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Investigation of biochemical properties and antifungal activity of pomegranate peel extract using different solvents

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ABSTRACT

The results showed that the Ferric reducing activity of plasma (RRAP) and free radical scavenging activity (DPPH) in extracts obtained with 70% methanol were higher than those with other solvents. Also, in water and methanol solvents, with increasing extraction time, the amount of polyphenol compounds and FRAP were the highest and the lowest, respectively, but the increase of DPPH was the highest in ethanol solvent and the lowest in methanol solvent. The results of the investigation of the antifungal effects of the methanol extract of pomegranate peel (MEPP) showed that this extract with a concentration of 0.3% had an antifungal effect nearly equivalent to that of potassium sorbate at a concentration of 0.1%, so that the mean diameter on the diameter of *Aspergillus niger* growth inhibition zone was 17.7 mm for pomegranate peel extract and 18.3 mm for potassium sorbate. Therefore, pomegranate peel extract can be used in food products as antioxidant compounds and preservatives as a natural and effective alternative to chemical preservatives like potassium sorbate.

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1-Introduction

Today, a wide range of additives are used with different purposes in the preparation of different food products. The importance of these additives is such that without using them, the production and consumption of many food products becomes almost impossible. One of the most important food additives is synthetic antioxidant and antimicrobial compounds, which play an important role in prolonging the shelf life of food and reducing waste. Hundreds of synthetic additive compounds have been introduced by responsible organizations as permitted compounds that can be used in food; But their use in food is limited due to not being safe. Natural additives are extracted from various plant and animal tissues, the amount of active compounds of which varies depending on the type of source. in In today's world, special attention has been paid to the optimal use of plant waste and the extraction of bioactive compounds (such as antioxidant and antimicrobial compounds). Pomegranate peel extract is a rich source of polyphenol compounds, tannins, anthocyanins and flavonoid compounds. Therefore, it seems that the use of this source of waste for the production of bioactive compounds, in addition to reducing the waste of agricultural products, will also create added value and reduce environmental pollution [1, 2].

Every year in Iran, millions of dollars of foreign currency are exported from the country to import substances such as essential oils, extracts and dyes, and this trend is increasing every year. The wastes and residues of farms in other countries are the main source of supply of the mentioned items for export to Iran and similar countries. This is while the amount of agricultural waste in the country's farms and food industries is considerable. This problem shows its importance when we know that in developed countries, by collecting and processing waste from cheap materials, they turn them into products with higher added value. The wastes of fruit juice factories are one of the most important sources for the production of food additives, antioxidants and synthetic antimicrobial compounds, which play an important role in prolonging the shelf life of food and reducing waste. Extracting effective compounds from waste with maximum efficiency is of special importance. The extraction method of plant extracts is one of the factors that can affect the properties of the effective compounds of the extracts [3-5].

Pomegranate with name scientific *Punikagaranaatom*[1] From the Ponikase family[2], is widely cultivated in many tropical and subtropical countries [3, 6]. Iran is the center of diversity of varieties and one of the largest producers and exporters of pomegranates in the world. According to the agricultural statistics of the Ministry of Agricultural Jihad, the cultivated area of pomegranate orchards in 1402 is about 97 thousand hectares (92 thousand hectares are fertile and the rest are non-fertile) and the number of scattered pomegranate trees is about 56 thousand. The average yield per hectare of pomegranate orchard in the same year was reported to be 9 tons. one of Pomegranate skin is a valuable resource for the production of natural additives. Pomegranate skin is one of the most important by-products of pomegranate fruit processing factories (mainly pomegranate juice factories). About 30 to 40% of the weight of pomegranate fruit is made up of its peel [7].. Due to the increase in the production of pomegranate fruit, because Increasing consumer awareness of its potential and health-giving properties, the need for industrial and optimal use of such a large amount of waste seems necessary. The composition of pomegranate peel is shown in Table 1 [8]. Pomegranate fruit contains various flavonoids that account for about 0.2 to 1% of the weight of the fruit. Pomegranate skin contains the highest amount of phenolic

1-*Pink garnet*

2- *Punic*

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compounds compared to other structural parts of the fruit, and about 30% of the total of these compounds, especially anthocyanidins.[3] (Dolphinidin[4], cyanidin, pelargonidin 3 glucoside[5] and pelargonidin 3 and 5 diglucoside) depending on the type and stage of fruit growth, accumulate in the skin of the fruit and cause skin color change [9]. [8].

Table 1. Approximate composition of pomegranate peel

Compositions	Amount
Moisture (% of dry matter)	69.93
Ash (%)	5.49
crude fiber (%)	3.95
Lignin (%)	4.29
Total phenols (mg/g)	40.53
Vitamin A (µg/g)	14.06
Sodium (mg/kg)	763.66
Calcium (mg/kg)	645.70
Magnesium (mg/kg)	1644.70
Phosphorus (mg/kg)	33.96
Iron (µg/g)	22.6
Copper (µg/g)	6.20
Zinc (µg/g)	8.03
Selenium (µg/g)	ND

ND: not detect

In general, the phenolic compounds of pomegranate skin are diverse and contain phenolic compounds with low or medium molecular weight, such as anthocyanins, gallotannins.[6], hydroxycinnamic acid, hydrobenzoic acid[7] or phenolic compounds with high molecular weight such as ellagitannin[8], galagyl esters[9] and proanthocyanidins. Phenolic compounds, due to their specific chemical structure, have antioxidant and antimicrobial properties [10]. Although the phenolic compounds of pomegranate skin have been noticed more from the point of view of the occurrence of antioxidant activity, these compounds have important biological activities in organisms and have shown beneficial effects in fighting diseases related to oxygen radical production with concentrations exceeding the antioxidant defense capacity of the human body and antimicrobial effects [11]. Pomegranate peels are byproducts of pomegranate juice processing. Nowadays, pomegranate peel and its extracts are used in several different products such as fish [12].، Bread [13]، Juice [14]، Yogurt powder [15] etc. have been tested. This is the growing interest in the consumption of pomegranate peel due to the different properties of the peel, especially its antioxidant properties. In fact, it is now well established that pomegranate peel extract has strong antioxidant activity [16].. The content of total phenolic compounds in skin extract has been reported to be approximately 10 times higher than that of pulp extract [17]. Studies have shown that the antioxidant activity of pomegranate skin is related to their phenolic content. Polyphenols are the most abundant and widespread groups of plant metabolites and are an integral part of human and animal diets. Extraction technology is a key element for the sustainable development of food-agricultural industries [18].. Polyphenols are traditionally extracted from plant materials by organic solvents. However, optimizing the extraction method before any qualitative and quantitative study will ensure the accuracy of the results. Among the extraction variables, solvent-to-sample ratio, solvent type, extraction time and temperature are very important to ensure extraction efficiency [19]. Solvent selection is an important step to obtain extracts with acceptable yield and strong antioxidant activity is [20].

purpose The present study of extraction of effective compounds from pomegranate peel It is by using different solvents and times so that the best solvent can be obtained in terms of extraction efficiency, amount of phenolic

3- Anthocyanidins

4 -Cyanidine

5 -Pelargonidin 3-Glucosides

6 -Gallotannins

7- Hydroxybenzoic Acids

8 -Ellagitannins

9 -Gallagyl Esters

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compounds, measurement of iron reducing power and antioxidant activities. Also, the antifungal effects of the methanolic extract of pomegranate peel were investigated as a suitable substitute for the chemical preservative potassium sorbate.

2- Materials and methods

1-2- Materials

Pomegranate skin was procured from Meles pomegranate juice company located in Mashhad city in the amount of 50 kg and after separating the waste, it was packed in low density polyethylene film with a thickness of 140 microns and kept at -18 degrees Celsius until the experiments were performed. The chemicals used include 70% ethanol, methanol, Folin Ciocalcu reagent, reagent DPPHGallic acid, sodium carbonate, TPTZIron sulfate II and iron chloride were obtained from Merck, Sigma-Aldrich, Charlot and Caldon.

2-2-Methods

1-2-2-Extraction of effective compounds of pomegranate peel

Extraction of the effective compounds of pomegranate peel was done using 70% ethanol, 70% methanol and water solvents. For this purpose, for each experiment, 100 grams of pomegranate peel was carefully weighed in a beaker and 400 ml of each solvent was added to the beaker and stirred at room temperature for 1, 12, and 24 hours. Then the solution was filtered under vacuum and evaporated with rotary evaporator under vacuum (model Laborota 4000 will be efficient, made in Germany) was concentrated under a temperature of 45 degrees Celsius until complete dehydration [21].

2-2-2- Measuring the phenolic compounds of the extract

To determine the amount of phenolic compounds, to 100 microliters of extract extracted with methanol, 6 milliliters of double-distilled water and 500 microliters of Folin Ciocalcho reagent were added, after keeping for 8 minutes at room temperature, 1.55 milliliters of sodium carbonate 20% by weight/volume was added and thoroughly mixed, and after keeping for 30 minutes at 40 degrees Celsius, Its absorption value was read at the wavelength of 765 nm.

To draw a standard curve, solutions with a concentration of 100 to 950 ppm of gallic acid were prepared, and the absorption of the solutions was read at a wavelength of 765 nm, and a standard curve was drawn. The amount of total phenolic compounds in the samples was determined from the standard curve and the results were reported in terms of milligrams of gallic acid per gram of dry sample [22].

2-3-2-Measuring the regenerative power of iron III [10] (FRAP)

First, 100 mg of the extracted extract was dissolved in 2 ml of methanol, and then 30 µl of it was mixed with 900 µl of the solution. FRAP And 90 microliters of distilled water was mixed in the test tube. After stirring, the test tube was placed in a bain-marie and after its temperature reached 37 degrees Celsius, the absorbance value was read against the control at a wavelength of 595 nm.

To prepare the standard curve of solutions with concentrations of 200 to 2000 micromol/liter of iron sulfate. II Its preparation and absorption was obtained at a wavelength of 595 nm and the amount of iron II It was determined using a standard curve [22].

2-2-4- Measurement of free radical inhibitory powerDPPH

First, 0.006% radical solution freeDPPH It was prepared in methanol. Then, to the test tubes containing one milliliter of methanol extract solution with different concentrations (depending on the free radical inhibitory power), one milliliter of the solution DPPH was added After complete mixing, the test tubes were kept in a dark place for one hour and then their absorbance was read at a wavelength of 517 nm against the control [23]..

10-Ferric Reducing Activity of Plasma

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5-2-2- Determining the minimum concentration of mold growth inhibitory using disk diffusion method

After choosing the most suitable concentration of pomegranate peel extract، A number of Whatman No. 7 filter paper was prepared using a punch with uniform holes and discs (the average diameter of each disc was 7 mm). The discs were autoclaved and dried in a suitable container. Then the disks were placed on a glass plate and coated with different volumes (5 to 25 microliters) of pomegranate peel extract with a concentration of 3000 mg/ml and potassium sorbate with a concentration of 1000 mg/ml and dried under sterile conditions.

Then mold *Aspergillus niger* 16404ATCC-، It was propagated in liquid culture medium containing 1% glucose, 0.5% yeast extract and 0.5% tryptone for 48 hours. Then, the liquid culture medium was uniformly spread on the specific solid culture medium of mold (dextrose chloramphenicol agar). The said solid culture medium was placed in the refrigerator for 10 minutes to absorb the liquid culture into the solid medium. Prepared discs with different volumes were placed on the solid culture medium and kept at 37 degrees Celsius for 24 hours. Then, the diameter of non-growth halos was measured with a caliper and the corresponding average was reported [2].

2-3- Statistical analysis

The related experiments were conducted in factorial format on a completely random basis and in three replications. The investigated factors included solvent (water, 70% methanol and 70% ethanol) and extraction time (1, 12 and 24 hours). The averages of the tests with the software SPSS Version 18 and were compared based on Duncan's test at the 5% level (0.05> P). To draw curves from the softwareMicrosoft Excel 2013 was used.

3- Results and discussion

1-3- Effect of solvent type and extraction time on phenolic compounds of pomegranate peel extract

Based on the results of analysis of variance, the independent effects of solvent and extraction time and their mutual effect on the amount of phenolic compounds of extracts extracted from pomegranate waste were significant (<0.05). P). According to Figure 1, with the increase of extraction time in all used solvents, the amount of polyphenolic compounds increased significantly, and this increase was the highest in water solvent, but the lowest in methanol solvent. Comparison of the averages showed that the amount of phenolic compounds increased about 2.96 and 2 times due to the use of methanol 70% (53.05) and ethanol 70% (36.11) compared to water solvent (17.87), respectively. Also, the amount of these compounds increased about 1.5 times due to the use of 70% methanol solvent (53.05) compared to 70% ethanol solvent (36.11). Because ethanol and methanol solvents are less polar than water and methanol than ethanol, and cause more destruction of the cell wall and result in increased extraction of polyphenolic compounds and anthocyanins [24].. Due to its suitable polarity, methanol has a high ability to extract flavonoids and other phenolic compounds from pomegranate skin [25].

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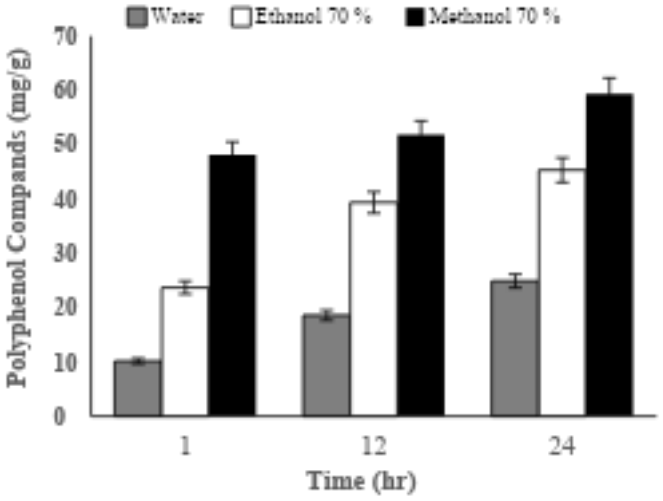

Figure 1. The interaction effect of solvent type and extraction time on the amount of phenolic compounds from pomegranate peel extract

2-3- Effect of solvent type and extraction time on antioxidant activity of pomegranate peel extract

Determining the reducing power of iron is a quick and convenient way to measure the reducing power of chemical compounds and it can be used as an indicator of antioxidant power. In this method, the ability of extracts to regenerate trivalent iron and convert it into divalent iron is measured. The presence of regenerating agents (antioxidants) leads to the regeneration of ferricyanide complexes and their conversion to the reduced form, which is accompanied by a color change from blue to various degrees of green and yellow, depending on the reductive capacity of the examined extracts. Antioxidants with higher iron-reducing power have a greater ability to terminate destructive radical chain reactions. Many antioxidants prevent lipid oxidation by deactivating free radicals.

Based on the results of analysis of variance, the independent effects of solvent and extraction time and their mutual effects on the reducing power of iron in extracts extracted from pomegranate waste were significant (<0.05). P). According to Figure 2, with increasing extraction time in all solvents used, the amount FRAP It increased significantly, and this increase was the highest in the use of water solvent, but the lowest in methanol solvent.

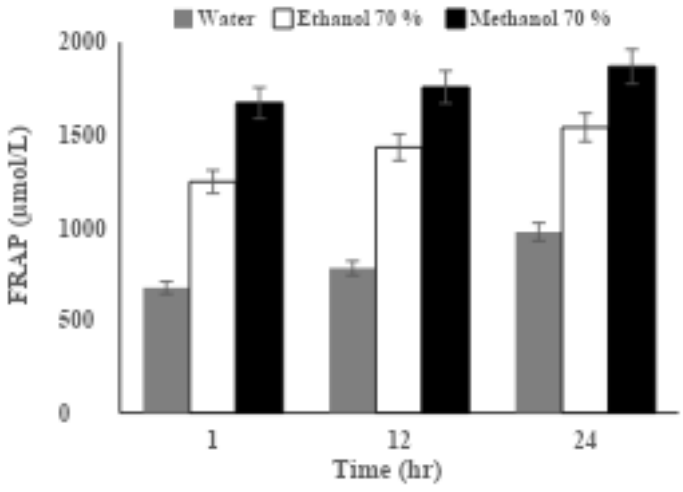

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Figure 2. The interaction effect of solvent type and extraction time on the amount of Ferric reducing activity of plasma (RRAP) from pomegranate peel extract

The comparison of the averages showed that the amount of phenolic compounds increased about 2.25 times and 1.23 times due to the use of methanol 70% and ethanol 70% solvent compared to water solvent, respectively. Also, the amount of these compounds increased about 1.83 times due to the use of 70% methanol solvent compared to 70% ethanol solvent.Extraction with methanol solvent for 24 hours had the highest iron reduction power in the extracts extracted from waste. These results are reported by Setlodi[11] and colleagues It was consistent [26].

To determine the free radical inhibitory power of antioxidants from various free radicals such as DPPH, proxy, hydroxyl and superoxy are used. Investigation of free radical scavenging activity DPPH It is one of the methods of determining antioxidant activity. In this method, the purple color of free radicals DPPH Due to the antioxidants present in the extract, it is neutralized and becomes colorless. Therefore, the degree of discoloration of this compound indicates the power of free radical trapping by existing antioxidants [27]..

The results of variance analysis of the data showed that the independent effects of solvent and time and their mutual effects on The free radical inhibitory power of antioxidants were significant (>0.05) in extracts extracted from pomegranate waste P). According to Figure 3, with increasing extraction time in all solvents used, the amount DPPH It increased significantly, and this increase was the highest in the use of ethanol solvent, but the lowest in methanol solvent.

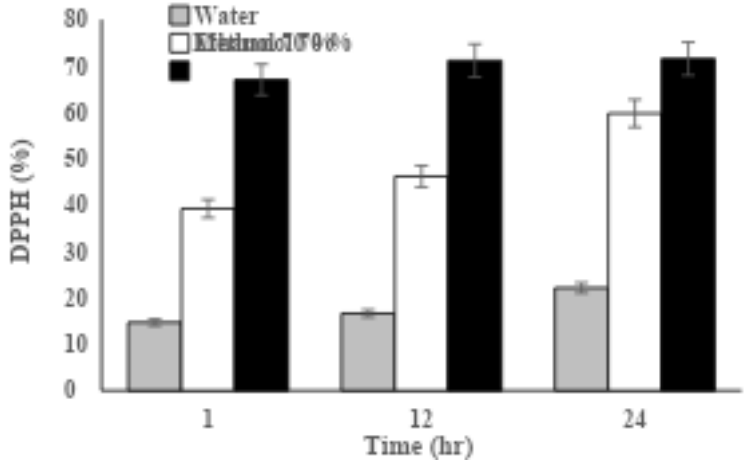

Figure 3. The interaction effect of solvent type and extraction time on the amount of DPPH free radical-scavenging from pomegranate peel extract

The comparison of the averages showed that the amount of phenolic compounds increased about 3.93 and 1.45 times due to the use of 70% methanol and 70% ethanol compared to the water solvent, respectively. Also, the amount of these compounds increased about 2.72 times due to the use of the 70% methanol solvent compared to the 70% ethanol solvent. These results were consistent with the report of Singh et al [28]. Extraction with methanol solvent for a maximum of 24 hours The regenerative

11- Filter

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power of iron in the extracts extracted from waste.

3-3- Investigating the effect of methanol extract of pomegranate peel (extracted under optimal conditions) on growth inhibition *Aspergillus niger*

Controlling the growth of microorganisms is one of the most important aspects of food preservation. Additives are among the substances that are intentionally added in the food production process to prevent adverse changes and spoilage caused by microorganisms and increase the shelf life of food. Potassium sorbate is one of the substances that is in the category of preservatives and prevents microbial spoilage in food and increases the shelf life. This substance can be used as a preservative in bread and other bakery products, dairy products, jams and syrups, pickles, juices, and dried fruits. Potassium sorbate tolerates the high temperature of the process and does not affect the flavor of food and does not react with vitamins, minerals and enzymes [29]. .The use of sorbic acid and their salts as preservatives in food is determined by international and national regulatory agencies, and their use is recommended in a very low concentration. Side effects of using such substances have been reported in the form of skin effects such as acne, hives, and contact dermatitis. Concerns related to the safety of chemical additives, restrictions on the permissible limits of consumption, as well as their possible risks to human health, have provided an increasing desire to replace this type of compounds with natural types. Therefore, in the present study, the antifungal effects of the methanol extract of pomegranate peel were studied as a substitute for the chemical preservative potassium sorbate. The effect of different volumes (5 to 25 microliters) of the extract extracted with 70% methanol of pomegranate peel (concentration 3000 mg/ml) compared to the synthetic preservative potassium sorbate, on the inhibition of mold growth. *Aspergillus Niger* After 24 hours of storage at 37 degrees Celsius, it is shown in Figure 4 and the results related to the diameter of the halo of non-growth of methanol extract of pomegranate peel are shown in Table 2.

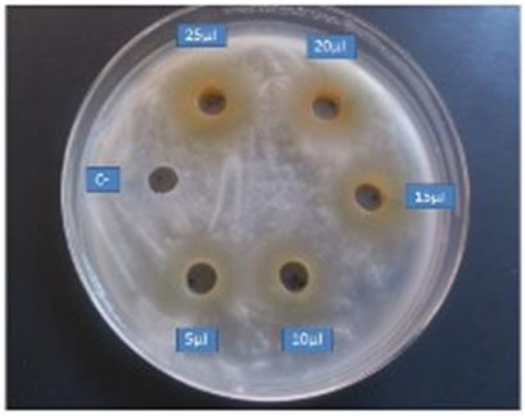

Figure 4- The effect of different amounts of 70% methanol extract of pomegranate peel (concentration 0.3%) on the growth and inhibition of *Aspergillus niger* after 24 hr at 37 °C

As can be seen from Table 2, the average diameter of the mold growth halo *Aspergillus niger* Under the effect of methanol extract of pomegranate peel and synthetic preservative potassium sorbate, it was 17.7 and 18.3 mm, respectively. therefore, The methanolic extract of pomegranate peel at a concentration of 0.3% had an antifungal effect almost equivalent to the antifungal effect of potassium sorbate (at a concentration of 0.1%). Similar results have been reported by other researchers [30, 31].

Table 2- Comparison of the mean effect of different treatments on the diameter of Aspergillus niger growth inhibition zone

Extract	Diameter of growth inhibition zone (mm)
Pomegranate peel methanolic extract (3000 mg/ml)	17.7±0.28[a]
Methanol 70% (negative control)	ND
Potassium sorbate (1000 mg/ml)	18.3±0.21[a]

The same superscript lower letters (a) beside mean values indicate a not significant difference from each other (t- test, P<0.05). ND: not detect.

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Potassium sorbate is effective against a wide range of molds and yeasts. The cause of this phenomenon is due to inhibition of dehydrogenase enzymes in fatty acid oxidation, inhibition of enzymes containing sulfhydryl, and as a result of uncoupling of oxidative phosphorylation and inhibition of catalase, and as a result, an increase in hydrogen peroxide in the cell [32].. The antifungal effect of the methanolic extract of pomegranate peel is also probably due to the phenolic compounds (flavonoids and tannins) contained in it. Phenolic compounds play an important role in preventing the growth of bacteria and fungi. These compounds exert their antimicrobial effect by disrupting the bacterial membrane through changes in the composition and permeability of the cell membrane, oxidative stress, inhibition of respiration and ion transport processes. The effectiveness of these compounds varies depending on the type of phenolic compounds, concentration of phenolic compounds, extraction method and solvent used for extraction, etc. [33].

4- General conclusion

In this research, the properties of pomegranate, the extraction of effective compounds from pomegranate skin, and the effect of solvents and different times on the amount of phenolic compounds and its antioxidant and antifungal activity have been investigated. The results showed that the amount of total polyphenolic compounds, iron reduction power and free radical inhibitory powerDPPH In the method of extraction with ethanol and methanol, it was more than extraction with water. Ethanol and methanol solvents, due to their lower polarity, destroy the cell wall and cause more extraction of polyphenols and anthocyanins. Also, with the increase of extraction time, the amount of phenolic compounds, iron reduction power and free radical inhibitory power DPPH increased in all solvents used. Also, the methanolic extract of pomegranate peel with a concentration of 0.3% had antifungal properties almost equivalent to potassium sorbate with a concentration of 0.1%, so that the average diameter of the halo of no mold growth *Aspergillus niger* It was 17.7 mm under the influence of pomegranate peel extract and 18.3 mm under the influence of potassium sorbate. Therefore, pomegranate peel extract can be used as a natural and effective substitute for chemical preservatives such as potassium sorbate in food products.

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5-Resources

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بررسی ویژگی‌های بیوشیمیایی و خاصیت ضدقارچی عصاره پوست انار با استفاده از حلال‌های مختلف

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چکیده

به منظور بررسی ویژگی‌های بیوشیمیایی ترکیبات عصاره پوست انار، آزمایشی در قالب فاکتوریل بر پایه طرح کاملاً تصادفی انجام شد. فاکتورهای مورد بررسی شامل نوع حلال (آب، متانول ۷۰ درصد و اتانول ۷۰ درصد) و زمان استخراج (۱، ۱۲ و ۲۴ ساعت) بود. نتایج نشان داد که قدرت احیاکنندگی آهن و فعالیت مهارکنندگی رادیکال آزاد در عصاره‌های استخراج شده با متانول ۷۰ درصد بالاتر از سایر حلال‌ها بود. همچنین با افزایش زمان استخراج در تمام حلال‌های مورد استفاده، مقدار ترکیبات پلی‌فنلی، قدرت احیاکنندگی (FRAP) و قدرت مهارکنندگی رادیکال آزاد (DPPH) به طور معنی‌داری افزایش یافت که افزایش مقدار ترکیبات پلی‌فنلی و FRAP در استفاده از حلال آب بیشترین مقدار اما در حلال متانول کمترین مقدار بود. اما افزایش DPPH در استفاده از حلال اتانل بیشترین مقدار و در حلال متانول کمترین مقدار بود. نتایج حاصل از بررسی اثرات ضد قارچی عصاره متانولی پوست انار نشان داد که عصاره متانولی پوست انار با غلظت ۰/۳ درصد، دارای خاصیت ضدقارچی تقریباً معادل با سوربات پتاسیم با غلظت ۰/۱ درصد بود، به طوری که میانگین قطر هالهٔ عدم رشد کپک *آسپرژیلوس نایجر* تحت تاثیر عصاره پوست انار ۱۷/۷ میلی‌متر و تحت تاثیر سوربات پتاسیم ۱۸/۳ میلی‌متر بود. لذا عصاره پوست انار می‌تواند به عنوان ترکیبات آنتی‌اکسیدنی و نگهدارنده طبیعی، جایگزین مناسبی برای نگهدارنده‌های شیمیایی مانند سوربات پتاسیم در محصولات غذایی استفاده شود.

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