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Antioxidant properties of *Ajwain* using square wave, cyclic voltammetry methods and DPPH method

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ABSTRACT
Ajwain is one of the medicinal plants, which the highest composition is thymol, as a strong antioxidant respect to the obtained chromatograms of GC/MS. The antioxidant activity of the *Ajwain* is measured by square wave voltammetry method and cyclic voltammetry method and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) method in at the specific concentrations of 1%, 1.5%, 2% and 2.5% and constant pH. The results of the present study indicated that the antioxidant activity of *Ajwain* obtained from cyclic voltammetry and square wave is almost equal to the results of the DPPH method, which can be used to measure the antioxidant activity. Compared to DPPH method, electrochemical method involves a number of advantages such as simplicity, cheap operating costs, fastness, low amount of solvent, and no need for chemical reagents. Furthermore, based on the results obtained, the square wave method includes a higher sensitivity and accuracy than cyclic voltammetry method and DPPH method.

Keywords: *Ajwain*; Square wave voltammetry; Cyclic voltammetry; Antioxidant; Radical trapping

INTRODUCTION
Ajwain is an herb, which is used as a spice in food belonging to the *Apiaceae* family and *Trachyspermum ammi L* is regarded as the scientific name. Some of the compounds of this plant are include thymol, carvacrol, alpha, beta-pinene, terpinen [1]. Phenolic compounds are the most important components including both antioxidant and antimicrobial properties, which are used as food preservatives. The essential oil of the herb is widely used in medicine and pharmaceutical industries. Furthermore, antioxidant capacity is one of the most important antioxidant parameters as it is used to prevent the oxidative degradation of different compounds such as lipid peroxidation [2-7]. Several methods are used to determine the antioxidant capacity, relying on various parameters such as temperature, time, antioxidant concentration and sample [8-14]. The antioxidant activity of pure compounds or the extracted herbal is measured by 2, 2'-azino-bisacid ($ABTS^{\cdot}$) and $DPPH^{\cdot}$ by spin trapping technique (ESR), as well as measuring the oxygen consumption in lipid/water emulsion, in which the oxidation lipid is initiated by metmyoglobin [15-20]. However, these methods are time-consuming sample

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preparation and tedious [21]. Electrochemical methods such as cyclic voltammetry and square wave are widely used to measure the antioxidant capacity including many benefits. These methods are simple, sensitive, low cost, and no preparation time is necessary for the related sample [22]. The calculated oxidation potential is used to compare the antioxidant activity of compounds such as phenolic acid, flavonoids, cyanic acids, and the like by using a cyclic voltammetry method [23-27]. However, glass-carbon electrodes are often used in electrochemical methods. A large number of studies have emphasized using cyclic voltammetry with glass-carbon electrode for measurement such as the antioxidant in wine, plant extracts, phenolic standards and human blood plasma [23, 27-29]. The oxidizing potential of the glass-carbon electrode is the parameter which was considered in these methods. In order to compare the results obtained by various methods, the parameters which can determine the antioxidant properties of the sample are evaluated with unique antioxidants such as gallic acid and ascorbic acid [30]. The present study aimed to antioxidant activity of *Ajwain* and ascorbic acid measure by DPPH method, square wave and cyclic voltammetry. The results show the electrochemical method can be useful for determination of antioxidant activity. In addition, the square wave method is higher sensitivity and accuracy than cyclic voltammetry method and DPPH method.

THEORETICAL METHOD

Instruments and chemicals

Ajwain (Trachyspermum ammi L) was purchased from the Karaj's plant research facility; 2, 2-Diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$**)** 95% was purchased from Sigma-Aldrich Company; sodium chloride, acetic Acid and phosphoric acid, vitamin C, ethanol and carbon tetrachloride were purchased from the Merck Company; Metrohm Autolab Instrument PGSTAT302N Model, equipped with NOVA 1.6 soft-ware was used for measure electrochemical method and the Spectrophotometer Cary 300 model, made by Australian variance was used to measure absorbance. GC (HP-6890model) Instrument of Hewlett Packard Company (USA), the MS Instrument model HP-5973 of the Hewlett Packard Company (USA), and FT-IR Instrument model 870 of the Nexus Company were used. All of the solutions were prepared using deionized water.

Hydrodistillation method

Floral water of *Ajwain* was obtained by using clevenger apparatus. 100 g of dried seed sample and 500 mL of deionized water was added into distillation balloons. Then, balloons were attached to the clevenger. Accordingly, the steam that contains floral water and the essence comes out of the balloon, and then was cooled into the cooling section of the apparatus. Then, a two-phase mixture was created, the essence (oil phase) in the top as supernatant and the floral water (water phase) in the bottom. Finally, the floral water was separated and kept in a dark glass in a cool environment.

Dispersive Liquid- Liquid Micro Extraction (DLLME) for Ajwain floral water

500 µL ethanol and 100 µL carbon tetrachloride were used as a disperser solvent and extraction solvent and then, were injected to 5 µL of the sample. After that, the sample was centrifuged at 3000 rpm for 5 minutes.

Analysis by gas chromatography - mass spectrometry (GC/MS)

In this study, 1µL of the extracted sample from floral water of *Ajwain* was injected to the GC/MS. the instrument specifications are tabulated in Table 1 and Table 2.

Table 1. GC Instrument specifications

GC Instrument	**HP-6890 GC Instrument of Hewlett Packard Company**
Column type	HP-5MS (5% phenyl dimethyl siloxane)
Column dimensions	Length 30 m; diameter 0.25 mm; film thickness 0.32 µm
Planning temperature	Initial temperature 60°C (3 minutes); Temperature gradient 5 °C/min;
Column	Final temperature 220 ° C
Injection site	Spilt / Spiltless Ratio (1 to 20)
Injection temperature	25 ° C
Carrier gas	Helium 99.999%

Table 2. MS Instrument specifications

Mass Instrument	**HP-5973 of the Hewlett Packard Company**
Ionization energy (EI)	70 eV
Ionization temperature chamber	230 °C
Mass analyzer	Quadruple
Mass analyzer temperature	150 °C

Determination of antioxidant activity by DPPH method

Different concentrations of floral water of *Ajwain* (1%, 1.5%, 2% and 2.5% V/V) were prepared, and 2 mL of each added to 1 mL of $DPPH^{\cdot}$. The initial concentration of $DPPH^{\cdot}$ was 6×10^{-5} M. Sample was kept in a dark environment at room temperature for 20 minutes. Then, the absorption of samples was measured in 517 nm by UV-Vis spectrophotometer. IC_{50} was used for better comparison. IC_{50} is the amount of antioxidant required to decrease the initial concentration of $DPPH^{\cdot}$ to 50%, which measures the antioxidant activity. Finally to compare results, ascorbic acid was used as standard. The RSA% was calculated through following formula:

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

Determination of antioxidant activity by cyclic voltammetry method and square wave methods

In this study, different concentrations of floral water of *Ajwain* (1%, 1.5%, 2% and 2.5% V/V) were prepared. Solutions of acetic acid and phosphoric acid 0.1 M, pH=3 were used as a supporting electrolyte. NaCl 0.5 M was used for adjusting the ionic strength. All electrochemical studies were accomplished by three installed electrodes, Ag/AgCl electrode as the reference electrode, platinum electrode as auxiliary electrode and the glassy carbon electrode was used as working electrode; which was cleaned by polishing with alumina powder**.** Cyclic voltammetry method and square wave method parameters were as follows:

Start potential; 0.0 V, Stop potential; 2.0 V, Step; 0.005 V, Upper vertex potential; 2 V, Lower vertex potential; 0 V, Scan rate; 0.1 V/S, Number of scans; 1.

Start potential; 0.0 V, Stop potential; 2.0 V, Modulation amplitude; 0.02 V, Step; 0.005 V and Frequency 25 Hz.

RESULTS AND DISCUSSION

Results obtained from the GC/MS

Following injection of the extracted sample from floral water of *Ajwain* to the GC/MS, the identified fractions was compared with the standard indices by considering the standard patterns of the normal alkanes, retention time and Kovats index of the compounds. Then, the chromatogram of each fraction was recognized. Fig 1 illustrates the obtained chromatograms of floral water of *Ajwain* and Table 3 indicates compounds percentages for each component. In the *Ajwain* sample, three compounds form the floral water was identified, among which thymol is regarded as the most important chemical composition. Moreover, thymol could be considered as an antioxidant and a strong antimicrobial agent.

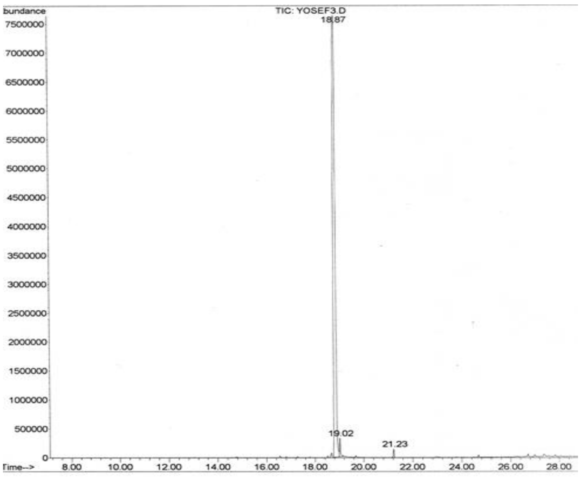


Fig. 1. The GC/MS total ion current of floral water of *Ajwain.*

Table 3. The compounds percentage in floral water of *Ajwain*

Compounds percentages	Kovats Index (K.I.)	Compounds
97.50%	1270	Thymol
1.78%	1277.5	Phenol
0.72%	1390.256	Coumarin

Results of floral water of Ajwain and Ascorbic Acid by DPPH Free Radical scavenging method

In DPPH method, $DPPH^{\cdot}$ react with the antioxidants leading to a decrease in the $DPPH^{\cdot}$amount, which causes a decrease in the absorption in 517 nm. Floral water of *Ajwain* was prepared in the concentration range of 1% to 2.5% V/V. Increasing the floral water concentration resulted in decreasing the remained $DPPH^{\cdot}$amount, which causes promoting the antioxidant activity of Floral water of *Ajwain*, excessively. Regarding Table 4 the absorption was decreased when concentration of sample increased and the amount of Radical scavenging activity (RSA%), would be increased with increasing the concentration. Fig 2 shows the amount of RSA% for *Ajwain* and ascorbic acid in different concentration as can be seen the antioxidant property of ascorbic acid is considered higher than *Ajwain*. As shown in Table 5, Inhibitory Concentration (IC_{50}) of the floral water for *Ajwain* is 1.28 % V/V, while it is 1.088 % V/V for ascorbic acid. Since the less the amounts of IC_{50} is better so, can be said the antioxidant activity of ascorbic acid has a little more than *Ajwain*. In general, the major problem with this method is decreasing of absorption after passing the time, subsequently leading to important changes in antioxidant reducing power (ARP) values.

Table 4. RSA% values and absorption measured by spectrophotometer for *Ajwain* and ascorbic acid in different concentrations

Sample	Concentration %(V/V)	Absorption		RSA%	
		Ajwain	Ascorbic acid	*Ajwain*	Ascorbic acid
1	1.0 %	0.086	0.118	36.624	51.102
2	1.5 %	0.055	0.110	59.469	54.436
3	2.0 %	0.037	0.045	82.311	81.149
4	2.5 %	0.009	0.021	93.367	91.225

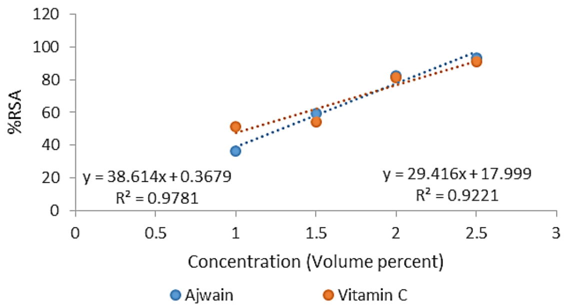


Fig. 2. RSA% in the concentrations 1%, 1.5%, 2% and 2.5% V/V for *Ajwain* and Ascorbic Acid.

Table 5. IC_{50} values for *Ajwain* and Ascorbic Acid

IC_{50}	
Ajwain	Ascorbic acid
1.285	1.088

Results of floral water of Ajwain and Ascorbic Acid by Cyclic voltammetry and square wave methods

The cyclic voltammetry were recorded in a certain potential (0 to 2.0 V), in different concentration (1%, 1.5%, 2% and 2.5%), in constant pH and in 25 °C for *Ajwain*. The cyclic voltammetry method reveals the reduction and reversibility of pure compounds. As can be seen Fig 3 voltammograms of cyclic voltammetry method do not show antioxidant properties in the *Ajwain* due to lack of appropriate limit of detection. Thus, the spike method was used (in the spike method, pure thymol was used with a concentration of 6 M) for improving the sensitivity. Fig 4 illustrates the voltammograms of floral water for *Ajwain* after the spike. As can be seen the intensities of the voltammograms would be increased with the increasing the concentration. The accuracy of this method is conclusive, due to the recovery percentages which were achieved from 92% to 108% (the percentage of the recovery calculated from the concentration of 20% (V/V) *Ajwain*).

The small variations in potential may be due to changes in temperature, concentration and dirty electrode.

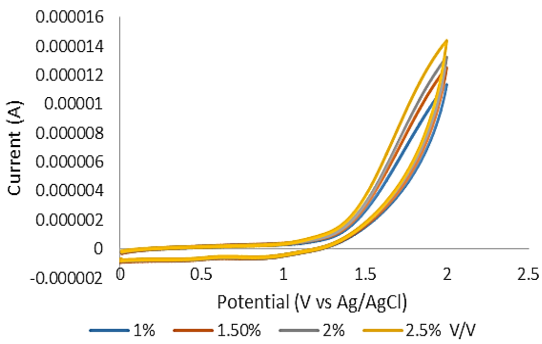


Fig. 3. Cyclic voltammetry of *Ajwain*; Experimental conditions: pH= 3.0, v= 0.1 Vs^{-1} in different concentration, potential = 0 to 2.0 V, temperature = 25 °C.

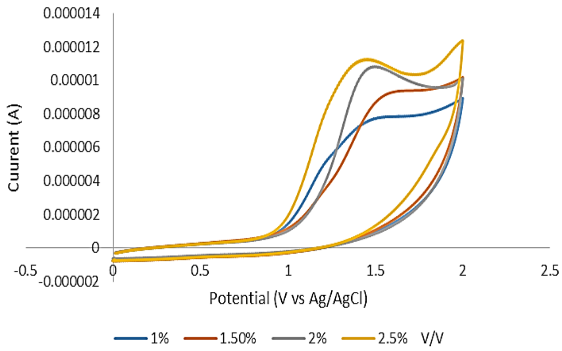


Fig 4. Cyclic voltammetry of *Ajwain* after spike; Experimental conditions: pH= 3.0, v= 0.1 Vs^{-1} in different concentration, potential = 0 to 2.0 V, temperature = 25 °C, The cathodic peak current (I_{pc}) increased with the increment thymol concentration.

In square wave method floral water of *Ajwain* was prepared in the concentration range of 1% to 2.5% V/V and voltammogram were recorded in a certain potential, in constant pH and in 25 °C. As shown in Fig 5, increasing the floral water concentration resulted in increasing intensities of the voltammograms. The accuracy of this method is conclusive, due to the recovery percentages which were from 95 % to 106 % (the percentage of the recovery calculated from the concentration of 1% (V/V) *Ajwain*).

One of the most important advantages of the square wave method over the cyclic voltammetry method is that Redox potentials of the compounds can be determined with appropriate accuracy, irrespective the antioxidant property of compounds, that voltammograms are well defined. Finally, the sensitivity and accuracy of this method is higher than cyclic voltammetry and DPPH methods.

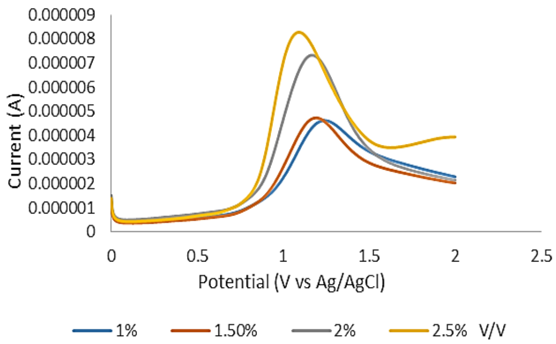


Fig. 5. Square wave voltammogram of *Ajwain*; Experimental conditions: pH= 3.0 in different concentration, potential = 0 to 2.0 V, temperature = 25 °C.

Ascorbic acid was used as proper standard for comparing *Ajwain* in the same experimental conditions. As illustrated in Fig 6 (a) and (b), the intensities of the voltammograms for ascorbic acid were much higher than *Ajwain* by electrochemical method. Therefore, antioxidant property of ascorbic acid was more than *Ajwain*, and does not need and added spike in cyclic voltammetry method.

In order to determine the samples concentration base on molar concentration, linear calibration curve was obtained by using square wave method of pure thymol with specific concentrations of 3, 4, 5 and 6 M (Fig 7 and Fig 8).

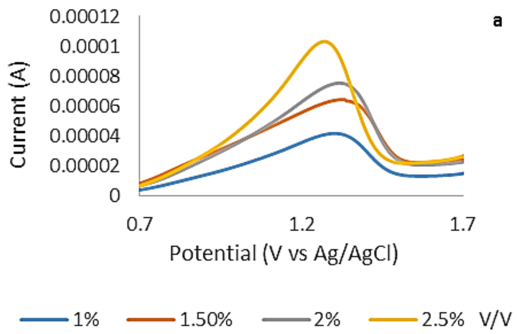
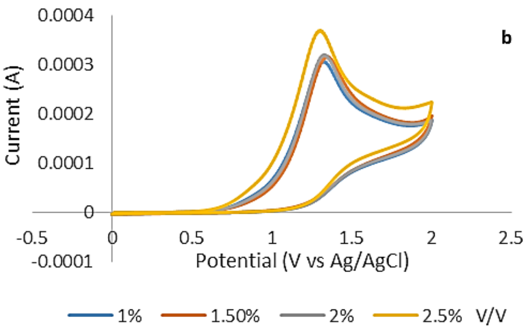


Fig. 6. a: Cyclic voltammetry of Ascorbic Acid, b: square wave voltammograms of Ascorbic Acid; The anodic peak currents (I_{pc}) vividly increased in the square wave voltammograms compared to the Cyclic voltammetry; Experimental conditions: pH= 3.0 in different concentration, potential = 0 to 2.0 V, temperature = 25°C.



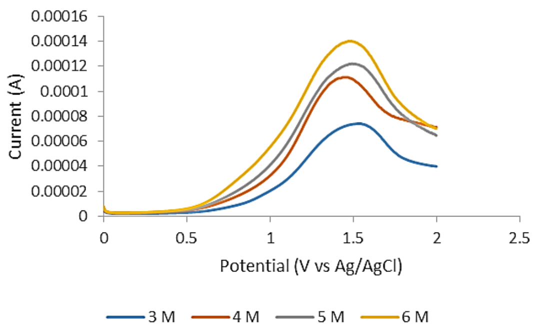


Fig. 7. Square wave voltammograms of pure thymol for calibration, Experimental conditions: pH= 3, potential = 0 to 2.0 V, temperature = 25°C.

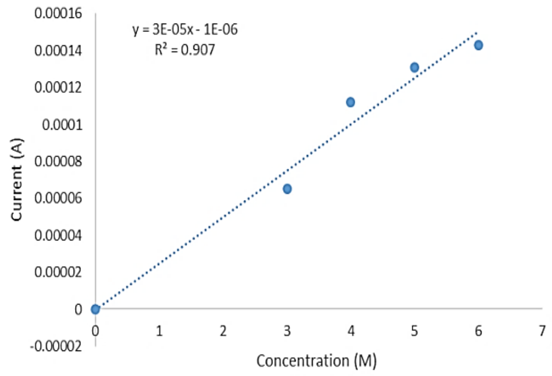


Fig. 8. Linear calibration curve.

CONCLUSION

In conclusion, square wave voltammetry method, cyclic voltammetry method and DPPH method were used for determination of *Ajwain* antioxidant activity and compared with ascorbic acid standard. The results of the present study indicated that the antioxidant activity of *Ajwain* obtained from cyclic voltammetry and square wave is almost equal to the results of the DPPH method, which can be used to measure the antioxidant activity. In conclusion, electroanalytical chemistry methods has many advantages over DPPH$^{\cdot}$ like high speed, simplicity, low operating cost, non-use of other reagents, low amount of solvent and short measuring time, Also electrochemical measurement can be done with different pH in any environments. As a final point, the most important advantage of the square wave method over the DPPH method and cyclic voltammetry method is antioxidant activity of the compounds can be determined with high sensitivity and accuracy.

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