BIOMEDICAL POTENTIAL OF HOREHOUND EXTRACT
(MARRUBIUM VULGARE, LAMIACEAE)

Nebojša SALAJ, Jelena BARJAKTAROVIĆ, Nebojša KLADAR, Neda GA VARIĆ and Biljana BOŽIN

Summary

Introduction. Horehound (Marrubium vulgare, Lamiaceae) is a widely used plant in traditional medicine used for prevention and treatment of various diseases. High content of phenolic compounds makes it a significant source of natural antioxidants. The aim of this research was to examine in vitro antioxidant properties and anticholinesterase activity of horehound water-alcoholic extract, followed by preliminary chemical characterization of horehound.

Material and Methods. The in vitro antioxidant potentials of horehound water-alcoholic extract were assessed using several antioxidant test systems (neutralization of 2,2-diphenyl-1-picrylhydrazyl, hydroxyl, and nitroso radical, determination of ferric reducing potential, as well as inhibition of lipid peroxidation). Preliminary chemical profiling of the extract included estimation of total phenolic and flavonoid contents, while anticholinesterase potential of the examined extract was evaluated by spectrophotometry.

Results. The amounts of total phenolics and total flavonoids in the obtained extract were 59.87 ± 7.31 mg gallic acid equivalents/g of dry extract and 14.47 ± 0.54 mg quercetin equivalents/g of dry extract respectively. Furthermore, significant antioxidant potential was noticed in the ferric reducing potential assay (64.07 ± 2.68 mg ascorbic acid equivalents/g of dry extract), while concentrations needed for neutralization of 50% (IC50) of generated 2,2-diphenyl-1-picrylhydrazyl, hydroxyl and nitroso radical were 13.41 μg/mL, 64.86 μg/mL and 63.99 μg/mL, respectively. The potential of the extract to inhibit lipid peroxidation process was moderate (IC50 = 823.82 μg/mL), while in the case of anticholinesterase potential, the recorded IC50 value was 2821.15 μg/mL.

Conclusions. Horehound represents a significant natural antioxidant, mostly as a result of high levels of phenolic compounds. In addition, the examined ethanol extract has a certain anticholinesterase activity.

Key words: Marrubium; Lamiaceae; Phenols; Antioxidants; Flavonoids; Spectrophotometry; Acetylcholinesterase; Cholinesterase Inhibitors; Biomedical Research

Sažetak

Uvod. Očajnica (Marrubium vulgare Lamiaceae) široko je primjenjivana u tradicionalnoj medicini u prevenciji i terapiji različitih oboljenja, a zbog visokog sadržaja fenolnih jedinjenja predstavlja i značajan izvor prirodnih antioksidanata. Cilj istraživanja bilo je ispitivanje in vitro antioksidativnog potencijala i antiholinesterazne aktivnosti očajnice, uz preliminarnu hemijsku karakterizaciju ekstrakta.

Materijal i metode. Određen je sadržaj ukupnih fenolova i flavonoida u vodenokaholnom ekstraktu Marrubium vulgare, in vitro, antioksidativni (neutralizacija 2,2-difenil-1-pikrilhidrazil, hidroksil i nitroso radikala, određivanje potencijala redukovanja Fe3+, inhibicija lipidne peroksidacije) i antiholinesterazni potencijal.

Rezultati. Sadržaj ukupnih fenolova iznosio je 59.87 ± 3.71 mg ekvivalenta galne kiseline/g suvog ostatka, a flavonoida 14.47 ± 0.54 mg ekvivalenta kvancetina/g suvog ostatka u ispitivanom ekstraktu. Testirana vrednost redukcionog potencijala u redukovanih Fe+3 testu iznosila je 64.07 ± 2.68 mg ekvivalenta ascorbinske kiseline/g suvog ostatka. Koncentracija pri kojoj je 50% slobodnih radikala inhibirano (IC50) za 2,2-difenil-1-pikrilhidrazil iznosila je 13.41 μg/mL, za nitroso radikal 64.86 μg/mL, za hidroksilni radikal 63.99 μg/mL, dok je u procesu inhibicije lipidne peroksidacije IC50 vrednost iznosila 823.82 μg/mL. Koncentracija ispitivanog ekstrakta neophodna za inhibiciju 50% aktivnosti acetylcholinesteraze iznosila je 2821.15 μg/mL.


Ključne reči: Marrubium; Lamiaceae; fenoli; antioksidanti; flavonoidi; spektrofotometrija; acetylcholinesteraza; inhibitori holinesteraza; biomedicinska istraživanja

Introduction

Horehound (Marrubium vulgare, Lamiaceae) is a widely used species in traditional medicine of many cultures. However, further researches are necessary to clarify the correlation between high levels of phenolic compounds and benefits in the treatment of certain pathological conditions. Genus Marrubium includes about 30 species, mostly growing in the Mediterranean region, and less in central Europe, northern Africa and temperate region of Asia. Marrubium vulgare (M. vulgare) is a typical representative of the genus [1, 2].

Due to significant pharmacological effects, horehound is also used in conventional medicine for the
preparation of bitter tonics, as an additional therapy for digestive disorders, loss of appetite and dyspepsia [3]. Secondary metabolites of M. vulgare exhibit the following potentials: antinociceptive [4], antihypertensive [5], antiedematogenic [6], analgesic [7], anti-inflammatory [8], antimicrobial [9] (Anti-Helicobacter pylori [10]), insecticidal [11] and citoprotective [12]. In addition, hypoglycemic and hypolipidemic effects have also been confirmed [13].Aglycosides and hydroalcoholic extracts of the aerial plant parts have been reported for treatment of cough and digestive and biliary disorders [14]. Recently, the potential role in the inhibition of cyclooxygenase-1 (COX) and acetylcholinesterase (AChE) has been demonstrated [15].

Previous investigations of the chemical composition of M. vulgare leaves revealed the presence of flavonoids like apigenin and luteolin and their 7-O-glucosides, quercetin and its 3-O-glucoside and 3-O-rhamnoside. Also, recently ladanein (5,6-dihydroxy-7-4'-dimethoxyflavone) was isolated, with confirmed vasodilatory effects. Reactive oxygen species (ROS) are produced by the physiological processes in all aerobic organisms. Various factors such are environmental factors, poisoning, increased physical activity, biotransformation of xenobiotics or inflammation processes, can lead to imbalance between production of ROS and antioxidative defense of the organism resulting in wide spectra of pathophysiological conditions [17, 18]. Furthermore, β-amyloid peptides, one of pathohistological markers of patients suffering from Alzheimer’s disease (AD), induce inflammatory processes and formation of free radicals. Antioxidants represent free radical scavengers and they can prevent or decrease the intensity of inflammatory processes. Drugs used in the treatment of AD, by inhibition of acetylcholinesterase (AChE), increase the level of acetylcholine (ACh), reducing the symptoms of AD, but hardly exhibit any antioxidant potential and therefore do not interfere with the inflammatory processes. Currently applied synthetic drugs used in the treatment of cognitive impairment and memory loss show severe side effects, which implies an increasing of interest in finding better AChE inhibitors from natural sources which could additionally, by several mechanisms, target different pathophysiological processes [19, 20].

Plants are known resources of phenolic compounds, terpenoids and vitamins. Recently, flavonoids have begun attracting attention of scientists because of their potential healing effects in diseases caused by free radical processes. Flavonoids, like many other polyphenols, are efficient free radical scavengers due to high reactivity and activity as hydrogen or electron donors [21].

The investigation of biological potentials of aqueous-alcoholic extracts of horehound included a hypothesis that active compounds possess significant antioxidative potentials and ability to inhibit the AChE.

The aim of the research was in vitro evaluation of antioxidant potentials (neutralization of 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (OH) and nitroso (NO) radicals, determination of reducing ability of ferric reducing potential (FRAP), and inhibition of lipid peroxidation) followed by preliminary chemical characterization of the horehound extract through quantification of the content of total phenolics and flavonoids. Also, the in vitro anticholinesterase potential of M. vulgare extract was evaluated.

Material and Methods

The chemicals used in this research: ethanol (p. a.) – Zorka Pharma (Serbia), DPPH radical, S-acetylthiocholine iodide, sulphanilamide (SA), N-(1-naphthyl)-ethylenediamine dihydrochloride (NEDA) – Alpha Aesar (Germany); Folin-Ciocalteu (FC) reagent – Merck (Germany); sodium bicarbonate, iron(II)-sulphate, acetic acid and methanol (pro analysis) – POCH (Poland); gallic acid, aluminium chloride, 2-deoxy-D-ribose, 2-thiobarbituric acid – Sigma Aldrich (USA); hydrogen peroxide – Lach-Ner (Czech Republic); quercetin – Extrasynthèse (France); 5,5'-dithiobis-(2-nitrobenzoic acid) – J. T. Baker (USA); commercial solution of acetylcholinesterase – Roche (Switzerland); sodium nitroprusside (SNP) – Centrohemia (Serbia) and distilled water.

The aerial parts of the horehound (M. vulgare, Lamiaceae) were collected at the full blossom stage in southeastern part of Republic of Srpska (locality: Korićka jama; Global Positioning System coordinates: 43.055518, 18.503914), in June 2015. The sample was identified at the Department of Biology and Ecology, Faculty of Natural Sciences, University of Novi Sad. The voucher specimen of M. vulgare was confirmed and deposited in the BUNS Herbarium (Herbarium of the Department of Biology and Ecology, Faculty of Natural Sciences and Mathematics, University of Novi Sad; Voucher No. 2-1510). The plant material was stored at room temperature, at the Laboratory of Pharmacognosy, Department of Pharmacy, Faculty of Medicine, University of Novi Sad until starting the experiments.

The extract was prepared by maceration with 70% ethanol during 24 hours, according to the instructions

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**Abbreviations**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>OH</td>
<td>hydroxyl</td>
</tr>
<tr>
<td>NO</td>
<td>nitroso</td>
</tr>
<tr>
<td>FRAP</td>
<td>ferric reducing potential</td>
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<tr>
<td>M. vulgare</td>
<td>Marrubium vulgare</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>FC</td>
<td>Folin-Ciocalteu</td>
</tr>
<tr>
<td>GAE</td>
<td>gallic acid equivalents</td>
</tr>
<tr>
<td>QE</td>
<td>quercetin equivalents</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>RSC</td>
<td>radical scavenging capacity</td>
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<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
</tr>
<tr>
<td>d.e.</td>
<td>dry extract</td>
</tr>
<tr>
<td>AsAE</td>
<td>ascorbic acid equivalents</td>
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</tbody>
</table>

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for the preparation of commercially available extracts of horehound available on the market [22] and recommendations proscribed by the European Pharmacopoeia (6th edition) [23]. The ethanol extract was filtered, and then evaporated to dryness to determine the content of dry extract (extraction yield 20.88%). For further experiments, 20% of aqueous solution of the extract was prepared and preserved at -20°C until the experiments were performed.

The total phenolics content (TPC) was determined by previously mentioned FC spectrophotometric method [24]. Phenolics in reaction with FC reagent (mixture of phospho-molybdic and phospho-wolframic acid) form a blue-colored compound, with a maximum of absorbance at 760 nm. The content of total phenolics was expressed based on previously designed calibration curve of standard solution of gallic acid as mg of gallic acid equivalents (GAE) per g of dry extract (d.e.) (mg GAE/g d.e.).

The content of total flavonoids was quantified by a previously mentioned spectrophotometric method [24]. The result was expressed as mg of quercetin equivalents (QE) per g of dry extract (mg QE/g d.e.).

**Determination of DPPH, OH and NO’ neutralization**

The ability of investigated extract to neutralize DPPH, OH and NO radicals was examined using the previously described spectrophotometric methods [25, 26]. Different concentrations of investigated extract were added in solution of purple colored stable DPPH and change of color was monitored spectrophotometrically at 515 nm. Neutralization of OH radicals, which were generated in a Fenton reaction, was also monitored spectrophotometrically, based on the degradation of 2-deoxy-D-ribose to malondialdehyde (MDA), whereby MDA forms a compound with thiobarbituric acid. The ability of the extract to inhibit generated NO radicals was examined by the use of Griess reagent.

All the measurements were carried out in three replications, while the free radical scavenging capacity (RSC) of different extract concentrations was calculated by the following equation (1):

$$- (1) \text{RSC} (%) = (1 - A/A_0) 100\% \; A \text{ was the absorbance of working solutions, and } A_0 \text{ the absorbance of blank solutions. Based on RSC value, IC}_{50} \text{ values (the extract concentration providing } 50\% \text{ inhibition of DPPH, NO’ and OH’)} \text{ were determined by applying regression analysis.}$$

**Determination of lipid peroxidation inhibition**

The extent of lipid peroxidation was determined by thiobarbituric acid (TBA) assay [25] measuring the absorbance of compound produced in the reaction between TBA and MDA, as the final product of lipid peroxidation. Liposome “PRO-LIPO S” emulsion was used as a model-system of biological membranes. All the measurements were carried out in three replications, while the percentage of lipid peroxidation inhibition was calculated by the following equation (2):

$$- (2) I (%) = 100 - 100 \left( A/A_0 \right); A_0 \text{ was the absorbance of control mixture, and } A \text{ was the absorbance of the test mixture.}$$

**Determination of ferric-reducing antioxidant power assay**

Determination of reducing potential as an indicator of antioxidative potential of the investigated extract was based on the spectrophotometric method of Benzie et al. Antioxidants at low pH values reduce iron(III)-2,4,6-tripyridyl-s-triazine complex to the iron(II)-2,4,6-tripyridyl-s-triazine complex [27]. All of the experiments were performed in triplicate. Ascorbic acid was used as a standard substance in this method, and results were expressed as mg of ascorbic acid equivalents (AsAE) per g of d.e. (mg AsAE/g d.e.).

**Inhibition of acetylcholinesterase activity**

The anticholinesterase activity of the extract was evaluated spectrophotometrically by modified Ellman’s method with S-acetylthiocholine iodide as a substrate [28]. All the measurements were carried out in three replications, while the percentage of AChE inhibition was calculated by the following equation (3):

$$- (3) I (%) = 100 - \left( A/A_0 \right) 100; A_0 \text{ was the absorbance of reaction mixture containing extract, and } A_0 \text{ the absorbance of control mixture.}$$

All data were processed using the Microsoft Excel, v. 2010 software package.

**Results**

**Determination of total phenolics and flavonoids content**

The amount of total phenolic content in the extract of *M. vulgare* was 59.87 ± 7.31 mg GAE/g d.e., while the total flavonoid content was 14.47 ± 0.54 mg QE/g d.e. (Table 1), which indicates high levels of these classes of secondary metabolites occurring in the examined species.

**Table 1. Content of total phenols and flavonoids in the extract of *M. vulgare***

<table>
<thead>
<tr>
<th>Phenols/Fenoli</th>
<th>Flavonoids/Flavonoids</th>
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<tbody>
<tr>
<td>A1</td>
<td>0,6301</td>
</tr>
<tr>
<td>A2</td>
<td>0,6595</td>
</tr>
<tr>
<td>A3</td>
<td>0,7679</td>
</tr>
<tr>
<td>As</td>
<td>0,6858</td>
</tr>
<tr>
<td>Content/Sadržaj</td>
<td>59,87 ± 7,31 mg GAE/g s.e.</td>
</tr>
</tbody>
</table>
Determination of antioxidative potential

Based on the obtained values for neutralization of DPPH, OH and NO radical, as well as inhibition of the lipid peroxidation process, which negatively correlated with the applied concentrations of the extracts (Graph 1 a, b, c and d), IC\textsubscript{50} values were calculated. The strongest antioxidant potential was recorded in DPPH-test system (IC\textsubscript{50}=13.41 μg/mL), while higher IC\textsubscript{50} values were noticed in OH- and NO-test systems (IC\textsubscript{50}=63.99 μg/mL and IC\textsubscript{50}=64.86 μg/mL, respectively). Furthermore, the potential of the examined extract to inhibit lipid peroxidation process was relatively modest (IC\textsubscript{50}=823.82 μg/mL), which was surprising, considering the strong reduction potential demonstrated in FRAP assay (64.07 ± 2.68 mg AsAE/g d.e.).

Inhibition of acetylcholinesterase activity

The potential of water–alcoholic extract of M. vulgare to inhibit AChE is important in pathophysiology of AD. The concentration of the extract required for the inhibition of 50% AChE activity (IC\textsubscript{50} value) was 2821.15 μg/mL (Graph 2).

Discussion

The results of previous studies regarding chemical characterization of M. vulgare vary significantly depending on the geographical origin of the plant material and the analytical parameters of the conducted analyses (type of extraction, solvent selection, duration of extraction) [29, 30].

Previous studies showed that the methanolic extract of horehound leaves contained slightly less amounts of total phenolics and flavonoids (40.57 ± 1.91 mg GAE/g d.e. and 10.25 ± 0.08 mg QE/g d.e., respectively) than the obtained results of our study [8].
other hand, great variations are present within the other Marrubium species considering the content of total phenolics. Very low amounts of the secondary metabolites are present in M. deserti [30], but high amounts of phenolics and flavonoids were present in extracts of M. peregrinum [31]. The study of M. parviflorum showed similar quantities of total phenolics and flavonoids in the methanolic extracts (49.8 ± 2.69 mg GAE/g d.e. and 9.77 ± 5.23 mg QE/g d.e., respectively) as in our study, but the amounts of secondary metabolites in hexane extract were 6.42 ± 2.66 mg GAE/g d.e. and 5.36 ± 1.08 mg QE/g d.e., respectively [32]. Generally, the extraction of phenolic compounds increases with the polarity of the used solvent, but variations in the content of secondary metabolites between the samples of the same species originating from different geographical locations must not be neglected because of the influence of abiotic (climatic, edaphic and orographic), as well as biotic (genetic influence on biosynthesis of active principles) factors [31].

A study dealing with horehound harvested in Algeria, where this plant is widely spread, showed a good correlation of antioxidant potential and the content of phenolic compounds [30]. The ethyl acetate extract of horehound showed similar potential of DPPH neutralization as in our study (IC50 = 11.67 ± 1.51 μg/mL), and stronger potential of OH neutralization (IC50 = 8.2 ± 0.09 μg/mL) [33]. Furthermore, the results obtained by FRAP assay in our study were similar to the previous results obtained for horehound methanol and acetone extract [30]. Current literature review showed no data for in vitro examination of lipid peroxidation inhibition. However, a study carried out in Canada, stated that horehound leaves extract significantly inhibited copper-induced low density lipoprotein peroxidation and enhanced reverse cholesterol transport. These antioxidant properties increase the anti-atherogenic potential of high density lipoprotein and thus offer an additional natural antioxidant source for prevention of cardiovascular diseases [34].

Inhibition of acetylcholinesterase, a key enzyme in the degradation of acetylcholine, is a significant strategy in the treatment of neurodegenerative disorders, such as AD, dementia, ataxia, myasthenia gravis and Parkinson’s disease. In support of this, galantamine - an alkaloid isolated from green snowdrop (Galanthus woronowii, Amaryllidaceae), has been approved recently, in the treatment of mild to moderate forms of AD [35]. Several studies demonstrated the potential of Marrubium vulgare extract to inhibit the activity of various enzymes, such as acetylcholinesterase and cyclooxygenase-1 [15]. It was noticed that Marrubium vulgare largely inhibits cyclooxygenase-1 as compared with the simultaneously investigated flowers of Globularia alypum and leaves of Eryngium maritimum. Data suggest that horehound possesses a large percentage of phenolic compounds (primarily flavonoids and coumarins), iridoids and monoterpenes, which could be responsible for the anti-inflammatory effects [8, 15]. The study of several Mediterranean plants, including Marrubium vulgare, revealed significant anticholinesterase potential of horehound, while the obtained IC50 value (3062.78 μg/mL) was comparable to our results [15].

Conclusion

The obtained results indicated significant biomedical potentials of Marrubium vulgare extract and suggested the possibility of its exploitation in pharmacy and phytotherapy in the future. It can be concluded that this plant is a potentially significant natural source of antioxidants, especially when polar solvents are being used for extraction. Also, the ethanolic extract of horehound exhibited an anticholinesterase potential. However, future investigations should be directed towards detailed chemical profiling followed by fractionation of extracts guided by different biological potentials, as well as towards future in vivo studies.

References
