Hormozgan Medical Journal

doi 10.5812/hmj.106093

Hormozgan Med J. 2021; 25(4): 180-186

Research Article

Antioxidant Activity and Phenolic Compounds Profile of Pistachio Skins (*Pistacia vera* L., cultivars Kallehghuchi and Ohadi)

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Abstract

Background: Pistachio with scientific name *Pistacia vera* L., a native plant in Asia, is a member of Anacardiaceae family. Pistachio nuts and skins are known as a rich source of phenolic compounds with antioxidant, anti-inflammatory and antimicrobial properties.

Objectives: In current study industrial production of phenolic compounds with antioxidant activity was investigated because of high mass production of Pistachio skin in Iran.

Method: The extraction of two pistachio cultivars, namely Kallehghuchi and Ohadi were carried out by using two methods (maceration and ultrasonic extraction) and four solvents (acetone 70%, ethanol 50%, methanol 50% and water). Antioxidant properties of pistachio skins were determined by three methods (Folin-Ciocalteu colorimetric method, DPPH assay, TLC/DPPH analysis).

Results: The results showed that the highest content of total phenolic compounds were measured by ultrasonic and maceration methods related to Ohadi and Kallehghuchi in acetone solvents 17.4 ± 0.04 and 17.26 ± 0.1 mg/g DW respectively. The highest antioxidant activity in were measured by ultrasonic and maceration methods related to Ohadi in acetone and water solvents IC_{50} = 0.057 ± 0.001 and 0.059 ± 0.002 µg/mL respectively. By TLC/DPPH analysis, gallic acid, 4-hydroxy-3, 5-dimethoxy benzoic acid, tannic acid and some unidentified compounds were determined. By HPLC analysis, gallic acid, coumaric acid, cinnamic acid, 4-hydroxy-cinnamic acid and 4-hydroxy benzoic acid were determined.

Conclusion: In conclusion, this study clarifies some special biochemical characteristics of pistachio skins. Therefore, according to results of the study pistachio skins could be successfully used in food and pharmaceutical industries.

Keywords: *Pistacia vera* L., Antioxidant activity, Extraction, Phenol compounds

Received June 2, 2020, **Accepted**: December 17, 2021, **Published Online**: December 29, 2021

Background

Plants contain many different bioactive compounds such as phenolic acids (1,2). Pistachio with scientific name *Pistacia vera* L., is a native plant in Asia and one of the members of Anacardiaceae family (3). In Iran, up to 400 000 tones/year pistachio skin is produced and it may be used as a source of food for ruminants (4). However, due to the toxicity of tannin content and the possibility of interaction with biomolecules, the usage of these products by ruminants restricted (5,6).

Pistachio nuts and skins' phenolic compounds possess antioxidant, anti-inflammatory and antimicrobial activities (4). Antioxidants in plasma are unable to neutralize free radicals in the body without assistance, therefore providing antioxidants from food sources is necessary (7). There is so much evidence that the toxicity and the effects of malnutrition of dietary synthetic antioxidants added to food such as butylated hydroxyanisole (BHA), butylated hydroxy-toluene (BHT) and tert-butylhydroquinone confirms. In addition, the risk of liver damage and cancer in laboratory animals from use of synthetic antioxidants is possible, so that the need for less toxic and more effective natural antioxidants is an inevitable necessity (8,9).

Natural antioxidants increase the antioxidant capacity of plasma, thus, reduces the risk of cardiovascular disease and stroke, as well as the development of cancer which causes DNA damage, will prevent (10,11).

The purpose of this study, the use of pistachio skins for the extraction of biochemical compounds such as phenolic compounds, flavonoids and anthocyanins, and as well as natural antioxidants pistachio which can be a good alternative to synthetic antioxidants.

Materials and methods

Reagents

Quercetin, aluminium trichloride, acetic acid, Folin-Ciocalteu phenol reagent, sodium carbonate, hydrochloric

acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), TLC silica-gel plates, toluene, ethyl acetate, formic acid, gallic acid, tannic acid, 4 -hydroxy 3, 5 dimethoxy benzoic acid, 4-hydroxy benzoic acid, 4-chloro benzoic acid, cinnamic acid, 4-hydroxy cinnamic acid, coumaric acid, vanillic acid, acetone, ethanol and methanol were purchased from Merck company (Germany).

Sample Preparation

Pistachio skins, (*Pistacia vera* L., Cultivars *P. vera* cv. Kallehghuchi and *P. vera* cv. Ohadi), were harvested in the summer of 2014, from Pistachio garden in city of Kerman (Iran) and were detected at Alzahra University herbarium. Pistachio skins were dried in the shade and was crushed and stored in the dark at 4°C.

Ultrasound-Assisted Extraction Procedure

Ultrasonic enhancement mechanism included Shear force created by the implosion of cavitation bubbles upon the propagation of the acoustic waves in the kHz range. The collapse of bubbles can have physical, chemical and mechanical effects, which resulted in disruption of biological membranes to facilitate the release of extractable compounds.

In ultrasound-assisted extraction (UAE) method, 5 mL of solvents (acetone 70%, ethanol 50%, methanol 50% and water) were added into 100 mg sample, then, the mixture was homogenized for 5 minutes and ultrasonicated at 25°C for 20 minutes. Afterwards, it was centrifuged for 10 minutes at 3000 g, and then the supernatant was kept in refrigerator 4°C (4).

Extraction by Maceration

In maceration method, 100 mg of sample with 5 mL of solvents (acetone 70%, ethanol 50%, methanol 50% and water) was macerated for 2 hours at room temperature on a shaker. homogenates were centrifuged for 10 minutes at 3000 g then supernatant kept in refrigerator 4°C (12).

Evaluation of Antioxidant Properties

Folin-Ciocalteu Colorimetric Method

Total phenolic content was measured by Folin-Ciocalteu colorimetric method using gallic acid as standard (the antioxidant capacity, expressed as content of total phenols) (13). Two tenths milliliters of Pistachio skin extracts to be tested with 1.8 mL of deionized water and 0.2 mL of diluted Folin-Ciocalteu reagent (1:15 v/v), after 5 minutes 2 mL of 7% sodium carbonate solution were added, then after 90 minutes, absorbance was measured at 750 nm (UNICO spectrophotometer UV/Vis 2100).

Evaluation of Antioxidant Activity by DPPH Radical

DPPH radical is Free radicals stable. In this way, the ability to extract hydrogen or electron to the DPPH radical, change of the solution color is measured from purple to yellow. Different volumes of extracts (0.1-10 µL) with absolute methanol were brought to volume 2 mL. After adding 1 mL solution DPPH 0.004 % mixture was kept in the dark at room temperature for 30 minutes. Absolute methanol as blank and sample containing absolute methanol 2 mL and 1mL DPPH was considered as control. The absorption of the samples was read in 517 nm (UNICO spectrophotometer UV/Vis 2100) and the percentage of free radicals cleared (RSA%) was calculated (14,15).

Thin Layer Chromatography to Confirm the Antioxidant Effect

The scavenging activity of the extracts also determined by thin layer chromatography (TLC), on TLC silica gel plates 60HF254 (16). Seven microliters of pure standard compounds (1 mg/mL) and 12 microliters of extracts of pistachio skins were put on the plates, ethyl acetate, toluene, formic acid, acetic acid and water (80-20-11-11-19 v/v) were used as mobile phase. Then the plates were dried and 0.4% methanolic DPPH solution sprayed on it. Plates after spraying were revealed as purple background with yellow bands due radical scavenging activity. Standards were including gallic acid, tannic acid, 4-hydroxy 3, 5 dimethoxy benzoic acid, cinnamic acid, 4-hydroxy cinnamic acid, coumaric acid, vanillic acid. R_f values were calculated for each spot.

R_f = distance traveled by the compound/distance traveled by the solvent front

Content of Total Flavonoids

Total flavonoid compounds were measured by aluminum chloride colorimetric method using quercetin as standard (17). Two tenths milliliters of Pistachio skin extracts were added to 0.2 mL of aluminum chloride, 0.1 mL acetic acid 33% and they were well mixed. Finally, the reaction mixture with ethanol 90% was brought to 5 mL volume, samples were kept for 30 minutes at room temperature then absorbance was measured at 414 nm (UNICO spectrophotometer UV/Vis 2100).

Content of Total Anthocyanins

One-tenth grams fresh plant tissue in a porcelain mortar with 10 mL of acidic methanol (pure methanol and pure hydrochloric acid volume ratio of 1: 99 v/v) was mixed completely. Homogenates were centrifuged for 10 minutes at 4000 g and the supernatant absorbance was measured at 550 nm (UNICO spectrophotometer UV/Vis 2100). The concentration of anthocyanins was calculated using the formula A = εbc that the extinction coefficient (ε) was considered 33000 M^{-1} cm^{-1}, the results were expressed as µmol for g of fresh material (µmol/g FW) (A: absorption, b: width of the cuvette, c: concentration) (18).

HPLC Analysis of Phenolic Compounds

Pistachio skins of Kallehghuchi and Ohadi were extracted by maceration and ultrasonic methods with aqueous acetone solvent (70%, v/v). After centrifugation, acetone was removed in vacuum. The remaining sample was added ethyl acetate in three stages. Upper phase, which was then ethyl acetate was lifted and dried. Then 10 mg of the residue was dissolved in 2 mL of acetonitrile and used for HPLC. Thirty microliters of sample volume was introduced onto the column and eluted under gradient conditions performed with re distilled water: acetonitrile: acetic acid in ratio 67:32:1 with the solvent flow rate was 1 mL/min, column temperature was set at 25°C and the chromatogram was recorded at 275 nm (19).

Statistical Analysis

Results were analyzed using the software SPSS version 20 and $P<0.05$. ANOVA for one factor designs, two-way analysis of variance for two or more factor designs and data were grouped with Duncan's multiple range test.

Results and Discussion

It is widely accepted that different methods validated benchmark methods are needed to describe the properties of antioxidants agents. Since the mechanism and characteristics of reactions involved in antioxidant systems are very diverse, no single method is not able to reflect all the antioxidant capacity in a complex biological system (20). In current study, three different assays include the DPPH assay, Folin-Ciocalteu colorimetric method and TLC/DPPH analysis were used in measuring the antioxidant properties. The present study shows which of the pistachio cultivars, solvents and extraction methods were suitable for extraction of phenolic compounds.

Evaluation of the Content of Phenolic, Anthocyanin and Flavonoids Compounds

The results showed that the highest and the lowest total phenolic content in ultrasonic method was measured 17.4 ± 0.04 and 16.26 ± 0.04 mg/g DW respectively related to Ohadi in acetone solvent and Kallehghuchi in methanol solvent that significant difference in the $P<0.05$ indicated (Figure 1). Whereas, highest and the lowest total phenolic content in maceration method was measured 17.26 ± 0.1 and 16.35 ± 0.15 mg/g DW respectively related to Kallehghuchi in acetone solvent and Ohadi in water solvent that significant difference in the $P<0.05$ indicated (Figure 1). These results in Kallehghuchi and Ohadi were lower than those reported by Nadernejad et al (12).

The results showed that the highest and the lowest total flavonoids content in ultrasonic method was measured 4.2 ± 0.6 and 0.7 ± 0.6 mg/g DW respectively related to Kallehghuchi in ethanol and water solvents that significant difference in the P < 0.05 indicated (Figure 2). Whereas,

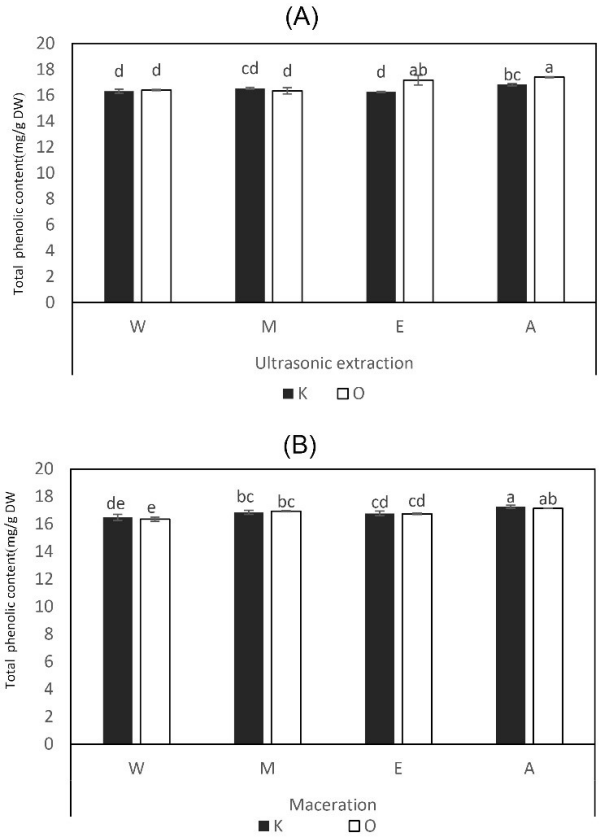

Figure 1. Total Phenolic Content in Different Extracts (W: Water, M: Methanol, E: Ethanol, A: Acetone). Values with different letters in the same column represent the difference significant. (P < 0.05). K: Kallehghuchi, O: Ohadi (A) ultrasonic extraction and (B) maceration method.

highest and the lowest total content phenol in maceration method was measured 4.63 ± 1.8 and 1.33 ± 0.22 mg/g DW respectively related to Ohadi in ethanol solvent and Kallehghuchi in water solvent that significant difference in the $P<0.05$ indicated (Figure 2). These results in Kallehghuchi and Ohadi are lower than those reported by Nadernejad et al (12).

Conventional extraction methods, such as maceration due to the large volume of organic solvents used and long extraction time has low efficiency (22). In this study, UAE and maceration methods have no significant difference in the extraction of bioactive compounds of cultivars Kallehghuchi and Ohadi. Therefore UAE Method is better for extracting antioxidant phenolic compounds. However the type of cultivar and solvent showed a significant difference in the extraction of antioxidant compounds that Ohadi cultivar and 70% aqueous acetone are more suitable for extracting antioxidant phenolic compounds.

Many factors such as extraction methods, solvents, extraction time, temperature, polarity of samples may affect the yield of chemical extraction (23). Extraction of phenolic compounds of pistachio skins decreased with increasing solvent polarity. According to Markom

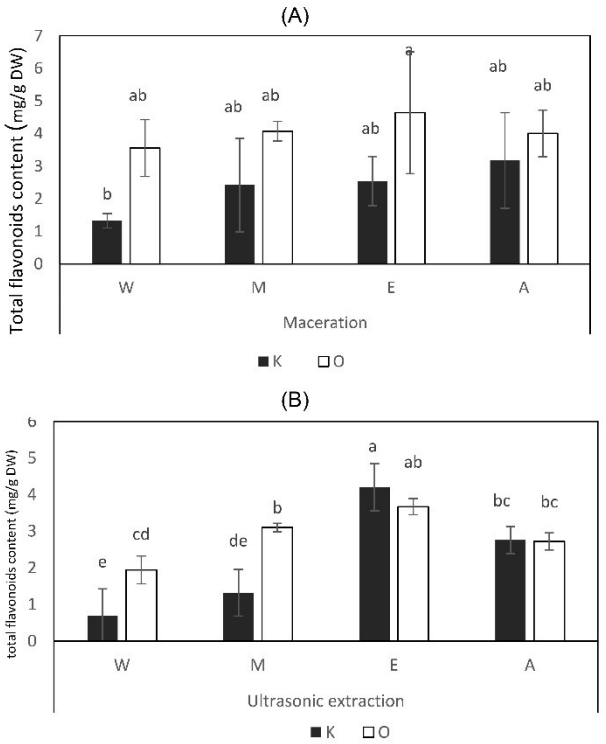

Figure 2. Total Flavonoids Content in Different Extracts (W: Water, M: Methanol, E: Ethanol, A: Acetone).Values with different letters in the same column represent the difference significant. ($P<0.05$). K: Kallehghuchi, O: Ohadi (A) ultrasonic extraction and (B) maceration method.

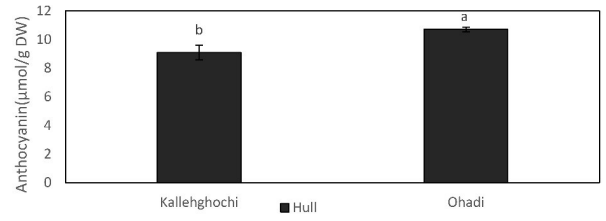

Figure 3. Anthocyanin Content in the Hull Two Cultivar Pistachio Kallehghuchi and Ohadi.

et al, Snyder's polarity indexes for 70% aqueous acetone, 50% aqueous ethanol, 50% aqueous methanol and water were 6.5, 7.1, 7.8 and 9 (24). Thus, phenolic compounds pistachio are often polar with some non-polar groups, therefore, the highest phenolic compounds were observed in 70% aqueous acetone. These results were similar to the results of the reported by Mokhtarpour et al (4).

Low polarity solvents such as benzene and chloroform are used for the flavonoids aglycones extraction whereas, high polarity solvents such as acetone and ethanol can be used for the extraction of flavonoid glycosides (25). Pistachio skins are often glycoside flavonoids that showed a maximum extraction in aqueous ethanol 50%. Turkmen et al showed that ethanol has a higher efficiency than other solvents, such as acetone, in extracting flavonoids from tea, which were consistent with our results (26).

The anthocyanins were measured in the skin of Kallehghuchi and Ohadi respectively, 9.09 ± 0.5 and 10.71 ± 0.15 µmol/g DW that significant difference in the $P<0.05$ indicated (Figure 3). Concentration anthocyanin in Kallehghuchi and Ohadi is lower than those reported by Nadernejad et al (12).

Evaluation of Antioxidant Activity by DPPH

The results showed that the highest and the lowest antioxidant activity in ultrasonic method was measured (IC_{50} $0.057 = \pm 0.001$ and 0.082 ± 0.002 µg/mL) respectively related to Ohadi in acetone and ethanol solvents that significant difference in the $P<0.05$ indicated. Whereas, highest and the lowest antioxidant activity in maceration method was measured (IC500.059 = ± 0.002 and 0.099 ± 0.003 µg/mL) respectively related to Ohadi and Kallehghuchi in water solvent that significant difference in the $P<0.05$ indicated. Also, ascorbic acid and gallic acid were used as standard that their antioxidant activity was measured respectively (IC_{50} $1.32 = \pm 0.09$ and 0.88 ± 0.014 µg/mL) (Figure 4). These results in Kallehghuchi and Ohadi were stronger than those reported by Tomaino et al and Ordoñez et al (16,21)

Thin Layer Chromatography to Determine Phenolic Antioxidant Compounds

The scavenging activity of the extracts was performed also TLC/DPPH method. Each component is identified by comparing their R_f value with recognized standards, radical scavenging activity showed that with the disappearance of purple background and convert it to the yellow spots and compounds of gallic acid, 4-hydroxy-3, 5-dimethoxy benzoic acid, tannic acid and other unidentified substances were detected (Figure 5). Tomaino et al (16) with TLC/DPPH analysis identified 5 phenolic compounds in *Pistacia vera* L., variety Bronte skins (gallic acid, catechin, cyanidin-3-O-galactoside, eriodictyol-7-O-glucoside and epicatechin).

High Pressure Chromatography to Determine Phenolic Compounds

Chromatogram peaks were detected through their

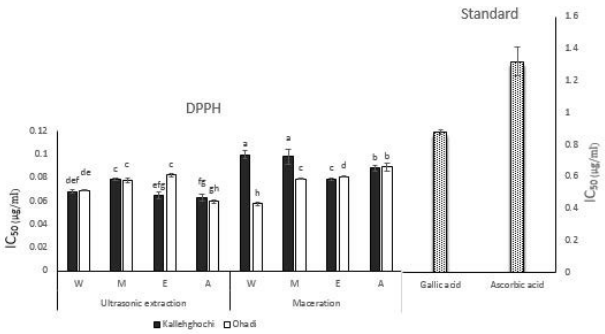

Figure 4 . DPPH Radical Scavenging Activity in Different Extracts (W: Water, M: Methanol, E: Ethanol, A: Acetone). Values with different letters in the same column represent the difference significant ($P<0.05$).

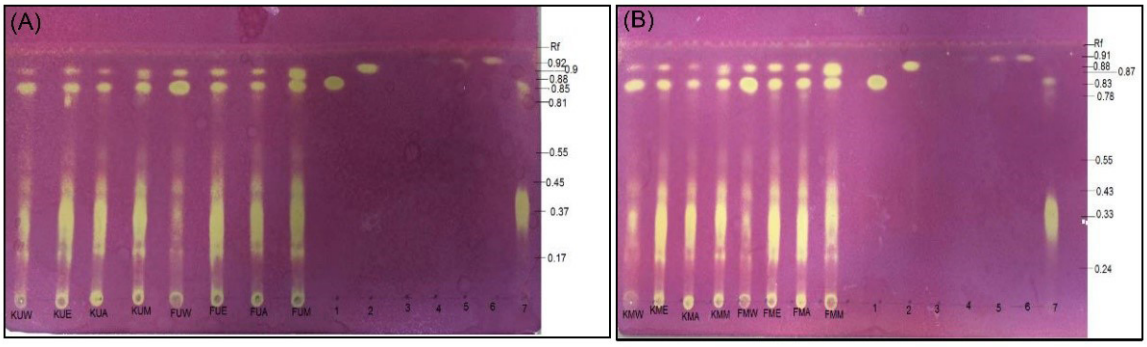

Figure 5. TLC Plate Stained With 0.4% Methanolic DPPH Solution. Extracts derived from two cultivars, K: Kallehghuchi and F: Ohadi in solvents, W: Water, M: Methanol, E: Ethanol, A: acetone with ultrasonic extraction: U(a), maceration method: M(b). The spots marked with 1-7 (1: gallic acid, 2: 4-hydroxy 3, 5 dimethoxy benzoic acid, 3: cinnamic acid, 4: coumaric acid, 5: vanillic acid, 6: 4-hydroxy cinnamic acid, 7: tannic acid) corresponds to the compounds with DPPH scavenging activity.

retention time, wavelength and UV properties with pure standards, 5 phenolic compounds in pistachio skins (gallic acid, coumaric acid, cinnamic acid, 4-hydroxy-cinnamic acid and 4-hydroxy benzoic acid) were identified (Table 1). Gallic acid and 4-hydroxy cinnamic acid was detected in all extracts, but cinnamic acid was detected in extracts derived from Kallehghuchi and 4-hydroxy benzoic acid in the extracts derived from Ohadi (Figure 6). The quantity amount of identified phenolic compounds (expressed as mg/g DW) is reported in Table 1. In fact in Kallehghuchi pistachio skins, gallic acid (0.11 mg/ g DW) is the most abundant compound, whereas in Ohadi pistachio skins, 4-hydroxy benzoic acid (0.0723 mg/ g DW) is the most abundant compound.

Tomaino et al (16) with HPLC analysis identified 13 phenolic compounds in *Pistacia vera* L., variety Bronte skins (gallic acid, catechin, epicatechin, eriodictyol-7-Oglucoside, naringenin-7-O-neohesperidoside, quercetin-3-O-rutinoside, eriodictyol, quercetin, naringenin, luteolin, kaempferol, cyanidin-3-O-galactoside and cyanidin-3-O-glucoside).

Conclusion

Current study clarify biochemical characteristics of some Kallehghuchi and Ohadi pistachio skins compound. The results of this study showed that pistachio skin can be used a cheap and available source of bioactive compounds. Given the importance of antioxidant compounds and their ability to inhibit the effects of free radicals, the antioxidant Pistachio can be a suitable option is to investigate the efficacy of these drugs. Antioxidants from fruits and vegetables play an important role in the prevention of cancer, inflammatory and cardiovascular disease. So, introduction of pistachios in daily diet may be in the protection of human health. On the other hand, pistachio can be used a significant product in industrial processes such as in the food, cosmetics, sanitary and pharmaceutical industry. It is recommended that other common pistachio cultivars, solvents and extraction methods be examined for antioxidant properties. Also, the phenolic compounds of pistachio skin be examined for anti-cancer and anti-inflammatory potential in vivo and in vitro.

Authors' Contribution

Study concept and design: PH and SA. Analysis and interpretation of data: PH and SA. Drafting of the manuscript: PH and SA. Critical revision of the manuscript for important intellectual content: PH and SA. Statistical analysis: SA.

Conflict of Interests

Table 1. Phenolic Compounds Identified in Pistachio Skins by HPLC

Peak no.	Compound	Retention Time (min)	The Area Under the Peak	Quantity Amount of Compounds (mg/g DW)
1	vanillic acid	0.5	0.750	—
2	4-chloro benzoic acid	0.7	2.985	—
3	4-hydroxy benzoic acid [3,4]	1.3	2.053	3 = 0.0704, 4 = 0.0723
4	Gallic acid[1,2,3,4]	2	7.823	1 = 0.063, 2 = 0.11, 3 = 0.043, 4 = 0.046
5	Cinnamic acid [1,2]	2.5	5.680	1 = 0.02, 2 = 0.017
6	4-Hydroxy cinnamic acid[1,2,3,4]	3.5	1.785	1 = 0.042, 2 = 0.022, 3 = 0.024, 4 = 0.016
7	3, 5 dimethoxy benzoic acid	5.5	3.787	—
8	Coumaric acid [2,3,4]	6.8	54.504	2 = 0.022, 3 = 0.023, 4 = 0.023

Note. 1: KM, 2: KU, 3: OM, 4: OU, K: Kallehghuchi, O: Ohadi, M: maceration method, U: ultrasonic extraction.

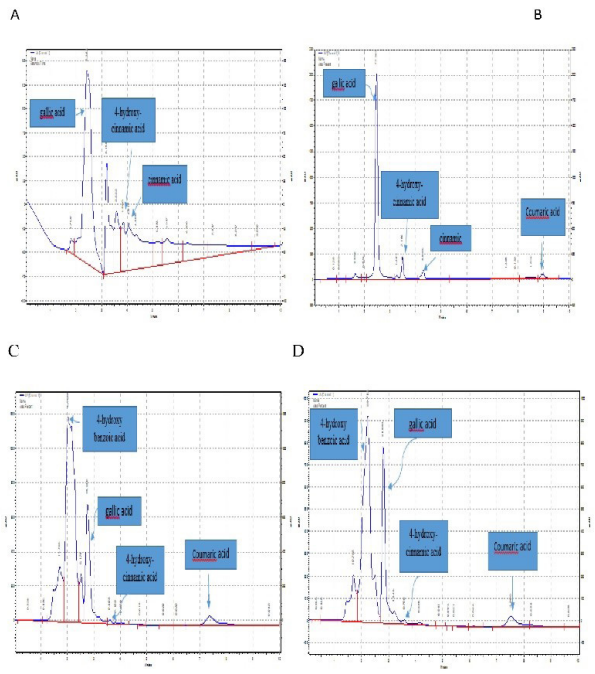

Figure 6 . HPLC chromatograms obtained from (A) Kallehghuchi skin in acetone solvent with maceration method (B) Kallehghuchi skin in acetone solvent with ultrasonic extraction (C) Ohadi skin in acetone solvent with maceration method (D) Ohadi skin in acetone solvent with ultrasonic extraction.

The authors have no conflict of interests.

Ethical Approval

This study does not need any ethical considerations.

Funding/Support

This work was funded by research administration University of Alzahra and was carried out at the Biochemistry Laboratory, Department of Biotechnology, Faculty of Biological Sciences, University of Alzahra, Tehran, Iran.

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