References

[1] Tang, S., Kerry, J., Sheehan, D., Buckley, D., and Morrissey, P. Antioxidative Effect of Added Tea Catechin on Susceptibility of Cooked Red Meat, Poultry and Fish Patties to Lipid Oxidation, *Food Res. International* 34:651(2001).

[2] Amarowicz, R., Naczk, M., and Shabidi, F. Antioxidant Activity of Various Fractions of Non-tannin Phenolics of Canola hulls. *J. Agric. Food Chem.* 48: 2755(2000).

[3] Demo, A., Petrakis, C., Kefalas, P., and Boskou, D., Nutrient Antioxidants in Some Herbs and Mediterranean Plant Leaves. *Food Res. International.* 31(5):351(1998).

[4] Decker, E. Strategies for Manipulating the Prooxidative/Antioxidative Balance of Foods to Maximize Oxidative Stability. *Trends in Food Sci. and Technol.* 9:241(1998).

[5] Prior, R. and Cao, G. Analysis of Botanicals and Dietary Supplements for Antioxidant Capacity: a Review. *JAOCS International* 83(4):1950(2000).

[6] Yen, G., Duh, P., and Tsai, C. Relationship Between Antioxidant Activity and Maturity of Peanut Hulls. *J. Agric. Food Chem.* 4:67(1999).

[7] Onyeneho, S. and Hettiarachchy, N. Antioxidant Activity of Durum Wheat Bran. *J. Agric. Food Chem.* 40:1496(1992).

[8] Duh, P., Yeh, D., and Yen, G. Extraction and Identification of an Antioxidative Component from Peanut Hulls. *JAOCS* 69(8):814(1992).

[9] Asamerai, A., Addis, P. Epley, R., and Krick, T. Wild Rice Hull Antioxidants. *J. Agric. Food Chem.* 44:126(1996).

[10] Anonymous, Iran and Its Special Position in Pistachio World Market. *Iran Daily newspaper* 8(2223)(aug/26/2003).

[11] Gutfinger, T. Polyphenols in Olive Oil. *JAOCS* 58(11):966(1981).

[12] Osawa, T. and Namiki, M. Natural Antioxidants Isolated from *Eucalyptus* Leaf Wax. *J. Agric. Food Chem.* 33(5):777(1985).

[13] Osawa, T. and Namiki, M. A Novel Type of Antioxidant Isolated from Leaf Wax of E*ucalyptus* leaves. *J. Agric. Biol. Chem.* 45(3)735(1984).

[14] Price, M. and Butler, L. Rapid Visual Estimation and Spectrophotometric Determination of Tannin Content of Sorghum Grain. *J. Agric. Food Chem.* 25(6):1268(1977).

[15] Graham, H. Stabilization of the Prussian Blue Color in the Determination of Polyphenols, *J. Agric. Food Chem.* 40:801(1992).

[16] Ramarathnam, N., Osawa, T., Namiki, M., and Kawakishi, S. Chemical Studies on Novel Rice Hull Antioxidants.l. Isolation, Fractionation, and Partial Characterization . *J. Agric. Food Chem.* 36:732(1988).

[17] Chen, Z., Wang, L., Chan, P., Zhang, Z., Chung, H., and Liang, C., Antioxidative Activity of Green Tea Catechin Extract Compared with that of Rosemary Extract. *JAOCS* 75(9):1141(1998).

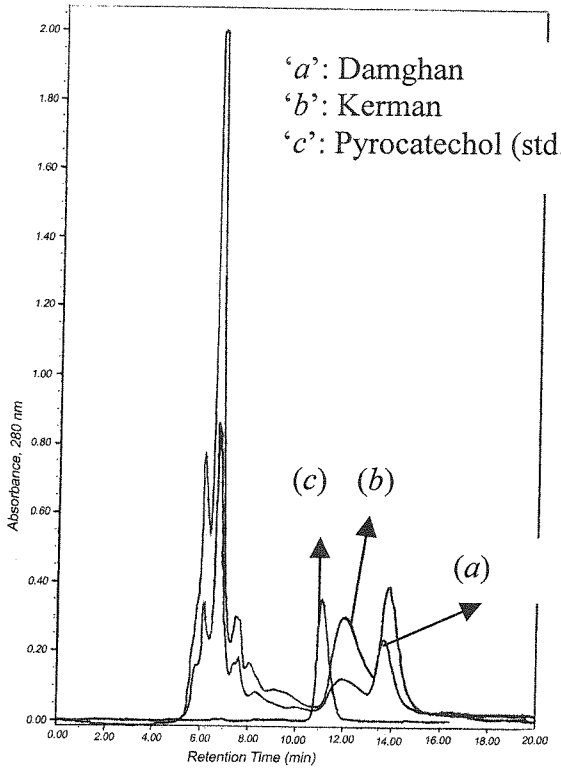
[18] Moreno, C., Gracia, M., and Frankel, E., Antioxidant Activity of Selected Spanish Wines in Corn Oil Emulsions. *J. Agric. Food Chem.* 48:5581(2000).

[19] Meza, N., Rodriguez, N., Juarez, M., Garcia, J., Casanova, R. and Guerrero, A. Antioxidant Activity in Soybean Oil of Extract from Thompson Grape Bagasse. *JAOCS* 76(2):1445(1999).

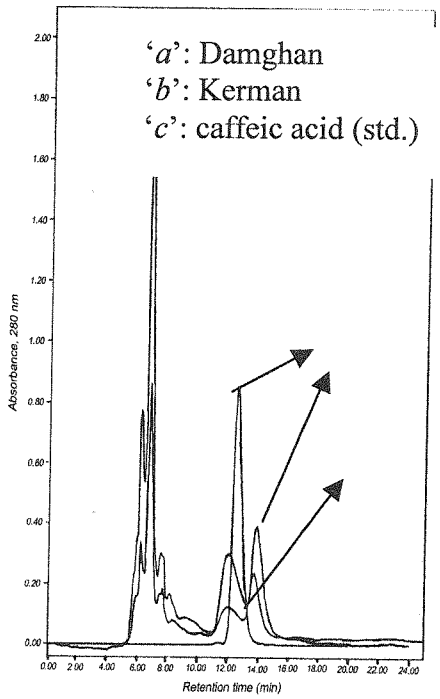
[20] Castillo, J.Garcia, O., Lorente, J., Alcaraz, A., Redondo, A., Ortuno, A., and Rio, J. Antioxidant Activity and Radioprotective Effects Against Chromosomal Damage Induced in Vivo by X-rays of Flavan-3-ols (Procyanidins) from Grape Seeds (*Vitis Vinifera*): Comparative Study Versus Other Phenolic and Organic Compounds. *J. Agric Food Chem.* 48(5):1738(2000).

[21] Madhavi, D., Deshparnde, S., and Salunkhe, D. (eds.), Food Antioxidants-Technological, Toxicological, and Health Perspectives, Marcel Dekher, Inc, 1996.

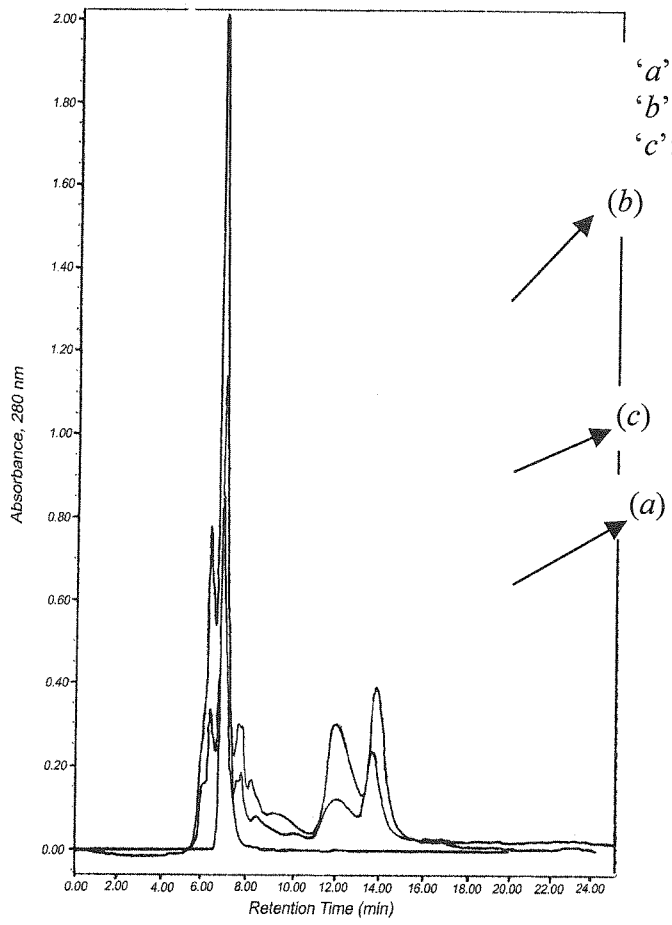
Appendix



Appendix



Appendix 3.



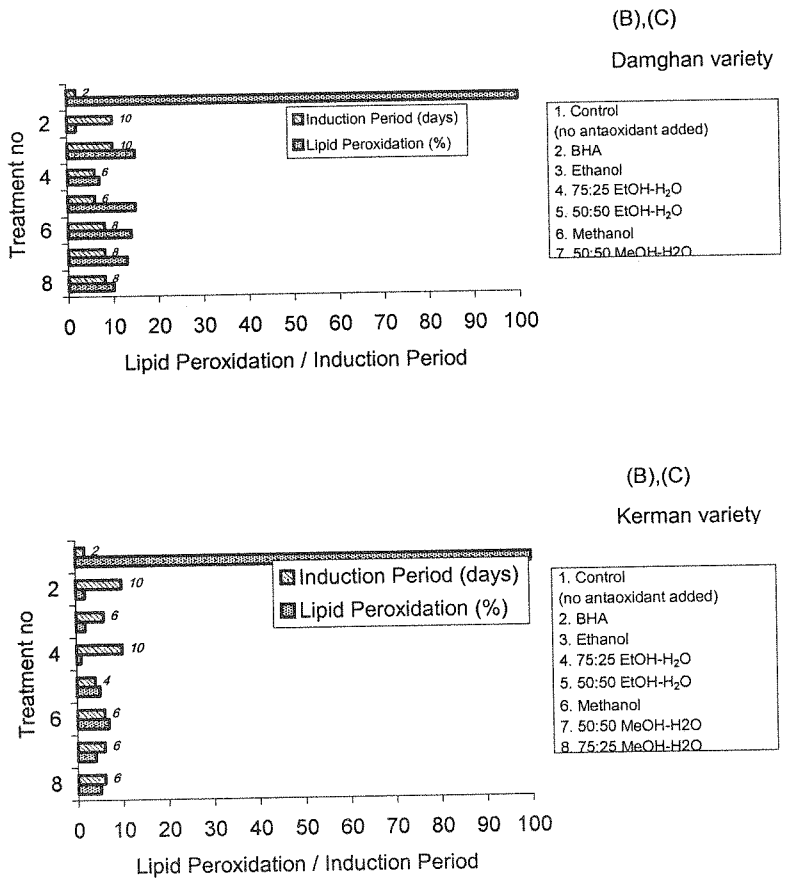


Figure (2) Antioxidant activities of pistachio hull extracts of different solvents determined by a thiocyanate method. The results are shown in terms of:A-spectrophotometric data (absorbance at λ507 nm versus time of incubation of reaction mixture at 40°C, B-degree of linoleic acid peroxidation(%), C-induction periods (elapsed time before detection of peroxidation) (days).

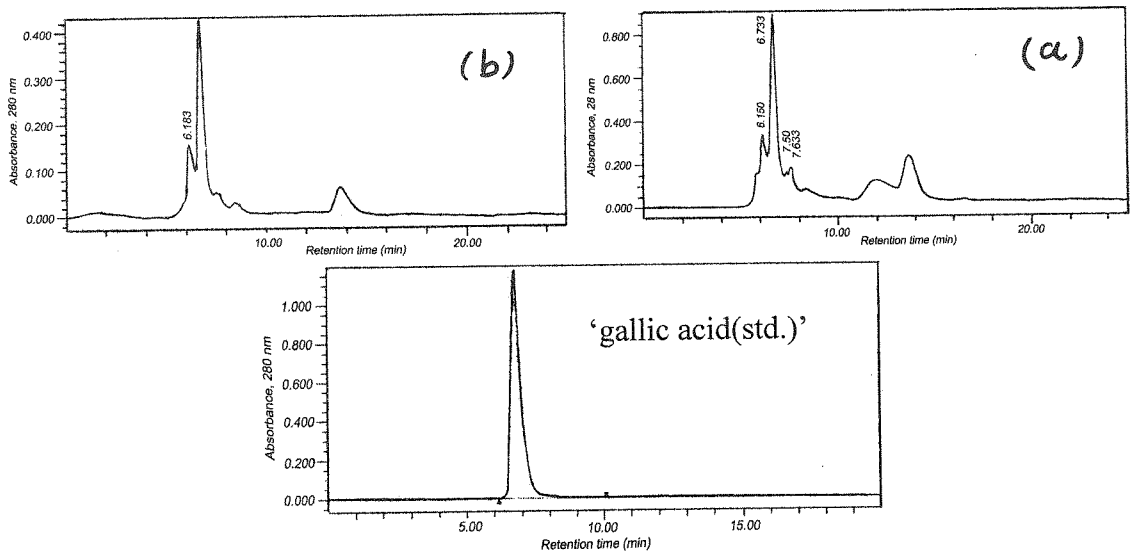


Figure (3) Hplc profile (at 280nm) of pistachio hulls extracts ('75:25' ethanol-H_2O): Damghan (*a*) and Kerman (*b*) commercial standards were: 3,4 dihydroxycinnamic acid, pyrocatechol, and gallic acid(caffeic acid). Each one of the standards was run with the extracts of the two varieties of the pistachio. see the text for the specifications given for Hplc analysis. Also see the appendices for details.

method as one the most reliable procedure for determining phenolics concentration was used in this study. While thiocyanate assay was used to assess the degree of the antioxidative activities of the phenolics extracts from pistachio hulls. The degree of inhibition of linoleic peroxidation of the extracts was found to be high and comparable with that of the synthetic type of antioxidant.

Acknowledgement

The research financially has been supported by a research grant from Amirkabir University of Technology (Office of Vice Chancellor in Research Affairs)- we deeply appreciate this support.

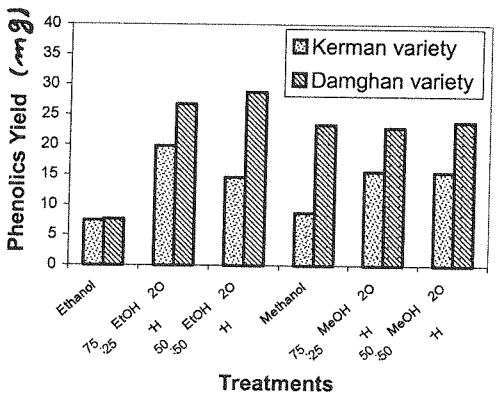
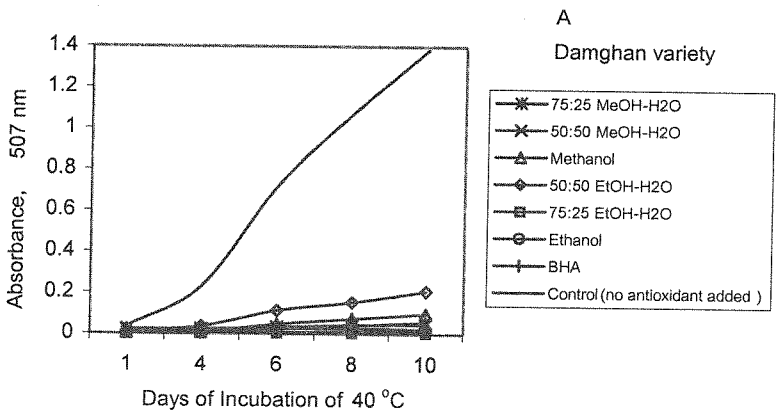
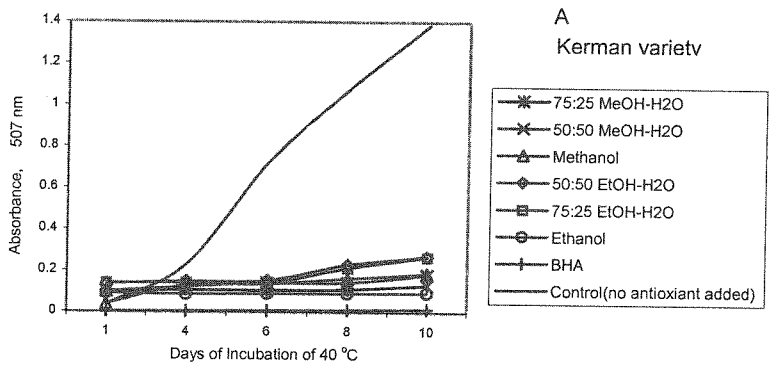


Figure (1) Amount of total phenolics recovered from pistachio hulls using methanol, ethanol, and some selected aqueous solutions of these solvents *Yield is calculated based on 1g dried pistachio hulls.





more phenolics but also the degree of inhibition of linoleic peroxidation has changed rather significantly (85% inhibition for ethanolic extract versus 93% for (75:25) EtOH-H_2O extract). Although, no change in antioxidant activity is seen when ethanol solvent for Damghan hulls extraction, changed from absolute to the ratio of (50:50) EtOH-H_2O (both, at the 85 percent, inhibition).

Extraction in the form of four successive steps were conducted to examine the possible role of the extraction step on the yield of polyphenolics and to estimate the overall recovery and percentage of the total extractable phenolics in the pistachio hulls based on the experimental conditions described in the present study. The results of (75:25) EtOH-H_2O solution used for Kerman hulls, showed that first extract contained more than 80% of the total extractable phenolics while in the case of Damghan hulls, the first extract gave 70% of the total extractable phenolics and each of (50:50) EtOH-H_2O and (50:50) MeOH-H_2O, showed that first and second extract both combined, contained 80-85% of the total extractable phenolics (data not shown). As was is mentioned above the phenolics of Damghan hulls obtained through extraction with (75:75) EtOH-H_2O solution, had highest antioxidant activity, 93%. The results presented in Figure 1 are based on the amounts obtained through four series of the extractions. Some dilution of the extract of (75:75) ethanol-H_2O from Kerman pistachio hulls were also made (1/10,1/20,1/50,1/100 of the extract). The antioxidant activities of the diluted extracts changed and the trend was decreasing, reaching to about less than 50% (data not shown). By reducing amount of the extract therefore, quantity of the component present in the pistachio hulls and responsible for inhibition of the peroxidation (*i.e.*, gallic acid) was decreased. Relationship between antioxidant activity and maturity of peanut hulls have been discussed (6) and for expressing a strong antioxidative effect, a definite amount of total phenolics compounds should be present in the hull. Presence of a such relationship for pistachio hulls would be quite probable, and more work is needed to discuss such a relationship. In the study with grape seeds it was found that in addition to the amount of the phenolic compounds, heterogenecity of the test system makes the physical properties to become important factor in evaluating the activity. The different activities observed during the procedure may not necessarily be related to the same phenolics compositions (18,19). On the other hand, presence of some nonphenolics in a natural plant source, may express even prooxidant activities such as trace elements, these could affect the antioxidant activity of the phenolics compounds. The level of copper and iron in the grape bagasse was found to be significantly higher than those present in the grape extract(19). In the present study the levels of iron and copper in the pistachio hulls, were measured and significant decrease (around 90%) in the amounts of these two trace elements was observed when the ethanolic extracts of these two varieties were tested (data not shown).

Hplc chromatograms of extracts of (75:25) EtOH-H_2O of Kerman and Damghan pistachio hulls are given in Figure 3. Both varieties show almost identical chromatograms with a major peak at retention time of 6 minutes (Figure 3). The strong antioxidative activity of the extracts could be due to the major component at this retention time which correspondes to gallic acid. Amount of gallic acid present in the recovered phenolics from the extracts were calculated from the peak area and is shown to be 79 and 82% for Damghan and Kerman varieties, respectively (see Fig. 3). Gallic acid was found to be the major active phenolics compound in expressing antioxidative activity in grape seeds, wine, and cloves (19,20,21).

Conclusion

Pistachio (Iranian type) is one of the principal agriculture plants in Iran. Regarding the presence of phenolics in pistachio hulls very few studies have been reported in general particularly considering Iran pistachio hulls. In the present study, polyphenols in the hulls of two most important Iran pistachio varieties (Kerman and Damghan) were extracted using methanol and ethanol solvents and various dilutions of these solvents with water. Folin

(10ml) and 0.2 M phosphate buffer pH7 (10ml) and the total volume was adjusted to 25ml with distilled water, and the mixed solution in a conical flask was incubated at 40°C. At appropriate time intervals during incubation up to 10 days period the peroxide value was determined spectrophotometrically using thiocyanate as a coloring reagent and reading the absorbance at 507nm(9,11).

High performance liquid chromatography (HPLC)

Ethanolic extract of each one of two pistachio varieties was filtered by passing through a 0.45μm membrane filter. The conditions of Hplc (Waters Chromatography) analysis were as follows: column, Partisil 10ODS (250 mm length×4.6mm(i.d)); UV detector (constant monitoring at 280nm). The mobile phase consisted of a methanol: water (50:50v/v) and was applied over a 30 min period at a flow rate of 0.5ml/min. The injecting sample volume was 10μL. The run was performed at ambient temperature. Peaks have been identified by comparing their retention times and the spectra obtained, with those of commercial standards. Standards of phenolics used were gallic acid, catechol; 3,4 dihydroxycinnamic acid(caffeic acid); and phenol and analyzed under the above mentioned Hplc conditions.

Results and Discussion

The concentration of total phenolic compounds present in the pistachio hulls was estimated by the Folin-Ciocalteau reagent(11). In our preliminary works however, we used the Prussian blue method which is indicated in the literature as the recommended procedure because of less interference from nonphenolic compounds in comparison with that of the Folin method(14). There are some drawbacks on Prussian method however, in fact we saw these weak points, also: formation of a precipitate and increase in the colour density, both after short periods of incubation of the testing solution. For handling these problems, some modifications in that experimental procedure have been suggested(15). Prominence and recognition of the Folin method have made this measuring technique for phenolics, very reliable for the present study. The phenolics yields of the different solvent extracts from hulls of two varieties of pistachio are given in Figure 1. The results show that efficiency of ethanol solvent to extract phenolics from Damghan hulls is alsow as that of methanol (7.68 versus 23.4). While the ethanolic and methanolic extracts of Kerman hulls give comparable results, the amount of phenolics are similar (7.43 and 8.76). By introducing water to these organic solvents and making aqueous solutions of ethanol or methanol, the capacity of the solvent for phenolics extraction was changed. The order of this change in the capacity for Kerman hulls is as follows: (75:25)EtOH-H_2O>(50:50) EtOH-H_2O≈(75:25) MeOH-H_2O≈(50:50) MeOH-H_2O. While the amount of phenolics extracted from Damghan hulls has the following order: (50:50) EtOH-H_2O>(75:25) EtOH-H_2O>(50:50) MeOH-H_2O≈(75:25) MeOH-H_2O. The results of several other studies on the isolation of phenolics from various plant sources show however, methanol and aqueous solution of this solvent were the solvents of the choice (9,16,17). For example the methanolic extract of the peanut hulls is about twice as that of the ethanolic extract and with decreasing polarity of the solvent the phenolics extractability decreases significantly(9).

Antioxidant activities of the different solvent extracts from hulls of Kerman and Damghan varieties of pistachio are shown in Figure 2. As it can be seen the ethanolic extract of Kerman hulls had higher antioxidant power as compared to that of the methanolic extract: 98 versus 93 persent of inhibition of linoleic peroxidation. The percentage of the inhibition obtained for this ethanolic extract is the same as antioxidant activity expressed by the synthetic type of antioxidant, BHA. By reducing the ethanol content of the aqueous solution from ratio of 75:25 to 50:50, the amount of extracted phenolics for Kerman hulls changes from 19.8 to 14.65 with decreasing the degree of the inhibition of the lipid peroxidation (4% decrease).

Use of aqueous ethanol solution (75:25) for Damghan hulls, results not only in extracting

therefore(6,7). Large amounts of researches have been conducted over the past years to find new sources of natural polyphenolics antioxidants. Durum wheat bran, rice bran, peanut hulls, are just some examples of numerous natural substances that have been searched for having antioxidant extracts(8,9). Pistachio (Iranian type) is one of principal agriculture plant in the Middle East region- the total Iran pistachio production in 2001 has reached 300000 metric tons, in which Kerman's production is in the vicinity of 250000 tons(10). Estimation of the exportable amount of pistachio from Iran in year 2001 was about 100,000 tons in which the Kerman's variety of pistachio made a figure around 85% of the total(10).

The present study as a part of the multistage research project is concerned mainly with quantification detection of polyphenolics in pistachio hulls with partial determination of the efficacy of the antioxidative effects of methanol/ethanol extracts of the hulls from two most prominent varieties of Iran's pistachio, Kerman and Damghan.

Materials and Methods

Sample preparation

Two Iran's pistachio varieties were selected :Kerman and Damghan. Immediately after hand picked pistachio at the time of harvest in the late summer ,the hulls were mechanically removed from the hard shell taking care not to cause any disturbance in the position of the kernel. The hulls were dried at room temperature in the dark for about 8-10 days, to a moisture level of about 2.5%. The hulls were ground in a laboratory mill and after passing it through 16-mesh sieve and the materials retained on the next finer sieve were used in this study. Preparation of the hull samples for polyphenol extraction was done within five months after harvest time.

Extraction

Hull samples (10g) from each of the two pistachio varieties were extracted overnight at room temperature with 60ml of the solvent, followed by filteration through Whatman No.1 filterpaper, after evaporatating the filterate to dryness in vacuum oven (80°C), weight of the extract was obtained to determine the amount of the soluble constituents in the hulls of pistachio varieties. When it was needed, the residue remaining on the filterpaper during the extraction step was extrarcted again applying the same procedure and the filterate was treated exactly as mentioned above. In this way the phenolics were extracted four times.

Determination of total phenolic content

The total polyphenols present in the pistachio hulls was determined spectrophotometrically using Folin-Ciocalteau reagent(11). The procedure consisted of first diluting the extract (up to 0.5ml ethanol) with ethanol (20%v/v) and after taking suitable aliquot of the extract and transferring it to a 10ml volumetric flask, water was added (*ca.* 3-4ml) and 0.5ml Folin-Ciocalteau reagent was then added, the contents of the flask were mixed throughly. Exactly after 3 minntes, 1ml of saturated (ca. 35%) Na_2CO_3 solution was added .The content after mixing, was diluted to the volume with distilled water. The mixture was let to stand for 60 minutes at room temperature in the dark. The blue color was measured at 725nm. The blue color was measured at 725nm (M350 double beam uv-visible spectrophotometer Camspec). Caffeic acid was used as the standard for preparation of the calibration curve in the range of 20-100 μg/10ml assay solution.

Measuring antioxidant activity

Antioxidant activity of the solvent extract of pistachio hulls was determined by thiocyanate method according to the procedure described by Osawa and Namiki (12,13). A suitable amount of the extract was put into a solution of linoleic acid (0.13ml), 99.5% ethanol

Antioxidant Activity of Polyphenols in Pistachio Hulls

F. Vahabzadeh
Associate Professor

M. Mehranian
Lab. Instructor

E. Mofarrah
Lab. Instructor

Food Engineering and Biotechnology Group,
Department of Chemical Engineering, Amirkabir University of Technology

Abstract

There are great interests in searching for natural compound, preferably from plant sources, exhibiting antioxidant activity in order to decrease/eliminate use of the synthetic antioxidants. Hulls from two most prominent varieties of Iran's Pistachio have been examined for the presence of components showing antioxidant activity. The polyphenolics yields of the different solvent extracts (ethanol, methanol, and aqueous solutions of these two) were measured using the Folin method. The methanol extract of Damghan hulls had higher amount of total phenolics as compared to that of the ethanolic extract. Although, recovery of the total phenolics from ethanolic and methanolic extract of hulls of Kerman pistachio, showed to be comparable. The highest yield of phenolics from Damghan variety using aqueous solution of ethanol (50:50(H_2O)) was 28.8mg per g of the dried pistachio hulls. While the yield of the ethanolic (75:25(H_2O)) extract from Kerman's pistachio hulls was 19.8 mg per g dried pistachio hulls. The extraction was carried out in the form of four successive steps, and the extracts were pooled to obtain an estimation of the total extractable phenolics. Thiocyanate assay was used to assess the degree of the antioxidative activities of the extracts. The aqueous ethanolic (75:25 EtOH-H_2O) extract for both of the pistachio varieties showed to have highest degree of inhibition of linoleic peroxidation at the levels of 93 and 99% respectively, very comparable with that of the synthetic type of antioxidant BHA. Hplc analysis revealed presence of gallic acid in the above-mentioned extracts. Percentage (%) of gallic acid in the recovered phenolics from the extracts were 79 and 82 for Damghan and Kerman varieties, respectively.

Keywords

Pistachio hulls, Antioxidant activity, Polyphenolics, Gallic acid

To whom the correspondence should be addressed. Fax:0098-216405847; E-mail: far@aut.ac.ir

Introduction

A wide range of antioxidants, both synthetic and of natural sources have been developed and are currently used as effective means to retard oxidative type of reactions and protect food and cosmetic products. Actually only butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) as the main synthetic antioxidants and tocopherol as natural one are practically used in food industries, because of their high efficiency and low cost(1,2,3). In fact BHA and BHT are found to be potentially toxic and suspected of causing serious cellular damages and thereby developing chronic diseases such as liver malfunction and even cancer(4,5). These circumstances stimulated scientific societies to search for isolating new antioxidant(s) from natural sources. The plant materials as the source of wide range of polyphenolics are on the focus of these research works. It is shown through several studies that natural polyphenols have some therapeutic effects and following many different mechanisms such as chelating metal ions and scavenging singlet oxygen, hydroxyl radicals,... act against presence of these unfavorable compounds and related tissue injuries,