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Influence of extraction methods on total phenolic, flavonoids and antioxidant activity of *Thymus kotchyanus* L. extract in Semnan Province, Iran

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ABSTRACT

Thymus kotchyanus L. is an aromatic plant belonging to Lamiaceae family and has promising traditional uses to treat some rural disorders. This species like other wild species of thyme is an aromatic plant and has been extensively used as an anti-inflammatory, expectorant, spasmolitic, sedative, antibacterial, antifungal, antioxidant and anti-infection agent. The present work concerned with ethnopharmacology as well as the influence of extraction methods on total phenolic contents (TPC), total flavonoids contents (TFC) and antioxidant activity of of *Thymus kotchyanus* L. extract in Semnan Province, Iran. In this regard, the aerial parts of plant were collected in the blooming period from Tash Mountain (2120 msl) in September 2015. Then, ethanol extracts of the plant were obtained by maceration and ultrasound-assisted methods. In addition, TPC and TFC were determined through the standard spectrophotometric methods using the Folin-Ciocalteu and $AlCl_3$ approaches, respectively. The antioxidant capacity was evaluated *in vitro* by the methods basing on 1,1-diphenyl-2-picryl hydrazyl radical scavenging (DPPH), total antioxidant capacity (TAC) and reducing power (RP) assays. The ultrasonic extract of plant was found to have higher amounts of TFC (81.17±1.07 mgQUE/g) and TPC contents (103.14±2.5 mgGAE/g) accounting for its high potential antioxidant activity (IC_{50}=14.12±0.1 mg/mL) especially using the DPPH method. There was a strong positive correlation between influence of extraction methods on TPC, TFC and antioxidant activity of plant indicating that *T. kotchyanus* posess remarkable antioxidant activity and can be used potentially as a good source of natural antioxidant activities.

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1. Introduction

On the average, around 300 spp from the genus *Thymus* are perennial, aromatic and herbaceous plants which are mainly found in some regions of Asia, Africa, and North America. These herbal plants possess a wide range of medicinal activities including anti-infection, anti-inflammation, expectorant, spasmolitic, sedative, antibacterial, antifungal, anti-aging and antioxidant behaviors (Nasapon et al., 2010; Eghdami et al., 2013; Hossain et al., 2013; Russo et al., 2013; Mohammadhosseini, 2016). *Thymus kotchyanus* has some similarities with some other *Thymus* species. This species as an important medicinal plant, has been used for centuries as spice, home remedy, and as a powerful antispasmolytic, antibacterial, antifungal, expectorant, antiseptic, anthehelmintic and antitusive remedy.

In traditional and folk medicine of many countries, some of *Thymus* spp. have been used in pure form or combined with other herbal species due to their expectorant, antiseptic, antispasmodic, antimicrobial, antifungal, antivirotic, carminative and sedative characteristics to cure rheumatic and skin diseases since the ancient times (Fachini-Queiroz et al., 2012). In many simillar investigations, it has been pointed out that the *Thyme* species contain high quantities of flavonoids and phenolic derivatives and natural

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compounds including thymol (12-61%), carvacrol (0.4-20.6%), 1,8-cineole (0.2-14.2%), *p*-cymene (9.1-22.2%), linalool (2.2-4.8%), borneol (0.6-7.5%), α-pinene (0.9-6.6%), and camphor (0-7.3%). These valuable monoterpenoids show promising antioxidative activity in the treatment of acne, hypertension, infections, and cancers (Takeuchi et al., 2004; Bazylko and Strzelecka, 2007; Al-Bayati, 2008; Golmakani and Rezaei, 2008; Tsai et al., 2011; Fachini-Queiroz et al., 2012; Moldovan et al., 2016).

Reactive oxygen species (ROS) can induce many current inflammatory and infectious disorders such as atherosclerosis, cancer, antibiotic resistantance, infections, arthritis, hypertension, diabetes, etc. So, there has been a great interest to identify natural antioxidant from medicinal herbs as pharmaceutical supplements and food additives. Many of these plant products are called secondary metabolites like flavonoids, tannins, coumarins, phenolics and terpenoids phenolic components and could be classified as potent antioxidant, anti-fungal and antibacterial agents. They can also play critical roles in inhibiting and scavenging of free radicals as anti-pathogen, anti-inflammatory and anticancer compounds (Pourmorad et al., 2006; Mazandarani et al., 2013; Mazandarani et al., 2015). Currently, synthetic antioxidants, such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) which are widely used in the food industries are reported to be carcinogenic. In view of this restriction, extensive investigations are undertaken to find effective natural antioxidant from natural plants sources (Sadeghi et al., 2013). The extracts from aromatic *Thymus* species have different anti-infection, anti-inflammation and other biological activities. Furthermore, in many reports, the components of the *Thymus* plants are greatly influenced in quality and quantity by different ecological factors, such as species, ecotype, collection processes, extraction type and processing technologies (Mancini et al., 2015). The natural antioxidants involving terpenoides, flavonoid and phenolic components in many Lamiaceae species, e.g. *T. kotchyanus* are very useful to prevent and treat many infections. In fact, the global trend is now toward screening of the natural antioxidants from wild endemic medicinal herbs in the treatment of many current infectious diseases (Tepe et al., 2005; Sadeghi et al., 2013; Mazandarani et al., 2015).

Phenolic, non-phenolic, diterpene and flavonoid compounds of *Thymus* extracts (thymol, carvacrol , gallic aicd, linalool, geraniol and *p*-cymene) are the major antifungal, antibacterial, antioxidant and antipathogenes components which are so prevalent in many species of thyme plants (Shan et al., 2005; Hashemi-Moghaddam et al., 2015; Bendif et al., 2017; Koksal et al., 2017; Tohidi et al., 2017; Vitali et al., 2017).

The widespread distribution of *T. Kotchyanus* L. in the mountainous regions of Semnan Province prompted us to establish this study. In this report, we have compared the impact of two different extraction methods, namely maceration and ultrasonic extractions on chemical composition and antioxidant activity of *T. kotchyanus* L. growing wild in Semnan Province.

2. Experimental

2.1. Chemicals and reagents

All chemicals used in this study were of analytical reagent grade and of highest purity. The chemicals were purchased from Merck and used without further purification.

2.2. Apparatus

The ultrasound extraction was carried out using a commercially available ultrasonic bath (Parasonic 2600s, 2.6 lit, 100W). The extraction process was performed by adding 10 g of the plant sample to a 100 mL ethanol (solvent) in a glass tube. Then, the resulting solution was placed in the ultrasonic bath at 25 ºC and continously sonicated for 10 min (Tavakolpour et al., 2016).

2.3. Plant material and extraction

The aerial parts of *T. kotchyanus* L. (Fig. 1) were collected in the blooming period from Tash Mountain (2120 msl), Semnan Province, Iran (Fig. 2) in early July 2015.

Fig. 1. Representation of *Thymus kotchyanus* L.

The plant was first identified by a local botanist and a voucher specimen was deposited at the Herbarium of RCMP (Research Center of Medicinal Plants, Islamic Azad University of Gorgan Branch, Golestan Province, Iran (Herbarium No. HRCMP:98). The plant samples were ground to a fine powder, and then in the next step, 30-g portions of the prepared powders were extracted with 500 mL of a hydroalcoholic (80%) solution by using the maceration method for 8 hours

and the resulting solution was filtered and stored at 4 ºC. The obtained solution was finally cooled down to the room temperature and stored prior to its use for the evaluation of antioxidant activities along with total phenolic and flavonoid contents.

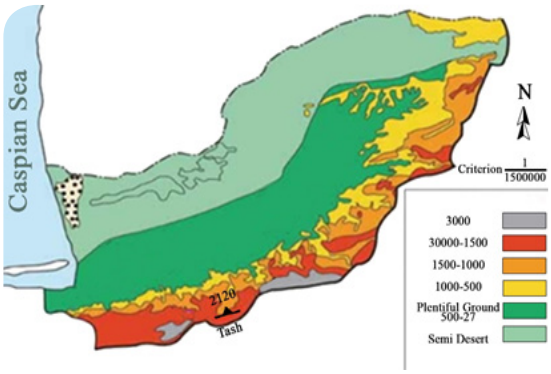

Fig. 2. The map of the sampling area.

2.4. Determination of total phenolic content (TPC)

The total phenolic content (TPC) of the *T. kotchyanus* L. extracts was determined using the Folin-Ciocalteu reagent (Eghdami et al., 2013). Accordingly, the reaction mixture contained 200 µL of a diluted thyme extract, 800 µL of freshly prepared and diluted Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate (Na_2CO_3). The final mixture was diluted to a final volume of 7 mL with deionized water. The obtained mixture was kept in the darkness at ambient conditions for 2 h to complete the reaction. In the next step, the absorbances of the solutions were measured at 765 nm. In our evaluations, gallic acid was used as the standard and the results were expressed as mg gallic acid (GAE)/g plant.

2.5. Determination of total flavonoid content (TFC)

Total flavonoid content (TFC) was determined using the aluminium chloride ($AlCl_3$) method according to a reliable approach using quercetin as the standard (Ordonez et al., 2006). In this regard, the plant extract (0.1 mL) was added to 0.3 mL of distilled water followed by addition of 0.03 mL of $NaNO_2$ (5% w/v). After 5 min. at 25 ºC, $AlCl_3$ (0.03 mL, 10%) was added. After further 5 min., the reaction mixture was treated with 0.2 mL of NaOH (1 mM). Finally, the reaction mixture was diluted to 1 mL with water and the absorbance was measured at 510 nm. The results were expressed as mg quercetin (QE)/g of *T. kotchyanus* L.

2.6. The DPPH assay for evaluation of antioxidant activity

The antioxidant activity of samples was measured by using the DPPH assay. *T. kotchyanus* L. extract (80 µL) was first diluted 15-fold with distilled water.The antioxidant activity was tested by the DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical scavenging method. In our evaluations, we prepared a diluted solution of DPPH (1.0 M). The absorbance of a mixture of 1 mL of the extract and 1 mL of the DPPH solution was measured at 517 nm. The radical scavenging activity was calculated from the equation:

Percentage of radical scavenging activity = (Abs control - Abs sample)/Abs control×100 (eqn.1).

2.7. Reducing power

The reducing power can be determined by a standard method given by Eghdami et al. (2013). To test the reducing power of the plant extract of *T. kotchyanus* L, 1.0 mL of this extract was mixed with 2.5 mL of a phosphate buffer (200 mM, pH 6.6) and 2.5 mL of potassium ferricyanide (30 mM). The resulting mixture was then incubated at 50 ºC for 20 min. Thereafter, 2.5 mL of trichloroacetic acid (600 mM) was added to the reaction mixture, centrifuged for 10 min. at 3000 rpm. The upper layer of solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (6.0 mM) and the respective absorbance was measured at 700 nm. Ascorbic acid was used as positive control, as well.

2.8. Total antioxidant capacity

The total antioxidant capacity of the extracts was evaluated according to the procedure described by Arabshahi-Delouee and Uroo (2007). These sequential steps were as follows. A 0.1 mL portion of sample (plant extract) was first mixed with 1 mL of a reagent solution composed of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The tubes containing the reaction solution were then incubated in a thermal block at 95 ºC for 90 min. The absorbance of each solution was finally measured at 695 nm using a UV-VIS spectrophotometer (UVmini-1240) against blank after cooling to room temperature. In our determinations, methanol (0.3 mL) was used as the blank. The total antioxidant activity was expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing a variety of ascorbic concentrations (1000, 500, 250, 125, 62.5 and 31.25 µg/mL) with ethanol (80%).

2.9. Statistical analysis

The results of statistical analysis were expressed as mean±standard error of the mean. The differences between the means were determined by one-way analysis of variance (ANOVA) using the SPSS package (Version 22). In all cases, differences were considered significant if $p<0.05$.

3. Results and Discussion

3.1. Ethnopharmacological properties

Our ethnopharmacological study in this study showed that in many field observations of the sampling area, Tash Mountain, Semnan Province, Iran. *T. kotchyanus* L. is among the most wild aromatic perennial herb, which has been used traditionally alone or in combination with other medicinal herbs as an antispasmoic, anti-inflammation, expectorant, sedative, antibacterial, antifungal, antioxidant and anti-infection to treat many dermal and intestinal infections, cold, flu, healing wounds such as burnt skins and some vaginal infections.

3.2. Phytochemical evaluations

According to Table 1, there is a strong positive correlation between the influence of extraction methods and TPC, TFC and their antioxidant activities. The ultrasonic extract of the plant (*T. kotchyanus* L.) was found to be a rich source of TFC (81.2±1.1 mg QUE/g) and TPC (103.1±2.5 mgGAE g^{-1}). It also exhibited a good potential antioxidant activity (IC_{50}=14.1±0.1 mg/mL) especially using the DPPH assay. Regarding the findings of this study, *T. kotchyanus* L. can be used potentially as a good source of natural antioxidants due to its traditional uses to treat the rural disorders.

The results exhibited that the types of extraction methods are effective on chemical composition of plant extracts (TPC and TFC) and on potential of scavenging of the DPPH free radicals in the reaction medium. Due to results, the ethanol extract which obtained by ultrasonic extraction method had remarkable TPC (103.1±2.5 mgGAE g^{-1}) and TFC (81.2±1.1 mg QUE/g). In addition, antioxidant power of the extract by using the ultrasonic method (IC_{50}=14.12±0.1 mg/mL) is comparable to IC_{50} of BHT 18.2±0.8 mg mL^{-1}) using the DPPH method. Regarding the obtained results, the IC_{50} values were according to the ultrasonic extract>maceration extract order (Table 1). Furthermore, the obtained correlation coefficient showed that TPC and TFC were responsible for antioxidant efficiency in *T. kotchyanus* extracts because the plant extract obtained by the ultrasonic method possessed more secondary metabolites. Therefore, this extract has more antioxidant activity especially in the DPPH method. Our findings are in agreement with previously published papers in the literature (Youdim et al., 2002; Tavakolpour et al., 2016). Different *Thymus* species particularly *T. vulgaris*, which are well-known herbs are widely cultivated in many regions of the world (Ahmad et al., 2014; Cerda et al., 2013). They are known to contain high contents of phenolic (caffeic acid, rosmarinic, thymol and carvacrol) and flavonoid compounds, which are found in their essential oils and organic extracts (Fecka and Turek, 2008). Several authors already reported that the essential oil with natural terpenoids like thymol, flavonoid and phenolic compounds (carvacrol) exhibited a wide range of biological activities such as antioxidant, anti-inflammatory, antimicrobial, anticancer, antitussive, expectorant, antispasmodic and anti-allergic effects (Barile et al., 2007; Höferl et al., 2009; Amin, 2012; Achour et al., 2012). Accordingly, it could be concluded that ethanol extract of *Thymus* species such as *T. kotchyanus* L. may be used as a natural antioxidant, but the type of extraction methods, type of solvent and another ecological stresses could be effective on quantity and quality of plant extract and their biological and medicinal effects. In many reports, the natural antioxidants found in herbal plants such as *Thymus* species with high antioxidant and anti-infection compounds, could positively reduce the potential risk of development of inflammation, diabetis, cancer, hypertension and other infectious diseases (Mazandarani et al., 2015). According to this report, in traditional medicine of Golestan Province, the *Thymus* products have been used to treat many dermal wounds and leishmaniasis. They have also been recognized as painkiller, anti-inflammation and anti-infection agents (Esmaeili et al., 2009; Mazandarani et al., 2015). Many similar researches showed that the antioxidant and antimicrobial activity of other *Thymus* species are considerably related to their main phenolic and terpene components like thymol, carvacrol, γ-terpinene, terpinene 4-ol and *p*-cymene representing a high inhibition against a wide range of microorganisms (Sonboli et al., 2004; Vagionas et al., 2007; Oke et al., 2009; Sadeghi et al., 2013). In some reports, monoterpene hydrocarbons and oxygenated monoterpenes such as thymol, terpinene, carvacrol, 1,8-cineole, γ-terpinene and *p*-cymene as the major constituents of *Thymus* species, *Satureja thymbra*, *Perovskia abrotanoides*, *Thymus carmanicus*,

Table 1
Total phenolic, flavonoids contents and antioxidant activities of hydroalcoholic extract of *Thymus kotchyanus* L.

Extraction method	Phenolic content (mg GAL/g)	Flavonoids content (mgQE)/g)	Antioxidant DPPH (IC_{50}=mg/mL)	Antioxidant (TAC) mmol/L	Reducing power
Maceration	67.8±0.1	43.3± 2. 5	25.1±0.2	34.9 ±1.4	51.2±0.5
Ultrasonic	103.1±2.5	81.2±1.1	14. 1±0.1	23.2±1.5	18.2±1.5

Artemisia sieberi and *Ferula gummosa* have shown strong antioxidant, antibacterial and antifungal activities (Mazandarani et al., 2015; Ben Jabeur et al., 2017). Regardless of these well-documented biological activities of *Thymus* plants, they have been traditionally used in North Provinces of Iran (Golestan and Semnan) as anti-spasmodic, anti-inflammation, antifungal and anti-infection .

4. Concluding remarks

The results of the current study showed that the ethanol extract of *T. kotchyanus* L. especially the extract which obtained by using the ultrasonic method had the highest scavenging activity. Furthermore, there was a direct correlation between the amounts of phenolic and flavonoid compounds of this plant and their subsequent scavenging effect. These results demonstrate that the antioxidant activities observed can be ascribed both to the type of extraction methods and a variety of quality and quantities of secondary metabolites of plant (*T. kotchyanus* L.). Therefore, the plant can be used as a potential source of natural antioxidants for pharmacological preparations which are well-documented by the present work. The highest values of free radical scavenging activity found in this attempt could be referred to their higher content of phenolic and flavonoid components, especially when using the ultrasonic extracting approach.

Conflict of interest

The authors declare that there is no conflict of interest.

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