CHEMICAL COMPOSITION, ANTIMICROBIAL, AND ANTIOXIDANT ACTIVITIES OF TWO MOROCCAN THYMUS ESSENTIAL OILS

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ABSTRACT

Objective: The aim of the present study is to investigate chemical constituents of Thymus riatarum and Thymus blecherianus essential oils (EOs) as well as their antioxidant and antibacterial activity against six bacterial strains responsible for nosocomial infections.

Methods: The chemical composition of EOs was analyzed using gas chromatography (GC) and GC-mass spectrometry, the antibacterial capacity of the two thymus species was evaluated against six bacteria species: Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus, Acinetobacter baumannii, and Citrobacter sp. using disk diffusion method and microdilution assay. Finally, the antioxidant activity was measured by four different test systems of assay, namely free radical scavenging activities, ferric reducing/antioxidant power assays, total phenolic, and flavonoid contents.

Results: A total of 15 compounds representing 99.6% of the total oil composition; the most abundant constituents were as follows: Carvacrol (45.9%), bornyl acetate (20.1%), and α-phellandrene (13.0%) as the main constituents. Eleven components of T. blecherianus were identified representing 98.2% of the total oil composition; the most abundant constituents were as follows: Carvacrol (45.9%), bornyl acetate (20.1%), and borneol (15.7%). Strong antibacterial activity of the two EOs was identified against all bacterial strains tested. Concerning the antioxidant results, T. riatarum EO exhibited higher antioxidant activity than T. blecherianus in the assays with an IC₅₀ value equal to 5.75±0.06 mg/ml, which was probably due to its high content of polyphenols (28.95±0.13 mg GAE/g DW). Total flavonoid content was found equal for the two EOs.

Conclusion: EO of T. riatarum and T. blecherianus from Morocco can be exploited as a natural antibacterial and antioxidant new potential sources.

Keywords: Thymus riatarum, Thymus blecherianus, Antibacterial activity, 2,2-Diphenyl-1-picrylhydrazyl, Ferric reducing/antioxidant power, polyphenol, Flavonoids.

INTRODUCTION

Herbs and species are natural plant products which have been used for thousands of years for flavoring, food preservation, and as a source of traditional medicines [1]. Therefore, the demand of aromatic plants is increasing through the world for their content of phytochemicals that can alleviate illness, antimicrobial, and antioxidant [2-6]. Antioxidant effect of plant essential oils (EOs) has been used as food preservation, natural therapies, functional food to promote health, and reduce oxidative stress and has a major role in the treatment of many human chronic diseases related to degenerative disorders, cardiovascular, diabetes, cancer, arthritis, and cancer, by acting as free radical scavengers [7-9]. In the other hand, the resistance of antibiotic provokes an increasing interest in evaluating the antimicrobial effects of plant secondary metabolites against many pathogens, to identify and evaluate natural products to assure consumers a safe, wholesome, and nutritious food supply [10].

The genus Thymus (Family Lamiaceae) comprises about 215 species of herbaceous perennials and subshrubs [11]. While the Mediterranean region has been recognized as the center of this genus [13], twenty one species of thyme have been identified in Morocco, twelve are endemic [12]. Some of these species have been used for their preservative and medicinal properties [2] and have been added to foods (condiment and spice) [14] their EOs has found applications in cosmetic, such as toothpastes and deodorants [15]. Previous studies have demonstrated that Thymus species have strong insecticidal, antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, and antispasmodic activity [11,16,17]. Different reports have shown that thymol and carvacrol are the main components of thymus EOs which are responsible for their high biological activities [18-21]. Among this genus, Thymus blecherianus and Riatarum locally called “zaatar” are the most important species from medicinal and aromatic properties and used worldwide for its antimicrobial activities.

To the best of our knowledge, there are no reports about antioxidant properties and antibacterial activity against bacteria responsible for nosocomial infections for the two thymus species (Blecherianus and Riatarum). Therefore, the objective of the present study is to investigate the chemical constituents of T. riatarum and T. blecherianus EOs as well as to evaluate, for the first time, their antioxidant and antibacterial activities.

METHODS

Plant material
Fresh leaves of T. blecherianus and T. riatarum were collected in Morocco at flowering stage (March 2016) from the region of Taza. They were identified by Professor Amina Bari, botanist Department of Biological Sciences, Faculty of Science, Sidi Mohammed Ben Abdellah University, Fez (Morocco). The leaves were dried for 7–10 days in the shade and then stored in cloth bags at 5°C until extraction.

Preparation and analysis of EO
Dried shoot was hydro-distilled for 3 h with 500 ml of water using a Clevenger-type apparatus, according to the European...
Pharmacopoeia [22]. The EOs obtained were dried over anhydrous sodium sulfate and stored in a refrigerator at 4°C before analysis. The oil yield was based on dry weight of the simple.

Gas chromatography-mass spectrometry analysis (GC–MS)

The EOs compounds were analyzed on a Thermo Fischer capillary gas chromatography directly coupled to a mass spectrometer system (model GC ULTRA S/NI 20062969; Polaris Q/NI 210729), HP-5MS nonpolar fused silica capillary column (60 m × 0.32 mm, 0.25 μm film thickness). The temperature was maintained at 40°C for 2 min, then increased at a programmed rate of 2°C per min to a final temperature of 260°C, which was maintained for 1 min; injector temperature 250°C. The helium was used as carrier gas at a flow rate of 1 ml per min. Each sample was run in hexane with a dilution ratio of 10:10. Compounds were identified by matching their MS and retention index with those reported in literature [23]. The injected specimen volume was 1 μl of diluted oil; split injection technique; ionization energy 70eV in the electronic ionization mode; ion source temperature 200°C, scan mass range m/z 40–650; and interface line temperature 300°C. The EO components were identified by comparing their retention times and mass fragmentations with NIST-MS and literature comparison [24].

Bacterial strains

Five Gram-negative bacteria (Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, and Citrobacter sp.) and one Gram-positive bacteria (Staphylococcus aureus). These bacterial strains which are responsible for nosocomial infections were isolated in a hospital environment from clinical patients in reanimation service (CHU, Morocco).

Antibacterial activity assessment

The antibacterial power of T. riatarum and T. blecherianus EOs was determined using the agar disk diffusion procedure was adapted from a method used earlier [25]. Each microorganism stock was suspended in Mueller–Hinton (MH) broth and incubated at 37°C for 18–24 h. The overnight cultures were diluted and adjusted to get a density of 1–5 × 10^8 CFU/ml (0.5 McFarland turbidity standards). They were flood-inoculated onto the surface of MH agar plates and 6 mm diameter, sterile filter discs of Whatman paper N°3, impregnated with 15 μg/disc of the EO and were delivered onto the inoculated agar MH. All plates were incubated for 18 h at 37°C. Antibacterial effect was tested by measuring the zones of inhibition. The antibiotic standards used were imipenem, ampicillin, pristinamycin, and ceftriaxone [26–28]. The tests were carried out in triplicates.

Determination of minimal inhibitory concentration

The minimum inhibitory concentration (MIC) was performed using a microdilution assay in 96-well plates according to the experiment of the National Committee for Clinical Laboratory Standards [29] with some modification; the different concentrations of thymus EO are prepared in a modification; the different concentrations of thymus EO are prepared in a microdilution assay in 96-well plates. The EO was added to the wells to determine MIC values. Finally, all plates were incubated at 37°C for 18–24 h. The MIC was determined by comparing the absorbance of the sample to the negative control (0.5 of absorbance) and the total was made up to 10 ml with distilled water. The solution of the EO and were delivered onto the inoculated agar MH. All plates were incubated for 18 h at 37°C. Antibacterial effect was tested by measuring the zones of inhibition. The antibiotic standards used were imipenem, ampicillin, pristinamycin, and ceftriaxone [26–28]. The tests were carried out in triplicates.

Reducing power capacity

The reducing capacity of the tested EOs was determined in accordance with the procedure of Oyaizu [34]. 100 μl of EO was mixed with 500 μl of phosphate buffer (0.2M, pH 6.6) and 500 μl of potassium ferricyanide [KFe(CN)₆] 1%. The obtained solution was incubated for 20 min at 50°C. The mixture was oxidized with 500 μl of trichloroacetic acid 1%, which was then centrifuged for 10 min at 3000 rpm. The upper layer of the solution (2.5 ml) was mixed with 500 μl of distilled water and 100 μl of FeCl₃ (0,1%) and the absorbance was measured at 700 nm (Jasco v-530). Quercetin was used as a standard. The results were expressed as I_{EC} (mg/ml). I_{EC} (concentration corresponding 0.5 of absorbance) was calculated by plotting absorbance against the corresponding concentration.

Total phenolic content (TPC)

TFC of the volatile extracts was determined by the Folin–Ciocalteu method [35]. The 0.5 ml of a known dilution of the EO and 2 ml of 7% sodium carbonate solution were added to 2.5 ml of 10% (v/v) Folin–Ciocalteu reagent. The absorbance was read at 760 nm (Jasco v-530) after 2H reaction at room temperature in the dark. Gallic acid was used as a standard for the construction of a calibration curve. Total phenolics contents were expressed as milligrams of gallic acid equivalents per gram of EO (mg GAE/g EO).

Total flavonoids contents (TFC)

TFC of volatile extracts were measured by the aluminum chloride colorimetric assay [36]. 1ml 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% NaNO₂ was added, after 5 min, 0.3 ml 10% AlCl₃ was added to react for 6 min. After that, 2 ml 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. TFC were expressed as milligrams of rutin equivalents per gram of EO (mg RE/g EO).

RESULTS AND DISCUSSION

EOs composition

The EO obtained from leaves of T. blecherianus and T. riatarum was yellow in color with a yield of 2% and 0.5% (v/w), on dry weight basis, respectively. The obtained yield is higher than the results of studies of the same species with a yield of 1.75% and 0.26% for T. blecherianus and T. riatarum, respectively [37,38].

The analysis of two EOs was carried out using GC and GC-MS (Table 1). 15 compounds constitute 99.6% of the total T. riatarum EO. The main compounds identified were thymol (28.8%), borneol (20.0%), and α-phellandrene (13%). A different composition has been reported recently for the same species, with the main compounds were bornol (41.67%), terpinene-4-ol (8.65%), and trans-caryophyllene (7.59%) [38]. Another study of this EO demonstrated that the major constituents were carvacrol (22.3%), p-cymene (17.5%), and α-terpinene (10.3%) [39]. Furthermore, Belmalha et al. revealed also the chemical composition of T. riatarum with 33 compounds identified (87.71%); borneol (31.3%) as the most abundant compound [40].

Regarding the EO compositions of T. blecherianus characterized by 11 compounds amounted to 98.2%. The oil was dominated by carvacrol (45.9%) followed by bornyl acetate (20.1%) and borneol (15.7%).
Amarti et al. revealed a different chemical composition for the same species, which the main constituents are α-terpinene (42.20%) and thymol (23.90%) [37]. The difference in the composition of EOs of the same species was attributed to various factors such as climate conditions, geographical location, harvesting period, and distillation method [41].

**Antibacterial activity**

The antibacterial effect of the EOs of *T. blecherianus* and *T. riatarum* was tested against six bacteria strains: *E. coli; P. aeruginosa; K. pneumonia; S. aureus; A. baumannii, and Citrobacter* sp. responsible for nosocomial infections in Center Hospital of Fez Morocco.

The results from the antibacterial activity tested by diffusion in agar disc method are summarized in Table 2. All bacterial strains were susceptible to Thymus EOs studied. According to our results (Table 2), the two EOs were generally more effective against Gram-positive than Gram-negative bacteria; the higher antibacterial effect of EOs of *T. blecherianus* and *T. riatarum* was observed against *S. aureus* strain with 20.1±0.1 mm for *T. blecherianus* and 21±0.5 mm for *T. riatarum*. However, the lowest inhibition was showed against *A. baumannii* and *Citrobacter* sp. respectively. On the other hand, the antibacterial effect of two thymus EOs showed stronger activity when compared to all standard antibiotics used as positive controls except IMP.

The antibacterial activity evaluation using the microdiffusion method (Table 2) showed that *T. blecherianus* and *T. riatarum* EOs are efficient at various concentrations ranging from 0.019 to 0.156 mg/ml. As presented in Table 2, the highest effective concentration was 0.019 mg/ml against *S. aureus* for two EOs and also against *K. pneumoniae* for only *T. riatarum*.

Our results are highest than those reported by El Bouzidi S. aureus in (2014). However, the lowest inhibition was showed against *A. baumannii* and *Citrobacter* sp. respectively. On the other hand, the antibacterial effect of two thymus EOs showed stronger activity when compared to all standard antibiotics used as positive controls except IMP.

Table 2: Volatile compounds (%) of EOs of studied *Thymus* species

| Component       | RI   | Tr (%) | Tb (%) |
|-----------------|------|--------|--------|
| a- thujene      | 930  | 2.6    | 1.4    |
| α-pinene        | 943  | 7.3    | 0.9    |
| Camphene        | 957  | 2.1    | 1.3    |
| α-Phellandrene  | 1003 | 13.0   | 0.6    |
| 2-carene        | 1007 | 0.8    | -      |
| 3-carene        | 1020 | 1.2    | 3.1    |
| Borneol         | 1170 | 2.0    | 15.7   |
| Bornyl acetate  | 1289 | 4.9    | 20.1   |
| Thymol          | 1297 | 28.8   | 5.0    |
| Carvacrol       | 1305 | 2.3    | 45.9   |
| Acetyl thymol   | 1357 | 4.6    | -      |
| Eugenol         | 1375 | 7.7    | 3.1    |
| Longifolene     | 1403 | 1.1    | -      |
| Caryophyllene   | 1420 | 1.7    | 1.1    |
| Caryophyllene oxide | 1457 | 1.5    | -      |
| Total           | 99.6 | 98.2   |

RI: Retention index, Tr: *Thymus riatarum*, Tb: *Thymus blecherianus*, EOs: Essential oils.

**Antioxidant activity**

**DPPH radical scavenging activity**

The antioxidant effect was often tested by the DPPH scavenging activity assay. Table 3 depicts DPPH results of *Thymus blecherianus* and *riatarum* EOs. As can be seen, *T. riatarum* had the greatest radical scavenging capacity with an IC₅₀ value of 5.75±0.06 mg/ml followed by *T. blecherianus* (IC₅₀ = 6.87±0.3 mg/ml). However, when compared to the pure reference antioxidant BHT (0.11±0.02 mg/ml), all the tested EOs showed a significantly lower antioxidant activity (p<0.05).

**Ferric reducing/antioxidant power (FRAP) capacity**

The reducing power capacity is a useful method for measuring antioxidant activities [51]. In this assay, hydroxyl radicals are generated by the reaction of Fe²⁺ and H₂O₂, and the antioxidant agents reduce the generation of hydroxyl radicals by chelating Fe³⁺ [52]. The results of this activity (Table 3) showed that the EOs of the two plants have a comparable reducing capacity against the ferric ion; 14.73±0.25 mg/ml for *Thymus blecherianus* and 11.86±0.15 mg/ml for *T. riatarum* which is lower than *T. serpyllum* and *T. algeriensis* EO (IC₅₀ = 0.66 µg/ml and 0.68 µg/ml, respectively) [49] and lowest than wild and cultivated *T. maroccanus* with IC₅₀ = 139.31±1.08 and 149.41±1.13 µg/ml, respectively [17]. Tóth et al. demonstrated that the reducing ability of the Thymus species was observed to increase with increasing EO concentration [53]. Previous studies have also reported the antioxidant activity of EOs from different Thymus species [48,49]. The antioxidant activity of Thymus oils is often attributed to the presence of thymol, carvacrol, phenolic acids, and flavonoids [54].

**TTC and TFC**

It is generally demonstrated that the antioxidant activity of plant EOs is correlated with the TTC and TFC [51]. Therefore, to determine the antioxidant activity of *Thymus blecherianus* and *riatarum* EOs, we analyzed the TTC and TFC of each test sample and results are presented in Table 4.

Table 4: Antimicrobial activity of *Thymus blecherianus* and *riatarum* EOs using disk diffusion assay and antibiotic standards

| Bacterial species | Thymus blecherianus EOs (15 µl/disc) | Thymus riatarum EOs (15 µl/disc) | Antibiotic standards |
|-------------------|-------------------------------------|----------------------------------|----------------------|
|                   | DD (mm) | CMI (mg/ml) | DD (mm) | CMI (mg/ml) | DD (mm) |
| *Escherichia coli*| 12±0.2  | 0.56        | 15±0.8  | 0.078      | 18 (IMP), 5 (AMP), 0 (CEC), 12 (PT) |
| *Klebsiella pneumoniae*| 14±0.8  | 0.078      | 18±0.6  | 0.019      | 30 (IMP), 0 (AMP), 12 (CEC), 10 (PT) |
| *Staphylococcus aureus*| 20.1±0.1 | 0.19     | 21±0.5  | 0.19       | 39 (IMP), 0 (AMP), 10 (CEC), 16 (PT) |
| *Pseudomonas aeruginosa*| 16±0.5  | 0.078      | 17±0.5  | 0.039      | 28 (IMP), 8 (AMP), 0 (CEC), 10 (PT) |
| *Acinetobacter baumannii*| 10±0.7  | 0.56      | 14±0.5  | 0.078      | 22 (IMP), 0 (AMP), 5 (CEC), 6 (PT) |
| *Citrobacter sp.*   | 12±0.5  | 0.078      | 15±0.9  | 0.078      | 17 (IMP), 8 (AMP), 15 (CEC), 8 (PT) |

MIC: Minimum inhibitory concentration, DD: Disk diffusion assay, diameter of inhibition zone including disk diameter of 6 mm, EOs: Essential oils.
Table 3: DPPH radical scavenging activity and ferric reducing power capacity of different EO from *Thymus blecherianus* and *Thymus riatarum*

| EO                     | DPPH IC₅₀ (mg/ml) | FRAP IC₅₀ (mg/ml) |
|------------------------|-------------------|-------------------|
| *Thymus blecherianus*   | 6.87±0.30a        | 14.73±0.25a       |
| *Thymus riatarum*       | 5.75±0.06a        | 11.86±0.15a       |
| BHT                    | 0.11±0.002c       | 0.03±0.006c       |
| Quercetin              |                   |                   |

All the results were expressed as mean±SD of three different trials. In each line, values followed by different letters are significantly different (p<0.05).

Table 4: Total phenolic and flavonoid contents of EO from *Thymus riatarum* and *Thymus blecherianus*

| EO                     | TPC (mg GAE/g) | TFC (mg RE/g) |
|------------------------|---------------|---------------|
| *Thymus blecherianus*   | 17.63±0.06a   | 24.17±0.21a   |
| *Thymus riatarum*       | 28.95±0.13a   | 24.20±0.23a   |

Data are reported as mean values±SD of three measurements. Means were significantly different when p<0.05; values followed by different letters are significantly different. TPC: Total phenolic content, TFC: Total flavonoid content, EOs: Essential oils.

TPC was determined in comparison with standard gallic acid, and the results were expressed in terms of mg GAE/g of sample. As we can see, *T. riatarum* demonstrated the highest phenolic content (28.95±0.13 mg GAE/g Eo).

On the other hand, TFC results were expressed in terms of mg RE/g weight extract. As shown in Table 4, the total flavonoid content has been found equal for the two spices of thyme.

The results of total phenols in our study were found to be higher than the values reported in literature for *Thymus* species such as *T. capitatus* (15.06±0.73 mg GAE/g) obtained by Jabri-Karoui et al. [55], *T. vulgaris* (8.55 mg QE/g) reported by Tohidi et al. [53], and *T. daenensis* subsp. *Daenensis celak* ranged from 18.82 to 18.97 (mg GAE/g) obtained by Aliazad et al. [56]. However, our total phenolic results are lowest than *T. numidicus* with 90.66±3.17 mg EAG/g reported by Ali et al. [57] and *T. satureioides* (69.05±0.01 mg GAE/g) reported by Wang et al. [58].

The observed antioxidant potential should be addressed to the phenolic oil constituents such as thymol and carvacrol [59].

Concerning TFC, *T. capitatus* showed a flavonoid content value of 10.62±0.24 mg QE/g [55]. Baharfar et al. recorded that the flavonoids content value of *T. kotschyanus* ranged from 32.04 to 74.60 mgQE/g [60]. It has reported that flavonoids have an interesting role in scavenging reactive oxygen species, which can counteract lipid oxidation in vitro and improve the body’s antioxidant enzyme activity, and decrease peroxide formation in vivo [61].

**CONCLUSION**

Our study can be considered as the first report on the antibacterial effect against nosocomial bacteria species and antioxidant capacity basing on free radical scavenging activity (DPPH), FRAP, TPC, and TFC of *T. riatarum* and *Blecherianus*. The tested EOs demonstrated a strong antibacterial and antioxidant activities which are conferred by high thymol and/or carvacrol content. Thus, this study suggests the possibility of using the oils of these *Thymus* species as natural antioxidant and food preservatives, as well as pharmaceutical and natural therapies.

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**AUTHORS’ CONTRIBUTIONS**

All the authors have contributed equally.

**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest regarding the publication of this paper.

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