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Evaluation the effect of *Cucurbita pepo* **seeds bioactive compounds obtained via ultrasoundassisted extraction on oxidative stability of ground mutton meat**

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1. Introduction

Oxidation of lipids is one of the important factors influencing the shortening of the shelf life of food products as well as the loss of nutritional value and its physical and chemical characteristics. One of the most effective methods of controlling the lipid oxidation process is the use of antioxidant compounds. These compounds in small amounts are able to prevent or greatly delay the oxidation process [1]. Among hundreds of compounds that have the ability to inhibit the oxidation of lipids, only a part of them are suitable for human and have food grade [2]. Several studies show that synthetic antioxidant compounds such as butyl hydroxytoluene (BHT), butyl hydroxyanisole (BHA) and propyl gallate (PG) have many negative effects on the health of human and animal cells [3]. Due to the growing awareness of consumers regarding health aspects and their interest in consuming healthy meals, the use of natural preservatives and environmentally friendly technologies has been considered [4]. Natural antioxidant compounds may be added directly to food products or by being included in packaging systems and edible films, they may increase the shelf life and quality of products [5]. Numerous researches have evaluated and investigated the effect of antioxidant compounds found in different parts of plants such as fruits, vegetables, flowers, stems, leaves, roots and seeds on maintaining and improving the quality of food products including meat and meat products [8-6]. Pumpkin with the scientific name *Cucurbita pepo*, belongs to the gourd family, is an annual plant. This plant is native to Mexico, North America, and East Asia. This plant has a high number of large and edible seeds [9, 10]. Based on previous studies, the oil obtained from pumpkin seeds is a rich source of linoleic acid (more than 38%). Also, the fatty acid linolenic acid has been identified in the oil obtained from this seed. Linoleic acid and linolenic acid are essential fatty acids that play a decisive role in maintaining human health. The human body is not able to synthesize essential fatty acids, so they must be supplied to the body through a

balanced diet [4]. Due to the high and significant value of this natural resource, unfortunately, they are often discarded as agricultural and industrial waste. Only in some regions, they are roasted and consumed in snacks and their oil is used for cooking applications. Since food security is the real challenge of the present and future, wasting any source of food is practically incorrect, so it is necessary to pay attention to the processing and extraction of valuable compounds from these seeds. Meat is the main source of proteins with high biological value in many countries and is an important part of human diet. The most common chemical change that causes a decrease in the quality of meat and meat products is the lipid oxidation. Oxidation of lipids and the production of primary and secondary products causes negative effects on the quality of meat and meat products such as changes in color, texture, flavor and taste, formation of toxic compounds, reduction of nutritional value and economic loss. Grinding is one of the conventional methods of meat processing, which improves its crispness and ease of use by consumers. Minced meat is divided into two groups, including minced meat without additives and those containing additives. Minced meats containing additives and artificial preservatives are used to produce various sausages and meat products. The small pieces of meat that are created in the primary and secondary cuts of the carcass, as well as the flank part, are among the most common sources of meat for the production of minced meat [11]. During the grinding process, the cell membrane is damaged and membrane lipids are mixed with pro-oxidant compounds such as metal ions. Also, the temperature increases during grinding, and oxygen, as one of the main factors of oxidation, is placed in the minced meat to a greater extent, and as a result, the possibility of oxidation reactions increases

[12]. In this study, the bioactive phenolic compounds extraction from pumpkin seeds has been investigated using ultrasound-assisted extraction (UAE). Then, the effect of the obtained bioactive compounds on oxidative stability of minced meat has been evaluated and compared with the synthetic antioxidant compound.

2. Materials and Methods

2.1. Materials

Organic, clean, and dry pumpkin seeds (with a moisture content of 7.46±0.21%) were obtained from the University of Zanjan and then, stored in a freezer (-18 ºC). Before the experiment, the seeds were ground by a laboratory grinder and then the size was determined using a sieve (18 mesh). The mutton meat (flank part) was purchased and transported to the laboratory in a cover of ice. After washing, the meat was divided into small pieces and ground twice by a meat grinder (Kenwood, MG 700, China). The prepared meat was kept in the refrigerator (4 ± 1) ºC) until the experiment. All the chemicals used in this research such as perchloric acid, butyl hydroxytoluene, thiobarbituric acid, potassium thiosulfate, acetic acid, chloroform, potassium iodide, trifluoroacetic acid, and high-purity methanol solvent were purchased from Merck and Sigma-Aldrich companies.

2.2. Bioactive compounds extraction using UAE

To perform the UAE process, an ultrasonic probe device with an input power of 200 W, a frequency of 24 kHz, and equipped with a probe with a radius of 1.5 mm was used. To carry out the recovery process, 5 g of the prepared sample was mixed with 60 ml of ethanol solvent with a solid-to-solvent ratio of 1 to 12 (w/v). Based on the results of preliminary studies, the operating conditions of the process were set as 50% ultrasound amplitude, 57 ºC temperature, 54 min of sonication time, and 100% duty cycle. After the process, the solid and liquid were separated using Whatman No. 4 filter paper. Next, the separation of the solvent was carried out using a rotary evaporator under vacuum at a temperature of 45 ºC. Then, the obtained

bioactive compounds were kept at -18 ºC for further analysis.

2.3. Preparation of meat sample

To investigate the effect of bioactive compounds on the oxidation process of minced lamb meat, first, the minced meat was divided into three equal parts. Three samples include *C. pepo*: containing bioactive compounds with a concentration of 1% (w/w), BHT: containing the synthetic antioxidant compound butyl hydroxytoluene with a concentration of 0.1% (w/w) as a positive control sample and C: The sample without any preservatives (of natural or synthetic origin) was considered as a negative control sample. All samples were kept in a polyethylene container for 15 days at a temperature of 4 ºC and the relevant tests were performed at 5-day intervals.

2.4. Determination of major phenolic compounds

To quantitatively and qualitatively identify the main phenolic compounds in the bioactive compounds obtained from pumpkin seeds, a high-performance liquid chromatography (HPLC) equipped with an ultraviolet-visible detector was used. Separation was done by reversed C18 column (25 cm, 4.6 mm, 5 μm). The mobile phase included solvent A (trifluoroacetic acid with pH 2.5) and solvent B (methanol). The washing operation was performed with a flow rate of 1 ml/min. All prepared solutions and samples were first passed through a 0.45 micrometer filter and then, injected into the device. Major phenolic compounds were identified according to the retention time against the standard compounds and quantitative measurement was done by calculating the area under the corresponding peak [13].

2.5. Determination of peroxide value (PV)

To measure the peroxide value (PV), first, 2 g of the sample was mixed with 30 ml of acetic acid-chloroform solution (2:3 v/v) and 0.5 ml of saturated potassium iodide. After 1 min of darkness, 30 ml of water and 0.5 ml of starch glue were added. This solution was titrated with 0.01 N sodium thiosulfate. As soon as the color of the solution changes, the titration is stopped. The amount of peroxide index was calculated in terms of milliequivalents of active oxygen per kilogram using equation 1 [14].

Equation 1:

$$
PV = \frac{(V_2 - V_1) \times C \times 1000}{m}
$$

Where C is the concentration of potassium thiosulfate (mol/L), V_1 : volume of potassium thiosulfate consumed by the control (ml), V_2 : volume of potassium thiosulfate consumed by the sample (ml), and m: weight of the sample (kg).

2.6. Determination of thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid index was measured according to the method of Aminzare et al. [15] with some modifications. For this purpose, 10 g of sample was homogenized with 35 ml of perchloric acid (4%) and 1 ml of BHT (0.5%) for 1 min by the vortex. The mixture was filtered after centrifugation (8000 rpm for 10 min). The filtered solution was made up to 50 ml with perchloric acid. 5 ml of this solution along with 5 ml of thiobarbituric acid reagent (0.02 M) were poured into the test tube and kept at a temperature of 85 ºC for 2 h. After cooling the solution, the absorbance of the sample was measured at 532 nm. The data were expressed in terms of milligrams of malondialdehyde (MDA)/kg.

2.7. Determination of pH

To measure the pH, 10 g of the sample was homogenized with 100 ml of distilled water, and then the pH was measured using a digital pH meter [4].

2.8. Sensory evaluation

Sensory evaluation was carried out by 20 students and employees of the University of Zanjan, Department of Food Science and Engineering, taking into account three properties of color, smell, and overall acceptability under the same conditions of light and temperature using a nine-point hedonic questionnaire.

2.9. Design of experiments and statistical analysis

The experimental data were statistically analyzed by the analysis of variance using the generalized linear model. The means were compared by the Tukey test at the 95% confidence level using Minitab Version 14.0 software (Minitab Inc. State College, PA, USA). Excel version 2013 software was used to draw the graphs. All experiments were performed with at least three replications and data were reported as mean ± standard deviation.

3. Results and Discussions

3.1. Major bioactive phenolic compounds

The major bioactive phenolic compounds obtained from pumpkin seeds were determined by high-performance liquid chromatography. Based on the results, six types of phenolic acids were identified, mainly including coumaric acid (14.36 \pm 0.17 mg/g), syringic acid (10.10 \pm 0.12 mg/g), ferulic acid (13.13 9.95 \pm 0 mg/g), caffeic acid $(6.55 \pm 0.11 \text{ mg/g})$, vanillic acid (6.28 \pm 0.10 mg/g) and protocatechuic acid $(2.34 \pm 0.12 \text{ mg/g})$. In the study conducted by Rezig et al. [16], all the phenolic compounds identified in the present study were also identified in pumpkin seeds (*Cucurbita maxima*); with the difference that these compounds are reported at lower levels. The difference between their quantitative results and the results obtained in the present study can be due to the difference in the variety, weather conditions, degree of ripening and the type of extraction method [17]. Phenolic compounds are produced as secondary metabolites in small amounts in plants, which play important roles in their defense systems and health. The benefits of phenolic compounds not only include the state of their producing organs, but also if they are included in diets; They have multiple effects in improving human health, well-being, and longevity. Phenolic compounds have high antimicrobial, anti-inflammatory, and anti-radical properties. These compounds consist of one or more aromatic rings and have one or more hydroxyl substituents [5].

Also, among the many methods used to control oxidation, the use of antioxidants is the most effective, appropriate, and economical method [18]. Therefore, in recent years, the tendency of researchers to identify natural antioxidant compounds and replace them instead of synthetic antioxidant compounds is increased [1]. Also, the method of UAE has been successfully used to recover bioactive compounds with antioxidant properties from other plant sources such as *Eryngium caucasicum* [19], fenugreek seeds [20], winter melon seeds [21], and garlic [22]. According to the results obtained in the present study, the compounds obtained from pumpkin seeds in UAE are a rich source of valuable phenolic compounds, which will be investigated in the following sections for their use as a natural preservative in meat products.

3.2. Evaluation of pH changes

The pH level of meat reflects glycolysis after slaughter and is an important factor for determining meat quality. Also, the pH value is used as an important indicator in determining the progress of spoilage and shelf life of meat products [1]. In the present study, the pH level in different prepared samples including sample C (sample without any additives), sample *C. pepo* (containing bioactive compounds), and sample BHT (containing BHT as an artificial antioxidant compound) at intervals of 5 days during 15 days were evaluated at 4 ºC. The results are shown in Figure 1. According to the results, the use of bioactive compounds obtained from pumpkin seeds and BHT had a significant effect on the pH of the samples $(p<0.05)$. On the first day of the study, the pH of all samples varied between 5.67 and 5.71, which is consistent with the results obtained by Emadzadeh et al. [23]. Then, gradually with the passing of the storage period, its amount increased with different intensities depending on the type of treatment. Based on the results, the highest increasing intensity was observed in the C sample, while in BHT and *C. pepo* samples, the increasing trend was slower and more controlled, and no significant difference was observed between them at the end of the storage period. As mentioned earlier, the BHT compound is used as an artificial antioxidant compound. In the present study, the samples with this compound showed a positive effect on inhibiting the pH changes of the meat during the cold storage period compared to the negative control sample. It should be noted that the use of bioactive compounds recovered from pumpkin seeds has significantly $(p<0.05)$ controlled the increasing trend in pH changes. On the fifteenth day of the cold storage period, there was a significant difference $(p<0.05)$ in the pH value of the sample containing bioactive compounds recovered from pumpkin seeds (6.52 ± 0.01) and the control sample (5.95 ± 1) 0.04). These findings indicated the favorable and successful effect of using bioactive compounds obtained from pumpkin seeds to control the increasing trend of pH during the storage period. The increase in pH can be seen as the result of the accumulation of nitrogenous alkaline compounds (such as ammonia and trimethylamine) that are produced as a result of microbial activities and enzymes during product storage [24]. Similar results were reported by Ansarian et al. [25].

Fig 1. Effect of bioactive compounds on pH changes during cold storage

3.3. Evaluation of the peroxide value

The peroxide value (PV) is widely used to measure the freshness quality of meat and meat products. This index shows the rate of formation of primary lipid oxidation products (hydroperoxides) [25]. In the present study, the PV of the prepared samples was evaluated at 5 day intervals during 15 days at a temperature of 4 ºC, and the results are shown in Figure 2. In the present study, at the beginning of the maintenance period, the value of this index was determined in the range of 2.23 to 2.40 milliequivalents of active oxygen per kilogram. The results showed that the PV increased with different intensities depending on the type of sample (control, containing recovered bioactive compounds and containing BHT antioxidant compound) at the beginning of the storage period and then gradually decreased at the end of the storage period. The maximum amount of peroxide was observed in all samples on the tenth day of storage at 4 ºC. Changes of the PV over time are related to the mechanism of lipid oxidation. Oxidation of lipids usually occurs in three stages: initiation (formation of free radicals), propagation (rapid increase of hydroperoxides) and termination (formation of non-radical products) [1]. There are other reports regarding the initial increase of the peroxide index in raw meat and then its

secondary decrease during the storage period [25, 26]. The increase in the PV indicates the progress of the production of peroxides and hydroperoxides as the primary products of lipid oxidation during storage. On the other hand, with the passage of storage time, hydroperoxides react with other metabolites such as ketones, aldehydes, proteins and epoxides [1, 25].

According to the results, sample C had the highest amount of PV during storage. The PV in the sample containing bioactive compounds recovered from pumpkin seeds was significantly lower than the negative control sample during 15 days of storage at 4 ºC. Based on the present results, the PV of the positive control sample in all storage days was not significantly different from the sample containing bioactive compounds obtained from pumpkin seeds, which indicates the significant ability of natural bioactive compounds to compete with the synthetic antioxidant compound BHT to inhibit spoilage. The amount of PV in the sample containing the recovered bioactive compounds, unlike the negative control sample, showed a milder increase during 10 days of storage at 4 ºC, which indicates a delay in the onset of oxidative stress and its spread. The cause of the increase in the peroxide index is the formation of hydroperoxide and the progress of lipid oxidation during the storage period. Minced meat is very sensitive to oxidation. Because the process of grinding or mincing meat causes damage to the muscle membrane and exposes the lipids of the membrane to oxidation. During this process, the reaction between internal and external prooxidants (such as air oxygen, metal enzymes, and proteins containing copper, zinc and heavy metals, and heme pigments containing iron) and unsaturated fatty acids is facilitated, which results in the production of free radicals and the release of oxidative reactions [27].

The hydroperoxides formed in the samples are decomposed when they are affected by various types of prooxidants (such as temperature, light, and metal ions) and thus the secondary products of lipid oxidation are formed [28]. Therefore, the decrease in the peroxide index of the samples is related to the decomposition of hydroperoxides. According to the results, adding bioactive compounds of pumpkin seeds to raw meat inhibited the oxidation of lipids, which can be caused by the presence of phenolic compounds in the bioactive compounds obtained from pumpkin seeds. In various studies, the anti-radical effects of phenolic compounds have been widely studied [13]. The antioxidant activity of phenolic compounds is due to their ability to inhibit free radicals, donate hydrogen atoms or electrons, or chelate metal cations and singlet oxygen. Due to the presence of hydrophobic benzoic rings and the ability to create hydrogen bonds by hydroxyl groups, phenolic compounds can react strongly with proteins, and therefore they can inhibit some enzymes involved in radical production [29]. Similar results have been reported by Roshni Neshat et al. [1] regarding the positive effect of using bioactive compounds obtained from lemon verbena leaves on inhibiting the peroxide index of fish meat during cold storage.

Fig 2. Effect of bioactive compounds on PV changes during cold storage

3.4. Evaluation of thiobarbituric acid reactive substances (TBARS)

TBARS index shows the amount of secondary oxidation products such as malondialdehyde. Therefore, the measurement of this index is considered an important criterion for evaluating the degree of oxidation and spoilage of fat [30]. In the present study, the thiobarbituric acid index of the prepared samples was evaluated at 5-day intervals for 15 days at a temperature of 4 ºC, and the results are shown in Figure 3. Based on the results, the TBARS index in all three types of experimental treatments, including sample C (sample without any additives), sample *C. pepo* (containing bioactive compounds) and sample BHT (containing BHT as an artificial antioxidant compound) was significantly $(p<0.05)$ increased during 15 days of storage at 4 ºC and reached the value of 1.53 ± 0.03 mg of malonaldehyde per kg in sample C. Meanwhile, its rate of increase was lower in BHT and *C. pepo* samples. In all storage days, sample C had the highest amount of thiobarbituric acid index. On the 15th day of storage, at the same time as the amount of peroxide index decreased in all three samples due to the decomposition of hydroperoxides, the amount of thiobarbituric acid index in them reached its maximum value. A comparative study between different treatments showed that the lowest value of thiobarbituric acid index was observed in samples with bioactive compounds obtained from pumpkin seeds. Since the index of thiobarbituric acid shows the formation of secondary lipid oxidation products; It can be concluded that the addition of bioactive compounds of pumpkin seed inhibits the oxidation of lipids. Inhibition of lipid oxidation can be related to the phenolic content and antiradical activity of the bioactive compounds recovered from pumpkin seeds. Antioxidant compounds (natural and synthetic) by inhibiting the oxidation of lipids have prevented the formation of small molecules of malondialdehyde and significantly reduced the index of thiobarbituric acid compared to sample C. The results of the research conducted by Banerjee et al. [7] showed that goat meat nuggets containing BHT (100 ppm) and goat meat nuggets containing extract recovered from broccoli powder by water solvent were significantly different from each other in terms of the rate of formation of substances reactive with thiobarbituric acid (malondialdehyde) during 16 days of storage at 4 ºC. The negative control sample contained the highest amount of malondialdehyde during the storage period.

The results of Yu et al. [26] research on investigating the antioxidant capacity of extract recovered from peanut skin using ethanol solvent on the oxidative stability of cooked beef showed that slight increase in the amount of peroxide index in samples containing peanut skin extract and samples containing the artificial antioxidant compound at the beginning of the storage period can be related to the ability to inhibit free radicals and chelate metals by the mentioned compounds. Similarly, in this research, the rate of increase of thiobarbituric acid index in the samples containing peanut skin extract and the sample containing synthetic antioxidant compound was reported to be milder than the negative control sample. In another study, Falowo et al. [31] investigated the effect of antioxidant compounds obtained from *Moringa oleifera* leaves on the oxidative stability of ground beef in cold storage. They also found that the

addition of natural antioxidant compounds (0.5 g/kg) significantly ($p<0.05$) controlled the lipid oxidation compared to control samples containing BHT.

Fig 3. Effect of bioactive compounds on TBARS changes during cold storage

3.5. Evaluation of Sensorial Properties

In this research, in order to evaluate the acceptance rate of the product, evaluation of sensorial properties including color, smell and overall acceptability at 5-day intervals during 15 days of storage at 4 ºC for all treatments including sample C (sample without any additives), sample *C. pepo* (containing bioactive compounds) and BHT sample (containing BHT as an artificial antioxidant compound) were performed and the results are shown in Figure 4 (a-c). According to the results of color evaluation, the addition of bioactive compounds recovered from pumpkin seeds to minced meat had a significant effect on color characteristics. On the first day of storage, the sample containing the bioactive compounds obtained a lower score for the evaluation of the color characteristic. This finding can be caused by the green color of the recovered bioactive compounds. But with the passage of time and on the 10th day of storage, the sample containing bioactive compounds and on the $15th$ day of storage, the same

sample along with the sample containing the artificial antioxidant BHT scored more in terms of color characteristics compared to the negative control sample. The free radicals formed during the lipid oxidation process lead to the oxidation of the iron atom in the heme group and accelerate the conversion of oxymyoglobin to metmyoglobin lead to an adverse effect on the color of meat products [4]. The bioactive compounds of pumpkin seeds improved the color of the sample on the 10th and 15th days of storage compared to the control sample. This result can be related to inhibiting free radicals and thus preventing the formation and accumulation of met myoglobin [32]. Based on the results of odor characteristic evaluation, the addition of bioactive compounds to the meat sample had a significant effect on the odor characteristic compared to other samples. On the first day of storage, no significant difference was observed in terms of the smell characteristic in the samples. On the fifth day of storage, the samples containing the bioactive compounds of pumpkin seeds and the sample containing BHT scored more than the negative control sample. This result can be caused by the increase of thiobarbituric acid index in the negative control sample. On the 10th day of storage, the sample containing bioactive compounds and also on the 15th day of storage, the same sample along with the sample containing BHT scored the highest score in the evaluation of odor characteristics. In general, the addition of bioactive compounds improved the scores related to the sensory characteristic of smell in the samples. Oxidation of lipids in meat is the most common chemical change that causes a decrease in the quality of meat and meat products [33]. Lipids are responsible for the pleasant or unpleasant smell of meat, and their oxidation causes changes in the color and smell of meat products. The decomposition of hydroperoxides and the production of secondary products of lipid

oxidation (aldehydes, ketones and alcohols) as well as the substances resulting from the decomposition of proteins (such as ammonia) cause the creation and spread of the unpleasant smell of meat and meat products [1]. Better preservation of odor in samples containing bioactive compounds can be related to postponing the lipid oxidation process and producing a lower amount of lipid oxidation secondary products. The delay in the oxidation of lipids can be caused by the presence of valuable phenolic compounds in the extract obtained from pumpkin seeds.

By increasing the stability of food components (especially unsaturated lipids) and preventing the destruction of color and oxidative sharpness, these compounds maintain the primary sensory characteristics of food. The results of the evaluation of the general acceptability are given in Figure 4-c. According to the results, the addition of bioactive compounds from pumpkin seeds to the meat sample had a significant effect on the overall acceptance of the treated samples. On the fifth day of storage, the samples containing bioactive compounds and the sample containing BHT scored the highest points in terms of overall acceptance. On the 10th day of storage, the sample containing the bioactive compounds obtained the highest score in terms of overall acceptability. The overall acceptability scores show that the addition of bioactive compounds of pumpkin seeds to minced meat did not have a negative effect on the sensory characteristics of the meat samples during the storage period. This result can be due to the presence of valuable phenolic compounds in the bioactive compounds recovered from pumpkin seeds. Similar results were reported by Maqsood et al. [34] regarding the positive effect of adding phenolic compounds on the sensory characteristics of raw camel meat during its cold storage period.

Fig 4. Effect of bioactive compounds on sensorial properties during cold storage (a) odor, (b) color and (c) overall acceptability

4. Conclusion

The bioactive compounds recovered from pumpkin seeds using UAE were a rich source of phenolic compounds such as coumaric acid, syringic acid, ferulic acid, caffeic acid, vanillic acid and protocatechuic acid. The results of the investigation of the oxidative stability of minced mutton meat showed that the use of bioactive compounds of pumpkin seeds compared to the control samples containing synthetic antioxidant compounds has a significant effect on inhibiting the oxidation of lipids during 15 days of storage at cold temperature. Also, according the sensory evaluation results, the use of bioactive compounds improves sensory characteristics including color, smell, and overall acceptability in the treated meat samples. These results show that the use of bioactive compounds of pumpkin seeds can be useful as a natural antioxidant in meat products.

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بررسی تأث یر افزودن ترکی بات زیستفعال دانهی کدو تخم کاغذی استخراج شده به روش حالل با کمک امواج فراصوت بر پایداری اکسایشی گوشت گوسفند چرخ شده فرشته نوروزی[٬]، ماندانا بیِمکر^۳ٌ، علی گنجلو^۲، مجید امین زارع^۳

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