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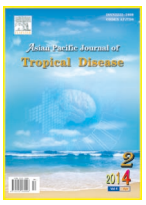

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Phytochemical screening and antioxidant capacity of the aerial parts of *Thymelaea hirsuta* L.

Nesrine Ouda Amari[1*], Mohamed Bouzouina[2], Abdellah Berkani[2], Brahim Lotmani[2]

[1]*University of Mostaganem, Faculty of Sciences and the Natural Sciences and Life, BP300, Mostaganem 27000, Algeria*

[2]*Plant Protection Laboratory, University of Mostaganem, Algeria*

PEER REVIEW

Peer reviewer

Dr. Noureddine Djebli, Professor, Laboratory of Pharmacognosy Api Phytotherapy, Department of Biology, Faculty SNV, University of Mostaganem Algeria.
Tel: +213772451866
E-mail: djebli_n@yahoo.fr

Comments

This is a good study in which the authors evaluated phytochemical qualitative and quantitative analysis of total phenols, flavonoids and antioxidant capacity of three part of *T. hirsuta*.
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ABSTRACT

Objective: To assess antioxidant activities of different aerial parts of *Thymelaea hirsuta* (*T. hirsuta*) from west Algeria, and to search for new sources of safe and inexpensive antioxidants.
Methods: Samples of leaves, stems and flowers from *T. hirsuta* were tested for total phenolic content, flavonoids content, and evaluation its total antioxidant activity, were done using the spectrophotometric analyses.
Results: Results of preliminary phytochemical screening of leaf, flower and stem of *T. hirsuta* revealed the presence of tannins, alkaloids, steroids, saponins, coumarins, reducteurs compound and anthraquinones. The total phenolics and flavonoids were estimated. The aqueous extracts of the aerial parts of *T. hirsuta* showed potent *in vitro* antioxydant activities using various models *viz*, DPPH scavenging assay, ferric reducing antioxidant power (FRAP) and ABTS radical scavenging activity.
Conclusions: On the basis of the results obtained, *T. hirsuta* extracts are rich sources of natural antioxidants appears to be an alternative to synthetic antioxidants and this justifies its therapeutic usage.

KEYWORDS

Phytochemicals, Flavonoids, Total phenolics, Antioxidant, *Thymelaea hirsuta*

1. Introduction

The large generation of free radicals, particularly reactive oxygen species and their high activity plays an important role in the progression of a great number of pathological disturbances such as inflammation, atherosclerosis, stroke, heart diseases, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, and Alzheimer's disease[1,2]. Therefore, the great interest has been recently focused on the natural foods, medicinal plants and phytoconstituents due to their well-known abilities to scavenge free radicals (*i.e.* antioxidant power)[3]. Plants constitute an important source of active natural products which differ widely in terms of structures, biological properties and mechanisms of actions. Various phytochemical components, especially polyphenols (such as flavonoids, phenyl propanoids, phenolic acids, tannins, *etc.*) are known to be responsible for the free radical scavenging and antioxidant activities. Polyphenols possess many biological effects, which are mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals. In general, polyphenols all share the same chemical patterns, one or more phenolic groups for which they react as hydrogen donors and in that way neutralize free radicals[4]. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity[5]. Phytochemicals are natural

*Corresponding author: Amari Nesrine Ouda, BP 300, Mostaganem 27000 Mostaganem, Algeria.
Tel: +213 770892174
Fax: +213 45214544
E-mail: nesrineamari@yahoo.fr; menaouar_31@yahoo.fr; berkani21@yahoo.fr

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bioactive compounds found in plants (flowers, leaves and shoots) that work with nutrients and fibers to act as defence system against disease or more accurately, to protect against disease. Some of the most important bioactive phytochemical constituents are flavonoids, tannins, terpenoids, saponins, phenolic compounds[6]. *Thymelaea hirsuta* (*T. hirsuta*), commonly known as "Methnane" in Algeria, is an evergreen shrubs belonging to the flowering plant family Thymelaeaceae, which is native to the Mediterranean region, north of central Europe and east of central Asia. The plant is traditionally used in Tunisia as an antiseptic, anti-inflammatory and for the treatment of hypertension[7]. The traditional medicinal use of this plant is not based on scientific research and few data dealing with its phytochemical composition are available[8,9]. Hypoglycemic, antidiabetic and antioxidant properties of this plant extracts were previously highlighted[10,11]. The antimelanogenesis effect of *T. hirsuta* extracts on B16 murine melanoma cells was reported by Kawano *et al*[12]. Phytochemical analysis showed the presence of several daphnane diterpenes[12,13]. The literature survey did not show any reference to previous work on the free radical scavenging and/or antioxidant properties of *T. hirsuta* (flowers, leaves and stem). Hence, the aim of the present study was designed to assess the *in vitro* antioxidant activity of aqueous extracts from flowers, leaves and stem of *T. hirsuta*, primary phytochemical screening of the main secondary metabolites classes of the aerial part of *T. hirsuta* and to determine the phenolic compounds and flavonoids contained in the aqueous extracts, in order to provide a scientific basis to justify its therapeutic usage and to search for new sources of safe and inexpensive antioxidants. The findings from this work may add to the overall value of the medicinal potential of the herb.

2. Materials and methods

2.1. Plant collection

The aerial parts (leaves, flowers, stems) of *T. hirsuta* were collected from the region of north of the wilaya of Relizane (Algeria) and located at 82 m of altitude, 35°55′33″ N latitude and 0°49′23″ E longitude during the period of March 2012. The specimens were identified at the National Institute of Agronomy in Algiers (INA). The plant materials were separated into leaves, flowers and stems. The plant parts were dried in shade away from sunlight and at room temperature (25-28 °C), then grinded to fine powder using electric blender and stored in clean labeled airtight bottles.

2.2. Preparation of the plant extract

A 10% aqueous decoction was performed separately on different parts of each plant[14]. A total of 100 g plant powder was placed in one liter of distilled water. After mechanical agitation, the preparation was carried in a water bath at 90 °C for 1 h. The cooled decoctions were vacuum filtered using glass microfiber filters Wattman No. 4 and concentrated under reduced pressure at 40 °C, using a rotary evaporator, and then frozen and lyophilized.

2.3. Phytochemical screening

The phytochemical tests to detect the presence of tannins, saponins, coumarins, terpenoids, anthraquinones, reducteurs compounds and alkaloids were performed according to the method described by Harborne and Raman[15,16]. The tests were based on the visual observation of color change or formation of a precipitate after the addition of specific reagents.

2.4. Determination of total phenolic content

The contents of total phenols aqueous extracts of different parts of *T. hirsuta* were determined by the modified Folin-Ciocalteu method[17]. Thus, 1 mL of the aqueous extract of the plant was mixed with 5 mL of Folin Ciocalteu (2 mol/L) diluted 10 times. After 5 min of incubation, 4 mL of sodium carbonate at a concentration of 75 g/L was added and the volume was adjusted to 10 mL. The resulting mixture was incubated at room temperature for 1 h. The absorbances of the samples were measured at 765 nm using UV-vis spectrophotometer. Results were expressed as mg of gallic acid equivalent/g of lyophilized extract. The same procedure was used for making standard curve using gallic acid and concentration range of 0-10 μg/mL was taken. All experiments were carried out in triplicates.

2.5. Determination of total flavonoids content

A volume of $AlCl_3$ methanolic solution (0.5 mL, 2%, w/v) was mixed with methanolic extract solution (0.5 mL, 0.1 mg/mL). After 10 min, the optical densities were recorded at 430 nm against a blank (mixture of 0.5 mL methanolic extract solution and 0.5 mL methanol) and compared to the quercetin calibration curve (0 to 200 mg/L)[18]. The data obtained were the means of three determinations. The amounts of flavonoids in the plant extracts were expressed as mg of quercetin equivalents (QE)/g of lyophilized extract.

2.6. Antioxidant capacity determined by diphenyl-1-picrylhydrazyl

Radical-scavenging activity of plant extracts against stable 1,1diphenyl-2-picryl hydrazyl radical (DPPH) was determined spectrophotometrically. The DPPH assay was carried out as described by Zuraini *et al*[19]. Stock solutions of crude extracts were prepared as 1 mg/mL in methanol. Fifty microlitres of different concentration samples were added to 5 mL of 0.004% methanol solution of DPPH. After 30 min of incubation in the dark at room temperature, the absorbance was read against a blank at 517 nm. Ascorbic acid (Sigma-Aldrich), a stable antioxidant, was used as a synthetic reference. The assay was carried out in triplicate and percentage of inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(AB-AA)}{AB} \times 100$$

Where AB is absorbance of blank; AA is absorbance of test.

2.7. Antioxidant capacity determined by ABTS radical cation assay

The ABTS radical cation was generated by reacting 7 mmol/L ABTS stock solution with 2.45 mmol/L potassium persulfate solution, and the mixture was kept in the dark at room temperature for 12-16 h before use. Prior the assay, the $ABTS^{+}$ solution was diluted with ethanol to an absorbance of (0.70±0.02) at 734 nm[20]. An aliquot (50 μL) of each sample at different concentrations (0.4, 0.6, 0.8 mg/mL) was added to 5 mL of diluted $ABTS^{+}$ solution. After 10 min, the absorbance was measured at 734 nm. The inhibition of ABTS radical cation in percent was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{AC-(AS-AB)}{AC} \times 100$$

Where AC is absorbance of control; AS is absorbance of sample; AB is absorbance of blank.

Ethanol (5 mL) plus each sample solution (50 μL) was used as a blank. $ABTS^{+}$ solution (5 mL) plus ethanol (50 μL) was used as a negative control. Also, Trolox solution (at the concentrations of 100, 80, 60, 40, 20, 0 μg/mL) was used as a positive control. All measurements were repeated in triplicate.

2.8. Antioxidant capacity determined by ferric reducing antioxidant power (FRAP)

The FRAP assay was done according to Benzie and Strain with some modifications[21]. The stock solutions included 300 mmol/L acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$) and 16 mL $C_2H_4O_2$), pH 3.6, 10 mmol/L 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol/L HCl, and 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 °C before using. An aliquot (0.3 mL) of each sample at different concentrations (0.4, 0.6, 0.8 mg/mL) were allowed to react with 9 mL of the FRAP solution and 300 μL of distilled water for 30 min in the dark condition. The absorbance of the reaction mixture was then recorded at 593 nm. The standard curve was linear between 0 and 300 μmol/L Trolox. All measurements were carried out in triplicate.

2.9. Statistical analysis

Correlations were established using Pearson's correlation coefficient (r) in bivariate linear correlations ($P<0.01$). All statistical analyses were performed with the Statistica 7.0 software for Windows.

3. Results

3.1. Phytochemical screening

The phytochemical qualitative and quantitative analysis of leaf, flower and stem of *T. hirsuta* revealed the presence of tannins, alkaloids, steroids, saponins, coumarins, reducteurs compound and anthraquinones (Table 1). Reducteurs compound were not detected in the stem.

Table 1
The phytochemical screening of the aerial part of *T. hirsuta*.

Test group	Leaf	Flower	Stem
Alkaloids	+	++	++
Tannins	+	+++	+
Saponins	+	++	++
Coumarins	++	++	+++
Terpenoids	++	+++	+
Reducteurs compound	+	+++	−
Anthraquinones	+	+	+

(−): not detectable; (+): low quantities; (++): high quantities; (+++): very high quantities.

3.2. Determination of total phenolic content and total flavonoids

The present study revealed relatively high level of total phenols content and flavonoids of the aqueous extract of the three parts of *T. hirsuta* as shown in Table 2. The concentrations of phenol [(113.96±9.97) mg gallic acid equivalent/g of lyophilized extract] and flavonoids content [(5.70±0.06) mg quercetin equivalent/g of lyophilized extract] were high in flower extract while in stem extract there was (93.78±3.12) mg gallic acid equivalent/g of lyophilized extract and (2.61±0.13) mg quercetin equivalent/g of lyophilized extract. The leaf extract showed the least concentration of phenol contents [(61.12±1.28) mg gallic acid equivalent/g of lyophilized extract]. The concentrations of flavonoids were comparatively low in the stem extract while in leaf extract were a value of (3.15±0.15) mg quercetin equivalent/g of lyophilized extract was recorded.

Table 2
Levels of total phenolic and flavonoids contents in aqueous extract of *T. hirsuta*.

Species	Total phenolic (mg GAE/g)	Total flavonoids (mg QE/g)
Leaf	61.12±1.28	3.15±0.15
Flower	113.96±9.97	5.70±0.06
Stem	93.78±3.12	2.61±0.13

Data are expressed as mean±SD. mg GAE/g: mg of gallic acid equivalent per g of lyophilized extract; mg QE/g: mg of quercetin equivalent per g of lyophilized extract.

3.3. In-vitro antioxidant activity

The leaf, flower and stem extracts *T. hirsuta* were found to possess concentration dependent scavenging activity on DPPH radicals and the results are presented in Table 3. So the aqueous extracts of flower and leaf exhibited great antioxidant effects at 8 mg/mL (90.83% and 71.90%, respectively) compared with the high antioxidant effect of ascorbic acid. ABTS stable free radical with the characteristic absorption at 734 nm was used to study the radical scavenging effect of *T. hirsuta* extract reacted with

ABTS at different concentrations (0.4, 0.6, and 0.8 mg/mL) and readings were recorded by measuring the reduction of radical cation generated by $ABTS^{+}$ at 734 nm. The flower and stem extracts of *T. hirsuta* showed maximum decoloration of 0.8 mg/mL with (68.63±0.53)% and (43.04±1.28)% respectively, and the leaf showed less decoloration (Table 3). The results obtained clearly implied that the aqueous extracts of the different organs inhibited the radical or scavenge the radical in a concentration dependent manner. The reducing power of the different parts extracts of *T. hirsuta* was found to be steadily increased in direct proportion to the increasing concentration of the extract in the following order, flowers>stem>leaves (Table 3). The reducing power of flower, stem and leaf extracts of *T. hirsuta* at 0.8 mg/mL concentration was found to be 0.810, 0.530 and 0.460 respectively.

Table 3
Total antioxidant activity, DPPH assay, $ABTS^{+}$ assay and FRAP assay of the aerial part of *T. hirsuta*.

Test group	Concentration (mg/mL)	Leaf	Flower	Stem
DPPH (% I)	4.0	40.15±0.96	72.47±0.29	34.35±0.41
	6.0	57.30±0.47	83.69±1.29	53.20±0.89
	8.0	71.90±0.29	90.83±0.32	62.34±2.01
$ABTS^{+}$ (% I)	0.4	14.52±1.50	28.54±5.74	21.69±1.07
	0.6	18.42±0.18	44.46±0.10	31.40±0.16
	0.8	24.52±1.08	68.63±0.53	43.04±1.28
FRAP absorption value	0.4	0.25±0.01	0.440±0.01	0.33±0.03
	0.6	0.35±0.02	0.660±0.02	0.41±0.01
	0.8	0.46±0.01	0.810±0.01	0.53±0.02

Data are expressed as mean±SD. (% I): Percentage inhibition of radical scavenging.

3.4. Correlation of different antioxydant assay of the aerial part of T. hirsuta

The Pearson correlation among the results of different antioxydant assay of leaves, flowers and stems from *T. hirsuta* are summarized in Table 4. There were good correlations among the antioxidant activities based on ABTS and FRAP assay and DPPH radical scavenging. The highest correlation coefficient found was for the relationship between antioxidant activity assessed by the ABTS stem and the FRAP stem (r=0.997). A similar correlation was observed among ABTS leaf and FRAP leaf, FRAP stem and ABTS stem, while the correlation coefficient for the relation between ABTS flower and FRAP flower, FRAP flower and ABTS flower were a little bit lower, equal to 0.782.

4. Discussion

The vital role of antioxidants is their interaction with oxidative free radicals. Because of its extra electron, DPPH gives a strong absorption band at 517 nm in visible spectroscopy. The efficacies of antioxidants are often associated with their ability to scavenge stable free radicals[22]. It has been shown that the scavenging effects on the DPPH radical increase sharply with the increasing concentration of the samples and standards to a certain extent[23]. In the present study, *T. hirsuta* extract (leaf, flower, stem) exhibited concentration dependent scavenging activity against hydroxyl radical in a reaction system, and the free radical scavenging potentials of the extracts of were found to be in the order of flower extract>leaf extract>stem extract. It appears that the three aerial parts extracts from *T. hirsuta* possess hydrogen donating capabilities to act as antioxidant.

The working mechanism of the ABTS method for the evaluation of antioxidant activity is the same as that of the DPPH method, but the ABTS method is more reliable than the DPPH method due to the solubility of the ABTS reagent in both aqueous solvents as compared to DPPH[24]. For that reason, ABTS assay is better than DPPH assay when applied to a variety of plant foods containing hydrophilic, lipophilic, and high-pigmented antioxidant compounds[25]. From the result, the flower extract of *T. hirsuta* possessed the highest ABTS scavenging capacity whereas the leaf extract of *T. hirsuta* showed the lowest ABTS scavenging activity. Higher concentrations of the extracts were more effective in quenching free radicals in the system[26]. For that reason, the results clearly imply that the aqueous extracts of different organs inhibit the radical or scavenge the radical in a concentration dependent manner. The scavenging activity of ABTS by the plant extract was found to be remarkably high. This implies that the plant extracts could be useful for treating radical-related pathological damages especially at higher concentrations. In the present study, the reducing power of the different parts extracts of *T. hirsuta* was found to be steadily increased in direct proportion to the increasing concentration of the extract. The presence of reductants like

Table 4
Pearson's correlation coefficients (r) of antioxidants activities of the aerial part of Thymelaea hirsuta

	$ABTS_{+}$stem	$ABTS_{+}$leaf	$ABTS_{+}$flower	FRAP stem	FRAP leaf	FRAP flower	DPPH stem	DPPH leaf	DPPH leaf
$ABTS_{+}$stem	1	–	–	0.997	–	–	0.987	–	−0.857
$ABTS_{+}$leaf	–	1	–		0.990	–	–	−0.841	–
$ABTS_{+}$flower	–	–	1	–	–	−0.782	–	–	–
FRAP stem	0.997	–	–	1	–	–	0.980	–	–
FRAP leaf	–	0.996	–	–	1	–	–	−0.783	
FRAP flower	–	–	−0.782	–	–	1	–	–	0.954
DPPH stem	0.987	–	–	–	–	–	1	–	–
DPPH leaf	–	−0.841	–	–	−0.783	–		1	–
DPPH flower	–	–	−0.857	–	–	0.954	–	–	1

antioxidant substances in the samples causes a reduction of the Fe^{3+} to Fe^{2+} form. Therefore, the ability of a compound to transfer electron is a significant indicator of its potential as an antioxidant[27]. This indicates that the antioxidant compounds are electron donors and could reduce the oxidized intermediate of lipid peroxidation processes; thus acting as primary and secondary antioxidants[28]. These findings verify the efficiency of the different parts extracts of *T. hirsuta* against the ABTS radical cation, DPPH radical as well as in FRAP assay. There were good correlations among the antioxidant activities based on ABTS and FRAP assay and DPPH radical scavenging because all these assays share the same principle (single electron transfer). High correlation is also found between these three assays in other plant derived samples[29]. Strong correlation of ABTS with FRAP confirms the authenticity of results of antioxidant potential for the three organ extracts of *T. hirsuta*. Quantitative phytochemical analysis indicated that the different organs of *T. hirsuta* contains significant amounts of phenolics compounds such as total phenolic and flavonoids. The amount of total phenolic compounds in all tested organs of the *T. hirsuta* was higher than some Asian vegetables[30], some herbs and medicinal plants such as *Armoracia rusticana*, *Fallopia convolvulus*, *Matricaria matricarioides*, *Trifolium hybridum* and *Typha latifolia*[31]. Thus, these classes of compounds were responsible for antioxidant and free radical scavenging effect of plant materials[32]. The determination of antioxidant activity of plant extracts is an unresolved problem. The results from different antioxidant assays are even difficult to compare because of the difference in substrates, probes, reaction conditions and quantification methods[33]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen. Flavonoids are wide spread in all natural compounds and possess a broad spectrum of biological activities. The chemical composition of flower, stem and leaf of *T. hirsuta* indicated the presence of phenolic compounds including flavonoids, which are known to possess antioxidant activities[34]. The high phenolic and flavonoids contents in *T. hirsuta* may be responsible for its free radical scavenging activity. Moreover, screening of phytochemical compounds in *T. hirsuta* revealed the presence of tannins, alkaloids, steroids, saponins, coumarins, reducteurs compound and anthraquinones. The antioxidant activities of *T. hirsuta* extract are due to the presence of these phytochemicals and thus the plant could serve as potential source in herbal medicine drugs. In the current investigation, the extracts from *T. hirsuta* flower, stem and leaf gave good results indicating that it possesses significant amount of phytochemicals and *in vitro* antioxidant activity. Phenolic compounds and other phytochemicals appear to be responsible for the *in vitro* antioxidant activity of the extracts and may contribute to the therapeutic activity observed. On the basis of the results obtained, *T. hirsuta* extracts are rich sources of natural antioxidants appears to be an alternative to synthetic antioxidants. This study was the first report about the antioxidant activities of different parts of *T. hirsuta*. Further investigation to determine antioxidant activity by *in vivo* methods could be considered.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Plants constitute an important source of active natural products which differ widely in terms of structures, biological properties and mechanisms of actions. Various phytochemical components, especially polyphenols (such as flavonoids, phenyl propanoids, phenolic acids, tannins, *etc.*) are known to be responsible for the free radical scavenging and antioxidant activities.

Research frontiers

Studies are being performed in order to determine that *T. hirsuta* extracts are rich sources of natural antioxidants, which appear to be an alternative to synthetic antioxidants and justify its therapeutic usage.

Related reports

Data regarding phytochemical qualitative and quantitative analysis are in agreement with more researchers in the same domain, the same remarque about antioxidant activities.

Innovations & breakthroughs

Results of preliminary phytochemical screening of leaf, flower and stem of *T. hirsuta* revealed the presence of tannins, alkaloids, steroids, saponins, coumarins, reducteurs compound and anthraquinones. The total phenolics and flavonoids were estimated. The aqueous extracts of the aerial parts of *T. hirsuta* showed potent *in vitro* antioxydant activities using various models *viz*, DPPH scavenging assay, FRAP and ABTS radical scavenging activity.

Applications

It may be significant to know antioxidant capacity of *T. hirsuta*. The results of the present study suggest relatively high level of total phenols content and flavonoids of the aqueous extract of the three organs of *T. hirsuta*.

Peer review

This is a good study in which the authors evaluated phytochemical qualitative and quantitative analysis of total phenols, flavonoids and antioxidant capacity of three parts of *T. hirsuta*.

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