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Studies on Chemical Composition, Phenolic contents and Antioxidant Activities of three Thymus Essential Oils from Morocco

L. El Hattabi[a], N. El Madani[c], Z. Charrouf[c], J. Costa[b], J. M. Desjobert[b] and M. Tabyaoui[a*]

[a]*Laboratoire des Matériaux, Nanotechnologies et Environnement, Université Mohammed V, Faculté des Sciences, Rabat, Maroc*
[b]*Laboratoire de Chimie des Produits Naturels, Faculté des Sciences et techniques, Université de Corse, UMR CNRS 6134, Corse, France*
[c]*Laboratoire de Chimie des Plantes et Synthèse Organique et Bio-Organique, Faculté des Sciences, Université Mohammed V, Rabat, Maroc*

ABSTRACT

The thymus belongs to the family Lamiaceae, commonly used as spice and medicinal herb, recognized by several pharmacological properties, such as antispasmodic, antiseptic, antibacterial and many other bioactivities. The chemical composition and the antioxidant activity of thymus's essential oils of three different Moroccan regions were studied. The essential oils were identified by chromatographic analysis (GC and GC/MS). Indeed, in essential oils of Thymus vulgaris and Thymus satureoides the main compound determined was the Carvacrol he represents 78.4% and 49.3% respectively. The quantitative determinations of total phenolics, total flavonoids, and various antioxidant activities (1,1-Diphenyl-l-2-picryl-hydrazyl radical and 2,2-azinobis 3 ethylbenz-thiazoline sulfonate) of plants extracts have carried out using colorimetric methods. The total phenolic content, expressed as µg of gallic acid equivalent (GAE) per mg of extract, was found varied between 121,44 and 143,17 µg GAE/mg and the IC50 values of 1,1-Dipheny l-2-picryl-hydrazyl radical (DPPH) free radical scavenging activity between 5,141 and 5,749 µg/mL. This study indicated the three oils of thymus possessed high antioxidant properties, therefore it can be considered as a bioresource of phenolic and source of natural antioxidant.

Keywords: Thymus vulgaris, Essential oils, ABTS, Antioxidant activity, 1,1-Dipheny l-2-picryl-hydrazyl radical.

INTRODUCTION

Plants such as vegetables, fruit and spices medicinal herbs..., have been used to cure many diseases since ancient time. Today in this modern world, even though synthetic drugs are readily available and highly effective in curing various diseases, there are people who still prefer using traditional folk medicines because of their less harmful effects. There is a wide diversity of compounds, especially secondary metabolites, found and isolated from plants and studies have shown that these compounds have anticancer [1,2], antibacterial [3,4], analgesic [5], anti-inflammatory [6], antitumor [7], antiviral [8] and many other activities to a greater or lesser extent. Antioxidants are significant regarding reducing oxidative stress which could affect and damage biological molecules [9]. Synthetic antioxidants such as butylated hydroxyl anisole (BHA), propyl gallate (PG), butylated hydroxyl toluene (BHT) which have been used to prevent oxidation have been found to cause internal and external bleeding in rats and guinea pigs at high dose [10,11]. Attention is therefore turned to the use of natural antioxidants such as bioactive flavonoids and polyohenols which are of great importance due to their high efficiency at trapping free radicals. Antioxidants such as flavonoids, phenolics, terpenoids, flavonols, proanthocyanidins and tannins are found in various plant products. The essential oils of thyme are widely used as antiseptics in various pharmaceutical fields and as flavoring for many types of food [12]. Several species of thymus have already proved their antibacterial, antifungal, antioxidant and other pharmacological activities [13-18]. In Morocco, the kind Thymus (family:

lamiaceae), is represented by 21 species of which 12 are endemic [19], the Mediterranean region is the center of this kind [20]. At present time, this plant is cultivated in large scale in Morocco. Evidently Thymus vulgaris continues to command a very important place in expanding world market. The composition of the essential oils varies strongly between thym plants. In recent years, a wide range of spectrophotometric assays has been adopted to measure antioxidant capacity of foods, the most popular being 2,2 0 -azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, among others such as oxygen radical absorbance capacity (ORAC) and ferric reducing ability of plasma (FRAP) assay [21-26]. The aims of the present paper are characterisation the chemical composition of essential oils of Thymus vulgaris from Morocco (Tafraout and Errachidia) and Thymus satureioides (Marrakech). The antioxidant activities were also determined by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity and Radical cation ABTS + scavenging activity methods. Furthermore, the evaluation purpose extended was use the thymus spice as a potential source of antioxidant compounds.

MATERIALS AND METHODS

Plant material

Plants of Thymus were collected in June 2014 from three region of Morocco: Tafraout, Marrakech and Errachidia. The plants were deposited at the Laboratory of the Scientific Institute of Plants in Rabat, Morocco. They were then ground to a powder with a special grinder and kept at room temperature until use.

Methanolic extract

A methanol extract of thymus (vulgaris and satureioides) was prepared by soxhlet apparatus (6 h extraction). The solvent was evaporated under reduced pressure at 45 °C using rotary evaporator (Heidolph G1, Germany). Prepared extracts were stored at -4 °C until further analysed. The experiment was performed at triplicate.

Essential oil extraction

The extraction of essential oils was carried out by steam distillation in a Clevenger-type apparatus [27]. Three distillations were carried out by boiling for 4 h, 200 g of fresh plant material with 800ml of water in a 1.5 l flask connected to a condenser. The essential oil yield was determined from the dry matter evaluated from three samples of 30 g dried for 48 hours in an oven at 50 ° C. The essential oil was stored at - 4 ° C in the dark in the presence of anhydrous sodium sulfate. It is diluted in methanol (1%, v/v) prior to GC analysis (gas chromatography) and GC/MS (gas chromatography coupled to mass spectrometry)

Gas chromatography analysis (GC-FID)

GC analysis was carried out using a Perkin-Elmer Autosystem XL GC apparatus (Waltham, MA, USA) equipped with a dual flame ionization detection (FID) system and the fused-silica capillary columns (60m*0.22mm I.D., film thickness 0.25µm) Rtx-1 (polydimethylsiloxane) and Rtx-wax (polyethyleneglycol). The oven temperature was programmed from 60 °C to 230 °C at 2 °C/min and then held isothermally at 230 °C for 35 min. Injector and detector temperatures were maintained at 280 °C. Samples were injected in the split mode (1/50) using helium as a carrier gas (1 mL/min) and a 0.2 µL injection volume of pure oil. Retention indices (RI) of compounds were determined relative to the retention times of a series of n-alkanes (C5–C30) (Restek, Lisses, France) with linear interpolation using the Van den Dool and Kratz equation [28] and software from Perkin-Elmer.

Gas chromatography mass spectrometry (GC-MS)

Samples were analyzed with a Perkin-Elmer turbo mass detector (quadrupole) coupled to a Perkin-Elmer Autosystem XL equipped with the fused-silica capillary columns Rtx-1 and Rtx-wax. Carrier gas: helium (1 mL/min), ion source temperature: 150 °C, oven temperature programmed from 60 °C to 230 °C at 2 °C/min and then held isothermally at 230 °C (35 min), injector temperature: 280 °C, energy ionization: 70 eV, electron ionization mass spectra were acquired over the mass range 35– 350 Da, split: 1/80, injection volume: 0.2 µL of pure oil.

Components identification

The identification of the essential oil constituents was based on: (i) comparison with the mass spectra of authentic reference compounds where possible and by reference to WILEY275,NIST 02 and Adams mass spectral libraries [29-32] (ii) comparison of their retention index (RI), calculated relative to the retention times of a series of C-5 to C-30 n-alkanes, with linear interpolation, with those of our own library of authentic compounds or literature data [32,33].

Determination of total phenolic content

The amount of total phenolics in methanolic plant extracts was determined with the Folin-Ciocalteau reagent using the method of Spanos and Wrolstad [34], as modified by Lister and Wilson [35]. 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 765nm 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 (0-300 mg/l). The concentration of total phenolic compounds in the extracts was determined as mg of gallic acid equivalent using an equation obtained from the standard gallic acid graph, and expressed as mg gallic acid/g of extract (mg GAE/g extract). The data were presented as the average of triplicate analyses.

Determination of total flavonoid content

Total flavonoids were measured by a colorimetric assay according to Dewanto V, [36] with some modification. One millilitre of dissolved sample (0.5g dried product in 50ml 80% aqueous methanol) was placed in a 10 ml volumetric flask. Distilled water was added to obtain a total volume of 5 ml and then 0.3 ml of $NaNO_2$ (50 $g{\cdot}L^{-1}$) was added. About 0.3 ml of $AlCl_3$ (100 $g{\cdot}L^{-1}$) was added after 5 min, the mixture was allowed to stand for another 6 min. About 2 ml of 1 M NaOH was added and the total volume was increased to 10 ml with distilled water. The mixture was allowed to react for 15 min and the absorbance was measured against prepared reagent blank at 510 nm. The amount of the total flavonoids was expressed as rutin equivalents (mg rutin/g sample) through the calibration curve of rutin. All tests were performed in triplicate

Antioxidant activity evaluation

The antioxidant activity of Thymus extracts from all regions was assessed using free radical-scavenging activity (RSA) with DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) radical assay.

DPPH radical scavenging activity

Oils were dissolved in methanol to get 500 µg/ml stock solutions separately, lower concentrations (5, 10, 15, 50, 75, 100 µg/ml) of oils were prepared by serially diluting stock solutions. Ascorbic acid was weighed (50 mg each) and dissolved in 100 ml of methanol to get 500 µg/ml stock solutions separately, lower concentrations of ascorbic acid (2, 5, 10, 15, 50, 75, 100 µg/ml respectively) were prepared by serially diluting stock solutions.

The stable DPPH radical was used for determination of free radical-scavenging activity of the oils; the tests were carried out as described by Mensor LI [37], with some modification. The 0.2 mM solution of DPPH in methanol (7.8 mg in 100 ml) was freshly prepared and 0.5 ml of this solution was added to 2.5 ml of different concentrations of oils prepared (5 - 100 µg/ml) and allowed to react at room temperature in the dark for 30 min. the absorbance was read at 517 nm against blank samples. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. BHT was used as a positive control and all measurements were done in triplicate. The radical-scavenging activity (RSA) or the percentage of inhibition of the extract was calculated using the following equation [38]:

$$\% \text{ RSA} = [(AD - AE)/ AD]*100$$

Where AD = absorbance of DPPH alone;
AE = absorbance of DPPH in the presence of various oils;

IC50 value was determined from the plotted graph of scavenging activity against the different concentrations of thymus oils, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. BHT was used as reference compound. The antioxidant activity was expressed as the antioxidant efficiency (EA) [39]: calculated as follows:

$$\text{EA} = \frac{1}{\text{IC50}}$$

ABTS radical scavenging assay

The scavenging activity of oil against ABTS radical was determined by following the method described by Roberta et al. [40]. 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 methanol 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 oil with different concentration (5 – 100 µg/ml) and the reaction mixture was vortexed and absorbance was measured at 734 nm after 1 min. The same was done for the Trolox standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) of various concentrations (2 – 100 µg/ml). The percentage inhibition of ABTS+ by the oil was calculated and compared with Trolox. The percentage inhibition of $ABTS^{\cdot+}$ by the oil was calculated using the equation:

ABTS radical scavenging activity (%): = (1- Abs_{sample}/ $Abs_{control}$) * 100

Where $Abs_{control}$ is the absorbance of the control ($ABTS^{\cdot+}$ solution without test sample)
Abs_{sample} is the absorbance of the test sample ($ABTS^{\cdot+}$ solution with oil).

RESULTS AND DISCUSSION

Chemical composition of Essential oil

The essential oil isolated by hydrodistillation of the whole plant of thymus vulgaris was purple yellow oil; determination of the percentage composition of the samples was based on peak area normalization without using correction factors. As shown in table 1. Thirty-one compounds were detected representing 96% of the total oil. The major compounds in T.satureioides were carvacrol (49,3%), Borneol (10,2%), p-Cymene (6%), Linalol (5,7%) and ϒ-Terpinene (5%). Other constituents (≥ 3.5 %) were Camphene, α-Pinene, E-Caryophyllene. These results are near to that of Bouzidi which carvacrol content (26.5%), followed of borneol (20,1%) reported the abundant compounds in T.satureioides [41]. However, in our study, Carvacrol was present at higher percentage of 49,3 %, whereas borneol only reached 10,2 % of the total essential oil.

Table1: Chemical compositions (%) of essential oils of T. vulgaris and T.satureioides (GC and GC-MS analysis)

	Thymus vulgaris			Thymus satureioides		
Components	**Ir apol**	**Ir pol**	**HE% apol**	**Ir apol**	**Ir pol**	**HE% apol**
Tricyclene	-	-	-	921	1012	0,2
a-Thujene	923	1023	0,1	923	1025	0,9
α-Pinene	931	1023	0,4	931	1025	2,4
Camphene	944	1068	0,1	945	1070	3,5
1-Octen-3-ol	961	1441	0,2	-	-	-
Octan-3-one	964	1248	0,2	-	-	-
β-Pinene	971	1111	0,1	971	1111	0,5
Octan-3-ol	979	1384	0,3	979	1384	0,5
Myrcene	981	1159	0,2	982	1159	1,3
α-Terpinene	1010	1179	0,3	1010	1179	1,0
p-Cymene	1014	1268	4,6	1014	1268	6,0
1,8-Cineole	1022	1209	0,2	1022	1209	0,3
Limonene		1200	0,4	1022	1200	0,5
Y-Terpinene	1049	1243	0,5	1050	1243	5,0
trans-Hydrate sabinene	1053	1455	0,1	1054	1455	0,2
Terpinolene	-	-	-	1080	1281	0,1
Linalol	1084	1538	0,6	1086	1538	5,7
Camphre	1122	1506	0,2	1122	1506	0,1
trans Pinocarveol	1124	1639	0,3	-	-	-
Borneol	1150	1688	0,8	1153	1690	10,2
Terpinen-4-ol	1162	1595	0,7	1163	1595	1,2
a-Terpineol	-	-	-	1174	1693	1,5
Carvacrylmethylether	1226	1597	0,3	-	-	-
Carvacrol	1286	2193	78,4	1286	2193	49,3
E-Caryophyllene	1418	1592	3,1	1418	1592	4,6
Caryophyllene oxyde	-	-	-	1570	1967	1,2
Aromadendrene	1437	1602	0,9	-	-	-
Alloaromadendrene	1457	1643	0,2	-	-	-
Ledene	1491	1684	0,6	-	-	-
Spathulenol	1563	2107	0,7	-	-	-
Caryophyllene oxyde	1569	1970	0,6	-	-	-
m-Camphorene	1964	2524	0,3	-	-	-
p-Camphorene	1999	2549	0,3	-	-	-
Total amount of compound	95,7			96		

Ir apol = retention indices on the apolar column (Rtx-1)
Ir pol = retention indices on the polar column (Rtx-Wax)
HE% apol = Relative percentages of components (%) are calculated on GC peak areas on the apolar column

The major constituents the essential oil of T.vulgaris are Carvacrol (78.4%), p-cymene (4.6%) and E-Caryophyllene (3.1%). These results are in agreement with that found by M.Boukhatem et al, reported the majority compounds are carvacrol (83.8%) and p-cymene (8.15%) [42]. Moreover, other results are in total contradiction with ours [43,44]. For example Shazia Shabnum indicates that the essential oil of T.vulgaris has a high rate in thymol (46.2%), γ-terpinene (14.1%), β-cymene (9.9%), linalool (4.0%), myrcene (93.5%), α-Pinene (3%) and α -thujene (2.8%) [45]. Zambonelli was raported thymol (22-38%), γ-terpinene and p-cymene [46].

Total phenolic and flavonoids contents determination

The concentration of total phenols was determined in the three methanolique extracts of Thymus plants. In Figure 2, the results of the colorimetric analysis are given; they were derived from the absorbance values of the extracts solutions compared to the standard solutions of gallic acid equivalents. The total polyphenol contents (TPC) of the three extracts from Marrekch, Tafrout and Errachidia are: 121,32; 138,75 and 143,17 mg gallic acid equivalents (GAE)/g of extract respectively. This values are relatives to the values reported in the literature for other Thymus species such as Thymus spathulifolius (141 mg/g of the polar subfraction of a methanol extract, reported by [47] and Thymus serpyllum (113 mg/g of an ethanol extract, reported by [48], T.vulgaris from Southern Italy (165.1 mg GAE/g) reported by [49] and T. caramanicus (124.30 ± 2.62 mg/g) reported by [50]

This level of total phenols were found to be higher than the values reported in the literature for Thymus species such as T.vulgaris harvested in Iran (5.82±0.42 mg gallic acid (GAE)/g) reported by [51] and T.satureioides collected from Rich in eastern High Atlas of Morocco (48.43 mg eqAG/g) reported by [52]

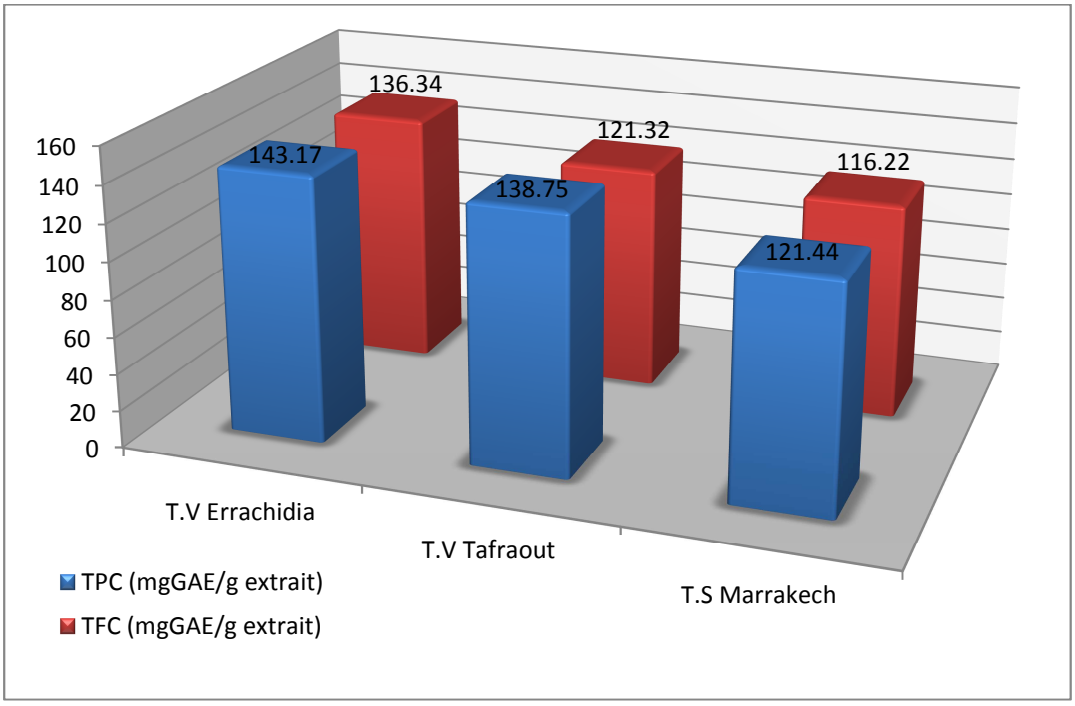

Figure 2: Phenolic and flavonoids contents in the studied three plant extracts

The result of total flavonoid contents of the three methanolique extracts of genus Thymus is given in Figure 2. The total flavonoid contents in the different extracts varied from 136,34; 121,32 and 116,22 mg rutin equivalent/g of extract. Among the three extracts, T.vulgaris from Errachidia contained the highest (136,22 mg/g) amount of flavonoids followed by T.vulgaris from Tafraout (121,32 mg/g) and T.saturioides from Marrekch (116.22 mg/g). These values were lower than those reported by [51], which found the total flavonoid content 186.93 ± 25.19 mg eq rutin/g and that of [53] (172.79 ± 2.12) mg rutin equivalent/g but higher than those reported by [54] 54.28 ± 1.6 mg RE/g DW. These variations in TPC and TFC could be related to various factors. One such factor may be the difference of genetic potential of individual cultivars for polyphenol biosynthesis [55]. Maturity, season, geographic origin, fertilizer, soil type, storage conditions and amount of sunlight received may also be critical in this respect [56].

Determination of antioxidant activity with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method.

The antioxidant activity of the different thymus oils was evaluated using methanolic solution of stable free radical, DPPH. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free radical attack on the DPPH molecule) and convert them to a yellow-colored product (i.e., 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance [57]. Figure 2 depicts DPPH radical scavenging activities (%) of thymus essential oils from: Errachidia, Tafraout and Marrakech (BHT witness: antioxidant reference). As shown in figure 2, all oils obtained from Thymus species showed antioxidant activity in these assay. Comparing data, it appears that the domestication did not greatly affect the

antioxidant property of the Thymus species studied, since the values did not differ significantly between thyme oils. T. Vulgaris from Errachidia had the important antioxidant activity, whit an inhibition reactively of (96.94 ± 0.11) %, similar to that of BHT with a maximal inhibition percentage of (96.99 ± 0.08)% at the same concentration (figure 2).

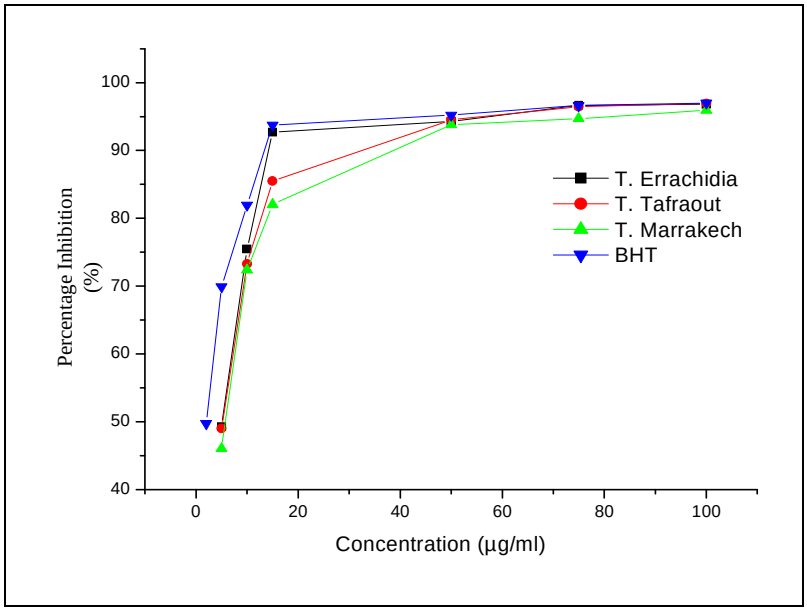

Figure 2: DPPH radical scavenging activities (%) of oils of thymus from Errachidia, Tafraout and Marrakech. BHT was used as positive control

Concentrations which provide 50% inhibition (IC50) were calculated from the curve of Figure 5 and are shown in Table 2. The values of IC50 calculated for Thymus oils confirmed the reactivity of these samples against DPPH. The most potent oil was obtained from T.vulgaris from Errachidia (IC50 = 5,141 µg/mL), but it was less potent than the pure compound used as positive control, namely the synthetic antioxidant BHT (IC50 = 2,041µg/mL) (table 2).

Another parameter that expresses anti-radical power was calculated from the first parameter (IC50) which is noted "EA" (Efficacity antiradical, equal to 1/IC50). EA values of all essential oils are significant. Moreover, these values are larger, more anti-radical power is high (Table1).

Table 2: IC50 and EA values of different thymus essential oils and BHT

	IC_{50} (µg/ml)	EA
Thym Errachidia	5,141	0,194
Thym Tafraout	5,204	0,192
Thym Marrakech	5,749	0,174
BHT	2,041	0,490

Indeed, the antioxidant activity of three thymus is very superior to that of essence T.spathulifolius (IC50 = 243.2 g/ml; [47]), the essence of T.caramanicus (IC50 = 263.09 µg/ml; [50]), the essential oil of T.zygis (IC50 = 75.97 ± 0.86 µg/ml [58]) and essential oil of T.bleicherianus (IC50 = 77.8 µg/ml; [59]). These results show that three essential oils of Thymus show an antioxidant activity very interesting.

Radical cation ABTS + scavenging activity

The ABTS radical is generated by the reaction of ABTS and potassium persulphate when an antioxidant is added, ABTS is converted to a non radical form. The ABTS method gives a measure of the antioxidant activity of oil by determining the reduction of the radical cation as the percentage of inhibition of absorbance at 734 nm. All of three oils were found to exhibit strong ABTS scavenging activities, and their activities were in concentration dependent manner, as shown in figure 3, this figure depicts a steady increase in the ABTS radical scavenging capacity of essential oils from T.Errachidia, T.Tafraout and T. Marrakech up to a concentration of 100 µl/ml (Fig. 3).

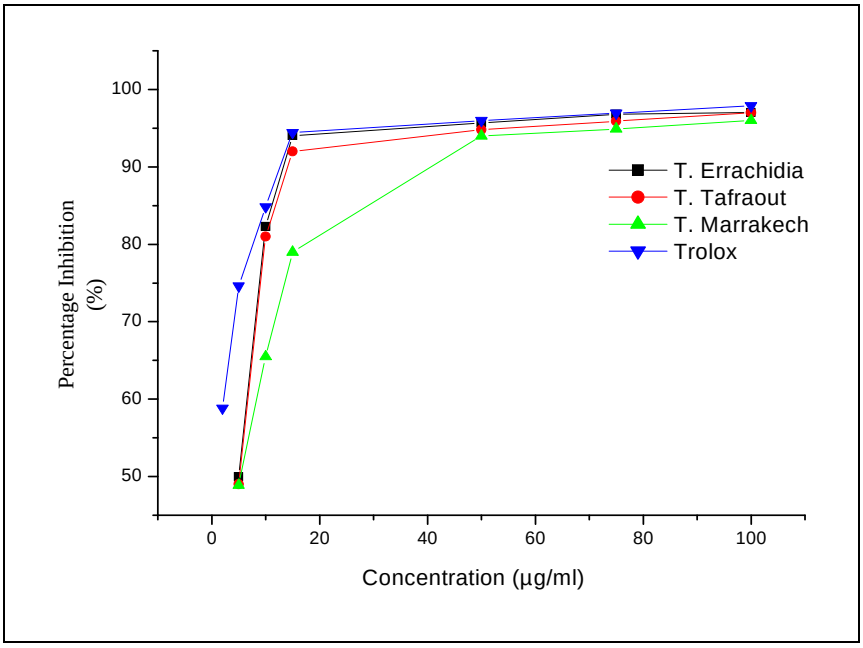

Figure 3: ABTS Radical scavenging activity of oils with different concentrations

The experimental data revealed that antioxidant activity of oils is proportional to phenolic compounds concentration. Table 3 presents the IC50 values of the studied oils; the highest reductive capacity resided with the oil obtained from Errachidia (IC50= 5,001 µg/mL). The oils from Tafraout and Marrakech exhibited lower scavenging activity with no grand difference between them (IC50= 5,156 µg/mL; IC50= 5,335 µg/mL), respectively. Trolox was used as a standard antioxidant. In this study, the IC50 values of the studied oils were less than the value of the reference antioxidant Trolox (IC50= 0.321 µg/mL). The efficacy antiradical (EA) values of three thymus in comparison with Trolox are significant.

Table 3: IC50 and EA values of different thymus essential oils and Trolox

	IC_{50} (µg/ml)	EA
Thym Errachidia	5,001	0,200
Thym Tafraout	5,156	0,194
Thym Marrakech	5,335	0,187
Trolox	0,321	3.115

Based on the above results, it appears that there was a very positive correlation between antioxidant activity of the oils and their content of the phenol, carvacrol. These results are consistent with the close relationship between carvacrol content and high antioxidant potential reported by many authors [60,47,61,62]. The high content of carvacrol in T.vulgaris and T.satureioides oils therefore likely explains their strong antioxidant activity compared other oils such as T.ciliatus, T.pallidus and T.broussonetii oils, which contained lower levels of this compound [62]. Monoterpene hydrocarbons, particularly Ɣ-Terpinene is also characterized by strong antioxidant activity which may be higher than that of phenols. The presence of strongly activated methylene groups in this molecule is probably the reason for this property [61,63]. The content of borneol does not appear to correlate with the antioxidant activity of T. satureioide oil studied. This result supports the idea that this volatile compound has a lower antioxidant activity than that of carvacrol [64,65]. In general, the antioxidant activity of essential oils is the product of additive and/or synergistic effects, as they are complex mixtures of several classes of compounds.

CONCLUSION

The essential oils of three Moroccan thymus populations sampled (T.vulgaris from Errachidia, T.vulgaris from Tafraout and T.satureioides from Marrakech) in different context climatic have been investigated and compared for their chemical composition. Carvacrol was found as a major compound (78.4 and 49.3%) in T.vulgaris oils and T.satureioides respectively, followed by borneol and p-cymene. Significant quantitative chemical variability was noted, with carvacrol levels being higher in the T.vulgaris from Errachidia and Tafraout (arid site). Moreover, this study focused on the phenolic fraction and the antioxydant activity of essential oils: T.V from Errachidia, T.V from Tafraout and T.S from Marrakech. The result of the present study suggests these plants can be a source of

antioxidants. It may be a potential used in many fields, such as natural preservatives of food, cosmetics, pharmaceutical fields and pharmacological preparations which is very well evidenced by the present work.

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