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INVESTIGATION OF THE ANTIOXIDANT PROPERTIES OF IRANIAN CHILLI PEPPER EXTRACT

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Abstract. Oxidative stress factors are known to cause diseases related to metabolic disorders. So, eliminating these factors, or at least decreasing their number can help prevent or improve metabolic problems. Recently, herbal medicines have been paid much attention to due to their high effectiveness, and application of synthetic antioxidants has been reduced because of their adverse effects on human health. This research aims at evaluating the antioxidant power of chilli pepper. Some solvents such as water, ethanol, and water-ethanol solution, with or without ultrasound wave treatment, have been used to obtain chilli pepper extracts. The amount of tocopherol and phenolic compounds in the extracts has been measured by the stoichiometrical method, and the antioxidant power of the extracts has been measured and analysed by the *beta*-carotene and DPPH tests. Finally, the extracts' oxidative stability has been determined. The data have been statically analysed by the Analysis of Variance (ANOVA) and Duncan's multiple range test. The P level <0.05 was considered statistically significant. The results have shown that the amounts of phenolic compounds in terms of Gallic acid in different extracts range 1066.3 to 1172.27 mg/kg, and the amounts of tocopherol compounds in terms of *alpha*-tocopherol are 693.52–867.65 mg/cm^3. The oxidative stability index in different extracts was 5.55 to 7.23 hours. The inhibitory percentage of linoleic acid oxidation in the extracts varied between 57.2 and 83.6%, and the inhibitory percentage of DPPH radicals in different extracts varied between 71.33 and 91.87%. The maximum and minimum efficiency for phenol and tocopherol compound extraction were obtained using ethanol and water solvents, respectively. With a high antioxidant power, chilli pepper can be widely used in food, pharmaceutical, and cosmetic industries.

Keywords: phenolic compounds, tocopherol, ultrasound, antioxidant activity, chilli pepper.

Introduction. Formulation of the problem

An antioxidant is a molecule capable of slowing down or preventing the oxidation of other molecules. The most common reactive oxygen species (ROS) include superoxide (O^{2-}) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals, and reactive hydroxyl (OH^-) radicals. The nitrogen-derived free radicals are nitric oxide (NO) and peroxynitrile anion (ONOO) [1]. Normally, the ROS generated are detoxified by the antioxidants nearby in the body, and there is symmetry between the ROS generated and the antioxidant present. However, due to ROS overproduction and/or an insufficient antioxidant response, this equilibrium is upset for more ROS, which culminates in an oxidative stress. The ROS actively attack and cause oxidative damage to various biomolecules including proteins, lipids, lipoproteins, and DNA [2]. This oxidative damage is a decisive etiological factor responsible for quite a lot of chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases. Besides, it affects the course of ageing [3]. Antioxidants prevent the production of such highly reactive species, remove free radicals, help repair oxidative damage, and contribute to effective functioning of antioxidants naturally prevailing in the body [4].

Analysis of recent research and publications

At present, most antioxidants are manufactured synthetically. Several synthetic antioxidants, e.g. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylated hydroquinone (TBHQ), and Gallic acid esters are commercially available. Such synthetic antioxidants are known to have potential side effects and toxicity

when taken in vivo, hence, their use is restricted nowadays, and there is an increasing interest to finding safer and bioactive natural antioxidants present in plants [5,6]. Thus, there is a demand for antioxidants of natural origin, because they can protect the human body from diseases caused by free radicals [7,8]. That is why in recent years, a lot of attention has been paid to receiving information about plants with antioxidant ability that can be useful for people.

Herbal medicines are widely used all over the world. They are often viewed as natural, and therefore harmless. Many herbal remedies, alone or in different combinations, in the form of leaves, powders, pastes, decoctions, infusions, etc., are recommended to treat various diseases. Many, if not most medicinal plants contain flavonoids. These compounds can have a number of beneficial effects, such as the antioxidant one, which is considered to be a fundamental property of vital importance [9].

Lipid oxidation is one of the most important factors of food spoilage. This phenomenon not only leads to a shorter shelf life, but also affects the nutritional value of food [10,11]. Various factors, including light, oxygen, heavy metals, and some types of fatty acids contribute to oxidation in foods [12].

At present, the most studied phytochemicals in plants are phenolic compounds, because they have various functions in the human body, mainly as antioxidants [13]. However, the composition and levels of certain phytochemicals with antioxidant potential present in vegetables do not necessarily reflect the total antioxidant capacity, which depends on the type and concentration of phytochemicals, and on the synergistic or inhibitory interaction of molecules in the matrix.

The best way to stabilise fats and prevent lipid oxidation is to use antioxidants. Usually, antioxidant compounds revive radical compounds by giving them hydrogen and thus preventing their oxidation. This phenomenon not only prolongs the shelf life of oils, but also preserves them by preventing the destruction of nutritious material such as vitamins and even proteins [14]. So it is necessary to identify antioxidants in natural resources.

Therefore, it is important to study phytochemicals present in vegetables widely consumed in some countries, such as peppers, in order to learn about their potential health benefits. Fruits of pepper (*Capsicum annuum* L.) are consumed fresh, processed, or as spice in various dishes around the world. It has also been found that peppers are a good source of polyphenol compounds with antioxidant properties [15,16].

In recent years, the pepper has grown in popularity, and a lot of varieties are now available in grocery shops. Almost all peppers turn from green to yellow, orange, red, or purple when they are fully ripe. Green bell peppers are often harvested before they are ripe, and changes in the maturity may affect the content of phytonutrients which are important in the diet as antioxidants. Fresh pepper is one of the vegetables that are high in vitamin C [17]. Phenolic compounds show good antioxidant ability [18], but they are relatively unstable [19]. The stability of phenolic compounds depends on various factors, such as the pH value and the temperature [20].

Antioxidants extracted from the chilli pepper belong to natural antioxidants. The chilli pepper contains 1.5% of oleoresin compounds. The most important oleoresin compound is kapsantyn (0.02% in a pepper), which has a phenolic structure and tastes very hot. The warmer the farming region of the pepper is, the more kapsantyn is produced, and the hotter the pepper [21]. Other important compounds found in pepper include some essence, carotenoids, vitamins A and C, which are strong antioxidants and affect free radicals [22].

To the best of our knowledge, there has been no report about the antioxidant activity of chilli pepper extract. That is why the **purpose** of this research was to study chilli pepper extract as a source of natural antioxidants using solvents, like water, ethanol, and water-ethanol solution, and ultrasound waves.

Objectives of the research:

1. Determining tocopherol and phenolic compounds in the extracts measured by spectrophotometrical methods;
2. Investigating *beta*-carotene for antioxidant power using the discolouring test and 2- and 2-diphenyl-1-pichrilhydrasine (DPPH) tests;
3. Determining the oxidative stability index (OSI) by adding pepper extract to sunflower oil and using a Rancimat device (was determined to be void of antioxidants).

Research materials and methods

Materials. The chilli pepper (*Capsicum frutesens*) was collected from Babolsar County, and stored in a fridge (4°C) until the start of the experiment. All the required chemicals (reagents and solvents) were provided by Merck Company and were of high purity. Antioxidant-free sunflower oil was collected from Behshahr Industrial Company, and was stored at 4°C.

Preparation of chilli pepper extracts. After being dried up at 60°C, the chilli pepper was ground with an electric food grinder (Moulinex 684, France), and stored at the freezing temperature until the start of the experiment.

The pepper powder was mixed with ethanol (in the ratio 1:5) by means of a two-stroke electric mixer (made in Iran, TAM Iran) at the speed 250 rpm for 48 hours [23]. Then it was cleaned by passing through a Whatman filter No.1.

According to the combined solvent and ultrasound method, the chilli pepper was extracted after being dried at 60°C and combined with the solvents (water and ethanol) in the ratio 1:5 at room temperature [23]. Then it was exposed to ultrasound waves of 75 Hz for

30 minutes in an ultrasonic device (Sonorex Digitec DT 510H, Germany). Then, the extract was passed through a Whatman filter No. 1. In order to make the solvent evaporate, a vacuum-based oven (made in Iran, Ariateb) was used, heated to 50°C. To prepare combined solvents (water and ethanol), 80% of ethanol and 20% of water were mixed [23].

In this research, 6 samples were prepared as follows:

Sample 1, prepared with ethanol and ultrasound. To combine ethanol and ultrasound, the chilli pepper was extracted after being dried at 60°C, and combined with 80% of ethanol at room temperature. Then it was exposed to ultrasound waves of 75 Hz for 30 minutes in the ultrasonic device.

Sample 2, prepared with ethanol, water, and ultrasound. To combine ethanol, water, and ultrasound, the chilli pepper was extracted after being dried at 60°C, and combined with water and ethanol in the ratio 1:5 at room temperature [23]. Then it was exposed to ultrasound waves of 75 Hz for 30 minutes in the ultrasonic device.

Sample 3, prepared with water and ultrasound. To combine water and ultrasound, the chilli pepper was extracted after being dried at 60°C, and combined with water at room temperature. Then it was exposed to ultrasound waves of 75 Hz for 30 minutes in the ultrasonic device.

Sample 4, prepared with water and ethanol. To prepare combined solvents (water and ethanol), 80% of ethanol and 20% of water were mixed [23]. In order to make the solvent evaporate, a vacuum-based oven was used heated to 50°C.

Sample 5, prepared with ethanol. The pepper powder was mixed with ethanol (in the ratio 1:5) in the electric mixer at the speed 250 rpm for 48 hours [23]. Then it was cleaned by passing through the Whatman filter No. 1. In order to make the solvent evaporate, the vacuum-based oven was used heated to 50°C.

Sample 6, prepared with water. The pepper powder mixed with water (1 to 5 ratio) and electric mixer at the speed of 250 rpm for 48 hours [23]. Then it was cleaned by passing through the Whatman filter No.1. Then, the vacuum-based oven was used heated to 50°C.

Measuring phenolic compounds. By Dorman et al.'s method (2003), a spectrophotometer (UV-VIS-2100) was used at the wavelength 765 nm, and the absorbance-density curve for Gallic acid (mg/kg) was drawn. 2.5 g of the extract was weighed in a centrifuge pipe, and after adding 2.5 ml of normal hexane, it was vortexed for 1 minute. Then, 2.5 ml of a methanol and water solution (in the ratio 80:20, respectively) was added and vortexed for another 40 seconds. The pipe containing the sample was centrifuged at 5000 rpm for 5 minutes (Heraeus Speatech Gmbh, Labofuge, Germany). The oily phase was extracted with a syringe and put into another centrifuge pipe. The water phase was also kept separately. 2.5 cm^3 of a methanol and water solution was added to the separated oily phase at the last step, and was centrifuged like at the first step. After centrifuging, the oily phase was extracted with a syringe and put in another pipe. The water phase was also kept separately. 2.5 cm^3 of a methanol and water solution was also added to the oily phase and centrifuged at 5000 rpm for 5 minutes. After that, the oily phase and the water phase were kept separately. The water phases kept in 50 ml volumetric flasks were combined. 2.5 cm^3 of the Folin–Ciocalteu reagent and 5 ml of 7.5% sodium carbonate were added to the water phase and brought to a volume of 50 cm^3 by diluting it with distilled water. The sample was stored overnight, and then its absorbance was read at the wavelength 765 nm. The quantity of phenolic compounds was calculated by the following formula (1) in terms of cm^3/kg of the oil sample [23].

$$P = \frac{Y}{W} \times 1000\,, \qquad (1)$$

where Y is the amount of phenolic compounds in the sample in terms of mg/cm^3, and W is the oil sample weight [23].

Measuring tochopherol compounds. By Wong et al.'s method (1988), a spectrophotometer (UV-VIS-2100) was used at the wavelength 520 nm, and the absorbance-alpha density curve (mg/ml) was drawn. 190–210 mg of an oil sample was weighted carefully in a 10 cm^3 volumetric flask. 5 cm^3 of toluene was added to the sample, which was then thoroughly stirred. Then 3.5 ml of a 2-2'bi-pyridine solution (0.07% volumetric weight in 95% hydrous ethanol) was mixed with 0.5 cm^3 of $FeCl_3.6H_2O$ (0.2% volumetric weight in 95% hydrous ethanol). Finally, the volume of the standard solutions was brought to 10 cm^3 by diluting it with 95% hydrous ethanol. The resultant solution was left to rest for 1 minute, and its absorbance was read at the wavelength 520 nm. The quantity of tocopherol compounds was calculated according to the following formula in terms of cm^3/kg of oil (2):

$$T = \frac{A - B}{M \times W}, \qquad (2)$$

where A and B are the absorbance in the 10 cm^3 solution for the sample and the control, respectively. M shows the slope of the alpha-tocopherol absorbance-density standard curve, and W is the weight of a sample in grams. M in this test was set to be 0.0039 with an explanation coefficient of 0.99. T denotes the tocopherol density in terms of cm^3/kg of oil [24].

Investigation of the antioxidant properties using the 2- and 2-diphenyl-1-pykryl-hydrazine (DPPH) test. By Kukic et al.'s method (2008), a spectrophotometer was used at 517 nm of wavelength. According to the method, DPPH, a stable radical compound, was used as a reagent, so that 50 μl of extracts with different densities (20, 40, 60, 80, and 100μg/cm^3) in ethanol was added to 5 Mmol of a 0.004% DPPH solution in ethanol. After 30 minutes of incubation (at room temperature), the light absorbance

of the samples was read at the wavelength 517 nm [25].

DPPH: percentage inhibition of free radicals (3):

$$I = (C_a - \frac{S_a}{C_a}) \times 100, \% \quad (3)$$

Where C_a is control absobance, S_a is sample absobance.

Sample absorbency. Sample absorbency denotes the amount of light absorbed by extracts with different densities. In this test, this characteristic of different extracts was measured by the amount of discolouring the 2- and 2-diphenyl-1-pykryl-hydrazine DPPH purple solution in ethanol [26].

Investigation of the anti-radical properties by *beta*-carotene discolouration (*beta*-carotene – linoleic acid). By Juntachote and Berghofer's method (2005), a spectrophotometer was used at 490 nm of wavelength. For the experiment, first a basic solution of *beta*-carotene and linoleic acid (Sigma-Aldrich) was prepared: 0.5 mg of *beta*-carotene was solved in 1 ml of chloroform, and then 25 µl of linoleic acid and 200 mg of Tween 40 was added to the solution and stirred. After that, by the vacuum evaporation method, the chloroform was separated and 100 ml of oxygen-saturated distilled water (30 minutes under the pressure of 100 ml/min) was added to it. 2.5 cm^3 of this solution was transferred to a test tube, and 350 µl extract was added to the test tube. After 48 hours of incubation at room temperature, the light absorbance of the samples was read at the wavelength 490 nm, and the antioxidant activity was measured by comparing the light absorbance of the samples to zero time and by the stability of the yellow colour of *beta*-carotene expressed as a percentage [27].

The oxidative stability index (OSI). In order to determine the oxidative stability, 600 ppm of each chilli pepper extract was added to antioxidant-free sunflower oil, and the oxidative stability was measured by a 743 Rancimat device, at 120°C and the airflow speed 20 L/h [28].

Statistical analysis. All the experiments were conducted in a purely random experimental format in three replications. The mean values were compared using MSTATC software and by Duncan's test and *t*-tests (with the significance level 5%). The diagrams were plotted using Microsoft Excel software.

Results of the research and their discussion

The quantities of phenolic compounds. As seen in Fig. 1, phenolic compounds in terms of Gallic acid in different extracts varied in quantity between 1066.3 and 1172.27 mg/kg. The maximum amounts of phenolic compounds were extracted by the ethanol-ultrasound, ethanol-water-ultrasound, and water-ethanol methods, which showed no significant difference, and were statistically similar. The minimum phenolic compound was extracted by sole water. In their research, Samadlooi et al. (2007) extracted the phenolic compounds of ten different species of pomegranate using acetone ($(CH_3)_2CO$) as the solvent and ultrasound waves, and measured them by the Folin–Ciocalteu method to investigate the antioxidant effect of the sample with the maximum content of phenolic compounds [29]. The results showed that the phenolic compounds of a pomegranate kernel varied by about 1.02%. The largest amount of phenolic compounds was found in black pomegranate species. It was added to the odourless soya oil sample at three concentration levels: 100 ppm, 200 ppm, and 350 ppm, and the BHA synthetic antioxidant was added at two concentration levels: 100 ppm and 200 ppm. How this treatment delayed the oxidation of raw oil (at 60°C) was investigated by measuring the quantity of peroxides and TBA. The results showed that the phenolic compounds of a pomegranate kernel had an antioxidant effect, and 350 ppm of the phenolic compounds extracted from pomegranate kernels had the highest antioxidant effect.

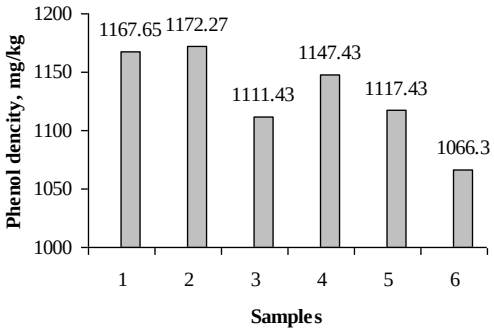

Fig. 1. Effect of the solvent type and ultrasound on phenolic compounds in the Iranian chilli pepper ($p<0.05$)

Zhuang et al. (2012) investigated the bioactive properties and antioxidant activities in 9 pepper species. The results showed that all fresh pepper samples were rich in vitamin C, carotenoids, and phenolic compounds. The content of phenolic compounds in chilli pepper was higher than in other peppers ($p<0.05$) [30].

Gorinstein et al. (2009) conducted a comparative study of phenolic compounds and antioxidant activity in consumable raw vegetables. The aim was to estimate the antioxidant value and anti-proliferative activity of some vegetables (the raw garlic, yellow or white onion, red onion, green and red pepper, and white cabbage) during one year and in the same geographical and weather conditions. The highest amount of bioactive compounds and antioxidant activity was found in the red onion [31].

Tochopherol content. As seen in Fig. 2, the amount of tochopherol compounds of different extracts was, in terms of alpha-tocopherol, between 693.52 and 867.65 mg/ml. The maximum of tocopherol compounds was extracted by the ethanol-ultrasound, ethanol-water-ultrasound, and water-ethanol methods, which were statistically similar, and the minimum

quantity of tocopherol compounds was extracted by the sole water method, without using ultrasonic waves.

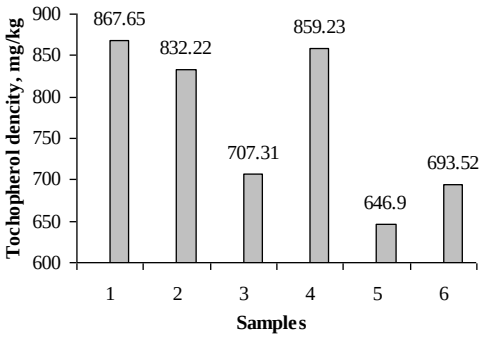

Fig. 2. Effect of the solvent type and ultrasound on the tocopherol content in the Iranian chilli pepper ($p<0.05$)

Lai et al. (2009) reported that the quantity of tocopherol in rice bran (Japonica variety), after extracting by the methanol method, was 537 mg/kg, and when the ethyl acetate (EtOAc) extracting method was used, this parameter reached 770 mg/kg, and for hexane it was 196 mg/kg [32]. Aguilar-Garcia et al. (2007) reported that the total amount of tocopherol in three different varieties of rice bran ranged 41–61.3 μg/kg [21].

Iqbal et al. (2005), using the HPLC method, estimated the tocopherol content in some Pakistani varieties as 392–512 mg/kg [22]. Chen et al. (2005) determined the tocopherol content of two rice bran varieties (Spirus and Bengal) using the HPLC method: for the Spirus variety, it was 3400 to 3900 mg/kg, and for the Bengal variety, 3800 to 4200 mg/kg [33].

Investigation of the antioxidant properties using the *beta*-carotene discolouration test (*beta*-carotene linoleic acid)

The percentage inhibition of linoleic acid for different extracts is shown in Fig. 3. The results show that the ethanol-ultrasound and ethanol-water-ultrasound methods provide the highest inhibitory power, and the sole water method has the lowest inhibitory power. This is probably due to a higher level of destruction of herbal tissues during ultrasonic treatment, and lower solubility of antioxidant compounds in polar solvents.

Iqbal et al. (2005) estimated the antioxidant activity of the linoleic acid system in some Pakistani rice varieties between 10 and 20%. The antioxidant activity in the linoleic acid system in some varieties was lower than the antioxidant activity in tocopherol, but it was higher than the BHT antioxidant activity [22].

Investigation of the anti-radical properties using the 2- and 2-DPPH test. As seen in Fig. 4, the percentage inhibition of DPPH radicals in different extracts varied between 71.33 and 91.87%. The ethanol-ultrasound and ethanol-water-ultrasound methods had the highest inhibitory power, and the sole water method had the lowest. The reason for this is probably a higher level of destruction of herbal tissues during ultrasonic treatment and a lower solubility of antioxidant compounds in polar solvents.

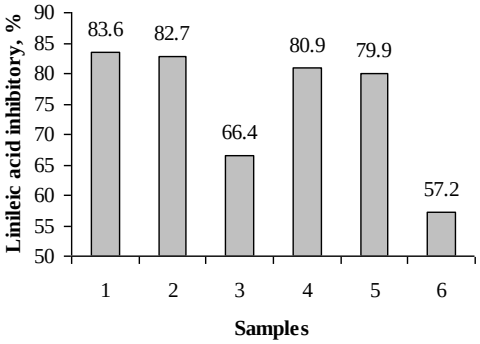

Fig. 3. Effect of the solvent type and ultrasound on the antioxidant properties of the Iranian chilli pepper ($p<0.05$)

Butsat et al. (2010) conducted an experiment on inhibiting DPPH free radicals of Thai rice bran. The inhibitory activity of DPPH in three different regions in Thailand varied between 85.9 and 86.7%, which was observed in the tested varieties [34].

Goli Movahed et al. (2008) investigated and compared the anti-radical and antioxidant activity in leaf vegetables eaten fresh (leek, cress, mint, basil, tarragon, and coriander) [14]. They collected methanol extract from dried leaves and investigated their antioxidant activity in linoleic acid systems. The anti-radical activity of the extracts obtained was also evaluated using a model system containing DPPH radicals. The results showed that cress had the highest (11.62%) and basil the lowest (4.42%) extraction yield. As such, it was established in the model system of a linoleic acid emulsion that the methanol extract of tarragon was the most effective in preventing oxidation (the induction period was 60.3 hrs, compared to 13.4 hrs for the control sample). In the system containing DPPH, mint and tarragon showed the highest activity. So the lowest IC_{50} (219 μg/ml) was that of mint. It was even lower than the IC_{50} of BHT synthetic compounds, although there was no significant difference between mint and tarragon. Generally, it was found that the methanolic extracts of tarragon and mint had the highest anti-radical and antioxidant activity.

Suja et al. (2004) compared the antioxidant activity of the major polyphenols of sesame oil with the BHA synthetic antioxidant using the DPPH method. The results showed that the rate of discolouration of DPPH radicals by sesamol is far higher than by BHT (about 11 times). Nevertheless, there is no significant difference between the rate of discolouration of DPPH radicals by other phenolic types of sesame oil (sesamin, sesamolin, and sesaminol) and by BHT [35].

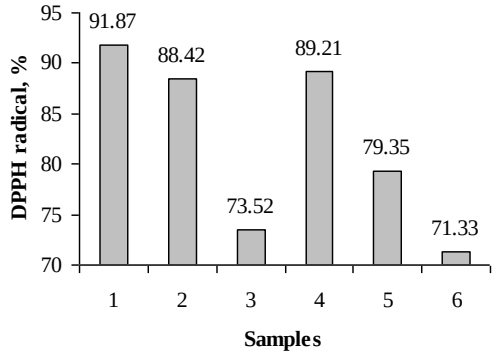

Fig. 4. Effect of the solvent type and ultrasound on the anti-radical properties of the Iranian chilli pepper (p<0.05)

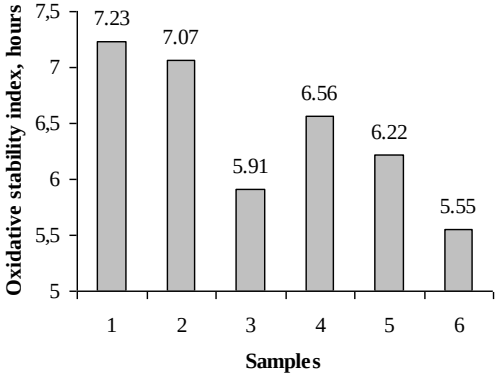

Fig. 5. Effect of the solvent type and ultrasound on the OSI of the Iranian chilli pepper (p<0.05)

The OSI experiment. The OSI value, depending on the extraction method, varied between 5.55 and 7.23 hrs. Of the methods of extraction, the ethanol-ultrasound method resulted in the highest, and the sole water method in the lowest OSI (Fig. 5). The OSI for the control sample, i.e. oil containing BHT, was 4.1, which is lower than with the studied extraction methods. This is due to the large amounts of tocopherol and phenolic compounds found in the extract, which can have an effect on the oil stability.

Under similar conditions, Anwar et al. (2005) obtained the values of 5.99 to 7.40 hrs for different samples of rice bran oil. As it can be seen, the reported numbers are similar to the numbers presented in this research [36].

Farag et al. (2004) investigated the effect of phenolic extracts obtained from olive oil on sunflower oil stability, which was more significant than the effect of BHT on sunflower oil stability [10].

Conclusion

According to the results of this research, there is no statistically significant difference between various extraction methods used for the chilli pepper. The ethanol-ultrasound, ethanol-water-ultrasound, and water-ethanol extraction methods do not differ significantly in the content of phenolic and tocopherol compounds and inhibiting DPPH radicals. Neither there is a significant difference between the ethanol-ultrasound and ethanol-water-ultrasound methods in their ability to inhibit linoleic acid oxidation. However, these methods differ significantly from the water-ethanol method. On the other hand, these three methods are significantly different when it comes to OSI. Generally, it can be concluded that among various extraction methods for the chilli pepper, the ethanol-ultrasound method is the most appropriate for extracting compounds with antioxidant properties. The chilli pepper is a rich source of tocopherols and phenolic compounds, and has high antioxidant activity, which makes it applicable in the food, medical, pharmaceutical, and cosmetic industries.

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