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Investigation of Cultivate Zone and Ultrasound on Antioxidant Activity of Fenugreek Leaf Extract

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ABSTRACT

According to the problems concerning synthetic antioxidants which the negative effects of this compounds on human health have been proved and also the oxidative stress which occurs in human's body due to several reasons, caused most of the researchers and industry replace synthetic antioxidants with the natural ones existing in plants, fruits and vegetables as a fact of defeating oxidative stress. In this study, the extraction of phenolic and tocopherols compounds and the antioxidants strength of methanol extracts and the fenugreek leaves methanol/water (50:50) in Ardebil and sari which have been under effect of ultrasound effects was estimated through Folin–Ciocalteu, 1,1-diphenyl-2-picrylhydrazyl (DPPH), β-carotene linoleic acid and Oxidative Stability Index (OSI) and compared with synthesis antioxidants of TBHQ. The results showed that the phenolic amount (1062 ppm) and tocopherols (403.243 ppm) of ultrasound methanol extract was high in Ardebil. Also ultrasound-methanol extract of fenugreek leaves in Ardebil with inhibition percent of DPPH (60.903 %) in 900 ppm concentration, β-carotene fading percentage of (91.327%) in 500 ppm concentration and oxidation stability index for 4.92 hour was the highest. The data shows that the fenugreek leaves extract concentration the solution kind and the planting location effects the antioxidant strength.

KEYWORDS: cultivate zone, ultrasound, antioxidants, fenugreek leaves, DPPH, β-carotene Linoleic acid, Oxidation stability index

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum L*) is one of the plants has been used in traditional medication in Iran and different nations for a long time and several medical qualities have recorded so far. It is worth mentioning that fenugreek has an expanded medical effects such as : antioxidant quality [9], painkilling, anti emphysema, anti spasm, anticancer, reducing the blood pressure, styptic, blinding bile, reducing blood cholesterol, reducing blood fat, reducing blood triglycerides, womb strengthening [32]. Most of physiology disorders pathologic events or illnesses effecting human beings have been concerned to unstable chemicals that are called free radicals or treatable oxygen kinds [33]. Free radicals are strong oxidants that contain non mated electrons. They are capable of damage in all body components (lipid, protein and DNA). Free radicals play a role in expanding dangerous illness such as cancer, heart attack, vessels obstruction, blood pressure, rheumatism and cataract [25]. Antioxidants are components that prevent chain reaction of oxidation by adding hydrogen atoms to free radicals. An antioxidant efficiency is related to how easy this hydrogen atom can be separated [13]. Although there exists antioxidants in plasma, the immunity system cannot destroy the free radical alone, therefor providing the antioxidants from external sources is necessary[41]. Oxidation is one of the main factors in fat and oil frustration, because of this the synthetic antioxidants such as BHA, BHT, TBHQ, and PG are added to the products in order to prevent oxidation [11]. These components are heat sensitive and are not good for food preserve and on the other hand can threat human health [40]. According to these conditions several acts should be done to prevent the side effects of synthetic antioxidants. One of these ways is extraction of natural antioxidants from fresh fruits and vegetables: containing high amount of antioxidant [45]. The plant extracts are full of antioxidants that are used as food resources and reduce the oxidative stress and prevent or delay the decreasing illnesses [34]. There are several ways to measure the antioxidants activity in natural materials, and the two methods of β-carotene discoloration [44] and oxidation stability index [18] have been used in this study.

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MATERIALS AND METHODS

Materials

The fenugreek was provided from two areas, Sari and Ardebil in 2013 the leaves were separated and clean by rubbing hands on them and then dried in room heat. After drying they turned into powder.

Extraction

In this methods, the fenugreek leaves powder was mixed with pure methanol and methanol/water(50/50) and put for 30 minutes in the ultrasound with the degree of 45℃ [30] and then at three stages of centrifuge the blue phase was refined with Whatman refining paper NO.1. After that the solution was evaporated and the fenugreek leaves extract was produced. The extract was covered by aluminum and kept in temperature of -18℃ [12].

Measurement and phenolic components

solution and turned blue[29]. 50 micro litter of the sample 4was thinned with 2.5 ml of Ciocalteu fooling The total amount of phenolic component of the extract was estimated by Folin–Ciocalteu method. Folin was recovered in alkaline with evaporated water. Then 2 ml carbon sodium was added and was put in temperature of 45℃ for 15 minutes to expand the blue phase and the absorb was read with spectrophotometric tocopherol components.

Estimating tocopherol components

The total amount of tocopherol components of the extract was estimated based on α- tocopherol. In this test 100mg of the extract was mixed with 5ml of toluene, then 3.5 ml of solution DPPH and 0.5 ml iron chloride 3 was added and mixed, and finally the standard solution amount was raised to 10 ml and was placed unmoved for 1 minutes and it's absorb was 520[38].

Estimating antioxidant activity

DPPH test

1,1-diphenyl-2-picrylhydrazyl(DPPH) is a purple component which have turned in to a radical due to the existence of phenyl group in its structure and is the free radical recourse. The ability to give hydrogen atoms or electrons by different extracts in the test is estimated with the discoloration amount of the purple solution of DPPH in methanol. In this study DPPH was used as stable radical component.

50 ml of extract concentration of 100, 300, 500, 700, 900 micro gram/5CC was added to 5 ml of 0.004% DPPH in methanol and stirred very fast. And put in heating room. For 30 minutes and then put back in a normal room temperature, light absorb of the sample was 517[4] and the DPPH free radical inhibition percentage was estimated by the following formula:

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

In this formula, A blank shows the light absorb without the extract and A sample shows the light absorb of extract with different concentration.

β -carotene Linoleic acid test

To start the test a base solution of β -carotene linoleic acid was provided through:0.5 mg β -carotene solved in 1ml chloroform and 25 micro litter linoleic acid and 200mg to in 40 was added and mixed well. Then the chloroform was extracted by vacuum evaporation. And 100ml of distilled water filled with oxygen was added to 2.5ml of the solution was added to 350 micro litter extract and put for 48 hours in heating room and then placed in a normal room. The light absorb of the samples were 490 nanometer and the antioxidant activity was measured by comparing the samples light absorb with total time of zero and the β -carotene yellow color stability [8].

$$\text{Inhibition percentage} = \frac{(Ac\ (0) - Ac\ (24)) - (As\ (0) - As\ (24))}{(Ac\ (0) - Ac\ (24))} * 100$$

Where:

Ac (0) : the absorb read for blank in a time equal zero

Ac (24) : the absorb read for blank after 24 hours

As (0) : the absorb of the sample in a time equal zero

As (24) : the absorb of the sample after 24 hours

Oxidation stability index (OSI)

To define the oxidation stability, 600ppm of fenugreek leaves extract was added to antioxidant free canola oil. The oxidation stability was done by Rancimat machine model 743, under the temperature of 120℃and airflow of 20L/h[14].

Statistical Analysis

All results are expressed in random of triplicate duplication. Different mean values (three factors) were analysed by software SPSS and the means were compared with danken test at P<0.05. The graphs of mean values and error bar were created using Excel version 2007.

CONCLUSIONS AND DISCUSSION

The amount of phenolic and tocopherol components of fenugreek leaves extracts it is show in table 1, the amount of phenolic and tocopherol of ultrasound methanol extract is the highest in Ardebil and has a reasonable difference comparing to other extracts. In extract method the ultrasound of 20 KH penetrates the material and causes frequent stretch and collection due to which several holes appear inside plant material, these holes attach each asymmetrically and make the materials come out of the cells. In addition, these waves can destroy the living cells and make the extract easier[20]. Extracting through the ultrasound reduces the process time noticeably and develops the returns comparing other methods. There for it seems that ultrasound waves releases phenol and tocopherl components from the fenugreek wall and fiber by destroying the leaf base fiber. as it can be seen the methanol/water(50/50) methods have the least amount of phenolic components. Using water in extracting provides a polarized environment. There for some of the phenol components with low polarity will be extracted less. In addition water extract contain impurities such as proteins and solution sugars [5]. In a research run by Dhanani et al.,(2013) to measure the antioxidant activity and the amount of phenolic components in the plant called *sumenifra* through the three methods of Soxhlet, ultrasound and microwave with three solutions of ethanol, ethanol/water and water, the amount of obtained phenolic components for the three methods was ethanol-ethanol/water-water. In 1994 Sotillo et al., studied the extract of phenolic from potatoes skin using methanol and water and found that the methanol solution in 4℃ is a more efficient extractor comparing to water in 25℃. Chung et al., (2010) while were extracting Soya reported that extraction with ultrasound due to a better return is a good extracting technique comparing with Soxhlet and traditional methods. In a method run by comparing with Soxhlet and traditional methods. In a method run by Esmaeilzadeh kenari et al., on sesame meal in 2014, the total phenol contain of the ultrasound-methanol (62.09) was move than ultrasound-methanol/water(37.24) which matches the research. Dua et al., obtained(2013) the amount of tocopherol for fenugreek seed in India using methanol 80% by shaker which was 2210±1.54 mg/g after 6 hours. But nazim ciftci etal., (2011) estimated the tocopherol existing in oil while they were studying the different kind of oils for fenugreek seeds. It was clear that the fenugreek seed contains oils.in different types the amount of alpha-tocopherol was between 620 to 910 mg/kg oil. The amount of pure phenol of fenugreek was equal 194.36 mg Gallic Acid in 100g of dry seed of the sample obtained by Souri et al., (2008) while Mirzaei et al., (2011) in estimating the antioxidant quality and pure phenol of fenugreek seed hydroalcoholic (Ethanol 70%) extract with maceration method obtained the pure phenol of 74 mg galic acid per extract geram using Folin–Ciocalteu method. The reason of these differences can be because of using difference of estimation, extract, different solutions, different standards to report the results, planting location, soil and weather [42].

Table 1-comparison of average of phenolic and tocopherol components in different fenugreek leaves extract (danken test p<0.05)

Extracting method-solvent- cultivate zone	Phenolic amount per ppm	Tocopherol amount per ppm
ultrasound-methanol-Sari	1014 ± 1.5011^{b}	**376.79 ± 0.51^{b}**
ultrasound-methanol-Ardebil	1062 ± 2.886^{a}	**403.243 ± 0.644^{a}**
ultrasound-methanol/water-Sari	748.467 ± 3.055^{d}	**242.057 ± 0.144^{c}**
ultrasound-methanol/water-Ardebil	764.8 ± 2.424^{c}	**233.03 ± 0.294^{d}**

Study of antioxidant activity

DPPH test

Using the spectrophotometric method, the absorb which present the amount of DPPH is being estimated. The more the amount will be, the less the antioxidant activity in removing free radicals will be. There for the amount of DPPH left is related to radical removal activity of antioxidant reverse [23]. The antioxidant of all extracts will increasing the concentration from 100ppm to 900ppm. This result agreement the researches done by Esmaeilzadeh kenari et al., (2014) and Igzia et al.,(2012) where an extract concentration of a factor was effective on increasing the antioxidant activity. According to table 2 the highest amount of antioxidant activity is related to synthesis antioxidant TBHQ (64.88%) with concentration of 100 ppm which has a reasonable difference comparing to other

extracts. After that is placed the extract using the ultra sound-methanol of Ardebil with inhibition percent of 60.903% with concentration of 900ppm. These results match the researches done by Esmaeilzadeh kenari et al., (2014) and Pinloo et al., (2005) that the ultrasound-methanol extract is the best for inhibition the free radical. They found that the antioxidant activities of methanol extract is more than ethanol extract and the ethanol extract and the ethanol extract is more than water extract and can inhibit more DPPH free radical generally, inhibition method for DPPH free radical in an organic environment is more suitable than a water place [19].

To compare an antiradical activity of different extracts EC50 is used. Which is defined as a extract concentration in which 50% of the DPPH Free radicals inhibited in the reaction environment [31]. There for the lower the concentration I, it shows that the specific extract has more antiradical activity. High correlation was reported for the ability of trapping the free radicals and the amount of phenol in fruits, vegetables and grains.

Table 2- comparing the average percentage of DPPH free radical inhibition in different fenugreek leaves extract in Sari and Ardebil(danken test $p<0.05$)

Different extract concentration / **Extracting methods**	**100 ppm**	**300 ppm**	**500 ppm**	**700 ppm**	**900 ppm**
ultrasound-methanol-Sari	40.313 ± 0.046^{m}	45.640 ± 0.08^{j}	50.683 ± 0.02^{g}	53.897 ± 0.02^{e}	58.687 ± 1.00^{c}
ultrasound-methanol-Ardebil	40.640 ± 0.04^{m}	46.853 ± 0.02^{i}	53.750 ± 0.04^{e}	56.673 ± 0.02^{d}	60.903 ± 0.56^{b}
ultrasound-methanol/water-Sari	31.770 ± 0.04^{q}	37.173 ± 0.02^{o}	43.547 ± 0.02^{l}	48.720 ± 0.04^{h}	51.097 ± 0.02^{g}
Ultrasound-methanol/water-Ardebil	33.327 ± 0.354^{p}	38.077 ± 0.56^{n}	44.043 ± 0.49^{k}	48.933 ± 0.64^{h}	51.723 ± 0.50^{f}
TBHQ	64.883 ± 354^{a}				

As you can see in figure1, TBHQ has the lowest amount of EC50(77.06±0.42). The ultrasound-methanol extract from Ardebil is placed after that with amount of (429.52±2.95). The higher amount of EC50 shows the lower amount of total phenolic components and the samples antioxidant qualities. Then the synthesis antioxidant sample has the most antioxidant quality. As mentioned be for, there exists a good cohesion between phenolic components and antioxidant quality. Methanol/water extracts have lower total phenol and tocopherol components and have lower antioxidant quality. With attention on EC50 amount for Sari and Ardebil it can be observed that EC50 data amount for Ardebil comparing to Sari in a equal extracting method is lower, which means the antioxidant strength of extracts obtained from Ardebil is more than Sari. Esmaeilzadeh kenari et al., (2014) in their study of sesame meal antioxidant quality showed that the lowest amount of EC50 relates to BHA synthesis antioxidant and after the extract using ultrasound-methanol is located, water/alcoholic extracts have the most amount of EC50 which means they have the lowest amount of antioxidant activity. Sweetie et al., (2007) from India studied on mint extract antioxidant effects and showed that the EC50 equals 28.5 micro grams per ml litter and for BHA equals 10.1 Mc.gr in ml.lit, while the EC50 obtained for Iranian Mint ethanol extract obtained by Kamkar et al., (2009) equal 12 micro gram per milliliter.

Also in a research done by Golluce et al., (2007) in Turkey on antioxidant quality of Bee balm essence and extract using DPPH the amount of EC50 for the essence was 10700 and for the extract was 74.4 micro gram per mili litter, but in a research which was done on Iranian Bee balm by Kamkar et al., (2011) the amount of EC50 for the essence was 1765 and for the methanol extract was 50 micro gram per milliliter. The researchers showed that the planting location effects the antioxidant strength matches the research results.

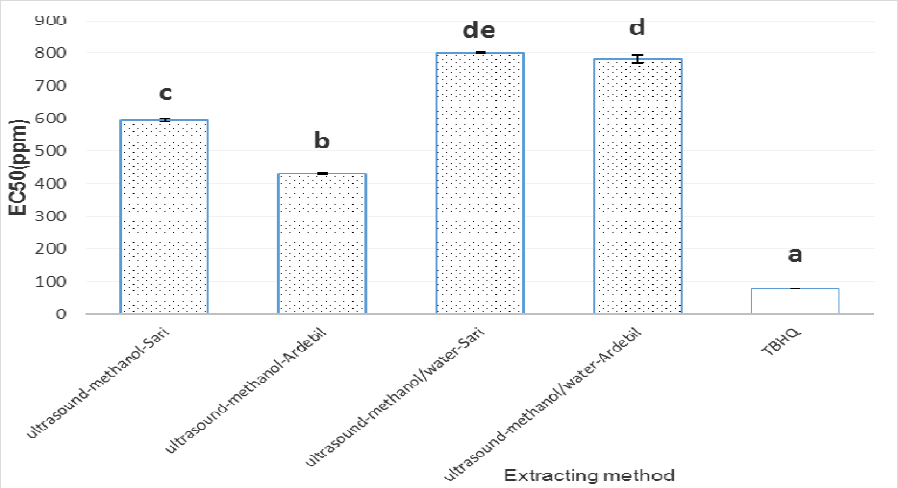

Figure 1- comparison of EC50(ppm) extracts and TBHQ synthesis antioxidants

Study of antioxidant quality with β-carotene colorless

The antioxidant activity of different Fenugreek extract is presented on table 3 and is compared with TBHQ synthetic antioxidant. The highest antioxidant effect in β-carotene colorless system relates to ultrasound methanol extract from Ardebil with concentration 500ppm(91.327%) which has a reasonable difference comparing to TBHQ and other extracts.

The results do not follow a certain pattern but according to the concentrations the extracting methods and planting locations are different.

Table 3- comparison of colorless percentage average of β-carotene in fenugreek leaves different extracts in Sari and Ardebil ($p<0.05$)

Different extract concentration / Extracting methods	100 ppm	300 ppm	500 ppm	700 ppm	900 ppm
ultrasound-methanol-Sari	53.327±1.65 ^{mn}	65.233±2.184^{g}	85.710±0.0^{c}	37.617±0.825^{q}	33.803±0.825^{r}
ultrasound-methanol-Ardebil	45.233±0.82^{p}	58.093±0.82^{ij}	**91.327±1.65^{a}**	62.850±1.43^{h}	66.187±0.82^{fg}
ultrasound-methanol/water-Sari	28.570±0.0^{s}	66.187±0.82^{fg}	76.187±0.82^{e}	53.327±1.65^{mn}	67.140±0.0^{f}
Ultrasound-methanol/water-Ardebil	55.710±1.43^{kl}	59.520±1.64^{i}	77.617±0.82^{d}	58.093±0.82^{ij}	51.423±1.42^{o}
TBHQ	88.270±0.0^{b}				

β-carotene exists in different plants and is a pre factor of producing Vitamin A, one of the strong antioxidant which prevents formation of oxygen free radicals in body, there for it has an independent process form Vitamin A. Fenugreek leaves contain carotene and has Vitamin A quality [23]. In addition Brewer (2011) showed that there exists a positive relation between antioxidant activity and vitamins C and E and β-carotene (vitamin A). the inhibition percentage decrease in this study may concern with the increase of B-carotene and other related antioxidants, antioxidant components keep the quality up to a specific range and after that by increasing the concentration change in to pro oxidants [2,26].

Oxidation Stability Index

Oxidation stability index is the time period needed for measurable speed expansion in oils and fats which can be a factor for comparing oil frustration [10]. To study the heat stability of treatments in Rancimat method amplification conditions of oxidations such as high temperature or airflow is used and increasing water electrical conductivity is assumed as an index in developing the oxidation, because during the oil\ oxidation organic acids like

formic acid will form which increases the electrical conductivity [27]. Figure 2 shows the oxidation stability index of fenugreek leaves extract and TBHQ synthetic antioxidant s on canola oil. As you can see the most amount relates to ultrasound methanol treatment in Ardebil (4.92±0.1 hours) which has a reasonable difference of 5 percent with TBHQ synthetic antioxidant (4.705±0.18 hours) and TBHQ is located after that. It can also be observed that ultrasound methanol extract in Sari is not statistically different from TBHQ. Generally, Fenugreek leaves different extracts show that the same as synthetic antioxidants and in some treatment more than that has contrasted canola oil oxidation which shows a well antioxidant strength of this plant extract.

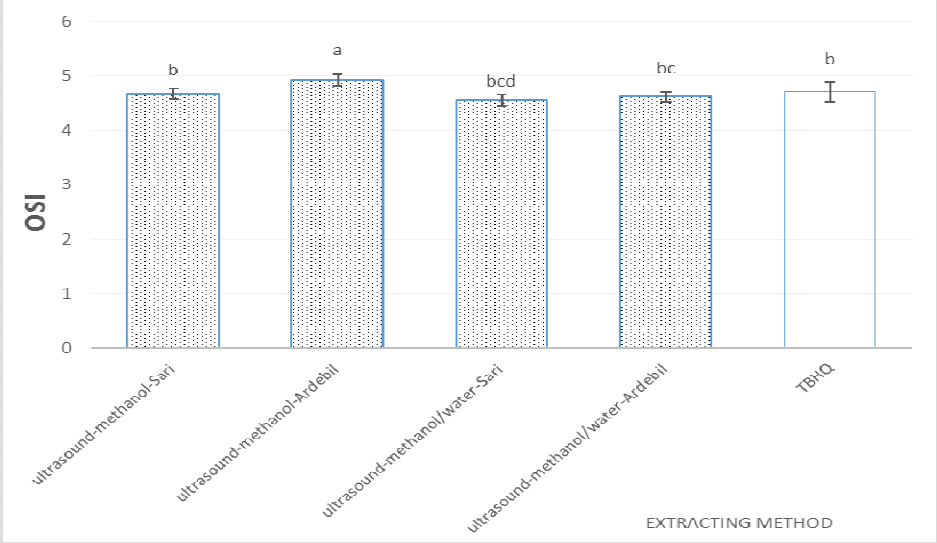

Figure 2- oxidation stability index of Fenugreek leaves different extracts and comparison with TBHQ synthetic antioxidants

Conclusion

Comparison of antioxidant activity of Fenugreek leaves different extract showed that among used solutions, the methanol solution and between the two planting location Ardebil was better than the others. Also concerning with the extract concentration in DPPH test the concentration of 900ppm and in β -carotene test the concentration of 500ppm the ultrasound methanol extract of Ardebil has the highest antioxidant strength. Mean while, using ultrasound as a helper for extract causes an outstanding decrease in extracting time and also increase the extract quality. There for it can be inferred that the solution type, extracting method planting location and the extract concentration effect the extract antioxidant strength. Generally, according to the obtained results from the research, Fenugreek leaves extract containing phenolic, tocopherol, strong antioxidant and antiradical components in most of cases is more effective than synthetic antioxidant and since it is known as a medicinal plant in most of countries and especially in Iran it can be a good replace for synthetic antioxidants in edible oils and fatness foods. To use the extract practically in industry more researches on estimating the extract and essence antioxidant strength in food types is recommended.

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