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EFFECT OF EXTRACTION MODE ON PHENOLICS CONTENTS AND BIOLOGICAL ACTIVITIES OF TWO SPECIES OF THYME EXTRACTS.

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Abstract

Description of the subject: The extraction of bioactive compounds from plants was influenced by several parameters like the mode of extraction and solvents used. Our interest in this report turns up from these parameters.
Object: The areal part of *Thymus Capitatus* and *Thymus fontanesii*, were subjected to a different mode of extractions, then the phenolics compounds were measured after that the biological activities were evaluated.
Methodology: The decoction, infusion, hydro-alcoholic and acidified hydro-alcoholic modes, and solvents were used for the extraction. The quantification of phenolics compounds was done by spectro-photometric tests. Antioxidant activities were done by scavenging of DPPH, reducing power test and total antioxidant capacity assay, furthermore, the antimicrobial activities were realized by disc diffusion and agar dilution methods.
Results: For both species, total phenolics and flavonoid contents were higher for the hydro-methanolic extracts. Concerning the antioxidant activities, the decoction of *T. capitatus* was the most active extract in DPPH and reducing power tests, but at the total antioxidant capacity, *T. fontanesii* acidified hydroalcoholic extract, has given the higher content. In the end, the hydroalcoholic extract of both species was more active than the aqueous extracts. **Conclusion:** The data from this study support the idea that each solvent and mode of extraction chosen has its characteristic effects on the yield of extraction, polyphenol quantities, and biological activities.
Keywords: *T.capitatus, T.fontanesii*, mode of extraction, antioxidant antimicrobial activities.

EFFET DUMODE D'EXTRACTION SUR LES CONTENUS PHÉNOLIQUES ET LES ACTIVITÉES BIOLOGIQUES DES EXTRAITS DE DEUX ESPÈCES DU *THYM*

Résumé

Description du sujet : L'extraction des composés bioactifs des plantes sont influencée par plusieurs paramètres comme le mode d'extraction et les solvants utilisés. Notre intérêt pour ce rapport découle de ces paramètres.
Objective : La partie areine de *Thymus Capitatus* et *Thymus fontanesii*, a été soumise à des modes d'extraction différent, puis les composés phénoliques ont été mesurés après quoi les activités biologiques ont été évaluées.
Méthodologie : La décoction, l'infusion, l'hydro-alcoolique et hydro-alcoolique acidifié ont été utilisés comme modèle pour l'extraction. Le dosage des poly-phénols a été accomplie par des tests spectro-photométrique. Le piégeage du DPPH, le test du pouvoir réducteur ainsi que la capacité antioxydant totale comme activité antioxidant. De plus, l'activité antimicrobienne réalisée par méthode de diffusion sur disque et aussi la dilution en gélose.
Résultats : Pour les deux espèces, les teneurs totales en composés phénoliques et en flavonoïdes étaient plus élevées pour les extraits hydro-méthanoliques. Concernant les activités antioxydants, le décocté de *T. capitatus* a été l'extrait le plus actif dans les tests de DPPH et du pouvoir réducteur, mais au pour le test de la capacité antioxydant totale, l'extrait hydroalcoolique acidifié de *T. fontanesii*, a donné la teneur la plus élevée. En fin, l'extrait hydroalcoolique des deux espèces a été plus actif que les extraits aqueux quant à l'activité antimicrobiens.
Conclusion : Les données issues de cette étude soutiennent l'idée que chaque solvant et mode d'extraction choisi, a ses effets caractéristiques sur le rendement d'extraction, les quantités de polyphénols et les activités biologiques.
Mots clés:*T. capitatus, T. fontanesii*, mode d'extraction, activités antimicrobiennes antioxydants.

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INTRODUCTION

The medicinal power of *al*. plants resides in phytochemical constituents that were the origin of pharmacological effects on the human body. Due to producing many benefits to society, especially in the line of medicine and pharmacology thus, medicinal plants have taken a lot of interest more than ever [1]. However, interest is growing internationally for herbal products, such as essential oils, or phenolic compounds to replace the synthetic anti-oxidants based on their emerging deleterious side effects, and due to an upsurge in antibiotic-resistant infections. Thus the search for new prototype drugs to combat infections is an absolute necessity [2]. Plant foods provide abundant natural bioactive compounds that possess biological activities, which have many proven health-promoting activities like antioxidant, antibacterial, antihypertensive, anti-inflammatory, etc…, where phenolics compounds are one of the most widely occurring groups of phytochemicals and are of considerable physiological and morphological importance for plants [3]. Recently there is an increasing interest among food researchers to distinguish antimicrobial and antioxidant compounds that have a natural origin and are safe to use. Numerous flavors and herbs are accounted to be suitable sources of antimicrobial and antioxidant agents. Over recent years, a number of studies have demonstrated that polyphenols present in dietary and herbal products hinder oxidative stress [4]. Differences in the structure of phenolic compounds also determine their solubility in solvents of different polarities and modes of extraction as well as the isolation procedures may have a significant impact on the yield of extraction polyphenols from plant material, thus the biological activities [3]. There are several methods established for the extraction of polyphenols from plant materials. Those methods vary in solvents and conditions used. The extraction method is essential for the accurate quantification of antioxidant content and capacity. This fact makes it hard to compare data from literature reports and identified a group of factors that influence the quantification of phenolics in plant materials [5]. The chemical nature of the phenolic compounds, the extraction method employed and the assay method were some of those factors. There are some reports concerning the optimization of extraction conditions of phenolic compound content and antioxidant activities of some plant foods but as some research indicated the optimal procedure is usually different for different plant matrices [3]. In Algeria, the empirical use of plants continues to maintain high popularity. The Algerian people are sometimes preying quackeries ignorant and dangerous to patients. Many plants are known for their therapeutic properties, especially for their antiseptic, antibacterial and antioxidant effect, like Rosemary, Sage, Thyme, Garlic, Anise, Chamomile, Eucalyptus, Grenadier, mild Orange, etc…[6]. The genus *Thymus* is a perennial herbaceous plant and shrub that are woody at the base and contain numerous branches. The leaves are about 3 to 8 mm long. The white pale purple, tubular, two-lipped flowers are arranged in whorled clusters. There are over 300 species of this hardy genus that are native to southern Europe and Asia [7], Over 11 species of this genus grow in Algeria [8]. Where *Thymus capitatus* Hoff, and *Thymus fontanesii* Boiss & Reut are two *Thymus* species growing wild in Algeria, several works were carried out on these two species [9-14]. Taking into account that, no extracting solvents or mode of extracting was the best in all of the parameters, Hence, the aim of this study was to evaluate the fluctuations in total phenolics content (TPC), and flavonoid content (FC) in extracts of *Thymus capitatus* Hoff. and *Thymus fontanesii* Boss et Reut, aerial parts, according to the different conditions (solvents and mode) of extraction and determination also the effect of those conditions on antioxidant, and antimicrobial activities, and compared the two species each other in order to provide a scientific basis for optimal extraction of *T. capitatus* and *T. fontanesii* actives compounds.

MATERIALS AND METHODS

1. Plants materials

The plants of this study were collected join 2016, at Azaba of Skikda City (36° 52' 0.001" N 6° 54' 0" E) for *T. capitatus* and at Sour El-Ghozlane of Bouira city (36° 8' 57.203" N 3° 41' 24.559" E), for both spices, the identified were done by Pr. Ben-Houhou from ENA El-Harrache Algiers Algeria.

2. Extraction procedure

The protocol of extraction was reported by Martins et *al*. [15]. Decoctions (TC1 and TF1), were performed by adding 200 mL of distilled water to the sample (1 g), heating (heating plate, VELP scientific), and boiling for 5 min.

The mixtures were left to stand for 5 min and then filtered under reduced pressure. The Infusions extracts (TC2 and TF2) were prepared by adding 200 mL of boiling distilled water to the sample (1 g) and were left to stand at room temperature for 5 min, and then filtered under reduced pressure. For both decoction and infusion, filtrates were evaporated at 30 ºC under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and the yield of extraction was calculated, after what the extracts were re-dissolved in distilled water to obtain a stock solution of 20 mg/mL. The hydroalcoholic extractions (TC3 and TF3) were performed by stirring the plant material (1g) with 30 mL of methanol/water (80:20, v/v) at 25 ºC and 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 30 mL portion of the hydroalcoholic mixture. The combined extracts were evaporated at 35 ºC under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland). The same steps were done for acidified hydroalcoholic (TC4 and TF4) extracts obtained by adding acetic acid (80:15:5 v/v/v). Hydro-alcoholic and acidified hydroalcoholic extracts were re-dissolved in methanol/water (80:20, v/v) and methanol/water/ acetic acid for the last. To obtain a stock solution of 20 mg/mL, the yield of extraction was calculated.

3. Assessment of phenolics contents

3.1. Total phenol contents

The total phenols contents (TPC) of extracts were determined spectrophotometrically, using the Folin–Ciocalteu assay [16]. Briefly, an aliquot (0.25 mL) of the extract was added to 3.75 mL of distilled water in a test tube, followed by 0.25 mL of Folin-Ciocalteu's reagent and allowed to react for 3 min, and then 0.75 mL of 20% sodium carbonate was added. Tube contents were vortexed and heated at 40°C for 40 min. The blue coloration was read at 760 nm. The test was repeated three times and the concentrations of phenolic compounds were calculated according to the following equation that was obtained from the standard gallic acid graph: Absorbance = 0.1035 gallic acid (µg/mL) + 0.1046 (R^2=0.98). And the results were expressed as mg GAE/g.d.w.

3.2. Total flavonoid contents

The total flavonoid contents in the extracts were determined by a colorimetric method described by Lamairson and Carnet [17].

1.5 mL of 2% $AlCl_3.6H_2O$ dissolved in methanol was added to equal volumes of the diluted extract. The mixture was shaken and the absorbance was read at 440 nm after 10 min incubation at room temperature. The test was repeated three times and the concentrations of flavonoid compounds were calculated according to the following equation that was obtained from the standard quercetin graph: Absorbance = 0.2829 quercetin (µg/mL) – 0.1155 (R^2=0.99). And the results were expressed as mg QE/g.d.w.

4. Antioxidant activity

4.1. DPPH assay (Radical scavenging activity assay).

The method of Braca et *al*. [18], was used for the determination of the scavenging activity of the DPPH free radical. From the different extracts serial dilution (5 µg/mL to 1000 µg/mL) of *T. capitatus* and *T. fontanesii* were mixed with equal volumes of freshly prepared DPPH in methanol solution (0.004 % w/v). The reaction mixture was vortexed thoroughly and then left to stand at room temperature in the dark for 30 minutes and the absorbance was read at λ=517 nm using a blank containing the same concentration of extracts without DPPH, α-tocopherol and ascorbic acid were taken as standard antioxidants. The scavenging activity of the free radical DPPH in percent (I%) was calculated based on the control reading, which contains equal volumes of DPPH solution and methanol without any test compound using the following equation: % scavenging activity = [(AC-AS)/AC] ×100 where AC is the absorbance of control reaction, and S is the absorbance of the sample. The extract concentration providing 50% scavenging activity (EC_{50}) was calculated from the graph of the scavenging effect percentage versus extract concentration. A lower value of EC_{50} indicates a higher antioxidant activity.

4.2. Reducing power assay

The ability of *T. fantanesii* and *T. capitatus* extracts to reduce Fewas revealed by the method of Oyaizu [19]. Different concentrations of plant extracts in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. Then, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3,000 rpm. An aliquot of the supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%).

The absorbance was measured at 700 nm against a blank. α-tocopherol and ascorbic acid were used as authentic standards. $EC_{0.5}$ value is the effective concentration of the extract which corresponds to 0.5 of absorbance for reducing power obtained from the linear regression analysis.

4.3. Total antioxidant capacity by phosphomolybdenum assay (TAC Assay)

The total antioxidant capacity (TAC) was evaluated according to the method described by Prieto et *al*. **[20].** This assay is based on the reduction of molybdenum (VI) to molybdenum (V) by the plant extracts, which produces a green phosphomolybdenum (V) complex under acidic conditions. An aliquot (0.2 mL) of plant extracts (1mg/mL) was combined with 2 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were incubated in heated at 95 °C for 90 min. Then, the samples were cooled to room temperature and the absorbance was measured at 695 nm against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram of dry weight (mg GAE/g DW). All samples were analyzed in triplicate.

5.Antimicrobial activity

5.1. Microbial strains

The different extracts of both plants were individually tested against pathogenic microbes including three Gram-positive bacteria *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (CIP 7625), *Enterobacter cloacae*, three Gram-negative bacteria *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (CIP A22), *Klebsiella pneumonia* E40; and four fungi: *Aspergillus flavus* (ATCC 200026), *Penicillium expansum; Fusarium culmorum* (ATCC 36017) and *Candida albicans* (M3). All microorganisms were obtained from The Microbiological Laboratory, Department of Natural's Sciences, ENS, Algiers, Algeria. Bacterial strains were cultured on Muller–Hinton agar (Institute Pasteur, Algeria), and fungi were cultivated on Sabouraud dextrose agar (Institute Pasteur, Algeria). All microbial strains were incubated for 24 h at 37°C.

5.2. Disc diffusion assay

The Antimicrobial tests were carried out using the disk diffusion **method [21].** The microbial cultures were harvested and then suspended in sterile saline (0.9% NaCl) and the cell density was adjusted to 0.5 McFarland.

Sterile 5.5 mm paper discs, impregnated with 10 µL of the extracts solutions (50 mg/mL) were placed on the inoculated surface. Before incubation, all Petri dishes were stored in the dark at +4°C for 1 hour, to allow the diffusion of the extracts from disc to medium without microbial growth. At the end of incubation time (18-48h between 25°C to 37°C), the diameter of the zones of inhibition around each disc (in millimeters, the diameter of the disc included) was used as a measure of antimicrobial activity. Levofloxacin (10 µg/disc) was used as a positive control against bacteria and nystatin (10 µg/disc) against fungi [22, 23].

5.3. Agar dilution method

The minimal inhibition concentration (MIC) of the extracts was carried out by the agar dilution method [24]. Appropriate amounts of the extract were added aseptically to the sterile medium to produce a concentration ranging from 25–0.097 mg extract/mL medium. The resulting agar solutions were immediately mixed and poured into Petri plates. The plates were spot inoculated with 3µl of microorganisms. At the end of the incubation period, the plates were evaluated for the presence or absence of growth. The MIC was defined as the lowest concentration of the extract needed to inhibit the growth of microbial strains tested.

6. Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means ± S.D. Differences were evaluated by a one-way analysis of variance (ANOVA) test completed by a Student's test. Differences were considered significant at $p<0.05$. The correlations between methods were determined using analysis of variance (ANOVA) and quantified in terms of the correlation factor.

RESULTS

1. Yielded of extraction

From the table 1, the yield of extraction (%), was very important for the alcoholic extracts than those of aqueous extracts (decoction or infusion), for both species. Whereas TF4 has given a higher yield of extraction (46.6%) and TC2 gives a lower yield (10.3%). But between the two species, *T. fontanessi* has a high yield of extraction than *T. capitatus* with the order of decreasing yield being TF4 < TF3 <TC3< TF1 < TC4 < TF2<TC1 <TC2.

2. Total phenolicand flavonoid

Concerning total phenolic, flavonoid, and tannin contents (Table 1). The results show for both species that these contents varied significantly as a function of solvent nature, mode of extraction, and plant species ($p < 0.05$). The analysis of variance performed on averages of these compounds showed significant species and solvent effects. For the two species, the alcoholic extracts had high phenolic contents than aqueous extracts, whereas the TC3 showed the highest phenolic contents (572.52±6.06 mg of GAE/g.d.e) than other extracts, and TF2 had given a lower content (48.96±0.14 mg of GAE/g.d.e). But between the two species *T. capitatus* had higher phenolic content than *T. fontanessi*. with the order of decreasing total phenolic content being TC3<TC4 < The same finding was made for total flavonoids contents where the alcoholic extracts have high flavonoids contents than aqueous extracts, notably TC3 showed the highest flavonoids contents (1.35±0.07 mg QE/g.d.e.), and the infusion extracts TF2 and TC2 were the lower flavonoid contents extracts (0.48±0.03 and 0.39±0.03 mg QE/g.d.e. respectively). Of the two species, *T. capitatus* have higher flavonoid content than *T. fontanessi*. But the aqueous extracts of *T. fontanesii* are better in total flavonoid content than those of *T. capitatus*, with the order of decreasing total phenolic content being TC3<TC4 TF4< TTF3< TF1< TF2< TC1<TC2. highest tannin content than *T. fontanesii* extracts with the order of decreasing total phenolic content being TF2<TC2< TC4< TC3< TF4<TF3 <TF1 <TC1.

Table 1. Yield of extraction, total phenolics and flavonoids contents (represented by mean ± S.D.) of *Thymus capitatus* and *Thymus fontanesii*

	TF1	TF2	TF3	TF4	TC1	TC2	TC3	TC4
Yielded of extraction (%)[a]	23.4±0.30	20.8±0.4	25±0.6	46.6±0.52	12.4±0.38	10.3±0.69	24.6±0.37	22.8±0.70
Polyphenolic content GAE/g.d.e[a b]	56.09±0.43	48.96±0.14	114.79±0.96	115.29±0.95	200.83±4.25	131.84±0.60	572.52±0.06	338.55±0.06
Flavonoid content mg QE/g.d.e[a c]	0.60±0.01	0.48±0.03	0.63±0.03	0.81±0.01	0.46±0.02	0.39±0.03	1.35±0.07	0.97±0.05

Controlled factor variance analysis at the 0.05% threshold is highly significant for the set of treatments where the smallest significant difference is still below the mean difference a Each value is presented as mean ± S.D. (N = 3). b Total phenolic content was expressed as mg gallic acid equivalents/g dried extract. c Total flavonoid content was expressed as mg quercetin equivalents/g dried extract.

3. Antioxidant activities

3.1. DPPH assay

As presented in table 2, the results of DPPH scavenging activity, showed for both species that varied significantly as a function of solvent nature, mode of extraction, and plant species ($p < 0.05$), considering the values of EC_{50}. *T. capitatus* extracts demonstrated a strong ability to scavenge the DPPH free radical, behind those of *T. fontanesii* extracts, except the acidified alcoholic (TC4, 180.03 ±2.46 µg/mL) wish was lower active than (TF4, 78.00 ±0.28 µg/mL). Another point, the aqueous extracts of *T. capitatus* are more active than alcoholic extracts (9.42±0.56, 9.52 ±0.22 µg/mL decocted and infused respectively), which was totally the opposite for the *T. fontanesii*, whereas the acidified alcoholic and the alcoholic extracts (78.00 ±0.28, 88.00 ±3.492 µg/mL, respectively), were more actives than decocted and infused (120.70 ±1.50 and 140.71 ±2.79 µg/mL, respectively), with the order of increasing scavenging activity by DPPH free radicals being TC4> TF2> TF1 > TF3> TF4> TC3>TC2 >TC1, compared with standards antioxidants α-tocopherol and ascorbic acid with the following IC_{50} 6.86± 0.05 and 4 ±0.1 µg/mL respectively we can that the activity of TC1 and TC2 was comparable with that of standards used.

Table 2. Antioxidant activities of extracts of *Thymus capitatus* and *Thymus fontanesii*, measured by different assays.

		DPPH (IC_{50} µg/mL)[ab]	RPA($IC_{0.5}$ µg/mL)[ac]	TAC (mg GAE/g d.w) [ad]
T fontanesii	TF1	120.70 ±1.50	1135.8 ±110.50	6277.78 ±532.10
	TF2	140.71 ±2.79	1125.5 ±116.67	7819.44± 106.91
	TF3	88.00 ±3.492	937.33± 37.6	10562.50± 130.10
	TF4	78.00 ±0.28	671.33 ±0.28	11569.44 ±294.87
T. capitatus	TC1	9.42±0.56	9.33± 0.25	6048.61 ±540.19
	TC2	9.52 ±0.22	1130.47±53.87	8298.61 ±411.60
	TC3	29.14± 2.90	1137.33 ±2.36	10333.33± 302.62
	TC4	180.03 ±2.46	990.94 ±3.18	10347.22 ±209.72
α-tocopherol		6.86± 0.05	106 ±1.15	
Ascorbic acid		4 ±0.1	47 ±0.28	

Controlled factor variance analysis at the 0.05% threshold is highly significant for the set of treatments where the smallest significant difference is still below the mean difference, a Each value is presented as mean ± S.D. (N = 3), b SC_{50}µg/mL scavenging concentration of the anti-radical activity, each value is presented as mean ± S.D. (N = 3).c SC_{05}µg/mL value is the effective concentration of the extract which corresponds to 0.5 of absorbance for reducing power, each value is presented as mean ± S.D. (N = 3). d Total antioxidant capacity (mg GAE/g d.w), each value is presented as mean ± S.D. (N = 3).

3.2. Reducing power assay

The RP assay was at all dominated (as presented in Table 2) by the high activity of alcoholic extracts than the aqueous extracts for both species, except decocted TC1 (9.33± 0.25µg/mL) which was the most active extract better than the standards used (α-tocopherol and ascorbic acid: 106 ±1.15, 47 ±0.28 µg/mL respectively), TF4 and TF3 (671.33 ±0.28, 937.33± 37.6 µg/mL respectively) have also shown good activity than the others extracts. Also, *T. fontanessi* was more active than *T. capitatus*, with the order of increasing RP assay being TF1 > TC3 >TC2 > TF2 > TC4 > TF3 > TF4 > TC1.

3.3. Total antioxidant capacity

From Table 2, the TAC (Total antioxidant capacity by phosphor-molybdenum assay), showed the highest content of the alcoholic extracts for *T. fontanesii* and *T. capitatus* better than the aqueous extracts (decocted and infused), Between the two species *T. fontanesii* had given the highest content than *T. capitatus* where TF4 had given the higher content 11569.44 ±294.87 mg GAE/g d.w. and TC1 had given the lowest content 6048.61 ±540.19 mg GAE/g d.w. with the order of increasing TAC test being TC1> TF1 >TF2> TC2> TC3> TC4>TF3> TF4.

4. Antimicrobial activities

4.1. Antibacterial activity

As presented in Table 3, The results of the Inhibition diameter zone (mm) and Mic (mg/mL) show For both species significant variation as a function of solvent nature, mode of extraction, plant species, and strain tested ($p < 0.05$).

From table 3, the aqueous extracts (decocted and infused extracts) of the two species were not active against *Pseudomonas aeruginosa*, while the alcoholic extracts of both species are active with interesting values of MIC for TF3 and TF4 (11±0.70 to11±0.90 mm and <0.19 mg/mL as CMI), while TC3 and TC4 had given an ID of 14 and 17 mm with 0.78 to 0.39 mg/mL respectively, compared with those of standard antibacterial (levofloxacin) ID 24.16±0.76 mm MIC 4 mg/mL. The extracts of *T. capitatus* are more active against *Escherichia coli* where the MIC value were all under 0.19 mg/mL, also and notably, the alcoholic extracts of this species showed an ID of 13 and 14 mm TC3 and TC4 respectively, compared to those of levofloxacin ID 29±1.00 mm and MIC of 0.78 mg/mL. The last Gram-negative strain *Klebsiella pneumonia* was more sensitive to all extracts of this study. *T. capitatus* extracts are more active against it than those of *T. fontanesii* where the alcoholic extracts (TC3 and TC4) demonstrated great antibacterial activity by the zones inhibition diameter or MIC values 18 mm, 0.19 mg/mL and 16 mm, 0.19 mg/mL respectively than the aqueous extracts compared to those of standard antibacterial ID 25.0 ± 1.8mm MIC 0.78 mg/mL. Concerning Gram-positive bacteria, *Staphylococcus aureus*, *Bacillus subtilus*, and *Enterobacter cloacae* with ID and MIC values by standard antibacterial as following and respectively (ID: 32 ± 1.00, 36 ± 1.00 and 27 ± 1.5 mm, MIC: 0.78, 1.56 and 0.78 mg/mL).

Enterobacter cloacae was more sensitive to the different extracts of this study both by ID value or MIC, the aqueous extracts of the two species were not active against *Staphylococcus aureus*, while the alcoholic extracts notably of *T. fontanesii*, are active with interesting values of MIC for TF3 and TF4<0.19 mg/mL. The results obtained against *Bacillus subtilus* seem to be comparable with those against *Staphylococcus aureus*, where the alcoholic extracts notably of *T. fontanesii*, showed an interesting antibacterial activity.

Table 3. Antibacterial activity of extracts of *Thymus capitatus* and *Thymus fontanesii*, and standard against tested bacterial strains.

	Pseudomonas aeruginosa		*Escherichia coli*		*Klebsiella pneumoniae*		*Staphylcoccus aureus*		*Bacillus subtilus*		*Enterobacter cloacae*	
	ID[a] mm	MIC[b] mg/ml	ID[a] mm	MIC[b] mg/ml	ID[a] mm	MIC[b] mg/ml	ID[a] mm	MIC[b] mg/ml	ID[a] mm	MIC[b] mg/ml	ID[a] mm	MIC[b] mg/ml
TF1	-	>25	-	>25	12±0.50	12.5	-	>25	8±0.50	6.25	12±0.50	12.5
TC1	-	>25	7±0.36	<0.19	13±0.20	<0.19	-	>25	7±0.31	6.25	11±0.25	6.25
TF2	-	>25	9±0.50	3.125	10±0.35	<0.19	6±0.50	>25	-	3.125	15±1.40	<0.19
TC2	9±1.76	>25	7±0.90	<0.19	15±0.53	<0.19	-	>25	6±1.6	0.78	12±1.30	25
TF3	11±0.70	<0.19	13±1.50	<0.19	13±0.44	<0.19	10±1.5	<0.19	10±0.5	<0.19	12±0.70	<0.19
TC3	14±0.30	0.78	13±0.70	<0.19	18±0.70	<0.19	10±2.5	0.78	10±0.65	<0.19	12±0.90	<0.19
TF4	11±0.90	<0.19	8±1.70	<0.19	13±0.90	<0.19	7±0.4	<0.19	10±0.72	<0.19	12±0.45	<0.19
TC4	17±0.50	0.39	14±0.80	<0.19	16±2.3	0.39	9±0.75	0.39	10±1.55	0.39	13±1.40	<0.19
Positive control	24.16±0.76	4	29±1.00	0.78	25.0±1.8	0.78	32±1.00	0.78	27±1.5	0.78	17±0.5	1.56

ID[a] mm: Inhibition zone in diameter around the impregnated disks and, values given as mg/mL, each value is presented as mean ± S.D. (N = 3), MIC[b] mg/mL: Minimal inhibition concentration; values given as mg/mLPositive control: [c]Levofloxacin (standard antibacterial)(–) indicate that there is no antimicrobial activity.

4.2. Antifungal activity

The antifungal activity of the different extracts of both species investigated in this report (results are presented in Table 4) generally was top than antibacterial activity. Against *Aspergillus flavus T. fontanesii* extracts were better than those of *T. capitatus* extracts where TF2 TF3 and TF4 had gives an ID of 30 mm and a MIC value of under 0.19 mg/mL, while between the aqueous and alcoholic extracts of *T. fontanesii* the alcoholic was leading. Compared with those of nystatin with ID of 17 ± 0.5 mm and MIC of 1.56 mg/mL those extracts were more active than this standard antifungal. Against *Fusarium culmorum* (standard antifungal: ID: 15 ± 1.5 mm and MIC:3.12 mg/mL), *T. capitatus* extracts have special antifungal activity than those of *T. fontanesii* extracts with 16, 24, 19, and 15 mm and 0.19 mg/mL for TC1, TC2, TC3, and TC4 respectively, wish are very higher than those of the nystatin notably those of TC1 and TC2, while between the aqueous and alcoholic extracts of this species, the alcoholic extracts were higher. On the contrary against *Penicillium expansum* (standard antifungal: ID: 19 ± 1.73 mm and MIC: 0.39 mg/mL), the *T. fontanesii* extracts have height activity even those of nystatin by an ID of 30 mm and 0.19 mg/mL for all extracts, and those of *T. capitatus* extracts, and always the alcoholic extracts were better than the aqueous extracts. In the end, its *T. capitatus* extracts which have activity against *Candida albicans* (TC4 27 mm and 0.39 mg/mL), than those of *T. fontanesii* extracts, also, this extract was more active than the nystatin (ID: 20 ± 0.0 mm and MIC: 3.12 mg/mL). Alcoholic extracts have more antifungal activity than aqueous extracts.

Table 4 . Antifungal activity of extracts of *Thymus capitatus* and *Thymus fontanesii*, and standard against tested fungi strains.

	Aspergillus flavuss		*Fusarium culmorum*		*Penicillium expansum*		*Candida albican*	
	ID[a] mm	MIC[b] mg/ml	ID[a] mm	MIC[b] mg/ml	ID[a] mm	MIC[b] mg/ml	ID[a] mm	MIC[b] mg/ml
TF1	14±0.74	<0.19	14±0.63	>25	30±0.10	<0.19	7±0.4	>25
TC1	15±1.53	<0.19	16±0.75	<0.19	10±1.5	<6.25	18±1.53	>25
TF2	30±0.35	<0.19	19±0.45	>3.125	30±0.57	<0.19	10±0.75	>25
TC2	11±0.90	<0.19	24±0.63	<0.19	8±0.40	<0.19	30±1.10	>25
TF3	30±0.44	<0.19	13±1.23	>0.17	30±0.63	<0.17	9±1.96	>0.17
TC3	9±0.50	<0.19	19±1.50	<0.19	10±0.25	<0.19	19±0.84	>1.65
TF4	30±1.00	<0.19	17±0.95	<0.19	30±0.79	<0.19	11±0.50	<0.19
TC4	10±2.5	<0.19	15±0.80	<0.19	9±1.16	<0.19	27±0.95	>0.39
Positive control	17±1.9	1.56	15 ±1.5	3.12	19±1.73	0.39	20±0.0	3.12

ID[a] mm: Inhibition zone in diameter around the impregnated disks, values given as mg/mL, and each value is presented as mean ± S.D. (N = 3), MIC[b]mg/mL: Minimal inhibition concentration; values given as mg/mL, Nystatin (standard antifungal); (–) indicate that there is no antimicrobial activity.

DISCUSSION

The extraction of bioactive compounds from plant materials is the first step in the use of phytochemicals from fresh, frozen, or dried plant samples in the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical, and cosmetic products [25]. Yields of extracting obtained by different methods appear statistically at least significant ($p \leq 0.05$), which means that there was a significant difference between yields obtained by each of the used methods (modes and solvents) from both plant materials. Variation in the yields of various extracts is attributed to the polarities of different compounds present in these two species [25]. The yield of extraction depends on the solvent with varying polarity, pH, temperature, extraction time, and composition of the sample. Under the same extraction time and temperature, the solvent and composition of a sample are known as the most important parameters. Compounds other than phenolics may have been extracted and contributed to higher yield [26]. Our results indicate that the alcoholic solvents combined with water give a height yield extraction than water only (decoction and infusion). The combined use of water and organic solvent may facilitate the extraction of chemicals that are soluble in water and/or organic solvent. This may be the reason why yields of aqueous methanol extracts are higher than yields of water extracts [27].

Commonly used extraction solvents are alcohols (methanol, ethanol), acetone, diethyl ether, and ethyl acetate. However, very polar phenolic acids (benzoic, cinnamic acids) could not be extracted completely with pure organic solvents, and mixtures of alcohol-water or acetone-water are recommended. Less polar solvents (dichloromethane, chloroform, hexane, benzene) are suitable for the extraction of nonpolar extraneous compounds (waxes, oils, sterols, chlorophyll) from the plant matrix [28].

Our results demonstrate that the phenolic extracts from plants exhibit large variations in TPC levels. The compounds of plants are always a mixture of different classes of phenols and others, which are selectively soluble in the solvents. Aqueous-alcohol solvents are the best solvents for the extraction of phenolic compounds for the two species ($p \leq 0.05$). Water solvents (decoction or infusion) are inefficient solvents for extraction of total phenols from studied plants ($p \leq 0.05$) since the larger part of polyphenols are not water-soluble and using water for extraction leaves a wide number of residual polyphenols that only a proper combination of solvents would extract [28, 29], that agree with our results. In general extractability of a particular component appeared to depend on the extract medium polarity and the ratio of solute to solvent [30, 5]. Hayouni et *al*. reported that polyphenol content was strongly dependent on the solvents used, and no effect of acid used in the mixture was signaled [31]. The extraction efficiency of compounds from plants are affected by the chemical nature of phytochemicals, the extraction method and solvents used, as well as sample particle size [32].

Złotek et *al*. reported that the composition of the extracting solvents significantly ($p < 0.05$) affected the measured polyphenolic content, and the choice of the most efficient solvent for phenolic compounds extraction must depend on the used food matrices, and no effect of acid used in the mixture was highlighted [3]. According to Alothman et *al*. it is hard to develop a standard extraction procedure suitable for the extraction of all plant phenols [5]. Mazandarani et *al*. reported that alcohol solvents were better solvents for flavonoid, phenol, and anthocyanin extraction compared to other solvents, notably water solvents [33]. This agrees with the data of this study. From the literature, Nouasri et *al*. with *T. hirtus* and *T. lanceolatus* had found as total phenols and flavonoids respectively 76.73±14.22 and 84.57±5.60 mg GAE/g.d.e., 2.04±0.03 2.01±0.02mg QE/g.d.e, these results were obtained with hydromethanolic extract [34], when compared to alcoholic extract, our results are better than those of *T. hirtus* and *T. lanceolatus*. Boubakour et *al*. [13] with *Thymus fontanesii*and different types of maceration obtained a low amount of phenolic and flavonoids contents than this study. Köksal et *al*. [27], have found 256.0, 158.0 mg GAE/g.d.e and 44.2, 36.6 mg QE/g.d.e of polyphenols and flavonoids respectively for the water extract and ethanolic extract, the water polyphenol of our study are low of those found by this author but the ethanol polyphenols of those authors are low compared with our funding, it appears from the results of flavonoids of those authors that they have found a height flavonoids content than our results. Msaada et *al*. [35], have studied *T. capitatus* of Tunisia, and found, their funding indicated the low polyphenol content (18.40 mg EAG/g dm) and the height flavonoid content (63.64 mg CE/g dm), compared with our results of *T. capitatus*. It seems to be very low compared to an alcoholic extract of our study. We can say, that there are a significant ($p \leq 0.05$) difference in polyphenol content according to the mode, solvents and species used.

Concerning antioxidant activity, several studies reported the scavenging activity of DPPH free radical, By extracts of thyme and other species, Muhammad et *al*. [4], have obtained an EC_{50} of 46.4 and 59.3µg/mL for methanol and aqueous extract respectively of *T. vulgaris*, that it's low compared to our results notably those of *T. capitatus*, but bitter than of *T. fontanesii* [4]. Nouasri et *al*. with *T. hirtus* and *T. lanceolatus* 35.24±0.95 and 34.09±0.34 µg/mL for hydromethanolic extract respectively [34], when compared with our results it's top than alcoholic extracts except that of TC3, which was very active. Msaada et *al*. [35], with *T. capitatus* extracted with pure ethanol obtained IC 50 of 10±0.98 µg/mL, this is comparable with our funding where the aqueous extract (infusion decoction) of *T. capitatus* was very active than all other extracts, even compared to those of *T. fontanessi*. Tabti et *al*. [36], with ethanolic and hexanoic extracts of *T. capitatus* obtained an IC50 of 31±0.92 µg/mL and 0.95±0.12 µg/mL respectively, these results are close for the ethanolic extract to our funding with *T. capitatus* but no for hexanoic extract. Megdiche-Ksouri et *al*. [37], with *T. algeriensis* and *T. capitatus* extracted by methanolic solvent found EC50 values respectively 7±0.02 µg/mL and 6±0.01 µg/mL if compared with the alcoholic extract of *T. capitatus* and *T fontanesii* of our finding their results were better. Martins et *al*. in their study of Decoction, infusion, and hydro-alcoholic extract of cultivated thyme (*T. vulgaris*), announced the height scavenging activity and reducing power by decocted and infused extracts greater than hydro-alcoholic [15], this is in agreement with our finding. A low correlation between, DPPH scavenging activity and TPC and TFC with R 0.20 and 0.53 respectively. As reported by Mohammedi and Atik [29], the anti-radical activity is due to the quality of the extract, not the quantity. Where for each solvent, taken individually total phenol content, determined by FC measurement present a good correlation with antioxidant activity, but it is not the case when we compare extracts obtained by various solvents, this was in agreement with our study. For reducing power assay, Megdiche-Ksouri et *al*. [37], with *T. algeriensis* and *T. capitatus* extracted by methanol solvent found as $EC_{0.5}$ value respectively 210±2.11 and 120±1.01 µg/mL, those results are very finer than our finding except TC1 9.33±0.25 µg/mL. Jabri-Karoui et *al*. [38], with *T. capitatus* flower extract obtained an $EC_{0.5}$ of 380±0.06 µg/mL, this result compared to our finding is very interesting to expect that of TC1 9.33±0.25. A strong correlation between RPA and TPC and TFC with r=0.78, and 0.53 respectively. (R=0.09) between the antiradical activity and total polyphenol content.

The antioxidant capacities of the plant extract largely depend on the composition of the extracts and the conditions of the test system. The antioxidant capacities are influenced by many factors, which cannot be fully described with one single method. Therefore, it is necessary to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action [28]. Differences in the antioxidant assay results could be due to the presence of compounds having a different affinity to react with DPPH or $K_3Fe(CN)_6$ [39]. Megdiche-Ksouri et *al*. [37], with *T. algeriensis* and *T. capitatus* extracted by methanol solvent 213.061±0.31 and 291.425±0.23 mg GAE/g.d.w respectively, those results were very highest than those of this study. A low correlation between TAC and TPC and TFC with R 0.36, and 0.61respectively, that correlation demonstrated that other compounds participate in total antioxidant capacity, that contrary to the funding of Kiselova et *al*. [40] as they found a positive correlation.

Thus, from all antioxidants tests realized in this work, it can be seen that extracts prepared by different solvents and different techniques, from different species exhibited varying degrees of antioxidant activity. Here we can easily see that the mode-solvents of extraction with plant species influenced the results of antioxidant tests. Statistical analysis revealed on a positive and not significant correlation coefficient was established between the three in vitro assays of antioxidant activity and phenolics, flavonoids, and condensed tannin (for all r<0.5 (p<0.05). The high correlation coefficient (r=0.78, was between RPA and TPC), thereby suggesting that other compounds may be contributors to the antioxidant activities of *Thymus* extracts, this is in conformity with Mssada et *al*. [34] but does not agree with numerous studies which correlate with high significant positive correlation the antioxidant activity of the plant extracts in the presence of phenolic compounds (p<0.05) Megdiche-Ksouri et *al*. [36], Efstathia et *al*. [41], Wojdylo et *al*. [42], Forough et *al*. [43]. The unclear relationship between antioxidant activity and total phenolics may be explained in numerous ways. In fact, the total phenolics content does not incorporate all the antioxidants. Their redox properties allow them to act as reducing agents, hydrogen donators, and singlet and triplet oxygen quenchers. Nevertheless, they may exhibit strong metal chelating properties.

In addition, the synergism between the antioxidants in the mixture makes the antioxidant activity, not only dependent on the concentration, but also on the structure and interaction between antioxidants [31]. As maintained by Kähkönen et *al*. [44], no significant correlations could be found between the total phenolic content and antioxidant activity of the plant extracts in any of the studied subgroups. It is known that different phenolic compounds have different responses in the Folin-Ciocalteu method. Similarly, the molecular antioxidant response of phenolic compounds in Melo varies remarkably, depending on their chemical structure. Thus, the antioxidant activity of an extract cannot be predicted on the basis of its total phenolic content. In the report of Ben El Hadj Ali et *al*. [45], the high free radical-scavenging inhibition activity, reducing power and TAC tests may be related to the presence of flavonoid-type compounds and other phenolics. So, the high contents of polyphenol compounds in the studied species contribute to their important antiradical and anti-oxidative activities. Other findings report the presence of different phenolic compounds with several biological activities in the *Thymus* genus such as rosmarinic acid, caffeic acid, ferulic acid, carnosic acid, quinic acid, p-coumaric acid, caffeoylquinic acid derivative, quercetin7-o-glucoside, cinnamic acid, methyl rosmarinate, naringenin, luteolin-7-o-rutinose and ferulic acid derivative, and flavonoids such as 5, 7, 4-trihydroxyflavone (apigenin); 4-dihydroxy-6, 7, 8trimethoxyflavone (xanthomicrol); 5, 7, 3, 4-tetrahydroxy-flavone (luteolin).

Antibiotics are generally associated with adverse effects that include hypersensitivity, depletion of the beneficial gut and mucosal microorganisms, immune-suppression, and allergic reactions. Therefore, the search for new antimicrobial substances exhibiting minimal side effects from natural sources is needed Kandil et *al*. [10]. The antibacterial and antifungal activity of these extracts displayed varying magnitudes of inhibition patterns with standard positive control depending on the susceptibility of the tested microorganism Megdiche-Ksouri et *al*. [37]. As stated by Martins et *al*. [15]. The majority of the studies available in the literature for thyme genus, report the antimicrobial activity of thyme essential oils, and a few rapports are for extracts.

The antimicrobial activity of the aqueous and alcoholics extract of the aerial part of *T. capitatus* and *T. fontanesii* was evaluated in vitro by the agar disk diffusion method against six different bacteria and four fungi strains. Their potencies were assessed qualitatively and quantitatively in the presence or absence of inhibition zone diameters and MIC values. Where they exhibit an interesting antimicrobial activity, the different extracts inhibited growth to variable extents. Kandil et *al*. [10], have studied the antimicrobial activities of ethanolic and aqueous extract from *T. capitatus* of Egypt by the method of well filled with different concentrations of extract, they found that for the antibacterial the alcoholic extract was top than the aqueous extract, but the antifungal was the opposite where the aqueous was better than the alcoholic**,** this finding agrees with our study.

Qaralleh et *al*. [9], in their study of the antibacterial activity of *T. capitatus* extract from Jordan, have found that alcoholic extract was more active than aqueous extract**,** these results are in agreement with our study but in disagreement with those of Hayouni et *al*. [31], worked on Tunisian *Quercus coccifera* L. and *Juniperus phoenicea* L. fruit extracts**.** Megdiche-Ksouri et *al*. [37], have found that polar extract (aqueous) has a weak to moderate antibacterial activity, this does not agree with our finding.

Hassan et *al*. [46], studying the antimicrobial activities of ethanolic extract of *T. capitatus* by different concentrations, reported that the results obtained demonstrated that generally there are no considerable variations in the intensity of the antimicrobial activity against Gram-negative and Gram-positive bacteria as well as eukaryotic microorganisms for the extract tested**.** Our results are different from their finding where the antibacterial activity of extracts tested is a little higher against Gram-negative bacteria than Gram-positive bacteria. Thus, compared with results found by Martins et *al*. [15], worked on *T. vulgaris***,** is close to our results. It is likely that the antimicrobial effect shown by the extracts tested may be related to the compounds known to be present in *Thymus* species plants such as polyphenols, and especially flavonoids [47]. The potent antibacterial activity of large thyme extracts isolated from *Thymus* depended on the plant chemotype, extract preparation, the solvent used, and finally, the sensitivity of bacteria [47] very corresponds with the finding of this study.

The larger the variety of compounds that are extracted by the extractant (solvent), the better the chance that biologically active components will also be extracted [48]. The mechanisms of antimicrobial activities of polyphenols inhibitory effect of bacterial growth by polyphenols, such as destabilization of the cytoplasmic membrane by the complex formation with the protein and some constituents of the cell wall leading to lyses, inhibition of enzymes extracellular microbial (enzymes leading to the formation of the extracellular polysaccharides); the direct actions on microbial metabolism, or deprivation of substrates required for microbial growth, especially essential mineral micronutrients such as iron and zinc (chelating of metals), whose depletion can severely limit bacterial growth [13]. In addition, the antimicrobial activities present in the extracts of *T. capitatus* and *T. fontanesii* indicate that the selection of a plant for evaluation based on its use in ethno-medicine is an important criterion and a useful predictive factor in identifying new anti-infective agents [9]. The different classes of polyphenols, mainly tannins, and flavonoids may increase the toxicity of extracts against microorganisms. This toxicity is dependent on the position and the number of hydroxyl groups present in the phenolic compound [49].

CONCLUSION

This study and compared with the literature survey reported that, the technique of extraction, as well as the extracting solvent, significantly affected extraction yield, total polyphenol, and biological activities (antioxidant and antibacterial) of different extracts from *T. capitatus* and *T. fontanesii*, the polyphenol, and flavonoids contents of those extracts, varied, depending on the nature of the solvent, the method of extraction used and the crude material (here aerial parts). The most efficient solvents and the best mode of extraction varied significantly with the biological test and the plant species, as finding no effect was observed of the addition of acid in extracting solvent. Regardless of the method used, the one-step extraction method allowed higher yields. As observed, extracts with higher antioxidant capacity and antimicrobial activities are not obvious the extract with higher polyphenol contents. These results indicate that selective extraction from natural sources, by appropriate solvents and suitable methods,

is important for obtaining fractions with high antioxidant and antimicrobial activities. We can suggest another investigation focused on the analysis of these different extracts for example HPLC UV-DAD or LC-MS to be more informed about the effects of the parameters considered in this study.

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