



In Vivo Antioxidant Activity of *Ruta montana* L. Extracts

M. Merghem, S. Dahamna, S. Khennouf

Laboratory of Phytotherapy Applied to Chronic Diseases, Ferhat Abbas University, Sétif, Algeria.

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mouniramergem@yahoo.fr
Phone: +213795792018;

Abstract

The aim of this study is to evaluate antioxidant activities of *Ruta montana* L. extracts. To do this, An *in vivo* activity was carried out on rats treated with aqueous extract (AqE) at doses of 100 and 300mg/Kg bw and methanolic extract (CrE) at a dose of 100 mg/Kg for 21 consecutive days, and one group was treated with vitamin C (Vit C 50 mg/kg) as a standard drug. The antioxidant activities of aqueous extract and methanolic extract *in vivo* were estimated using the antioxidant plasma capacity (APC), 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and reducing power tests. All extracts did not show any significant change. The activity of catalase (CAT), the level of MDA and GSH were evaluated in liver and kidney homogenate. The results showed an increase of CAT activity in the groups treated with AqE (300mg/kg) and CrE (100mg/kg) by 30.76% and 32.07% for liver tissue and 24.48% and 32.11% for kidney tissue, respectively. The GSH levels were increased by 18.57%, 22.97% for liver tissue and 12.3%, 37.36% for kidney tissue, respectively. However, the level of MDA was decreased for the treated groups with AqE (300mg/kg) and CrE (100mg/kg). These results suggest that *Ruta montana* L. extracts have active substances contributing to the increase of antioxidant potential.

1. Introduction

The production of oxidants is a typical event associated with aerobic metabolism. When oxygen is supplied in excess or its reduction is insufficient, reactive oxygen species or free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide are generated [1]. Accumulation of the free radicals in body organs or tissues can cause oxidative damage to biomolecules and membranes of cell, eventually leading to many chronic diseases, such as inflammatory, cancer, diabetes, aging, cardiac dysfunction and other degenerative diseases [2].

Antioxidants are capable of stabilizing or deactivating, free radicals before they attack cells [3]. Reactive oxygen species can be eliminated by a number of enzymatic and non-enzymatic antioxidant mechanisms. Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase, and catalase. Non-enzymatic antioxidants include ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione, carotenoids, flavonoids, and other antioxidants [4]. However, under oxidative stress conditions, enzymatic antioxidants may not be sufficient, and non-enzymatic antioxidants (dietary antioxidants) may be required to maintain optimal cellular functions [5]. Antioxidants may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress.

Medicinal plants were used all over the world to treat various diseases including inflammation, heart diseases, cancer, etc. A great number of medicinal plants contain compounds exhibiting antioxidant properties as phenolic compounds, which possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals [6]. They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers. Antioxidants from plant materials terminate the action of free radicals thereby protecting the body from various diseases [7].

The genus *Ruta* (Rutaceae) encompasses more than 1800 species, mainly found in tropical and temperate regions with major centres of diversity in Southern Africa and Australia. Members of this genus have been used since antiquity in traditional medicine. The infusions/decoctions of the species *Ruta montana* L. are widely used as a tonic, a febrifuge and a treatment of malaria as well as inflammatory, antioxidant and microbial processes [8].

The aim of the present investigation was the evaluation of antioxidant activity *in vivo* of the aerial part of *Ruta montana* L., using different extracts.

2. Material and Methods

2.1. Animals

Albino Wistar rats weighing between 150 and 200 g were used for *in vivo* antioxidant activity. All experimental animals were purchased from Pasteur Institute (Algiers, Algeria). These animals were kept in the animal house, at a temperature of 20°C and a natural photoperiod cycle. The animals were housed in plastic cages (6 rats per cage) and had free access to standard commercial diet and tap water.

2.2. Plant material

Ruta montana L. was collected in October, from Beniiaziz region, Wilaya of Sétif in Northeast of Algeria, and it was identified by Pr. Laouar H. (Department of Ecology & Plant Biology, Faculty of Natural and Life Sciences, Ferhat Abbas university, Sétif Algeria).

2.3 Preparation of plant extract

2.3.1. Aqueous extract

The aerial parts of plant material were cleaned with tap water, dried in the shade at room temperature for 2 weeks and ground into powder using an electric grinder. Briefly, 100g of *Ruta montana* L. powder was mixed with 1L of boiled distilled water (100 °C) and was placed at room temperature during 72h, The resulting mixture was filtered using Wattman filter paper n°3 and then evaporated in rotary vacuum evaporator at 45°C.

2.3.2. Methanolic extract

The methanolic extract was obtained by maceration in water/methanol mixture (25:75) for 72 h. The resultant extract was filtered through Wattman paper n°3 and the solvent was removed by rotary evaporator under reduced pressure at 45°C. The resulting crude extract was then stored at -20°C until further analysis [9].

2.4. Evaluation of *In vivo* antioxidant activity

Thirty male rats were randomly divided into five groups, each consisting of 6 animals, which included:

- Group 1: Untreated control rats which received distilled water for 21 days.
- Group 2: Positive control received 50mg/kg bw of vitamin C for a period of 21days.
- Group 3: Received 100 mg/kg bw of *Ruta montana* L. aqueous extract for 21 days.
- Group 4: Received 300 mg/kg bw of *Ruta montana* L. aqueous extract for 21 days.
- Group 5: Received 100 mg/kg bw of *Ruta montana* L. methanolic extract for 21days.

Administrations were done orally, the extract dissolved in 1 mL of distilled water per 100 g of body weight. 24 h after the last dose, all animals were anesthetized with diethyl ether and blood samples were obtained by retro-orbital puncture and collected into tubes containing heparin and it was centrifuged at 3000g for 15 min at 4°C to obtain plasma. Plasma stored at -20°C until used for DPPH and reducing power test. After the sacrifice the liver and kidney of each animal were dissected out, washed in ice-cold saline, patted dry and weighed and homogenized in Tris HCL buffer (pH 7.4), to prepare 10% (w/v) homogenate. Then, the homogenate was centrifuged at 4000 rpm at 4 °C for 15min and the supernatant was collected and used for the estimation of MDA, reduced glutathione (GSH), and catalase (CAT).

2.4.1. Effect of extracts on plasma antioxidant capacity using DPPH radical

The ability of plasma to scavenge DPPH radical was evaluated by the method of Hassani and al, [10] with some modifications, based on the same principle as the DPPH test previously conducted *in vitro*. Briefly, a volume of 50 µL of plasma was added to 1250 µL of methanolic DPPH solution (2.4 mg / 100 mL methanol). After 30 min of incubation in the dark, followed by centrifugation the absorbance is measured at 517nm. The plasma antioxidant capacity is then calculated according to the equation below:

$$\text{Radical scavenging activity (\%)} = \{(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}\} \times 100.$$

2.4.2. Effect of extracts on plasma reducing power

Reducing power of the test samples was determined on the basis of the ability of their antioxidant principles to form colored complex with potassium ferricyanide, TCA and FeCl₃ and it was measured by the method reported by Jayaprakasha, and al [11]. 1mL of plasma was mixed with 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide (1% w/v). The mixture was incubated at 50°C for 20 min. After terminating the reaction by adding trichloroacetic acid (10% w/v), the mixture was centrifuged at 3000 rpm for 10 min. The supernatant of solution 0.5 mL was mixed with 0.5 mL distilled water and 0.1 mL FeCl₃ (0.1% w/v). After 5 min later, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

2.4.3. Estimation of lipid peroxidation

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) formation, using the method of Okhawa, and al [12]. Malondialdehyde (MDA), the end product of lipid peroxidation, is a good marker of free radical-mediated damage and oxidative stress. The principle of this method consists in the reaction of MDA with thiobarbituric acid (TBA) in acidic conditions and at a higher temperature (90-100°C) to form a pink MDA-(TBA)₂ complex, which can be quantified spectrophotometrically at 530 nm. In the procedure, 0.5mL of 20% TCA was added to 0.5mL of the tissue homogenate, then there was an addition of 1 mL of 0.67% TBA. The mixture was incubated at 100°C for 15 min in a water bath, cooled and then added with 4 mL of *n*butanol and centrifuged at 3000rpm for 15min. The absorbance of the clear pink supernatant was then read against a blank at 532nm spectrophotometrically. The concentration of MDA is expressed in nmol / g of the tissue.

2.4.4. Determination of reduced glutathione (GSH)

Reduced glutathione was determined by the method of Ellman [13]. The assay is based on the oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid (TNB) which has yellow color. Therefore, GSH concentration can be determined by measuring absorbance at 412 nm. For this assay, 50 µL of the tissue homogenate was diluted in 10 mL of phosphate buffer (0.1 M, pH 8). To 3 mL of the mixture of dilution, 20 µL of DTNB (0.01 M) was added. Absorbance is read at 412 nm against a blank prepared under the same conditions.

2.4.5. Determination of catalase activity

The enzymatic activity of catalase was determined by the method of Clairborne [14]. The principle is based on the hydrogen peroxide H₂O₂ degradation in the presence of the enzyme. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.4, 19 mM H₂O₂ and 16.5 uL tissue homogenate. The consumption of H₂O₂ was monitored spectrophotometrically at 240 nm for 1 min. and the enzymatic activity is calculated according to the formula:

$$K = 2.303/T \times \log (A1/A2) \quad (4)$$

Where:

K: Rate of reaction

T : Time interval (minutes)

A1 : Absorbance at time zero

A2 : Absorbance at 60 seconds interval

2.4.6. Statistical Analyses

The results are expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) followed by the Tukey test was performed to assess differences between groups. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Effect of extracts on plasma antioxidant capacity using DPPH radical

The obtained results (Figure 1) showed that oral administration of AqE (300 mg /ml) and CrE (100 mg / ml) leads to increased plasma antioxidant capacity ($18.13 \pm 5.366\%$ and $17.57 \pm 5.869\%$, respectively). This increase is not significant statistically compared with the control group $13.65 \pm 1.46\%$. However, the administration of AqE at the dose 100 mg/kg did not cause any change $13.28 \pm 3.253\%$ compared with the control group.

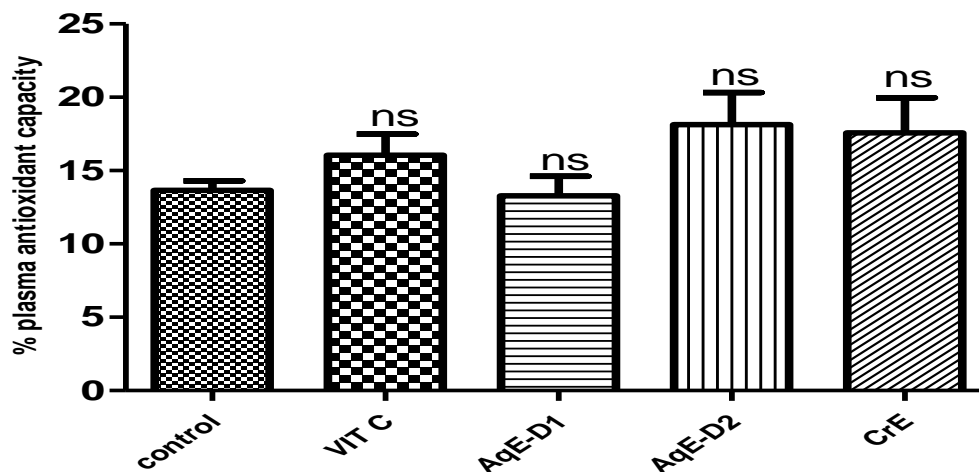


Figure 1. Plasma antioxidant capacity toward DPPH radical for different groups. Values are means \pm SD (n = 6). Comparisons are made relative to the control group, ns: not significant.

3.2. Effect of *Ruta montana L.* extracts on plasma reducing power

The obtained results (Figure 2) showed that oral administration of AqE (100 and 300 mg / ml) and CrE (100 mg / mL) did not show any significant changes compared with the control group.

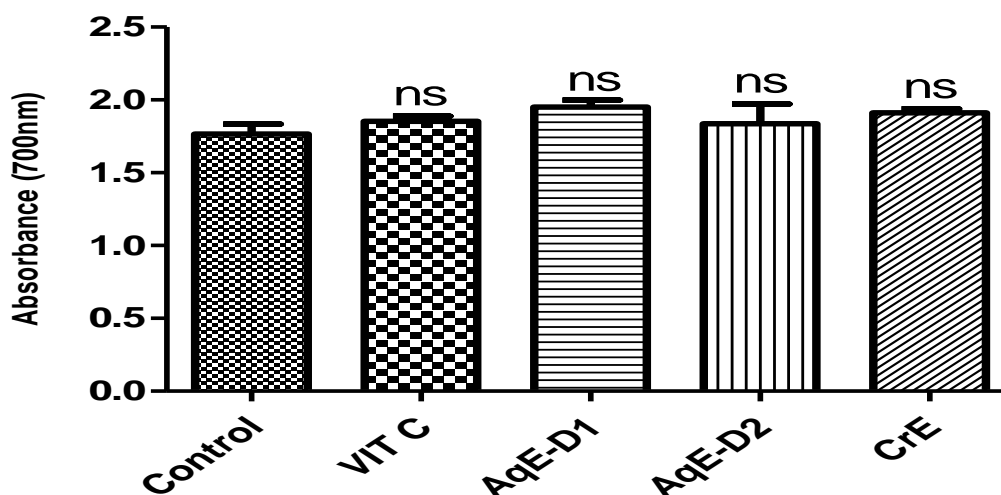


Figure 2. Reducing power of plasma for different groups. Values are means \pm SD (n = 6). Comparisons are made relative to the control group, ns: not significant.

Plasma antioxidant capacity (PAC) is one of the most commonly used biomarkers to assess the effectiveness of dietary supplementation or antioxidant treatment. In fact, the plasma contains a network of endogenous antioxidants such as (albumin, bilirubin, reduced glutathione and uric acid) as well as exogenous antioxidants derived from food. These antioxidants may act in a complementary and synergistic manner to provide a better protection against ROS. Due to the large number of antioxidants present in plasma, several methods have been

developed: the ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), total radical absorption potential (TRAP) and the DPPH radical scavenging activity [15, 16]. In our study, two complimentary tests were used to evaluate the antioxidant activity of *Ruta montana* L. extracts in plasma: DPPH scavenging activity and reducing power.

3.3. Effect of *Ruta montana* L. extracts on lipid peroxidation in liver and kidney

The results of lipid peroxidation (Table 1) revealed a decrease in liver and kidney tissues for the group treated with AqE 300mg/kg and CrE 100mg/kg with the value of $11,20 \pm 2,56$, $14,99 \pm 3,70$ nmol/g tissue and $11,85 \pm 3,59$, $11,15 \pm 3,30$ nmol/g tissue respectively, compared to group treated with distilled water $18,50 \pm 3,31$ and $16,54 \pm 3,45$ nmol/g tissue.

Table 1: Effect of *Ruta Montana* L. extracts on lipid peroxidation in liver and kidney

MDA concentration (nmol/g tissue).		
Group	Liver	Kidney
Group (distilled water)	18.50 ± 3.31	16.54 ± 3.45
Group (Vit C 50mg/kg)	12.90 ± 5.18^{ns}	14.59 ± 4.95^{ns}
Group (AQE 100mg/kg)	18.90 ± 5.49^{ns}	17.88 ± 3.22^{ns}
Group (AQE300mg/kg)	11.20 ± 2.56^{ns}	11.85 ± 3.59^{ns}
Group (CrE 100mg/kg)	14.99 ± 3.70^{ns}	11.15 ± 3.30^{ns}

Each value represents the mean \pm SD (n = 6). ns: not significant

3.4. Effect of *Ruta montana* L. extracts on reduced Glutathione (GSH)

The obtained results (Table 2) indicated that AqE at the dose of 300mg/kg and CrE at the dose of 100mg/kg presented an increase in GSH in liver tissue 0.7 ± 0.16 and 0.74 ± 0.15 $\mu\text{mol} / \text{g}$ tissue, respectively compared with the control group $0.57 \pm 0.15 \mu\text{mol} / \text{g}$ tissue.

In the kidney tissue AqE at the dose of 300mg/kg and CrE at the dose of 100 mg/kg showed an increase with 0.65 ± 0.06 and 0.91 ± 0.09 $\mu\text{mol} / \text{g}$ tissue, respectively, compared to the control group 0.57 ± 0.06 $\mu\text{mol} / \text{g}$ tissue.

Table 2: Effect of *Ruta montana* L. extracts on reduced Glutathione (GSH)

GSH concentration ($\mu\text{mol} / \text{g}$ tissue)		
Group	Liver	Kidney
Group (distilled water)	0.57 ± 0.15	0.57 ± 0.06
Group (Vit C 50mg/kg)	0.79 ± 0.11^{ns}	1.02 ± 0.11^{ns}
Group (AqE 100mg/kg)	0.44 ± 0.13^{ns}	0.58 ± 0.09^{ns}
Group (AqE 300mg/kg)	0.7 ± 0.16^{ns}	0.65 ± 0.06^{ns}
Group (CrE 100mg/kg)	0.74 ± 0.15^{ns}	0.91 ± 0.09^{ns}

Each value represents the mean \pm SD (n = 6). ns: not significant.

3.5. Effect of *Ruta Montana* L. extracts on catalase activity

The results of catalase (Table 3) revealed an increase in liver and kidney tissue for the groups treated with AqE (300 mg/kg) and CrE (100 mg/kg) with the value of 0.52 ± 0.038 , 0.53 ± 0.13 and 0.98 ± 0.17 , 1.09 ± 0.02 $\mu\text{mol/g}$ tissue, respectively compared to group treated with distilled water 0.36 ± 0.02 and 0.74 ± 0.14 $\mu\text{mol/g}$ tissue.

The body has evolved a complex defence strategy to minimize the damaging effects of various oxidants. Central to this defence are the non-enzymatic and enzymatic antioxidants. These include reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) respectively, which act in concert to protect the organism from oxidative damage [17]. Thus, In present work, we evaluated the effect of extracts on

oxidative stress parameters MDA, GSH and CAT at certain organs (liver and kidney) which oxidative stress appears to play a crucial role.

Table 3.Effect of *Ruta Montana* L. extracts on catalase (CAT) activity

Group	Catalase activity ($\mu\text{mol/g}$ tissue)	
	Liver	Kidney
Group(distilled water)	0.36 \pm 0.02	0.74 \pm 0.14
Group (Vit C 50mg/kg)	0.53 \pm 0.11 ^{ns}	0.99 \pm 0.12 ^{ns}
Group (AqE 100mg/kg)	0.31 \pm 0.031 ^{ns}	0.75 \pm 0.10 ^{ns}
Group (AqE 300mg/kg)	0.52 \pm 0.038 ^{ns}	0.98 \pm 0.17 ^{ns}
Group (CrE 100mg/kg)	0.53 \pm 0.13 ^{ns}	1.09 \pm 0.02 ^{ns}

Each value represents the mean \pm SD (n = 6). ns: not significant.

Lipid peroxidation is an auto-catalytic, free-radical mediated, destructive process, where by polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides [18]. Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde [19]. This compound is a reactive aldehyde, and is one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein adducts referred to as advanced lipoxidation end-products. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism [20].

The non-enzymic antioxidant, glutathione is one of the most abundant tripeptides present in the liver. Its functions are mainly concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals, and maintenance of membrane protein thiols and as a substrate for glutathione peroxidase and GST [21].

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues. It decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals [22]. Catalase activity varies greatly from tissue to tissue, the highest activity is found in liver and kidney, whereas the lowest activity is seen in the connective tissue [23]. Inhibition of this enzyme may enhance sensitivity to free radical-induced cellular damage. Therefore reduction in the activity of CAT may leads to deleterious effects as a result of superoxide and hydrogen peroxide assimilation.

Similar research indicated that in *in vivo* model, *Ruta graveolens* L. has been found to reduce oxidative stress by decreasing TBARS level and increasing antioxidant enzymes activities such as SOD, catalase and GPX in liver and heart in hypercholesteromic rats [24]. Preethi, and al [25] also indicated in *in vitro* experiments that *Ruta graveolens* L. extract was found to scavenge hydroxyl radical and inhibit lipid peroxidation.

In addition, [26] showed that treatment with the ethanolic extract of *Ruta chalepensis* L. could reduce oxidative stress as well as inflammation in hypercholesteromic rats. Flavonoids widely distributed in plants have the ability to inhibit oxidative damage. Indeed, these flavonoids have the potential to function as *in vitro* antioxidants by scavenging superoxide anion [27], singlet oxygen [28] lipid peroxy-radicals [29, 30], and/or stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species [31]. Flavonoids existing in *Ruta chalepensis* L., in addition to their free radical scavenging properties also enhance the expression of intracellular endogenous antioxidants such as superoxide dismutase (SOD), catalase, and GPX [32].

According to [33], the aerial part of *Ruta Montana* L. plant contains rutin. Recently, a study by Mahmoud [34] demonstrated that rutin, the major active constituent of *Ruta graveolens*, markedly decreased lipid peroxidation, increased glutathione concentration and ameliorated the antioxidant enzyme activities in liver and brain of AC-induced hyperammonemic rats.

Conclusion

The present study was carried out to evaluate the Antioxidant activity of aqueous and methanolic extracts prepared from the aerial parts of *Ruta montana* L., this activity was tested *in vivo* using *Albino Wistar* rats. The results showed that oral administration of extracts leads to increased plasma antioxidant capacity toward DPPH radical, and caused amelioration in the liver and kidney antioxidant status, by decreasing the MDA concentration and increasing the rate of reduced glutathione (GSH) and catalase activity (CAT).

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