51 ± 4.94 µg/mL), with positive correlation between scavenging activity on DPPH and TPC and TFC values. This indicates that polyphenolic compounds from *H. humifusum* aerial parts contribute to their antioxidant effects.

Considering these results, the following order in antioxidant activities was established: *H. humifusum* ME < *H. humifusum* EE < BHT < quercetin. According to this method, the ethanolic extract of *H. humifusum* aerial parts showed a high antioxidant activity ($IC_{50} \leq 50$ µg/mL) [1]. Similar results have been obtained for other analysed *Hypericum species*, and they are in accordance with other published data, where different units have been used for data expression [3].

For further applications in the pharmaceutical industry or as a food supplement of this natural product, further investigations are required for a better understanding of the antioxidant mechanisms involved.

Antimicrobial activity

The results of antibacterial evaluation of *H. humifusum* aerial parts extracts and standard antibiotic streptomycin against both Gram-positive and Gram-negative bacteria are presented in Table IV. The *in vitro* antimicrobial activity was tested by the microdilution assay, and MIC and MBC were assessed. The MIC values ranged from 0.078 to 1.25 mg/mL for the ethanolic extract and from 0.15 to 1.25 mg/mL for methanolic extract of *H. humifusum* aerial parts. The results show that both extracts had similar effects against three bacterial strains: *B. cereus, P. aeruginosa, E. coli* and *S. typhimurium*. The best antimicrobial activity was determined for *H. humifusum* ethanolic extract against *S. aureus* and *L. monocytogenes* (MIC = 0.078 mg/mL, MBC = 0.15 mg/mL), followed by the effect against *B. cereus*, *P. aeruginosa* and *E. coli* (MIC = 0.62 mg/mL, MBC = 1.25 mg/mL). Less sensitive strains were *S. typhimurium* for both *H. humifusum* extracts. According to Salvat *et al*. [12], plant extracts with MIC value less than/or around 0.5 mg/mL indicate good antimicrobial activities, thus *H. humifusum* aerial parts can be considered a valuable source of antibacterial compounds.

Table IV
Antimicrobial effect for *H. humifusum* extracts

Bacterial Strains	MIC (mg/mL)		MBC (mg/mL)		MIC Streptomycin
	H. humifusum ME	*H. humifusum* EE	*H. humifusum* ME	*H. humifusum* EE	
S. aureus	0.15	0.078	0.3	0.15	0.03
B. cereus	0.62	0.62	1.25	1.25	0.015
P. aeruginosa	0.62	0.62	1.5	1.5	0.06
L. monocytogenes	0.15	0.078	0.3	0.15	0.015
E. coli	0.62	0.62	1.25	1.25	0.12
S. typhimurium	1.25	1.25	2.5	2.5	0.06

ME = methanolic extract, EE = ethanolic extract

The results of our study showed an important antibacterial effect against the tested bacterial strains.

Conclusions

The study presents for the first time the analysis of polyphenolic substances and hypericins, as well as the evaluation of antioxidant and antimicrobial potential of *H. humifusum* aerial parts.
The current results show that aerial parts of *H. humifusum* are a rich source of polyphenolic compounds and suggest the use of this natural product for antioxidant and antimicrobial formulations based on their proven activities.

Acknowledgement

This research received funding from UEFISCDI, Romania, project no. PNII-RU-TE-2014-4-1247.

References

1. Andriamadio J.H., Rasoanaivo L.H., Benedec D., Vlase L., Gheldiu A-M., Duma M., HPLC/MS analysis of polyphenols, antioxidant and anti-microbial activities of *Artabotrys hildebrandtii* O. Hffm. Extracts. *Nat. Prod. Res.*, 2015; 29(23): 2188-2196.
2. Arsene A.L., Rodino S., Butu A., Petrache P., O., Butu M., Study on antimicrobial and antioxidant activity and phenolic content of ethanolic extract of *Humulus lupulus*. *Farmacia*, 2015; 63(6): 851-857.
3. Božin B, Kladar N, Grujić N, Anačkov G, Samojlik I, Gavarić N, Conić BS. Impact of origin and biological source on chemical composition, anti-cholinesterase and antioxidant properties of some St. John's wort species (*Hypericum spp., Hypericaceae*) from the Central Balkans. *Molecules,* 2013; 18(10): 11733-50.
4. Hernandez MF, Fale PLV, Araujo MEM, Serralheiro MLM. Acetylcholinesterase inhibition and antioxidant activity of the water extracts of several *Hypericum* species. *Food Chem.,* 2010; 120: 1076–1082
5. Jiang L, Numonov S, Bobakulov K, Qureshi MN, Zhao H, Aisa HA. Phytochemical Profiling and Evaluation of Pharmacological Activities of *Hypericum scabrum* L. *Molecules*, 2015; 20(6): 11257-11271.
6. Michiels J.A., Kevers C., Pincemail J., Extraction conditions can greatly influence antioxidant capacity assays in plant food matrices, *Food Chemistry*, 2012; 130: 986-993.
7. Mocan A., Crişan G., Vlase L., Crişan O., Vodnar D.C., Raita O., Comparative studies on polyphenolic composition, antioxidant and antimicrobial activities of *Schisandra chinensis* leaves and fruits. *Molecules,* 2014; *19*: 15162-15179.
8. Mocan A., Vodnar D.C., Vlase L., Crişan O., Gheldiu A.-M., Crişan G., Phytochemical Characterization of *Veronica officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz from Romania and Their Anti-oxidant and Antimicrobial Properties. *Int. J. Mol. Sci.,* 2015; *16*: 21109-21127.
9. Patel A., Shenouda N., Tallis G., PDR for Herbal Medicines. Ed. 4, Montvale: Ed. Thomson Healthcare, 2007; p. 797-820.
10. Pirvu L., Nicorescu V., Hlevca C., Udeanu D.I., Nicorescu I., Antimicrobial and synergistic activity of some whole and selective *Epilobium hirsutum* l. (great willowherb) extracts tested on standard and wild *Staphylococcus aureus* strains. *Farmacia*, 2015; 63(5): 690-695.
11. Radulović N, Stankov-Jovanović V, Stojanović G, Šmelcerović A, Spitelle, M, Asakawa Y., Screening of *in vitro* antimicrobial and antioxidant activity of nine *Hypericum* species from the Balkans. *Food Chem.* 2007; 103: 15-21
12. Salvat A., Antonacci L., Fortunato R.H., Suarez E.Y., Godo H.M., Antimicrobial activity in methanolic extracts of several plant species from Northern Argentina. *Phytomedicine,* 2004; 11: 230-234.
13. Tămaş M, Toiu A, Oniga I, Deliu C, Oltean B, Coldea G., Quantitative determination of total polyphenols and flavonoids from indigenous species of *Epilobium* of wild origin and in vitro regenerates plantlets. *Contribuţii Botanice,* 2009; XLIV: 119-123.
14. Tămaş M., Drăgulescu C., Oniga I., Gliga F., Comparative Chemical Research on some *Hypericum* species and *Hypericum perforatum L.* populations from Sibiu County flora. *Acta Oecologica*, 2001; 8(1-2): 25-33.
15. Vlase L., Benedec D., Hanganu D., Damian D., Csillag I., Sevastre B., Evaluation of antioxidant and antimicrobial activities and phenolic profile for *Hyssopus officinalis*, *Ocimum basilicum* and *Teucrium chamaedrys*. *Molecules,* 2014; 19: 5490-5507.
16. Vlase L., Parvu M., Parvu E.A., Toiu A., Chemical constituents of three Allium species from Romania. *Molecules*, 2013; 18(1):114-127.
17. xxx - European Pharmacopoeia, 8th Ed., Council of Europe, Strasbourg, 2014.
18. xxx - Farmacopeea Română, ed. a X-a, Ed. Medicală, Bucureşti, 1993.
19. Zheleva-Dimitrova D Nedialkov P, Kitanov G. Radical scavenging and antioxidant activities of methanolic extracts from *Hypericum* species growing in Bulgaria. *Pharmacogn. Mag*., 2010; 6(22):74-78.