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Antioxidants and Antibacterial Activities of Extract from Thyme and Mint Leaves

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ABSTRACT

Thyme (Thymus vulgaris L.) and mint (Mentha spicata L.) are the mint family (Lamiaceae) herbs that usually applied in culinary and medicinal uses due to the health benefits contributed by various natural antioxidants. Thus, this study was aimed to evaluate the antioxidant and antibacterial activities in these two herbs. The total phenolic content (TPC) of aqueous extracts of Thymus vulgaris L. and Mentha spicata L. herbs were determined spectrophotometrically according to the Folin Ciocalteau method and the results were expressed as gallic acid equivalents (GAE). The extracts also were screened for their antioxidant activities by two methods namely Free Radical Scavenging Activity (DPPH) and Ferric Reducing Antioxidant Power (FRAP) where the activity expressed as per percent inhibition of oxidation (%) and µmol Trolox Equivalent/g (µmol TE/g) respectively. For antibacterial test, the powdered dried leaves were extracted using absolute methanol. The disc diffusion method was used to screen the antimicrobial activity as reported by Duraipandiyan. All of the three antioxidant test assays showed that Thymus vulgaris L. significantly ($p<0.05$) had higher antioxidant activities compared to Mentha spicata L. The extract of Thymus vulgaris L. contained 0.350mg GAE/g total phenolic compounds and 52.8% inhibition of oxidation activity for DPPH assay which were significantly higher than those in extract from Mentha spicata L. (TPC: 0.219 mg GAE/g and DPPH: 27.5%). For the antioxidant activity of FRAP assay, Thymus vulgaris L. also showed the higher significant ($p<0.05$) result compared to Mentha spicata L. with 339. 2µmol TE/g and 121.1 µmol TE/g respectively. The highest inhibitory effect for Thyme was observed against Staphylococcus aureus (zone of inhibition: 12 mm) while the weakest activity was demonstrated against Listeria monocytogene and Salmonella typhi (zone of inhibition: 7 and 7 mm) respectively. It can be concluded that the Thymus vulgaris L. can be regarded as promising candidates for natural plant sources of antioxidants with high value and the most antibacterial activity against all the isolates tested of Staphylococcus aureus, Bacillus cereus, Escherichia coli, Listeria monocytogene and Salmonella typhi.

Keywords: *Thymus vulgaris* L.; *Mentha spicata* L.; Natural antioxidant; Phenolic compounds; Antibacterial

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INTRODUCTION

Thyme is herbaceous plant of the platoon species, grows in mountainous areas, used as a beverage instead of or with tea, added to some food to give it an acceptable flavour, the plant is used in folk medicine frequently where it is prescribed to treat mouth infections, stomach, intestine and airways, coughing and gastroenteritis and expel intestinal worms, as well as to strengthen the heart [1]. Extracts from thyme have been used in traditional medicine for the treatment of several respiratory diseases like asthma and bronchitis and for the treatment of other pathologies thanks to several properties such as antiseptic, antispasmodic, antitussive antimicrobial, antifungal, antioxidative, and antiviral [2]. Thyme essential oil constitutes raw material in perfumery and cosmetics due to a special and characteristic aroma [3]. The potential source of natural antioxidants is plants, fruits and vegetables [1]. Thyme

contains many flavonoids, phenolic antioxidants like zeaxanthin, lutein, pigenin, naringenin, luteolin and thymonin. Fresh Thyme herb has one of the highest antioxidant levels among herbs. It is packed with minerals and vitamins that are essential for optimum health. Its leaves are one of the richest sources of potassium, iron, calcium, manganese, magnesium and selenium [4]. Thymol is the main phenolic components that are primarily responsible for its antioxidative activity [5]. The herb Thymus vulgare Linn, Labiatae is pungent in taste and contains moisture, protein, fat, crude fibre, Ca, K, Na, Fe, P, vitamin A, B and vitamin C. The main constituent of the oil extracted from Thyme is thymol [6]. The herb is also a rich source of many important vitamins such as B-complex, folic acid, beta carotene, vitamin A, K, E and C. Thyme provides 0.35 mg of vitamin B-6 or pyridoxine; furnishing about 27% of daily recommended intake. Pyridoxine keeps up gamma-aminobutyric acid (GABA) (beneficial neurotransmitter in the brain) levels in the brain, which has stress buster function [4]. Vitamin C helps body develop resistance against infectious agents and scavenge harmful, pro-inflammatory free radicals. Vitamin A is a fat soluble vitamin and antioxidant that is required maintaining healthy mucus membranes and skin and is also essential for vision. Consumption of natural foods rich in flavonoids like vitamin A and beta-carotene helps protect from lung and oral cavity cancers [3,6]. Mentha species are commonly used as herbal tea, flavoring agent, and medicinal plant. Infusion, decoction, and distillated water of the aerial parts of Mentha species have been used for centuries as tonics, carminative, digestive, stomachic, antispasmodic, and anti-inflammatory agents in Iranian Traditional Medicine [7]. The literature data on the antioxidant activities of Mentha species is frequently scattered throughout the papers and the data available is often difficult to compare because of the differences in the methodologies [8-10]. Thus, comparison of the antioxidant activities of the species, using a similar approach is necessary. Species, using a similar approach is necessary. The objective of the research was to determine antioxidants and antibacterial activities of extract from Thyme and mint leaves.

MATERIALS AND METHODS

Materials

The chemicals used for phenolic content and antioxidant analysis are Folin Ciocalteu (FC) reagents were obtained from Merck (Darmstadt. Germany), sodium carbonate (Na_2CO_3), gallic acid (GA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyltriazine (TPTZ) were supplied by Sigma (Steinheim, Germany), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), Ferric Chloride, herbs and methanol. All other reagents were analytical grades.

Preparation of Sample for Antioxidants Activity

Thyme leaves (*Thymus vulgaris* L.) were purchased from retail shop in Yemen, while mint leaves (*Mentha spicata* L.) were purchased from super market in Kajang, Selangor. Both thyme and mint leaves were sun dried in few days until constant weight were reached before used as samples. Dried leaves were powdered by using laboratory grinder prior to extraction. Then, water extraction was conducted by adding 1 g of each dried herbs leaves with 10 ml of distilled water in a universal bottle according to Abdelfadel et al. [11] with modification. The mixture was boiled for 15 min then allowed to stand for 24 hours at 25 ± 5ºC while stirred vigorously. After that, the mixture was centrifuged at 4000 rpm for 20 min by using laboratory centrifuge model 2420 (Kubota, Japan) and the supernatant was pipetted and kept in 4ºC until further analysis.

Determination of Total Phenolic Content (TPC)

Total phenolic content were measured using the modified Folin Ciocalteu (FC) method reported by Musa et al. [12]. An aliquot (100 µl) of each extract was mixed with 0.5 ml diluted FC Reagent. After 5 min, 0.4 ml of 7.5% sodium carbonate was added to the mixture. The absorbance was read at wavelength 765 nm using EPOCH Microplate Reader Spectrophotometer (Vermont, USA) after 2 hours incubation at room temperature. Analysis was done in triplicate for each extracts. Standard solutions of gallic acid with concentration 20-100 µg/ml were used to obtain a standard curve for the phenolic content estimation in extracts samples. The total phenolic content was reported as mg gallic acid equivalents (GAE)/g samples. All determinations were carried out in triplicate.

Determination of Antioxidant Activity by DPPH

Antioxidant activity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method that was according to Musa et al. [12] with modification. Methanolic solution of DPPH (2.0 mM) also known as stock solution was prepared fresh daily before UV measurements. The working solution was prepared by diluting stock solution with methanol until absorbance obtained approximately 1.0 ± 0.01. Then, 1 ml of this solution was mixed with 100 µl of individual blank and extract solution as well as herbal preparation. After 1 hour incubation in

the dark, antioxidant compound reaction with DPPH was measured at 516 nm by using EPOCH Microplate Reader Spectrophotometer (Vermont, USA). Analysis was done in triplicate and antioxidant activity of each extracts was determined based on the reduction of DPPH absorbance by calculating percentage of DPPH scavenging activity:

$$\% \text{ DPPH scavenging activity} = [(\text{Absorbance}_{\text{Blank}} - \text{Absorbance}_{\text{Sample}})/\text{Absorbance}_{\text{Blank}}] \times 100$$

Determination of Ferric Reducing Antioxidant Power (FRAP) Capacity

Preparation of FRAP solution was adopted from Musa et al. [12] with modification. FRAP solution were prepared fresh using 300 mM acetate buffer pH 3.6 (0.31 g sodium acetate trihydrate mixed with 1.6 ml glacial acetic acid made up to 100 ml with distilled water), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1 to produce working solution. 50 µl of each extracts and standards was pipetted into 1.95 ml of freshly prepared FRAP solution to initiate the reaction. The absorbance was read by using EPOCH Microplate Reader Spectrophotometer (Vermont, USA) at 595 nm wavelength after 30 min incubation. Distilled water was used as a blank and different concentrations of Trolox (0.2 mM to 1.0 mM) were used as standard. Analysis was done in triplicate for standard and each extracts. Antioxidant capacity of each extracts was determined based on absorbance by expressing the results as micromole of Trolox equivalents per gram of sample (µmol TE/g).

Determination of Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was carried out according to Huang et al. [13] using FLUOstar Omega Multi-Detection Microplate Reader (BMG LABTECH, Germany). Trolox was used as standard and different dilutions of Trolox (6.25 µM to 100 µM) and proper dilution of herb leaves were prepared in phosphate buffer (10 mM, pH 7.4). All solutions should be prepared fresh daily. For ORAC assay run, in every working well of a 96-well plate was prepared containing 25 µl of Trolox dilution (standards), 25 µl of sample dilution (samples), 25 µl of phosphate buffer (blank) and 150 µl of a 10 nM Fluorescein solution (all wells). All assays were pipetted in triplicate and performed at 37 ºC with 30 min incubation that was held in the microplate reader. After incubation, fluorescence measurements (excitation 485 nm, emission 520 nm) were taken every 90 sec to determine the background signal. After 3 cycles, 25 µl of 240 mM AAPH was injected using the onboard injectors into each well for a final volume of 200 µl. The microplate reader and data points are summarized over the time controlled by the Omega MARS data analysis software (BMG LABTECH, Germany). The ORAC values were calculated using the regression equation between Trolox concentration (6.25–100 µM) and the net area under the curve and were expressed as micromole Trolox Equivalents per gram of fresh weight sample (µmol TE/g of sample).

Extraction of Thyme and Mint Leaves for Antibacterial Activity

Powdered dried leaves were extracted as reported by Abdelfadel et al. [11] with modification by adding 1 g of each dried herbs leaves with 10 ml of absolute methanol in a universal bottle. The mixture was left while stirred vigorously for 24 hours at 25 ± 5°C. After that, the mixture was centrifuged at 4000 rpm for 20 min by using laboratory centrifuge model 2420 (Kubota, Japan). Then, the supernatant containing the plant extract was then concentrated by evaporating the solvent at 50°C using water bath according to Mukhtar and Ghori [14] method with modification. The crude extract was weighed and dissolved in a known volume of absolute methanol to obtain a final concentration of 200 mg/ml and sterilized by filtration through nylon syringe filter 0.22 µm (Membrane solutions). The Aqueous extracts were stored in sample bottles at 4°C prior to use.

Preparation of Inoculum

Preparation of inoculum was conducted as referred to method Duraipandiyan et al. [15] with modification. Stock cultures were maintained at 4°C on nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to universal bottle of 10 ml Nutrient broth (NB) for bacteria that was incubated for 24 hrs at 37°C. The cultures were then diluted with fresh Nutrient broth to achieve optical density of a McFarland 0.5 turbidity standard, approximately corresponding to 2×10^8 colony forming units (CFU/ml) of bacterial strains.

Antimicrobial Susceptibility

The disc diffusion method was used to screen the antimicrobial activity as reported by Duraipandiyan et al. [15] with modification. *In vitro* antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained from Oxoid (USA). The MHA plates were prepared by pouring 15 – 20 ml of molten media into sterile petri plates. The plates were allowed to solidify for 5 minutes and 0.1 ml inoculum suspension was swabbed uniformly by using sterile cotton bud and the inoculum was allowed to dry for 5 minutes. The leaves extract of thyme and mint were

loaded on 6 mm sterile disc of filter paper Whatman no. 1. The compound on discs was allowed to remain at room temperature until complete diluent evaporation for 5 minutes. Discs loaded with natural products were placed onto the surface of the agar and the plates were kept for incubation at 37°C for 24 hrs. Commercial Ampicillin discs (10 mg) were used as positive control. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimetre. These studies were performed in triplicate.

Statistical Analysis

All data were expressed as means ± standard deviation. Data were analyzed using IBM Statistical Package for Social Science (SPSS) version 22 (California, USA). Total phenolic, DPPH, FRAP and ORAC values between thyme and mint leaves were evaluated by using Independent T test to determine the difference. A significant difference was considered at the level of $p<0.05$.

RESULTS AND DISCUSSION

In this study, all samples which are *Thymus vulgaris* L. (thyme) and *Mentha spicata* L. (spearmint) was extracted by using hot water as a solvent. Hot water was choosing as a solvent through study on several previous researches. Phenolic constitutes is one of the major groups of compounds acting as primary antioxidants found in herbs plant. The phenolic content increased with the polarity of the solvent which means the highest amount of total phenolic content corresponded to water [16]. So, the amount of total phenolic was higher in polar solvents extract and weaker in non-polar solvents extracts. This result is in agreement with the report of Lee et al. [17] who showed that water is the most suitable solvent for extraction of phenolic compounds from *Pleurotus citrinopileatus*. Ozsoy et al. [18] also reported that the highest content of total phenolic compounds in *Smilax Excelsa* leaves was found in water extract and the lowest was found in ethyl acetate. In addition, the percentage and maximum recovery extractable total phenolic content was recorded in water extracts followed by other solvent extracts [16]. Therefore, it was reasonable to use water as extraction solvent for herbs sample.

Total Phenolic Content

Water extracted samples was further tested by measuring the total phenolic content (TPC) and antioxidant activities namely DPPH, FRAP, and ORAC assays. All tests were conducted and measured in triplicate to test the reproducibility of the assays. The results obtained were simplified in Table 1. The TPC of thyme and spearmint was determined by using Folin - Ciocalteu method where the content of phenolic compounds is expressed as mg of Gallic acid equivalent (GAE) /g extract (mg GAE/g). The reaction generally provides accurate and specific data for several groups of phenolic compounds, because most of the compounds change color differently due to differences in unit mass and reaction kinetics [11]. Phenols are very important plant constituents because of their scavenging ability of free radicals due to their hydroxyl groups. Table 1 showed that TPC in thyme extract was significantly ($p<0.05$) higher than mint extract which were 34.22 mg GAE/g and 21.48 mg GAE/g respectively (standard curve equation: $y=0.0105x + 0.0239$, $R^2 = 0.9998$ data not shown).

Table 1: Total phenolic content (TPC) and antioxidant activities of *Thymus vulgaris* L. (thyme) and *Mentha spicata* L. (spearmint)

Antioxidative Activities	Type of herbs	
	***Thymus vulgaris* L.**	***Mentha spicata* L.**
TPC (mg GAE/g of sample)	34.22 ± 0.17^a	21.48 ± 0.58^b
DPPH (%)	87.17 ± 1.78^a	56.90 ± 0.60^b
FRAP (μmol TE/g of sample)	330.63 ± 2.67^a	113.09 ± 0.4^b
ORAC (μmol TE/g of sample)	240.73 ± 28.95^a	79.96 ± 8.68^b

[a,b]Different alphabet within the same row indicates significant difference ($p<0.05$)

Kratchanova et al. [19] reported that the TPC value for thyme (85.83 mg GAE/g) is higher compared to spearmint (37.13 mg GAE/g). In another related study, the highest total phenolic content was observed in thyme out of all different herbs tested (21.52 mg GAE/g) [20]. The high value of TPC in thyme due to contribution of the main phenolic compounds such as e- vanillic, pyrogall, caffeic, cinnamic and salicylic [11]. These compounds that can delay or prevent the oxidative damage of lipids or other molecules caused by free radicals, can probably be protective against the development of major diseases such as coronary heart disease and cancer in human and plant extracts containing antioxidants including phenolic compounds may prevent from free radical damage [21-24].

Even the value of TPC obtained from spearmint considered lower than thyme, the phenolic components of caffeic acid, eriocitrin, luteolin- 7-O-glucoside, naringenin-7-O-glucoside, isorhoifolin, rosmarinic acid, eriodictyol, luteolin, and apigenin were identified in water-soluble extracts from different *Mentha* species by Dorman et al. [21].

The dried samples of spearmint was described with higher phenolic content (24.89 mg GAE/ g) which means spearmint also can be used as herbs with good natural of antioxidant [25].

DPPH

The DPPH assay or radical scavenging activity was observed when discoloration of samples occurred. DPPH, a stable free radical with a purple color, changes into a stable yellow compound on reacting with an antioxidant. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity [26]. The effect of antioxidants on DPPH radical-scavenging is thought to be due to their hydrogen-donating ability; DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable molecule [27]. Table 1 shows that the thyme extract had shown higher activity (87.17%) to detoxify DPPH free radicals as compared to spearmint (56.90%) extract. This result suggests that the major part of the antioxidant activity in Thyme results from the phenolic compounds. Thyme was observed to have high discoloration followed by spearmint. When the difference in the results was high between the DPPH solution and sample, the percentage of free radical activity is high or the sample had high potential to scavenge the free radical of DPPH.

According to our result, the radical scavenging activity of thyme extract obtained has the percentage that not too much different from the activity of thyme extract reported by Abdelfadel et al. [11] where the inhibition percentage were 87.17% and 91.93% respectively. Heim et al. [28] mentioned that the antioxidant activity of plant extracts is mainly due to the concentration of phenolic compounds in the plant. Moreover, Arabshahi-Delouee and Urooj [29] observed that, the antioxidant activity was correlated with the amount of total phenolic present in the respective extracts in each assay. The related extract contained the highest amount of phenolic compounds and also exhibited the strongest antioxidant capacity in all the assays used. Antioxidant constituents of plant origin are vital substances which protect the body from coronary diseases and from damage caused by free radical-induced oxidative stress [26].

By referring to the study from Ahmad et al. [26], it was observed that antioxidant activity recorded for spearmint was (61-71%) where the percentage not showing big different compared to our results (56.90%). This percentage may be due to the antioxidant and antiperoxidant properties found in spearmint extract. Spearmint has been found to have eugenol, caffeic acid, rosmarinic acid and a-tocopherol and it could enhance error-free repair for DNA damage and hence could be antimutagenic [30,31]. Consequently, Mentha species which is spearmint enable to prevent cell damage through its strong antioxidant activity, by scavenging free radicals and neutralizing toxic invaders [26].

FRAP

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2- pyridyl)-s-triazine (TPTZ), forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content. Intensity of blue color is depending on amount of Fe^{3+} that is reduced to Fe^{2+}. If a sample reduces Fe^{3+} to Fe^{2+}, at the same time it will be oxidized, so that sample can act as antioxidant [32].

In FRAP capacities among water herbs extracts, thyme and spearmint extract were significantly different from each other ($p<0.05$). The results obtained were expressed as μmol TE/g of sample where thyme showed the stronger antioxidant power compared to spearmint extract with 330.63 μmol TE/g and 113.09 μmol TE/g respectively. FRAP assays depend on the mechanism that involves oxidation and reduction reactions.

ORAC

The ORAC method is based on the measuring of the antioxidant scavenging activity against peroxyl radical generated by thermal decomposition of AAPH at 37°C. Fluorescein (FL) was used as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical [19]. As referred to the Table 1, thyme (240.73 μmol TE/g) indicates the significantly higher ORAC value than spearmint (79.96 μmol TE/g). From these values we can conclude that thyme considered rich in antioxidant compared to spearmint. Ninfali et al. [33] was performed a comprehensive evaluation of different foods and spices using the ORAC method. On the basis of fresh weight, they reported ORAC values for thyme is 274.26 μmol TE/g where the value is quite close to our results (240.73 μmol TE/g).

In another recent study, Kratchanova et al. [19] investigated the ORAC antioxidant activity of 25 Bulgarian medicinal plants subjected to water or 80% acetone extractions. The higher ORAC values was obtained by water extraction of thyme (1434 μmol TE/g) compared to spearmint (598 μmol TE/g). According to other research also, thyme (274.26 μmol TE/g) showed the higher ORAC values [34], than spearmint (12.70 μmol TE/g) [35]. Therefore, all of these previous studies were in line and able to support our data regarding the absolutely higher ORAC value of thyme as compared to spearmint even with different values. The differences in the antioxidant

activity between the same materials can be attributed to some environmental factors such as climate, location and temperature which can significantly affect the accumulation of the antioxidant components in plant material [19].
The ORAC assay has several advantages over other available antioxidant scavenging capacity assays that explain its popularity. Unlike DPPH scavenging capacity assays, ORAC measures scavenging activity against a physiologically relevant radical, the peroxyl radical, which is known to play a role in the oxidation of lipids in human bodies and food systems [36]. Moreover, the ORAC assay is conducted under physiological pH and is adaptable for high throughput analysis. Lastly, the ORAC assay takes into account both kinetic and thermodynamic properties of antioxidant-radical reactions [37].

Table 2: Diameter of inhibition zone (mm) of Antimicrobial extracted from thyme and mint leaves against bacterial strains

Bacterial strain	Inhibition zone (mm)		
	Amp 10	Thyme	Mint
Staphylococcus aureus	36	12	8
Bacillus cereus	11	11	7
Escherichia coli	18	8	ND
Listeria monocytogene	7	7	ND
Salmonella typhi	23	7	ND

ND: growth inhibition not detected

Table 2 showed the susceptibility pattern of the methanolic extract of Thyme and Mint against the bacterial isolates. The extract of Thyme was the most efficient extract as they showing the most antibacterial activity against all the isolates tested of *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Listeria monocytogene and Salmonella typhi* with inhibition zones (mm) of 12, 11, 8, 7 and 7 respectively. While the extract of Mint exhibited inhibition against *Staphylococcus aureus* and *Bacillus cereus* isolates with inhibition zones (mm) of 8 and 7 respectively. Positive control of Ampicillin 10 µg (Amp 10) showed positive inhibition towards all bacterial strains means this antimicrobial test was valid. The highest inhibitory effect for Thyme was observed against *Staphylococcus aureus* (zone of inhibition: 12 mm) while the weakest activity was demonstrated against *Listeria monocytogene and Salmonella typhi* (zone of inhibition: 7 and 7 mm) respectively.

CONCLUSION

Thyme (*Thymus Vulgaris L*.) is an important medicinal plant which belongs to the *Lamiaceae* family. *Thymus vulgaris L*. can be regarded as promising candidates for natural plant sources of antioxidants with high value and the most antibacterial activity against all the isolates tested of *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Listeria monocytogene* and *Salmonella typhi*.

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