Journal of Physical and Theoretical Chemistry
of Islamic Azad University of Iran, 14 (4) 353-360: Winter 2018
(J. Phys. Theor. Chem. IAU Iran)
ISSN 1735-2126

Evaluation of the antioxidant activity of *Thymus* Using Electrochemical Methods and radical scavenging method

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Received April 2018; Accepted May 2018

ABSTRACT

Thymus is regarded as one of the medicinal species, which according to obtained chromatograms of floral water of *Thymus*; one of the main components is thymol as strong antioxidant. In this study the appropriate and physically powerful correlation was found between the anti-radical and oxidation potentials. The antioxidant activity of the *Thymus* was measured by using square wave and cyclic voltammetry methods and 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) method in the concentration range of 1% to 2.5% V/V. Electrochemical methods are used based on the relationship between oxidation power and antioxidant strength, which compared with ascorbic acid as standard. Based on the results of electrochemical methods, these methods have several advantages over the $DPPH^{\cdot}$ method such as simplicity, cheaper operating costs, short integration time, high speed, low solvent and sample usage. Moreover, the square wave voltammetry method is more sensitive method for evaluation the antioxidant activities compared to DPPH and cyclic voltammetry methods.

Keywords: Antioxidant activity; Square wave voltammetry method; Cyclic voltammetry; Free radical scavenging method; DPPH

INTRODUCTION

Thymus (*Thymus vulgaris L.)* is a several-year-old plant from *Lamiaceae* family and native Mediterranean [1], including small green spear-shaped leaves with a length of 6-12 mm and short and rectangular stems at the bottom of the woody stem, which are green in younger part. Essential oil is available in different parts of *Thymus* although it is mostly observed in blooming shoots. Further, *Thymus* is used as a spice and condiment, having anti-bacterial, antimicrobial and anti-oxidant effects, due to thymol and carvacrol phenolic compounds [2]. During the last decades, an increasing consumption of natural substances as well as the prevalence of diseases has motivated a large number of researches to conduct their studies on using essential oils and floral waters. Essential oils and floral waters include anti-parasitic and anti-toxin properties, as well as antimicrobial ones. These characteristics are related to the type of active ingredient included in essential oils and floral waters [3]. In addition, essential oils and floral waters are widely used in various industries such as food, pharmaceutical, cosmetic, sanitary, industrial, and other

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industries. Further, thymol and carvacrol are considered as the main components of the essential oils of the Lamiaceae family, which are chemically similar although the position of the hydroxyl group is differs in these two compounds [4]. Furthermore, the antimicrobial effect is related to the permeability of the cell membrane through these compounds, which can chelated with membrane surface cations and disturb vital activities [5]. Thymol (2-isopropyl-5-methylphenol) is a natural and antioxidant compound, which is found in various herbs such as *Thymus* and Ajwain. Generally thymol is the main Phenol component in *Thymus* and carvacrol is the secondary one. Thymol includes many uses in different areas such as perfume production, food stuffing, mouthwash and cosmetics.

Some methods such as UV spectrophotometry, high performance liquid chromatography, and liquid chromatography were used to determine the thymol [6, 7].

Antioxidant capacity is one of the most important antioxidant parameters, which is defined as the combination ability of preventing the oxidative degradation of various compounds such as lipid peroxidation. Generally, these methods are based on the direct reaction between the studied compounds and free radicals or based on the reaction with transition metals [8-13]. Different assays have been used to evaluate the antioxidant activity of natural products, but a comparison of the results is very difficult because of the different experimental methods adopted. Antioxidant activities of pure compounds and floral water have been determined, among others, by an accelerated test , by using radical species such as ABTS+• and DPPH• , by the ESR spin trapping technique and by measuring the oxygen consumption in a heterogeneous lipid/water emulsion with lipid oxidation initiated by metmyoglobin [14-19]. However, all of these methods include some disadvantages since they require the use of specific reagents and a time-consuming process of preparing the samples. However, these methods rely on some parameters such as temperature, time of analysis, a combination of extracts, the concentration of antioxidants and pro-oxidants and the like [20, 21].

Electrochemical methods are still being developed to specify antioxidant capability. Electrochemical measurement methods (cyclic voltammetry method and square wave voltammetry method) include many benefits such as a quick determination of the antioxidant capacity of many compounds. Based on these experiments, glassy carbon electrode (GCE) is often used. Low oxidation potentials represent the greater power and the ease of electron donation in molecules. Therefore, it is regarded as a stronger antioxidant [22, 23]. Electrochemical methods provide a fast, simple, and sensitive method to analyze the bioactive compounds, along with refining the radicals as well as the antioxidant capacity. These methods have low cost and often do not require a time-consuming preparation of samples [24-29]. The Redox potential of glass-carbon electrode is the main parameter considered in these methods. In order to compare the results by various methods, the parameters characterizing the antioxidant properties of the sample could be evaluated by unique antioxidants such as tannic and ascorbic acid.

Consequently, the present study aims to determine the antioxidant activities of *Thymus* through comparing DPPH free radical scavenging method and cyclic voltammetry and square wave voltammetry method as an electrochemical method.

MATERIALS AND METHODS

Instruments and chemicals

The following materials and instruments

were used in order to obtain the antioxidant property by electrochemical and DPPH free radical scavenging method:

Thymus (*Thymus vulgaris*) and DPPH were purchased from Karaj Botanical Institute, Sigma Aldrich Company, respectively. NaCl, acetic acid and phosphoric acid, vitamin C, and ethanol and carbon tetrachloride were purchased from Merck Company, Auto lab Instrument model PGSTAT302N. Cary 300 Spectrophotometer, manufactured by the Varian Corporation of Australia, was used to measure the absorbance. All of the electrochemical studies were conducted by three installed electrodes, Ag / AgCl as the reference electrode, the platinum as auxiliary electrode, and the glassy carbon as a working electrode. In the present study, HP-6890 GC Instrument of Hewlett Packard Company (USA), the MS Instrument model HP-5973 of the Hewlett Packard Company, and FT-IR Instrument model 870 of the Nexus Company used.

Hydrodistillation method

Distillation with water is appointed to extract the floral water. 100 g of dried plant sample was placed in distillation flask, then deionized water was added until two third of the flask was full; afterwards, the flask was attached to Clevenger apparatus. The steam that contains the essence and floral water comes out of the flask after heating it, and then is cooled in the cooling section of the apparatus, turns into liquid. Then, a two-phase mixture is created with the oily essence on the top as a supernatant, and the floral water that is mixed with water is at the bottom. Finally, the water phase is separated and kept in refrigerator in dark glass.

Dispersive Liquid- Liquid micro extraction for of Thymus floral water (DLLME)

5 µL of *Thymus* extract was added to 500 µL of ethanol as a disperser solvent and 100 µL carbon tetrachloride as extraction solvent. Then, the cloudy solution was centrifuged for 5 minutes at 3000 rpm. Finally, 1 µL of the extracted sample was injected to the GC/MS Instrument.

Evaluation of antioxidant activity by DPPH method

In this study, the antioxidant property was determined using 2, 2-diphenyl-1-picrylhydrazyl hydrate (the initial concentration of DPPH was 6×10^{-5} µg/ml), different concentrations of floral water and ascorbic acid (1%, 1.5%, 2% and 2.5% V/V) were prepared and 2 mL of each certain concentration added to 1 mL DPPH. The reaction mixtures were kept in the absence of light for 25 minutes at 30 ° C. Then, the absorbance of the samples at 517 nm was read by a UV-Vis spectrophotometer. Finally, Radical scavenging activity was calculated through the following formula:

$$RSA\% = (A\ Blank - A\ Sample / A\ Blank) \times 100$$

RSA%= Radical scavenging activity;
A Blank = Blank absorption in 517 nm;
A Sample = Sample absorption in 517 nm

Inhibitory Concentration (IC_{50}) was used to compare antioxidant activity of *Thymus* floral water than standard. IC_{50} is concentration of floral water, which is used to trap 50% of free radicals. The experiment was repeated on ascorbic acid for the comparison.

Measuring the antioxidant activity by electrochemical method such as cyclic voltammetry and square wave voltammetry

Cyclic voltammetry and square wave voltammetry were performed with AUTOLAB Analyzer and with three electrodes. At the first, concentration of floral water and ascorbic acid were prepared in 1%, 1.5%, 2% and 2.5% V/V.

Then, 0.1M solutions of acetic acid and phosphoric acid were used in pH= 3 as the supporting electrolyte. In the next stage, ionic strength was obtained with NaCl 0.5M. Finally, all potentials were measured by the Ag/AgCl electrode, and platinum electrode was considered as the auxiliary electrode and the glassy carbon electrode was used as working electrode. Notice that, the glassy carbon electrode was polished with alumina powder to be cleaned. Cyclic voltammetry method and square wave method parameters were tabulated as follows (Table 1 and Table 2):

Table 1. Cyclic voltammetry method parameters

Parameters	Values
Start potential	0.01 V
Upper vertex potential	2 V
Lower vertex potential	0 V
Stop potential	0.01 V
Number of scans	1
Scan rate	0.1 V/S
Step	0.00244 V

Table 2. Square wave method parameters

Parameters	Values
Start potential	0.0 V
Stop potential	2.0 V
Step	0.005 V
Modulation amplitude	0.02 V
Frequency	25 Hz

RESULTS AND DISCUSSION

Results of the GC/MS

Subsequent to injection of the extracted sample from floral water of *Thymus* to the GC/MS, the chromatogram of each compound was identified; Fig 1 shows the obtained chromatograms of floral water of *Thymus.* Then, chromatogram was compared with the standard indices like the normal alkanes, Kovats index of the compounds. Two compounds were identified in floral water of *Thymus*; Table 3 tabulates the percentages of compounds for each component.

Table 3. The compounds percentage in *Thymus* water

Compounds percentages	Kovats index (K.I.)	Names of the compounds
%80.27	1286	Thymol
%12.32	1277	Phenol

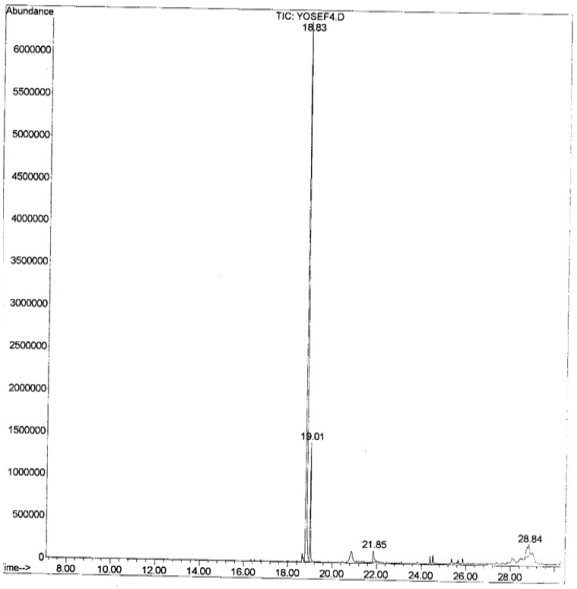

Fig. 1. The GC/MS total ion current of floral water of *Thymus.*

Results of comparing Thymus floral water and ascorbic acid by applying the DPPH-free radical trapping method

Fig 2 shows the amount of RSA % (Radical scavenging activity) for ascorbic acid and *Thymus*. As can be seen antioxidant property ascorbic acid is little stronger than floral water of *Thymus* sample. Regarding Table 4 the absorption would be decreased when concentration increased also, the results show the amount of RSA% for *Thymus* is slightly smaller than the amount of RSA% for ascorbic acid in different concentrations by DPPH method. Subsequently, it can be said that

ascorbic acid has somewhat more antioxidant activities by DPPH method (Table 4). Afterward, IC_{50} was determined by the slope of equation, since the less amounts of is IC_{50} desirable the results indicated the antioxidant activity of ascorbic acid higher than *Thymus*. (Table 5).

Table 4. Values of RSA% and absorption for *Thymus* and ascorbic acid in different concentrations

Samples	Concentration (V/V %)	Absorption the remained DPPH		RSA%	
		Thymus	**Ascorbic Acid**	***Thymus***	**Ascorbic Acid**
1	1.0 %	0.080	0.168	41.219	30.434
2	1.5 %	0.066	0.081	51.506	66.304
3	2.0 %	00.31	0.042	75.753	82.608
4	2.5 %	0.006	0.013	95.591	94.565

Table 5. Values of IC_{50} obtained for *Thymus* and ascorbic acid

IC_{50}	
Thymus	Vitamin C
1.322	1.089

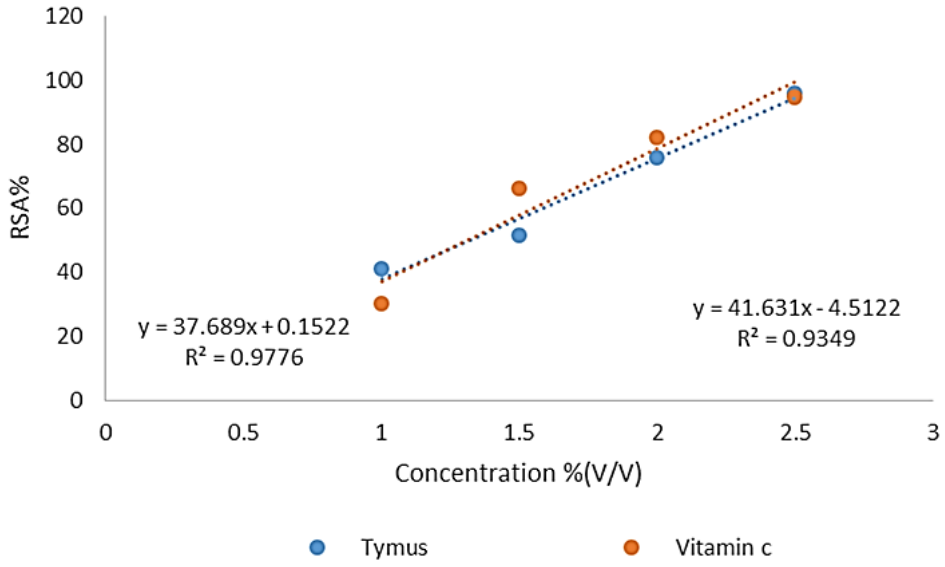

Fig. 2. RSA% in different concentrations for *Thymus*; determination coefficient for *Thymus* R^2=0.9776 and for ascorbic acid; R^2=0.9349.

Results of comparing Thymus floral water and ascorbic acid by square wave voltammetry method and cyclic voltammetry method (CV)

Electrochemical measurement methods include some benefits such as a rapid determination of antioxidant capacity of many compounds. As demonstrated in Fig 3 (a), the cyclic voltammetry method is not always directly related to the antioxidant level of the sample, which is the major problem of this method. Thus, the spike method was implemented for promoting the sensitivity. In spike method, thymol was used with a concentration of 6 M. Fig 3 (b) demonstrated the cyclic voltammetry

Thymus floral water after the spike. As can be seen the intensities of the Voltammograms were increased with the increasing the concentration of *Thymus* floral water. The accuracy of this method is satisfactory, due to the recovery percentages which were 88%, 95% and 121% (the percentage of the recovery calculated from the concentration of 20% (V/V) *Thymus*).

The square wave voltammetry method is in high-quality shape from scanning speed and sensitivity point of views. As demonstrated in Fig 4, the intensity of Voltammograms would be increased with increasing the concentration of sample. The accuracy of this method is reliable, due to the recovery percentages which are 106%, 112% and 119%. The percentage of the recovery calculated from the concentration of 1% (V/V) *Thymus*. Finally, the sensitivity and accuracy of this method is higher than of the previous methods and this method does not need any spike.

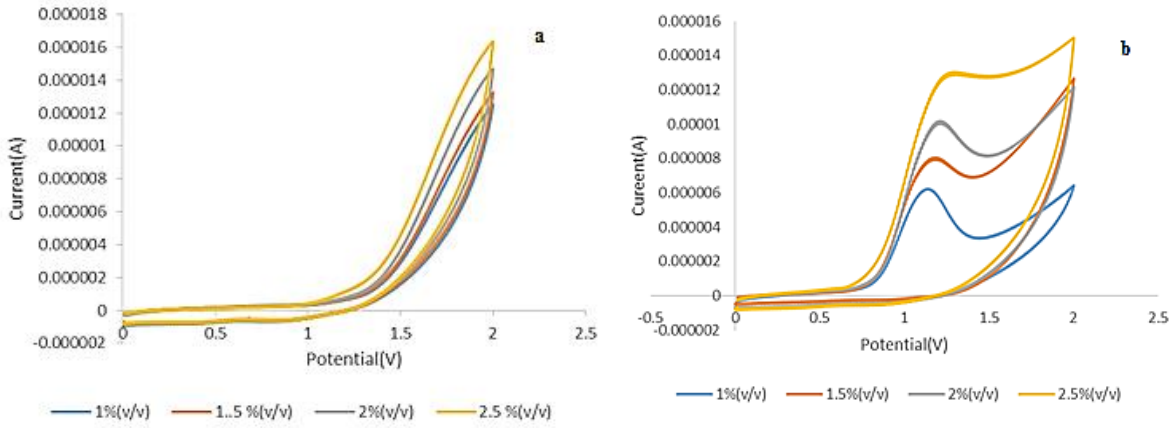

Fig. 3. Cyclic voltammetry of **a:** *Thymus*, **b:** *Thymus* after spike Potential was swept between 0 and 2.0 V. Scan rate was 0.1 Vs^{-1}. pH was set at 3.0. Cathodic peak current (I_{pc}) increased with the increment thymol concentration.

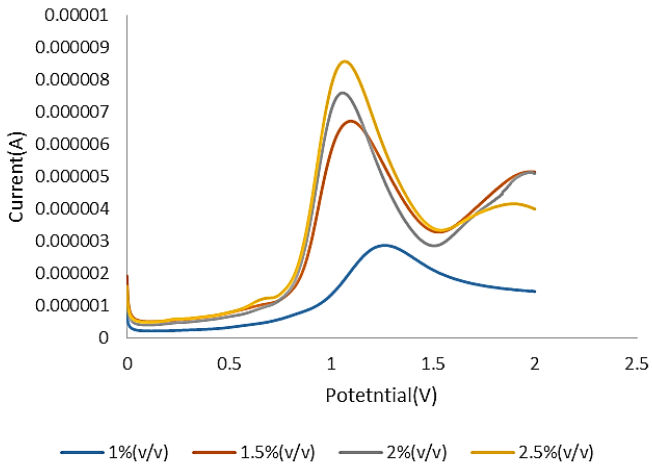

Fig.4. Square wave voltammetry of *Thymus* in different concentrations. Potential was swept between 0 and 2.0 V. pH was set at 3.0.

In order to evaluate the original sample, ascorbic acid was used as a standard. As showed in Fig 5 (a) and (b), the intensities of the voltammograms for ascorbic acid were much higher than *Thymus* by electrochemical method. Like DPPH method, all the results obtained from this method indicated that the antioxidant property of ascorbic acid is more than *Thymus.* Nevertheless, the difference of antioxidant property between *Thymus* and ascorbic acid was revealed properly in electrochemical method than DPPH method.

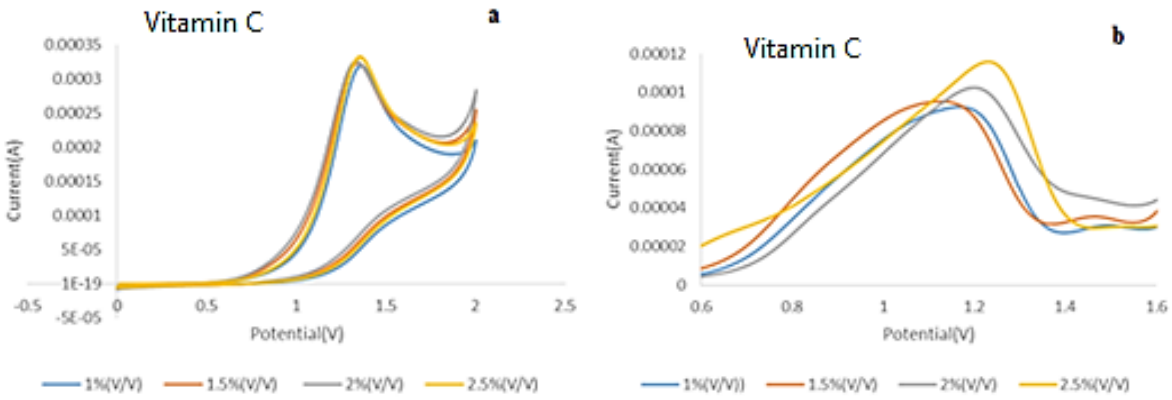

Fig. 5. a: Cyclic voltammetry of ascorbic acid **b:** Square Wave voltammetry of ascorbic acid.

Experimental conditions were the same in both experiments. pH was 3.0 and temperature was 25 °C. The anodic peak currents (Ipc) vividly increased in the SWV compared to the CV.

CONCLUSION

In this study, the electrochemical method can be used for the antioxidant capability, because of the correlation found between the anti-radical and oxidation potentials. Square wave voltammetry, cyclic voltammetry and DPPH methods were used for determination of *Thymus* antioxidant property and compared with ascorbic acid as standard. The results indicated the antioxidant capacity of *Thymus* obtained from square wave voltammetry method is more sensitive than the DPPH and cyclic voltammetry methods. Moreover, electrochemical method have many advantages compared to DPPH method, including high sensitivity, cheaper operating costs, high speed, short integration time, using no certain reagents and time consuming process of sample preparation, also electrochemical measurement can be accomplished with different pH and in many environments. Furthermore, it is possible to compare the antioxidant activity of molecules in a wide range of different conditions. Consequently, can be said the square wave voltammetry method was considered as sensitive method for evaluation the antioxidant activities compared to DPPH method and cyclic voltammetry method.

AKNOWLEDGMENTS

The authors are grateful to Laboratory Complex of Islamic Azad University for valuable technical assistance.

“The authors declare no conflicts of interest”

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