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In vitro Antioxidant Properties of Extracts from *Thymus vulgaris* and *Thymus fragrantissimus*

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This work has been performed to explore the *in vitro* antioxidant activities of extracts from *Thymus vulgaris* and *Thymus fragrantissimus*. The samples were respectively extracted with ethanol and water, and the antioxidant effects were employed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and β-carotene bleaching experiment. It was found that the ethanol rather than water extracts possess the antioxidant profiles, with a concentration-dependent relationship. The respective antioxidant capacities decreased in the order of *T. vulgaris* ethanol extract > *T. fragrantissimus* ethanol extract > *T. vulgaris* water extract > *T. fragrantissimus* water extract. Further, the antioxidant activities of extracts from *T. vulgaris* were more efficient than those of *T. fragrantissimus*. At the concentration of 4.0 mg/mL, in the DPPH assay and β-carotene bleaching systems, the values of *T. vulgaris* ethanol extracts were reached to 88.97 ± 0.76% and 81.00 ± 0.41%, respectively. On the basis of the results, we conclude that *T. vulgaris* represents a valuable natural antioxidant source and may potentially be applicable in health food and pharmaceutical industries.

Key words: *Thymus vulgaris*, *Thymus fragrantissimus*, Ethanol extraction, Water extraction, Antioxidant activities.

Reactive oxygen species (ROS) are highly reactive and toxic molecules, formed as a natural byproduct of the normal metabolism of oxygen, including singlet oxygen ($^1O_2$), superoxide ion (O_2^-), hydroxyl ion (OH^-), and hydrogen peroxide (H_2O_2)[1]. In general, harmful effects of ROS on the cell are most oxidative damages to DNA, proteins, enzymes, and lipid peroxidations, being related with pathogenesis of oxidative diseases[2]. Dietary supplementation of synthetic antioxidants will lessen the cellular injury induced by ROS. Recently, due to toxicological concerns associated with synthetic compounds and the development of natural antiseptic, there have been many attempts to use natural substances as food preservatives and antioxidants, such as the extracts from genus *Thymus*[3, 4]. Bioactive constituents from theses plants usually have at least one benzene ring with a hydroxyl functional group, associated with the single electron donating potential[3].

The genus *Thymus*, belongs to the family of *Lamiaceae*, has the aromatic and perennial characteristics, flowering with a strong aroma and attracting bees, flies etc.[5]. About 215 species of this genus are grown in the world, and abundantly distributed in Asia, Africa and North America, with most species being endemic[6]. Generally, the plants are small shrubs about 25 cm high, with quadrangular stem and branches, as well as 6-12 mm long leaves. *Thymus* species are well known as medicinal plants, because that their leaves and flowering parts are widely used as herbal tea,

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antiseptic, antitussive, and treating colds[7, 8]. Meanwhile, extracts from *Thymus* are beneficial to health and valuable natural antioxidants in cosmetic and pharmaceutical industries, also for flavoring and keeping food products[4]. The identified main active ingredients of *Thymus* extracts show high concentration of phenolic monoterpenes, thymol and carvacrol, especially thymol being about 20-54%[6, 9]. Besides, the essential oil from *Thymus* species also contains p-cymene, myrcene, borneol and linalool, commercially used to produce antiseptic and mouthwash, because of the pharmacological properties[4, 6, 9]. In China, although wild thyme despites the strong resistance, ease of cultivation, but the active ingredient and ornamental value are lower than those introduced from abroad, such as *Thymus vulgaris* and *Thymus fragrantissimus*[10].

To the best of our knowledge, a systematic comparison of *in vitro* antioxidant activities of *T. vulgaris* and *T. fragrantissimus* has not been performed yet. In this study, the extracts will be respectively obtained using ethanol and water, and the differences of activities will be evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and β-carotene bleaching test. We anticipate that the results will be of value in the development of antioxidant agents.

MATERIALS AND METHODS

Preparation of extract

T. vulgaris and *T. fragrantissimus* were air dried at room temperature and ground in a grinder with a mesh of 2 mm in diameter. Subsequently, they were passed through a 0.5 mm sieve to obtain a fine powder. The extracts were filtered and concentrated via the water and ethanol extraction methods, respectively[11,12]. Finally, the extracts were lyophilized and kept in dark at 4 °C.

DPPH radical scavenging assay

The stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used as a reagent in the spectrophotometric assay[13]. The sample (100 µL) was mixed with 1.4 mL ethanol and then added to 1.0 mL ethanol, including 0.004% DPPH (Sigma–Aldrich). The mixture was vigorously shaken and then immediately placed in a UV–Vis spectrophotometer (AWARENESS) to monitor the absorbance value at 517 nm, with the reference of ascorbic acid (Sigma–Aldrich). Inhibition percentage of DPPH (radical-scavenging activity) was estimated as follows,

$$\text{Inhibition percentage (Ip)} = [(AB-AA)/AB] \times 100^{14} \quad ..(1)$$

where AB and AA are the absorbance values of the blank sample and the tested samples, respectively. Each experiment was repeated in triplicate.

β-carotene/linoleic acid bleaching assay

Antioxidant activity of the extracts was also determined throughout β-carotene bleaching test[13]. Firstly, 10.0 mg of β-carotene (type I synthetic, Sigma–Aldrich) was dissolved in 10.0 mL chloroform. The carotene–chloroform solution of 0.2 mL was pipetted into a boiling flask containing 20.0 mg linoleic acid (Sigma–Aldrich) and 200 mg Tween 40 (Sigma–Aldrich). Chloroform was evaporated under vacuum and 50 mL distilled water was then added to the residue, with the aim to form an emulsion. 5.0 mL emulsion was added to a tube containing 0.2 mL sample solution, and the absorbance was immediately measured at 470 nm in contrast to the blank without β-carotene. The test tubes were incubated in a hot water bath at 50 °C for 60 min, and the oxidation of the emulsion was monitored at 470 nm on an ultraviolet spectrometer. Negative controls were filled by 200 µL distilled water instead of the extracts, and the antioxidant butylated hydroxytoluene (BHT, Sigma–Aldrich) was used as a positive control. Antioxidant activities (AA) of the extracts were expressed throughout the following equation:

$$AA = 100(DRC - RS)/DRC \quad ...(2)$$

where DRC = degradation rate of the control = [ln(a/b)/60]; DRS = degradation rate in the presence of the sample = [ln(a/b)/60]; a = absorbance at time 0; and b = absorbance at 60 min. All tests were carried out in triplicate.

RESULTS AND DISCUSSION

DPPH assay

The antioxidant activities of *T. fragrantissimus* and *T. vulgaris* extracts,

respectively obtained by the water and ethanol extraction methods, were determined by DPPH assay. Regarding as the water extraction, free radical-scavenging activities of *T. fragrantissimus* and *T. vulgaris* extracts (4.0 mg/mL), were determined to be 46.11 ± 1.21% and 52.13 ± 0.79%, respectively; whereas the extracts obtained by ethanol extraction were 77.03 ± 0.99% and 88.97 ± 0.76% (Fig. 1). It indicated that the dependence on solvent is relatively large, which seemed the ethanol extraction is more fitness than water extraction, for the *Thymus* extracts. Besides, the free radical-scavenging rates were increased in concentration-dependent manner. Among the four extracts, the scavenging activity of *T. vulgaris* ethanol extracts was much higher than others, at the concentration of 4.0 mg/mL, with the value of 88.97 ± 0.76%. The respective scavenging activities decreased in the order of *T. vulgaris* ethanol extract > *T. fragrantissimus* ethanol extract > *T. vulgaris* water extract > *T. fragrantissimus* water extract (Fig. 1).

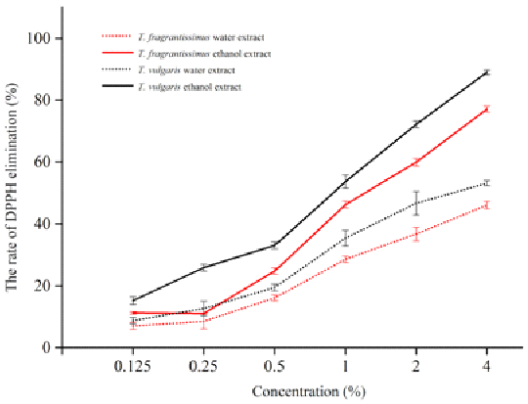
β–carotene bleaching test

The antioxidant activities of *T. fragrantissimus* and *T. vulgaris* extracts were further determined by the β-carotene bleaching test. The results were consistent with the data obtained from the DPPH test, with the same decreasing order (Fig. 2). The effects of *T. fragrantissimus* extracts obtained by water or ethanol extraction were 40.23 ± 2.28% and 75.15 ± 1.11%, respectively, at the concentration of 4.0 mg/mL; meanwhile, the values of butylated hydroxytoluene (BHT), *T. vulgaris* extracts obtained by water or ethanol extraction were 94.89 ± 3.12 %, 49.89 ± 3.21% and 81.00 ± 0.41%, respectively. It seemed that the inhibition capacities of all the tested samples were mostly related to their concentrations, and the values of the four extracts were both lower than that of the synthetic antioxidant BHT (Fig. 2).

Implications from antioxidant activity

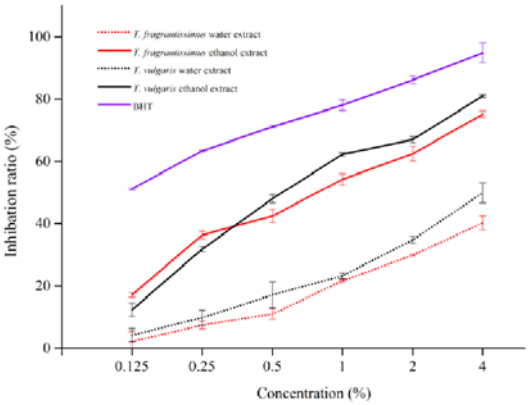
Recently, *Thymus* species are commonly known as medicinal plants due to the pharmacological properties. It has been confirmed that their extracts have relatively strong antibacterial or antioxidant activities, with the main active chemical ingredients of thymol and phenolic secondary metabolites (flavonoids and carvacrol etc.)[4, 6-9]. It was also found that phenolic and flavonoid compounds derived from *Thymus* species exhibited concentration-dependent antioxidant and free radical scavenging activities[15]. Moreover, an inverse correlation was found between the antioxidant effects and the amount and diversity of polyphenols[16], and water/ethanol is the most efficient for the extraction of antioxidant phytochemicals[17].

In our works, the antioxidant activities have also been demonstrated in *T. fragrantissimus* and *T. vulgaris* extracts obtained by the water and ethanol extraction methods, and ethanol rather than water fits the extraction of antioxidant compounds from *Thymus* (Figs. 1 and 2). Although capacities of test samples were generally lower more than that the standard antioxidant (BHT), they showed marked antioxidant activities. It can be explained by the fact that the concentration of the active components, which comprise only a fraction, should be much lower than the standard antioxidant we used. Therefore, if the active



Values of each curve are means ± SD (n = 3). $p < 0.01$.

Fig 1. The rate of DPPH elimination.



Values of each curve are means ± SD (n = 3). $p < 0.01$.

Fig 2. β-Carotene bleaching test.

components were isolated and purified, they would probably show higher activities than those observed here. Based on the results described above, it is suggested that the *T. fragrantissimus* and *T. vulgaris* extracts have antioxidant properties, especially *T. vulgaris* ethanol extracts, by enhancing the cell viability, reduction of production of ROS, inhibition of oxidative damage, mitochondria dysfunction and ultimately inhibition of cell apoptosis[18].

CONCLUSIONS

In summary, our study showed that *T. fragrantissimus* and *T. vulgaris* extracts obtained by the water and ethanol extraction methods all had the *in vitro* antioxidant properties, especially *T. vulgaris* ethanol extracts, with the aid of DPPH assay and β-carotene bleaching test. The respective antioxidant effectiveness decreased in the order of *T. vulgaris* ethanol extract > *T. fragrantissimus* ethanol extract > *T. vulgaris* water extract > *T. fragrantissimus* water extract. Besides, the ethanol extraction is more fitness than water extraction, for the *Thymus* extracts. Due to the virulence of *T. vulgaris* ethanol extract, it can work as natural antioxidant, which is a promising alternative to the use of synthetic antioxidants in food supplement or in pharmaceutical and cosmetic industry.

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