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ORIGINAL ARTICLE

Effect of Ultrasound on the Rate of Antioxidant Extraction from *Anethum graveolens*

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ABSTRACT
The benefits of antioxidants in preventing cardiac diseases and decreasing eye and brain damage have been proved yet. In addition, antioxidants scavenge the function of the free radicals and neutralize them. So, providing antioxidants reserves for decreasing the oxidative stress' effects is very important. Dill is a plant resource, rich of polyphenol compounds. Therefore, this research performed to investigate the rate of antioxidants of dill plant of Khorassan region extracted by ultrasound technology. For this purpose, we performed ten concentrations (from 48.875 to 9000 µg/ml) and three repetitions to produce dry dill methanolic essence. The antioxidants activity of each dilution was determined by DPPH. This research the effect of strong ultrasound waves (produced by a 750 W power device) on the rate of extracted antioxidants of dill plant in Khorassan region was investigated. Temperature was 25 ◦c, time duration was 5 minutes and the sound intensities were 30, 60 and 90 %. The result indicated that the scavenging percentage of dill plant developed by the increase of the concentration. In different concentrations of ascorbic acid from 46.87 to 375µg/ml, the rate of scavenging has been developed by the increase of concentration. For the range of concentration from 375 to 9000 µg/ml, no significant difference happened in scavenging rate. Regarding to the scavenging free and stable radicals, the 50 percent scavenged concentration of the methanolic essence in different intensities of 30, 60 and 90 percent were respectively 2503.35, 2217.49 and 1961.08 µg/ml. The results revealed that in the temperature of 25 ◦c and during 5 minutes, the rate of extracting antioxidants develops by the increase of intensity.
Keywords: Antioxidant activity, Methanolic Essence, DPPH, Dill

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INTRODUCTION

The antioxidants are some compounds which effectively restrain the reaction of the free radicals in the forms of active oxygen and nitrogen with biomolecules like protein , amino acid , lipid and DNA and consequently result in decrease of cellular damage or death, Cardiovascular diseases and different kinds of cancer [1]. Nowadays, natural antioxidants produced from plants and spices are used widely because of their antioxidant properties [2]. Moreover, adding antioxidants to most of foodstuffs including foods with plenty of unsaturated fat prevents the loss of nutritional quality, color and unsavory arising from forming poisonous compounds.Antioxidants divide into two general groups of natural and chemical (3). Chemical antioxidants which generally are used in food industry, including butylateddehydroxyanisol [BHA], butylateddehydroxytoluene [BHT], tertiary butyl hydroquinone [TBHQ] and propyl gallate have Carcinogenic property and their negative effects on human health have been approved (4). Researchers took into consideration the usage of a wide range of medical plants and aromatic compounds as natural sources of antioxidants [5].

Most of the acceptable natural antioxidants are usual food components which a person uses in his own food regime. Nowadays, most of the researches have been focused on using new and Safe antioxidants of herbal, animal, microbial and food resources .To refer to the most important antioxidants resources in food regime we can mention tocopherols, glutathiones, ascorbic acid , ascorbate salts, carotenoids and phenol compounds[6].

Anethum Graveolens plant is called shebet (shevid) in Farsi and dill in English. It belongs to Apiaceaefamilia. Dill is an annual or biennial plant widely cultivated in different regions in Iran. The

leaves and seeds of dill are used as spice and seasoning and their essence is used in producing gums, confectioneries and pickled spices. The color of dill essence is light yellow and its smell is relatively spicy and very similar to cumin [7]. Dill essence flavors the food and makes it savory because of the existence of de caron [8]. Dill plant was used to cure diseases of digestive system from old time. Antimicrobial, Anti-spasmodic and Anti-fat effects of dill are the result of existence of Flavonoid and other phenolic compounds in dill essence. In initial studies, ethanolic antioxidant property of dill was approved in a laboratorial mouse fed by full of fat foods [9].

Antibacterial activity of marine dill essence on the pure species of several bacteria (Staphylococcus aureu, EscherichiaColi, Pseudomonas aeruginosa, salmonella typhimurium, shigellaflexneri) was examined by Arora and Kuar. The results indicated that the essence of this plant has a significant antibacterial activity on all the studied species .Extracting the essence by use of ultrasound waves is one of the most important methods of producing valuable compounds of plants [11] and is executable in small and large scales(industrial and laboratorial) [12]. Comparing with other methods, extracting on the basis of microwave i.e. using ultrasound waves, is cheaper and easily applicable [13].Studying the influence of using strong ultrasound waves on extracting oil from olive grinded seeds, indicated that these waves demolished the cell walls and plant tissue and more antioxidants compounds (polyphenols and tocopherols) and pigments (Carotenoid and chlorophyll) entered into the oil and increased the nutritional value [14]. In most of the cases because of the mechanical effects of ultrasound waves, they usually used as assisting factors in extraction process [15].The main mechanism of extraction by ultrasound waves is related to cavitation phenomenon. In this phenomenon very small bubbles are made in the liquid and grow fast and critically and then blow up. This bubble explosion frees a lot of energy in form of shear stress and impresses the surrounding environment [16]. Dill as an Iranian native plant is easily accessible and cheap and used for preparing food and medicine from old times. This study can be an introduction to practical usage of the essence of this plant as an antioxidant in medical and food industries. In this way, the possibility of using an accessible and economical resource will be provided and also wasting and the resulted losses will be prevented. Finally, this study is an effort for the purpose of promotion of the food health and safety of the society.

This study is a laboratorial research including collection, recognition and extraction of dill plant using ultrasound technology. The aim of this study was measuring the rate of antiradical activity and considering the rate of the activity of antioxidant compounds of dill plant essence and also free radicals scavenging activity of this essence using radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) indicators.

MATEREALS AND MTHODS

Chemical materials: The chemical materials used in this study consisted of methanol with the purity grade 99.5% made in Iran, DPPH indicator made in Sigma-Aldrich and ascorbic acid.

Providing sample: Fresh dill named scientifically *Anethum graveolens* harvested in average size from the farms around Mashhad in August 2013. They have been washed and dried in dry weather and far from sunlight and then grinded in industrial mill of the laboratory of Jahad Daneshgahi of Mashhad.

Providing the essence of dill plant using ultrasound technology

The ultrasound waves' energy was used in extraction of the essence of the plant. We used the ultrasound device model FRIGOMIX CV33 with Fixed Frequency 30 KH and the Max power of 750 W to prepare the condition of extraction and consider the effect of each treat. We controlled the temperature around the container of extracted essence by using circulator and rotating warm liquid around the container. The essence extracted from the leaves and tip of the branches of dill plant by using methanol solvent and ultrasound technology. To produce dill essence, we added 2.5 gr dried grinded dill to 50 ml methanol and then influenced this solution by ultrasound process in three different factors of temperature, time and intensity and each one was examined in three different level to determine the optimum temperature, time and sound intensity for antioxidants extraction. We performed this extraction in different temperature (25, 35 and 45◦c), time (5, 10 and 15 minutes) and intensities (30, 60 and 90 percent). We filtered the solution by Whatman number 1 filter paper, and dried it in vacuum to reach the stable weight and then kept the produced essence in refrigerator adjusted on 4◦C until the time of examining.

Measuring the scavenging power of free radical of DPPH

The speed of regeneration of chemical reaction by adding free radicals DPPH used as a method to specify the nature of the very specific reaction's radical. We can obtain some information about reaction between compounds and free radicals by doing this experiment. Free radicals of DPPH in 516 nanometers wavelength of visible spectrum, indicate a strong violet absorption band.

Absorption will stop, when electron couples in presence of free radical scavenger. Consequently discoloration or color transformation of the solution into pale yellow will be happened regarding to the number of scavenged electrons. Discolored DPPH presents the ability of the antioxidant essence for

regenerating free radicals which are independent from enzyme activity [17]. First of all, 0.05 % solution of free radical DPPH in methanol and then for each of different treats, different concentrations (from 9000 µg/ml to 46.87 µg/ml in methanol and in ten concentrations) were prepared. In this research, we used ascorbic acid to compare antioxidant activities of different produced essences. To do spectrophotometry we poured 100 µl of different produced concentrations in a 96 cell plate for three times. Then we add 100 µl of DPPH solution to each sample in the plate and kept them in dark place for thirty minutes. Then absorption rate of the essences were read in the wave length of 517 nanometers. Three cell of the plate which contained only 100 µl of DPPH and 100 µl of methanol, were considered as control. The following formula used to calculate free radicals scavenging percentage after reading the absorption rate by spectrophotometry device:

I % = (A blank- A sample)/ A blank ×100

In this formula, I is the scavenging percentage, A blank is negative control photo absorption which lacking the essence and A sample is the rate of photo absorption of the different Essence 's concentrations .After sketching the percentage of free radicals scavenging activity against the concentrations, the proper curve according to the data was fitted. Afterwards, using the above mentioned equation, IC_{50} or the concentration in which the antioxidant compound is able to scavenge 50% of free radicals of DPPH, was calculated. The IC_{50} defined as the rate of the concentration of the essence in which 50 percent of free radicals of DPPH existing in reaction are scavenged or neutralized [9]. Therefore, lower concentration means more powerful antioxidant or antiradical activity of the essences.

Statistical method of the plan

We used Excel software to record and arrange data. After testing normality of data distribution, factorial analysis of variance in form of completely randomized design with three repetitions was performed using SPSS19 software. The averages were compared by using the least significant differences method and diagrams were sketched by Excel.

DISCUSSION AND CONCLUSION

Old and traditional methods of antioxidant extraction like flood irrigation method performed by other researchers, need a long period of time. For example in the dill extraction method of Abolfazl Kamkar, he has added grinded dill to ethanol solution and has put it on shaker for 48 hours. The other steps have been done after this long period [18]. But in ultrasound extraction method the time period is very shorter. Other researchers also reported the shortening effect of ultrasound waves on time period of extraction [19, 20].Ultrasound waves cause bubbles to produce, grow and then explode and also create vortex and Flow disturbances throughout the fluid mass and in this way shorten the time period of extraction. In addition, damage of tissue and permeation of the solution into it causes the essence and the solution touch each other and in result the rate of extraction will increase.

Make use of medicine plant properly requires accurate scientific information and recognition of their chemical compounds which are the reason of the therapeutic effect of the plant. Antioxidant activity of all the plants is directly related to the rate of phenyl and Flavonoid compounds. Mint essence includes high level of phenyl and Flavonoid compounds and presents a proper antioxidant activity (21). Another plant with high level of antioxidant activity is rosemary and its activity is directly related to its phenyl content [22].

In this research we evaluated, free radicals scavenging power of methanolic essence of dill plant in laboratorial method. The power of scavenging free radicals was tested by DPPH examination. In this examination, increasing the concentration of the essences developed the scavenging of the radicals.

One of the valid, accurate, easy and economical methods of measuring the rate of free radicals scavenging is DPPH. Tis method which has high repeatability is used in examining the antioxidant activity of the plants in laboratorial conditions [23]. The results of the analysis of variance revealed that the kind and concentrations of the essences and ascorbic acid has significant influence on the measure of scavenging free radicals. The results show that, the ability of the essences in scavenging free radicals is related to their concentration and increasing the rate of concentration develops the antiradical influence of them.

The rates of scavenging free radicals of DPPH in ten different concentrations between 9000 to 46.87 µg/ml of the essence of dill plant and ascorbic acid as the control have been presented in figure1. The results indicate that the percentage of the essence of the dill plant decreases when the rate of concentration decreases. In concentration less than 375 µg/ml there is no change in scavenging rate. No significant difference will be observed in ascorbic acid with concentration more than 375µg/ml and this acid in this concentration scavenges one hundred percent of free radicals of DPPH. Increasing the concentration from 46.875 µg/ml to 375 µg/ml develops the rate of scavenging.

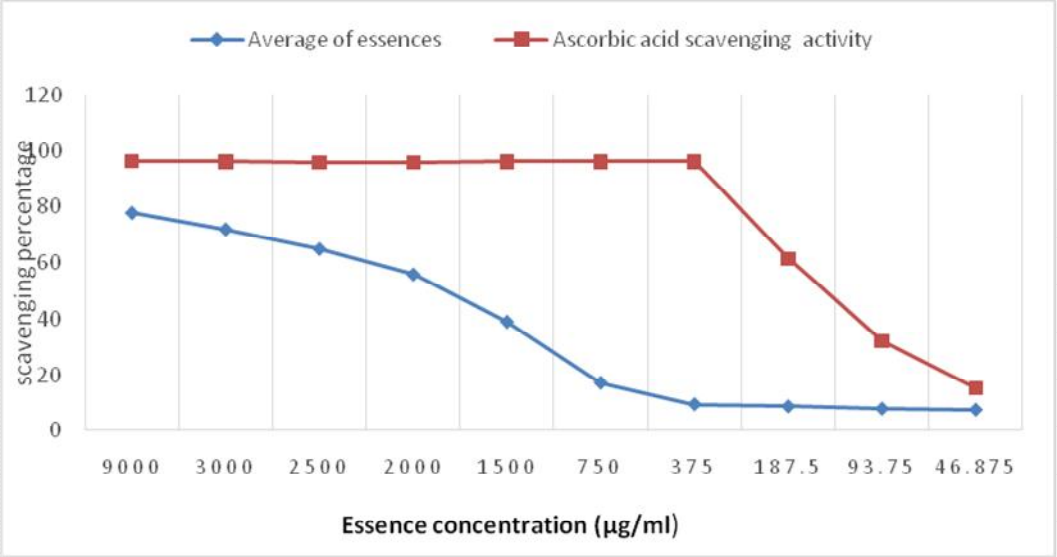

Figure 1- Radical scavenging activity of DPPH

In this study, we can relate the observed differences between the scavenging percentages of the essences to the rate of phenol compounds (figure 2). Increasing the rate of concentration of phenolic compounds is directly develops the rate of the abilities of different essences in scavenging free radicals.

Increasing the concentration of the phenolic compounds causes the raise of hydroxyl groups existing in reaction medium and increases the possibility of giving hydrogen to free radicals and as a result the scavenging power of the essence will develop (24).Scavenging power of the different essences is largely related to the number and location of the hydroxyl groups and the molecular weight of the phenolic compound. Hydroxyl groups are more easily available in phenolic compound with lower molecular weight (25). In the case of scavenging influence of ascorbic acid in concentrations more than 375 µg/ml because of creation of a kind of saturation, increasing concentration does not make a significant influence on the rate of scavenging free radicals.

According to this result, a critical concentration of phenolic compounds is enough for scavenging free radicals. Considering the essences with the above mentioned concentrations, we can see increasing the concentration cause development of scavenging and there was no significant difference in concentration rates lower than 375 µg/ml. This rate of concentration did not have any significant influence on scavenging free radicals of DPPH or in other words the essences with concentration lower than 375 µg/ml were not efficient in scavenging free radicals of DPPH.

The results of the variance analysis of the influence of each of the main effects (intensity, temperature, time and ultrasound) on the rate of scavenging and also interplays between factors are significant ($p<0.05$) according to the table1.

We can observe the percentage of the essence which caused 50% radical scavenging of (IC_{50}) in table 2. It is clear, if lesser amount of the essence neutralize the fifty percent if the free radicals of DPPH, the antiradical property of the essence is greater and there are more antioxidants in the essence.

n this experiment by increasing the concentration of the essence, radical scavenging will happen more strongly. Therefore, the ability of scavenging free radicals on Methanolic essence in 25◦c and in the time duration of 5 minutes by using ultrasound process with 30, 60 and 90 intensity respectively resulted in 2503.35,2217.49 and 1961.08 µg/ml of the essence.

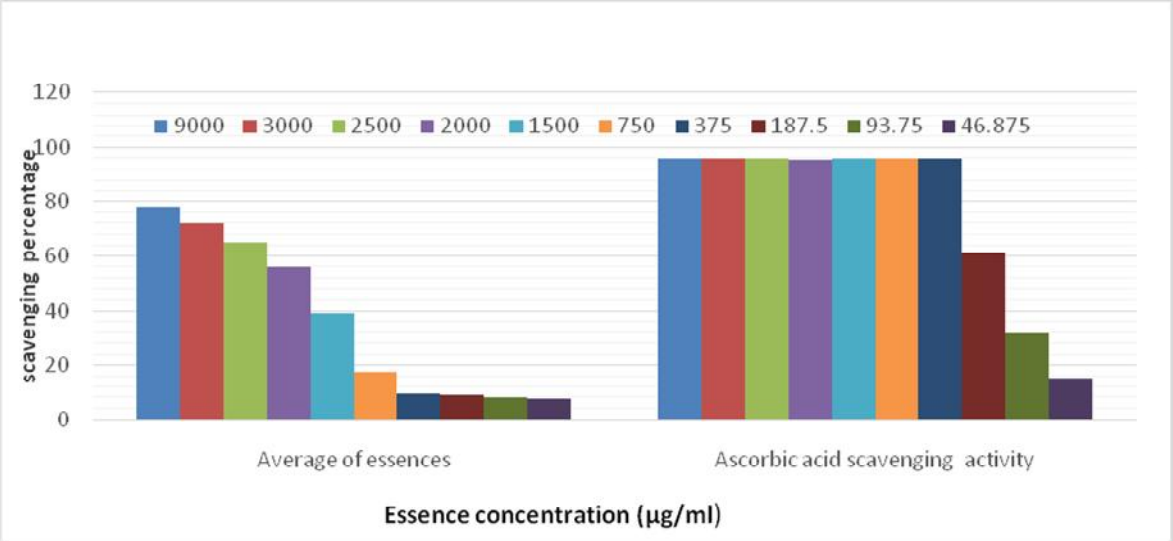

Figure 2- Anti radical activity of the methanolic essence of dill plant comparing with ascorbic acid

Table 1 – variance analysis of the rate of scavenging in 517 nanometer wavelength for studied factors

Source of changes	Rate of freedom	Mean-square IC_{50}
intensity	2	540057*
Time	2	441326*
Temperature	2	135732*
Intensity*Time	4	188612*
Intensity*Temperature	4	351222*
Time*Temperature	4	201921*
Time*Intensity*Temperature	8	244649*
Test error	27	14912

Rate of significance 0.05

Table 2: The influence of methanolic essences and ascorbic acid on scavenging free radicals of DPPH

sample	DPPH. IC_{50}(µg/mL)
Intensity30%	2503.35
Intensity60%	2217.49
Intensity90%	1961.08
Ascorbic acid	169.51

According to the diagram 3, in this research the rate of antioxidants extraction and after that the power of scavenging free radicals of DPPH have been developed by increasing ultrasound intensity on tested treats in 25◦c and during 5 minutes. As you see, the time duration for extraction by ultrasound is 5 minutes which is very short. In addition, plant essence extraction is performed in high temperature in common methods which lead to quality fall of this valuable materials and deduction of the antioxidant activity of the essence.
Wong et al (2008) realized that the frequency has positive linear effect on extraction of phenolic compounds and increasing it causes more extraction [26].
Touma et al (2001) found out that increasing frequency causes more essence extraction and the reason is exceeding the rate of mass transition between solution and herbal material [27]. Cavitation result in better cell fractionation and facilitate releasing of its contamination. One of the privileges of ultrasound method is better penetration of the solution in low temperature, faster extraction and more output of the product [28].

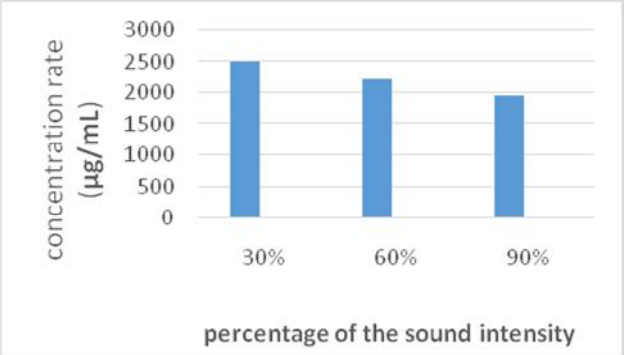

Figure 3- diagram of the rate of ic50 in different intensities, time duration 5 minutes, temperature 25 ◦c

CONCLUSION

This research indicated that the methanolic essence of dill plant contains notable antioxidant influence. Using ultrasound method in extracting methanolic essence of dill plant decreased the time of extraction, facilitated the essence extracting and saved the materials. Increasing the intensity of ultrasound in 5 minutes and 25◦c developed the antioxidant extraction. The result demonstrated high antioxidant potential of the dill plant. Regarding the undesirable influences of Synthetic antioxidants on the human body, availability and low price of dill plant in our country, using the essence of dill is proposed for producing foodstuffs which contain fat. Extending the dill essence to the edible oils increases their stability against antioxidants. Considering this matter that the rate of antioxidant compounds impressed by different factors like genetic factors, postharvest condition and environmental factors and is various in different parts of the plant, more researches on different parts of the plant is necessary to use this plant's antioxidant benefits in different fields.

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