Influence of chemical composition on the antioxidant and anti-inflammatory activity of *Rosmarinus officinalis* extracts

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Abstract

Inflammation, especially the chronic inflammation, plays an important role in the pathogenesis and progression of major diseases. The aim of this study is to explore the antioxidant-anti-inflammatory potential of *Rosmarinus officinalis* L. extracts with different polyphenol content and obtained by distinct processing methods from the same vegetal material. The two rosemary extracts isolated by acetone maceration (R1) and hydroalcoholic extraction under reflux (R2), contain 3.14% and respectively 8.84% total polyphenols as determined by Folin-Ciocalteu method. Moreover, HPLC analysis proves that R2 extract have a higher content of rosmarinic and ferulic acids. Correlated to higher polyphenol content, R2 extract exerts a good scavenging activity on DPPH radical (20-79% at 1-10mg/mL concentrations), inhibits ICAM-1 production on TNF-α-stimulated endothelial cells culture and reduces rat paw edema fresh egg-white-induced on the entire experimental period, comparable to acetylsalicylic acid. In contrast, R1 extract show more weaker and shorter effects. As the triterpenic content was found similar for the two extracts, it can be concluded that polyphenols exhibit not only antioxidant effects but also contribute to antiinflamatory activity of rosemary extracts.

Keywords: Rosemary, Lamiaceae, ICAM-1, DPPH, polyphenols

1. Introduction

Inflammation, especially the chronic inflammation, plays an important role in the pathogenesis and progression of major diseases including the life-threatening degenerative diseases such as cancer, cardiovascular disease (CVD), stroke, renal failure and chronic obstructive pulmonary disease (COPD) (WU&WU, 2007 [1]). Generation of ROS (reactive oxygen species) is one of the most important aspects of the inflammatory process because these molecules are able to over-ridden proinflammatory gene expression by activating transcription factors such as NF-kB.

Inflammation seems to be the root of many chronic diseases and the need to develop effective drugs has become imperative. With the current understanding of the complexity of the inflammatory process, there are hundreds of potential therapeutic targets.

*Rosmarinus officinalis* L. (Lamiaceae) – rosemary – is a native species to the Mediterranean region of Europe, and cultivated world-wide. It contains up to 2.5% of...
essential oil, the main constituents of which are camphor (5-21%), 1,8-cineole (15-55%), -pinene (9-26%), borneol (1.5-5.0%), camphene (2.5-12.0%), -pinene (2.0-9.0%) and limonene (1.5-5.0%). Phenolic compounds are represented by flavonoids with a methylated aglycone (e.g. genkwanin) and by phenolic acids (> 3%), particularly by rosmarinic, chlorogenic and caffeic acids. Tricyclic diterpenes such as rosmaridiphenol, carnosol, carnosic acid and rosmanol, and diterpenes, including seco-hinokiol are also present (WHO Monograph, 2009 [2]).

Bioactive compounds in rosemary extracts exert antioxidant, antimicrobial, anti-inflammatory and chemopreventive activities. A good correlation between the antioxidant activities and total phenol content in the extracts was found (MORENO & al., 2006 [3]) and one possible explanation for this activity was suggested by membrane biophysics models which showed that polyphenols stiffen plasma membrane and prevent the diffusion of free radicals (PEREZ-FONS & al, 2006 [4]). Antiinflammatory activity is mostly correlated to triterpenic compounds content, the effects being attributed to inhibition of different stages of inflammatory reaction – histamin release, COX and LOX activity, NO production (ALTINIER & al, 2007 [5]), but a high polyphenol content could be useful for selective inhibition of COX-2 (YI & WETZSTEIN, 2010 [6]).

The aim of the present study is to explore the antioxidant-antiinflammatory potential of rosemary extracts with different polyphenol content and obtained by distinct processing methods from the same vegetal material.

2. Materials and Methods

Cell lines, chemicals and biochemicals
Folin-Ciocalteu’s phenol reagent, 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH), rosmarinic acid, caffeic acid, ferulic acid, gallic acid, quercetin and apigenin were purchased from Sigma Aldrich-Fluka. Acid acetylsalicylic acid was purchased from Zentiva, Romania. HUVEC (human umbilical vein endothelial cells) cell line was purchased from ATCC. ELISA kit for ICAM-1 quantification was purchased from Invitrogen USA and TNF- from Alexis, Switzerland. The assay was performed according to manufacturer’s instructions. All other reagents used in cell culture assay were from Sigma.

Extraction and Isolation
Rosmarinus officinalis L. aerial parts were obtained from indigenous crops (Bucharest, Romania) and dried and ground as a fine powder. A voucher specimen was deposited at the manufacturer (079/2013).

Method consisted of extraction of the active substances from 200g Rosmarini herba with acetone (vegetal material / solvent ratio = 1/7 m/v) at room temperature for 3 hours with occasional stirring, followed by filtration of the extract, solvent evaporation at reduced pressure until a precipitate appeared. After filtration, the precipitate was dried at 40°C yielding R1 sample (1,34g). The filtrate was concentrated to dryness under reduced pressure and re-dissolved in 150ml methanol. In the meantime, wet plant material was re-extracted with 20% ethanol (vegetal material / solvent ratio = 1/10 m/v) under reflux for 2 hours and continuous stirring. After filtration, the extract was concentrated under reduced pressure for alcohol removal and precipitated with the methanolic solution obtained previously. The resulting precipitate was dried at 40°C yielding R2 sample (9,60g).
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Determination of total phenolics

Total phenolics content was determined according to the Folin-Ciocalteu method (JAVANMARDI & al, 2003 [7]). The total phenolic content was expressed as gallic acid by reference to the gallic acid standard calibration curve in milligrams per gram sample. Results are presented as percentage.

Free radical scavenging assay

The extracts were diluted in methanol solution at the concentration of 5mg/mL and 1mg/mL. 50 μl aliquots of the extract were mixed with 2950 μl of the methanolic DPPH solution (0.025g/L).

The reduction of the DPPH free radical was measured by reading the absorbance at 517nm and related to the absorbance of the control without the herbal drugs. Inhibition ratio (percent) was calculated from the following equation: % inhibition = [(absorbance of control – absorbance of sample)/absorbance of control] x 100%. Concentration which decreased initial concentration of DPPH by 50% was defined as EC50. Quercetin (0.2mg/mL) was used as positive control.

HPLC analysis

A method for quantification of polyphenols was developed. A modular Shimadzu system comprised of a LC-20AD pump, a CTO-20A column oven, a DAD-MS detector with SPD-M20A diode array and MS Shimadzu, a DGU-20As degasser and a LC-MSsolution software were used. Phytochemicals were separated on a Kromasil C18 analytical column for polyphenols (4.6 mm x 100 mm).

Separation of polyphenols was done using a mobile phase comprised of water acidified with 1% formic acid (A component) and methanol acidified with 1% formic acid (B component) at an initial flow rate of 0.1 mL/ min; with an injection of 15 μL. The following gradient was applied: initially 5% B for 15 min; to 30% B in 20 min and flow another 20 min; to 50% B in 50 min; to 5% B in 2 min and continuing at 0% B until completion of the run.

Before use, all mobile phases were filtered through a 0.20 μm membrane (CHROMAFIL ® O-20/25) and dis-aerated in an ultrasonic bath.

ICAM-1 assay

Human umbilical vascular endothelial cells (HUVEC) were cultivated on 96 well-plates (Corning, USA) in Dulbecco's Modified Eagle's Medium low-glucose containing 10% fetal bovine serum and 1% penicillin-streptomycin-neomycin solution. The cultures were maintained at 37°C and 5% CO2. After reaching a subconfluent stage, the cells were treated with various concentrations of extracts (50-200 μg/mL dissolved in culture medium without serum) and 10mM acetylsalicylic acid (Zentiva, Romania) which were maintained in culture for 2 hours. After this, extracts were discarded and one experimental group received 50ng/mL TNF-α dissolved in medium with 10% serum and another group received medium with 10% serum. Cells cultivated in culture medium untreated with extracts but stimulated with TNF-α and cells untreated and unstimulated were used as controls. After 20 hours incubation, supernatant were taken for measuring adhesion molecule expression by an ELISA kit. All samples were tested in triplicate.

Rat paw edema

The assay was carried out using fresh egg-white as phlogistic agent – induced rat hind paw edema as a model of acute inflammation. Wistar rats (150-200g) of either sex were
purchased from Cantacuzino Institute animal house. The animals were kept under controlled condition (temperature: 22±2°C, humidity: 50-60%, 12h light-dark cycle) and had free access to food and water. The animals were acclimatised for 7 days to the laboratory conditions before doing experiments and were deprived of food 12 hours before doing experiments.

The experiment was carried out in accordance with the guidelines for the care of laboratory animals and ethical guidelines, and were approved by the ethics committee for research on laboratory-animal use of the institution. The number of the animals was the minimum necessary to show consistent results.

The extracts (250mg/ml/100gbw) were administered by gastric gavage 1 hour before inducing inflammation. Control mice received equivalent amount of normal saline and the reference group administered acetylsalicylic acid (Zentiva, Romania) 100mg/kg.

Inflammation of the hind paw was induced by injection of 0.1ml of fresh egg white into the subplantar surface of the right hind paw of the rats. The thickness (mm) of the paw was measured by a plethysmometer (Ugo-Basile, Italy) immediately before the administration of the phlogistic agent (“zero” time) and 30, 60, 120 and 180 minutes thereafter. Thus, inflammation was assessed as the difference between zero time paw diameter and that 30, 60, 120 and 180 minutes after administration of phlogistic agent.

Antiinflammatory effect was calculated as percent of edema inhibition according to the following ecuation:

\[
\text{Antiinflammatory effect} \% = 100 \times \left[ 1 - \frac{(a-x)}{(b-y)} \right]
\]

Where: \(a\) = mean paw volume of treated group (mL); \(b\) = mean paw volume of untreated group (mL); \(x\) = mean initial paw volume of treated group (mL); \(y\) = mean initial paw volume of untreated group (mL).

Inflammatory activity against phlogistic agent is obvious if the inflammatory index is higher than 20%. The results were processed by CUB soft for data acquisition and storing.

All the results were expressed as mean ± standard error for each measurement time. Data were analyzed using one-way analysis of variance test (ANOVA) followed by Student’s test. P-values < 0.05 were considered as statistically significant.

3. Results and discussion

**Determination of total phenolics and HPLC analysis**

For R1 sample it was found a 3.14% total polyphenols content and for R2 sample a content of 8.84%, as determined by Folin-Ciocalteu method. It was also showed by HPLC that R2 contains not only a higher content of phenols than R1 but higher contents of polyphenolcarboxylic acids like ferulic, caffeic and rosmarinic. (Table 1, Figure 1)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>not detected</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>-</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.603 mg/g</td>
</tr>
<tr>
<td>Rosmarinic acid</td>
<td>14.87 mg/g</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.704 mg/g</td>
</tr>
<tr>
<td>Apigenin</td>
<td>0.277 mg/g</td>
</tr>
</tbody>
</table>

**Table 1. Major compounds of Rosmarinus samples quantified by HPLC**
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Figure 1. Mass spectrum *Rosmarinus* samples (inset rosmarinic acid normalization)

a) R1

b) R2

**DPPH inhibition**

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to discolor in the presence of antioxidants. The DPPH radical contains an odd electron that is responsible for the absorbance at 540 nm and also for the visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Table 2 lists the total phenolics content and antioxidant capacity of the extracts.

**Table 2.** Correlation of total phenolics content and antioxidant capacity of the extracts.

<table>
<thead>
<tr>
<th></th>
<th>Total polyphenols (% expressed as gallic acid)</th>
<th>% DPPH inhibition 0.1 mg/mL</th>
<th>1 mg/mL</th>
<th>5 mg/mL</th>
<th>10 mg/mL</th>
<th>0.2 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>3.14</td>
<td>1.18±0.07</td>
<td>4.98±0.12</td>
<td>31.8±0.07</td>
<td>53.6±0.5</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>8.84</td>
<td>5.4±0.1</td>
<td>20.1±0.08</td>
<td>70.3±0.11</td>
<td>79.8±0.18</td>
<td>86±0.08</td>
</tr>
<tr>
<td>Quercetin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The polyphenol content seems to be correlated with scavenging activity. DPPH inhibition was dose-dependent and effective especially for R2 sample which proved the highest polyphenol content. EC50 value was found to be 8.4mg/mL for R2 and 10.76mg/mL for R1. From previous studies was determined that triterpenic acids content is similar for both samples (data not shown) and it can be concluded that antioxidant activity could be due to phenolic content.

**ICAM-1 assay**

*Rosmarinus* extracts can inhibit TNF-α-induced ICAM-1 expression. HUVEC were preincubated for 2 h with either control medium (Control), or medium containing *Rosmarinus* extracts of various concentrations. Following the 2-h pre-incubation, TNF-α (50ng/ml) was added to the cultures where indicated. After 20 hours incubation, all reagents were removed and ICAM-1 expression of the cells was determined by ELISA. A dose-effect correlation is obvious for both extracts. Extracts effect on ICAM-1 production is comparable to reference
substance only at maximum concentration tested (200μg/mL, p< 0.05) for R1 but also for 100μg/mL (p< 0.05) in the case of R2 (Figure 2 and 3). Level of ICAM-1 production is very similar for R1 TNF-α-stimulated and non-stimulated cells.

![Figure 2](image_url)  
**Figure 2.** Effect of R1 sample, acetylsalicylic acid 10mM, on ICAM-1 production TNF-α-induced on HUVEC. Each bar indicates the mean ± SD of three measurements.

![Figure 3](image_url)  
**Figure 3.** Effect of R2 sample, acetylsalicylic acid 10mM, on ICAM-1 production TNF-α-induced on HUVEC. Each bar indicates the mean ± SD of three measurements.

**Anti-Inflammatory assay**

We examined the anti-inflammatory effect of *Rosmarinus* extracts (at a dose of 250mg/100gbw) using the fresh egg-white-induced edema model. Paw volumes of all groups are presented in Table 3. Maximal edema inhibition was observed at 30 minutes after the edema induction for both extracts and for aspirin, the tested reference compound, maximal edema inhibition was recorded at 120 min.
Table 3. Paw volumes variation in rat paw edema assay.
Values are mean ± SD. *p< 0.05, significantly different from control, n=3

<table>
<thead>
<tr>
<th>Time</th>
<th>Samples</th>
<th>Mean±SD</th>
<th>VC %</th>
<th>Mean±SD</th>
<th>VC %</th>
<th>Mean±SD</th>
<th>VC %</th>
<th>Mean±SD</th>
<th>VC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>R1</td>
<td>1,428±0,139</td>
<td>9,702</td>
<td>2,207±0,292</td>
<td>13,234</td>
<td>2,319±0,330</td>
<td>14,251</td>
<td>2,421±0,295</td>
<td>12,188</td>
</tr>
<tr>
<td>30</td>
<td>R1</td>
<td>1,790±0,023</td>
<td>1,280</td>
<td>2,443±0,169*</td>
<td>6,920</td>
<td>2,499±0,083*</td>
<td>3,302</td>
<td>2,235±0,057*</td>
<td>2,558</td>
</tr>
<tr>
<td>60</td>
<td>R1</td>
<td>1,506±0,172</td>
<td>11,394</td>
<td>2,862±0,071</td>
<td>2,493</td>
<td>2,511±0,254</td>
<td>10,108</td>
<td>2,273±0,306</td>
<td>13,475</td>
</tr>
<tr>
<td>120</td>
<td>R1</td>
<td>1,744±0,297</td>
<td>31,25</td>
<td>2,489±0,241</td>
<td>19,735</td>
<td>2,318±0,161*</td>
<td>17,326</td>
<td>2,216±0,197*</td>
<td>20,015</td>
</tr>
<tr>
<td>180</td>
<td>R1</td>
<td>1,428±0,139</td>
<td>9,702</td>
<td>2,207±0,292</td>
<td>13,234</td>
<td>2,319±0,330</td>
<td>14,251</td>
<td>2,421±0,295</td>
<td>12,188</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>1,790±0,023</td>
<td>1,280</td>
<td>2,443±0,169*</td>
<td>6,920</td>
<td>2,499±0,083*</td>
<td>3,302</td>
<td>2,235±0,057*</td>
<td>2,558</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1,506±0,172</td>
<td>11,394</td>
<td>2,862±0,071</td>
<td>2,493</td>
<td>2,511±0,254</td>
<td>10,108</td>
<td>2,273±0,306</td>
<td>13,475</td>
</tr>
<tr>
<td></td>
<td>ASP 100</td>
<td>1,744±0,297</td>
<td>31,25</td>
<td>2,489±0,241</td>
<td>19,735</td>
<td>2,318±0,161*</td>
<td>17,326</td>
<td>2,216±0,197*</td>
<td>20,015</td>
</tr>
</tbody>
</table>

In particular, treatment with R1 extract reduced the edema rate only at 30 min, similar to aspirin treatment but showed no anti-inflammatory effect after. (Table 4). On the other hand, R2 extract exhibits anti-inflammatory activity on the entire period of 180 minutes (p< 0.05).

Table 4. Edema inhibition percentage relative to positive control

<table>
<thead>
<tr>
<th>Time</th>
<th>R1</th>
<th>R2</th>
<th>ASP 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>42.59</td>
<td>51.81</td>
<td>42.98</td>
</tr>
<tr>
<td>60 min</td>
<td>11.36</td>
<td>29.44</td>
<td>58.20</td>
</tr>
<tr>
<td>120 min</td>
<td>-</td>
<td>41.96</td>
<td>64.25</td>
</tr>
<tr>
<td>180 min</td>
<td>-</td>
<td>50.39</td>
<td>54.40</td>
</tr>
</tbody>
</table>

4. Conclusion
Rosemary is a well known species which exhibits various pharmacological effects. In this study we showed the antioxidant and anti-inflammatory effects of two extracts having different polyphenol content. The sample R2 which had a higher polyphenol content exhibited good antioxidant and anti-inflammatory effects on all assays. The activity was prolonged in time and exerted at smaller doses comparing to the other rosemary extract tested. The reported protective effect against the oxidative stress is in accordance with other experimental data obtained by KISS & al. [8] using two other methods for evaluation of antioxidant activity.

It can be concluded that polyphenols play an important role not only in antioxidant action but also in anti-inflammatory effects of rosemary extracts.

5. Acknowledgements
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