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ANTIOXIDANT ACTIVITY OF TWO WILD *TEUCRIUM* SPECIES FROM MOROCCO

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ABSTRACT: The aim of the present study was to investigate antioxidant activity, total polyphenols and total flavonoids contents of extracts from two Moroccan *Teucrium* species (*Teucrium polium* and *Teucrium aurum*). The Antioxidant activity was evaluated *in-vitro* by three assays namely, free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and total antioxidant capacity. Total polyphenol content was measured using Folin-Ciocalteu assay. The aluminum chloride colorimetric test measured total flavonoids contents. The total phenols contents, flavonoids contents, and the antioxidant activity of *T. polium* extracts were higher than *T. aurum.* The phenols contents ranged from 109.28 to 20.61 mg GA E/ g dry extract. The total flavonoids varied between 102.99 and 10.33 mg RE/g dry weight. The extracts showed significant scavenging activity of DPPH, with IC_{50} values ranging between 0.40 and 2.12 mg/ml and a good ferric reducing power, with IC_{50} values varying from 0.15 to 4.23 mg/ml. The total antioxidant capacity assay showed that the water extract of *T. polium* had a highest activity with a value of 153.89 mg Vit C E/g dry weight. The ethyl acetate extract has a weak antioxidant activity in the three tests. A good correlation between antioxidant activities and contents of polyphenols and flavonoids was found. These results show that Moroccan *Teucrium* species, especially *T. polium,* is a rich source of phenols and natural antioxidant compounds, which can be used as a natural food preservative.

INTRODUCTION: Oxidative stress is characterized as an imbalance between the production of reactive species and antioxidant defense activity [1]. This imbalance has been associated with numerous diseases such as, neurodegenerative and Alzheimer's disease [2, 3], cardiovascular disease [4], cancer [5] diabetes [6, 7] and inflammatory diseases [8].

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Medicinal and aromatic 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 various diseases [9]. The genus *Teucrium* (Lamiaceae family) includes 300 species distributed all over the world, particularly in the Mediterranean basin [10].

It usually develops on hillsides, sands, semi-arid and in arid places [11]. In Morocco folk medicine, Germander (*T. polium* and *T. aurum*) locally called "Jaada," it is used for the treatment of a variety of diseases, including digestive disorders, liver problems, [12] hypertension, fever, diabetes, rheumatism, parasitic diseases such as amoebicide [13].

Numerous studies showed therapeutic properties of some *Teucrium* species such as anti-cancer [14, 15], anti-allergic [16], antibacterial [17], antidiabetic [7], anti-inflammatory, anti-nociceptive [18] and antioxidant [19] effects. It was reported that the therapeutic ability of *T. polium* extracts is generally attributed to their propriety to suppress oxidative processes [20]. It was also reported that the alcoholic extract of *T. polium* possesses a suppressing effect on hydrogen peroxide-induced lipid peroxidation in red blood cells [21].

To the best of our knowledge, no data are available on the antioxidant activity, total polyphenol and flavonoid contents of *T. aurum* species extracts. However, the objective of our study was to evaluate, for the first time, the antioxidant activity of methanol, aqueous, ethanol and ethyl acetate extracts of *Teucrium polium* and *Teucrium aurum* which are growing in South Morocco.

MATERIALS AND METHODS:

Reagents and Standards: 2,2-Diphenylpicryl-hydroxyl radical (DPPH), Butylated hydroxyl-toluene (BHT), ammonium molybdate, aluminum chloride ($AlCl_3$), sodium phosphate, quercetin, 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 grade.

Plants Materials: Aerial parts of *Teucrium polium* and *Teucrium aurum* were collected in April 2015 from the region of Kser Lahri, Midelt (Morocco). They were identified by Professor Amina Bari, Botanist at the Department of Biological Sciences (Faculty of Sciences, Sidi Mohammed Ben Abdellah University, Fez, Morocco).

Preparation of *Teucrium* Extracts: The plant material was dried at room temperature, powdered (10 g) and macerated with 100 ml of solvent (water, methanol, ethanol and ethyl acetate). The resultant macerate was filtered and then concentrated 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.

Statistical Analysis: Data statistical analyses of scavenging activity and reducing capacity assays were performed by using One-way ANOVA followed by Tuckey-test. The results statistical analyses of total phenol, flavonoids content and total antioxidant capacity tests were realized by using the Student's t-test. Correlations between antioxidant activity and total phenolic or flavonoid content were realized by a Pearson correlation coefficient (r). The level of significance was set at $P<0.05$.

Determination of Total Phenolic Content: Total phenolic content of the extract was determined by the Folin - Ciocalteu method [22]. 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 2 h of reaction at room temperature in the dark. Gallic acid was used as a standard for the construction of a calibration curve. Total phenols contents were expressed as milligrams of gallic acid equivalents per gram dry weight of extract (mg GAE/g DW).

Determination of Total Flavonoids Contents: Total flavonoids contents of extracts were measured by the aluminum chloride colorimetric assay [23]. 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 the calibration curve. Total flavonoids contents were expressed as mg Rutin equivalents per gram dry weight of each extract (mg RE/g DW). All samples were analyzed in triplicate.

***In-vitro* Antioxidant Activity:**

DPPH Radical Scavenging Activity: 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 the extracts or standard was added to 1.5 ml of the 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 the dark. The percentage inhibition was calculated by the following equation:

$$I\ (\%) = (1-(A_s/A_C)) \times 100$$

Where A_c is the absorbance of the negative control, and A_s is the absorbance of the sample. BHT served as positive control. The IC_{50} values were calculated as the concentration of causing a 50% inhibition of DPPH radical.

Ferric Reducing Antioxidant Power: The reducing power of the tested extracts was determined by the procedure of Oyaizu [25]. 200µl of the extract was mixed with 500 µl of phosphate buffer (0.2M, 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 Trichloroacetic (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). Quercetin was used as standard. The result was expressed as IC_{50} (mg/ml). The extract concentration corresponding 0.5 of absorbance (IC_{50}) was calculated by plotting absorbance against the corresponding extract concentration. All samples were analyzed in triplicate.

Total Antioxidant Capacity: 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, 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 vitamin C (mg vit C E/g DW).

RESULTS AND DISCUSION:

Total Phenolic Contents: The distribution of phenolic compounds in *T. polium* and *T. aurum* extracts is shown in **Fig. 1**. Results demonstrated that the methanol and ethanol extracts from *Teucrium polium* contained the highest amounts 109.26 and 100.94 mg GAE/g dry weight of extract, respectively, and the lowest phenolic content was observed in water *Teucrium aurum* extract (20.61 mg GAE/g dry of extract) **Fig. 1**. In all extracts, the contents of phenolics were higher in *T. polium* than *T. aurum.* Similarly, based on the results of selected *Teucrium polium* from Serbia [27], the authors also found that the highest total phenolic concentration of different parts of *T. polium* is noticed with the methanol extract of leaves (157.84 mg of GA/g dry extract). Methanol is therefore the best solvent to extract phenolic compounds from medicinal plants.

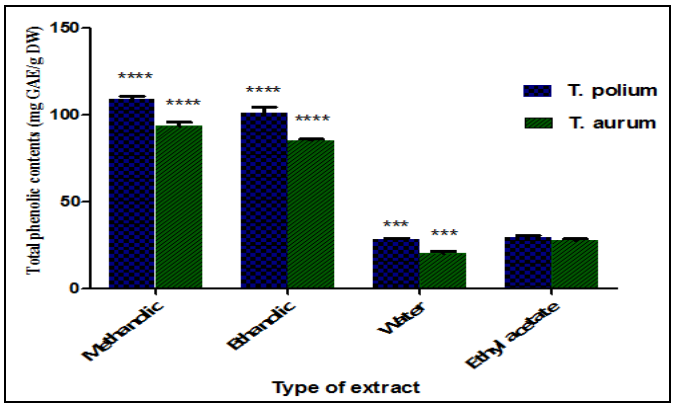

FIG. 1: TOTAL PHENOLIC COMPOUNDS OF EXTRACTS FROM *T. POLIUM* AND *TEUCRIUM AURUM*. Results were expressed as mg GAE/g dry weight. Each value represents means ± SD of tree experiments. (*p<0.01, **p<0.01, ***p<0.001 and ****p<0.0001)

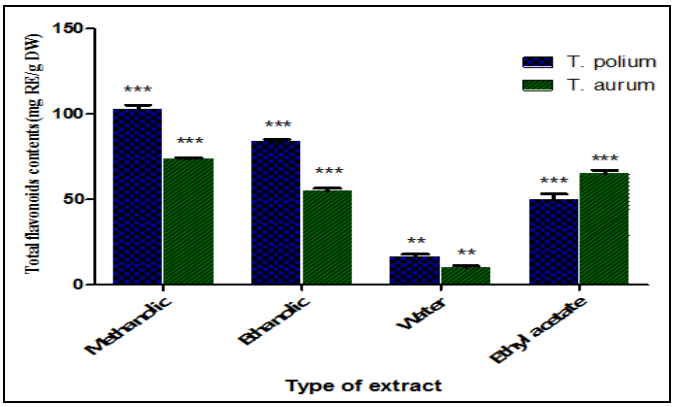

FIG. 2: TOTAL FLAVONOID CONTENT OF EXTRACTS FROM *T. POLIUM* AND *TEUCRIUM AURUM*. Results were expressed as mg RE/g dry weight. Each value represents means ± SD of tree experiments. (*p<0.01, **p<0.01, ***p<0.001 and ****p<0.0001)

Total Flavonoids Contents: Total flavonoids contents of *T. polium* and *T. aurum* extracts were determined in comparison with rutin standard and the results were expressed regarding mg RE/g dry

weight extract. As depicted in **Fig. 2**, the total flavonoid content of two species extracts ranged from 102.99 to 10.33 mg RE/g DW. The methanol extract of *T. polium* contained significantly a higher concentration of flavonoids (102.99 ± 2.35 mg of RE/g DW) than the other tested extract. Comparing the flavonoid concentration of *T. polium* and *T. aurum*, all extracts of *T. polium* (except ethyl acetate) had a significantly greater concentration of flavonoids than *T. aurum* extracts obtained using the same solvent. The total flavonoids content results obtained are higher than the results reported by Bakari *et al*., (2015) [20]. According to another study, the flavonoids content values ranged from 6.48 to 139.87 mg ER/g in the leaves, flowers and stemmed from *T. polium* extracts with the acetone extract giving a higher amount of flavonoids [27]. Like polyphenols, flavonoids have been confirmed to have a strong antioxidant activity [28]. Chemical studies on the *Teucrium* genus revealed the presence of flavonoids, saponins, polyphenols, sterols and tannins [20]. The *T. polium* species also contains essential oils, iridoids, flavonoids and diterpenoids [10].

Antioxidant Activity: The antioxidant activity of *Teucrium* extracts was investigated by the DPPH radical scavenging assay, the ferric reducing antioxidant power, and the total antioxidant capacity.

DPPH Radical Scavenging Activity: $DPPH^{\bullet}$ is a stable free radical that can receive hydrogen or electron from an antioxidant to become a stable molecule. Results in **Table 1** show DPPH radical scavenging activity of *Teucrium polium* and *Teucrium arum* extracts. In general, the IC_{50} values of all tested samples through the DPPH scavenging activity test ranged from 0.4 to 2.12 mg/ml, and all extracts inhibited the DPPH radical as follows: methanol > ethanol > ethyl acetate. These results clearly indicate that *T. polium* extracts had higher activity than the *T. aurum* extracts in comparison to the same solvent extracts. We found that, the methanol extract from *T. polium* had the greatest radical scavenging capacity in all samples tested with IC_{50} values of 0.40 ± 0.03 mg/ml, followed by ethanol (0.41 ± 0.031 mg/ml) then water (0.48 ± 0.012 mg/ml) and ethyl acetate extracts (1.60 ± 0.15 mg/ml). With *T. aurum*, we found also that the methanol extract had the greatest capacity with an IC_{50} value of 0.49 ± 0.009 mg/ml followed by water extract (0.51 ± 0.021 mg/ml), then ethanolic extract (0.53 ± 0.035 mg/ml).

However, when compared to the pure reference antioxidant BHT (0.11 ± 0.0001 mg/ml), all the tested extracts showed a significantly lower antioxidant activity ($p<0.05$). An antioxidant agent is considered to be active against free radicals if IC_{50} is less than 5 mg/ml [29]. All the extracts studied of two *Teucrium* species have $IC_{50}<5$ mg/ml, therefore all our extracts are a possible good source of antioxidant compounds. Also, extracts with high scavenging activity should have a low IC_{50} value.

Previous studies demonstrated that extracts of *Teucrium* plants have strong antioxidant activity [17, 27]. The *Teucrium polium* extract showed significant free radical scavenging activity [19, 30]. Methanolic extract of *T. polium* exhibited an IC_{50} value of 20.1 µg/ml [31], which is below that found in our study. Another study found that IC_{50} of *Teucrium polium* extracts were ranging from 14.50 to 238.25 µg/ml and the higher activity noticed with polar solvent extracts [27]. These differences observed can be attributed to the different extraction types used.

TABLE 1: DPPH RADICAL SCAVENGING ACTIVITY (mg/ml) OF *T. POLIUM* AND *T. AURUM* EXTRACTS COMPARED TO THAT OF BHT (IC_{50} = 0.118 ± 0.0001)

Type of extract	Methanolic	Ethanolic	Water	Ethyl acetate
Teucrium polium	0.40 ± 0.03	0.41 ± 0.031	0.48 ± 0.012	1.6 ± 0.15
Teucrium aurum	0.49 ± 0.009	0.53 ± 0.035	0.51 ± 0.021	2.12 ± 0.87

Values are given as mean ± SD (n=3). The extracts of the same solvent and BHT are significantly different by the Tuckey-test ($P<0.05$)

Ferric Reducing Antioxidant Power: The FRAP assay evaluated the ferric reducing capacity of investigated extracts. The reductive activity is generally associated with the presence of antioxidant agents which exert their effect by breaking the free radical chains *via* hydrogen atom donation [32]. Therefore, the ferric reducing power assay is often used to evaluate the capacity of extracts to transform the Fe^{3+} to Fe^{2+}; this capacity is compared to that of quercetin. Extracts with high reducing power should have a low IC_{50} value. The results in **Table 2** showed that the methanolic

extract of *T. aurum* had the stronger ferric reducing power than all other extracts with an IC_{50} value of 0.15 ± 0.003 mg/ml but this was still significantly ($p<0.05$) lower than that of the synthetic antioxidant quercetin (0.033 ± 0.0004 mg/ml). The ethyl acetate extract from *T. aurum* possessed the lowest ferric reducing power with an IC_{50} value of 4.23 ± 0.11 mg/ml.

Previously published papers demonstrated that *Teucrium* genus possesses a high ferric reducing power [19, 30]. In this study, we found that methanolic extract of *T. aurum* showed the highest ferric reducing power propriety. Several studies reported the reducing power of the extracts of *T. polium* and that the activity increased with concentration [19, 20].

TABLE 2: FERRIC REDUCING ANTIOXIDANT POWER (mg/ml) OF *T. POLIUM* AND *T. AURUM* EXTRACTS COMPARED TO THAT OF QUERCETIN (IC_{50} = 0.033 ± 0.0004)

Type of extract	Methanolic	Ethanolic	Water	Ethyl acetate
Teucrium polium	0.28 ± 0.014	0.32 ± 0.01	0.48 ± 0.01	3.89 ± 0.081
Teucrium aurum	0.15 ± 0.003	0.36 ± 0.001	0.6 ± 0.041	4.23 ± 0.11

Values are giving as mean ± SD (n=3). The extracts of same solvent and quercetin are significantly different by the Tukey-test ($P<0.05$).

Total Antioxidant Capacity: Total antioxidant capacity of investigated *T. polium* and *T. aurum* extracts were determined by the phospho-molybdenum 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 vitamin C equivalents (Vit C E) are presented in **Fig. 3**. They revealed that the most solvent extraction of antioxidant capacity was water and the highest level of antioxidant capacity was found in the water of *T. aurum* with value of 153.89 ± 12.7 mg vitamin C equivalent to 1 g dry weight.

The antioxidant capacity was significantly higher in *T. aurum* than *T. polium* in methanol, ethyl acetate, and water extracts. According to a study performed by Ljubuncic *et al.*, [33] the aqueous extract of *T. polium* had a substantial antioxidant activity *in-vitro*.

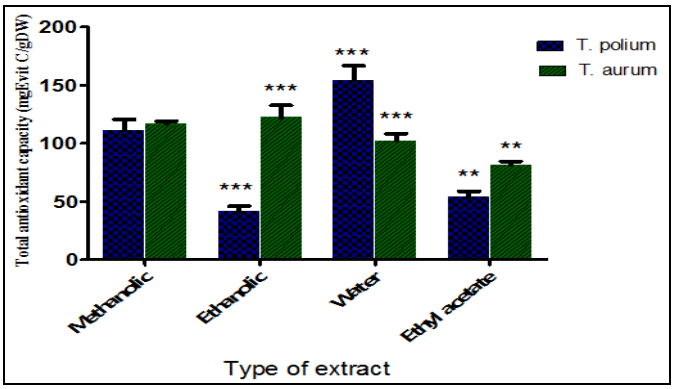

FIG. 3: TOTAL ANTIOXIDANT CAPACITIES OF DIFFERENT EXTRACTS FROM *T. POLIUM* AND *T. AURUM*. Results were expressed as mg vit C E/g dry weight. Each value represents the means ± SD of three experiments. (**p<0.01 and ***p<0.001).

Correlations of Antioxidant Activities with Phenolic and Flavonoids Contents: To find the influence of polyphenols on the antioxidant activity of *Teucrium* extracts, we studied the correlation between the results of three antioxidant tests and the contents of total phenols and flavonoids. As illustrated in **Table 3**, a significant and negative correlation was found between DPPH and total phenols (r^2 = -0.78). As a consequence, DPPH was also significantly correlated with total flavonoids (r^2 = -0.7). For the ferric reducing power, the IC_{50} values were significantly and negatively correlated with total phenols (r^2 = -0.81) and with total flavonoids (r^2 = -0.73). Weak and non-significant correlation between total antioxidant capacity and totals phenols could be detected as well for total flavonoids.

Similar to our results, a high relationship between the total phenol and flavonoids contents with two of the antioxidant assays, like free radical scavenging activity and ferric reducing power of *Teucrium* extract has been reported [34]. The weak and non-significant correlation was found between the total antioxidant capacity and polyphenols and flavonoids contents of two *Teucrium* species extracts, indicating that total antioxidant capacity, in these extracts, measures the activity of some other phytochemicals than the polyphenols, a similar result was also observed by Brantner [35] with Lavandula extracts. 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 [9, 36, 37] and they are considered as anti-cancer, anti-inflammatory, antiviral, and anti-

bacterial agents due to their antioxidant and free radical scavenging properties [38].

TABLE 3: CORRELATION OF ANTIOXIDANT ACTIVITIES WITH PHENOLS AND FLAVONOIDS CONTENT OF EXTRACTS FROM *T. POLIUM* AND *T. AURUM*

Compounds	DPPH	Ferric Reducing power	Total antioxidant capacity
Phenols contents	-0.78	-0.81**	0.44
Flavonoids contents	-0.7*	-0.73*	0.34

* Correlation is significant at the P<0.05 level
** Correlation is significant at the P<0.01 level

CONCLUSION: Based on the findings from this study, methanolic extracts of *T. polium* and *T. aurum* displayed significant antioxidant effect and a remarkable levels of total phenols and flavonoids content. The highest antioxidant propriety is noticed with polar solvent extracts. Significant correlations were found between the total phenol and flavonoids contents and two of the antioxidant tests, such as free radical scavenging activity and ferric reducing power.

The Moroccan Germander (*T. polium* and *T. aurum*) is a rich source of phenols and natural antioxidant compounds which can be used as a natural food preservative agent. To the best of our knowledge, this is the first report on the antioxidant activities of Moroccan Germander extracts.

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CONFLICT OF INTEREST: There is no conflict of interest.

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