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Influence of Ecological Conditions on the Phenolic Composition and Antioxidant Activity of Thymelaea hirsuta Extracts from Two Tunisian Regions

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Abstract:
Thymelaea hirsuta is a medicinal plant widely distributed in North Africa and traditionally used for the treatment of various diseases. This study aimed to investigate the phenolic composition and antioxidant potential of methanolic extracts obtained from the aerial parts of T. hirsuta collected from two ecologically distinct regions of Tunisia: Medenine (arid climate) and Mahdia (semi-arid climate). Plant materials were subjected to solvent extraction, and the resulting extracts were analyzed for total polyphenol and flavonoid contents using spectrophotometric methods. Antioxidant activity was evaluated in vitro using DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assays. In addition, high-performance liquid chromatography (HPLC) was employed to characterize the phenolic profiles of the extracts.
The results revealed significant variations in phenolic and flavonoid contents depending on both the extraction method and the geographical origin of the plant material. Crude extracts exhibited higher concentrations of bioactive compounds compared with extracts obtained from dry powder. The crude extract from Mahdia showed the highest total polyphenol and flavonoid levels, whereas the Medenine extract displayed greater diversity in phenolic composition. All extracts demonstrated antioxidant activity, although their reducing power was lower than that of ascorbic acid. Notably, extracts from dry powder exhibited stronger radical scavenging capacity in the DPPH assay.
HPLC analysis revealed complex phenolic profiles with numerous detectable compounds, confirming that the antioxidant activity is likely due to synergistic effects among multiple constituents. Environmental stress associated with arid conditions appears to enhance the synthesis of secondary metabolites. Overall, T. hirsuta represents a promising natural source of phenolic antioxidants with potential applications in pharmaceutical, nutraceutical, and food industries.

Keywords: Thymelaea hirsuta; Polyphenols; Flavonoids; Antioxidant activity; HPLC; DPPH; FRAP; Medicinal plants; Environmental stress.

تأثير الظروف البيئية على التركيب الفينولي والنشاط المضاد للأكسدة لمستخلصات نبات *Thymelaea hirsuta* من منطقتين تونسيّتين

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الملخص
يُعد نبات Thymelaea hirsuta من النباتات الطبية المنتشرة في شمال إفريقيا ويُستخدم تقليديًا في علاج العديد من الأمراض. هدفت هذه الدراسة إلى تقييم التركيب الفينولي والنشاط المضاد للأكسدة لمستخلصات ميثانولية من الأجزاء الهوائية للنبات جُمعت من منطقتين مختلفتين بيئيًا في تونس: مدنين ذات المناخ الجاف، والمهدية ذات المناخ شبه الجاف. تم تحضير المستخلصات باستخدام المذيبات العضوية، ثم قياس محتوى البوليفينولات الكلية والفلافونويدات بالطرق الطيفية. كما تم تقييم النشاط المضاد للأكسدة باستخدام اختبار تثبيط الجذور الحرة DPPH واختبار القدرة الاختزالية للحديد .FRAP إضافة إلى ذلك، استُخدم كروماتوغرافيا السائل عالية الأداء (HPLC) لتوصيف المركبات الفينولية.
أظهرت النتائج وجود فروق واضحة في محتوى المركبات الفينولية والفلافونويدية تبعًا لطريقة الاستخلاص والمنطقة الجغرافية. فقد احتوت المستخلصات الخام على تراكيز أعلى من المركبات النشطة مقارنة بالمستخلصات الناتجة عن المسحوق الجاف. وسجل مستخلص المهدية الخام أعلى محتوى من البوليفينولات والفلافونويدات، بينما أظهر مستخلص مدنين تنوعًا أكبر في التركيب الفينولي. كما أبدت جميع المستخلصات نشاطًا مضادًا للأكسدة، إلا أن قدرتها الاختزالية كانت أقل من حمض الأسكوربيك. وتميزت مستخلصات المسحوق الجاف بقدرة أعلى على تثبيط الجذور الحرة.
كشفت نتائج HPLC عن تركيب فينولي معقد يتضمن عددًا كبيرًا من المركبات، مما يشير إلى أن النشاط المضاد للأكسدة ناتج عن تأثير تآزري بين عدة مكونات. ويبدو أن الظروف البيئية القاسية في المناطق الجافة تعزز من إنتاج المستقلبات الثانوية. وبناءً على ذلك، يُعد هذا النبات مصدرًا واعدًا لمضادات الأكسدة الطبيعية ذات التطبيقات المحتملة في المجالات الدوائية والغذائية والتغذوية.

الكلمات المفتاحية: نبات Thymelaea hirsuta؛ البوليفينولات؛ الفلافونويدات؛ النشاط المضاد للأكسدة؛ HPLC؛ DPPH؛ FRAP؛ النباتات الطبية؛ الإجهاد البيئي.

Introduction

In recent decades, growing scientific interest has focused on natural antioxidants due to their significant therapeutic potential in preventing and managing diseases associated with oxidative stress. Medicinal plants represent a major source of these bioactive compounds, and extensive research has been devoted to the extraction, identification, and quantification of plant-derived antioxidants, particularly polyphenols and flavonoids. These compounds are known for their diverse biological activities, including anti-inflammatory, antimicrobial, antidiabetic, and cardioprotective effects. Consequently, the exploration of medicinal plants has become an important strategy in the search for safe and effective natural alternatives to synthetic drugs.

Thymelaea hirsuta (family: Thymelaeaceae) is an evergreen shrub native to North Africa and widely distributed in Mediterranean regions. The plant has long been used in traditional medicine for the treatment of various ailments, including hypertension, diabetes, skin disorders, and infections. In Tunisia, where it is commonly known as "Methnane," preparations made from its aerial parts are traditionally employed as antiseptics and as decoctions for managing metabolic and cardiovascular conditions. These therapeutic applications are largely attributed to the plant's high content of antioxidant compounds.

Previous phytochemical investigations have revealed that *T. hirsuta* contains a wide range of secondary metabolites, such as polyphenols, flavonoids, tannins, diterpenes, coumarins, anthocyanins, and phytosterols. Many of these constituents exhibit strong antioxidant activity and contribute to the plant's pharmacological properties. Secondary metabolites play a crucial role in plant defense mechanisms against environmental stresses, including drought, high temperature, intense radiation, and pathogen attack. Therefore, variations in ecological conditions can significantly influence the synthesis and accumulation of these compounds.

Environmental factors such as climate, soil composition, water availability, and altitude are known to affect the phytochemical profile of medicinal plants. Arid and semi-arid environments, in particular, can induce physiological stress that enhances the production of phenolic compounds as part of the plant's adaptive response. Understanding how environmental conditions shape the chemical composition of medicinal plants is essential for optimizing their therapeutic use and ensuring consistency in bioactive compound content.

Despite the recognized medicinal importance of *T. hirsuta*, comparative studies examining the influence of different ecological environments on its phytochemical composition and antioxidant activity remain limited. Tunisia provides an ideal setting for such investigations, as it encompasses diverse climatic zones ranging from arid desert regions to semi-arid coastal areas.

Therefore, the present study aims to analyze the chemical composition and antioxidant activity of methanolic extracts obtained from the aerial parts of *Thymelaea hirsuta* collected from two ecologically distinct regions in Tunisia—Medenine (arid climate) and Mahdia (semi-arid climate). By comparing the levels of total polyphenols, flavonoids, and antioxidant capacity using established assays, this research seeks to elucidate the impact of environmental conditions on the phytochemical properties of this medicinal plant and to contribute to the understanding of its potential therapeutic value.

Literature Review

Medicinal plants have long been recognized as important sources of biologically active compounds with therapeutic potential. In recent decades, scientific interest in plant-derived antioxidants has intensified due to their role in preventing oxidative stress–related diseases. Traditional herbal medicine, particularly in the Mediterranean and Arab regions, remains a rich reservoir of pharmacologically active species that continue to be investigated using modern scientific approaches [1]. Among these plants, *Thymelaea hirsuta* (family Thymelaeaceae) has received increasing attention because of its ethnomedicinal uses and diverse indication of biological activities.

Ethnobotanical studies demonstrate that medicinal plants are widely used for treating chronic and infectious diseases, especially in regions where access to modern healthcare is limited [2]. In North Africa and the Middle East, *T. hirsuta*—known in Arabic as "mithnan"—has been traditionally employed for managing diabetes, hypertension, skin disorders, and infections [3,4]. Surveys of traditional medicine practices confirm that many of these therapeutic uses are linked to the plant's bioactive secondary metabolites, particularly phenolic compounds and flavonoids [1]. Furthermore, ethnopharmacological investigations highlight the continued reliance on herbal remedies and emphasize the importance of validating their efficacy and safety through scientific research [2].

Botanical and ecological studies describe *T. hirsuta* as an evergreen shrub adapted to arid and semi-arid environments of the Mediterranean basin [5,6]. Taxonomic and karyosystematic analyses have documented significant variability among populations across different geographical regions, suggesting that environmental conditions may influence the plant's morphology and chemical composition [7]. Such variability is especially relevant for phytochemical research, as the concentration of secondary metabolites often depends on climate, soil characteristics, and ecological stress factors [8].

Phytochemical investigations reveal that *T. hirsuta* contains a wide range of secondary metabolites, including polyphenols, flavonoids, diterpenes, tannins, coumarins, and phytosterols [9]. Secondary metabolites play essential roles in plant defense against environmental stressors and pathogens, while also contributing to pharmacological properties in humans [10]. Studies on related species in the Thymelaeaceae family have identified daphnane diterpenes as characteristic compounds with potent biological activity [11]. More

recent research isolated novel diterpenoids, such as hirseins A and B, which exhibit inhibitory effects on melanogenesis in melanoma cells, indicating potential applications in dermatology and oncology [12].

Polyphenols represent one of the most extensively studied classes of plant secondary metabolites due to their antioxidant capacity. These compounds can neutralize free radicals, chelate metal ions, and modulate enzyme activity, thereby protecting cells from oxidative damage [13]. Dietary intake of polyphenols has been associated with reduced risk of cardiovascular diseases, cancer, and neurodegenerative disorders [14,15]. Moreover, bioavailability studies suggest that although absorption varies among compounds, many polyphenols exert beneficial effects through metabolites formed during digestion [16].

Flavonoids, a major subclass of polyphenols, exhibit a wide spectrum of biological activities, including anti-inflammatory, antimicrobial, and anticancer effects [17]. Their antioxidant action is primarily attributed to their ability to scavenge reactive oxygen species (ROS) and regulate oxidative pathways. Experimental studies demonstrate that flavonoids interact with cytochrome P450 enzymes and influence cellular signaling mechanisms, further highlighting their pharmacological relevance [17]. Quercetin, one of the most abundant flavonoids, has been investigated extensively for its antioxidant and potential pro-oxidant properties, depending on concentration and cellular context [18].

Environmental stress conditions significantly influence the biosynthesis of phenolic compounds in plants. Factors such as drought, temperature extremes, nutrient availability, and carbon dioxide concentration can alter secondary metabolism [19]. Research indicates that plants subjected to water stress often accumulate higher levels of phenolics and flavonoids as part of their adaptive response [20]. Similarly, mineral deficiencies, particularly boron deficiency, have been shown to increase flavonoid content in plant tissues [21]. Elevated atmospheric CO_2 levels can also enhance antioxidant compound production by increasing carbon availability for biosynthesis [22].

The biological importance of plant-derived antioxidants is closely linked to their ability to counteract oxidative stress. Reactive oxygen species are continuously generated during normal cellular metabolism and can cause damage to lipids, proteins, and DNA if not neutralized [23]. Oxidative stress has been implicated in the pathogenesis of numerous diseases, including diabetes, cardiovascular disorders, and cancer [24]. Antioxidants from dietary sources and medicinal plants form a complex defense network that protects against these harmful effects [25].

Assessment of antioxidant activity typically involves chemical assays that measure the ability of compounds to neutralize free radicals. The DPPH assay is among the most widely used methods due to its simplicity and reliability in evaluating radical-scavenging capacity [26]. Other analytical techniques, such as the Folin–Ciocalteu method, are employed to quantify total phenolic content in plant extracts [27]. Advances in chromatography and mass spectrometry have further enabled precise identification and characterization of polyphenolic compounds in complex plant matrices [28].

Several studies have reported significant antioxidant activity in plant extracts rich in phenolic compounds. Investigations of Algerian medicinal plants demonstrated strong correlations between phenolic content and free radical scavenging activity [29]. Similar findings have been observed in various plant species used in traditional medicine, reinforcing the hypothesis that phenolics are major contributors to antioxidant potential [30]. Moreover, tannic acid and other phenolic derivatives have shown potent radical-scavenging properties in vitro, supporting their therapeutic relevance [31].

Beyond antioxidant effects, *T. hirsuta* exhibits additional pharmacological activities. Experimental studies have demonstrated hypoglycemic effects in diabetic animal models, suggesting potential use in diabetes management [32]. Antimelanogenic activity observed in

melanoma cell lines indicates possible applications in skin disorders and cosmetic formulations [33]. Other research has reported antiparasitic effects, including activity against *Leishmania* species, further expanding the plant's pharmacological profile [34].

Despite these promising findings, the toxicity of certain compounds within the Thymelaeaceae family must be considered. Some diterpenes are known to possess irritant or tumor-promoting properties, highlighting the need for careful evaluation of safety and dosage [35]. Consequently, future research should focus on isolating specific bioactive compounds and assessing their therapeutic index.

Research Gap

Despite extensive research on medicinal plants as sources of natural antioxidants, important gaps remain regarding *Thymelaea hirsuta*. Most studies have focused on its traditional uses or general phytochemical screening, with limited investigation of how environmental conditions and extraction methods influence its phenolic composition and biological activity. In addition, few studies have combined quantitative phytochemical analysis with multiple antioxidant assays and detailed compound identification. Therefore, further research is needed to clarify the variability of bioactive constituents and to identify the specific molecules responsible for the plant's antioxidant properties.

Materials and Methods

Sampling

The plant material used is obtained from the aerial parts of the *Thymelaea hirsuta* plant. The plant was harvested in March 2015 in the Medenine region (Beni-Khedache) and the comparative plant was harvested in the Mahdia region (Sidi Massaoud) in February 2015 during the inflorescence season. The different organs of the plant were cut up and sorted in order to recover the leaves and flowers. The leaves and flowers were dried in the shade and away from moisture. The dry plant material was ground to obtain a powder ready for our phytochemical study.

Preparation Methods

Preparation of the F0 crude extract

The powder obtained is distributed in packets of filter paper made in such a way as to facilitate the diffusion of the compounds of biomedical interest into the solvent. Each pack contains 12g of *T.hirsuta dry powder*.

Powdered	Filter assembly	A desiccant

Figure 1: Sample preparation

The packets are then macerated in a mixture of methanol (MeOH) / dichloromethane (DCM) (50/50) for 48 hours at room temperature in a desiccant. After 48 hours, the maceration is recovered and after evaporation away from light, the crude extract called F0 is obtained.

Figure 2: Preparation of the crude extract

Preparation of methanolic extracts

a) Methanolic extract from dry powder of *T. hirsuta*

In a bottle, 0.50 g of dry powder is put in 20 ml of MeOH. The mixture is stirred for 24 hours, after two hours of decantation, the supernatant is filtered using filter paper. The filtrate is placed in a clean, dry brown flask.

Figure 3: Methanolic extract from the dry powder

b) Methanolic extract from the crude fraction F0

After evaporation 0.4g of the crude fraction F0 is recovered and stirred in 16 ml MeOH for 24 hours, after two hours of decantation, the filtrate is placed in a clean and dry brown flask.

Determination of total polyphenols

Principle

Phenolic compounds or polyphenols are secondary metabolites characterized by the presence of an aromatic ring carrying free hydroxyl groups or committed to a carbohydrate. The most represented are anthocyanins, flavonoids and tannins. The determination of total polyphenols by the Folin-Ciocalteu reagent was described as early as 1965 (Singleton and Rossi).

The reagent consists of a mixture of phosphotungstic acid ($H_{3PW12O40}$) and phosphomolybdic acid ($H_{3PMo12O40}$). It is reduced, during the oxidation of phenols, to a mixture of blue oxides of tungsten and molybdenum**.** The colouring produced, whose maximum absorption is between 725 and 750 nm, is proportional to the amount of polyphenols present in the plant extracts. [106].

Technical

In a tube, 400µL of the methanolic extract obtained from the dry powder and the crude extract F0 (diluted to 1/10th) are placed. Then add 1.6 ml of Bi-distilled H2O and 10 ml of folin-Ciocalteu reagent (diluted to 1/10th in distilled water). After 1 min, 8 ml of sodium carbonate (Na2CO3 7.5% (w/v) is added). The tubes are shaken and kept for 2 hours at room temperature and in the dark. The absorbance is measured at 765 nm using a spectrophotometer against a blank made following the same protocol but replacing the methanolic extract with pure methanol.

The total polyphenol content is calculated according to the following formula:

$$\text{Polyphenol levels} = \frac{Abs(765\ nm)}{0.001441} \times FD$$

Abs: Sample absorbance
0.001441: constant
FD: Dilution Factor

The results are expressed in milligrams (mg) of hydroxytyrosol equivalent per kilogram of dry matter, All measurements are repeated 3 times.

Flavonoid Determination

1) Principle

The quantification of flavonoids was carried out by a method adapted by Zhishen et al, (1999) with aluminum trichloride and soda. Aluminum trichloride forms a yellow complex with flavonoids and soda forms a pink-colored complex absorbed in the visible range at 510 nm.

2) Technical

1 ml of the methanolic extract obtained from the dry powder and the crude extract F0 (diluted to 1/10th) are mixed with 4 ml of distilled water and 0.3 ml of sodium nitrite (NaNO2) at 5%. After 5 min, 0.3 ml of 10% aluminium trichloride (AlCl3) (w/v) is added to the mixture. After 5 min of incubation at room temperature, 2 mL of 1M sodium hydroxide (NaOH) and 2.4 mL H2O are added. Immediately, the mixture is completely stirred in order to homogenize the contents. The absorbance of the pinkish-coloured solution is determined to be 510 nm against a white.

The total flavonoid content of the plant extracts studied is calculated according to the formula:

$$\text{Flavonoid content} = \frac{Abs\ éch\ (510)}{0.0015 \times m(g)} \times FD$$

Abs: Sample absorbance
0.0015 —constant
FD: Dilution Factor
Mass of dry matter in g

The results are expressed in catechin equivalent milligram(g) per kilogram of dry plant matter (g EC/kg). All measurements are repeated 3 times

Separation of phenolic compounds by High Performance Liquid Chromatography (HPLC)

HPLC is one of the most widely used techniques in chemical analysis laboratories. It allows the separation and dosing of chemical compounds in a mixture.

Principle

The sample to be analyzed is pushed by a liquid (called the mobile phase) into a column filled with a stationary phase of fine particle size (the "grains" are very small). The flow rate of the mobile phase is high, which leads to an increase in the pressure in the system. This high throughput reduces the time required to separate the components along the stationary phase. The fine particle size of the stationary phase allows for better separation of components. The mobile phases used are mixtures of water and a miscible organic solvent (acetonitrile, methanol) or combinations of organic solvents (alcohols, hexane, dichloromethane, etc.) that are miscible with each other.

Technical

During the analytical HPLC, we used a Phenomenex chromatograph (Luna 5 µm PFP) pentafluorophenyl, equipped with a UV detector with a variable wavelength from 260 to 310 nm and equipped with an Agilent 1110 C18 column with a double pump A and B (250cm x 5µm x 4.60µm) at room temperature.

The mobile phase consists of 2 solvents: 100% water and 0.1% TFA (trifluoroacetate) (Phase A) and acetonitrile 100% with 0.1% TFA (Phase B).

The gradient chosen is as follows:

- 100% to 100% B; in17min.→
- 100% B between 17 and 22 min.
- 100% B 100% A in 30 min.→

The flow rate is maintained at 1 ml/min.

Then we will perform a preparative chromatography using an X-Bridge (Waters) C18 5µm – 19 x 250 mm column according to the following gradient:

- 100% to 100% B; in 15min.→
- 100% B between 15 and 18 min.
- 100% B 100% A between 18 and 22 min→
- 100% A between 20 and 22 min

The flow rate is maintained at 1 ml/min.

Preparative chromatography is a chromatography used to purify and recover samples, whether after an organic synthesis or an extraction step. The recovered samples will then be identified by analytical methods such as mass spectrometry.

In *vitro antioxidant activity*

In order to evaluate the activity of the extracts of the two varieties studied, two *in vitro* tests were performed: the DPPH test, and the FRAP test.

The DPPH radical scavenging test

a) Principle

The chemical compound 2,2-diphenyl-1-picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyle) was one of the first free radicals used to study the antioxidant structure-activity relationship of phenolic compounds [28,29]. It has an unpaired electron on an atom in the nitrogen bridge (Fig 11). Due to this delocalization, the molecules of the radical do not form dimers, DPPH• remains in its monomer form relatively stable at ordinary temperature. The relocation also causes the characteristic blue-violet color of the DPPH● solution. The measurement of the effectiveness

of an antioxidant is done by measuring the decrease in blue staining, due to a recombination of DPPH● radicals, measurable by spectrophotometry at 515-518 nm.

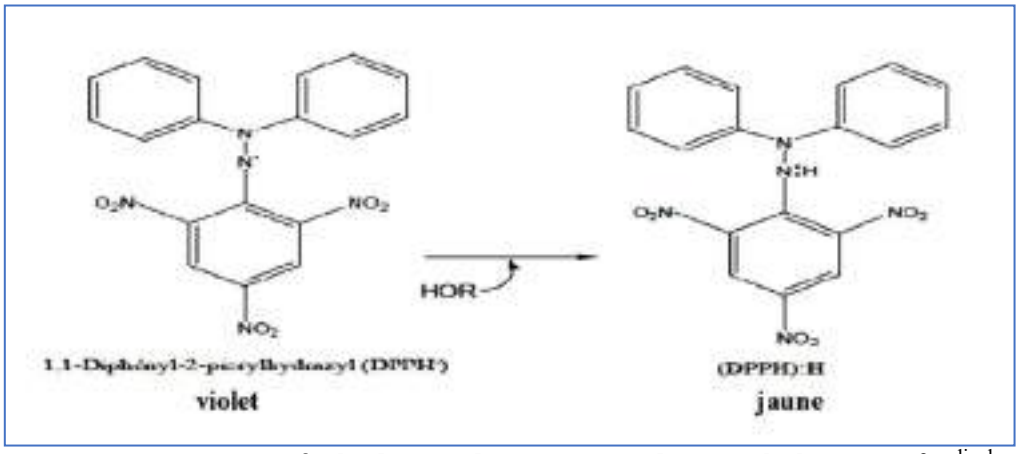


Figure 4: Reaction of a hydrogen donor (antioxidant) with the DPPH• radical

b) Technical

In a tube containing 100µl of methanolic extract of the dry powder (diluted 1/10th), of the crude fraction F0 also diluted 1/10th, 400 µl of Tris HCl and 500 µl of DPPH● (1.9 mg DPPH diluted in 100 ml of methanol) of purple colour are added. The white is prepared by mixing 600µl of methanol with 400µl Tris HCl. The control is prepared by mixing 100µl of methanol, 400µl of Tris HCL and 500µl of DPPH•. After incubation in the dark for 30 min and at room temperature, the absorbances are read at 517 nm using a spectrophotometer.
The percentage of inhibition (trapping) of DPPH● is determined using the following formula:

$$\% \text{ inhibition} = \frac{Abs\,(517)(C) - Abs(517)(éch)}{Abs(517)(C)} \times 100$$

Abs(c): control absorbance
Abs (ech): absorbance of the sample
All measurements are carried out three times.

Iron reduction: FRAP (*Ferric reducing antioxidant power*)

a) Principle

The antioxidant activity of the extracts of the study plant was evaluated using the FRAP method. The latter is a simple, fast and reproducible test. It is universal, can be applied to plants as well as plasmas and in organic and aqueous extracts. The presence of reductants in the extracts causes the reduction of iron Fe^{3+} ferricianide complex to the ferrous form. Therefore, Fe^{2+} can be assessed by measuring and monitoring the increase in density of the blue-green color in the reaction medium at 700 nm. In other words, the $FeCL_3$ /K3Fe(CN)$_{6\ system}$ gives the method the sensitivity for the "semi-quantitative" determination of the concentrations of polyphenols, which participate in the redox reaction.

b) Technical

250 µl of different methanolic extracts (diluted 1/100th) are mixed with 250 µl of the phosphate buffer solution (0.2M) and 250 µl of potassium ferricyanide ($K3Fe(CN)_6$) at 1%. The mixtures are incubated at 50°C for 20 min. 250 µl of trichloroacetic acid (10%) is added. The whole is centrifuged at 3750 revolutions for 10 min. 500 µl of the supernatant is mixed with 125 µl

FeCl3 (0.1%). After incubation in the dark, absorbance is measured at 700 nm using a spectrophotometer.
Ascorbic acid is used as a positive control in this experiment under the same operating conditions.

To explore the results obtained, the most common way used is to draw graphs of the absorbances obtained as a function of the different concentrations used. The increase in absorbance corresponds to an increase in the reducing power of the fractions tested.

Statistical analyses

The curves and histograms are plotted by Microsoft Excel 2007. The results of the tests performed are expressed as an average ± mean standard error (SEM). Statistics were calculated using SPSS software. Version 18.0.

Results

1-Determination of total phenols

The determination of total phenols was performed by the spectrophotometric method adapted from Singleton and Ross (1965) with the Folin-Ciocalteu reagent.
The results obtained are expressed in mg equivalent of hydroxytyrosol per kilogram of dry plant matter. Hydroxytyrosol is a molecule that belongs to the polyphenol family, it is a powerful ubiquitous antioxidant. It was used as a reference substance in the laboratory and calibration from a series of dilutions.
The polyphenol levels for the plants studied are shown in the following figure:

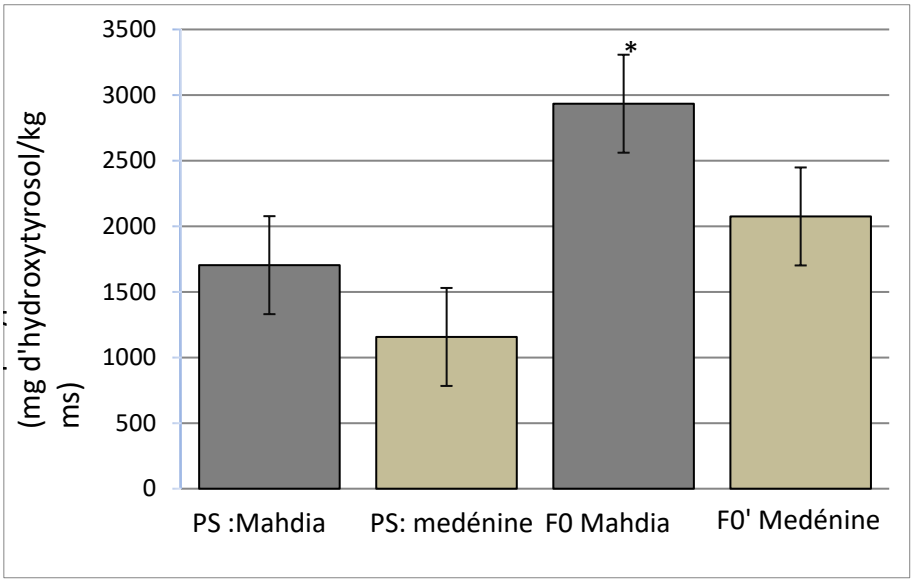


Figure 5: Variation in the level of total polyphenols in methanolic extracts of *Thymelaea hirsuta* according to the raw material used.
The difference at the P0.05 threshold was considered statistically significant<

The four extracts of *T. hirsuta* studied show a variability in the total phenol contents. The level of polyphenols is higher in methanolic extracts obtained from the crude fraction of the two plants compared to extracts obtained from PS (almost 2 times). The methanolic extract of the crude fraction F0 Mahdia contains the highest level of polyphenols, in the order of 2934 ± 0.034 mg equivalent of hydroxytyrosol per kilogram of dry plant matter followed by the methanolic extract of the crude fraction F'0 Medenine, with a content of 2074.9 ± 0.153 mg equivalent of hydroxytyrosol per kilogram of dry plant matter. Finally, the two extracts of the

dry powder Mahdia and Medenine have a content of 1703,60,046 and 1156,60,021 mg respectively.

2- Flavonoid assay

The determination of flavonoids was carried out by the colorimetric method described by Zhishen *et al*, (1999). The catechin considered as a positive control made it possible to carry out a calibration curve, making it possible to calculate the flavonoid content of the different plant extracts expressed in g catechin equivalent (EC) per kilogram of dry plant matter.

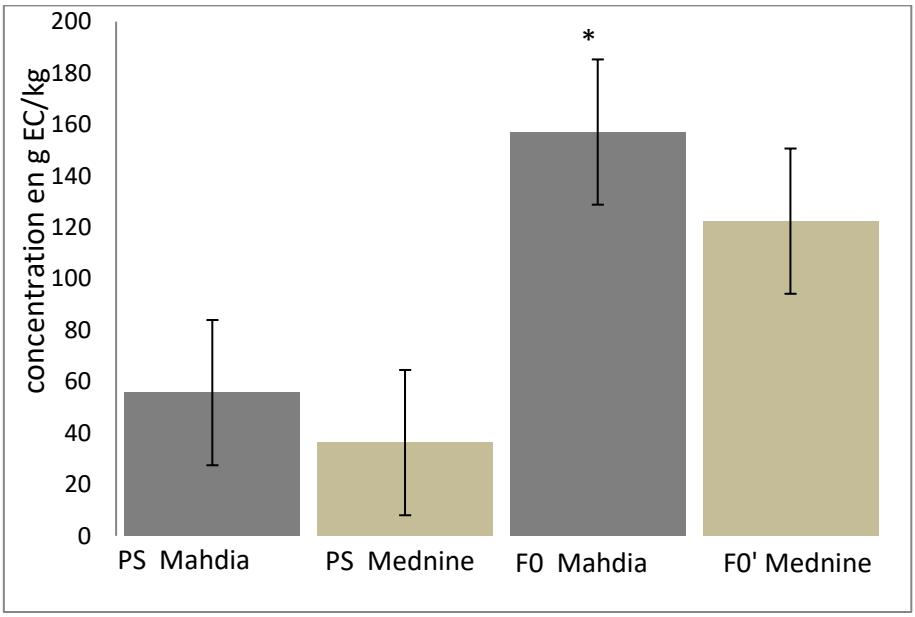


Figure 6: Variation in the level of total flavonoids in methanolic extracts of *Thymelaea hirsuta* as a function of the raw material used.
The difference at the P0.05 threshold was considered statistically significant<

We observe high flavonoid contents in the methanolic extracts of the crude fraction F0 Mahdia and F0' Medenine (3 times significant) compared to those obtained in the extracts from the dry powder. They represent a value of 157,060,021 and 122,40,002 g EC/kg respectively, compared to methanolic extracts of dry powder Mahdia with a content of 55.7 ± 0.024g EC/kg.and the methanolic extract of dry powder Medenine which represents a value of 36,260,002 g EC/kg.

3- Measurement of antioxidant activity

Antioxidant activity is determined by the decrease in the absorbance of an alcoholic solution of DPPH· at 515 nm, due to its reduction to a non-free form DPPH-H by the hydrogen-donating antioxidants (HA) present in the plant extract.

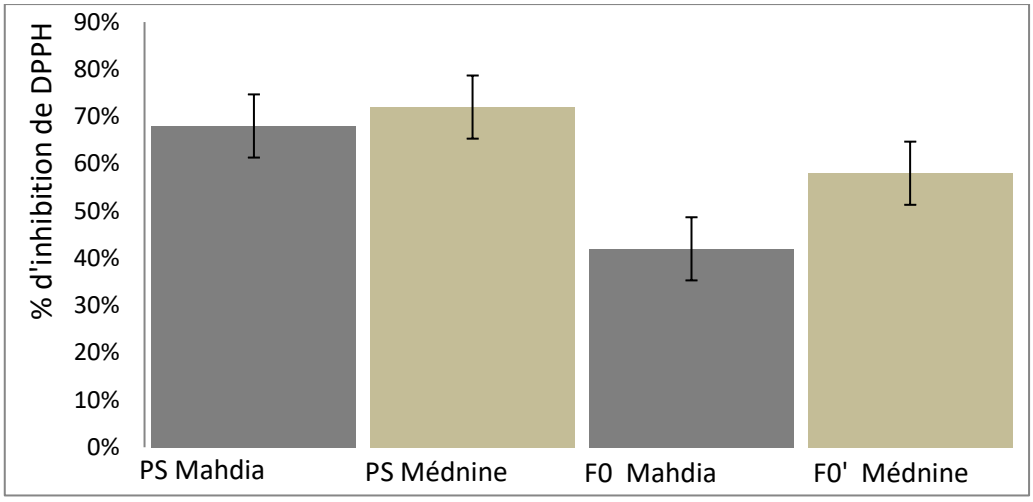


Figure 7: Percentage of DPPH inhibition by *Thymelaea hirsuta* depending on the raw material used.

The antioxidant activity of a compound corresponds to its ability to scavenge free radicals and therefore resist oxidation. This activity is expressed as a percentage of DPPH inhibition. The methanolic extract of the Medenine dry powder and the Mahdia dry powder showed a greater DPPH● scavenging power than the F0 Medenine/Mahdia crude methanolic extract. The percentages of inhibitions are in the order of 72% and 68% followed by those obtained for the F0 crude extracts with a percentage of 58% and 42% respectively.

4-Iron reduction: FRAP (Ferric reducing antioxidant power)

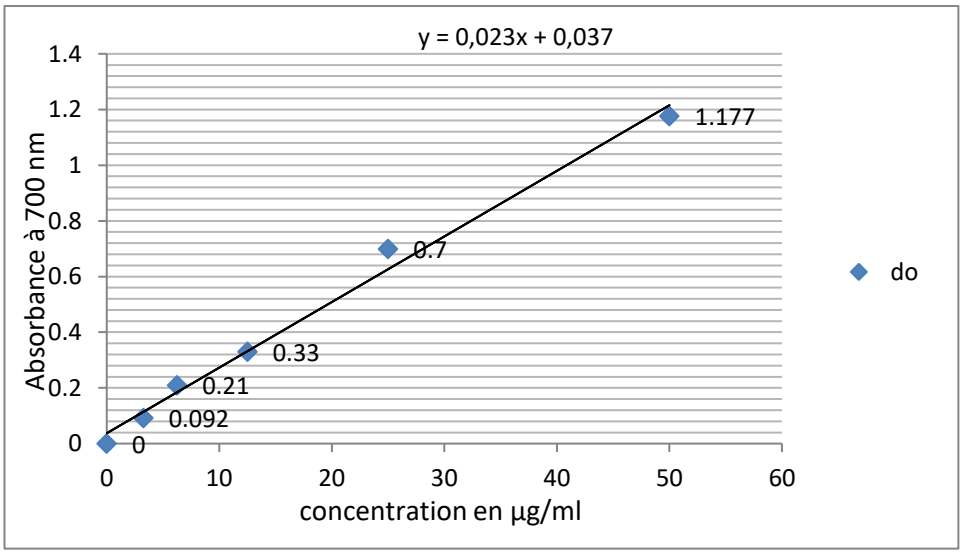


Figure 8: Ascorbic acid calibration curve

The absorbance of the samples corresponds to a concentration of 25µg/ml of the dry powder or crude fraction. The results are shown below:

Table 1 :p reducing works of methanolic extracts of *Thymelaea hirsuta* according to the raw material used.

	Vitamin C	PS Mahdia	PS Medenine	F0 Mahdia	F0' Medenine
DO	1,17	0,452	0,309	0,730	0,520
% PR	100	38	26	62,4	44,4

According to our results, all our extracts have lower antioxidant activities than ascorbic acid, for the latter the reduction is considered total from a concentration of 0.5mg/ml.
Crude extracts of *T. hirsuta* are more active (1.5 times) compared to the same methanolic extracts in dry powder.

5- Separation of phenolic compounds by High Performance Liquid Chromatography (HPLC)

- The analysis carried out at a maximum of 310 nm of absorption of phenolic compounds makes it possible to separate the condensed phenolic compounds of the flavonoid type from the two crude extracts F0 and F0' of the T. *hirsuta* plant. The chromatograms obtained are represented by the figures below.

Mahdia Crude Extract
Analytical HPLC

- **Detection of total phenolic compounds at 260 nm**

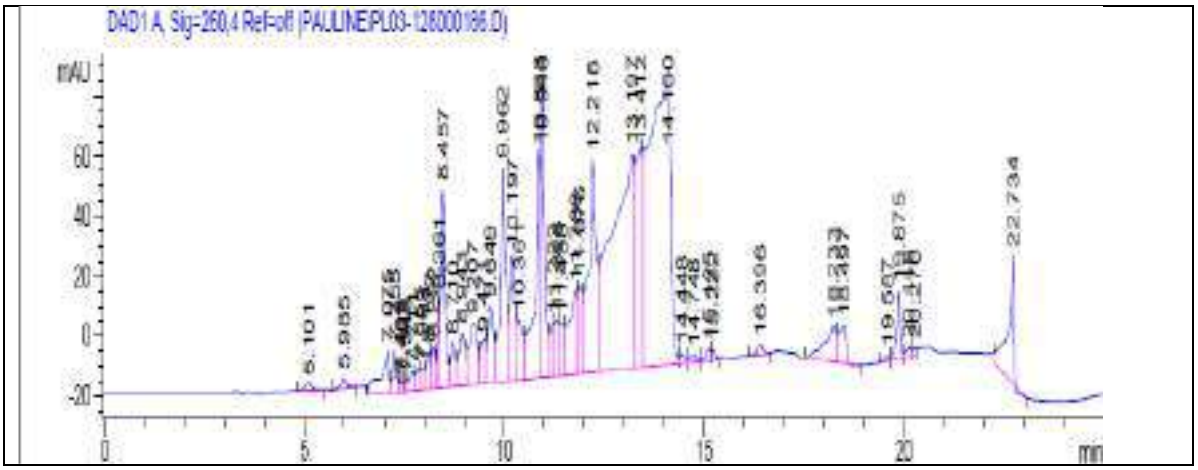


Figure 9: analytical chromatogram of phenolic compounds in crude Mahdia extract (F0)

- **Detection of condensed phenolic compounds such as flavonoids, anthocyanins at 310 nm**

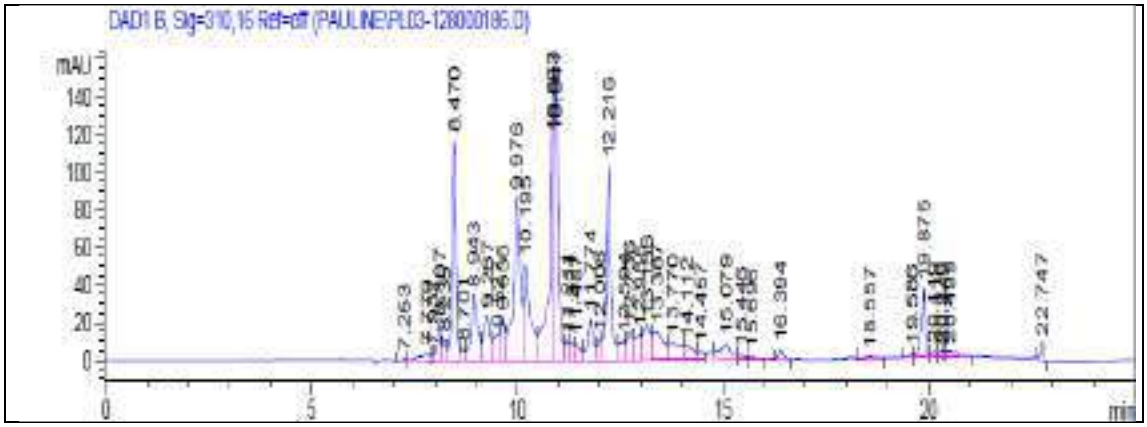


Figure 10: Analytical chromatogram of phenolic compounds in crude Mahdia extract (F0)

Table 2: Main Phenolic Constituents Detected by HPLC in Thymelaea hirsuta Extract

Peak #	Retention Time (min)	Height (mAU)	Area %
6	8.470	117.29	8.24
8	8.943	34.96	4.23
12	9.976	87.11	11.35
13	10.195	51.00	6.86
14	10.863	157.58	13.04
15	10.941	144.92	9.94
21	12.216	103.31	8.59

HPLC analysis revealed a complex chromatographic profile with 41 detectable peaks. However, only the major peaks (Area ≥ 4%) were considered for interpretation, as they represent the predominant phenolic constituents of the extrac.
The results show at 310 nm, 4 products of which 4 are the majority products: N° 6 - 12 - 14 - 21. They will be collected by preparatory HPLC

Preparative HPLC

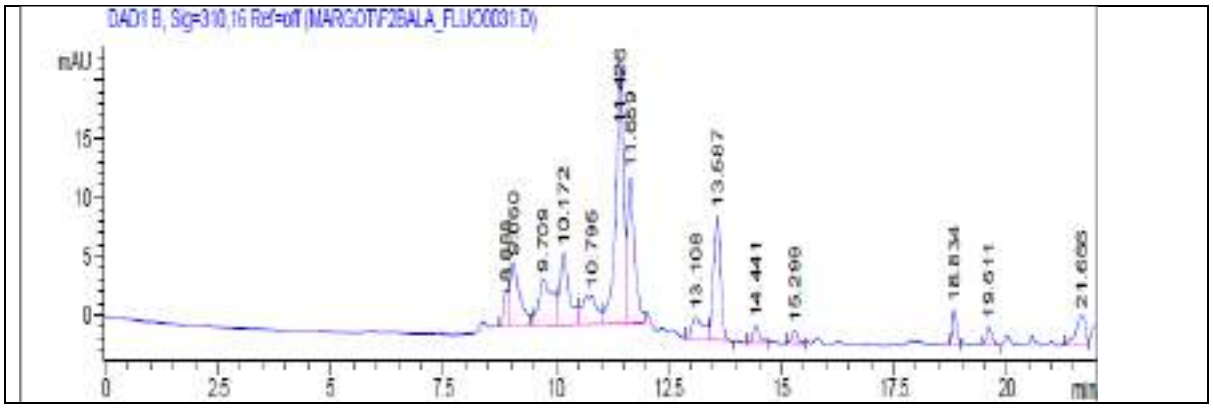


Figure 11: 310 nm preparative HPLC chromatogram of phenolic compounds in crude Mahdia extract

Table 3: Major HPLC Peaks of Phenolic Compounds in the Methanolic Extract of Thymelaea hirsuta

Peak #	Retention Time (min)	Height (mAU)	Area %
2	9.060	5.35	8.50
3	9.709	3.90	8.94
4	10.172	6.10	8.37
5	10.795	2.37	5.69
6	11.426	22.31	26.98
7	11.659	12.44	14.44
9	13.587	10.63	12.16

HPLC analysis revealed 14 detectable peaks. However, only the major peaks (Area ≥ 5%) were considered for interpretation, as they represent the predominant constituents of the extract.

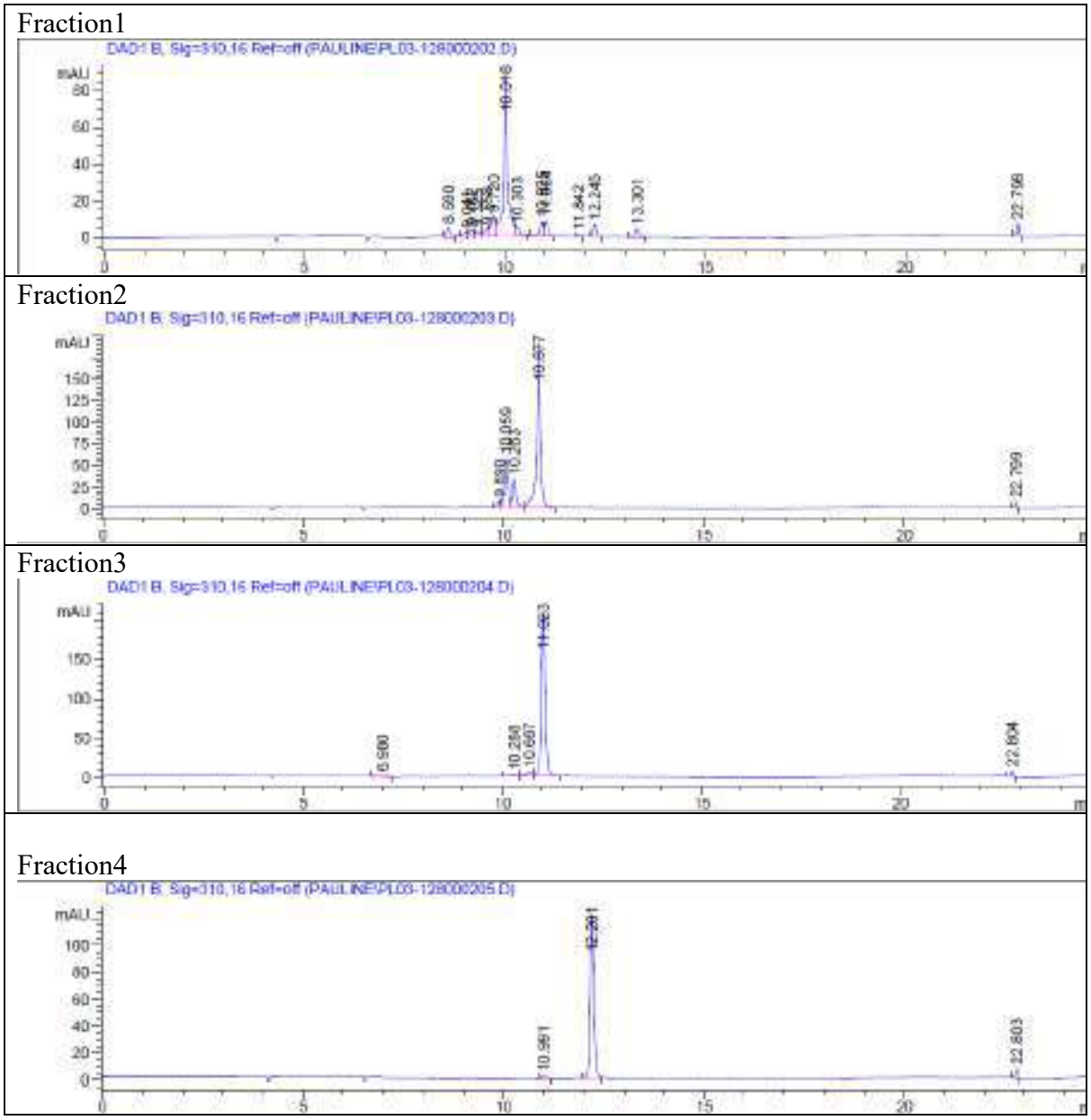


Figure 12: Chromatographic profiles of the different fractions collected analyzed by analytical HPLC

Fraction 1 collected contains a majority product of 57.7%, i.e. 8.9% of the total extract. Fraction 2 contains a majority product of 66.98%, i.e. 26.9% of the total extract. Fractions 3 and 4 are pure since the majority products represent 90.8 and 94.7% of the collected compounds but represent 14.4 and 12.16% of the total extract respectively.

Medenine Crude Extract

Analytical HPLC

- **Detection of total phenolic compounds at 260 nm**

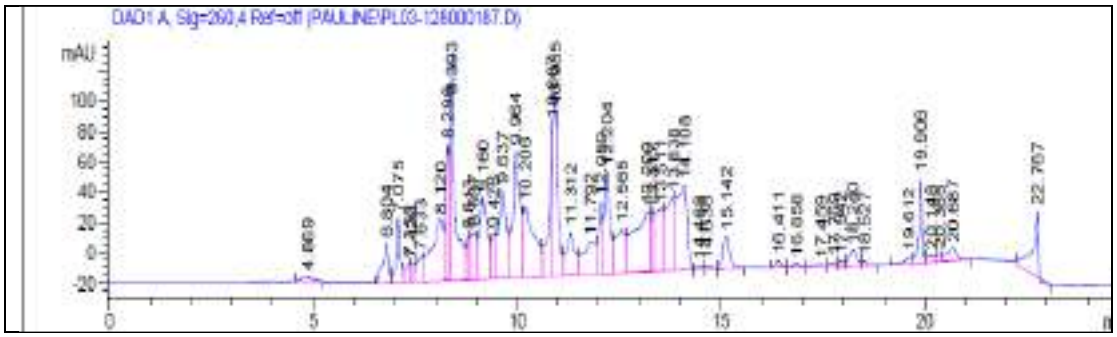


Figure 13 : Analytical chromatogram of phenolic compounds in crude Medenine extract (F'0)

Detection of condensed phenolic compounds such as flavonoids, anthocyanins at 310 nm

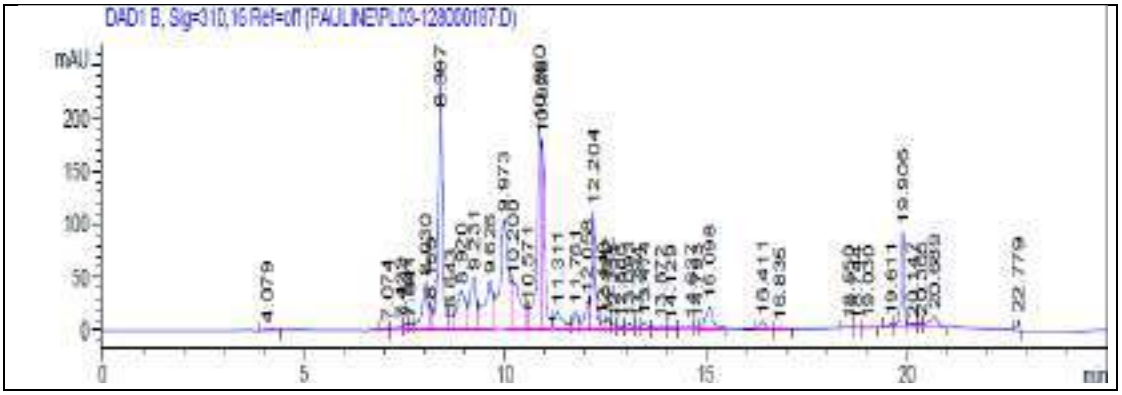


Figure 14: chromatogram of the phenolic compounds of the crude extract from the Medenine plant.

Table 4: Phenolic compounds of the crude extract of Medenine determined by analytical HPLC.

Peak #	Retention Time (min)	Height (mAU)	Area %
8	8.397	256.44	14.76
11	9.231	49.84	4.48
12	9.626	45.90	5.56
13	9.973	104.44	12.19
14	10.200	44.92	6.16
16	10.860	197.35	11.21
17	10.928	176.64	8.27
21	12.204	111.04	6.38

HPLC analysis revealed a complex chromatographic profile with 44 detectable peaks. However, only the major peaks (Area ≥ 4%) were considered for interpretation, as they represent the predominant constituents of the extract.

The table of the retention times of the different products of the crude extract from the Mahdia plant separated by preparative HPLC and the % of presence of the different phenolic compounds shows 44 products, 5 of which are the majority of compounds which represent a fraction collected and then analyzed by analytical HPLC.

reparative HPLC

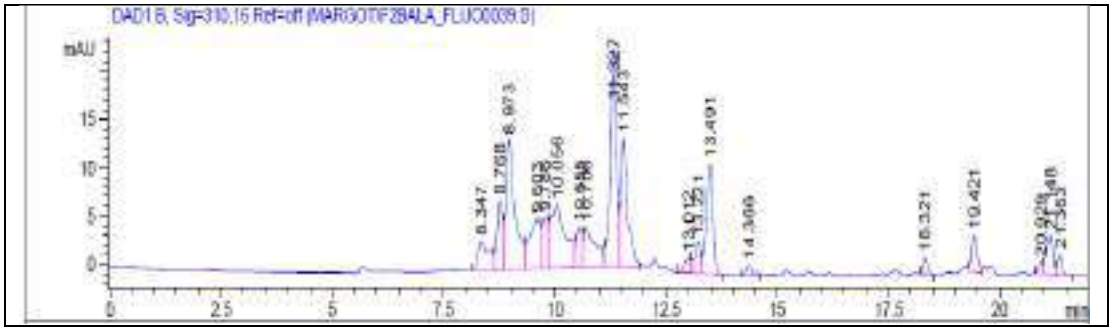
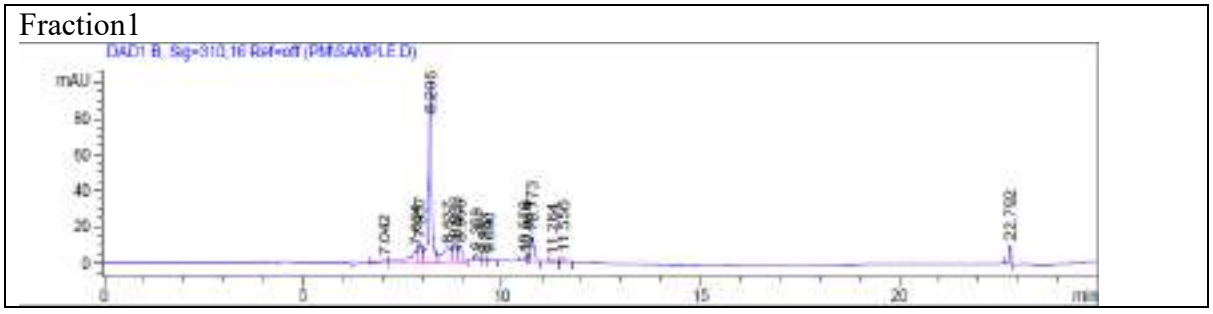


Figure 15: chromatogram obtained by preparative HPLC at 310 nm of the phenolic compounds of the crude Medenine extract

Table 5: phenolic compounds of the crude extract from Medenine separated by preparative HPLC.

Peak #	Retention Time (min)	Height (mAU)	Area %
3	8.973	13.37	14.30
6	10.056	6.59	11.48
9	11.327	22.64	15.62
10	11.543	13.12	10.51
13	13.491	11.35	8.51
8	10.706	4.11	5.93
4	9.603	5.16	6.22

Preparative HPLC analysis of the crude extract of Thymelaea hirsuta collected from the Medenine region identified 19 distinct components based on retention times and relative peak areas. HPLC analysis revealed 19 detectable peaks; however, only the major peaks (Area ≥ 5%) were considered for interpretation, as they represent the predominant constituents of the extract. Among these, five major compounds were predominant, forming the principal fraction of the extract, and were therefore collected and subsequently analyzed by analytical HPLC for further characterization.



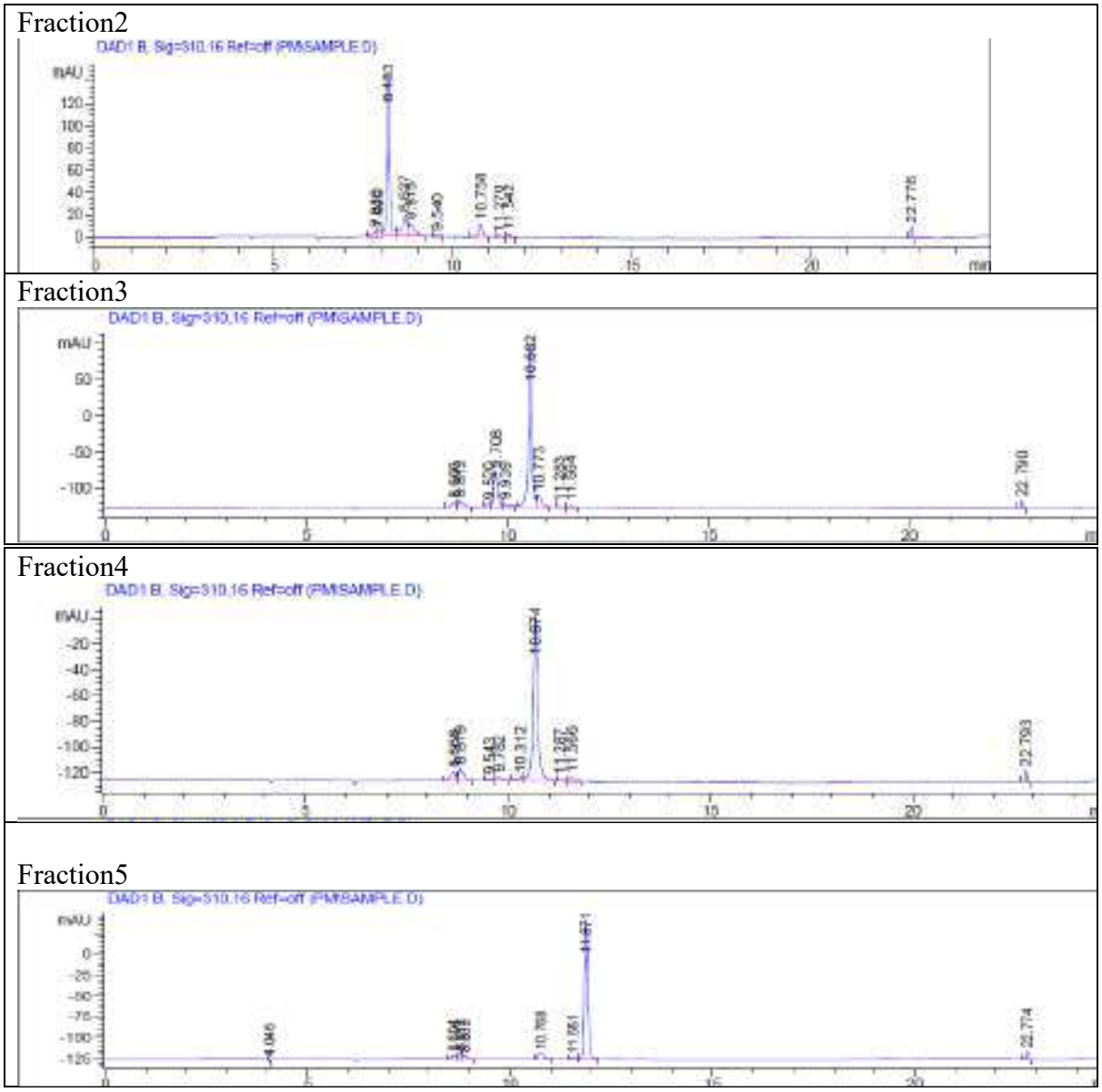


Figure 16: Fractions obtained from the preparative HPLC of Medenine extract

Fraction 1 collected contains a majority product of 44.89%, i.e. 14.3% of the total extract. Fraction 2 contains a majority product of 60.98%, i.e. 11.4% of the total extract. Fraction 3 contains a majority product of 65.84%, i.e. 15.6% of the total extract. Fraction 4 contains a majority product of 78.18%, i.e. 10.5% of the total extract, and finally fraction 5 contains a majority product of 81.08%, i.e. 8.5% of the total extract.

Discussion

The present study investigated the phenolic composition and antioxidant potential of methanolic extracts obtained from the aerial parts of *Thymelaea hirsuta* collected from two different regions. The findings demonstrate that extraction conditions, plant origin, and the nature of the raw material significantly influence both the quantity of bioactive compounds and the antioxidant activity of the extracts.

Preparation of Extracts

Methanol is widely recognized as an effective solvent for extracting phenolic compounds due to its polarity and ability to dissolve a broad range of plant metabolites. In this study, a

methanol–dichloromethane mixture was employed to obtain crude extracts, enabling the recovery of compounds with varying polarities. The use of dried plant material appears to be particularly important, as fresh tissues are susceptible to enzymatic degradation and microbial fermentation, which may reduce the stability of sensitive compounds such as flavonoid glycosides. Drying in the absence of light further helps preserve phytochemicals by preventing photo-induced degradation and structural alterations.

Grinding the plant material into powder enhances extraction efficiency by increasing the surface area and improving solvent penetration into cellular structures. Maceration under continuous stirring at room temperature for an extended period facilitates the diffusion of solutes into the solvent while minimizing thermal degradation. These methodological considerations collectively contribute to maximizing the recovery of phenolic compounds and maintaining their bioactivity.

Total Polyphenols and Flavonoids

The results indicate that *T. hirsuta* is a rich source of phenolic compounds, including both total polyphenols and flavonoids. Extracts obtained from the crude fractions exhibited substantially higher phenolic contents than those derived from powdered material, suggesting that extraction conditions and sample preparation strongly affect compound recovery. The highest total polyphenol concentration was observed in the crude extract from Mahdia, followed by that from Medenine, while extracts from dry powder showed comparatively lower values.

Similarly, flavonoid analysis revealed markedly elevated concentrations in crude extracts relative to powdered samples, with values approximately three times higher. These findings support the notion that flavonoids constitute a major component of the phenolic profile of *T. hirsuta* and contribute significantly to its biological activity. The variability observed between regions may reflect environmental influences such as climate, soil composition, and water availability, which are known to modulate secondary metabolite production in plants.

Antioxidant Activity

The antioxidant capacity of the extracts was assessed using both radical scavenging and reducing power assays, providing complementary insights into their mechanisms of action.

Radical Scavenging Activity

The DPPH assay demonstrated that extracts derived from dry powder exhibited stronger radical scavenging activity than crude extracts, despite their lower total phenolic content. This apparent discrepancy suggests that antioxidant activity is not determined solely by the quantity of phenolics but also by their qualitative composition and structural characteristics. Certain phenolic compounds may possess higher radical-quenching efficiency than others, leading to enhanced activity even at lower concentrations.

The observed activity can be attributed primarily to the presence of flavonoids and other phenolic molecules capable of donating hydrogen atoms or electrons to stabilize free radicals. The correlation between phenolic content and antioxidant activity has been reported in numerous studies, although synergistic interactions among multiple constituents may also play a significant role. The ability of these compounds to neutralize reactive oxygen species underscores their potential for preventing oxidative damage in biological systems.

Reducing Power

The reducing power assay revealed that crude extracts exhibited stronger electron-donating capacity than powdered extracts, although their activity remained lower than that of the reference antioxidant, ascorbic acid. This finding indicates that crude extracts contain compounds capable of reducing ferric ions to ferrous form, reflecting their potential to act as reductants in oxidative processes. Phenolic hydroxyl groups are known to play a crucial role in this mechanism, enabling electron transfer and stabilization of oxidized intermediates.

The enhanced reducing power of crude extracts may result from the presence of a complex mixture of phenolic compounds that act synergistically to produce a stronger overall effect.

Such interactions highlight the importance of studying whole extracts rather than isolated components when evaluating antioxidant properties.

Phenolic Composition by HPLC

High-performance liquid chromatography analysis revealed a highly complex phenolic profile in *T. hirsuta*, with more than forty detectable compounds in the crude extracts. The plant material collected from Medenine exhibited a slightly greater number of phenolic constituents than that from Mahdia, suggesting regional variation in chemical composition. Preparative HPLC further enabled the isolation of major fractions, indicating the presence of dominant phenolic compounds that contribute substantially to the overall extract composition.

The diversity of phenolic compounds observed is consistent with the adaptive strategies of plants growing in arid environments. Water deficit and environmental stress are known to stimulate the biosynthesis of secondary metabolites, including polyphenols, which function as protective agents against oxidative stress and other adverse conditions. Consequently, differences in environmental factors between regions likely account for the variations in phenolic profiles observed in this study.

Overall Implications

Taken together, the results confirm that *Thymelaea hirsuta* is a rich source of phenolic antioxidants with significant biological potential. Both quantitative and qualitative differences in phenolic composition influence antioxidant activity, highlighting the importance of extraction methods and environmental conditions in determining bioactive properties. The presence of numerous phenolic compounds suggests that synergistic interactions may enhance the overall antioxidant effectiveness of the extracts.

These findings support the traditional use of *T. hirsuta* in herbal medicine and underscore its potential as a natural source of antioxidants for pharmaceutical, nutraceutical, and food applications. However, further studies are needed to isolate individual active compounds, elucidate their mechanisms of action, and evaluate their safety and efficacy in biological systems.

Conclusion and Future Perspectives

Aromatic and medicinal plants represent an important natural source of antioxidants and remain insufficiently explored for pharmaceutical applications. Given the significant role of antioxidants in preventing oxidative stress–related diseases, the development of therapeutic agents derived from natural antioxidant compounds is of considerable scientific interest.

In this study, the phytochemical composition and antioxidant activity of various extracts obtained from the aerial parts of *Thymelaea hirsuta* collected from central and southern regions of Tunisia were investigated. The results revealed substantial variability in total phenolic and flavonoid contents among the different extracts. The crude extract from the Mahdia region exhibited the highest levels of total polyphenols and flavonoids. However, high-performance liquid chromatography analysis of the crude fractions indicated a greater diversity and abundance of phenolic compounds in the extract from the Medenine region, which may be attributed to environmental stress conditions associated with arid climates.

The findings suggest that *T. hirsuta* is a promising source of natural antioxidant compounds. Evaluation of antioxidant activity using both DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assays demonstrated that all tested extracts possessed antioxidant potential, although their reducing capacity remained lower than that of the reference antioxidant, ascorbic acid. Notably, the dry powder extract from the Medenine region showed particularly strong radical scavenging activity in the DPPH assay, highlighting the important contribution of flavonoids and other phenolic compounds to the antioxidant properties of the plant.

Chromatographic analysis further confirmed the complex phenolic composition of *T. hirsuta*, supporting the hypothesis that the observed biological activity results from the combined effects of multiple bioactive constituents. Environmental factors, particularly water stress in arid regions, appear to play a significant role in modulating the synthesis and accumulation of these secondary metabolites.

Despite these promising results, additional investigations are required to fully characterize the bioactive molecules responsible for the antioxidant activity. Future research should focus on the isolation and structural identification of individual compounds using advanced analytical techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy. Moreover, evaluating antioxidant activity through complementary in vitro assays and in vivo models would provide a more comprehensive understanding of the therapeutic potential of *T. hirsuta*.

In conclusion, this study highlights the significant phytochemical richness and antioxidant capacity of *Thymelaea hirsuta*, supporting its traditional use and suggesting its potential as a source of natural compounds for pharmaceutical and nutraceutical applications. Further detailed phytochemical and pharmacological investigations are warranted to validate its efficacy and safety as a potential therapeutic agent.

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Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.