

THE EVALUATION OF ANTIOXIDANT POTENTIAL OF *VERONICA OFFICINALIS* AND *ROSMARINUS OFFICINALIS* EXTRACTS BY MONITORING MALONDIALDEHIDE AND GLUTATHIONE LEVELS IN RATS

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Abstract

Malondialdehyde and glutathione are two of the main biomarkers of oxidative stress. These biomarkers were used to evaluate the antioxidant potential of two plant extracts, obtained from *Rosmarinus officinalis* and *Veronica officinalis*, in Wistar rats. In the case of malondialdehyde the assay was based on derivatization with dinitrophenylhydrazine, followed by chromatographic analysis with UV detection at 307nm. The reduced and total glutathione were quantified from rat plasma, after derivatization with o-phthalaldehyde, using a HPLC method with fluorescence detection. The results indicated a potential protective effect of these plants against oxidative stress.

Rezumat

Malondialdehida și glutathionul reprezintă doi biomarkeri importanți ai stresului oxidativ. Acești biomarkeri au fost utilizați pentru a evalua potențialul antioxidant al extractelor obținute din *Rosmarinus officinalis* și *Veronica officinalis*, la șobolani Wistar. În cazul malondialdehidei, metoda de dozare s-a bazat pe derivatizarea cu dinitrofenilhidrazină, urmată de analiza cromatografică cu detecție în UV la 307nm. Cuantificarea glutathionului redus și total din plasma de șobolan s-a realizat printr-o metodă HPLC cu detecție în fluorescență, după o prealabilă derivatizare cu o-ftalaldehida. Rezultatele obținute au indicat un potențial efect protector al plantelor testate față de stresul oxidativ.

Keywords: *Rosmarinus officinalis*, *Veronica officinalis*, malondialdehyde, glutathione, oxidative stress, chromatography

Introduction

Oxidative stress is due to some very reactive species, mainly oxygen radical derivatives and peroxides. This process becomes obvious when the antioxidant defense mechanisms of the cell are overwhelmed by the levels of free radicals. Reactive free radicals can oxidize biomolecules, such as proteins, lipids and nucleic acids. It is well known the involvement of the oxidative stress in the pathogenesis of multiple diseases (e.g. neurodegenerative, cardiovascular, inflammatory diseases) [3-5,7].

There are many cases when a disease is treated through the modulation of the antioxidants level or by using drugs with antioxidant activity. For this reason, there is a great need to identify also plant materials which may develop antioxidant potential.

The plants chosen to be tested in this work were *Veronica officinalis* (Lamiaceae) and *Rosmarinus officinalis* (Scrophulariaceae). These species were selected based on their content in some compounds with antioxidant potential, such as flavonoids and rosmarinic acid [1]. Flavonoids are phenolic components, present in many plant species, which have beneficial effects in preventing some cardiovascular and inflammatory diseases [6, 10-12]. In order to perform the tests on animals, alcoholic extracts were prepared from *Veronica officinalis* leaves and *Rosmarinus officinalis* herba.

The influence of these extracts on oxidative stress can be evaluated by monitoring the specific biomarkers of radical induced damage and toxicity [2].

In previous papers we presented two chromatographic methods for the quantification in rat microsomal preparations of malondialdehyde and glutathione as markers of oxidative stress [8, 9].

The aim of this work was to evaluate the antioxidant effect of these two plant extracts, through the quantification of malondialdehyde (MDA, a lipid peroxidation end-product) and glutathione (GSH, a very potent endogenous protective agent against oxidative stress) in rat plasma samples exposed to carbon tetrachloride, an agent capable of inducing oxidative stress. In order to perform these tests, we made some changes in the assays for MDA and GSH from microsomes, in order to quantify the two biomarkers, from plasma samples [8, 9].

Materials and methods

Chemicals

1,1,3,3-tetraethoxypropan (TEP) were purchased from Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany). HPLC grade reduced and

oxidized glutathione, were obtained from Fluka (Fluka, Buchs SG, Switzerland). HPLC grade acetonitrile, methanol, formic acid, acetic acid, acetone and hexane were purchased from Merck (Merck, Darmstadt, Germany). All other chemicals were analytical reagent grade and they were obtained from Merck (sodium hydroxide, hydrochloric acid, sulfuric acid, perchloric acid, 2,4-dinitrophenylhydrazine (DNPH), sodium tetraborate decahydrate, o-phthalaldehyde, acetaldehyde, TRIS hydrochloride (Tris hydroxymethyl aminomethane hydrochloride) and 1,4-dithiothreitol (DTT)).

Deionised water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA).

Animals

Male Wistar rats (mean body weight of 200g) were obtained from the Animal Breeding Station of University of Medicine and Pharmacy Cluj-Napoca. During the experiment, the animals were housed in standard conditions and were allowed free access to standard food and water.

Four groups of animals were used, three groups of 30 rats each (*Rosmarinus*, *Veronica* and positive control group) and a group composed of six rats (negative control). The plant extracts were standardized in active compounds. The total flavonoid content of *Veronica officinalis* extract was of 2.95 mg/mL, expressed as rutoside and the content of phenyl propane derivatives was of 170 mg/mL, expressed as caffeic acid. *Rosmarinus officinalis* extract contained 77.89 µg/mL rosmarinic acid and 28 mg/mL phenyl propane derivatives, expressed as caffeic acid.

The plant extracts and the other substances were administered according to the following protocol:

- For 7 days, alcoholic extract (1part plant material / 2 parts 50° alcohol) of *Rosmarinus officinalis* or *Veronica officinalis* were administered orally after a 1/4 dilution with water (10mL diluted extract/kg body weight/day). In case of positive and negative control group, the extracts were substituted with 50° alcohol. On the 7th day, one hour after the last dose of extract or 50° alcohol, CCl₄ was administered (1200 mg/kg bodyweight or 120 mg/100 g rat in 0.5 mL sunflower oil). The negative control did not receive CCl₄.

Six animals were sacrificed at 30 minutes after the administration of CCl₄, while at 60, 90 and 120min, 8 animals were sacrificed at each time point. Blood samples were collected in order to quantify the studied biomarkers of oxidative stress.

Sample preparation**Total MDA**

In order to quantify total MDA, 75µL plasma, 75µL 1% H₂SO₄ (or TEP working solution in case of calibration standards) and 25µL 6M NaOH were pipetted in a 1.5mL Eppendorf tube. The sample was maintained at 60°C, for 30min in a waterbath, in order to hydrolyse the MDA bound to proteins. After the hydrolysis, 100µL internal standard (IS) (acetaldehyde) solution was added, followed by a deproteinisation step with 63µL 36% HClO₄. The sample was vortexed thoroughly and centrifuged at 10000rpm for 10min. 150µL of the supernatant was derivatized with 20µL 5mM DNPH 5mM in 2M HCl, at room temperature, protected from light. The obtained hydrazone was extracted in 1.2mL hexane and the organic layer was evaporated in a centrifugal concentrator at 30°C. The residue was dissolved in 150µL mobile phase and 50µL of the obtained solution was injected into the chromatographic column.

Reduced and total glutathione

100µL rat plasma spiked with 50µL GSH working standard was pipetted into 1.5mL Eppendorf tubes; the sample was deproteinized with 50µL sulfosalicylic acid, vortexed and centrifuged at 10000rpm for 5min. 50µL of the supernatant were treated with 50µL OPA solution (a mixture of 54mg OPA/1mL methanol and 343mg Na₂B₄O₇·10H₂O/9mL deionized water, pH 9.5) by vortexing at room temperature for 1 min. After derivatization 5µL were injected into the chromatographic column.

In case of total GSH, after deproteinization, the oxidized glutathione (GSSG) was reduced by incubating 50µL supernatant with 100µL DTT 100mM, for 5 min at room temperature. In the final step the sample was diluted with 800µL deionized water and 5µL were injected into the chromatographic column.

Instrumentation and chromatographic conditions

The analysis were performed using a 2695 Waters Alliance HPLC (Waters, Milford, MA, USA) system composed of a quaternary pump, autosampler, column heater and solvent degasser. The HPLC unit was linked to a 996 PDA (photodiode array) detector and a 2475 fluorescence detector (Waters, Milford, MA, USA).

Total MDA

Chromatographic separation was achieved on a Spherisorb ODS column (250mmx4mm, 5µm) and a Spherisorb ODS (20mmx4mm, 3µm) guard column, maintained at 25°C. The mobile phase consisted in a mixture

of 1% formic acid/acetonitrile (62/38, v/v). The flow rate was 1mL/min and the absorbance of the eluent was monitored at 307nm, corresponding to the maximum in the UV spectra of the MDA hydrazone obtained after derivatization.

Reduced and total glutathione

The chromatographic analysis was performed on a Supelcosil LC-18 analytical column (150mmx4.6mm, 3 μ m particle size), maintained at 30°C and with a mobile phase consisting in a mixture of 0.25% acetic acid (pH 6.91, with 6M NaOH) /methanol (85/15, v/v). The flow rate was set at 0.8mL/min. The sample compartment was maintained at 4°C. The fluorescence detection was performed at λ_{em} = 420nm with λ_{exc} = 350nm.

Results and discussion

Determination of MDA

The elution times of MDA hydrazone and acetaldehyde hydrazone in the specified chromatographic conditions were 9.96 min and 20.15 min, respectively (Fig.1).

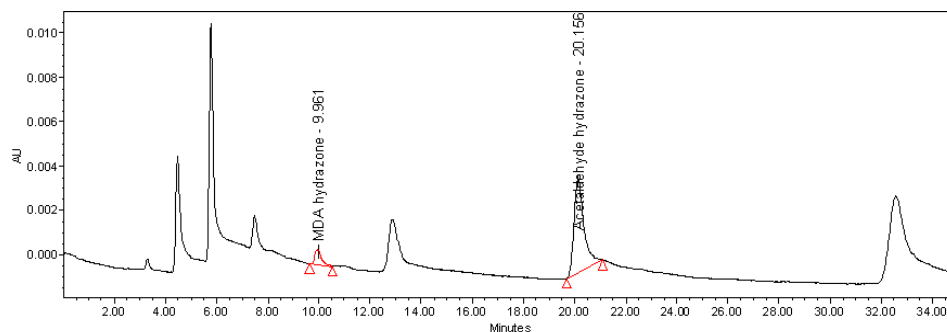


Figure 1

The chromatogram of a plasma sample spiked with 5 nmol/mL MDA and 354nmol/mL acetaldehyde, respectively

A full validation of the method was not performed, but we studied the selectivity and linearity of the assay over the concentration range of 1-40ng/mL MDA.

Selectivity was assessed through the analysis of blank rat plasma samples coming from 6 independent sources in a single analytical run. Since MDA is an endogenous compound it can be detected and quantified in all blank samples. Regarding the linearity, the calibration curves were constructed based on the relation between peak area ratios and concentration

ratios of MDA and internal standard. The method proved to be linear over the studied concentration range, with a coefficient of correlation of 0.9898.

Determination of glutathione

The elution time of GSH after derivatization with OPA was 3.98 min, with the total analysis time being of 8 min in case of reduced GSH and 23 min for total GSH.

Fig. 2 shows a chromatogram for the reduced GSH from a spiked plasma sample (2.7nmol/mL).

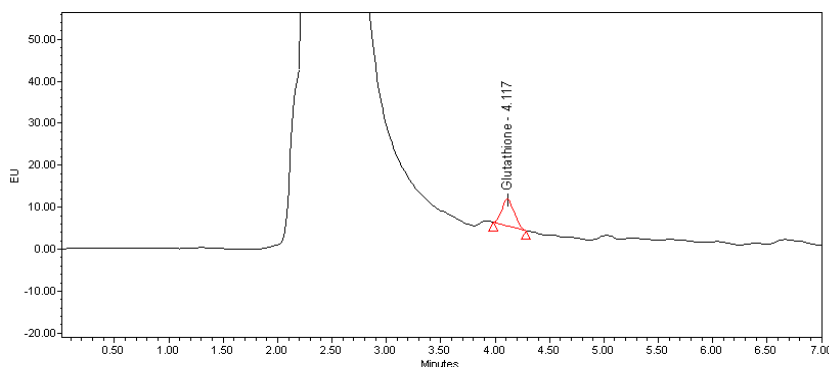


Figure 2

The chromatograms for the determination of reduced GSH in spiked plasma samples (2.7 nmol GSH/mL plasma)

The method was linear over the studied concentration range (1-15 nmol/mL for reduced glutathione and 1-30nmol/mL for total glutathione).

Evaluation of the antioxidant properties of the plant extracts

MDA is the most frequently used biomarker of oxidative stress, due to the fact that it is a lipid peroxidation end-product, a process which is very intense at cellular level because of the great abundance of membranary lipids.

Another important marker is glutathione, the decrease of its reduced form indicating an increase of the oxidative stress. Usually, in case of this biomarker it is possible to work with the reduced glutathione levels or with the reduced glutathione/total glutathione ratio.

In order to evaluate the antioxidant potential of the selected plants, *Veronica officinalis* and *Rosmarinus officinalis*, total MDA and reduced/total glutathione were quantified in all plasma samples obtained from the four groups of rats used during the experiment. The statistical evaluation of the results was performed using student's t-test, the results obtained in case of the two groups treated with the studied plant extracts being compared to those obtained for the positive control group.

The mean total MDA levels (nmol total MDA/mL plasma) obtained for the different animal groups at different time points are presented in Table I.

Figure 3 indicates the effect of the *Veronica officinalis* and *Rosmarinus officinalis* extracts on the total MDA formation.

Table I

Mean total MDA levels (nmol total MDA/mL plasma)

Total MDA (mean $\pm$ SD)				
Time (min)	Negative control	Positive control	<i>Rosmarinus officinalis</i> test group	<i>Veronica officinalis</i> test group
0	2.131($\pm$ 0.312)	-	-	-
30	-	1.819($\pm$ 0.409)	1.093($\pm$ 0.466)	0.745($\pm$ 0.292)
60	-	4.619($\pm$ 1.817)	1.938($\pm$ 1.031)	0.843($\pm$ 0.547)
90	-	1.942($\pm$ 0.837)	1.792($\pm$ 1.095)	1.175($\pm$ 0.563)
120	-	2.158($\pm$ 0.664)	0.902($\pm$ 0.201)	2.426($\pm$ 0.875)

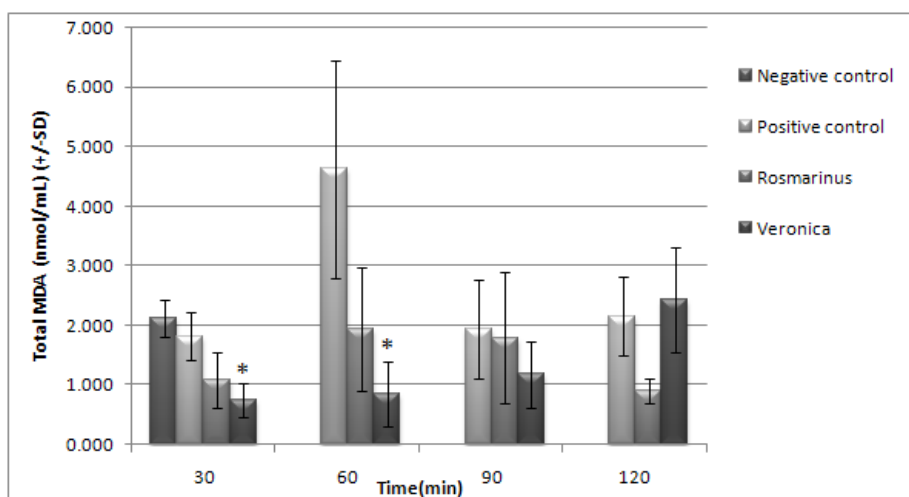


Figure 3

The influence of *Veronica officinalis* and *Rosmarinus officinalis* extracts on the total MDA levels in rats treated with carbon tetrachloride (*, $p < 0.05$)

The mean reduced/total glutathione ratio obtained for the different animal groups at different time points are presented in Table II.

Figure 4 indicates the effect of the *Veronica officinalis* and *Rosmarinus officinalis* extracts on the reduced/total glutathione ratio.

Table II

Reduced/total glutathione ratio in plasma				
Reduced GSH/Total GSH ($\pm$ SD)				
Time (min)	Negative control	Positive control	<i>Rosmarinus officinalis</i> test group	<i>Veronica officinalis</i> test group
0	0.0295($\pm$ 0.0022)	-	-	-
30	-	0.0250($\pm$ 0.0035)	0.0454($\pm$ 0.0070)	0.0259($\pm$ 0.0027)
60	-	0.0182($\pm$ 0.0025)	0.0305($\pm$ 0.0031)	0.0200($\pm$ 0.0029)
90	-	0.0293($\pm$ 0.0043)	0.0238($\pm$ 0.0017)	0.0282($\pm$ 0.0032)
120	-	0.0311($\pm$ 0.0018)	0.0163($\pm$ 0.0020)	0.0276($\pm$ 0.0013)

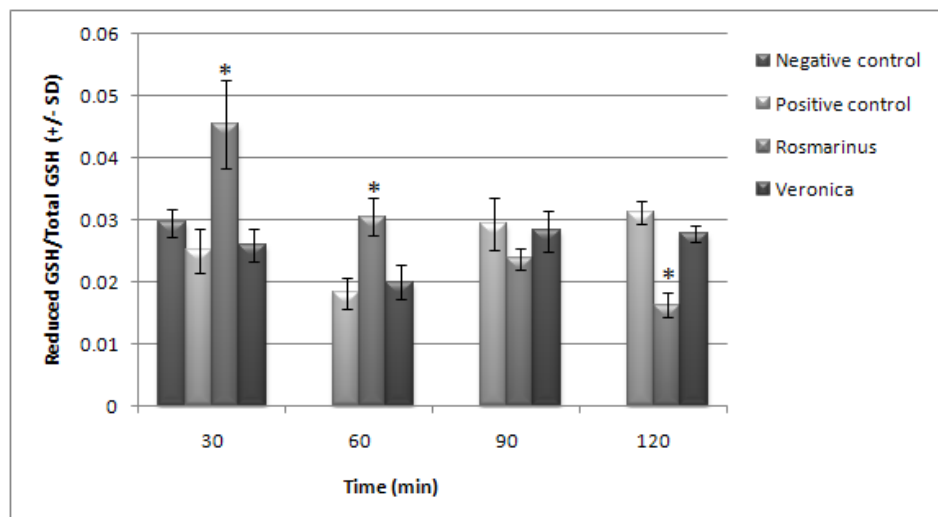


Figure 4

The influence of *Veronica officinalis* and *Rosmarinus officinalis* extracts on the reduced/total GSH ratio in rats treated with carbon tetrachloride (*, $p < 0.05$)

The chromatographic analysis of MDA after derivatization with DNPH showed that CCl_4 induced oxidative stress and lipid peroxidation with the maximum of this effect at 60 minutes. The results obtained in case of the *Rosmarinus officinalis* and *Veronica officinalis* test groups showed that both extracts have protective effects against the hepatotoxicity of CCl_4 , but with statistically significant results ($p < 0.05$) only in case of the *Veronica* extract. At 60 min (corresponding to the maximum intensity of the effect of CCl_4) the *Veronica* extract showed superior efficiency regarding the protective effect against the oxidative stress. It is also noteworthy that, based on the evolution of MDA levels in time, the *Rosmarinus* extract showed a less intense antioxidant effect than *Veronica*, but with a longer duration.

The determination of reduced/total glutathione ratio confirmed that the effect of CCl₄ presented maximum intensity at 60 minutes after administration. The results obtained for GSH indicated great differences regarding the effects of the two tested extracts. While *Veronica* showed only a weak protective effect (the mean values were not significantly different from those obtained for the positive control group) against the toxicity of CCl₄, the *Rosmarinus* extract induced a significant delay in the onset of the toxic effect of the same agent (the maximum decrease of the reduced/total GSH ratio was observed at 120 min, instead of 60 minutes, as it was shown in case of the positive control and Veronica groups).

Conclusions

The results of this study indicate that it is not possible to use only one biomarker in order to evaluate oxidative stress or the protective effect of some agents, because there are different mechanisms of inducing the oxidative stress or to fight against it. Probably the two tested plant extracts present different mechanisms of protection due to their different chemical composition. However, based on these results, both plant materials could prove efficient in protecting the living organisms against oxidative damage.

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