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Phytochemical Screening and Antioxidant Activity of Moroccan *Thymus satureioïdes* Extracts

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Abstract

Phytochemical screening and antioxidant activities in different solvent extracts of *Thymus satureioïdes* was carried out. The extracts were subjected to various chemical test for phytochemical constituents, total phenolic contents were evaluated using Folin Ciocalteu method and their antioxidant activity was assayed through *«in vitro»* radical scavenging activity using DPPH· assay, FRAP and ABTS. The phytochemical screening of this study indicate the presence of steroids, flavonoids, alkaloids, saponnins, and catechic tannins. The average total phenol content of hydroethanolic extracts were significantly ($P<0.05$) higher when compared with the total polyphenol contents in the hexane, ethyl acetate and dichloromethane extracts. In order of effectiveness (IC50) of the plant extracts the potent inhibitors was hydroethanolic extract, followed by Dichloromethane, ethyl acetate, the least was the hexane extract, for all the methods (DPPH, ABTS and FRAP). This shows that *Thymus satureioïdes* solvent extracts especially the hydroethanolic extracts may be a potent source of natural antioxidant and its use in the management of diseases associated with oxidative stress is justified.

1. Introduction

The genus Thymus comprising of around 300 species of perennial, herbs, aromatic and subshrubs is predominantly found in Mediterranean region, Southern Europe and Asia **[1].** Thymus species are considered as medicinal plants due to their pharmacological and biological properties. In native medicine, flowering parts and leaves of Thymus species have been extensively used as herbal tea, tonic, carminative, antitussive and antiseptic, as well as for treating colds **[1-6].** According to the literature reports the composition and biological activities of extract from various Thymus species are rarely investigated.

Nowadays, in Morocco, Thymus species are threatened due to their overexploitation, such as *T. satureioïdes*, which is widely exported as herbs or essential oil. As a result, this species are now endangered, and the conservation of this valuable plant is imperative **[7]**. *Thymus satureioïdes*, a member of Lamiaceae 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 [8], antifungal [9], antibacterial [10], and anti-inflammatory properties after topical or oral administration [11].

Antioxidants are substances that, at low concentrations, prevent or retard the oxidation of easily oxidisable biomolecules such as lipids, proteins or DNA. They counteract free radicals and thus prevent oxidative damage **[12].** There are two basic categories of antioxidants, namely, synthetic and natural. Natural antioxidants, particularly in fruits and vegetables have gained increasing interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer [13- 14]. The defensive effects of natural antioxidants in fruits and vegetables are related to three major groups: vitamins, phenolics, and carotenoids. Ascorbic acid and phenolics are known as hydrophilic antioxidants, while carotenoids are known as lipophilic antioxidants [15].

To the best of our knowledge there are no earlier reports yet available regarding the detailed evaluation of antioxidant principles of extract from plants of *Thymus satureioïdes*, native to Morocco.

2. Experimental

2.1. Plant material

The aerial parts (stems and leaves) of *T. Satureioiïdes* were collected during the flowering period in the outskirts of the city of Marrakech; the plant material was dried at room temperature, ground to a powder using a blender. Crude extracts were prepared by Soxhlet extraction with solvents of increasing polarity: hexane, dichloromethane, ethyl acetate and water-ethanol (1-4 V/V) for 8 hours.

2.2 Phytochemical screening

Chemical tests for the screening and identification of bioactive chemical constituents in *T. Satureioïdes* were carried with extracts prepared using the standard procedures.

2.2.1 Detection of Sterols, Polyterpenes [16]

Using LIEBERMANN reagent allows identifying these compounds, Blue-green ring between layers indicates the presence of steroids and pink- purple ring indicates the presence of terpenes.

2.2.2 Detection of Reducing Sugars

Reducing sugars have been identified in crude extracts by the Fehling reagent, and then confirmed their presence by Tollens test.

To realize the Fehling tests, 5 ml of crude extract are added to 5 ml of Fehling's solution. The formation of a precipitate red brick after 2-3 min of heating bath at 70 °C indicates a positive reaction.

The detection of reducing sugars by the Tollens test consisted of adding 5 ml of crude extract to 5 ml of the Tollens reagent. Forming a silver mirror after a few minutes indicates a positive reaction.

2.2.3 Detection of Alkaloids [17]

Alkaloids were characterized from Bouchardat reagent (reagent iodo-iodized) and Dragendorff (reagent iodobismuthate of potassium). 6 ml of each solution were evaporated to dryness. The residue is taken up in 6 ml alcohol at 60 °. The addition of 2 drops of reagent Dragendorff on the alcoholic solution caused a precipitate or orange color. Adding 2 drops of Bouchardat reagent on the alcoholic solution caused a color precipitate reddish brown and indicated a positive reaction.

2.2.4 Detection of Proteins [18]

The proteins were identified in the extracts by the biuret reaction. To an aliquot of extract dissolved in 2 ml of 20% aqueous NaOH in a test tube are added 2 -3 drops of an aqueous solution of $CuSO_4$ to 2%. The appearance of a purple color, sometimes with a reddish tinge, indicates a positive reaction.

2.3.5 Detection of Coumarins[19]

The coumarins have been identified in the extracts by the reaction with the lactone ring. In two test tubes, are introduced 2 ml ethanolic solution obtained from each residue. In one of test tubes, are added 0.5 ml of 10% NaOH, then the test tubes are heated in a water bath until boiling. After cooling, were added to each test tube 4 ml of distilled water. If the liquid from the test tube in which was added the alkaline solution is transparent or more transparent compared to the control test tube liquid (without alkaline solution), then the reaction is positive. Acidifying the clear solution with a few drops of concentrated HCl, it loses its yellow color, is troubled or it forms a precipitate.

2.2.6 Detection of Tannins [19]

Search for catechic tannins is made from reagent Stiasny. 5 ml of each extract were evaporated to dryness. After adding 15 ml of reagent Stiasny the residue, the mixture was kept in a water bath at 80°C for 30 min. The observation of a precipitate in large flakes characterized catechic tannins.

For gallic tannins, we filtered the previous solution. The filtrate was collected and saturated with sodium acetate. The addition of $FeCl_3$ drops causes the appearance of a blue-black coloration intense, indicating the presence of gallic tannins.

2.2.7 Detection of Flavonoids [20]

0.5 g of various extract was shaken with petroleum ether to remove the fatty materials (lipid layer). The defatted residue was dissolved in 20 ml of 80% ethanol and filtered. The filtrate was used for the following tests: 5 ml of the dilute ammonia solution was added to the portion of the aqueous filtrate of each plant extract followed by the addition of concentrated H_2SO_4. The appearance of the yellow coloration indicated the presence of flavonoids.

2.2.8 Detection of Saponosides[17]

To find saponnins, we contributed in a test tube, 10 ml aqueous total extract. The tube was shaken for 15 s and allowed to stand for 15 min. A height of persistent foam greater than 1 cm indicated the presence of saponnins.

2.3 Determination of total phenolic content

The amount of total phenolic contents was determined according to Folin-Ciocalteu method as described by Lister and Wilson [21]. Briefly, 0.5 ml of sample solution was mixed with 2.5 ml of Folin-Ciocalteu reagent diluted with distilled water 1:10, followed by the addition of 4 ml of Na_2CO_3 (7.5 %, w/v). The mixture is then incubated in a water bath at 45°C for 30 min and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer against blank sample. The standard curve of Gallic acid is obtained under the same conditions as above using a range of concentrations (0-200 mg/l). The total phenolic content was measured as Gallic acid equivalents (mg GAE/g extract).

2.4 Determination of flavonoids content

Flavonoid contents were measured using a modified colorimetric method [22]. 0.25 mL of extract solution was added to a test tube containing 1.25 mL of distilled water. Sodium nitrite solution (5%, 0.075 mL) was added to the mixture and maintained for 5 min. Then, 0.15 mL of 10% aluminum chloride was added. After 6 min, 0.5 mL of 1 M sodium hydroxide was finally added. The mixture was diluted with 0.275 mL of distilled water. The absorbance of the mixture at 510 nm was measured immediately in comparison to a standard curve prepared by quercetin. The flavonoid contents were expressed as mg quercetin equivalent (QE)/g of extract.

2.5 Antioxidant Activity (AA)

2.5.1 Free radical scavenging activity (DPPH)

The free radical scavenging activity of the plant extracts was measured by 1.1-diphenyl-2-picryl-hydrazil (DPPH) [23], with some modifications. Briefly, 0.2 mM solution of DPPH in ethanol was prepared and 0.5 ml of this solution was added to 2.5 ml of plant extract and was allowed to stand at room temperature for 30 min, and then absorbance was read at 517 nm against blank samples. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. IC50 value was determined from the plotted graph of scavenging activity against the different concentrations of Thymus extracts, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. Ascorbic acid was used as reference compound.

2.6. Determination of reducing/antioxidant power (FRAP)

The ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) method [24] was used to measure the reducing capacity of the plant extracts with a slight modification, which involves the presence of extracts to reduce the ferricyanide complex to the ferrous form. Various concentrations of extracts from the stock solutions and the standard (ascorbic acid) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1% w/v). The mixture was incubated at 50°C for 20 min. Then 2.5 ml of trichloroacetic acid (10% w/v) was added to the reaction mixture. Afterwards, it was centrifuged at 1000 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with deionised water (2.5 ml) and ferric chloride (0.5 ml 0.1% w/v). The absorbance was measured at 700 nm at the reaction time of 30 min. The reducing power of the extracts was represented as ascorbic acid equivalent (mg AAE/ g of extract). [24], Studies on products of browning reaction prepared from glucoseamine.

2.6.3 ABTS radical scavenging assay

The scavenging activity of extracts against ABTS radical was determined by following the method described by Roberta et al [25]. Briefly the stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate ($K_2S_2O_8$) in equal volumes were allowed to stand in the dark for 12-16 h at room temperature. Prior to assay, ABTS solution was diluted in ethanol to give an absorbance of 0.700 ± 0.02 at 734 nm. 2 ml of the resulting solutions was allowed to react with 200µl of the plant extracts with different concentrations, reaction mixture was vortexed and absorbance was measured at 734 nm after 30 min. The same was done for the ascorbic acid standard (oxo-3-gulofuranolactone acid) of various concentrations (1 – 100 µg/ml). The amount of sample necessary to decrease the absorbance of DPPH by 50% (IC50) was calculated graphically.

3. Results and discussion

3.1. Yields of extract

The extraction yield of various solvent showed significant differences ($P<0.05$). The values of extraction yield varied from 0.66% for the hexane extract to 5.35% for the hydroethanolic extract. The extraction yield increased in the following order: water/ ethanol > ethyl acetate > Dichloromethane > Hexane. Few researches have been reported about the extraction yield of *Thymus satureioïdes* extracts [26]. Variation in the various extracts yield was due to the polarities of different compounds present in the plants, and such differences have been reported in the literature concerning fruit seeds [27], three *Mentha* species [28], Red Clover plant [29] Moroccan macro algae species [30] and Moroccan Flowers and seeds [31]. The highest yield in the sequential extractions was achieved with polar solvents.

Table 1: The extraction yield of four solvent extracts

Extract	Yield (%)
Hexane	0.66
Dichloromethane	1.79
Ethyl acetate	2.48
Water/ethanol	5.35

3.2 Phytochemical screening

From the phytochemical studies, it has evaluated in all extracts remarkable presence of Steroids, flavonoids and alkaloids. Others metabolites and bioactive compounds were identified such as saponosides, catechic tannins. They are present in water/ethanol extracts, while they are absent in the other extracts, Coumarin, protein, and hydrolysable tannin are absent in all the extracts (Table 2).

The presence of flavonoids in all extract is likely to be responsible for the free radical scavenging effects observed. Flavonoids are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers [32].

All *Thymus satureioïdes* extracts were also revealed to contain steroids, which are known to produce an inhibitory effect on inflammation [32] and alkaloids that have been reported to exert analgesic, antispasmodic and antibacterial activities [32, 34]. The phytochemical screening results of the extracts are consistent with the results reported by Nema *et al* for *Thymus vulgaris* from Egypt [35].

3.3 Total phenolic and flavonoid content

Phenolic is a kind of polyphenols that can be divided into tannin, propanoid and flavonoid. Phenolic compounds are known as powerful chain breaking antioxidants [36], which may contribute directly to antioxidative action [37]. These compounds are very important constituents of plants and their radical scavenging ability is due to their hydroxyl groups [38].

Table 2: Phytochemical screening of *Thymus satureioïdes* extracts

	Hexane	Dichloromethane	Ethyl acetate	Water/EtOH
Steroids	+++	+++	+++	++
Reducing sugars	-	-	-	-
alkaloids	++	++	++	++
Proteins	-	-	-	-
Coumarins	-	-	-	-
Hydrolysable tannins	-	-	-	-
Catechic tannins	-	-	-	++
Flavonoids	+	++	+	+++
Saponosides	-	-	-	+

Table 3: Total phenolic content (mg GAE.g^{-1}) and flavonoid content (mg QE.g^{-1}).

Extract	TPC (mg GAE /g)	TFC (mg QE/g)
Hexane	97.90 ± 6.45	92.133 ± 1,006
Dichloromethane	243.06 ± 0.80	159.6 ± 2.6
Ethyl acetate	129.08 ± 2.02	114.4 ± 1.2
Water/EtOH	403.01 ± 3.63	267.8 ± 2.6

The content of phenolic compounds in various extracts was determined from regression equation of calibration curve of Gallic acid and expressed as milligrams equivalent of Gallic acid per gram of dry extract (mg GAE/g). Flavonoids content was expressed as milligrams equivalent of Quercetin per gram of dry extract (mg QE/g). Total phenolic and flavonoid contents are shown in Table 3. From these results, Hydro-alcoholic extract showed high phenolic and flavonoid compounds (403.01 ± 3.63 mg GAE/g and 267.8 ±2.6 mg QE/g respectively) followed by Dichloromethane extract (243.06 ± 080 mg GAE/g of phenolics and 159.6 ± 2.6 mg QE/g of flavonoids). Ethyl acetate extract showed lower values of phenolic content (129.08 ± 2.02 and 114.4 ±1.2 respectively). Lowest phenolic and flavonoid content was seen in Hexane extracts (97.90 ± 6.45 and 92.13 ± 1.006 respectively).

These results clearly show that the solvent influences the extractability of the phenolic compounds. The phenolic extracts of plants are always a mixture of different classes of phenols, which are selectively soluble in the solvents. The use of an alcoholic solution provides satisfactory results for the extraction process [39]. The use of mixture alcohol and water present the advantage of modulating the polarity of alcohol solvents, also adding that solubility of polyphenols depends mainly on the hydroxyl groups, the molecular size and the length of hydrocarbon [40]. Hydro-alcoholic solvents are the best solvents for extraction of phenolic compounds from *Thymus satureioïdes* plant. Ethyl acetate and Hexane are inefficient solvents for extraction of total phenols from plant part studied.

Our results are almost similar to those reported by Roby *et al*., for *Thymus vulgaris L.* from Egypt [41], the lower polarity solvents, particularlyhexane and diethyl ether showed much lower ability in extracting the phenolic compounds as compared to the polar solvents.

3.4 Antioxidant activity

Antioxidant activity of the antioxidants is concerning with those compounds capable of protecting the organism system against the potential harmful effect of oxidative stress [42]. In this study, the antioxidant capacity of extracts from *Thymus satureioïdes* was accessed by three different assays: Ferric Reducing Antioxidant Power (FRAP), DPPH scavenging activity and ABTS assay. IC50 of ABTS and DPPH scavenging activities of each extracts were compared to IC50 of Ascorbic Acid.

Table 4: Antioxidant activity of *Thymus satureioïdes* extracts

Extracts	DPPH (IC_{50} µg/ml)	ABTS (IC_{50} µg/ml)	FRAP (mg equivalent Ascorbic acid/g of extract)
Hexane	275.71 ± 11.26	127.38 ± 3.83	97.819 ± 0.377
Dichloromethane	8.18 ± 0.07	80.09 ± 0.65	153.457 ± 0.247
Ethyl Acetate	23.75 ± 0.67	85.16 ± 3.22	123.004 ± 0.377
Water-Ethanol	3.86 ± 0.07	51.27 ± 0.82	233.292 ± 0.377
Ascorbic Acid	1.27 ± 0.01	25.29 ± 0.27	-

3.4.1 DPPH scavenging activity

The DPPH method was evidently introduced nearly 50 years ago by Blois [43] and it is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant capacity. The parameter IC50, is used for the interpretation of the results from the DPPH method and is defined as the concentration of substrate that causes 50% loss of the DPPH activity. Some plant extracts and essential oils were examined in relation to their IC50 value, while others were tested for their antioxidant capacity.

Table 4 showed the DPPH radical scavenging activity of the extracts of *Thymus satureioïdes*. DPPH is a stable free radical which is reduced in the presence of hydrogen donating antioxidants. The scavenging ability of different solvents extracts of *Thymus satureioïdes* for free radicals of 1, 1-diphenyl 1-2-picrylhydrazyl (DPPH) showed remarkable scavenging activities. Hydro-ethanolic extract showed the highest scavenging activity (lowest IC50; 3.86 ± 0.07 µg/ml) followed by Dichloromethane extracts (IC50; 8.18 ± 0.07 µg/ml). The lowest activity was found in hexane extract (275.71 ± 11.26 µg/ml). Phenolic compounds are hydrogen donating antioxidants, thus higher radical scavenging activity of hydro-alcoholic extract may be attributed to higher amount of hydrogen donating phenolic antioxidants in ethanol extract. Ramchoun *et al* [11] have found for the same plant from Tafilalet Region an IC50 of 0.48 mg/mL. Same result was found by Roby *et al* for *Thymus vulgaris* [41], who conclude that the antioxidant activities of plant extracts expressed as antiradical power (ARP) are affected by solvents used for extraction.

3.4.2 ABTS assay

ABTS assay is better to assess the antiradical capacity of both hydrophilic and lipophilic antioxidant because it can be used in both organic and aqueous solvent system as compared to other antioxidant assay. This method is based on the ability of antioxidants to reduce the ABTS radical cation [44]. In the present work, different solvent extracts of *Thymus satureioïdes* were evaluated for their ABTS radical cation scavenging activity. Ascorbic acid was used as standard and its IC50 values was 25.29 ug/ml. IC50 values ranged from 51.27 to 127.38 ug/ml. Hydro-ethanolic extract showed good ABTS radical cation scavenging activity with IC 50 values of 51.27 ug/ml. Ethyl acetate and dichloromethane extracts showed moderate activity and its IC 50 values were 80.09 and 85.16 ug/ml respectively. Hexane extract showed poor ABTS radical cation scavenging activity with IC 50 values of 127.38 ug/ml. Moderate to weak antioxidant activity by ABTS method was shown by some medicinal plant extracts [45].

3.4.3 Ferric Reducing Antioxidant Power (FRAP)

The antioxidant compounds are responsible for the reduction of ferric (Fe^{3+}) form to ferrous (Fe^{2+}) form. The addition of $FeCl_3$ to the ferrous form led to the formation of blue colored complex. So the reduction ability can be determined by measuring the colored complex at 700 nm [46]. The reducing properties associated with the presence of compounds exert their action by breaking the free radical chain through donating a hydrogen atom [47]. hydroethanolic extracts of *Thymus satureioïdes* showed greater FRAP value as 233.292 (mg equivalent of ascorbic acid /g of extract). The other extracts dichloromethane, ethyl acetate, and Hexane showed FRAP value 153.457, 123.004 and 97.819 (mg equivalent of ascorbic acid /g of extract) respectively. The ability of extract to reduce iron (FRAP) suggests that they contain compounds that are electron donors, which can react with free radicals to convert them to more stable products and terminate radical chain reaction. FRAP assay showed positive correlation between reducing power and phenolic content in *Thymus satureioïdes* extracts (Table 3). So these compounds are phenolic

compounds. It was reported by Rice-Evans et al. [47] that phenolic compounds have redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. The redox potential of phenolic compounds played an important role in determining the antioxidant potential.

Conclusion

Based on the results obtained in the present study, it is concluded that the hydroethanolic extracts of *Thymus satureioïdes* exhibit considerable antioxidant radical scavenging activity on all tested assays (DPPH, ABTS and FRAP) and they possess substantial amounts of phenolic compounds. Thus, *Thymus satureioïdes* can be considered as good source of antioxidants which might be beneficial for combating oxidative stress.

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