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Phenolic Contents and Antioxidant Capacities of Various Extracts of *Thymus Riatarum* from Morocco

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Abstract

Thymus riatarum (Family Lamiaceae) is endemic Moroccan species used at numerous medicinal and culinary effects. The objective of the current study is to determine, for the first time, antioxidant activity, total flavonoid and total phenolic content of four extracts (water, methanol, ethanol, and ethyl acetate) of *Thymus riatarum* aerial parts from Morocco. The antioxidant properties were evaluated in vitro using five separate methods, inhibition of free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), measurement of total antioxidant capacity, ferric reducing/antioxidant power (FRAP) assays, Total phenolic, and total flavonoid contents. The ethanol extract was able to reduce the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) with an IC_{50} value equal to 0.11±0.016 mg/ml and showed an important total antioxidant, which was probably due to its high polyphenols content (328.5±3.216 mg GAE/g DW). The reducing power of water extract was found superior than those of synthetic antioxidants (0.150±0.013 mg/ml). However, flavonoids were determined at the highest level in ethyl acetate extract. These findings suggest that *Thymus riatarum* may be a new potential source as a natural antioxidant and recommended for further food applications.

Keywords: Antioxidant activity, DPPH, Flavonoids contents, FRAP, *Thymus riatarum,* Total phenolic content.

INTRODUCTION

Various plants have used as both traditional medicine and food throughout the world. Therefore, investigation of natural antioxidant has been a major research interest for the past two decades as many studies interest institutions have been screening plant materials for possible antioxidant properties [1].

Oxidative stress is important from a biomedical point of view because it caused a variety of human chronic diseases, such as diabetes, allergies, cardiovascular, neurodegenerative and inflammatory diseases.

The Mediterranean region can be described as the center of the genus Thymus (family Lamiaceae) [2]. This genus is represented in Morocco by twenty one species, twelve are endemic [3]. Some of these species have been used for their preservative and medicinal properties [2] and have been added to foods (condiment and spice) [4] their oil and extracts has found applications in cosmetic, such us toothpastes, and deodorants [5]. It is reported in the previous research that Thymus species have strong antibacterial [6], antioxidant [7], antifungal [8, 9] antispasmodic carminative [10], diuretic effects [11] and antiviral activities [2]. In the present paper, we report the results of a study aimed to evaluate the antioxidant activities of methanol, ethanol, aqueous and ethyl acetate extracts of *Thymus riatarum*. These activities were determined by using five in vitro assays: inhibition of free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH), measurement of total antioxidant capacity, ferric reducing/antioxidant power (FRAP) assays, Total phenolic, and flavonoid. To the best of our knowledge, the antioxidant activities of *Thymus riatarum* extracts have not been carried out.

MATERIAL AND METHODS

Reagents and standards

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), Butylated hydroxytoluene (BHT), aluminum chloride ($AlCl_3$), sodium phosphate, ammonium molybdate, iron III chloride ($FeCL_3$), potassium ferricyanide ($K_3Fe(CN)_6$), sodium nitrite ($NaNO_2$), ascorbic acid, gallic acid and Folin-Ciocalteu were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate (Na_2CO_3) was purchased from Prolabo (Paris, France). All the other chemicals and solvents used were of analytical grade.

Plant materials

Aerial parts of *Thymus riatarum* were collected in January 2016 in a winery located in hills in the region of Taza, 100 km from Fez city, Morocco. They were identified by Professor Amina Bari, botanist (Department of Biological Sciences, Faculty of Science, Sidi Mohammed Ben Abdellah University, Fez (Morocco). The plant material was dried for 7 to 10 days in the shade, and then stored in cloth bags at 5°C until extraction.

Preparation of extracts

Plant material was powdered (5g) and extracted with 100 ml of solvent (water, methanol, ethanol, and ethyl acetate) for 8 h using a Soxhlet extractor. Afterwards, all the resulting extracts were filtered and evaporated under reduced pressure at 40°C using rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C.

Statistical analysis

Means of triplicate analysis were calculated and data was expressed as mean ± SD. One-way ANOVA statistical analysis followed by Tuckey-test was performed using the GraphPad Prism 5 (Microsoft Software) for comparison between the extracts. A difference was considered to be statistically significant when $P < 0.05$.

Total phenolic content

Total phenolic content of the extract was determined by Folin-Ciocalteu method [12]. The 0.5 ml of a known dilution of the extract and 2 ml of 7% sodium carbonate solution was added to 2.5 ml of 10% (v/v) Folin-Ciocalteau reagent. The absorbance was read at 760 nm (Jasco v-530) after 2H of reaction at room temperature in the dark. Gallic acid was used as standard for the construction of calibration curve. Total phenols contents were expressed as milligrams of gallic acid equivalents per gram powder (mg GAE/g DW).

Total flavonoids contents

Total flavonoids contents of extracts were measured by the aluminium chloride colorimetric assay [13]. 1 ml of sample or rutin standard solution was added into a 10 ml volumetric flask containing 4 ml of distilled water. To the flask 0.30 ml 5% $NaNO_2$ was added, after five minutes 0.3 ml 10 % $AlCl_3$ was added to react for six min. After that, 2 ml NaOH was added and the total was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm (Jasco v-530). Rutin was used as standard for the construction of calibration curve. Total flavonoids contents were expressed as mg rutin equivalents per gram dry weight of each extract (mg RE/g DW). All samples were analyzed in triplicate.

Antioxidant activity

DPPH radical scavenging activity

The DPPH method was introduced 50 years ago by Blois [14]. The ability extracts to scavenge the DPPH radical was measured

using the method by Wu et *al*. [15]. 0.1 ml of various concentrations of extracts or standard was added to 1.5 ml of ethanolic solution containing 0.1 mmol of DPPH (2, 2-diphenyl-1-picrylhydrazyl). The absorbance of the mixture was measured at 517 nm with a spectrophotometer (Jasco V-530) after 30 min of incubation time at room temperature in dark. The percentage inhibition was calculated by the following equation:

$$I\ (\%) = (1-(A_s / A_0))*100$$

Where A_0 is the absorbance of the negative control, and A_s is the absorbance of the sample. BHT served as positive control. The IC_{50} values were calculated as the concentration of causing a 50 % inhibition of DPPH radical.

Reducing power capacity

The reducing capacity of the tested extract was determined in accordance with the procedure of Oyaizu [16]**.** 100 µl of extract was mixed with 500 µl of phosphate buffer (0.2 M, pH 6.6) and 500 µl of potassium ferricyanide [$K_3F_e\ (CN)_6$] 1%. The obtained solution was incubated at 50 °C for 20 min. The mixture was acidified with 500 µl of Trichloracetic (TCA) 10 % which was then centrifuged at 3000rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 500 µl of distilled water and 100 µl of F_eCl_3 (0.1 %), and the absorbance was measured at 700 nm (Jasco v-530). Quercitine was used as standard. The results were expressed as IC_{50} (mg/ml). IC_{50} (concentration corresponding 0.5 of absorbance) was calculated by plotting absorbance against the corresponding concentration.

Total antioxidant capacity

The assay was based on the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo (V) complex in acid pH [17]. A total volume of 25 µl oils dissolved in ethanol was added to 1 ml of reagent solution (0.6 mol/l sulphuric acid, 28 mmol/l sodium phosphate and 4 mmol/l ammonium molybdate). The mixtures were incubated at 95 °C for 90 min then cooled to room temperature. The absorbance was measured at 695 nm (Jasco v-530). The total antioxidant activity was expressed as the number of equivalence of ascorbic acid (mg AAE/g DW).

RESULTS AND DISCUSSION

Total phenolic (TPC) and total flavonoids (TF) contents

Table 1: Total phenolic and flavonoid contents of extracts from *T.riatarum*

Type of extracts	*Total phenolic content (mg GAE/g DW)*	Total flavonoid content (mg RE/g DW)
Ethanolic	328.5±3.216[a]	212.3±1.528[b]
Methanolic	291.2±1.767[b]	192±1.732[c]
Ethyl acetate	3.850±0.196[d]	349.7±1.155[a]
Aqueous	170.9±1.863[c]	13.78±0.347[d]

All the results were expressed as mean ± SD of three different trials. a –d Column wise values with same superscripts of this type indicate no significant difference (P<0.05). In each line, values followed by different letters are significantly different (P < 0.05).

Phenolic compounds are considered to be the major contributors to the antioxidant capacity of plants. Among the four extracts of *Thymus riatarum* (Table 1), the ethanol significantly demonstrated the highest phenolic content (328.25 ± 3.216 mg GAE/g DW), followed by methanol (291.2 ± 1.767 mg GAE/g DW), water (170.9 ± 1.863 mg GAE/g DW) and ethyl acetate value of (3.850 ± 0.196 mg GAE/g DW).

The total flavonoid contents significantly varied among the studied extracts (Table 1). The uppermost amounts were observed for ethyl acetate (349.7 ± 1.155 mg QE/g DW). Whereas minimum one was noticed for water (13.78±0.347 mg QE/g DW). This level of total phenols was found to be higher than the values reported in the literature for *Thymus* species such as *T. spathulifolius* (141 mg GAE/g of a methanol extract, obtained by Sokmen et *al*. [18], *T. serpyllum* (113 mg/g of an ethanol extract, reported by Mata et *al*. [19], *T. vulgaris* harvested in Iran (5.82±0.42 mg gallic acid (GAE)/g) reported by [20], *Thymus caramanicus* (124.30 mg GAE/g) reported by Safaei-Ghomi et *al* .[21], *T. satureioides* collected from Morocco (48.43 mg eqAG/g) reported by [22] and in Italy, *T. vulgaris* (165.1 mg GAE/g) reported by Mancini et *al.* [23]. While *T.capitatus* showed a total flavonoid content value of (10.62±0.24 mg QE/g DW) [24]. Baharfar et *al*. [25] recorded that the flavonoid contents value of *T. kotschyanus* ranged from 32.04–74.60 mg QE/g DW. Other research demonstrated that the aqueous extract of *T. satureioides*, *T. atlanticus*, and *T. zygis* had a greater flavonoid contents (182.79 ± 3.23 mg QE/g DW) (155.11 ± 3.90 mg QE/g DW) and (208.13 ± 4.20 mg QE/g DW) respectively [35]. This difference in results depends on the type of solvent and the methods of extractions [26]. Previous studies have shown that the amount of phenol compounds in plants depend also on biological factors and environmental conditions (temperature, water stress and salinity). In other hand, a recent research by Pereira and Cardoso [27] summarized the polyphenolic compositions of various Thymus species. In addition, various experimental investigations have shown that many secondary metabolites such as polyphenol compounds extracted from medicinal and aromatic plants had a high antioxidant activity due to their hydroxyl groups and protected more efficiently against many diseases caused by free radical [28].

DPPH radical scavenging activity

Table 1: DPPH radical scavenging activity and ferric reducing power capacity of different extracts from *Thymus riatarum.*

Type of extracts	*DPPH (mg/ml)*	FRAP (mg/ml)
Ethanolic	0.110±0.016[c]	0.312±0.017[b]
Methanolic	0.085±0.010[c]	0.179±0.013[c]
Ethyl acetate	0.766±0.028[b]	0.639±0.053[a]
Aqueous	1.079±0.016[a]	0.150±0.013[c]
BHT	0.119±0.002[c]	-
Quercitine	-	0.03±0.006[d]

Data are reported as mean values ± SD of three measurements. Means were significantly different when P<0.05; values followed by different letters are significantly different.

Table 2 depicts DPPH radical scavenging activities of extracts of *Thymus riatarum*. The lowest IC_{50} values indicated the highest free radical scavenging activity of the extract. As shown in Table 2, all extracts obtained from *Thymus riatarum* showed antioxidant activity in this assay with an IC_{50} ranged from 0.085 to 1.079 mg /ml for methanol and aqueous extracts, respectively. The IC_{50} value of methanolic (0.085 ± 0.010 mg/ml) and ethanolic extracts (0.110 ± 0,016 mg/ml), were significantly similar to that BHT, positive control, (0.119 ± 0.002 mg/ml). This antioxidant propriety may be attributed to the presence of certain compounds in our extracts with hydrogen donating ability. The methanolic extract of *T.riatarum* showed higher activity than that founded by Ben El Hadj Ali [29] (IC_{50} = 0.011 mg/ml) and lower than the IC_{50} value of *Thymus caramanicus* reported by Safaei-Ghomi [21] (IC_{50} = 0.043 mg/ml). In addition, previous research attested an important antioxidant activity of various extracts from Thymus species such as *T. praecox* [30] and *T. vulgaris* [31].

Ferric reducing capacity

The ferric reducing power assay does not involve free radicals, but involves reduction of the ferricyanide to ferrocyanide, by the reducing agent [17]. This capacity is compared to that of quercetin. The results (Table 2) showed a range for FRAP

antioxidant capacity from 0.150 ± 0.013 to 0.639 ± 0.053 mg/ml. The aqueous extract significantly contained the highest ferric reducing power (0.150 ± 0.013 mg/ml) than the other extracts.
This strong ferric reducing power of *T. riatarum* extracts demonstrate the good hydrogen and/or electron donor abilities of these extracts which probably owed to their antioxidant propriety. Previous studies had indicated that the Thymus species possessed the most effective capacity for ferric reducing power [32- 34].

Total antioxidant capacity

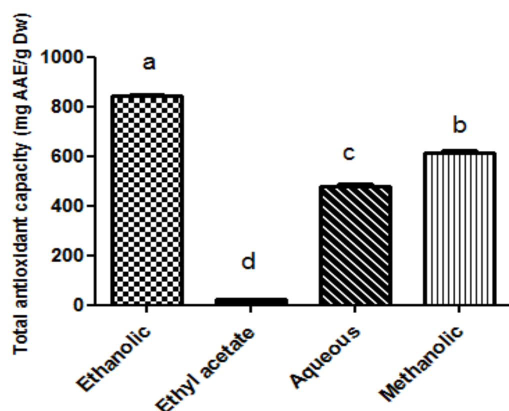

Figure 1: Total antioxidant capacity of different extracts from *Thymus riatarum*
Each value represents means ± SD (n=3). Different letters are significantly different ($P < 0.05$)

The total antioxidant propriety was evaluated by the formation of phosphomolybdenum complexes. This assay is based on the reduction of Mo (VI) to Mo (V) by the extract; the reaction of this method is accompanied by the appearance of a green color. The results of the studied extracts were expressed as ascorbic acid equivalents (mg AAE/g DW) (Figure 1). The ethanol significantly revealed the highest total antioxidant capacity (845.7±4.619 mg AAE/g DW) followed by methanol (615.3±16.80 mg AAE/g DW), then water (479±6.928 mg AAE/g DW) and ethyl acetate ((22.67±0.5777 mg AAE/g DW). Previous published papers demonstrated that extracts of Thymus spices have strong ability to act as antioxidant [29,34,35]. In another study, they evaluated the ability of *T. vulgaris* oil to protect the laboratory animals from the toxic effects of AFs [36].

CONCLUSION

The present study is the first characterization of the antioxidant capacities of *Thymus riatarum* extracts. All investigated extracts showed remarkable antioxidant potential and important phenolic and flavonoid contents of this Moroccan thymus. Thus, the present study suggests this plant can be a source of natural antioxidants. It may be used in many fields, such as cosmetics, pharmaceutical fields and natural food preservatives, which is very well justified by the present work.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest related with this manuscript.

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