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Evaluation the chemical composition, phenol, flavonoid and antioxidant activity of walnut skin extract (*Juglans regia L.*)

RafieiDehkordi, Z. [1], Sedaghat, N. [2*], Sabahi, S.[3]

1. MS.c in Food Science and Technology , Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.
2. Professor, Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.
3. Assistant Professor, Department of Nutrition, School of Allied Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

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*Corresponding Author E-Mail:
sedaghat@um.ac.ir

ABSTRACT

Today, consumers are more inclined to use healthy and natural additives. Phenolic compounds obtained from natural products are a good option to minimize lipid oxidation. Walnut is one of the most important nuts that is widely cultivated all over the world. Due to the fact that the green skin of the walnut contains about 64% of the wet weight of the walnut fruit, an amount equal to 240 thousand tons of green skin of the walnut is produced from this fruit every year. The walnuts used in this study were purchased in 1400 from the gardens of Saman city located in Chaharmahal and Bakhtiari province. After powdering the dried green peel, extracting with methanol solvent was done by Soxhlet method. In order to identify chemical compounds and functional groups, GC-MS and FTIR tests were performed. Also, the amount of phenol and flavonoids in the extract of walnut skin was measured. Finally, the antioxidant activity of green walnut skin extract was investigated by measuring the reduction of radical capacity with the help of 2-2-diphenyl-1-picrylhydrazyl (DPPH). A total of 89 compounds were identified, representing 99.9% of the total compounds in the extract. The most important identified compounds that had the highest area under the curve included polyphenols, organic compounds and phytosterols. FTIR results indicate the presence of hydroxyl, alkane and aromatic functional groups of lignin. In the present study, the amount of phenolic compounds in walnut peel extract was 96.07±0.22 (mg of gallic acid per gram of dry weight of the sample). Also, the flavonoid compounds of green walnut skin extract were 349 µg/g of quercetin. EC50 values obtained in this study were equal to 0.15. The results showed that the methanolic extract of green walnut skin can be introduced as a substitute for synthetic antioxidants.

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1. Introduction

Phenolic compounds are secondary metabolites that exist in different parts of all plant species. They belong to a large and heterogeneous group of biologically active molecules, and their production depends on various enzymes involved in metabolic pathways. Phenolic compounds play a role in the physiological mechanisms of fruit and tree growth and affect different characteristics of the periods before and after fruit harvest. Also, phenolic compounds play an important role in the defense mechanisms of plants [1 and 2]. In addition, phytochemical compounds such as phenolics are beneficial for human well-being. They can reduce the risk of cardiovascular and degenerative diseases by preventing oxidative stress and oxidation of biological macromolecules. Phenolic compounds can eliminate free radicals and, in addition to the described anticancer activities, they also have metal chelating properties. In recent years, much attention has been paid to describe the polyphenol content and evaluate the antioxidant activity of various plant materials, especially nuts, because their regular consumption is associated with a reduction in the risk of some diseases such as cancer and cardiovascular diseases (CVDs) [3].

sex*Juglans*Or more specifically, the Juglandaceae family includes several species and is widely distributed throughout the world. Iranian, English, normal or walnut*Juglans regia L.* , is its well-known member, which includes significant forms of deciduous trees that are mainly identified in temperate regions and are commercially cultivated in Asia, Western South America, the United States, the States, and Central and Southern Europe [4]. Walnut is a large tree species that is traditionally cultivated for its valuable wood and fruit, with a height of 25 to 35 meters and a trunk up to 2 meters in diameter. Walnut is a valuable product and green walnut skin, shell, kernel, root and leaf are widely used in pharmaceutical and cosmetic industries. In this regard, it can be used from all parts of the walnut tree as an excellent source of various compounds expressing antioxidant and antimicrobial potential, as well as pain reliever, antihistamine, antiwound, antiasthma, antidiabetic, immune modulator, antifertility, liver protector, Central nervous system stimulant, pain reliever. Anti-inflammatory, wound healing, lipolytic, larvicidal, insecticidal and many other properties that have a positive effect on human health [5]. Walnut is classified as a strategic species for human nutrition because the Food and Agriculture Organization has placed it in the group of priority plants. It has been reported that the health benefits of walnuts are generally related to their chemical properties. According to previous studies, walnuts have one of the highest antioxidant content among all studied seeds and nuts. Walnuts are a good source of tocopherols and essential fatty acids, including linoleic, the primary fatty acid, followed by linolenic, palmitic, oleic, and stearic acids. . It is known that the high concentration of polyunsaturated fatty acids reduces the risk of cardiovascular diseases by reducing the concentration of low-density lipoprotein (LDL) and increasing the level of high-density lipoprotein (HDL). It has been reported that the concentration of oil, tocopherols and fatty acids It can fluctuate significantly among different types of walnuts and environmental conditions. In addition, it has been shown that walnut has a high concentration of α-tocopherol, a compound of the vitamin E family, which shows antioxidant properties, especially in inhibiting lipid oxidation processes [6]. The green covering of the walnut fruit that surrounds the walnut is called husk and it is an agricultural waste that is widely used in folk medicine to treat skin diseases and relieve pain. Currently, in the country, green skin is mostly used for burning. However, in recent years, mainly due to its antioxidant properties, it has received considerable attention in modern pharmacology. The green shell of the walnut is separated during the harvesting and processing of the walnut fruit. Antioxidant and antimicrobial properties have been valued. Effective use of the shell can be a critical issue because its use as a rich source of phytochemicals highlights the importance of walnut production, as well as a by-product that is produced in large quantities. It can have many uses. The number of 13 phenolic compounds including: hydroxycinnamic acids (chlorogenic acid, caffeic acid, ferulic acid and sinapic acid), hydroxybenzoic acids (gallic acid, lactic acid, protocatechuic acid, syringic acid and vanillic acid), flavonoids (catechin, epicatechin, myrsten) and jaglone 5-hydroxy 1 and 4 naphthoquinone)

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have been identified in walnut. Compounds such as naphthoketone, flavonoids and jaglonase are important antioxidant compounds of walnut leaves. Jaglone is one of the most important phenolic compounds found in walnuts and has strong antioxidant activity. Also, walnut skin is a cost-effective source for the preparation of jaglon, which can be a precursor for the preparation of vitamin K. In order to use these phenolic compounds from the green skin of walnut, it is necessary to extract these compounds. Different methods are used to extract phenolic compounds. Among these methods, soaking, Soxhlet, ultrasound, and microwave can be mentioned [7-9]. is.

2- Materials and methods

2-1-Preparation of green walnut skin extract

2-1-1-Preparation of green walnut skin powder

The walnuts needed for this research were randomly collected and purchased from the orchards of Saman city located in Chaharmahal and Bakhtiari province in 1400. Purchased walnuts must be completely healthy and without any damage to the green skin. It should be noted that no chemical pesticides were used during the growing stages of the trees. After purchase, the samples were washed and rinsed and then peeled, and the separated green skins were completely dried in an environment away from direct light and at room temperature under a fan. After the green skins were completely dried, they were ground by an electric mill (Moulinex, France) and then sieved with a 20 mesh sieve (to make the size of the particles uniform) and then the powder obtained from the dried green skin The walnuts were stored in a brown glass container with a lid (to prevent light and moisture) and at the temperature of the refrigerator [10].

It should be noted that the time of harvest has an effect on the amount of phenolic compounds and the highest amount of these compounds was at the first time of harvest (Tir). So that at this time the walnut fruit is not fully ripe and the wooden skin is getting hard. The second harvest (August) compared to the third harvest (September) showed higher phenolic compounds in green walnut skin, but there was no significant difference between the phenolic compounds of these two harvests.

2-1-2-Preparation of walnut green skin extract by Soxhlet method

Methanol solvent was used to prepare the extract, because the results of previous researches have shown that methanol is more effective in extracting phenolic compounds compared to other solvents such as water, ethanol, acetonitrile, hexane and ethyl acetate. Extraction was done by Soxhlet method and according to the method of Liu et al. (2008). In this method, 30 grams of the sample was placed in the cartridge and connected to the Soxhlet device Foss model 2050, and after adding the appropriate amount (about 400 ml) of solvent (60% methanol) to the sample, the extraction was carried out for 48 hours. . The solvent was then completely evaporated in a Heidolph model 4001 vacuum rotary evaporator. The obtained extract was kept in a glass container of colored coffees and kept in the refrigerator (about 4 degrees Celsius) until the end of the experiments. [11]

2-2-Identification of the components of green walnut skin extract

2-2-1-Analysis of walnut extract compounds

The sample of green walnut skin extract using an Agilent model 7890 GC-MS machine available in Jundishapur University of Medical Sciences, Ahvaz, with a system equipped with a HP-5MS capillary column (30 meters long, 0.25 mm diameter, and 25/25 inner layer thickness) 0 micron) was analyzed. Helium carrier gas was used with a speed of 1.5 milliliters per minute and an ionization energy of 70 electrons. The temperature program of the column was set from 40 to 246 degrees Celsius at a speed of 2.5 degrees per minute. Extract compounds were identified as a result of comparing their mass spectrum with a spectrum and comparing their inhibition coefficients with reference values. Inhibition coefficients (Quatz index) were calculated using the inhibition times of normal alkanes that were injected with the same device and under the same conditions. The relative values of the components were calculated from the total surface of the peaks by the device software [12].

2-2-2-FTIR test

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To identify functional groups of possible biomolecules of green walnut skin extract and its molecular characteristicsInfrared spectrometer (FT-IR) is used. The infrared spectrum (IR) of green walnut peel extract was measured and recorded in the CM range of 400-4000 using a Bruker FTIR device, model Vertex70, available at the Faculty of Pharmacy, Jundi Shapur University of Medical Sciences, Ahvaz. It should be noted that the samples were placed in the dissector until the analysis [13].

2-2-3- Determination of total phenol content

The amount of phenol in walnut green skin extract was measured by the method of Behbahani et al. (2018). According to this method, 1 ml of the diluted extract of green walnut skin was mixed with 1 ml of Folin Ciocaltio reagent. After a period of 3 minutes, one milliliter of saturated sodium carbonate solution was added to the mixture of extract and reagent, and this mixture was made up to 10 milliliters with distilled water. The reaction was carried out for 90 minutes in a dark environment. After the completion of the reaction, the absorbance at 725 nm was read by a spectrophotometer. Gallic acid was used as a standard to draw the calibration curve and the amount of phenolic compounds of the samples was expressed as milligrams of gallic acid (GAE) per gram of dry weight [14].

Equation 1

$$x = \frac{y + 0.022}{0.0041}$$

Y = absorbance read, X = amount of phenol

2-2-4- Determining the amount of flavonoids in walnut peel extract

The amount of flavonoids in the extract of the green skin of walnut was measured according to the method of Chango et al. (2002). First, 0.1 milliliter of 10% methanolic aluminum chloride was mixed with 0.1 milliliter of one molar potassium acetate, and then 2.8 milliliters of distilled water was added to it. In the next step, 0.5 ml of the extract solution mixed with 1.5 ml of methanol was added to the mixture containing aluminum chloride, potassium acetate and distilled water. The resulting final mixture for each extract (with a volume of 5 ml) was placed at room temperature for 30 minutes. Then, the absorbance of the reaction mixture was measured at 415 nm wavelength by BEL brand Uv-M51 spectrophotometer. Quercetin was used as a standard to draw the calibration curve. The obtained result was obtained based on micrograms per gram of quercetin [15].

Equation 2

$$Y=ax+b$$

Y= absorbance read from the sample

X= I made a mistake

2-2-5-the rate of DPPH free radical trapping

The antioxidant activity of walnut green skin extracts was investigated by measuring the reduction of radical capacity with the help of 2-diphenyl-1-picrylhydrazyl (DPPH). DPPH is a purple compound that easily becomes a radical due to the presence of phenyl groups in its structure and is a source of free radicals. The ability to donate electrons or hydrogen atoms by different extracts and compounds was investigated in this test by the amount of decolorization or reduction of the optical absorption of DPPH purple solution in methanol. Different concentrations (0.1-1 mg/ml) of green walnut peel extract and synthetic antioxidant TBHQ were prepared in methanol solvent. 0.3 milliliters of the prepared solutions were mixed with 2.7 milliliters of methanol solution containing DPPH reagent (concentration 0.1 mM). The mixture was stirred and placed in the dark for 60 minutes. Then, the absorbance was measured at 517 nm using a BEL spectrophotometer, model Uv-M51. The radical scavenging activity was calculated by the following equation [16].

Equation 3

$$\text{به دام اندازی رادیکال آزاد (٪)} = [(A_C-A_S)/A_C] \times 100$$

In this regard:

AC: control absorption (which contains 1 ml of methanol in 1 ml of DPPH solution).

AS: sample absorption (which contains different volumes of green walnut skin extract (antioxidant), methanol and DPPH solution).

2-4-Statistical analysis

Data analysis was done by one-way analysis of variance using spss version 22 software. To compare the averages, Duncan's multiple range

test method was used at a confidence level of 95% [17].

3. Results and Discussion

3-1-Identification of the components of green walnut skin extract

3-1-1- GC-MS test results of walnut green skin extract

Investigating the profile of volatile compounds with gas chromatography coupled to the mass GC-MS Agilent model 7890 available at Jundishapur University of Medical Sciences, Ahvaz with a system equipped with a capillary column HP-5MS (30 meters long, 0.25 mm diameter and inner layer thickness 25 0.0 micron) was done and the compounds were identified and the amount of each individual compound was expressed as a percentage. Analysis between the mass spectra of materials using the area under the peak curve by comparing it with the data available in the NIST database. 62 lib. and FFNSC 2 were performed.

The compounds in walnut shell extract are listed in Table 1. A total of 89 compounds were identified, representing 99.9% of the total compounds in the extract. The abundance of the constituent compounds is shown on the vertical axis of the chromatogram and the separation time on the horizontal axis.

In the present study, the comparison of the results of the chemical composition of green walnut skin extract with previous studies in different parts of the world has been shown. Although the main components of the extract are somewhat similar to other studies, they have a series of differences, especially in the percentage and type of chemical compounds. The difference in the amount of the main composition of walnut can be attributed to reasons such as ecological differences such as latitude and longitude, altitude, temperature, humidity, climate and soil, metabolic pathways and biosynthesis of effective substances in these plants, which result in various secondary metabolites under different environmental conditions. It is biosynthesized. Various researches have confirmed this [18].

The most important identified compounds that had the highest area under the curve included polyphenols, organic compounds and phytosterols. Most of the volatile compounds are usually present in the green skin of the walnut. Many of the identified phytochemicals have been reported to have interesting biological activities. Dihydroxynaphthalene, for example, is a bioactive metabolite with phytotoxic activity commonly found in plants and fungi. Stigmasterol is a plant sterol in the extract that has strong antifungal, antibacterial and anti-inflammatory activity. It is also considered as a medicinal plant for treating wounds, bronchitis, diabetes and heart diseases [19].

Compounds such as Hexenal and Hexaborane in walnut extract have been reported in various studies. Kosmolska et al. (2010) reported that six compounds (ferulic acid, vanillic acid, coumaric acid, syrinic acid, myristicin and juglone) were identified using reverse phase liquid chromatography [20].

The most effective compounds known in this research are polyphenolic compounds, which according to research has been determined that phenolic compounds along with organic acids and hexamethyls have high antioxidant and antimicrobial potential due to their chemical structure. As mentioned, some differences in the content and type of polyphenolic compounds may be related to environmental factors and variation in the sample variety. From a pharmacological point of view, antioxidant, anti-inflammatory and immunogenic properties are dependent on polyphenolic compounds. In addition, unsaturated fatty acids such as hexadecanoic acid and octadecanoic acid are able to influence the function of platelets and the cardiovascular system. Other components such as tocopherol as methylated phenol are capable of modulating the oxidative status [21].

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Table 1 Compounds in skin of walnut extract

Identification of components of green walnut skin extract			
Library/ID	Peak number	Retention Time	Area
Butanal, 3-methyl-	1	3.6018	1.4955
4-Hexenal	2	5.7418	3.1450
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	3	9.0663	2.5859
4,5-Diamino-2-hydroxypyrimidine	4	12.0760	5.2923
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	5	13.9071	5.8259
2-Furancarboxaldehyde, 5-(hydroxymethyl)-	6	16.3675	10.4701
2,3-.MU.-TRIMETHYLSILYL-CC'-DIMETHYL-4,5-DICARBA-NIDO- HEXABORANE(8)	7	25.3796	14.2348
Phenol, 2-ethoxy-5-(1-propenyl)-	8	27.0905	7.8954
Benzoic acid, 4-hydroxy-3,5-dimethoxy- $$ Syringic acid $$ 3,5-Dimethoxy-4-hydro	9	30.4893	6.9227
1,7-Dihydroxynaphthalene $$ 1,7-Naphthalenediol $$ C.I. 76635 $$ Naphthalene-1,7		30.6381	7.0173
n-Hexadecanoic acid	10	33.2874	1.7561
9,12-Octadecadienoic acid (Z,Z)-	11	36.4688	1.9727
Stigmasterol, 22,23-dihydro-	12	53.6461	4.5169

3-1-2- Interpretation of infrared spectrum with Fourier transform FT-IR extract of green walnut skin

In order to investigate the absorption characteristics and functional groups present in green walnut peel powder, Fourier transform infrared spectrum (FT-IR) and KBr tablet method were used. The information obtained from infrared spectroscopy with Fourier transform is based on the vibrational frequency in terms of time, and the resulting spectrum indicates the percentage of absorption or the percentage of transmission in terms of wave number. Finally, the examination and analysis of this spectrum shows the presence or absence of functional groups and links between elements [22].

In Figure 1, the infrared spectrum with Fourier transform of green walnut shell powder can be seen. this

The spectrum shows a number of absorption bands, which indicates the complex nature of the compounds present in green walnut skin. In the spectrum of walnut green bark powder, broad absorption band in cm^{-1} 3402 indicates the presence of hydroxyl group (O-H) bond and can prove the functional group of alcohol or phenol. Absorption peak at about cm^{-1} 2927, 1725 and 1049 are assigned to -CH (alkane group), C=O and C-O stretching, respectively. Absorption observed in cm^{-1} 1603 is related to the C=C stretch, which may be attributed to the aromatic group of lignin [23 and 24].

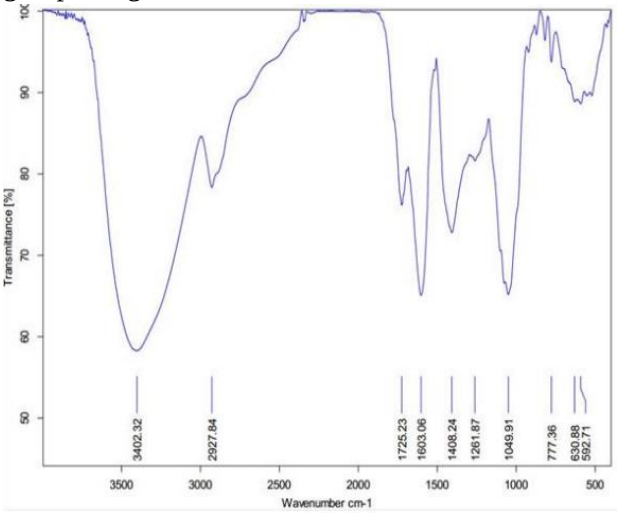

Fig 1 FTIR spectrum of green walnut skin powder

3-1-3- Phenolic compounds of green walnut skin extract

Phenolic compounds are substances that have antioxidant properties. These compounds are present in all parts of the walnut tree (fruit, skin and leaves) and its antimicrobial and antioxidant

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effects are known. Therefore, different parts of the walnut tree can be valuable as a source of natural antioxidants and antimicrobial agents. 13 phenolic compounds including: hydroxycindamic acids (chlorogenic acid, caffeic acid, ferulic acid and sinapic acid), hydroxybenzoic acids (gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, flavonoids catechin, epicatechin and myrsten) and Juglone (hydroxy and naphthoquinone) has been identified in walnut. Among these compounds, juglone has the highest amount and is the main compound found in the skin of walnut fruit [20].

In the present study, the amount of phenolic compounds in walnut peel extract was 96.07±0.22 (mg of gallic acid per gram of dry weight of the sample). Oliveira et al. (2008) evaluated the amount of total phenol, antioxidant and antimicrobial activity of 5 different varieties of walnut extract and showed that green walnut skin can be considered as an important source of health protective and antimicrobial compounds [7]. .

3-1-4- Flavonoid compounds of green walnut skin extract

In this study, the flavonoid compounds of walnut green peel extract were 349 micrograms/gram of quercetin. As it was determined in previous studies, there is an effective and positive relationship between phenolic content and flavonoid content [25]. Flavonoids have high antioxidant properties and by trapping free radicals and reducing oxidative stress as well as inhibiting oxidation macromolecules, the risk of degenerative diseases[1]reduces [26]. Flavonoids can increase the antioxidant function inside the cell by removing oxygen free radicals. It should be noted that the most important feature of flavonoids is their role in preventing heart diseases [27].

3-1-5- DPPH free radical trapping rate

The EC50 values obtained in this study were equal to 0.15, which is consistent with the research of Wang et al. (2011) [28] and Pereira et al. (2007) [29]. Comparison of EC50 values for green walnut skin extract and TBHQ shows that TBHQ has higher antioxidant activity than walnut skin extract obtained by Soxhlet method. Phenols have antioxidant activity due to the presence of hydroxyl groups. Therefore, the content of phenolic compounds in plants is directly related to their antioxidant properties [30]. Many researchers have reported a direct relationship between phenolic content and antioxidant activity in fruits and vegetables. The direct correlation of polyphenol concentration with antioxidant activity has been shown in the study of Oliveira et al. (2008) [7] and Vema et al. The results of evaluating the antioxidant activity of the extract by the DPPH radical inhibition method showed that with the increase in the concentration of the extract, especially in concentrations less than 0.2 mg/ml, the DPPH radical inhibition percentage increases, which is related to the increase in the phenolic compounds present in the extract. Probably, in very high concentrations, due to the emergence of a kind of saturation state, increasing the concentration does not have a significant effect on the level of inhibition of free radicals. These results show that a critical concentration of phenolic compounds is sufficient to inhibit free radicals [32].

Table 2 The amount of EC50 according to DPPH

EC50	Antioxidant compound
0.15±0.0008	Green walnut skin extract
0.08±0.0006	TBHQ

4- General conclusion

Today, the green skin of walnut, which is produced in large quantities every year, is unused and mostly considered as waste. While the bioactive compounds present in it can be used as a natural antioxidant compound. This research was carried out with the aim of determining the exact chemical structure and bioactive functional groups present in the green skin of walnut. Also, the amount of phenol, flavonoid and antioxidant activity of the extract of this product has also been determined. It is suggested to investigate the use of this compound as an antimicrobial substance with high antioxidant activity in increasing the shelf life of food products, especially meat products.

[1]. Degenerative

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6-Resources

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مطالعه ترکیبات شیمیایی، میزان فنل، فلاوونوئید و فعالیت آنتی‌اکسیدانی عصاره پوست سبز گردو (*Juglans regia L.*)

زهرا رفیعی دهکردی[۱]، ناصر صداقت[۲*]، سحر صباحی[۳]

۱- کارشناسی ارشد، گروه علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه فردوسی مشهد، مشهد، ایران.

۲-استاد، گروه علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه فردوسی مشهد، مشهد، ایران.

۳-استادیار، گروه تغذیه، دانشکده پیراپزشکی، دانشگاه علوم پزشکی جندی شاپور اهواز، اهواز، ایران.

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* مسئول مکاتبات:

sedaghat@um.ac.ir

چکیده

امروزه مصرف‌کنندگان تمایل بیشتری به استفاده از افزودنی‌های سالم و طبیعی دارند. ترکیبات فنولی که از محصولات طبیعی به دست می‌آیند، گزینه خوبی برای به حداقل رساندن اکسیداسیون لیپیدها هستند.گردو یکی از مهم‌ترین خشکبارهایی می‌باشد که به طور وسیع در سراسر جهان کشت می‌گردد. با توجه به اینکه پوست سبز گردو حدود ٦٤٪ وزن مرطوب میوه گردو را شامل می‌شود، سالانه رقمی برابر با ۲٤۰ هزار تن پوست سبز گردو از این میوه تولید می‌شود. گردوهای استفاده شده در این مطالعه در سال ۱٤۰۰ و از باغات شهرستان سامان واقع در استان چهارمحال و بختیاری خریداری شدند. پس از پودر شدن پوست سبز و خشک شده، عصاره‌گیری با حلال متانول و به روش سوکسله انجام پذیرفت. جهت شناسایی ترکیبات شیمیایی و گروه‌های عاملی آزمون‌های GC-MS و FTIR انجام پذیرفت. همچنین میزان فنل و فلاوونوئیدهای عصاره پوست گردو نیز اندازه‌گیری شدند. در نهایت فعالیت آنتی اکسیدانی عصاره پوست سبز گردو با استفاده از روش اندازه گیری کاهش ظرفیت رادیکالی به کمک ۲-۲دی فنیل -۱-پیکریل هیدرازیل (DPPH) مورد بررسی قرار گرفت. در مجموع ۸۹ ترکیب شناسایی شدند که نشان دهنده ۹۹/۹ در صد و کل ترکیبات داخل عصاره بودند. مهمترین ترکیبات شناسایی شده که بیشترین سطح زیر منحنی را داشتند شامل پلی فنول ها، ترکیبات آلی و فیتو استرول‌ها بودند. نتایج FTIR نشان دهنده وجود گروه های عاملی هیدروکسیل،گروه آلکان وآروماتیک لیگنین بودند. در مطالعه حاضر مقدار ترکیبات فنولی در عصاره پوست گردو ۰/۲۲±۹٦/۰۷ (میلی گرم اسید گالیک بر گرم وزن خشک نمونه) به دست آمد. همچنین ترکیبات فلاوونوئیدی عصاره پوست سبز گردو ۳٤۹ میکروگرم بر گرم کوئرسیتین بود. مقادیر EC50 به‌دست‌آمده در این پژوهش برابر با ۰/۱۵ بود. نتایج نشان داد عصاره متانولی پوست سبز گردو می‌تواند به عنوان جایگزینی برای آنتی‌اکسیدان های سنتزی معرفی گردد.