Variability of phenolic contents in ethanolic extracts of *Teucrium polium* L. populations and effect on antioxidant and antimicrobial activities

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**ABSTRACT**

This study was designed to compare phenolic contents, antioxidant and antimicrobial activities of *Teucrium polium* L. ethanolic extract of two Algerian populations. This study indicates that the two plants are rich in total polyphenol with values ranging from 146.13 ± 18.95 to 206.54 ± 58.17 mg GAE/g extract in Setif and Biskra plant extracts respectively. The same tendency was observed for flavonoid levels (4.99 ± 0.39 to 7.30 ± 0.90 mg QE/g respectively). Accordingly, population of Biskra presented the highest antioxidant capacities based on, both DPPH and FRAP methods. Significant correlations were observed between the total phenols and flavonoid contents and the estimated antioxidant activities. Moderate antimicrobial and inhibitory activities were observed against the tested bacterial strains notably Gram-negative bacteria: *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). However, the Biskra plant extract exhibited the highest antimicrobial activities levels. In this study, the antibacterial and antioxidant properties of *Teucrium polium* extract approved the traditional uses of this plant, and can be used for medicinal and therapeutic applications.

**Keywords:** *Teucrium polium* L., phenolic compounds, DPPH assay, FRAP assay, inhibitor concentration, bactericidal concentration.

**INTRODUCTION**

Medicinal plants have always been a significant source of natural products having therapeutic potential. *Teucrium polium* (Lamiaceae) is a wild-growing flowering plant, found abundantly in South-Western Asia, Europe and North Africa. The *Teucrium polium* contains a number of compound classes that include phenylpropanoid glycosides [1], neoclerodane diterpènes [2], essential Oils [3,4] and flavonoids [5], which have been reported in the literature to have antimicrobial [6-8] and antioxidant properties [9, 10]. However, there is not any information about relationship between its medicinal activities and the different Algerian populations.

The main aim of our study was to compare total phenolic and flavonoid contents, with respect to their antimicrobial and antioxidant activities in two Algerian populations of *Teucrium polium* L. used in traditional medicine.

**MATERIALS AND METHODS**  

**Plant material and preparation of extracts**

Flowering branches of *T. polium* were collected during June 2012 from two regions in East of Algeria: Setif and Biskra. Then, they dried in dark and powdered. 5 g of each plant material was extracted with ethanol 70% (v/v) for 72 h at room temperature. The extracts were filtered using Whatman filter paper and then concentrated in a rotary evaporator and kept in sealed dark flasks. The residues obtained were stored at 2 °C until further tests.
Determination of total phenolics

Total soluble phenolic compounds in the T. polium extracts were determined with Folin–Ciocalteu reagent according to the method of [11] using gallic acid as a standard phenolic compound. Briefly, 25 µl ml of the T. polium extracts solution in a volumetric flask was diluted with distilled water (475 µl). 250 µl of Folin–Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 10 min, 1.25 ml of Na₂CO₃ (2%) was added and was then allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 725 nm in a spectrophotometer. The total content of phenolic compounds in the extract in gallic acid equivalent (gallic acid Equivalent) was calculated by the following formula: 

\[ Y = 0.0012 x + 0.0324, \quad R^2 = 0.99. \]

Total flavonoids determination

Aluminium chloride colorimetric method is used for flavonoids determination [12]. Two milliliters of 2% AlCl₃ in ethanol is added to 2 ml of the test sample. The UV absorption is measured at 420 nm after 30 minutes at room temperature. Determinations were performed in triplicates. Total flavonoid contents were obtained from the regression equation of the calibration curve of quercetin (\( Y = 0.066x, \quad R^2 = 1 \)).

Antioxidant activity assays

The antioxidant activities of extracts compound were evaluated in vitro through the use of two different methods: DPPH scavenging test and reducing power assay (FRAP).

DPPH decoloration assay

The free radical scavenging capacity of T. polium extracts was measured by 2,2-diphenyl-1-picryl-hydrazil (DPPH•) using the method of [13]. Briefly, 1.95 ml methanolic solution of DPPH• (0.025 g/ml) was added to 50 µl of T. polium extracts solution in water at different concentrations (0.5, 1, 1.5 and 2 mg/ml). 15 minutes later, the absorbance was evaluated spectrophotometrically at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH• radical was calculated using the following equation:

\[ \text{DPPH• scavenging effect %} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \]

where \( A_0 \) is the absorbance of the control reaction and \( A_1 \) is the absorbance in the presence of the sample of T. polium extracts. Ascorbic acid was used as positive control and as reference.

Determination of reducing power

The reducing power of T. polium extracts was determined by the method of [14]. Different concentrations of T. polium extracts (0.5, 1, 1.5 and 2 mg/ml) in 0.5 ml of distilled water were mixed with phosphate buffer (1.25 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (1.25 ml, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged for 10 min at 3000 rpm.

The upper layer of solution (1.25 ml) was mixed with distilled water (1.25 ml) and FeCl₃ (250 µl, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid was used as positive control and as reference.

Antibacterial activity assay

In this work, the antibacterial activity of the isolated flavonoids are determined by the disc diffusion method on Mueller-Hinton Agar (MHA) medium [15]. The crude ethanolic were screened against two Gram-negative bacteria: Escherichia coli (ATCC 25922) and, Pseudomonas aeruginosa (ATCC 27853), two Gram-positive bacteria: Staphylococcus epidermidis (ATCC 12228) and Bacillus megaterium (ATCC 14581) obtained from the bacteriology department of the hospital of Tebessa, Algeria, are used for this study.

Disc diffusion method

The antibacterial activity of the extracts was carried out by disc diffusion test [15]. The microbial cultures were harvested and then suspended in sterile saline (0.9% NaCl) and the cell density was adjusted to 0.5 McFarland standard (10⁶ CFU/ml). Sterile 6 mm diameter filter paper discs were impregnated with 10 µL of the extracts solutions, were placed on the inoculated surface. Before incubation, all Petri dishes were stored in the dark at 4 °C for 1 h, to allow the diffusion of the extracts from disc to medium without microbial growth, and then incubated at 37°C for 24 h to ensure the growth of bacterial strains tested. After incubation, the diameters of inhibition zones were measured in millimeter. Negative controls were prepared using the same solvents employed to dissolve the plant extracts. The evaluation of antimicrobial activities of samples was carried out in three repetitions.
Determination of the minimum inhibitory and bactericidal concentrations

MIC assay of extracts were tested against bacterial strains [16, 17]. Three to four colonies of overnight cultures of each microorganism was inoculated into 5 ml of sterile nutrient broth and incubated for 3 to 5 h. 50 μl of this suspension was diluted in twice concentrated Mueller-Hinton Broth for bacteria to adjusted the culture to 0.5 McFarland. Then, 2 ml of the inoculum, initially adjusted to the density cited above, was spread onto 18 ml Mueller–Hinton agar supplemented with the extract at concentrations ranging from 2000 - 5 μg/ml in Petri dishes. These serially cultures were then incubated at 37°C for 24 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of extracts inhibiting visible growth of the tested strains. Referring to the results of the MIC assays, the wells showing complete absence of growth were identified and 10 μL of each well was transferred to agar plates and incubated at 37 °C for 24 h. Plates without growth were considered as minimum bactericidal concentrations (MBC) and those with growth as bacteriostatic concentrations of the extracts.

Statistical analysis

All the experiments were conducted in triplicate unless stated otherwise and statistical analysis of the data was performed by analysis of variance (ANOVA), using STATISTICA 7.0. The correlations between antioxidant activities and total phenolic and/or flavonoid contents were determined using analysis of variance (ANOVA) and quantified in terms of the correlation factor. A probability value of \( p \leq 0.05 \) was considered to denote a statistically significance difference. All data are presented as mean values ± standard deviation (SD).

RESULTS AND DISCUSSION

Total phenolic and flavonoids contents determination

The amount of total phenolic, measured by Folin–Ciocalteu method, varied widely in herb materials and ranged from 146.13 ± 18,95 to 206.54 ± 58,17 mg GAE/g extract (Figure 1). The highest total phenolic content was detected in Biskra population, while Setif population was the lowest one.

The range for total flavonoid content was from 4.99 ± 0.39 to 7.30 ± 0,90 mg QE/g extract (Figure 1). Biskra population also showed the highest flavonoid content while Setif population showed the lowest one.

![Figure 1. Phenolic and flavonoids contents in the studied plants extracts](image_url)

It is well known that the constituents of medicinal herbs can vary greatly as a result of genetic factors, climate, soil quality and other external factors [18], particularly the semiarid climate from northeast of Algeria (Biskra population) causes the production of secondary metabolites different from those found in the same species grown under less extreme conditions (Setif population).

Antioxidant activity of the extracts

DPPH radical scavenging activity

The hydroethanolic crude extracts of 2 plant extracts were tested for antioxidant activity using the DPPH assay. The evaluation of the antioxidant activity between the two plant extracts is showed in Figure 2. We obtained a higher antioxidant activity in Biskra plant extract (2 mg/ml induces a 64.46% ± 17.21 of DPPH inhibition) in comparison...
with Setif plant extract (2 mg/ml induces a 23.32% ± 0.32 of DPPH inhibition). Also we used a synthetic antioxidant ascorbic acid as standard for the determination of the antioxidant activity (2 mg/ml induces a 92.31% ± 0.21 of DPPH inhibition).

Figure 2. Comparison of the DPPH radical scavenging activity in the two plant extracts

The results were expressed as IC50, which is defined as the concentration of substrate at 50% inhibition. There is a wide range of free radical scavenging activity between the plant analyzed. The values of IC50 ranged from 2.82 mg/ml and 3.31 mg/ml. IC50 values were found to be in the following order: Biskra plant extract > ascorbic acid > Setif plant extract. The DPPH radical scavenging effect of Biskra plant extract was higher (IC50= 2.87 mg/ml) than ascorbic acid and Setif plant extract. A higher DPPH radical scavenging activity is associated with a lower IC50 value.

The variations in the antioxidant activity of different Teucrium polium extracts were statistically significant (p < 0.001). When these results were compared with standard ascorbic acid, Setif plant extract showed significantly (p < 0.001) less antioxidant activity.

Many studies on medicinal plants confirm the relation between the antioxidant activity and the presence of polyphenolics content. Different flavonoids and phenolic compounds react with free radical to reduce the degradation of membranes by preventing the reaction between free radicals and phospholipids [19]. Flavonoids antioxidants function as scavengers of free radicals by rapid donation of a hydrogen atom to radicals [20]. Other workers also reported that antioxidant activity of plants is higher than that of synthetic antioxidant [21, 22].

**Ferric reducing antioxidant power**

The evaluation of the reducing power of two extracts at different concentrations was plotted against positive control ascorbic acid. Figure 3 showed a higher antioxidant activity in Biskra plant extract (2 mg/ml induces a 63.43% ± 3.45 of reducing ability) in comparison with Setif plant extract (2 mg/ml induces a 62.58% ± 3.73 of reducing ability).

The hydroethanolic crude extracts of the 2 plant extracts were tested for antioxidant activity using the ferric reducing power. It can be seen from Figure 3 the reducing ability of two extracts of Teucrium polium. The results clearly indicate that from two plant extract tested, Biskra plant extract exhibited higher reducing activity (IC50= 0.954 ± 0.30 mg/ml) than Setif plant extract which showed next higher scavenging activity (IC50= 1.079 ± 0.196 mg/ml). ANOVA showed no significant difference (P>0.05) in reducing power among hydroethanolic extracts analyzed.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity [23, 24]. Compounds with reducing power indicate that they are electron donors or hydrogen to Fe3+ to reduce Fe2+ and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants [25]. However, the reducing capacity does not necessarily reflect antioxidant activity, as has been suggested by [26] and [27].
Correlation between antioxidant activity and phenolic content
A moderate correlation between the flavonoids contents and antiradical properties tested by DPPH assay was observed ($R^2 = 0.50$). Another hand, there was a notable correlation between the content of total phenolic and antioxidant activity in FRAP assay ($R^2 = 0.66$).

Some authors [28, 29] have demonstrated a linear correlation between the content of total phenolic compounds and their antioxidant capacity, while others [30, 26] show poor linear correlation or report total antioxidant activity and phenolic content with no comment. Different types of phenolic compounds have different antioxidant activity, which mainly depends on their chemical structure and substitution pattern of hydroxyl groups [31]. Some authors demonstrated that antioxidant was not solely dependent on phenolic content but it may be due to other phytoconstituents as triterpenoids or combine effect of them [21].

Antibacterial activity determination
The results of the antimicrobial screening of 2 plant extracts against four bacteria species are summarized in Table 1 (inhibition zones in the agar diffusion assay). All the tested extracts revealed antimicrobial activity showing different selectivity for each microorganism. Gram negative bacteria; Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853) was the most sensitive to Biskra plant extract, the average zones of inhibition were $10.0 \pm 2.64$ to $11.0 \pm 1.00$ mm respectively. Concerning Gram bacteria positive, the two plant extracts were found to be active against Bacillus megaterium (ATCC 14581) and Staphylococcus epidermidis (ATCC 12228) with diameter of inhibition zones ranging from $7.00 \pm 1.73$ to $9.00 \pm 4.35$ mm respectively.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Gram Negative</th>
<th>Gram Positive</th>
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<tbody>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>Pseudomonas aeruginosa</td>
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<tr>
<td>Biskra plant extract</td>
<td>$10.0 \pm 2.64$</td>
<td>$11.0 \pm 1.00$</td>
</tr>
<tr>
<td>Setif plant extract</td>
<td>$7.16 \pm 0.70$</td>
<td>$6.66 \pm 0.57$</td>
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The determination of the antibacterial activity in the two plant extracts by the minimal inhibitory concentration showed the presence of activity against the four bacterial strains tested. A high antibacterial activity was obtained by Biskra plant extracts against the two bacterial strains Bacillus megaterium (ATCC 14581) and Pseudomonas aeruginosa (ATCC 27853) (MIC 2000 µg/ml). Same minimal inhibitory concentration was obtained by Setif plant extracts against Escherichia coli (ATCC 25922) (MIC 2000 µg/ml).
Minimal bactericide concentration corresponds to the lowest concentration of plant extract that is able to kill 99.99% of the bacterial strains tested. The two plant extracts showed no bactericide activity on the tested bacteria (MBC 0%).

One of the undisputed functions of flavonoids and related polyphenols is their role in protecting plants against microbial invasion. The presence of a phenolic group in a natural flavonoid would be expected to provide antimicrobial activity and the addition of further phenolic groups might be expected to enhance this activity [19]. Our finding is in agreement with other study; [31] assumed that flavonoids lacking the free hydroxyl groups have more antimicrobial activity compared to those who are filled, which leads to an increase of the chemical affinity for membrane lipids.

In general, the gram positive bacteria were found to have more susceptibility as compared to gram negative bacteria species. This is in line with early studies which attribute the observed differences to the variation in chemical composition and structure of cell wall of both types of microorganisms [33, 34]. The Gram-bacterial resistance against antibacterial agents is related to the hydrophilic character of their outer membrane which is rich in lipopolysaccharide molecules, that serves as a barrier to the penetration of these antibacterial agents.

CONCLUSION

Our study revealed that the highest amount of total phenolic and flavonoids were observed in Biskra plant extract (206.54 ± 58.17 mg GAE/g extract and 7.30 ± 0.90 mg QE/g extract respectively).

In this report, the DPPH and FRAP methods were used for the determination of antiradical activity and vitamin C as standard. The DPPH test is already reported to evaluate in relatively short time the antiradical activity. Setif plant extract showed low antioxidant activity (23.32% ± 0.32 of DPPH inhibition), whereas the strong antiradical property was estimated in Biskra plant extract (64.46% ± 17.21 of DPPH inhibition). Meanwhile, ascorbic acid possess strongest antiradical activity (92.31% ± 0.21 of DPPH inhibition). Indeed, the results of the present study confirm the high reducing power in the two extracts from Biskra and Setif populations which exceed 62.58% ± 7.37 and 63.43% ± 3.45 of reducing ability respectively.

The results of this study indicate that the polyphenolic content and the antioxidant activity significantly contributes to of Teucrium polium extracts. In addition, the contribution of phenolics to the antioxidant activity in the two samples confirms their important role in the bioactivity of the plant material and its physiological response to abiotic stress.

Antibacterial activity was also detected in the two plant extracts against the bacterial strains tested Staphylococcus aureus (ATCC 12228), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853) and Bacillus megaterium (ATCC 14581). The extract of Biskra plant revealed antimicrobial properties. Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853) were the most sensitive among four tested Gram-negative and Gram-positive bacteria: inhibitory zones were 10.0 ± 2.64 to 11.0 ± 1.00 mm respectively.

In conclusion, while plant of Teucrium polium seem to be the appropriate for industrial use as sources of natural antioxidants and antimicrobial agents.

REFERENCES