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Research Article

In-Vitro Antioxidant Activity and Total Phenolic Content of *Ruta montana* L.

Extracts

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ABSTRACT

The aim of this study is to evaluate *in vitro* antioxidant activities of *Ruta montana* L. extracts. This activity was evaluated by three methods : DPPH (2, 2'-diphenyl- 1- picrylhydrazyl), bleaching of β -carotene and chelation of ferrous iron. Results showed that ethyl acetate extract (EAE) represents the highest amount of total polyphenols, tannins and flavonoids with $257,1 \pm 0,703 \mu\text{g}$ gallic acid equivalent/mg of extract, $251 \pm 1,41 \mu\text{g}$ tannic acid equivalent /mg of extract, $117,4 \pm 3,451 \mu\text{g}$ quercetin equivalents/mg of extract, $139,5 \pm 4,107 \mu\text{g}$ rutin equivalents/mg of extract, respectively. In the DPPH assay, ethyl acetate extract showed the higher scavenging capacity ($\text{IC}_{50} = 0.044 \pm 0.001 \text{ mg/ml}$) followed by methanol, aqueous and chloroform extract. Whereas, AqE showed the best chelating effect and the best inhibitory capacity of the coupled oxidation of linoleic acid/ β -carotene.

Keywords: *Ruta montana* L; polyphenols; antioxidant activity; free radical scavenging.

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1. INTRODUCTION

Free radicals are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune function[1]. Oxygen is an element indispensable for life. When cells use oxygen to generate energy free radicals are produced by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process. The free radicals have a special affinity for lipids, proteins, carbohydrates and nucleic acids [2].

Some of these free radicals play a positive role *in vivo* such as energy production, phagocytosis, regulation of cell growth and intercellular signaling and synthesis of biologically important compounds [3]. At high concentrations, free radicals leads to damaging the lipids in the cell membranes, proteins in tissues as well as enzymes, carbohydrates, and DNA to induce oxidation. This oxidative damage may play a causative role in aging and several diseases which are cancer, cardiovascular disease, cataracts, and cognitive dysfunction [4].

Antioxidants are defined as any compounds or materials capable of delaying or preventing the oxidation of lipids, proteins, or other molecules by inhibiting initiation or propagation of free radical reaction [5]. Various compounds which have antioxidant activity have been isolated from plants, many of them are polyphenols including phenols, phenolic acids, flavonoids, tannins, and lignans [4].

Ruta species (*Rutaceae*) are sources of different classes of natural products such as flavonoids, alkaloids, essential oils, coumarins, phenols, saponins lignans, and triterpenes, with biological activities, including antifungal, antioxidant, phytotoxic, abortive depressant, antidotal and anti-inflammatory. *Ruta montana* in Arabic "*fidjla*", is a perennial aromatic herb originated from North Eastern Algeria. The plant has been used in Algeria as a cure for emmenagogue, antispasmodic and rubefiant [6].

The aim of the present study was the evaluation of the antioxidant activity of different extracts from the aerial part of *Ruta montana* L. using different *in vitro* tests and the total phenolic, flavonoids and tannins contents.

2. MATERIALS AND METHODS

2.1. Chemicals

Folin-Ciocalteu, aluminum chloride (AlCl_3), gallic acid, quercetin, rutin, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), ethylenediaminetetraacetic acid (EDTA), gallic acid and tween 40 were purchased from Sigma Chemical Co. (St. Louis, MO). Linoleic acid, β -carotene and butylated hydroxytoluene (BHT) were obtained from Fluka Chemical Co. (Buchs, Switzerland). Ferrous and ferric chloride were obtained from Merck. All other reagents were of analytical grade.

2.2. Plant material

Ruta montana L. was collected in October, from Beniaziz region, Wilaya of Sétif in Northeast of 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 (Crude 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 [7].

2.3.3. Fractionation of methanolic extract

Fractionation of the crude extract is performed using a series of solvents of increasing polarity. The crude extract was initially mixed with the hexane (V / V) to eliminate lipids, and after separation, the upper organic phase was recovered. This step is repeated several times with renewal of the solvent until it becomes transparent. The hexane is then evaporated and the resulting extract is considered as the fraction of hexane. The lower aqueous phase was subjected to another fractionation with chloroform to give the chloroform extract (ChE), and finally by ethyl acetate to give the fraction of ethyl acetate (EAE). All fractions were preserved at -20°C until use.

2.4. Determination of total polyphenol content

Total phenolic content was determined using Folin-Ciocalteu method, according to Li and al, [8] with slight modifications. A volume of 100 μl of the extract was mixed with 500 μl of Folin-Ciocalteu (diluted 10% in distilled water). After 4 min, 400 μl of sodium carbonate solution Na_2CO_3 (75 g/l) was added to the mixture, the reaction mixture was incubated at room temperature for 1h 30 min and the absorbance of the mixture was measured at 760 nm, Gallic acid (20-140 mg/l) was used as standard for the calibration curve. The total polyphenols content was expressed as micrograms of gallic acid equivalents (GAE) per milligram of extract. All samples were analyzed in three replications.

2.5. Determination of total flavonoids contents

The total flavonoids in plant extracts were determined using the aluminum trichloride (AlCl_3) method [9]. Briefly, 1 ml of 2% AlCl_3 in methanol was mixed with 1 ml of the extract. After incubation in dark at room temperature for 10 min, the absorbance of the reaction mixture was measured at 430 nm. Quercetin and rutin (1-40 mg/l) were used as standards for calibration curve and the total Flavonoids content was expressed as micrograms quercetin and rutin equivalents (QE

and RE, respectively) per milligram of extract.

2.6. Determination of tannins contents

The test of haemoglobin precipitation by tannins compounds was used [10]. Briefly, a volume of each plant extract was mixed with an equal volume of hemolysed blood (absorbance (576 nm) = 1.6). After 20 minutes incubation, this mixture was centrifuged for 10 minutes at 4°C and the absorbance of the supernatant was measured at 576 nm. Different concentrations of tannic acid were also mixed with an equal volume of hemolysed blood and the absorbance was measured in the same manner. The tannins content was expressed as micrograms tannic acid equivalent per milligram of extract.

2.7. Evaluation of antioxidant activity

2.7.1. DPPH free radical-scavenging assay

The free radical scavenging activity of the extracts was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [11]. After dissolving the aqueous extract in distilled water, the methanol, chloroform and ethyl acetate extracts in methanol, the solution of DPPH in methanol (0.04 mg/mL) was prepared and 1250 µL of this solution was added to 50 µL of extracts solution at different concentration. The mixture was shaken vigorously and then kept in the dark for 30 minutes at room temperature. Then, the absorbance was measured at 517 nm. BHT, rutin, quercetin and gallic acid were used as standards. All tests were performed in triplicate. Radical-scavenging activity was calculated using the following equation:

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

A_{blank} : Absorbance of the control

A_{sample} : Absorbance of the reagent with extract.

2.7.2. β -carotene/linoleic acid assay

In this test, the antioxidant capacity of the extracts was determined by measuring the inhibition of the oxidative degradation of β -carotene (discoloration or bleaching) by the oxidation products of the acid linoleic [12]. The β -carotene solution was prepared by dissolving 0.5 mg β -carotene in 1 mL of chloroform. One milliliter of this solution was pipetted to a flask covered with aluminum foil. Then 25 µL of linoleic acid and 200 mg of tween 40 were added. The chloroform was evaporated using evaporator at 45°C. Then 100 mL of distilled water saturated with oxygen was added. 2.5 mL of

this prepared β -carotene solution were transferred to test tubes, and 350 µL of the extracts (2 mg/mL methanol) were added before incubation for 48 h at room temperature. The same procedure was repeated with butylated hydroxytoluene (BHT) as a positive control and with distilled water and methanol as a negative control. The absorbance was reading at 490 nm after 1 h, 2 h, 6 h, 24 h and 48 h. The antioxidant activity of extracts was calculated using the following equation:

$$AA\% = A_{\text{sample}} / A_{\text{BHT}} \times 100.$$

A_{sample} : Absorbance in the presence of the Extract; A_{BHT} : Absorbance in the presence of positive control BHT.

2.7.3. Chelation of ferrous iron

The chelating ability of the extracts is determined according to the method of Le and al, [13], which is based on the inhibition of the formation of Fe^{2+} -ferrosine complex after treatment of samples with Fe^{2+} ions. Briefly, 250 µL of extract solutions at different concentrations were added to 50 µL of FeCl_2 (0.6 mM in distilled water) and 450 µL of methanol. After 5 min of incubation, 50 µL of ferrosine (5 mM in methanol) was added, and the mixture was stirred and allowed to react for 10 min to allow the complexation of residual iron. The absorbance of the Fe^{2+} -ferrosine complex was measured at 562 nm against a blank contained all the reaction reagents except ferrosine. Moreover, the negative control containing all reagents except that test sample is replaced by the same volume of methanol. EDTA was used as reference chelator.

The chelating effect was calculated as a percentage, using the same equation as that in the DPPH assay.

Statistical Analyses

The results are expressed as the mean \pm 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. Total polyphenols, flavonoids and tannins contents

The contents of total polyphenols, flavonoids and tannins in extracts are shown in Table 1. The results showed that EAE contain the highest amount of phenolic compounds ($257.1 \pm 0.703 \mu\text{g GAE/mg}$), followed by ChE ($245.6 \pm 6.005 \mu\text{g GAE/mg}$), CrE ($81.56 \pm 0.766 \mu\text{g GAE/mg}$) and AqE ($76.90 \pm 0.681 \mu\text{g GAE/mg}$).

Table 1. Total polyphenols, flavonoids and tannins content of *Ruta montana* L. extracts.

Extract	Polyphenols		Flavonoids	Tannins
	µg GAE/mg extract	µg QE/mg extract	µg RE/mg extract	µg TAE/mg extract
CrE	81,56 ± 0,766	50,80 ± 1,247	60,10 ± 1,484	80.33 ± 0.70
ChE	245,6 ± 6,005	35,81 ± 1,118	42,23 ± 1,330	83 ± 0.98
EAE	257,1 ± 0,703	117,4 ± 3,451	139,5 ± 4,107	251 ± 1.41
AqE	76,90 ± 0,681	22,54 ± 0,2205	26,54 ± 0,3002	33.6 ± 0.84

CrE; methanolic extract, ChE; chloroform extract, EAE; ethyl acetate extract, AqE; aqueous extract; GAE: gallic acid equivalent; QE: quercetin equivalent; RE: rutin equivalent; TAE: tannic acid equivalent. Each value represents the mean ± SD (n = 3).

The highest value of flavonoids found in EAE was 117,40 ± 3,45µg QE/mg, 139,5 ± 4,11µg RE/mg and the lowest was 22,54 ± 0,22 µg QE/mg, 26,54 ± 0,30µg RE/mg for AqE with the following decreasing order: EAE >CrE>ChE>AqE. On the other hand, EAE contained the highest amount of tannins (251 ± 1.41µg TAE/mg E), followed by ChE (83 ± 0.98µg TAE/mg E), CrE (80.33 ± 0.70 µg TAE/mg E) and the AqE (33.6 ± 0.84 µg TAE/mg E).

3.2. Antioxidant activity evaluation

3.2.1. DPPH radical scavenging activity

The scavenging ability of the extracts was expressed as IC₅₀ value, which is the concentration of substrate that causes 50% loss of DPPH activity (color) [14]. Low IC₅₀ value indicates strong ability of the extract to act as DPPH scavenger. The DPPH scavenging activity of the extracts is represented in table 2. The results revealed that the EAE exhibited the highest radical scavenging activity with IC₅₀ of (0.044± 0.001mg/ml), which was significantly near to that of BHT (0.032± 0.000mg/ml), followed by CrE with 0.067± 0.002mg/ml, AqE with 0.083±0.003mg/ml and ChE with 0.146±0.015mg/ml.

Table 2: DPPH scavenging activity of *Ruta montana* L. extracts and standards.

Standards	IC ₅₀ (mg/mL)	Extracts	IC ₅₀ (mg/mL)
BHT	0.032 ± 0.000	CrE	0.067 ± 0.002**
Gallic acid	0.056 ± 0.001#	ChE	0.146 ± 0.015***
Quercetin	3.491 ± 0.001#	EAE	0.044 ± 0.001 ^{ns}
Rutin	4.179 ± 0.000#	AqE	0.083 ± 0.003***

#: µg/ml. Comparison was realized against BHT, **: p ≤ 0.01, ***: p ≤ 0.001. Each value represents the mean ± SD (n = 3).

3.2.2. β-carotene/linoleic acid bleaching assay

As can be seen in figure 1 and figure 2, all the extracts were capable of inhibiting the bleaching of β-carotene by scavenging linoleate derived free radicals.

AqE and ChE exhibited the highest antioxidant activity (90.34 ± 0.46% and 89.92 ± 0.29%, respectively), which was near to that of BHT (100 ± 0.52%), following by EAE and CrE with an antioxidant activity (73.56 ± 0.63 % and 63.27 ± 2.27%, respectively).

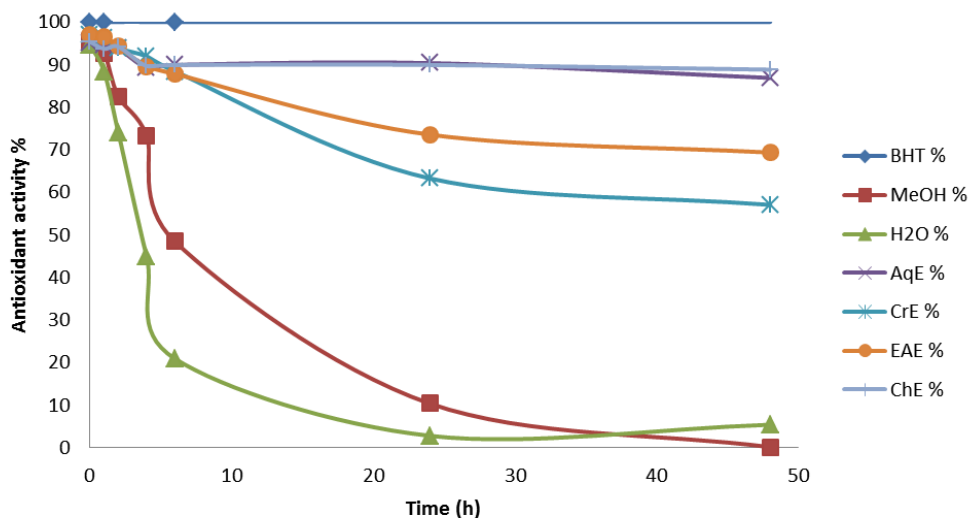


Fig.1. The changes in the percentage of the inhibition ratios of linoleic acid oxidation under the influence of *Ruta montana L.* extracts (2mg/mL), compared to BHT as a positive control during 48 h. AqE: aqueous extract, CrE: crude extract, ChE: chloroform extract, EAE: ethyl acetate extract.

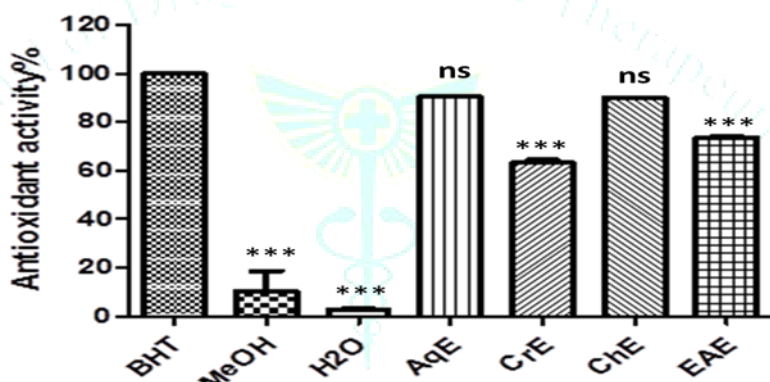


Fig.2. Inhibition percentage of extracts of *Ruta montana L.* in β -carotene/linoleic acid assay after 48 h compared with BHT, methanol and water as standards. Results represent the means \pm SD (n = 3). High inhibition percentage indicates higher antioxidant activity. AqE: aqueous extract, CrE: crude extract, ChE: chloroform extract, EAE: ethyl acetate extract.

3.2.3. Metal chelating activity

Table 3 list IC₅₀ values of *Ruta montana L.* extracts obtained from measurement of metal chelating activity. The smaller IC₅₀ value represents the higher metal chelating activity. The

AqE and CrE appear to have the most important chelating activity with IC₅₀ values of 0.005 \pm 0.004 mg / ml and 0.021 \pm 0.005mg/ml, respectively. The chelating effect of ChE and EAE is very low (IC₅₀ = 0.146 \pm 0.015 and 0.771 \pm 0.021mg / ml, respectively).

Table 3. Metal chelating activity of *Ruta montana L.* extracts and EDTA.

Extracts	IC ₅₀ (mg/ml)
CrE	0.021 \pm 0.005 ^{ns}
ChE	0.146 \pm 0.015 ^{***}
EAE	0.771 \pm 0.021 ^{***}
AqE	0.005 \pm 0.004 ^{ns}
EDTA	5.32 \pm 0.03 [#]

#: μ g/mL. Comparison was realized against EDTA; ***: p \leq 0.001. Each value represents the mean \pm SD (n = 3).

Plant phenolics, tannins and flavonoids represent major groups of plant constituents that work predominantly as powerful antioxidants or scavenger of free radicals. They play beneficial role in human health and cure or prevent ailments such as inflammatory disorders, cardiovascular diseases, cancer and diabetes which occur due to the deregulation of free radicals generation in the cells [15].

There have been two opinions on the correlation between phenolics and total antioxidant activity. Some reports demonstrated positive correlation between them [16,17] and the others showed no correlation [18]. Our results agree with the latter, it seems that no correlation exists either between the percentage inhibition (% scavenging effect) assayed by DPPH and total phenolics, between the antioxidant activity assayed by β -carotene bleaching method and total phenolics, or between the antioxidant activity assayed by ferrous ion chelating ability and total phenolics. [19] thought this phenomenon can be explained on the basis of high antioxidant activity of some individual phenolic units, which may act as efficient antioxidants rather than contributing to high total phenolics, while [20] thought that total phenolics content did not include all the antioxidants, such as ascorbic acid, carotenoid and tocopherol [21] held the idea that the synergism among the antioxidants in the mixture made the antioxidant activity not only dependent on the concentration of antioxidant, but also on the structure and interaction among the antioxidants. This probably is the reason why samples with similar concentrations of total phenolics may vary remarkably in their antioxidant activity.

In general the antioxidant activity of phenolic compounds reportedly varies with the structure and degree of hydroxylation of the aromatic ring [22,23]. It is associated with the number of hydroxyl groups and the most active possess from 3 to 6 hydroxyl groups. Hydroxylation in the C3 position seems to be detrimental for their antioxidant potency [24]. [25] reported that for benzoic and cinnamic acid derivatives, flavonols and anthocyanidins, an increase in the number of hydroxyl groups on the aromatic ring lead to higher antioxidant activity *in vitro*. Compounds with three hydroxyl groups on the phenyl ring of phenolic acids or the B-ring of flavonoids had high antioxidant activity. The loss of one hydroxyl group decreased activity slightly.

3' 4' ortho dihydroxy configuration (catechol) in B ring and 3-OH group in C ring is very important for antiradical efficacy. Catechol ring and C2 – C3 double bond conjugated with 4-

keto moiety are essential for electron delocalization from the ring B, and it increases the antiradical activity [26]. Many studies showed that when these structural features were removed from flavonoids structure their antioxidant activity was decreased [27, 28].

4. CONCLUSION

The present study aimed to evaluate the Antioxidant activity of extracts prepared from the aerial parts of *Ruta montana* L., this activity was tested *in vitro* using three methods: DPPH (2, 2'-diphenyl- 1- picrylhydrazyl), bleaching of β -carotene and chelation of ferrous iron. The results showed that *Ruta montana* L. has an important antioxidant activity. The ethyl acetate extract exhibited the highest scavenging activity, whereas aqueous extract showed the best chelating capacity and the best inhibitory capacity of the coupled oxidation system of linoleic acid/ β -carotene.

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