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Antioxidant activities, total phenol an flavonoid contents of two *Teucrium polium* subspecies extracts

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Abstract

The soxhlet extracts of two subspecies from Moroccan *Teucrium polium* were examined for their antioxidant activities, total polyphenol and total flavonoids contents. The Antioxidant activities was evaluated *in vitro* spectrophotometrically using four methods, such as $ABTS^{+\bullet}$, 1,1-diphenyl-2-picrylhydrzyl ($DPPH^{\bullet}$), ferric reducing antioxidant power and phosphomolybdenum assay. Total polyphenol and flavonoid contents were measured using Folin-Ciocalteu test and aluminum chloride colorimetric method, respectively. The total phenol contents, flavonoids contents and antioxidant activities of *T. polium subsp. polium* extracts were higher than *T. polium subsp. aurum*. The phenols contents ranged from 112.27 to 4.38 mg GA E/ g dry extract. The total flavonoids varied between 174.57 and 40.42 mg RE/ g dry weight. The extract showed significant scavenging activity of $ABTS^{+\bullet}$ and $DPPH^{\bullet}$, with IC_{50} values ranging from 0.38 to 5 mg/ml and from 0.23 to 4.02 mg/ml, respectively. Moreover all extracts showed a good ferric reducing antioxidant power with EC_{50} values varying between 0.19 and 3.94 mg/ml. The total antioxidant capacity assay revealed that the water extract of *T. polium subsp. aurum* had a high activity with a value of 220 mg VitCE/g dry weight. The ethyl acetate extract has a weak antioxidant activity in the four tests. These results shows that Moroccan *T. polium* subspecies are a rich source of phenols and natural antioxidant compounds, which can be used as a natural food preservative.

Keywords: *T. polium; phenolic compounds; flavonoids; antioxidant activity; DPPH*

1. Introduction

Antioxidants are compounds that eliminate the oxidation of some molecules. Antioxidant activity has been based on neutralizing the oxidative damage resulting from the action of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. These species are mainly, produced in vivo following exposure to exogenous factors or for specific metabolic reasons [2]. Accumulation of these species (ROS and RNS) lead them to react with biological macromolecules such as protein, lipids, nucleic acids and carbohydrates [3]. High levels of certain reactive species can contribute to cell damage and development of many pathologies [4]. Moreover, when a greater imbalance between the production of reactive species and antioxidant defense activity, oxidative stress develops [5]. This imbalance has been associated with numerous diseases [4, 6].Medicinal plants are considered as an important source of active principles with high antioxidant potential. Polyphenols, usually referred to as antioxidant compounds, play a major role in the prevention and protection against many diseases [7]. The genus Teucrium (Lamiaceae family) includes 300 species distributed all over the world, particularly in the Mediterranean basin [8] . It usually develops on hillsides, sands, semi-arid and in arid places [9]. In Morocco folk medicine, Germander (*T. polium*) locally called "Jaada", it is used for the treatment of a variety of diseases, including digestive disorders, liver problems [10] inflammation, hypertension, fever, diabetes, rheumatism, parasitic diseases such as amoebicide [11]. Numerous studies showed therapeutic properties of some Teucrium species such as, anti-inflammatory, anti-cancer [12] antiulcer [13], and anti-allergic [14], antibacterial [15,16], antidiabetic, anti-spasmodic, anti-nociceptive [17] and antioxidant [18] effects.

It was reported that the therapeutic ability of *T. polium* extracts are generally attributed to their proprieties to suppress oxidative processes [19]. It was also reported that the alcoholic extract of *T. polium* possess a suppressing effect on hydrogen peroxide-induced lipid peroxidation in red blood cells [20]. However to our knowledge, no data is available on the antioxidant activity, total polyphenol and flavonoid contents of *T. polium subsp. aurum* species extracts.

The objective of our study was to evaluate, the antioxidant activities of methanol, aqueous ethanol and ethyl acetate extracts of two *Teucrium polium* subspecies which grow in wild habitats in southeast of Morocco.

2. Materials and methods

2.1. Plant samples and reagents

Aerial parts of two *Teucrium polium* subspecies were collected in april, 2015 from the regions of Errachidia (Morroco). Butylated hydroxytoluene (BHT), azino-bis (3- ethylbenzthiazoline-6-sulfonic acid) 2,2-Diphenylpicrylhydrazyl radical (DPPH), ammonium molybdate, aluminum chloride ($AlCl_3$), sodium phosphate, quercitin, vitamin C, rutin, gallic acid, iron III chloride ($FeCl_3$), potassium ferricyanide ($K_3Fe(CN)_6$), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals and solvents used were of analytical grad.

2.2. Soxhlet extraction

The plant material was dried at room temperature and powdered (5 g) and extracted with 100 ml of solvent (water, methanol, ethanol and ethyl acetate) using a Soxhlet extractor. The extracts were filtered and to dryness under vacuum at 40°C using Rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C.

2.3. Statistical analysis

All tests were performed in triplicate and results were expressed as mean ± SD. The results were compared by one-way ANOVA followed by Tuckey-test, using the GraphPad Prism 5 (Microsoft Software). Differences at $P < 0.05$ were considered significant.

2.4. Determination of total phenolic content

Total phenolic content of the extracts was determined by Folin-Ciocalteu method [21]. The 0.5 mL of a known dilution of the extract and 2 ml of 7% sodium carbonate solution were added to 2.5 mL of 10% (v/v) Folin-Ciocalteau reagent. The absorbance was read at 760 nm (Jasco v-530) after 2H of reaction at room temperature in the dark. Gallic acid was used as standard for the construction of calibration curve. Total phenol contents were expressed as milligrams of gallic acid equivalents per gram dry weight of extract (mg GAE/g DW).

2.5. Determination of total flavonoids contents

Flavonoids are considered as the most important natural phenols. They have a broad spectrum of biological and chemical effects including radical scavenging activities. Total flavonoids contents of T. polium subsp. polium extracts were measured by the aluminum chloride colorimetric assay [22]. 1 ml of sample or rutin standard solution was added into a 10 mL volumetric flask containing 4 ml of distilled water. To the flask 0.30 ml 5% $NaNO_2$ was added, after five minutes 0.3 ml 10 % $AlCl_3$ was added to react for 6 min. After that, 2 ml of NaOH (1M) was added and the total was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm (Jasco v-530). Rutin was used as a standard for the construction of calibration curve. Total flavonoids contents were expressed as mg Rutin equivalents per gram dry weight of each extract (mg RE/g DW).

2.6. In vitro antioxidant activities

2.6.1. ABTS radical scavenging activity

ABTS is a green colored radical, usually used to evaluate the antioxidant capacity of natural products. In the present study, $ABTS^{+\bullet}$ radical was generated from oxidation of ABTS by potassium persulfate using the method described by Adedapo et al. [23]. The radical was prepared by mixing equal amounts of 7 mM ABTS and 2.4 mM potassium persulphate. These were later allowed to react for 12 h in the dark at room temperature. The resulting solution (1,5 ml) was further mixed with methanol to obtain an absorbance of 0.7 at 734 nm. Moreover, 1 ml of extract or standard prepared in methanol at different concentrations was added to 1 ml of the $ABTS^{+\bullet}$ methanolic solution. The absorbance of was then measured spectrophotometrically at 734 nm after 7 min. The percentage ABTS scavenging activity of the extract or standard was calculated using the formula:

$$I(\%)= (1-(As/Ac))*100 \qquad (1)$$

Where I (%) is the percentage of inhibition, Ac and As are the absorbencies of the negative control and sample, respectively. Trolox served as positive control. The IC_{50} values were calculated as the concentration of causing a 50% inhibition of ABTS radical.

2.6.2. DPPH radical scavenging activity

DPPH is a stable radical, largely used to determine the antioxidant effect of natural and synthetic compounds The ability of the extracts to scavenge the DPPH radical was measured using the method described by Wu, Chen [24]. 0.1 ml of various concentrations of each extract or standard was added to 1.5 ml of ethanolic solution containing 0.1 mmol of DPPH (2, 2-diphenyl-1 picrylhydrazyl). The absorbance of the mixture was measured at 517 nm with a spectrophotometer (Jasco V-530) after 30 min of incubation time at room temperature in dark. The percentage inhibition was calculated by the following the formula (1). BHT served as positive control. The IC_{50} values were calculated as the concentration of causing a 50% inhibition of DPPH radical.

2.6.3. Ferric Reducing antioxidant power (FRAP)

The reducing power capacity of the tested extracts was determined in accordance with the procedure of Oyaizou [25]. 200 µl of extract was mixed with 500 µl of phosphate buffer (0.2 M, pH 6.6) and 500 µl of potassium ferricyanide [$K_3Fe(CN)_6$] 1%. The obtained solution was incubated at 50°C for 20 min.The mixture was acidified with 500 µl of trichloracetic acid (TCA) 10% which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 500 µl of distilled water and 100 µl of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm (Jasco v-530). Quercitin was used as standard. The results were expressed as EC_{50} (mg/ml). EC_{50} (concentration corresponding 0.5 of absorbance) was calculated by plotting absorbance against the corresponding concentration.

2.6.4. Phosphomolybdenum assay

The assay was based on the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate Mo (V) complex in acid pH [26]. A total volume of 25 µL extract was added to 1 mL of reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The mixtures were incubated at 95 °C for 90 min, and then cooled to room temperature. The absorbance was measured at 695 nm (Jasco v-530). The total antioxidant activity was expressed as the number of equivalence of ascorbic acid (mg Vit C E/g DW).

3. Results

3.1. Total phenolic and flavonoids contents

The distribution of phenolic compounds in *Teucrium polium* subspecies (figure 1) demonstrated that the ethanol and methanol extract from *T. polium subsp. polium* contained the highest amounts 112.27 ± 4.75 mg GAE / g dry weight and 112.04 ± 1.78 mg GAE / g dry weight of extract, respectively. The lowest phenolic content was observed in ethyl acetate *T. polium subsp aurum* extract (4.38 ± 0.12 mg GAE / g DW) (Figure 1). In all extracts (except for water extract), the contents of phenolics were higher in *T. polium subsp. polium than T. polium subsp. aurum*.

Total flavonoid content was determined in comparison with rutin standard and the results were expressed in terms of mg RE/g dry weight extract..

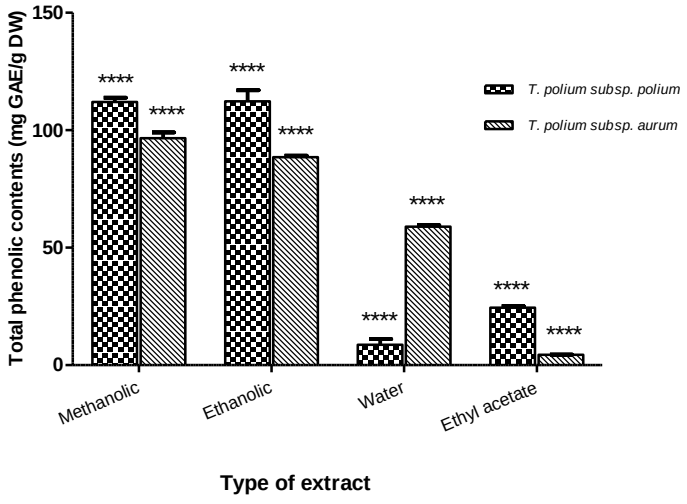

Figure 1. Total phenolic compounds of different extracts from two *Teucrium polium* subspecies. Results were expressed as mg GAE/g dry weight. Each value represents means ± SD of tree experiments. (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001).

As shown in figure 2, the total flavonoid content of two plants extracts ranged from 174.54 to 8.66 mg RE/g. The ethanol extracts of *T. polium subsp. polium* contained significantly a higher concentration of flavonoids (174.54 ± 3.04 mg of RE/g) than the other tested extract. Comparing the flavonoid concentration of *T. polium subsp. polium* and *T. polium subsp. aurum*, all extracts of *T. polium subsp. polium* (except water) had significantly greater concentration of flavonoids than *T. polium subsp. aurum* extract obtained using the same solvent

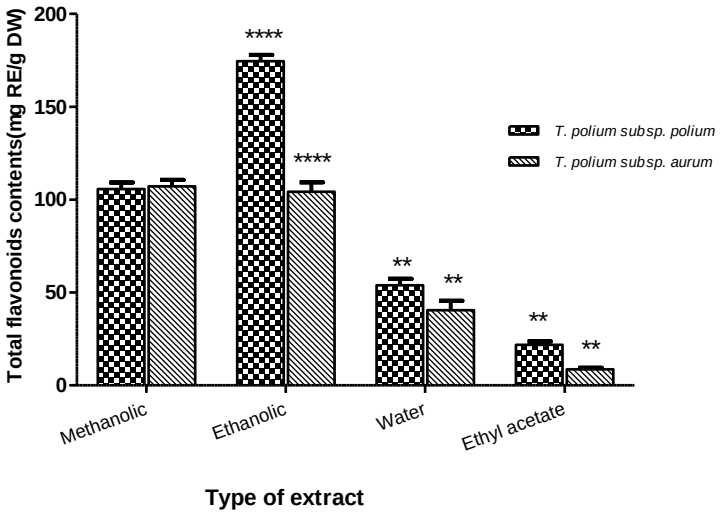

Figure 2. Total flavonoid content of different extracts from two *Teucrium polium* subspecies. Results were expressed as mg RE/g dry weight. Each value represents means ± SD of tree experiments. (*p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001).

3.2. Antioxidant activities

The antioxidant activities of *Teucrium polium* extracts was investigated by $ABTS^{+\bullet}$, $DPPH^{\bullet}$, ferric reducing power, and total antioxidant capacity assays. All extracts showed a noticeable effect which varied significantly among species.

3.2.1. ABTS radical scavenging activity

The $ABTS^{+\bullet}$ radical assay was generated by potassium persulfate in order to determine its hydrogen donating propriety of tested extracts. As depicted in figure 3, the both essential from *T. polium* subspecies extracts presented a potent antiradical effect in a concentration dependent manner. The concentrations of the tested samples (IC_{50}), needed to remove 50% of the initial ABTS radical concentration, are calculated and presented in table 1. The IC_{50} values of all tested extracts through ABTS scavenging activity test ranged from 0.23 to 4.02 mg/ml. The ethanolic and methanolic extracts of *T. polium subsp. polium* were more efficient in the reduction of $ABTS^{+\bullet}$ with IC_{50} values of 0.23 ± 0.029 and 0.3 ± 0.01 mg/ml, respectively than all other extracts. Whereas lowest values were recorded with ethyl acetate extracts in the two subspecies. When compared to the pure reference antioxidant Trolox (0.01 ± 0.002 mg/ml), all the tested extracts showed a significantly lower antioxidant activity ($p < 0.05$).

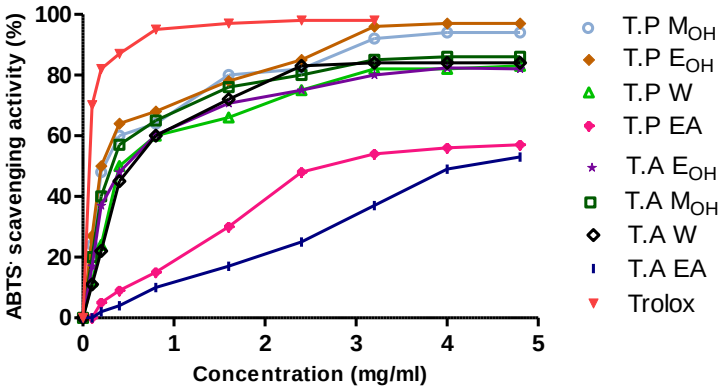

Figure 3: Dose-dependent ABTS radical scavenging activity of the studied extracts. Each point represents the mean of three experiments. T.P: *T. polium subsp polium*; T.A: *T. polium subsp aurum;* M_{OH}: methanol; E_{OH}: ethanol; W: water; EA: ethyl acetate.

Table 1. ABTS radical scavenging activity (mg/ml) different extracts from two Teucrium polium subspecies compared to that of Trolox (IC_{50} = 0.01 ± 0.002). Values are given as mean ± SD (n=3). The extracts of the same solvent and Rutin are significantly different by the Tuckey-test (P<0.05)

Type of extract	Methanolic	Ethanolic	Water	Ethyl acetate
T. polium subsp. polium	0.3 ± 0.01	0.23 ± 0.029	0.42 ± 0.022	2.70 ± 0.31
T. polium subsp. aurum	0.33 ± 0.013	0.38 ± 0.003	0.5 ± 0.04	4.02 ± 1.06

3.2.2. DPPH radical scavenging activity

Free radical scavenging capacity were defined as the concentration of antioxidant necessary to reduce the initial DPPH radical concentration by 50% (IC50). The highest antioxidant activity was indicated by the lowest IC50 value. Results in table 2 shows DPPH radical scavenging activity of *Teucrium polium* and *Teucrium arum* extracts. In general, all extracts inhibited the DPPH radical as follows: methanol > ethanol > ethyl acetate. These results clearly indicate that *T. polium subsp. polium* extracts had higher activity than the *T. polium subsp. aurum* extract in comparison to the same solvent extracts. We found that, the methanol extract from *T. polium subsp. polium* had the greatest radical scavenging capacity in all samples tested with IC_{50} value of 0.38 ± 0,06 mg/ml, followed by ethanol (0.39 ± 0.04 mg/ml) then water (0.49 ± 0.01 mg/ml) and ethyl acetate 3.50 ± 0.17 mg/ml. With *T. polium subsp. aurum*, we found also that the methanol extract had the greatest capacity with an IC_{50} value of 0.46 ± 0.007 mg/ml followed by water extract (0.49 ± 0.01mg/ml), then ethanolic extract (0.51 ± 0.04 mg/ml). However, when compared to the pure reference antioxidant BHT (0.11 ± 0.0001mg/ml), all the tested extracts showed a significantly lower antioxidant activity ($p < 0.05$).

Table 2. DPPH radical scavenging activity (mg/ml) of different extracts from two *Teucrium polium* subspecies compared to that of BHT (IC_{50} = 0.118 ± 0.0001). Values are given as mean ± SD (n=3). The extracts of the same solvent and BHT are significantly different by the Tuckey-test (P<0.05)

Type of extract	Methanolic	Ethanolic	Water	Ethyl acetate
T. polium subsp. polium	0.388 ± 0.06	0.397 ± 0.042	0.459 ± 0.012	3.502 ± 0.179
T. polium subsp. aurum	0.469 ± 0.007	0.512 ± 0.046	0.491 ± 0.011	5.003 ± 0.921

3.2.3.Ferric reducing antioxidant power

The reducing power of investigated extracts were evaluated by the FRAP assay. The reductive capacity is generally associated with the presence of antioxidant agents which exert its effect by breaking the free radical chains via hydrogen atom donation [27]. Therefore, the reducing power assay is often used to evaluate the ability of extracts to transform the Fe^{3+} to Fe^{2+}, this capacity is compared to that of quercetin. The results in table 3 showed that the methanolic extract of *T. polium subsp. aurum* had the strongest ferric reducing power than all other extracts with an EC_{50} value of 0.193 ± 0.006 mg/ml but this was still significantly ($p < 0.05$) lower than that of the synthetic antioxidant quercetin (0.033 mg/ml). The ethyl acetate from *T. polium subsp. aurum* possessed a lowest ferric reducing power with an EC_{50} value of 3.947 ± 0.15 mg/ml.

Table 3. Ferric reducing power capacity (mg/ml) of two *Teucrium polium* subspecies compared to that of quercetin (EC_{50}=0.033 ± 0.0004). Values are giving as mean ± SD (n=3). The extracts of same solvent and quercetin are significantly different by the tuckey-test (P<0.05).

Type of extract	Methanolic	Ethanolic	Water	Ethyl acetate
T. polium subsp. polium	0.319 ± 0.012	0.395 ± 0.011	0.456 ± 0.011	3.293 ± 0.059
T. polium subsp. aurum	0.193 ± 0.006	0.381 ± 0.009	0.588 ± 0.031	3.947 ± 0.15

3.2.4. Phosphomolybdenum assay

Total antioxidant capacity of investigated both *T. polium* subspecies extracts were determined by the phosphomolybdenum method which is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the subsequent formation of a green phosphate Mo (V) complex at acidic pH [26]. The found results, expressed as ascorbic acid equivalents (vit C E) are presented in Figure 4. They revealed that the most solvent extraction of antioxidant capacity was water and the highest level of antioxidant capacity was found in water extract from *T. polium subsp. aurum* with 220 mg ascorbic acid equivalent to /g dry weight. In methanol and ethyl acetate extracts, the antioxidant capacity was significantly higher in *T. polium subsp. polium* than *T. polium subsp. aurum.*

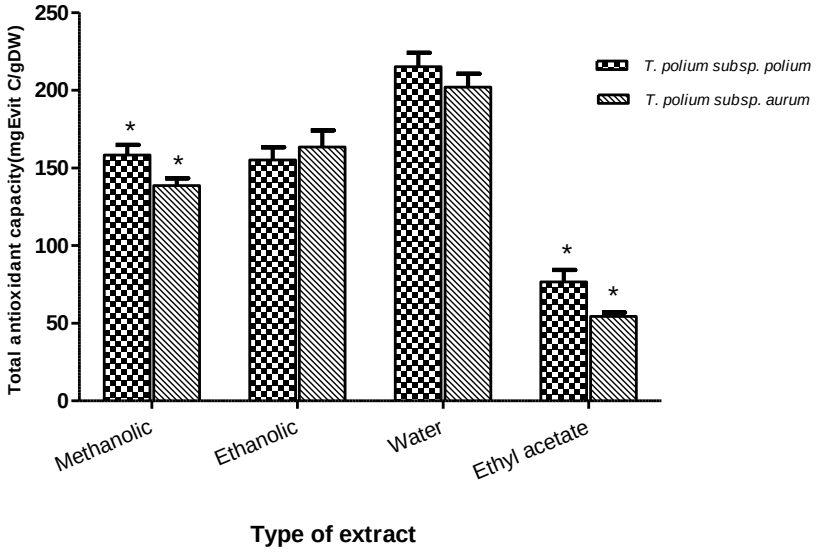

Figure 4. Total antioxidant capacity of different extracts from two *Teucrium polium* subspecies. Results were expressed as mg vit C E/g dry weight. Each value represents means ± SD of tree experiments. (*$p < 0.05$).

5. Discussion

The antioxidant activities has been evaluated using four different methods based on various mechanisms of action. It is important to use numerous tests to take in consideration the chemical composition of extract which act through different mechanisms. The present study is the first to demonstrate that extracts, obtained by Soxhlet, from *T. polium subsp. aurum* and *T. polium subsp. polium* from Morocco had a good antioxidant effects. However, there are some studies have determined the antioxidant activity of *T. polium* but they did not specify its subspecies. Based on the results of selected *T. polium* from Serbiae [28], the authors found that the highest total phenolic concentration of different parts of this plant is noticed with the methanol extract of leaves (157.84 mg of GA/g dry weight). Similarly, our study revealed also that methanol extract of *T. polium subsp. polium* contained the highest total phenolic content (112.27 mg GAE / g DW (figure 1). Methanol is therefore the best solvent to extract phenolic compounds from medicinal plants. In our study the total flavonoids content results obtained are higher than the results reported by Tepe et al [29]. According to another study, the flavonoids content values ranged from 6.48 to 139.87 mg RE/g in the leaves, flowers and stem from *T. polium subsp. polium* extracts with the acetone extract giving a high amount of flavonoids [28]. Like polyphenols, flavonoids have been confirmed to have a strong antioxidant activity [30,31]. In the other hand, Chemical studies on the Teucrium genus revealed the presence of flavonoids, saponins, polyphenols, sterols and tannins [32]. The *T. polium* specie contains essential oils, iridoids, flavonoids and diterpenoids [12].

The $ABTS^{+\bullet}$ radical assay was generated by potassium persulfate in order to determine its hydrogen donating propriety. In this study, both *T. polium* subsp. extracts scavenged $ABTS^{+\bullet}$ radical in a concentration dependent-manner. The high percentage of ABTS scavenging activity founded in this study can be attributed to a high content of phenolic compounds, which is similar to the results reported in previous studies [4]. An antioxidant agent is considered to be active against free radicals if IC_{50} is less than 5 mg/ml [33]. All the extracts studied have IC_{50} less than 5 mg/ml, therefore all tested extracts are a possible good source of antioxidants compounds. In addition, extracts with high scavenging activity should have a low IC_{50} value. Previous studies demonstrated that extracts of Teucrium plants have strong antioxidant activity [15,34]. Extracts of *Teucrirum polium* showed significant free radical scavenging [18,35]. Methanolic extract of *T. polium* exhibited an IC_{50} value of 20.1µg/ml [35], which is below that found in our study. Other study found that the IC_{50} values of *Teucrium polium* extracts were ranging between 14.50 and 238.25 µg/ml and the highest activity noticed with polar solvents extracts [28]. These observed differences could be attributed to the different extraction types used. In addition, extracts with high reducing power should have a low EC_{50} value. Previous published papers demonstrated that Teucrium genus possess a high reducing power [36]. In this study we found that methanolic extract of *T. polium subsp. aurum* showed the highest reducing power propriety. Several studies reported the reducing power of the extracts of *T. polium* and that its activity increased with concentration [29,37]. The phosphomolybdenum assay of is often used as an indicator of antioxidant capacity for plant extracts. According to a study performed by Ljubuncic et al. [38], the aqueous extract of *T. polium* had a substantial antioxidant activity *in vitro*. A previous in vivo study reported that rats treated with a *T. polium subsp. polium* ethanolic extract showed significant antioxidant activity in the DPPH test compared to the positive control α-tocopherol [39]. Acoording to our previous study[18], the ethanolic, mathanolic and water extracts of *T. polium* obtained by the maceration technique showed a good antioxidant potential but it's low than that founded in this work. Moreover, the free radical scavenging activity of the extracts, in our case, could be attributed to the phenolic content. According to the literature phenol compounds can contribute to the antioxidant potent [40,41] and they are considered as anti-cancer, anti-inflammatory, antiviral, and anti-bacterial agents due to their antioxidant and free radical scavenging properties [42].

6. Conclusion

In this study, the extracts of aerial parts of two Moroccan *Teucrium polium* subspecies (*T. polium subsp. polium* and *T. polium subsp. aurum*), were investigated and compared for their, antioxidant activities and total polyphenol and flavonoid contents. Considerable antioxidant activity of methanolic extracts of the two subspecies showed that *T. polium subsp. polium* has the greatest antioxidant properties and the highest level of total phenols and flavonoids content. The highest antioxidant propriety is noticed with polar solvents extracts. The Germander (*T. polium*) is a rich source of phenols and natural antioxidant compounds which can be used as a natural food preservative. Our results on antioxidant tests justified it uses in folk medicine.

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