Phenolic content, antioxidant and antifungal activities of acetonic, ethanolic and petroleum ether extracts of *Hypericum perforatum* L.

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Abstract

The objective of this study was to evaluate antifungal and antioxidant activities of *Hypericum perforatum* L. extracts against the growth of certain fungi. The ethanolic, acetonic and petroleum ether extracts of the plant were evaluated for phenols, flavonoids and non-flavonoids. The highest amounts of phenols (17.6 mg EPC/g dry extract) and flavonoids (16.85 mg EPC/g dry extract) were found in the acetonic extract. The highest inhibitory effect on the growth of *Penicillium canescens*, *Fusarium oxysporum*, *Alternaria alternata, Aspergillus glaucus* and *Phialophora fastigiata* by the disk diffusion method was exhibited by the ethanolic extract at the concentration of 25 mg/disk. The minimum inhibitory concentration (*MIC*) of the ethanolic and petroleum ether extracts was 20 mg/mL. The acetonic extract did not affect the growth of the tested fungi. Antioxidant activity was assessed by determining 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH) free radical scavenging activity. The results showed that the ethanolic extract of *Hypericum perforatum* L. possesses antioxidant activity. The IC_{50} values, defined as the concentration of the test sample leading to 50% reduction of the free radical concentration, determined for each measurement were <7.8125, 105.9, 5.99 and 12.77 µg/ml for the ethanolic extract, the acetonic extract, ascorbic acid and BHT, respectively, for DPPH free radical scavenging activity.

Keywords: Antifungal activity • *Hypericum perforatum* • Total phenols • Antioxidant

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In recent years, the consumption of products derived from *Hypericum perforatum* L. *(Hypericaceae = Clusiaceae*) has increased dramatically, the plant being one of the most popular of medicinal plants worldwide [1]. These products derived from *Hypericum perforatum* L. are available as phytopharmaceuticals, nutraceuticals, teas, tinctures, juices, and oily macerates [2]. *Hypericum perforatum* L. has a wide range of medicinal uses, including skin wounds, eczema, burns, diseases of the alimentary tract, and psychological disorders [3]. Ethanolic extracts of this plant are known to contain a number of phenolic compounds, including hypericin, hyperforin and their derivatives, rutin, hyperoside, quercetin, chlorogenic acid, flavonols and flavones. This can serve as an indicator of their potential antioxidant properties [4]. Hypericin exhibits antibacterial, antiviral and anti-inflammatory activities [5], and hyperforin is the major antidepressive component [6]. Hyperforin shows effects against methicillin-resistant strains of *Staphylococcus aureus* with a minimum inhibitory concentration (*MIC*) value of 1.0 µg/ml [7]. Apart from having therapeutic properties, *Hypericum perforatum* L. is also used as a flavouring substance for foods and alcoholic beverages [8]. Additionally, infusions, alcoholic tinctures and fluid extracts of the plant are used in the flavouring industry to prepare liqueurs, especially digestive and tonic bitters [9]. In view of the above literature data confirming the use of *H. perforatum* as both an effective antimicrobial substrate and a taste corrigent, this study was aimed at evaluating the inhibitory effect of the selected *Hypericum perforatum* L. extracts against fungal growth in foodstuffs i.e. determining the potential use of the plant extracts as natural preservatives. Various studies suggested that total phenolic compounds are closely associated with antioxidant [10] and antimicrobial activities of phenols and phenolic extracts [11]. Phenols take part in biological oxidation-reduction reactions following the quinone/ /hydroquinone mechanism.

EXPERIMENTAL

Chemicals

1,1-Diphenyl-2-picrylhydrazyl hydrate (DPPH), Folin–Ciocalteu, Muller–Hinton broth, ascorbic acid, butylated hydroxytoluene (BHT), nystatin and pyrocate-

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chol were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Hydrochloric acid, formaldehyde, anhydrous sodium carbonate, potato-glucose agar, methanol, ethanol, acetone and petroleum ether were purchased from Centrohem (Centrohem, Stara Pazova, Serbia). All other chemicals and reagents were of analytical reagent grade.

Spectrophotometric measurements

Spectrophotometric measurements were performed using a UV–Vis spectrophotometer MA9523-Spekol 211 (ISKRA, Horjul, Slovenia).

Plant material

Aerial flowering parts of the plant *Hypericum perforatum* L. (*Hypericaceae = Clusiaceae*) were collected in the region of Kragujevac, central Serbia, in June 2006. The plant material was air-dried at room temperature (20±2 °C). The identity and purity of the plant were checked at the Department of Botany, Faculty of Biology, University of Belgrade. A voucher specimen (voucher 0799HP) of the plant was deposited in the Herbarium of Department of Botany, Faculty of Biology, University of Belgrade, Serbia.

Preparation of extracts

The air-dried plant *H. perforatum* L. (40 g) was broken into small 2–6 mm pieces by a cylindrical crusher, and extracted with ethanol:water solution (80:20, v/v, 180 ml) using a Soxhlet apparatus. The mixture was filtered through filter paper (Whatman, No. 1) and evaporated. The residue (5.8 g) was stored in a dark glass bottle for further processing. Conventional microbial culture media were used, which included potato-glucose agar for the disk diffusion method, and the Mueller–Hinton broth for the dilution method.

Determination of phenolic compounds in plant extracts

The acetonic, ethanolic and petroleum ether extracts of the test plant were evaluated for phenolic content. Spectrophotometric methods were employed using Folin–Ciocalteu reagent in $NaHCO_3$ solution by measuring the absorbance at 765 nm [12]. The phenolic content of the test extracts was compared to the amount of pyrocatechol standard and the results were expressed as mg pyrocatechol per 1 g of dry extract. The flavonoid fraction was determined after precipitation by diluted HCl (1:3) and formaldehyde. Upon filtration, the filtrates were examined for non-flavonoids [12]. The obtained amount of flavonoids was the difference between total phenols and the amount of non-flavonoids.

Test microorganisms

Fusarium oxysporum (FSB91), *Penicillium canescens* (FSB24), *Aspergillus glaucus* (FSB32), *Alternaria alternata* (FSB51) and *Phialophora fastigiata* (FSB81) were collected from deteriorating fruit samples (lemon and orange) for microbiological analysis. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Biology, Faculty of Science, University of Kragujevac, Serbia. All fungi were identified by standard mycological methods through determination of macroscopic culture characteristics (SDA, HA and PDA) and physiological characteristics (germination, urease production, capsules).

Disk diffusion assay

The paper disk-agar diffusion method was used to evaluate the antifungal activity of methanol and dichloromethane extracts of each sample [13]. Test plates were prepared with potato glucose agar inoculated with a cell suspension in sterile water. The concentration of the inoculum was adjusted spectrophotometrically to 10^6 CFU/mL to reach a final concentration of 10^5 CFU/mL. Paper disks (6 mm in diameter) were impregnated with 5 and 10 mg of both ethanolic and water extracts per disc. Nystatin was used as a standard antimycotic at a starting concentration of 5 mg/mL with twofold serial dilutions subsequently made. Nystatin was dissolved in sterilized water.

Modification of dilution method

The dilution method is a microbiological method designed to test a series of cultures using various concentrations of an extract to determine the minimum inhibitory concentration (*MIC*) of the extract [14–16]. Twofold serial dilutions of the acetonic, ethanolic and petroleum ether extracts were prepared. The extracts were dissolved in phosphate buffer (pH 8) to obtain an initial concentration. A total of eight test tubes were used and each was filled with 1 mL Czapek broth. The first test tube was filled with 1 mL extract of certain concentration. Twofold dilutions of the extracts were subsequently made to obtain the following test concentrations of the extracts: 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 and 0.09 mg/mL. Then, up to 0.1 mL spore suspension at an inoculum density of 0.2×10^{-4} CFU/mL was added to each test tube. *MIC* readings were performed after a 48-hour period [17]. Three controls were used: C1 medium, C2: medium + 0.1 mL standardized fungal suspension (1×10^{-2} CFU/mL) and C3: medium + 1 mL phosphate buffer + 0.1 mL standardized fungal culture suspension. Nystatin was used as a standard antimycotic at a starting concentration of 5 mg/mL with twofold serial dilutions subsequently made. Nystatin was dissolved in sterilized water.

Determination of DPPH free radical scavenging activity

The method used by Takao *et al*. [18] was adopted with suitable modifications from Kumarasamy *et al*.

[19]. DPPH (8 mg) was dissolved in MeOH (100 mL) to obtain a concentration of 80 μg/mL. Serial dilutions were carried out with stock solutions (1 mg/ml) of methanol extract to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.95 and 0.97 μg/mL. Diluted solutions (2 mL each) were mixed with DPPH (2 mL) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. Ascorbic acid and butylated hydroxytoluene (BHT) were used as reference standards and dissolved in distilled water to make the stock solution with the same concentration (1 mg/mL). The control sample was prepared containing the same volume without test compounds and reference ascorbic acid or BHT. Methanol (95%) was used as a blank. The DPPH free radical scavenging activity (%) was calculated using the following equation:

$$\text{Inhibition} = 100(Ac - As)/Ac$$

The percentage inhibition values were calculated from the absorbance of the control, *Ac*, and of the sample, *As*, where the controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses. The IC50 value, defined as the concentration of the test sample leading to 50% reduction of the free radical concentration, was calculated as μg/ml through a sigmoidal dose-response curve.

RESULTS AND DISCUSSION

The amounts of phenols, flavonoids and non-flavonoid compounds were determined in 1ml solution of 0.02 g extract concentration. The calculated values of total phenols, flavonoids and non-flavonoid compounds expressed as mg per 1 g extract of the test plant, *i.e.*, per 100 g of the plant are summarized in Table 1. They represent mean values of three successive measurements with an error being ±0.5. The above results on the quantitative determination of total phenols, flavonoids and non-flavonoids in the tested *Hypericum perforatum* L. extracts suggest that the highest contents of phenols and flavonoids were found in the acetonic extract (17.6 mg EPC/g dry extract and 16.85 mg EPC/g dry extract). The contents of total phenols and flavonoid compounds were, respectively, 50 and 60% lower in the ethanol extract of the plant, which however had the highest measured content of non-flavonoid components. The contents of total phenols and flavonoids were almost identical in the petroleum ether extract, which was also found to have the lowest content of non-flavonoid compounds. The antifungal activity of the *H. perforatum* L. extract was evaluated by the disk diffusion and dilution methods. The test samples included the spore suspension at a density of 0.2×10^4 CFU/mL for the disk diffusion and dilution methods. The duration of test culture growth was 48 and 72 h, respectively [20]. The test results are given in Table 2. The acetonic extract of *H. perforatum* L. showed poor antifungal activity. The fungi exhibited resistance to all extract concentrations tested. The minimum in-

Table 1. The amount of phenolic compounds expressed as mg per g dry extract

Extract	Phenolic compounds, mg EPC/g dry extract		
	Total phenols	Flavonoids	Non-flavonoids
Acetone	17.6	16.85	0.75
Ethanol	8.03	6.58	1.45
Petroleum ether	4.40	4.15	0.25

Table 2. The antifungal activity of the acetonic, ethanolic and petroleum ether extracts of H. perforatum *as evaluated by the disk-diffusion and dilution methods*

Fungal species	Dilution method, *MIC* / mg ml^{-1}						Disk-diffusion method; inhibition zone, mm														
	Acetonic extract		Ethanolic extract		Petroleum ether extract		Acetonic extract					Ethanolic extract					Petroleum ether extract				
							Extract concentration, mg/disk														
	Ex.[a]	Nys.[b]	Ex.	Nys.	Ex.	Nys.	25	10	5	2.5	Nys. (12.5)	25	10	5	2.5	Nys. (12.5)	25	10	5	2.5	Nys. (12.5)
Aa[c,d]	–[e]	2.50	20	2.50	10	2.50	–	–	–	–	78	25	–	–	–	78	44	20	–	–	78
Pc[f]	–	1.25	20	1.25	10	1.25	–	–	–	–	80	21	–	–	–	80	36	18	–	–	70
Ag[g]	–	2.50	–	2.50	20	2.50	–	–	–	–	88	20	–	–	–	88	50	20	–	–	80
Pf[h]	20	2.50	–	2.50	20	2.50	–	–	–	–	64	18	–	–	–	64	46	18	–	–	64

[a]Extract; [b]nystatin; [c]*Alternaria alternate* (Fr. Keissler); [d]fungal spore suspension density, 0.2×10^4CFU/mL; [e]the extract does not exhibit antifungal activity; [f]*Penicilium canescens* (Soop); [g]*Aspergills glaucus* (Link); [h]*Phialophora fastigiata* (Lagneb)

hibitory concentration of 20 mg/mL acetonic extract was determined for *Phialophora fastigiata*. The ethanolic extract of *H. perforatum* L. showed antifungal activity at a concentration of 25 mg per disk, with the inhibition zone measuring 25 mm. In terms of the temporal effect, following a 72-h growth period, all fungi exhibited resistance to the extract. The highest susceptibility to the concentration of 25 mg per disk was recorded for *Alternaria alternata*, with the inhibition zone measuring 25 mm. The minimum inhibitory concentration for the fungi *Alternaria alternata* and *Penicillium canescens* following a 48-h period was 20 mg/mL. The petroleum ether extract of *H. perforatum* L. showed antifungal activity against all five fungi at concentrations of 25 and 10 mg extract per disk, with the inhibition zone measuring 25 and 10 mm. Following a 72-h growth period, the effect of the extract decreased and inhibition zones were reduced by up to 50%. The highest susceptibility to the effect of the petroleum ether extract was detected for *Aspergillus glaucus* and *Alternaria alternate* in dilution methods, with the obtained value being 10 mg/mL for both fungi. Minimum inhibitory concentration was determined in all five fungi and it ranged from 10–20 mg/mL. The free radical scavenging capacity of the acetonic, ethanolic and petroleum ether extracts of *H. perforatum* L. was measured by DPPH assay and compared to the activity of ascorbic acid and BHT. The results are given in Figure 1. The DPPH radical scavenging capacity of the ethanolic extract was lower than the two antioxidants, but as the concentration increased, the inhibition values of the extract grew closer to their values. Petroleum ether extracts did not show antioxidant activity. The IC_{50} value, defined as the concentration of the test material leading to 50% reduction of the free radical concentration, was <7.8125, 105.9, 5.99 and 12.77 µg/mL for ethanolic extract, acetonic extract, ascorbic acid and BHT, respectively.

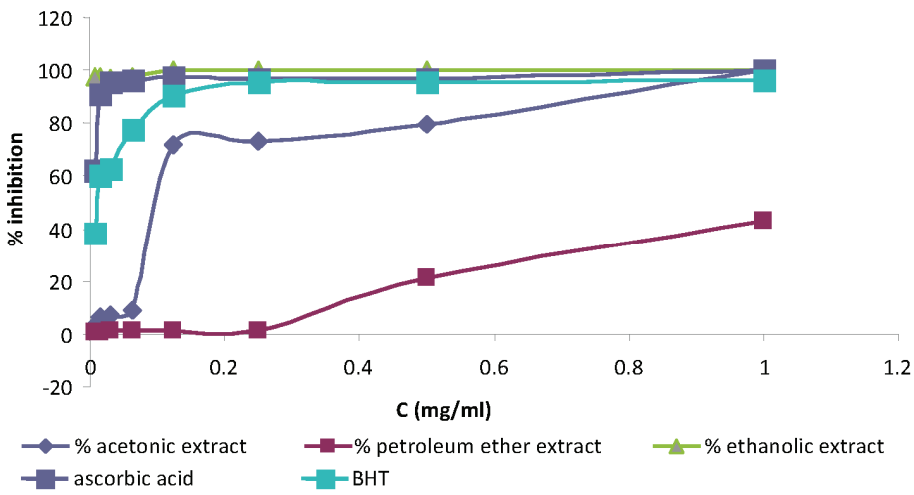

Figure 1. DPPH free radical scavenging activity. Each value is expressed as mean±standard deviation.

CONCLUSION

The highest amounts of phenols (17.6 mg EPC/g dry extract) and flavonoids (16.85 mg EPC/g dry extract) were found in the acetonic extract. The results revealed antioxidant activity of the ethanolic extract of *H. perforatum.* The IC_{50} values determined for each measurement were <7.81, 105.90, 5.99 and 12.77 mg/ml for ethanolic extract, acetonic extract, ascorbic acid and BHT, respectively, for DPPH free radical scavenging activity. The highest inhibitory effect on the growth of *Penicillium canescens*, *Fusarium oxysporum*, *Alternaria. alternata, Aspergillus glaucus* and *Phialophora fastigiata*, as identified by the disk diffusion method, was exhibited by the ethanolic extract at a concentration of 25 mg/disk. The *MIC* of the ethanolic and petroleum ether extracts was 20 mg/mL for the fungi *Alternaria alternata* and *Penicillium canescens*. Antioxidant and antifungal properties of the extracts and various extracts obtained from many plants are of great interest in both fundamental science and food industry, since their use as potential natural additives emerged from a growing tendency to replace synthetic antioxidants by natural ones. The present study confirmed the antifungal and antioxidant activities of *H. perforatum*. The obtained results suggest that *H. perforatum* has moderate antifungal activity under *in vitro* conditions against the test fungi as well as antioxidant activity relative to the control antioxidants.

REFERENCES

[1] O. Stark, Clinical Laboratory Analysis, Medicinska knjiga, Belgrade, Serbia, 1969.

[2] F. Gaedcke, St. John's wort herb extracts: manufacturing, standardization and characterization, In: Hypericum, Medicinal and Aromatic Plants-Industrial Profiles 31, E. Ernest, Ed., 2003, pp. 49–64.

[3] V. Butterweck, Mechanism of action of St. John' s wort in depression, CNS Drugs. **17** (2003) 539–562.

[4] A.B. Silva, F. Ferreres, J.O. Malva, Phytochemical and antioxidant characterization of *H. perforatum* alcoholic extracts, Food Chem. **90** (2005) 157–167.

[5] A. Singh Pal, Hypericin-A naphthodianthrone from *Hypericum perforatum*, Eur. J. Clin. Pharmacol. **62** (2006) 225–233.

[6] M.A. Couceiro, F. Afreen, S.M.A. Zobayed, Variation in concentrations of major bioactive compounds of St. John's wort: effects of harvesting time temperature and germplasm, Plant Sci. **170** (2006) 128–134.

[7] J. Reichling, A. Weseler, R. Saller, A current review of the antimicrobial activity of *Hypericum perforatum* L., Pharmacopsychiatry **31** (2001) 116–118.

[8] G.E. Meral, N.U. Karabay, *In vitro* antibacterial activities of three Hypericum species from west Anatolia, Elect. J. Biotechnol., Special Issue (2002) 6–10.

[9] G. Fenaroli, Aromatizzazione, Pirola, Milan, 1985.

[10] P.D. Duh, G.C Yed, Antioxidative activity of three herbal water extracts, Food Chem. **60** (1997) 639–645.

[11] M. Fernández, M. Garcia, M. Sáenz, Antibacterial activity of the phenolic acid fraction of *Scrophularia frutescens* and *Scrophularia sambucifolia*, J. Ethnopharmacol. **53** (1996) 11–14.

[12] Ph. Jug. IV, Pharmacopoeia Jugoslavica 4th ed., National Institute for Health Protection, Belgrade, 1984.

[13] NCCLS, Performance Standards for Antimicrobial Disc Susceptibility test. Approved Standard NCCLS Publication M2/A4, Villanova, PA, USA, 1990.

[14] S.K. Agarawal, S.S. Singh, S. Verma, Antifungal activity of anthraquinone derivatives from Rheumemodi, J. Ethnopharmacol. **72** (2000) 43–46.

[15] NCCLS, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard NCCLS Publication M7/A2., Villanova, PA, USA, 1992, 14–18.

[16] J. Hindler, Special antimicrobial susceptibility test, in: Textbook of Diagnostic Microbiology, C. Mahon, G. Manuselis, Eds., Elsevier Health Sciences, 1995.

[17] B. Dulger, A. Gonuz, Antibacterial activity of the endemic Hypericum kazdaghensis, Fitoterapia **76** (2005) 237–239.

[18] T. Takao, N. Watanabe, I. Yagi, K. Sakata, A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. Biosci. Biotechnol. Biochem. **58** (1994) 1780–1783.

[19] Y. Kumarasamy, M. Byres; P.J. Cox, M. Jaspars, L. Nahar, S.D. Sarker, Screening seeds of some Scottish plants for free-radical scavenging activity. Phytother. Res. **21** (2007) 615-621.

[20] F. Candan, M. Unlu, B. Tepe, Antioxidant and antimicrobial activity of the essential oil and methanol extracts of Achillea millefolium subsp. millefolium Afan. (Asteraceae), J. Ethnopharmacol. **87** (2003) 215-220.

IZVOD

SADRŽAJ FENOLNIH JEDINJENJA, ANTIOKSIDATIVNA I ANTIFUNGALNA AKTIVNOST ACETONSKOG, ETANOLSKOG I PETROL-ETARSKOG EKSTRAKTA BILJKE *Hypericum perforatum* L.

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(Naučni rad)

Cilj ispitivanja bio je da se utvrdi antifungalna i antioksidativna aktivnost nekih ekstrakata biljke *Hypericum perforatum* L. u suzbijanju razvoja nekih gljiva. Ispitivan je sadržaj ukupnih fenola, flavonoida i neflavonoida u etanolskom, acetonskom i petrol-etarskom ekstraktu ove biljke. Najveća količina fenola (17,6 mg EPC/g suvog ekstrakta) i flavonoida (16,85 mg EPC/g suvog ekstrakta) utvrđena je u acetonskom ekstraktu. Najveće inhibitorno dejstvo na rast Penicillium canescens, Fusarium oxysporum, Alternaria alternata, Aspergillus glaucus i Phialophora fastigiata utvrđeno na osnovu disk-difuzione metode ispoljeno je od strane etanolskog ekstrakta pri koncentraciji od 25 mg/disku. MIC etanolskog i petrol-etarskog ekstrakta iznosila je 20 mg/mL. Acetonski ekstrakt nije uticao na razvoj ispitivanih gljiva. Antioksidativna aktivnost ispitana je utvrđivanjem aktivnosti hvatanja slobodnih radikala (DPPH). Dobijeni rezultati pokazali su da etanolski ekstrakt *H. perforatum* L. poseduje najveću antioksidativnu aktivnost. Vrednosti IC_{50} utvrđene za svako merenje iznosile su <7,8125; 105,9; 5,99 i 12,77 µg/ml za etanolski ekstrakt, acetonski ekstrakt, askorbinsku kiselinu, odnosno BHT, za aktivnost hvatanja slobodnih radikala (DPPH).

Ključne reči: Antifungalna aktivnost • *Hypericum perforatum* • Ukupni fenoli • Antioksidans