CHEMICAL, ANTIOXIDANT AND TOXICITY EVALUATION OF ROSEMARY LEAVES AND ITS DRY EXTRACT

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Abstract

Rosmarinus officinalis L. is an aromatic herb widely used in traditional medicine, cosmetics and gastronomy. The aim of our research was to obtain, chemically characterize, and evaluate the antioxidant activity and cytotoxic properties of an ethanolic dry extract from Romanian Rosmarinus officinalis L. (rosemary) leaves. The flavonoids, phenolcarboxylic acids, total phenol and rosmarinic acid contents of the raw material and of the extract were established using spectrophotometric and HPLC methods. The antioxidant activity of the extract was assessed using two methods (DPPH and ferric reducing assay). The extract contains: 3.22% of flavonoids (expressed as rutin), 34.30% phenolcarboxylic acids (expressed as chlorogenic acid), 31.86% total phenols (expressed as tannic acid) and 3.29% rosmarinic acid. The antioxidant activity of the extract was good (EC_{50} = 4.63 µg/mL - for DPPH method; EC_{50} = 63.85 µg/mL - for ferric reducing power). Cytotoxic effects of the extract upon Daphnia magna invertebrate were registered only at very high doses (> 500 µg/mL).

Rezumat

Rosmarinus officinalis L. este o plantă aromatică, mult folosită în terapeutică, cosmetică și gastronomie. Obiectivul acestei cercetări a constat în obținerea, caracterizarea chimică, stabilirea capacității antioxidante și a toxicității unui extract etanolic uscat din frunze de Rosmarinus officinalis (rozmarin), produs indigen. Flavonene, acizii fenolici polifenoli totali și acidul rozmarnic s-au determinat prin metode spectrototométrice (UV-VIS) și cromatografice (HPLC) în materia primă vegetală și în extract. Activitatea antioxidantă a extractului a fost evaluată prin două metode (DPPH și reducerea ferului). Extractul etanolic uscat are un conținut de 3,22% flavone (exprimate în rutozidă), 34,30% acid clorogenic (exprimați în acid clorogenic), 31,86% polifenoli totali (exprimați în acid tanic) și 3,29% rosmarinic acid. Capacitatea antioxidantă este importantă (EC_{50} = 4,63 µg/mL - metoda DPPH; EC_{50} = 63,85 µg/mL - reducerea ferului). Toxicitatea testată asupra invertebratului Daphnia magna a fost observată la doze mari (peste 500 µg/mL).

Keywords: rosemary, rosmarinic acid, antioxidant activity, Daphnia magna

Introduction

Rosmarinus officinalis L. is an aromatic herb, native from the Mediterranean basin, with culinary, medicinal and fragrance uses [14]. Phytochemical studies regarding rosemary showed that the major family of chemical constituents are phenolcarboxylic acids (rosmarinic acid, caffeic acid, quinic acid, vanillic acid, syringic acid) [5]. Other compounds are: flavones (homoplatantin, cirsimaritin, genkwanin, galloclatechin, nepetrin, hesperidin, 6-hydroxyluteolin-7-glucoside, luteolin-3’-glucuronide and isomers etc.) [5, 26], diterpenes (carnosic acid, carnosol, rosmanol, its isomers epirosorosmanol and epirosmanol, rosmadial, rosmariniphenol, as well as other derivative compounds such as methylcarnosate, epirosmanolmethyl ether and 5,6,7,10-tetrahydro-7-hydroxyrosmaquinone) [5, 22], triterpenes (ursolic, betulinic and micromeric acids, anemosapogenin) [5] and essential oil (with significant variations in the chemical composition, in relation to vegetative stage, botanical origin and environmental conditions) [40].

Previous pharmacological researches regarding rosemary reported numerous health benefits such as neuroprotective effect (through the inhibition of apoptosis-related genes expression, Bax, Bak, Caspase-3 and -9) [29] and improvement on long-term memory by inhibition of acetylcholinesterase in rats frontal cortex and hippocampus (the inhibitory activity is higher for carnosic acid than for rosmarinic acid) [3, 28]. According to Azad N. et al., carnosic...
acid could decrease neuronal death in the *cornu ammonis* region of the hippocampus due to its antioxidant properties and may ensure protection against neurodegenerative effects induced by β-amyloid plaques [2]. Another study showed that carnosic acid protects neuronal cells under ischemia/hypoxia through scavenging or reducing ROS (reactive oxygen species) and inhibiting COX2 (cyclooxygenase type 2) and MAPK (mitogen-activated protein kinase) pathways [17].

Considering all the above, the aim of our study was the obtaining, phytochemical characterization and evaluation of the antioxidant activity and cytotoxic properties of a selective dry extract from indigenous rosemary leaves, in view of further including it in a phytomedicine, with potential neuroprotective activity.

**Materials and Methods**

**Raw material.** Rosemary leaves (*Rosmarini folium*) were harvested from an ecological crop, from Hunedora District, Romania, in 2015. A voucher specimen is deposited in the official Herbarium of the Pharmacognosy, Phytochemistry and Phytotherapy, Department of the University of Medicine and Pharmacy, Bucharest, Romania. The raw material was packed in paper bags and stored for 2 weeks, until the beginning of the experiments.

**Reagents and solvents.** All reagents and solvents were purchased from Roth. (Germany), unless otherwise stated.

The research was performed in the following steps: 1. quality assessment of the raw material; 2. evaluation of the antiradical activity of rosemary leaves using two tests: DPPH method and ferric reducing power assay; 3. obtaining of the dry selective extract and its phytochemical characterization; 4. *in vitro* evaluation of rosemary dry extract antioxidant properties; 5. assessment of the cytotoxic properties of the dry extract (*Daphnia magna* bioassay).

**The quality of the raw material** was assessed by spectrophotometric methods (quantification of flavonoids, phenolcarboxylic acids and total phenolic compounds) and HPLC analysis (used for identification and quantification of rosmarinic acid).

**Preparation of stock solutions used for spectrophotometric and chromatographic analysis of rosemary leaves:** 2.00 g of *Rosmarini folium* were extracted under a reflux condenser with 100 mL of 50% (v/v) ethanol, for 30 minutes. After cooling and filtration, the filtrates were brought into volumetric flasks and completed with 50% (v/v) ethanol until 100 mL.

**Spectrophotometric assays.** The flavonoids content (F) was determined based on the chelating reaction with aluminium chloride [4]. Phenolic acids (PAC) were assessed based on formation of oxymes in the presence of sodium nitrite/ hydrochloric acid and sodium hydroxide [41]. The total phenolic content (TP) was evaluated based on the capacity to reduce molybdenic compounds (VI) [32]. For all spectrophotometric determinations a Jasco V-530 spectrophotometer (Jasco, Japan) was used. The following calibration curves were used to calculate the content of active compounds: rutin (linearity range: 5.0 - 35.0 µg/mL, r² = 0.9998, n = 11), chlorogenic acid (linearity range: 0.0113 - 0.0527 mg/mL, r² = 0.9998, n = 6) and tannic acid (linearity range: 2.0 - 12.0 µg/mL, r² = 0.9990, n = 10).

**HPLC analysis** was carried out using a previously published external standard method with gradient elution and UV detection [12, 13].

**Evaluation of the antiradical activity of rosemary leaves** was assessed using DPPH radical scavenging method conducted according to Brand-Williams W. et al. [6], and ferric reducing power performed according to Oyaizu method [27]. The detailed techniques were previously published [12]. The concentration ranges were 8.00 - 80.00 µg/mL (for DPPH assay) and 64.9 - 3239.00 µg/mL (for ferric reducing power method), respectively.

The results were expressed as EC₅₀ factor (µg/mL) that represents the concentration of the test solutions that inhibited 50% of the DPPH radical activity (for DPPH method)/concentration of the test solutions providing 0.5 of absorbance (for ferric reducing assay). The antioxidant activity of our samples was compared to that of gallic acid (used as standard reference).

**Obtaining and phytochemical characterization of the dry extract**

For the preparation of rosemary dry extract (*Rosmarini extractum*), the dried and finely ground raw material was extracted by 50% (v/v) ethanol, as previously described [12]. The extraction yield was expressed as the percentage of the total mass of the dry extract (*Mext*, without the water content) with respect to the mass of the raw material (*Mpv*) loaded onto to the flask for solvent extraction:

\[ Y\% = (\frac{Mext}{Mpv}) \times 100 \] [39].

For the phytochemical analysis of the rosemary dry extract there were used the same methods employed for the raw material characterization, as presented above. The stock solution was a 0.1% solution, prepared by dissolving 0.1 g extract in 100 mL of 50% ethanol.

**In vitro evaluations of rosemary dry extract antioxidant properties**

The antioxidant activity of the extract was evaluated using the same methods, previously described above. The concentration range was 2.0 - 250.0 µg extract/mL for DPPH method and 4.0 - 250.0 µg extract/mL for ferric reducing power method, respectively.

Furthermore, a correlation between the antioxidant activity (expressed as gallic acid equivalents) and
total phenolic content was carried out using Pearson coefficient.

Assessment of the cytotoxic properties of the dry extract

The cytotoxicity of the extract was assessed using Daphnia magna bioassay, according to the methods described by Fan W. et al. with some modifications [10, 25, 34]. The method consists in the exposure of the invertebrates to serial dilutions of the test substance/compound and counting the survivors after 24 and 48 h of exposure. The data regarding the protocol technique was previously presented, including the same concentration range [12]. The cytotoxic activity is presented as lethality percentages.

Statistical analysis. The statistical analysis was performed using Microsoft Excel 2010 software (Microsoft Corp., USA) and GraphPad Prism v. 5.0. (GraphPad Software, USA).

Results and Discussion

Ethanol is extensively used to extract antioxidant compounds from herbal products like rosemary, lemon balm or oregano [1, 30, 35]. 50% ethanol gives higher polyphenolic content and higher extraction yield, than absolute ethanol, therefore we choose it for extraction [20]. The extraction yield was 21.65% and was in agreement with Juntachote T. et al. results (20.60%) [19].

According to the scientific literature [38], rosmarinic acid is the main phenolic acid found in rosemary. Attempts regarding optimization of extraction of rosmarinic acid from Melissa officinalis indicate that 50% ethanol was the optimum extraction solvent [1]. All results, regarding the quantitative assays (HPLC and spectrophotometric analysis) of the active substances from raw material and rosemary dry extract are presented in Table I.

Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>g% PCA</th>
<th>g% F</th>
<th>g% TP</th>
<th>Rosmarinic acid (g%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosmarini folium (raw material)</td>
<td>8.00 ± 0.24 g chlorogenic acid/100 g raw material</td>
<td>0.90 ± 0.31 g rutin/100 g raw material</td>
<td>7.88 ± 0.13 g tannic acid/100 g raw material</td>
<td>0.09 g/100 g raw material</td>
</tr>
<tr>
<td>Rosmarini extractum (dry extract)</td>
<td>34.30 ± 2.21 g chlorogenic acid/100 g extract</td>
<td>3.22 ± 1.11 g rutin/100 g extract</td>
<td>31.86 ± 0.13 g tannic acid/100 g extract</td>
<td>3.29 g/100 g extract</td>
</tr>
</tbody>
</table>

PCA = phenolic acids, F = flavonoids; TP = total phenolic content.

Results are expressed as average with standard deviation after five independent experiments.

The flavonoids content of raw material (0.90 g rutin/100 g vegetal product, equivalent to 0.44 g quercetin/100 g vegetal product) may be compared (or slightly higher) to other authors’ results, like Ben Jemia M. et al. (0.22 - 0.63 g quercetin/100 g leaves) and Švarc-Gajic J. et al. (0.04 - 0.35 g rutin/100 g leaves) [3, 36]. The flavonoids content determined in the dry extract (3.22 g rutin/100g dry extract, which might be equivalent to 1.59 g quercetin/100 g dry extract) shows a higher value than that found by other authors (0.02 g rutin/100 g extract [21], 0.31 g quercetin/100 g extract [23]).

Regarding the content of total phenols of rosemary extract, our results are higher than that reported by other authors in ethanolic extracts (14.20 g of tannic acid/100 g of extract) [11]. We assume that the quantitative differences are the consequence of different extraction methods.

Our results indicate a high content of phenol-carboxylic acids for the raw material and for the extract (8.00 g chlorogenic acid/100 g raw material and 34.30 g chlorogenic acid/100g rosemary extract). Rosmarinic acid was identified in both rosemary leaves and dry extract. According to scientific data, the rosmarinic acid content of rosemary leaves and of rosemary extract vary widely: 0.07 g/100 g raw material [8], 1.02 - 1.45 g/100 g extract (depending on the extraction procedure) [16] and 5.46 g/100 g extract [28]. Our results (0.09 g/100 g raw material, 3.29 g/100 g extract) are within these ranges of concentrations, which prove a good quality of the raw material and an adequate extraction procedure.

In many studies, the antioxidant activity of rosmarinic acid is linked to its neuroprotective, photoprotective and antiinflammatory activity [17, 29, 31]. Therefore, in order to obtain an extract with neuroprotective activity it was important to evaluate its antioxidant activity and that of the corresponding raw material. The results of the antioxidant evaluation are found in Table II.

Table II

Antioxidant activity of Rosmarini folium and Rosmarini extractum

<table>
<thead>
<tr>
<th>Sample</th>
<th>EC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH</td>
</tr>
<tr>
<td>Rosmarini folium (raw material)</td>
<td>83.75 ± 1.76</td>
</tr>
<tr>
<td>Rosmarini extractum (dry extract)</td>
<td>4.63 ± 0.30</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>15.15 ± 0.33</td>
</tr>
</tbody>
</table>

Results are expressed as average ± standard deviation after three independent experiments.
As it was expected, the rosemary extract has a higher antioxidant activity than the raw material. Considering the scientific literature, the antioxidant activity of the rosemary extract, expressed as EC$_{50}$ varies greatly (EC$_{50}$ = 24.00 µg/mL [37], EC$_{50}$ = 54.00 µg/mL [9], 151.00 µg/mL - 597.00 µg/mL [18]). However, these differences might be the consequence of different work protocols. Our results show that the dry extract has a better scavenger capacity upon DPPH free radical compared to gallic acid. Probably, the high content of rosmarinic acid and the presence of other compounds like carnosic acid [9] may explain the higher DPPH scavenging effect of rosemary extract. The antioxidant activity of rosemary extract was found to be higher than that of rosmarinic acid [9]. Considering the ferric reducing power assay, gallic acid had a higher antiradical activity than rosemary leaves and extract. It is well-known that polyphenols are highly effective free-scavenging donors, hydrogen donors and metal chelators. Also, their antioxidant activity is related to the chemical structure, particularly to the number and position of the hydroxyl groups [7, 33]. The relationship between the total phenolic content and the antioxidant activity has been investigated using the Pearson correlation, expressed as $r$ (Figure 1). $r$ values indicate a positive correlation (DPPH: $r = 0.84$; ferric reducing assay: $r = 0.74$). These results suggest that polyphenols and other compounds act as antiradical for both vegetal product and extract. Although, in our study we found a positive correlation between rosemary (both leaves and extract) polyphenol concentration and its antioxidant activity, the scientific data are controversial [11, 20, 21, 24].

![Figure 1.](image)

Relationship between TP (total phenolic content) and the antioxidant activity expressed as gallic acid equivalents (Eq. gallic acid): A) DPPH method; B) ferric reducing assay

The cytotoxicity evaluation of rosemary extract (Table III, Figures 2 and 3) showed a high toxicity, inducing lethality in the range of 90% - 100%, at concentrations between 500 and 1500 µg/mL after a 24 h exposure. At concentrations lower than 250 µg/mL, the toxicity did not exceed 20%. The toxicity increased significantly at 250 µg/mL from an average of 50% at 24 h to 90% after 48 h of exposure.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time of exposure</th>
<th>LC$_{50}$ (µg/mL)</th>
<th>IC95% (µg/mL)</th>
<th>Goodness of fit ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosmarini extractum</td>
<td>24 h</td>
<td>246.6</td>
<td>202.3 - 300.5</td>
<td>0.9605</td>
</tr>
<tr>
<td>(rosemary dry extract)</td>
<td>48 h</td>
<td>109.0</td>
<td>78.47 - 151.3</td>
<td>0.9729</td>
</tr>
</tbody>
</table>

The values above 0.85 of the $r^2$ indicate a good correlation between the concentration and the biological effect. By analysing the lethality curves plotted for the rosemary extract, we assume that the toxicity is cumulative, the curves being almost parallel.

![Figure 2.](image)

Lethality on Daphnia magna versus the concentration of extract
Concentration-lethality curves for cytotoxic activity on Daphnia magna

These results are in accordance with literature data that suggest that polyphenols behave at higher concentrations as pro-oxidants and can induce apoptosis [15]. Further studies are needed in order to establish the pharmacological profile and the therapeutic doses.

Conclusions

A sample of Romanian rosemary leaves was chemically characterized and its antioxidant activity was determined. It contains 0.90 g flavonoids (expressed as rutin)/100 g raw material, 8.00 g phenolcarboxylic acids (expressed as chlorogenic acid)/100 g raw material, 7.88 g total phenolic compounds (expressed as tannic acid)/100 g raw material and 0.09 g rosmarinic acid/100 g raw material. The antioxidant activity of the rosemary leaves (towards DPPH free radical and in the ferric reducing test) were lower compared to gallic acid (used as standard reference). Using ethanol 50% as extraction solvent and an in-house preparation protocol, a dry extract was obtained. The extraction yield was 21.65%.

This extract was chemically characterized and their antioxidant activity and cytotoxic properties were assessed. The dry extract has a high content of flavonoids (3.22 g rutin/100 g extract), phenol-carboxylic acids (34.30 g chlorogenic acid/100 g extract), total phenolic compounds (31.86 g tannic acid/100 g extract) and rosmarinic acid (3.29 g/100 g extract).

The extract has an important antioxidant activity (EC$_{50}$ = 4.63 µg/mL - for DPPH method; EC$_{50}$ = 63.85 µg/mL - for ferric reducing power). The scavenger activity towards DPPH free radical was higher compared to that of gallic acid. Considering the ferric reducing power assay, the extract had lower antioxidant activity than gallic acid. It is a positive correlation between the content of total phenols and antioxidant activity (DPPH: $r$ = 0.84; ferric reducing assay: $r$ = 0.74).

The cytotoxicity of the extract upon Daphnia magna invertebrate appears only at very high doses (at least 500 µg/mL).

Further research is needed in order to establish the pharmaco-toxicological profile of the extract, and after that to obtain a phytomedicine with potential neuroprotective activity.

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