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Comparison of novel assisted extraction techniques of antioxidants in *Pimpinella anisum* seed

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Abstract

In recent years, the use of natural antioxidants from various sources, in the food industry has been increased. In this study, with the aim of improving the extraction of antioxidants from aniseed using ethanol asGRAS (general recognize as safe) and green solvent, assisted extraction techniques of microwave and ultrasound were studied. Comparison of extracts was performed based on the evaluation of extraction yield, evaluation of antioxidant activity usingDPPH(2,2-Diphenyl-1-picrylhydrazyl) scavenging assay andHPLC(High-performance liquid chromatography) analysis of represent ative phenolic compounds (Gallic acid and Quercetin).Results showed that in case of antioxidants extraction, top treatmentsof microwaves were more efficient and more selective than top ultrasound treatments, despite of the lower extraction yields and shorter times.According to the results, it was provedthat microwaves technique can be used successfully as a pretreatment for increasing the extraction efficiency of antioxidants from anise using ethanol.

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Introduction

Anise (Pimpinella anisum) from family of *Umbelliferae* is an aromatic and grassy annual plant with white flowers and small green to yellow seeds which grows in Turkey, Iran, India, Egypt and many other warm regions of the world (Pourgholami *et al.,* 1999). Fruit of this herb is used as flavouring in the food industry (Gulcin *et al.*,2003) and antioxidative properties of its extract has been proved in several studies. Gulcin*et al.*(2003), investigated antioxidant and antimicrobial activities of water and ethanol extracts of anise (*Pimpinella anisum* L.) seed. The antioxidant properties of both extracts of anise , were evaluated using different antioxidant tests. various antioxidant activities were compared with synthetic antioxidants such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and α-Tocopherol. The water extract of anise exhibited greater antioxidant capacity than that of ethanol. Al-Ismail *et al.*(2004), investigatedthe antioxidant activities of water and alcohol extracts of anise .In that study, extracts had the marked antioxidant activity in both linoleic acid and liposome model systems. This indicates that could be used as antioxidants in fat-containing foods. Nickavar*et al.*(2009), studied antioxidative activities of ethanol extracts from aniseand six other *Umbelliferae* fruits by the DPPH radical scavenging test. The extracts were also investigated regarding their total flavonoid contents by the $AlCl_3$ technique. All the studied extracts showed antioxidant capability and anise extract exhibited the strongest activity. Marques*et al.* (2009) ,determined the chlorogenic acids composition of fourteen dried medicinal plants by chromatographymethods. In that study total chlorogenic acids contents in the infusions were similar to those in the methanolic extracts and indicated that a satisfactory extraction occurs during the preparation of infusions. Topal *et al.*(2008),studied Chemical compositions and antioxidant activities of essential oils from anise and eight other different species. Essential oils were obtained by supercritical carbon dioxide extraction and steam distillation, and were analyzed by gas chromatography –mass spectrometryand their antioxidant activities was tested by means of DPPH assay. In that study,essential oils extracted by supercritical carbon dioxide and steam distillation showed different compositions in different species.Hence,it seems that anise contains extractable antioxidants which can be substitute for chemicals in different industries such as food, pharmaceutical,hygienic and cosmetics.

The use of antioxidants, either synthetic or natural, is one of the most effective ways for reduction of oxidation of fats and oils. But at present time, the increasing interest of consumers towards more natural foods, and concerns of some health experts about potential risk of long term use of synthetic antioxidants such as BHA and BHT, have led to development of more efficient and more perfect extraction processes in order to isolation of natural antioxidants from various sources. In most extraction processes ,organic solvents such as methanol and acetone are usually and primarily used.Although extraction yield of antioxidant can be increased by these solvents because of their ability to link with hydrogen (Tena *et al.*, 1997) but due to the toxicity,their use can make problems for producers and consumers. thus, the discussion of using GRAS solvents and environmentally friendly or green which are accepted in the food industry, such as boiling water (Chen *et al.*, 2007; Dorman *et al.*, 2003) and ethanol at low temperature (Navarrete *et al.*, 2011; Visentin *et al.*, 2011) has been posed. However, when using these solvents, due to the low mass transfer and extraction yield(Rodriguez-Rojo *et al.*, 2012),extraction time is relatively increased. In this situation, there is the possibility of uncontrolled reactions. so in order to have a more efficient and economical solvent extraction, it is necessary to use assisted extraction techniques such as ultrasound, microwave, pressurized fluid, etc as well as the pretreatments such as grinding, maceration, stirring, etc. Extraction by microwaves (Krishnaswamy*et al.*, 2012) and ultrasounds at a frequency of 20 to 100 kHz(Khan *et al.*, 2010) has been introduced as new methods of extraction of bioactive compounds. Extraction by microwaves reduces the internal mass

transfer limitations. Ultrasound mainly reduces the external mass transfer limitations and also breaks cell membrane which leads to a reduction of control of internal mass transfer (Rodriguez-Rojo *et al.,* 2012).
When plant material along with extraction solvent are exposed to microwaves, their molecules that have permanent or induced dipoles, move and rotate(Sun *et al.*, 2007). In the microwave field, Molecules attempt to adjust themselves to the oscillating electromagnetic field by means of diversion or distribution of their electron cloud or by physically rotating their molecular dipoles. As a result, the system is heated quickly (Dandekar and Gaikar., 2002). Enhancement of extraction utilizing ultrasounds is mainly attributed to the passage of an ultrasound wave through the solvent and production of acoustic cavitations (Ma*et al.*, 2008;Velickovic*et al.*, 2008), greater penetration of solvent into sample matrix and an increase in the contact surface between them because of offering mechanical effects (Hossain *et al.*, 2012; Mason, 2003; Toma*et al.*, 2001). However, the high temperature and long time of extraction in these techniques may lead to unwanted reactions or destruction of targeted compounds (Biesaga,2011).So before using these techniques as a treatment or pretreatment for an extraction process, appropriate conditions of extraction should be determined.The aim of this study was to evaluate the efficacy of the two extraction techniques, ultrasound and microwave, in different conditions and to find a route for a more efficient extraction of antioxidant compounds from aniseed.To prevent from probable changes of antioxidants, some variables have been selected which using them the extraction system is not exposed to high temperature for a long time.

Materials and methods

Materials

Aniseeds were obtained from farms in Sabzevar (Iran). After cleaning, they were maintained in a freezer at -18°C until extraction.Commercial standards of phenolic compounds (Gallic acid and Quercetin) and DPPH reagent (2, 2-diphenyl-1-picrylhydrazyl) were prepared from sigma (Aldrich-Sigma). All solvents which were used in the extraction processes and chromatography, were HPLC grade and were purchased from merck (Germany).

Methods

Methodsof extraction

Aniseeds were grinded by an electrical mill for 20 seconds and then were passed through a 40 mesh sieve. For each treatment (including microwave, ultrasound, maceration for 8 hours with stirring and for 24 hours without stirring),10 g of grinded seeds were blended with pure ethanol(with a ratio of 1 to 10 w/v) and then extraction process was performed. Then extracts were filtered over whatman No. 1 paper under vacuum.The mixture of extracts and solvent was concentrated by a rotary evaporator (laborota4001-efficient; Germany) at 40^{oC} and under vacuum. Then the solvent which was remained in extract was removed by vacuum oven (memmert Vo200;Germany) at a temperature of 45^{oC}.For each treatment, Extraction was performed in triplicate.Finally the dried extracts were stored in freezer at -18 ^{oC}.

Microwave assistedsolventextraction

A domestic microwave oven (Panasonic, NN-S651 WF with frequency of 2450 MHz) was applied for extraction. The oven was equipped with a rotating surface on it the samples were slowly rotating.The oven was programmable for powers of 100 and 1000 watts and also for desirable times. Samples were prepared in glassy containers with a diameter of 5 and a height of 10 cm. Then powers of 100 and 200 watts and times of 40, 80 and 120 seconds were applied for extraction.

Ultrasound assistedsolvent extraction

An ultrasound device (Dr.hielscher up 200H, Germany with Frequency 24 KHz) with a 2 mm diameter probe was used for extraction.In time of extraction, the probe was immersed for 1 cm in the center part of the sample containers (with a diameter of 5 and a height of 10 cm, as mentioned before). Extraction was performed at amplitudes of 50 and

100%, cycle of 0.5 and times of 10, 20 and 30 minutes at room temperature (25 ° C).

Extraction using maceration with stirring for 8 hours andwithout stirring for 24 hours:In extraction by maceration with stirring, samples in the glassy containers (with a diameter of 5 and a height of 10 cm) were moderately stirred on a magnetic stirrer for 8 h at room temperature (25 °C). In extraction by maceration without stirring, samples containers were placed in darkness for 24 h at room temperature without stirring.

Analysis methods

Extraction yield:

One ml of each filtered extract was weighed and then dried in 50°C oven for 24 hours. After calculating net weight of dried extracts and regarding the ratio of dried aniseed to solvent, extraction yield was reported asgram of dried extract per 1 kg of dried aniseed.Calculations for each treatment were performed in triplicate.

DPPH scavenging assay

Solution of 0.006% of DPPH in methanol was prepared.Then, 1 ml of DPPH solution was added to the tubes containing 1 ml extract with different concentrations. The test tubes were vortexed for 15 seconds, kept in dark place for 1 hour and then their absorbance value was measured at 512 nm by uv-vis spectrophotometer (T70+, England).Percentage of inhibitionof the free radical was calculated by the following formula:

$$\%A = \frac{Ac - As}{Ac} \times 100$$

Where %A is percentage of inhibition of free radical and Ac and As are absorbance of control sample and samples with different concentrations, Respectively. Percentage of inhibition offree radical was plotted against concentration of antioxidative solution. Then appropriatecurve was fitted andthe concentration in which the antioxidative solution inhibited 50% of free radicals was reported as IC_{50} (Siger *et al.*, 2008).

HPLC analysis of phenolic compounds

For this purpose,the extract of each treatment was filtered but was not concentrated and dried. Then, it was kept in a freezer at -18°C for 24 hours. Finally, their HPLC analysis and gallic acid and quercetin standards were performed according to the method of Chen, Zhou and Deng (2001).In each test,20 microliters of sample was injected onto the HPLC system.The device was reverse-phase type (Knauer-ASI, Germany) and included a pump system (smartLine pump 1000 Gradient) and a detector(smartLine uv-vis 2600). Compounds were detected at wavelengths of 280 and 360 nm. The column model was vertex;Eurospher 100-5 C18 (250 × 4.6 mm, 5 µm with precolumn). A gradient solvent system was applied which consisted of solvent A (H2O/CH3COOH, 97:3, v / v) and solvent B (MeOH) was used. This system was such that the solvent A decreases from100% to 90% during the time period of 0 to 10 min, and decreases to 30% in the time interval of 10 to 32 min and finally would reach to 0 over the time period of 32 to 45 min. The flow rate value of 1.0 ml/min was employed and all tests were performed at 25 °C.

Statistical analysis

A completely randomized factorial design was applied to analyse the effect of variables on extraction yield and antioxidant activity. The comparison of techniques and comparison of concentrations of representativephenolic compounds were analysed by completely randomized design.Means were compared using MStatC software and Duncan's test (p <0/05). Graphs were plotted using microsoft excel software.

Results and discussion

In extraction of antioxidants from aniseeds, two novel techniques, microwave and ultrasound, were compared. The effect of these techniques on extraction yield, antioxidant activity and extraction of representative antioxidative compounds (gallic acid and quercetin) were examined.In this study, maceration for 8 hours with stirring and for 24 hours without stirring was used as control.

Effect of different treatments on the extraction yield

In case of microwaves, power increment did not have a significant effect on extraction yield, except in time of 80 seconds. But in each power, with timeincreasing, the extraction yield of extracts was significantly increased. Although in power of 200 w, there was no significant difference between time of 80 and 120 seconds(Fig. 1).In case of ultrasounds,increasing of amplitude of ultrasound waves significantly led to an increase in extraction yield,except in time of 10 minutes,But in each amplitude of ultrasound,increasing of process time did not have a significant effect on extraction yield.Although inamplitude of100%,there was significant difference between time of 10 and 20 minutes(Fig. 2).Generally,comparison of meansshowed that ultrasound was significantly more effective than microwaves in case of extraction yield and there was no significant difference between mean of extraction yield of microwaves and maceration without stirring for 24 hours (Fig. 3).

Table 1. Influence of extraction techniques on the concentration of gallic acid and quercetin. Means with similar letters statistically have no significant difference(p <0/05).

	microwave						Ultrasound							Maceration With stirring for 8 h	Maceration Without stirring for24 h
Power	100w			200w			amplitude	50%			100%				
time	40sec	80sec	120sec	40sec	80sec	120sec	time	10min	20min	30min	10min	20min	30min		
Gallic acid	134019^{h*}	218537^{g}	249867^{e}	151788^{h}	223543^{fg}	346228^{d}	Gallic acid	245084^{ef}	360239^{d}	385279^{c}	490548^{b}	568753^{a}	573910^{a}	Gallic acid	406871^{c}	226468^{fg}
Quercetin	ND•	93103^{E}	183466^{C}	ND	173049^{D}	268825^{B}	Quercetin	ND	ND	ND	ND	ND	ND	Quercetin	435140^{A}	ND

* The peak area as a function of concentration.

• ND: not detected.

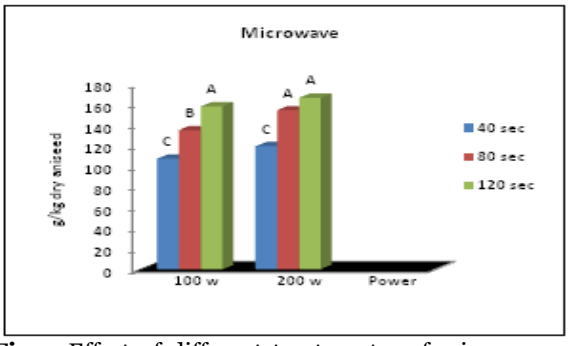

Fig. 1. Effect of different treatments of microwaves on the extraction yield. Means with similar letters statistically have no significant difference (p <0/05).

Effect of different treatments on the antioxidant activity

In case of microwaves,power increment did not significantly affect on the antioxidant activity,except for time of 120 seconds.But in each power, with time increasing, the antioxidant activity of the extracts was significantly increased (Fig.4).In case of ultrasounds, amplitude of ultrasound waves and process time caused an increase in the antioxidant activity.In each amplitude of ultrasound waves, an increase in process time significantly increased the antioxidant activity of extracts(Fig. 5).Mean of antioxidant activity of extracts prepared by microwaves was higher than that of prepared by ultrasounds but it was not statistically significant. Results showed that in each extraction technique,there was positive relationship between extraction yield and antioxidant activity i.e. with an increase in extraction yield the antioxidant activity increased .Butcomparisionof techniques indicated that there was no relationship between antioxidant activity and extraction yield. Probably because the entrance of non-antioxidant ingredients into the extracts prepared with ultrasound was higher than their entrance into extracts prepared with microwaves. Mean of antioxidant activity of extracts prepared by microwaves and ultrasounds were significantly higher than that prepared with maceration without stirring for 24 hours (Fig. 6).

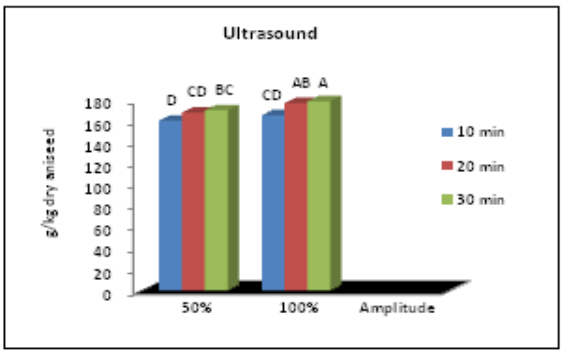

Fig. 2. Effect of different treatments of ultrasound waves on the extraction yield. Means with similar letters statistically have no significant difference (p <0/05).

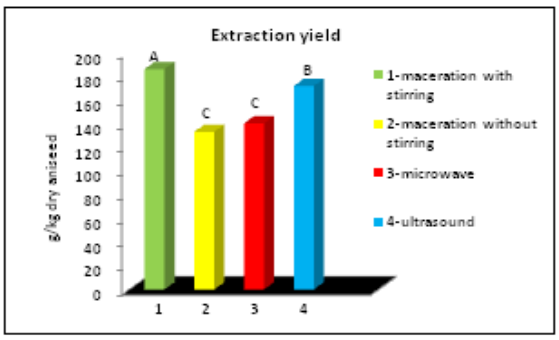

Fig. 3.comparison of effect of extraction techniques on the extraction yield. Means with similar letters statistically have no significant difference (p <0/05).

Results of HPLC

Retention time of gallic acid (3.23 min) and quercetin (33.83 min) at standard chromatograms was determined and compared with retention time of extracts compounds. In this manner, peaks belonging to those compounds were determined and area of the peak was utilized as a function of concentration of these compounds in the extracts.As it is shown in Table 1, the effect of microwaves and ultrasounds on the extraction of gallic acid and quercetin is different. In all treatments, mean of concentration of gallic acid in the extracts prepared by ultrasounds was more than that of prepared by microwaves, except for extracts prepared in the amplitude of 50% and time of 10 minutes. The concentration of quercetin in extracts prepared by ultrasounds was undetectable, while it was detectable in the extract prepared by microwaves, except for extracts prepared at time of 40 seconds. These results along with the higher mean of antioxidant activity of extracts prepared by microwaves than that of prepared by ultrasounds show that in each condition, there is the possibility of incongruity between the data obtained from measurement of gallic acid as a method of measuring antioxidant activity (e.g. Folin-Ciocalteu method) and the data obtained from other methods of measuring antioxidant activity.

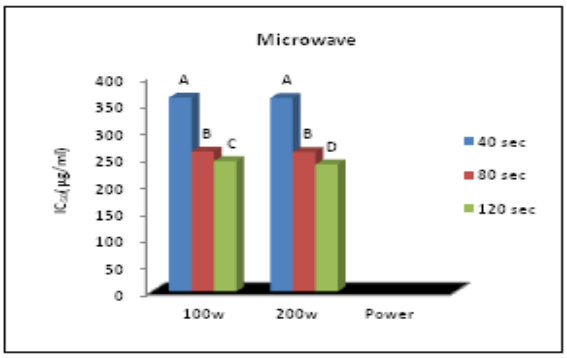

Fig. 4. Effect of different treatments of microwaves on the antioxidant activity. Means with similar letters statistically have no significant difference (p <0/05).

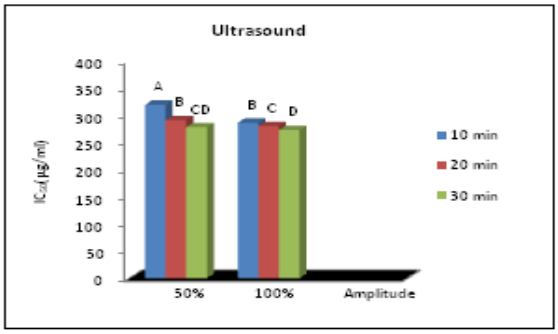

Fig. 5. Effect of different treatments of ultrasound waves on the antioxidant activity. Means with similar letters statistically have no significant difference (p <0/05).

Gulcin*et al.* (2003) studied the antioxidant properties of aniseed and found that there is no correlation between the total phenolic compounds and antioxidant properties of the extracts.Difference in extraction yield of gallic acid and quercetin may be due to the differences in mechanism of action and test conditions of the two techniques and their effect on these substances.It seems that the mechanical effect of ultrasounds and process time on extraction of gallic acid were more than the effects of microwaves(e.g. heating).but in case of quercetin, ultrasounds not only

were effective, but also may had destructive influences on quercetin.In contrast with ultrasound waves, increasing the power and time in of microwave treatments increased the concentration of quercetin in the extracts. This can be due to an increase in solvent temperature and a decrease in the viscosity of aniseed particle soil which results in better penetration of hot solvent into the particles and increasing quercetin solubility and its entrance into extracts.Wang *et al.* (2010) also indicated that enhancement of recovery and solubility of targeted compounds such as flavonoids by microwaves is attributed to thermal effects.

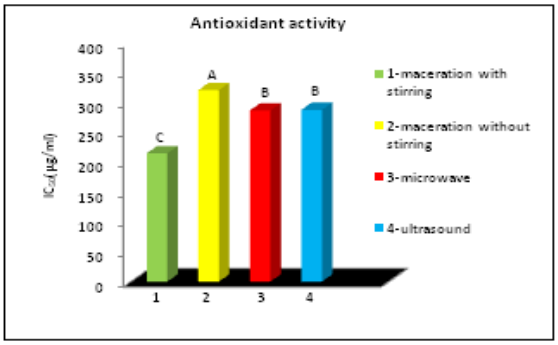

Fig. 6.comparison of effect of extraction techniques on the antioxidant activity. Means with similar letters statistically have no significant difference (p <0/05).

Conclusions

Means ofextraction yield and antioxidant activity of extracts prepared by extraction techniques of microwaves and ultrasounds were compared and the following results were obtained: Although the extracts prepared by ultrasound waves had a higher extraction yield than the extracts prepared by microwaves ,their antioxidant activity was not higher. Maximum antioxidant activity belonged to extracts prepared by microwaves (power of 200 w, time of 120 sec) and representative phenolic compounds were also detectable in most of microwaves treatments. Therefore, in case of the extraction of anti-oxidative substances, microwaves acted more efficient andselective.Although the antioxidant activity of extracts prepared by microwaves was significantly lower than that of prepared by maceration of aniseed particles with stirring, but in appropriate conditions,microwave can be used as a pretreatment of antioxidant extraction of aniseed in order to reduction of extraction time, preservation of antioxidants and reduction of process costs.Finally, it is suggested that the effect of particles size, fat content and solvent temperature on the extraction of antioxidants be studied.Also we suggest to use different ranges of variables, to take into account other variables and standard compounds and optimization of antioxidants extraction from aniseed.

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