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Research Article

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 to evaluate, for the first time, their antioxidant effect 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 *T. riatarum* oil were identified with thymol (28.8%), borneol (20.0%), 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 three assays with an IC_{50} 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.

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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 insecticide, 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 proprieties 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 (Mars 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 chromatograph directly coupled to a mass spectrometer system (model GC ULTRA S/N 20062969; Polaris QS/N 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 10 min; injector temperature 250°C. The helium was used as carrier gas at a flow rate of 1ml per min. Each sample was run in hexane with a dilution ratio of 10:100. 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 baumani*, 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 × 10^6 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 suspension containing 0.2% agar in sterile distillate water to disperse the compounds without adding solvent or detergent [30]. They are carried out by successive dilutions 1/2 ranging from 20 to 0.039 mg/ml. The concentrations obtained in the well were between 5 and 0.0097 mg/ml. The bacterial suspensions were prepared in the same manner described previously and diluted in MH broth and plated in 96 well plates at a density of 1–5 × 10^6 CFU/ml. Thymus EO was added at different concentrations at the corresponding wells to determine MIC values. Finally, all plates were incubated at 37°C for 18–24 h, bacterial growth was visually by adding to each well 20 µl of 2,3,5-triphenyl tetrazolium chloride aqueous solution (1%), with additional incubation for 1 h. MIC was the lowest concentration that does not produce red color [31].

Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH test was introduced 50 years ago by Blois [32]. The ability of EOs to scavenge the DPPH radical was measured using the method described by Wu *et al.* [33]. 0.1 ml of EOs or standard was added with 1.5 ml of an ethanolic solution containing 0.1 mmol of DPPH. After 30 min of incubation time at room temperature in the dark, the absorbance of the mixture was measured at 517 nm with a spectrophotometer (Jasco V-530). The inhibition percentage was calculated by the following equation:

$$I\ (\%) = (1-(A_s/A_0))*100$$

Where A_0 and A_s were the absorbances of the negative control and the sample, respectively. Butylated hydroxytoluene (BHT) served as positive control. The IC_{50} values were defined as the concentration of causing a 50% inhibition of DPPH radical.

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 $[K_3Fe(CN)_6]$ 1%. The obtained solution was incubated for 20 min at 50°C. The mixture was acidified with 500 µl of trichloroacetic 10%, 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 $FeCl_3$ (0, 1%) and the absorbance was measured at 700 nm (Jasco v-530). Quercetin was used as a standard. The results were expressed as IC_{50} (mg/ml). IC_{50} (concentration corresponding 0.5 of absorbance) was calculated by plotting absorbance against the corresponding concentration.

Total phenolic content (TPC)

TPC 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 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 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% $NaNO_2$ was added, after 5 min, 0.3 ml 10% $AlCl_3$ 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). All samples were analyzed in triplicate.

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 then 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 borneol (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 University 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 microdilution 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. pneumonia* for only *T. riatarum*.

Our results are highest than those reported by El Bouzidi *et al*. about the effective activity of wild and cultivated EO of *T. maroccanus* against *S. aureus* with MIC = 0.46 and 0.96 mg/ml, respectively [17]. Rota *et al*. showed an increased sensitivity of *S. aureus* for the Thymol chemotype of *Thymus zygis* ssp. Gracilis [42]. However, Kaloustian *et al*. found that *E. coli* is more susceptible than *S. aureus* to Thyme [43]. Previous studies are also demonstrated that *T. riatarum* and *T. numidicus* EO exhibited a significant antibacterial activity which *S. aureus* showed higher inhibition [38,44]. This great antibacterial activity of thyme EO can be explained by the presence of carvacrol and thymol [45,46], it is possible that other minor constituents are also contributing to the activity, as well as the synergistic impact of their combination in EO [47].

Table 1: Volatile compounds (%) of EOs of studied *Thymus* spices

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	20.0	15.7
Bornyl acetate	1289	4.9	20.1
Thymol	1297	28.8	5.0
Carvacrol	1305	2.3	45.9
Acethyl 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 *T. blecherianus* and *riatarum* EOs. As can be seen, *T. riatarum* had the greatest radical scavenging capacity with an IC_{50} value of 5.75±0.06 mg/ml followed by *T. blecherianus* (IC_{50}= 6.87±0.3 mg/ml). However, when compared to the pure reference antioxidant BHT (0.11±0.002 mg/ml), all the tested EOs showed a significantly lower antioxidant activity ($p<0.05$). *T. bleherianus* EOs showed the lowest activity than that founded by Amarti *et al*. (IC_{50} = 77.8 µg/ml) [48], and also lowest than *T. serpyllum*, *Thymus algeriensis,* and *Thymus vulgaris* with IC_{50}= 0.96 µg/ml, IC_{50} = 1.64 µg/ml, and IC_{50} = 4.80 µg/ml, respectively, reported by Nikolić *et al*. [49].

Recently, the demand for natural antioxidants for food conservation is one of the important trends in the food industry. Synthetic antioxidants such as BHT are toxic and carcinogenic [50]. This is why the use of EOs as natural antioxidants has attracted increasing interest in recent decades.

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 Fe^{2+} and H_2O_2, and the antioxidant agents reduce the generation of hydroxyl radicals by chelating Fe^{2+} [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 *T. blecherianus* and 11.86±0.15 mg/ml for *T. riatarum* which is lowest than *T. serpyllum* and *T. algeriensis* EO (IC_{50} = 0.66 µg/ml and 0.68 µg/ml, respectively) [49] and lowest than wild and cultivated *T. maroccanus* with IC_{50} = 139.31±1.08 and 149.41±1.13 µg/ml, respectively [17]. Tohidi *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].

TPC and TFC

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

Table 2: Antimicrobial activity of *Thymus blecherianus* and *Thymus 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	0.156	15±0.8	0.078	18 (IMP), 5 (AMP), 0 (CEC), 12 (PT)
Klebsiella pneumoniae	14.3±0.8	0.078	18±0.6	0.019	30 (IMP), 0 (AMP), 12 (CEC), 10 (PT)
Staphylococcus aureus	20.1±0.1	0.019	21±0.5	0.019	39 (IMP), 0 (AMP), 10 (CEC), 16 (PT)
Pseudomonas aeruginosa	16±0.5	0.078	17.6±0.8	0.039	28 (IMP), 8 (AMP), 0 (CEC), 10 (PT)
Acinetobacter baumannii	10.6±0.7	0.156	14.3±0.1	0.078	22 (IMP), 0 (AMP), 5 (CEC), 6 (PT)
Citrobacter sp.	12±0.5	0.078	15.5±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*

	DPPH IC_{50} (mg/ml)	FRAP IC_{50} (mg/ml)
Thymus blecherianus	6.87±0.30[a]	14.73±0.25[a]
Thymus riatarum	5.75±0.06[b]	11.86±0.15[b]
BHT	0.11±0.002[c]	-
Quercetin	-	0.03±0.006[c]

All the results were expressed as mean±SD of three different trials. In each line, values followed by different letters are significantly different ($p<0.05$). FRAP: Ferric reducing/antioxidant power, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, BHT: Butylated hydroxytoluene, EOs: Essential oils

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±0.06[a]	24.17±0.21[a]
Thymus riatarum	28.95±0.13[b]	24.20±0.23[a]

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 Alizadeh *et al*. [56]. However, our total phenolic results are lowest than *T. numidicus* with 98.66±3.17 mg EAG/g reported by Ali *et al*. [57] and *T. satureioides* (69.05±0.01 mg/g GAE) 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 flavonoid contents 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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