Antioxidant and antineurodegenerative properties of St. John's-wort dry extract

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Received: September 21, 2017
Accepted: September 28, 2017
Published on-line: September 29, 2017
Published: December 25, 2017

Different biological activities of *Hypericum perforatum* L. dry extract was tested in this study. Antioxidant activity was evaluated by four different assays. Antineurodegenerative activity was tested using acetylcholinesterase (AChE) and tyrosinase inhibitory activity assays. In all four antioxidant assays, concentration dependent manner was noticed in the activity of tested extract. The most active was the highest applied concentration (500 µg/ml). In DPPH test, extract applied in concentration of 200 µg/ml showed similar or higher radical scavenging activity compared to the concentration of 100 µg/ml applied for standard substances BHA and BHT. The lowest antioxidant activity for the extract was detected in ABTS test. All applied concentrations of dry extract were significantly more potent than vitamin C in β-carotene test. Moreover, the extract reached 40.31 and 45.56% of the inhibition in AChE and TYR assays, respectively. In both cases, the most active concentration of the extract was 200 µg/ml.

Key words: *Hypericum perforatum, dry extract, biological activity*

http://dx.doi.org/10.5937/leksir1737005S

1. INTRODUCTION

Hypericaceae includes 8 genera, with about 360 species, but only *Hypericum perforatum* (St. John's wort) is officially accepted for its positive pharmacological activities. It is included in European Pharmacopoeia 8.0 as well as in monographs of the European Medicines Agency, World Health Organization or European Scientific Association for Phytotherapy (Ph.Eur.8.0., 2014; EMA, 2009; WHO, 2002; ESCOP, 2003). Flowering aerial parts of St. John's wort have been commonly used in a form of tea, hydroalcoholic extracts or oil extracts, while standardized, dry hydroalcoholic extracts are used for the preparation of herbal products, i.e. capsule. Mainly, such solid preparations are recommended in the treatment of mild to moderate depressive episode (Butterweck and Schmidt, 2007). Moreover, St. John's wort has been used traditionally for the treatment of mild gastrointestinal complaints and stomach ulcer, or externally as oil extract for skin problems and wound healing agent (Miller et al., 1993; Tucakov, 1996). Chemical composition of *H. perforatum* is very complex and the several groups of compounds are considered as the active principles, i.e. naphthodianthrone derivatives hypericin and pseudohypericin, phloroglucinol derivatives hyperforin and adhyperforin, flavonoids and xanthones (Del Monte et al., 2015; Zdunić et al., 2017; WHO, 2002). Those compounds are connected with variety of biological activities such as antibacterial, antioxidant, antiviral, antiinlammatory (Šavikin et al., 2007; Zdunić et al., 2009; Saddiqe et al., 2010; Zdunić et al., 2017). Moreover, *H. perforatum* extract is proved to be an antidepressant for the treatment of depression with less side effects then synthetic drugs (Volz, 1997; Peron et al., 2013). Also, it is considered as neuroprotective agent that can prevent neurodegenerative pathologies by regulating neurotransmitter release in animal model, as well as by its antioxidant and antiinflammatory activity (Grundmann et al., 2010; Jungke et al., 2011; Klusa et al., 2001).

The aim of this work was to study antioxidant and antineurodegenerative properties of St. John's-wort dry extract obtained for commercial purposes in the Production Sector of the Institute for Medicinal Plant Research "Dr. Josif Pančić".

2. MATERIALS AND METHODS

1. Plant material

St. John's-wort dry extract (4-6.5 : 1) was obtained from Production Sector of the Institute for Medicinal Plant Research "Dr. Josif Pančić". Extraction was done using 60% EtOH. Extract was brown colored powder, with 5% percentage of moisture, 0.3 mm degree of fragmentation and it contained 0.14% of

hypericin. The amount of hypericin was analyzed according to Ph. Eur. 8 and the amount corresponded to the requirement. Also, the amount of total phenolics was 58.11±0.94mg GAE/g (Singleton and Rossi, 1965) while the amount of total flavonoids was 28.55±0.65mg QE/g (Park et al., 1997).

2. Evaluation of antioxidant activity

2.1. DPPH assay

The scavenging activity of extract was evaluated using 2.2-dyphenyl-1-picrylhydrazyl (DPPH) assay (Blois, 1958) with slight modifications. One hundred µL of extract solutions in methanol (concentrations of 100, 200 and 500 µg/mL) and 900 µL of methanolic solution of DPPH (40 µg/mL) were mixed. BHA, BHT and ascorbic acid in concentrations of 50 and 100 µg/mL were used as positive controls (standards). Methanol was used as a blank, while control was prepared to contain methanol instead of extract/standard. Absorbance of the reaction mixture was measured after 30 minutes in the dark at room temperature at 517 nm. The decrease of absorption of DPPH radical at 517 nm was calculated using equation:

$$\text{Inhibition of DPPH radical (\%)} = \frac{AC - AS}{AC} x100\%$$

, where AC is the absorbance of control (without test sample) and AS is the absorbance of the test sample at different concentrations.

All experimental measurements were carried out in triplicate and the results are expressed as average of three measurements ± standard deviation.

2.2. ABTS assay

The scavenging activity of extract was evaluated using ABTS assay using procedure of Miller et al. (1993) with some modifications. Stock ABTS+ solution (7 mM) was prepared 12-16 hours before experiment in 2.46 mM potassium-persulfate and stored in the dark at room temperature, and then diluted by distilled water to obtain an absorbance of working solution 0.700 ± 0.020 at 734 nm. Extract solutions in methanol (25 µL) in concentrations of 100, 200 and 500 µg/mL were mixed with 1 mL of working ABTS+ solution and incubated for 30 min at 30ºC. The same procedure was applied for positive controls BHA, BHT and ascorbic acid in concentrations of 50 and 100 µg/mL. Absorbance was recorded at 734 nm, and distilled water was used as blank, while control contained distilled water instead of sample. The decrease of ABTS radical absorption at 734 nm was calculated using equation:

$$\text{Inhibition of ABTS radical (\%)} = \frac{AC - AS}{AC} x100\%$$

, where AC is the absorbance of control (without test sample) and AS is the absorbance of the test sample at different concentrations. All experimental measurements were carried out in triplicate and the results are expressed as average of three measurements ± standard deviation.

2.3. Ferric-reducing ability of plasma (FRAP) assay

Ferric-reducing ability of plasma (FRAP) assay evaluates total antioxidant power of the sample using reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a test sample at low pH. The FRAP assay was performed according to Benzie and Strain (1996) procedure with slight modifications. FRAP reagent was prepared freshly to contain sodium acetate buffer (300 mmol/L, pH 3.6), 10 mmol/L TPTZ in 40 mmol/L HCl and FeCl3 x 6H2O solution (20 mmol/L), i.e. in proportion 10:1:1 (v/v/v), respectively. Working FRAP solution was warmed to 37°C prior to use. Extract solutions in methanol (40 µL) in concentrations of 100, 200 and 500 µg/mL were added to 1200 µL of FRAP reagent and absorbance was recorded at 593 nm after 4 minutes. BHA, BHT and ascorbic acid (in concentrations of 50 and 100 µg/mL) were used as standards. Distilled water was used as blank, while control was prepared to contain distilled water instead of extract. The same procedure was repeated for standard solution of FeSO4 x 7H2O (0.2-1.6 mmol/L) in order to construct calibration curve. FRAP values of sample was calculated from standard curve equation and expressed as µmol FeSO4 x 7H2O/g dry extract. All experimental measurements were carried out in triplicate and the results are expressed as average of three measurements ± standard deviation.

2.4. β-carotene bleaching (ß-CB) assay

β-carotene bleaching assay, designed to evaluate the capacity of the antioxidants to reduce the oxidative loss of β-carotene in a β-carotene linoleic acid emulsion, was performed according to slightly modified procedure of Dapkevicius et al. (1998). β-carotene (1 mg), linoleic acid (50 µL) and Tween 40 (400 mg) were dissolved in 2 mL of chloroform. Chloroform was removed using a rotary evaporator at 40°C, and 200 mL of distilled water was added with vigorous shaking. The solutions of extract (100, 200 and 500 µg/mL) and positive controls BHA, BHT and ascorbic acid (50 and 100 µg/mL) were prepared in methanol. Aliquots of 1000 µL of the emulsion and 140 µL of sample (extract/standard) were mixed. Distilled water was used as blank, while control contained distilled water instead of sample. The absorbances were measured immediately (t=0 min) and after 2 h incubation (t=120 min) at 490. The antioxidant activity of the sample was evaluated in terms of inhibition of β-carotene bleaching using the following equation:

$$\text{\% Inhibition} = \frac{A120 - C120}{C0 - C120} x100\%$$

, where A120 and C120 are the absorbances measured in t=120 minutes for sample and control, respectively, while C0 is absorbance of control in t=0 min. All experimental measurements were carried out in triplicate and the results are expressed as average of three measurements ± standard deviation.

3. Evaluation of antineurodegenerative activities

3.1. Acethylacholinesterase (AChE) inhibitory activity assay

AChE inhibitory activity assay was performed according to spectrophotometric method (Ellman et al., 1961) using 96-well plates as described befor (Orhan et al., 2012) with slight modifications. The AChE activity was measured by monitoring of increase of yellow color produced from tiocholine when it reacts with DTNB ion. The test reaction mixture (S) was prepared by adding 140 µL of sodium phosphate buffer (0.1 M, pH 7.0), 20 µL of DTNB, 20 µL of extract-buffer solution containing 5% DMSO (concentration of 100, 200 and 500 µg/mL) and 20 µL of AChE solution (5 units/mL). The mixture without extract was used as the control (C), while blank (B) did not contain AChE solution. The commercial anticholinesterase alkaloid-type of drug galanthamine was used as reference. After incubation (15 min, 25°C), the reaction was initiated with the addition of 10 µL of acetylthiocholine iodide and absorbance was measured at wavelength of 412 nm using Tecan Sunrise SN microplate reader equipped by XFluor4 software. Percentage of inhibition of AChE by sample was determined using the formula:

$$\text{Inhibition of AChE (\%)} = \frac{C - (S - B)}{C} x100\%$$

All experimental measurements were carried out in triplicate and the results are expressed as average of three measurements ± standard deviation.

Table 1. Antioxidant activity of St John's-wort dry extract

Sample	Concentration	DPPH radical scavengy activity[a]	ABTS radical scavengy activity	FRAP activity	β-carotene bleaching activity
	(µg/mL)	% of inhibition	% of inhibition	µmol Fe(II)/g	% of inhibition
SJW dry extract	100	25.50±0.69a	12.94±0.73a	193.03±5.71a	23.68±1.02a
	200	44.69±0.39b	22.83±0.75b	361.98±3.80b	29.99±1.36b
	500	87.80±0.64c	48.13±0.80c	663.76±7.78c	59.85±1.94c
BHA	50	28.21±0.60d	56.18±0.80d	231.22±4.71a	50.79±2.76d
	100	43.33±0.87b	64.95±0.63e	572.85±5.71d	57.70±1.91c
BHT	50	20.86±0.81e	37.47±0.67f	364.05±5.62b	48.74±1.79d
	100	34.31±0.43f	55.11±0.44d	413.03±3.13be	56.29±1.44c
Vit C	50	58.99±1.01g	35.14±0.58g	427.56±5.18e	1.73±1.19e
	100	91.65±0.21h	55.70±0.69d	576.17±7.61d	2.99±2.13e

[a]Values with the same letter (a-h) in each column showed no statistically significant difference (P <0.05); Statistical analysis was based on a one-way ANOVA and Bonferroni tests.

3.2. Tyrosinase inhibitory activity assay

Tyrosinase inhibitory activity assay was performed according to slightly modified spectrophotometric method of Masuda et al. (2005) using 96-well plates. Samples (extract and standard kojic acid) were dissolved in sodium phosphate buffer (0.1 M, pH 7.0) containing 5% DMSO and phosphate buffer, respectively, in concentration of 100, 200 and 500 µg/mL. The wells were designed as: A (containing 120 µL of sodium buffer and 40 µL of tyrosinase in the same buffer (46 units/L), B (containing only buffer), C (containing 80 µL of buffer, 40 µL of tyrosinase and 40 µL of sample) and D (containing 120 µL of buffer and 40 µL of sample). After addition of 40 µL of L-DOPA and incubation (30 min, 25°C), absorbance was measured at 475 nm using Tecan Sunrise SN microplate reader equipped by XFluor4 software. Percentage of inhibition of tyrosinase was determined using the formula:

$$\text{Inhibition of tyrosinase (\%)} = \frac{(A-B)-(C-D)}{(A-B)} x100\%$$

All experimental measurements were carried out in triplicate and the results are expressed as average of three measurements ± standard deviation.

4. Statistical analysis

Results are presented as the mean value ± standard deviation of three independent replicate experiments (n = 3). Statistical analysis was based on a one-way ANOVA test.Statistically significant effects were further analyzed and means were compared using Bonferroni test. A level of P<0.05 was taken as statistically significant.

3. RESULTS AND DISCUSSION

1. Evaluation of antioxidant activity

Antioxidant activity of St. John's-wort dry extract was measured using four parallel tests and the results are presented in Table 1. Extract was tested in three concentrations (100, 200 and 500 µg/ml), while standard substances BHA, BHT and ascorbic acids, in concentrations of 50 and 100 µg/ml.

In all four assays, concentration dependent manner was noticed in the activity of tested extract. The most active was the highest applied concentration of 500 µg/ml. In DPPH test, extract applied in concentration of 200 µg/ml showed similar or better radical scavenging activity compared to the concentration of 100 µg/ml applied for standard substances BHA and BHT . The lowest antioxidant activity for the extract was detected in ABTS test. Considering FRAP assay, only concentration of extract of 500 µg/ml was more active (663.76 µmol Fe(II)/g) compared to the tested standard substances applied in concentration of 100 µg/ml. Similar was in ß-carotene test, but taking into account vitamin C, all applied concentrations of dry extract were significantly more potent than that standard substance in 100 µg/ml.

Del Monte et al. (2015) reported higher DPPH radical scavenging activity of extracts they analyzed compared to our extract. Briefly, methanol and chloroform–methanol extracts they analyzed showed 73.07% and 77.23% of scavenging activity, respectively but, chloroform extract did not exhibit DPPH scavenging activity (IC_{50} more than 100 µl). This difference is probably due to different solvents used in their study (methanol and chloroform–methanol)compared to 60% EtOH in our. Moreover, Heydarian et al. (2017), showed that crude polysaccharides extracted from St John's-wort have DPPH and OH free radicals scavenging activity.

1.1. Evaluation of antineurodegenerative activities

Enzyme inhibition activity of St. John's-wort dry extract was tested against two enzymes, acetylcholinesterase (AChE) and tyrosinase (TYR), which are connected with development of the different neurodegenerative disorders. Loss of memory that occurs in Alzheimer's disease (AD) is characterized by reduced level of neurotransmitter acetylcholine. Consequently, cholinesterase inhibitors are proved to be effective in treating patients suffering of mild to moderately level of AD (Ahmed et al., 2013). Tyrosinase, a key enzyme in the synthesis of melanin, is also involved in the formation of neuromelanin which hyperproduction is associated with Parkinson's disease (PD) (Greggio et al., 2005). The current therapy for neurodegenerative diseases dominantly reduce progression of the disease but do not lead to complete cure. Also, such therapy is often accompanied by side effects. Plants or natural products which could prevent, slow down or cure various disorders, including neurodegenerative diseases are of great

Table 2. Antineurodegenerative activity of St John's-wort dry extract

	Concentration (µg/mL)	AChE inhibitory activity[a] % of inhibition	Tyr inhibitory activity % of inhibition
SJW dry extract	100	35.44±1.01ab	41.08±4.60a
	200	40.31±4.73a	45.56±0.60ab
	500	31.32±2.22b	43.87±2.51a
Galanthamine	100	57.11±1.68c	-
Kojic acid	100	-	51.81±2.55b

[a]Values are means ± standard deviation. n = 3; Mean values within a column with different letters are significantly different at P<0.05; Statistical analysis was based on a one-way ANOVA and Bonferroni tests.

interest (Morzelle et al., 2016).
In our study, three concentrations (100, 200 and 500 µg/ml) of St. John's-wort dry extract were tested in AChE and TYR inhibition assays and compared with the activity of 100 µg/ml reference drugs galanthamine and kojic acid, respectively (Table 2). Both standard substances achieved an inhibition of over 50% at the concentration of 100 µg/ml, while our extract reached 40.31 and 45.56% in AChE and TYR assays, respectively. In both cases, the most active was concentration of 200 µg/ml of extract.
Although antineurodegenerative activities of *H. perforatum* extract were evaluated as weaker comparing to standards, it could be connected to the type of extragens applied for the production of extract.
Hernandez et al. (2010) tested water extracts of three Hypericum species from Portugal on AChE inhibitory activity and all species possessed activity but the lowest were detected for the extract of *H. perforatum*. Moreover, Cao et al. (2017) reported neuroprotective activity of 80% ethanolic *H. perforatum* extract against AlCl3-induced AD like pathology in rats. They concluded that beneficial effect of the extract could be due to its known antioxidant and anti-inflammatory activities.

CONCLUSION

In the present study, the results for antioxidant and antineurodegenerative activities of St. John's-wort dry extract are presented. The highest antioxidant activity has been noticed in DPPH test while the weakest was in ABTS test. All applied concentrations of dry extract were significantly more potent than vitamin C in β-carotene test. Antineurodegenerative activities of *H. perforatum* extract were evaluated as moderate. Future research will be focused on extraction optimization aimed to enrich the extract with compounds that could contribute to AChE and TYR inhibitory activities.

ACKNOWLEDGMENTS

Acknowledgment. This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, project number 46013, and 173029.

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