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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Document heading doi: 10.1016/S2221-1691(15)30151-9

Investigation of antioxidant and antihemolytic properties of *Thymus satureioides* collected from Tafilalet Region, south-east of Morocco

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PEER REVIEW

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Comments

This is a valuable research work in which authors have analyzed, using the HPLC approach, the aqueous extract of the *T. satureioides*. In addition, *in vitro* and *in vivo* studies clearly showed an antioxidant and antihemolytic properties.

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ABSTRACT

Objective: To examine the antioxidant and antihemolytic activities of the aqueous extract, total polyphenols and total flavonoids of *Thymus satureioides* (*T. satureioides*).

Methods: This plant was collected from Tafilalet Region of Morocco. The aqueous extract was obtained by cold maceration, and the components were obtained by Soxhlet extraction using solvents of varying polarity. The identification and quantification of phenol (caffeic and rosmarinic acids) and flavones (luteolin 7-glycoside and hesperetin) were carried out by high performance liquid chromatography analysis.

Results: Total polyphenol and flavonoids contents in the aqueous extract of *T. satureioides* were (456.73±6.94) mg caffeic acid equivalent/g of dry plant and (172.79±2.12) mg rutin equivalent/g of dry plant, respectively. Different extracts showed good antioxidant activity. IC_{50} for 1,1-diphenyl-2-picrylhydrazil radical scavenging activity was (0.480±0.010), (0.418±0.005), (43.891±2.467) and (0.510±0.010) mg/mL for the aqueous extract, total polyphenol, flavonoids and trolox, respectively. Also, the extracts showed ferric reducing antioxidant power and the values were (50.79±2.02), (117.51±6.46), (7.03±0.29) and (44.33±7.55) mmol trolox/g for the aqueous extract, total polyphenol, flavonoids and trolox, respectively. Serum levels of malondialdehyde was significantly decreased in comparison with the oxidized control (P<0.001). They showed good activity against 2,2,-azobis 2-amidinopropane dihydrochloride induced hemolysis in erythrocytes of rabbit blood. In addition, they ameliorate the half time of hemolysis.

Conclusions: Our results provide evidence that aqueous extract, total polyphenols and total flavonoids of *T. satureioides* exhibit marked antioxidant and antihemolytic activities, thus confirming and justifying the popular uses of this plant to relieve some pains.

KEYWORDS

Thymus, Polyphenols, Flavonoids, Antioxidant, Antihemolytic, Lipid peroxidation

1. Introduction

Thymus species are known to have significant amounts of phenolic and flavonoid compounds and exhibit strong antioxidant activities[1]. Also, the previous studies have evaluated and demonstrated four varieties of thyme with the important antioxidant capacity[2,3]. Many current human health problems relate to diets. Micronutrients are involved in numerous biochemical processes and

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Article history:
Received 29 Aug 2014
Received in revised form 9 Sep 2014
Accepted 8 Oct 2014
Available online 16 Oct 2014

an adequate intake of certain micronutrients relates to the prevention of deficiency diseases. Malnutrition is a major concern in many tropical developing countries. Iron deficiency anemia, for example, affects one third of the world population[4,5].

Phenolic compounds can prevent oxidative damage via a number of different mechanisms, such as free radical scavenging, transition metal chelation and interactions with lipid membranes, proteins and nucleic acids[6]. The spectrum of phenolic compounds isolated from plants depends on several factors, such as the nature of tissue matrix, the extraction time and temperature, and the polarity of solvent system[6]. Water, ethanol, methanol, acetone and aqueous/organic solvent mixtures are frequently used to extract phenolic compounds from plants[7-9]. Plants of the mint family (Lamiaceae) produce many metabolites with bioactive properties. There is a great interest in the production of specific metabolites under controlling environmental conditions that maintain a given phytochemical profile[10,11]. Polyphenolic compounds have been reported to have multiple biological effects, including antioxidant activity[12].

In Morocco, the genus of *Thymus* is represented by many species which some of them are endemic. The flowered stem contains essentially flavonoids (derived of apigenol and luteolin), and acids phenols (in particular, caffeic and rosmarinic acids)[2,13].

Thymus satureioides (*T. satureioides*), a member of Limiaceae family, is an aromatic plant of Mediterranean flora and is commonly used as spices and traditional medicine remedies. They are reported to possess some biological effects such as antioxidant and antispasmodic[14-16], antibacterial[17,18], antifungal[19,20], and anti-inflammatory properties after topical or oral administration[21].

Among various medicinal plants, some endemic and edible species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and high content of minerals with health benefits. Herbs are used in many domains, including medicine, nutrition, flavouring, beverages, dyeing, repellents, fragrances and cosmetics[22]. Many species have been recognized to have medicinal properties and beneficial impact on health, *e.g.* antioxidant activity, digestive stimulation action, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic effects and anticarcinogenic potential[23]. To the best of our knowledge, there is no scientific reports about antioxidant and antihemolytic activities of polyphenols and flavonoids extracted from *T. satureioides*. In this study, the antioxidant activity of aerial parts of this plant at flowering stage is examined by different *in vitro* assays in order to understand the usefulness of this plant in traditional medicine.

2. Materials and methods

2.1. Plant material

T. satureioides was collected in April-May 2009 in the Tafilalet Region, South-east of Morocco. The plant was determined and confirmed by Dr. Ibn Tatou and a voucher specimen was deposited at the herbarium of the Scientific Institute, University Mohammed V, Rabat, Morocco (No.: RAB 77497).

2.2. Preparation of the aqueous extract

The aqueous extracts of *T. satureioides* were prepared using a manner similar to that used by patients with some modifications. The dried powder from aerial parts (50 g) of the plant was collected by a Soxhlet extractor for 4 h, filtered, and the obtained solution was concentrated in a rotatory evaporator under vacuum at 60 °C. The yield of extracts in terms of starting dried plant material was 14% (w/w). The crude extract was suspended in distilled water and the aliquots were stored at -20 °C before use.

2.3. High performance liquid chromatography (HPLC) analysis

Gradient HPLC analysis of thyme extracts was carried out on a ReproSil Pur C18 column equipped with a photodiode array detector. Chromatographic separation of extract (100 μL) was conducted using C18 analytical column (250 mm, 3 mm) with a particle size of 5 μm thermostated at 28 C. The flow rate was 0.5 mL/min and the absorbance changes were monitored at 215, 250 and 280 nm. The mobile phase consisted of 0.2% glacial acetic acid in methanol/water (20/80) (solvent A) and 0.2% glacial acetic acid in methanol/water (80/20) (solvent B). The following gradient profile was used: initially 100% (A) and 0% (B) at 0 min, 50% (A) and 50% (B) for 10 min, 17% (A) and 83% (B) for 20 min [which was changed to 100% (A) and 0% (B) in 5 min untel completion of the run]. The retention time of phenolic standards and the corresponding UV spectra were used for identification of the compounds in *T. satureioides* extracts.

2.4. Polyphenols extraction

The polyphenols extraction has been made by the method of Jordan *et al.* with some modifications[24]. The dried powder from aerial parts (50 g) of the plant was defatted with *n*-hexane (C_6H_{14}) in a Soxhlet extractor. Afterwards, the residue was air-dried and extracted with methanol 80% (at 45 °C for 16 h). The resulting residue was suspended in distilled water and extracted with *n*-butanol. The aqueous fraction (polyphenolic fraction) was concentrated in distilled water for a battery of biologic tests.

2.5. Flavonoids extraction

The flavonoids extraction has been made by the method of Lee *et al.* with some modifications[25]. The dried aerial parts of herbs (50 g) were extracted by Soxhlet in distilled water and ethanol (1/1) for 4 h at 60 °C, and then filtered. The aqueous phase obtained was extracted by the *n*-butanol and then acidified by HCl 10% (pH 3). The butanolic fraction has been concentrated in rotator evaporator under vacuum at 40 °C. The obtained product has been extracted three times by the mixture of distilled water/ethyl acetate (1/1) for 1 h and the organic fraction has been basified by $NaHCO_3$ (pH 9). After 15 min, the organic fraction (total flavonoids) has been evaporated at 40 °C, and weighed and the residue was suspended in ethanol for the biologic tests.

2.6. Determination of total phenol contents

Total polyphenol contents in aqueous extracts were assayed using the Folin-Ciocalteu reagent, following Singleton and Rosis method[26], based on the reduction of a phosphowolframate-phosphomolybdate complex by phenolics to blue reaction products and slightly modified by Dewanto *et al.* with some modifications[27]. An aliquot of diluted sample extract was added to 3.5 mL of distilled water and 0.25 mL of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before addition of 0.5 mL of 7% Na_2CO_3. The solution was then adjusted with distilled water to a final volume of 5 mL and mixed thoroughly. After incubation in dark for 30 min, the absorbance was read at 725 nm versus the prepared blank. Total polyphenols content of the aqueous extract was expressed as milligrams of caffeic acid equivalents per gram of dry plant through the calibration curve with caffeic acid. All samples were analyzed in three replications.

2.7. Determination of flavonoid contents

The flavonoid contents in extract was determined spectrophotometrically according to Jay *et al.* using a method based on the formation of a complex flavonoids-aluminium[28], having the maximum absorbance at 430 nm. Rutin was used to make the calibration curve. The flavonoid contents were expressed in milligrams per gram of rutin equivalent (mg/g extract). The analyses were done in triplicate.

2.8. Antioxidant study of T. satureioides aqueous extract, polyphenols and flavonoids

2.8.1. Radical scavenging activity (RSA) assay

1,1-Diphenyl-2-picrylhydrazil (DPPH) is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The model of scavenging the stable DPPH radical is widely used for relatively rapid evaluation of antioxidant activities compared to other methods[29]. The capacity to scavenge the stable free radical DPPH was monitored according to the method of Zhang *et al.* with slight modifications[30]. Various concentrations of aqueous, polyphenol and flavonoid extracts of *T. satureioides* (100 μL) were mixed with 1.9 mL of methanolic solution containing DPPH radicals (0.06 mg/mL). The mixture was shaken vigorously and left to stand for 30 min at room temperature. The reduction of the DPPH-radical was measured by monitoring continuously the decrease of absorption at 517 nm. The RSA was calculated as a percentage of DPPH discolouration using the equation:

$$\%RSA=[(A_{DPPH}-A_S)/A_{DPPH}]\times 100$$

Where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The IC_{50} of the extract concentration was calculated from the graph of RSA percentage against extract concentration. Trolox were used as reference compound.

2.8.2. Ferric reducing antioxidant power (FRAP assay)

The method is based on the reduction of the Fe^{3+} tripyridyl triazine complex to the ferrous form at low pH[31]. This reduction is monitored measuring at 37 °C, the absorption change at 593 nm under in acid conditions (pH 3.6).

2.8.3. Malondialdehyde (MDA) assay

The determination of thiobarbituric acid reactants is a widely used method for investigating overall lipid peroxidation. With plasma and lipid fractions, an assay would facilitate standardization of the method. The level of lipid peroxidation products in tissues or plasma lipid was measured as thiobarbituric acid reactive substances according to the modified method of Park *et al*[32]. Briefly, lipid-rich plasma obtained from mice injected with Triton WR-1339 at a dose of 600 mg/kg body weight was used as substrate for oxidative process. In brief, in the control tube, plasma was incubated with distilled water only. In the second test, oxidation was inducted with 10 μL of copper sulphate ($CuSO_4$; $5H_2O$) solution (0.33 mg/mL). In the third assay oxidation was inducted by copper, then aqueous extract, total polyphenols and flavonoids at the concentration of 25, 50 and 100 mg/mL, respectively, to evaluate their possible antioxidant property at 30 °C for 24 h. Then, to each assay with 100 μL of 8.1% (w/v) sulphate dodecyl sodium, the mixture was stirred and incubated for 60 min at room temperature. Afterward, the reaction mixture was heated at 95 °C for 60 min after the addition of 250 μL of 20% trichloroacetic acid (pH 3.5) and 250 μL of 0.8% (w/v) thiobarbituric acid. After cooling, 1 mL of *n*-butanol was added and vortexed. The solution was centrifuged at 4 500 r/min for 15 min and the absorbance of resulting coloured layer was recorded at 532 nm. The amount of thiobarbituric acid reactive substances was calculated as a MDA equivalent from the calibration curve of 1,1,3,3-tetramethoxypropane standard solutions and expressed as mmol/L MDA. All measurements were done in triplicate.

2.8.4. Inhibition of the 2,2,-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced erythrocyte oxidative hemolysis

The antioxidant activity of plant extracts was measured by the inhibition of AAPH-induced oxidative erythrocyte hemolysis according to the procedure established by Prost with slight modifications[33]. Blood was obtained from a rabbit and diluted with heparined 10 mmol/L phosphate buffer saline (PBS) at pH 7.4. In order to induce free radical chain oxidation in the erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH in oxygen[34]. A rabbit erythrocyte suspension in PBS was used to make different control and samples shown in Table 1.

The reaction mixture was shaken gently and incubated at 37 °C. The absorbance was read at 540 nm. The optical density is read

Table 1

Rabbit samples in two tests.

Test	Control samples	Plant extract samples	Haemolysis sample
Test one: rabbit 1	Erythrocyte suspension in PBS	Erythrocyte suspension in PBS +AAPH + plant extract	Erythrocyte suspension in PBS+AAPH
Test two: rabbit 2	Erythrocyte suspension in PBS	Erythrocyte suspension in PBS + plant extract	-

every 5 min, with the aim of measuring most correctly possible time of half hemolysis. The percentage of hemolysis inhibition was calculated by the equation[35]:

$$\%\text{Hemolysis inhibition}=[(A_{AAPH}-A_S)/A_{AAPH}]\times 100$$

Where A_S is the absorbance of the sample containing the extract, and A_{AAPH} is the absorbance of the control sample containing no extract. The IC_{50} of the extract concentration was calculated by the graph of hemolysis inhibition percentage against extract concentration.

The half-time of hemolysis corresponds in necessary time so that the initial optical density decreases in 50%. It corresponds to 50% of hemolysis of the initial erythrocytes. A high half-time of hemolysis corresponds to a good resistance of erythrocytes. The addition of an antiradical substance will lead to an increase of the half-time of hemolysis. Trolox was used as standard.

2.9. Statistical analysis

Data obtained were analyzed using the student's *t*-test and a *P* value less than 0.05 was considered statistically significant. Our results are expressed as means±SEM.

3. Results

3.1. Total polyphenol and total flavonoid contents

The yields of extraction are 14%, 6% and 7% for the aqueous extract, polyphenols and flavonoids, respectively. The total polyphenols content in aqueous extract were (456.73±6.94) mg caffeic acid equivalent/g of dry plant. The flavonoids content were (172.79±2.12) mg rutin equivalent/g of dry plant. The extract is dissolved in a minimum amount of double distilled water for biological tests.

3.2. HPLC analysis of T. satureioides extract

According to the retention time of calibration standards, thyme extracts presented a chemical profile composed of six phenolic compounds, including caffeic, rosmarinic acids, quercetin, hesperetin, luteolin-7-glycoside and apigenin-7-glycoside. The analysis of the typical HPLC chromatogram (Figure 1) depicted that rosmarinic acid is the major phenolic acid. Luteolin-7-glucoside and hesperetin were also identifed in this plant. Rosmarinic acid was expected to represent the single most antioxidative constituent (Table 2).

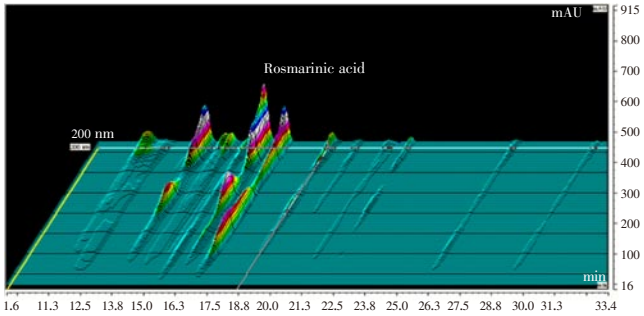

Figure 1. HPLC profiles of *T. satureoides* aqueous extract detected at 280 nm.

Table 2
Identified compounds of *T. satureioides* and their retention times.

Identified compound	Retention time (min)
No identified compound	13.70
Luteolin-7-glycoside	15.96
Rosmarinic acid	17.00
Hesperetin	22.00

3.3. Antioxidant activities

3.3.1. RSA and FRAP assay

The RSA of the different extracts was evaluated using methanolic solution of stable free radical, DPPH. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (*i.e.*, by providing hydrogen atoms or by electron donation, conceivably via a free radical attack on the DPPH molecule) and convert them to a colourless/bleached product (*i.e.*, 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation.

The antioxidant activities of *T. satureioides* are shown in Table 3. Using the RSA method the IC_{50} of aqueous extract is (0.480±0.010) mg/mL, IC_{50} of total polyphenols is (0.418±0.005) mg/mL and IC_{50} of total flavonoids IC_{50} is (43.891±2.467) mg/mL. The use of FRAP assay shows that the values of antioxidant activity are (50.79±2.02), (117.51±6.46), (7.03±0.29) mmol of trolox/g dry plant for the aqueous extract, total polyphenols and total flavonoids respectively.

Table 3
Antioxidant activities of aqueous extract, total polyphenols and the flavonoids from *T. satureioides*.

Extracts	RSA [IC_{50} (mg/mL)]	FRAP (mmol trolox/g)
Aqueous extract	0.480±0.010*	50.79±2.02^{ns}
Total polyphenols	0.418±0.005***	117.51±6.46***
Flavonoids	43.891±2.467***	7.03±0.29***
Trolox	0.510±0.010	44.33±7.55

***: $P<0.001$; *: $P<0.05$; ^{ns}: Not significant.

The scavenging effects of total polyphenols on DPPH radicals increased with increasing the concentration and were high (87%-100% at 0.78 mg/mL), comparable to the RSA values obtained for the standard Trolox (96% at 0.97 mg/mL).

3.3.2. MDA assay

The results of this test are given in Figure 2. The results reported here indicated that aqueous extract, total polyphenols and total flavonoids extracts were demonstrated the significant ($P<0.001$) reduction of MDA formed by the oxidative action of $CuSO_4$. But the total phenolic substances are the major compound with relived the higher antioxidant activity ($P<0.001$ against the oxidized control).

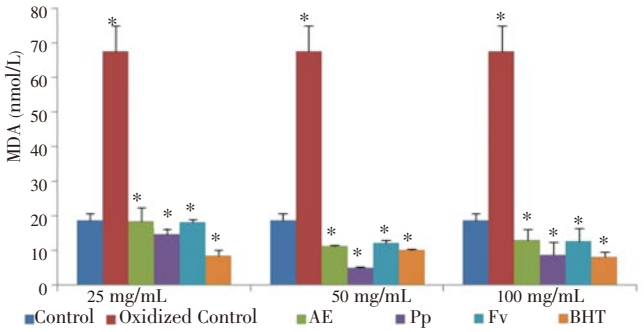

Figure 2. Level of serum MDA at different concentrations of aqueous extract, total polyphenols and total flavonoids from *T. satureioides*.
Control: no oxidized control; AE: Aqueous extract; Pp: Polyphenols; Fv: Flavonoids; BHT: Butylated hydroxytoluene; *: $P<0.001$ (oxidized control is compared with no oxidized control; AE, Pp and Fv are compared with oxidized control).

3.3.3. Assay for erythrocyte hemolysis

AAPH is a peroxyl radical initiator that generates free radicals by its thermal decomposition and will attack erythrocytes, to induce the chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to hemolysis. In this study, the protective effect of the compound solution on erythrocyte hemolysis by peroxyl RSA was investigated. Tables 4, 5, 6 and 7 show the inhibition of aqueous extract, total polyphenols and total flavonoids of *T. satureioides* on AAPH induced erythrocyte oxidative hemolysis and the percentage deviation of hemolysis induced by AAPH in ram erythrocytes in comparison with the positive and negative control. Also, it shows the effects of plants' extract samples when to added with the erythrocyte suspension in PBS.

Table 4
Antihemolytic activity of different extracts.

Plant samples	Hemolysis half-time (min)	% Diviation[a]	% Diviation[b]
Control	220.00±20.10	-	-
AAPH sample	90.00±8.31*	-144.44	-144.44
AAPH+Pp 10.0%	395.00±26.86a* b*	+79.54	+338.88
AAPH+Pp 7.5%	365.00±12.74a* b*	+65.90	+305.55
AAPH+Pp 5.0%	345.00±5.78a* b*	+56.81	+283.33
AAPH+Pp 2.5%	250.00±14.04a^{ns} b*	+13.63	+177.77
AAPH+Pp 1.0%	190.00±18.30a^{ns} b*	-13.63	+111.11
AAPH+AE 10.0%	260.00±17.32a^{ns} b*	+18.18	+188.88
AAPH+Trolox 1.0%	250.00±13.26a^{ns} b*	+13.63	+177.77

AE: Aqueous extract; Pp: Polyphenols; *$P<0.001$, ^{ns}: Not significant. Control *vs* AAPH; [a]: Pp 10.0%, Pp 7.5%, Pp 5.0%, Pp 2.5%, Pp 1.0%, AE 10.0% and Trolox *vs* Control; [b]: Pp 10.0%, Pp 7.5%, Pp 5.0%, Pp 2.5%, Pp 1.0%, AE and Trolox *vs* AAPH sample.

The addition of AAPH decreases the half time of hemolysis by 144.44%. While, the aqueous extract, total polyphenols and total flavonoids extracts to the erythrocytes suspension with AAPH increase the half time of hemolysis by 188.88%, 338.88%, and 150.00%, respectively.

The inhibition percentage of the standard Trolox on hemolysis of red blood cell was 177.77% at 10 mg/mL compared with the half time of hemolysis by aqueous extract and total flavonoids.

A relationship between the inhibition percentages of hemolysis and reducing power of RSA was found, suggesting that the mechanism of action of the extracts in their antioxidant activity may be identical, being related to the content in total phenols.

In conclusion, total polyphenols from *T. satureioides* revealed better antioxidant properties than total flavonoids and the aqueous extract, which is in agreement with the higher content of phenols found in the control. The IC_{50} values obtained for reducing power and scavenging effects on DPPH radicals were higher than for hemolysis inhibition mediated by peroxyl free radicals.

Table 5
Antihemolytic activity of flavonoids from *T. satureioides* at different concentrations.

Plant samples	Hemolysis half-time (min)	% Diviation[a]	% Diviation[b]
Control	220.00±20.10	-	-
AAPH sample	90.00±8.31*	-144.44	-144.44
AAPH+Fv 10.0%	225.00±13.26a^{ns} b*	+2.27	+150.00
AAPH+Fv 7.5%	220.00±9.15a^{ns} b*	0.00	+144.44
AAPH+Fv 5.0%	215.00±4.93a^{ns} b*	-2.27	+138.88
AAPH+Fv 2.5%	170.00±11.64a*** b*	-22.72	+88.88
AAPH+Fv 1.0%	160.00±5.60a** b*	-27.27	+77.77
AAPH+AE 10.0%	260.00±17.32a^{ns} b*	+18.18	+188.88
AAPH+Trolox 1.0%	250.00±13.26a^{ns} b*	+13.63	+177.77

AE: Aqueous extract; Fv: Flavonoids; *$P<0.001$; **$P<0.01$; ***$P<0.05$; ^{ns}: not significant; Control *vs* AAPH; [a]: Fv 10.0%, Fv 7.5%, Fv 5.0%, Fv 2.5%, Fv 1.0%, AE 10.0% and Trolox *vs* Control; [b]: Fv 10.0%, Fv 7.5%, Fv 5.0%, Fv 2.5%, Fv 1.0%, AE 10% and Trolox *vs* AAPH sample.

Table 6
Antihemolytic activity of total polyphenols from *T. satureioides* at different concentrations.

	Hemolysis half-time (min)	% Diviation
Control	125.00±13.22	-
Blood+Pp 10%	186.66±35.11***	+49.32
Blood+Pp 7.5%	188.00±12.52**	+50.40
Blood+Pp 5%	160.00±13.22***	+28.00
Blood+Pp 2.5%	130.00±5.00^{ns}	-4.00
Blood+AE 10%	228.33±17.55**	+82.66

AE: Aqueous extract; Pp: Polyphenols; **$P<0.01$; ***$P<0.05$; ^{ns}: Not significant.

Table 7
Antihemolytic activity of flavonoids from *T. satureioides* at different concentrations.

	Hemolysis half-time (min)	% Diviation
Control	125.00±13.22	-
Blood+Fv 10%	203.33±18.92**	+62.66
Blood+Fv 7.5%	170.00±5.00**	+36.00
Blood+Fv 5%	161.66±17.55***	+29.32
Blood+Fv 2.5%	143.33±11.54^{ns}	+14.66
Blood+AE 10%	228.33±17.55**	+82.66

AE: Aqueous extract; Fv: Flavonoids; **$P<0.01$; ***$P<0.05$; ^{ns}: Not significant.

4. Discussion

Many experimental investigations have demonstrated that a number of secondary metabolites such as polyphenol compounds extracted from medicinal and aromatic plants possess a high antioxidant potential due to their hydroxyl groups and protect more efficiently against some free radical-related diseases[36]. In this

work, we demonstrated that *T. satureoides* plant originating from Errachidia area is rich in total polyphenols and total flavonoids. Furthermore, the polyphenol-rich extracts show a marked radical FRAP and scavenging activity against DPPH radical. In addition, the decomposition of the water-soluble azo compound (AAPH) at physiological temperature generates free radicals *in vitro* that attack erythrocyte membranes and induce lipid peroxidation leading to hemolysis. Natural antioxidants can scavenge and react with free radicals and then terminate the free radical reaction. In this field, the suppression of the oxidative modification of plasma lipid and lipoprotein by antioxidants constituted one of the major targets of many antiatherogenic agents and the preferable strategy to prevent the crises of cardiovascular diseases[37,38]. Hemolysis has a long history of use in measuring free radical damage and its inhibition by antioxidants, but few studies have been performed with erythrocytes in whole blood. In this study, we used a biological test based on free radical-induced erythrocyte lyses in rabbit blood. This assay is useful for screening studies on various molecules and their metabolites, especially those having an oxidizing or antioxidizing activity, and molecules having a long-term action[39].

T. satureioides extracts present strong antioxidant activity by both FRAP and RSA tests. In hemolysis test this extract is able to neutralize the free radicals liberated by the AAPH. This antioxidant activity protects the erythrocyte membrane from lesions and lead to an increase of half-time hemolysis. The antioxidant activity of this extract can be linked up to the high polyphenols and flavonoids content. Diverse studies mentioned the effect of the polyphenols and flavonoids in the antioxidant activity of different plant extracts[40,41]. Phenolics have shown to possess an important antioxidant activity toward these radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure[42,43]. It has been established a highly positive relationship between total phenols and antioxidant activity in many plant species[44]. We also noted that total flavonoids, although it posts only one weak antioxidant capacity, present an antihemolytic activity equivalent to that exhibited by aqueous extract and Trolox. Moreover, the addition of the aqueous extract, total polyphenols and total flavonoids in hemolysis test induct an increase of the half-time hemolysis which is superior to that showed by the witness. This indicates an increase of the level of erythrocytes membranes stability. The actions of aqueous extract, total polyphenols or total flavonoids are not limited to inhibit the free radicals, but it also seems to have an influence on the structural stability of the erythrocyte membrane.

In the present antihemolysis test, the addition of AAPH induced the decrease of hemolysis half-time. However, the treatment with the *T. satureioides* extracts induce an increased erythrocyte resistance to hemolysis provoked by the free radicals. This protective activity can be explained by the scavenging effect of thyme extracts against free radicals induced membrane lipid peroxidation[45]. On the other hand, several authors provided evidence that flavonoids could be incorporated in erythrocyte membranes and improve their stability[38,46]. In addition, De Freitas *et al.* revealed that the exacerbation of the van der Waals contacts inside the lipid bilayer to flavonoids that could be a source of membrane stabilization[46]. Thus, we suggested that the main antioxidant activity induced by the aqueous extract of this plant can be linked to the presence of polyphenol compounds as demonstrated by Zhao *et al*[47]. In fact, a highly positive relationship has been established between total phenols and antioxidant activity in many plant species[44]. Many investigators have proposed some mechanisms to explain the antioxidant activity of phenolic natural antioxidants; these compounds may directly scavenge free radicals and break the oxidative reactions[48,49]. Secondly, they may also chelate pro-oxidant metal ions stimulating free radical formation[49,50]. Our results clearly demonstrate that the bioactive compound(s) contained in this plant have a polar character since they are soluble in water.

Biological membranes can be affected by many natural products present in medicinal plants[51]. Various authors mentioned that flavonoids and the widely distributed subgroup of polyphenols had beneficial effect on the erythrocyte membrane stability[46,52,53].

Flavonoids can be incorporated into the erythrocyte membranes[53]. Furthrmore, De Freitas *et al.* relate that the exacerbation of the van der Waals contacts inside the lipid bilayer by the flavonoids could be a source of membrane stabilization[46]. A good part of the antioxidant activity and consequently the resistance of the erythrocytes to hemolysis inducted by different extract of *T. satureioides* can be linked up to the content of polyphenols and flavonoids. Our results show that this plant has a protective and stabilizing effect of cell membrane, and therefore we will consider studying their anti atherogenic effect.

Besides, HPLC profile of *T. satureioides* polyphenol-rich extracts showed that this plant contains rosmarinic acid as the major polyphenol compound which can be probably involved in the observed pharmacological activities.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Plant-derived natural products still make up a considerable portion of currently available drugs. Thus, *T. satureioides*, a member of Limiaceae family, is an aromatic plant of the Mediterranean flora and is commonly used as traditional medicine remedies. Nevertheless, there is no scientific study reported today on the biological activity of the *T. satureioides*.

Research frontiers

The present research work allowed the identification and quantification of phenolic and flavonoids contents of the aqueous extract from *T. satureioides*. The results also showed that aqueous extract exhibited marked antioxidant and antihemolytic activities.

Related reports

Plants harbor a plethora of so-called secondary metabolites, which often represent the biologically active constituents of phytopharmaceutical preparations that have been used traditionally for the treatment of numerous ailments. Even today, plant-derived natural products and their derivatives and synthetic mimics still make up a considerable portion of currently available drugs. Thus, terpenoids are the largest known class of secondary metabolites in plants and possess a broad range of biological

activities and potential therapeutic effects on several diseases. Based on their structural diversity and biological activity, from the phytopharmaceutical point of view, specifically sesquiterpenes have been suggested to be of special interest among the terpenoids.

Innovations and breakthroughs

This the first study demonstrating that *T. satureioides* is rich of antioxidant and antihemolytic molecules.

Applications

It is well known that *Thymus* species are safe to humans. This scientific study supports and suggests the use of the aqueous extract of *T. satureioides* as an adjuvant along with other antioxidant agents.

Peer review

This is a valuable research work in which authors have analyzed, using the HPLC approach, the aqueous extract of the *T. satureioides*. In addition, *in vitro* and *in vivo* studies clearly showed an antioxidant and antihemolytic properties.

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