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Scientific Research

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 in the preparation of various food products for numerous purposes. These additives are so essential that producing and consuming many food products would be nearly impossible without them. One of the most important food additives includes synthetic antioxidant and antimicrobial compounds, which play a crucial role in extending the shelf life of food and reducing waste. Hundreds of synthetic additives have been approved for use in food products by regulatory bodies, but their application is limited due to safety concerns. Natural additives are extracted from various plant and animal tissues, with the active compound content varying depending on the source. In modern times, there has been a significant emphasis on utilizing plant waste optimally and extracting bioactive compounds (e.g., antioxidants and antimicrobials). Pomegranate peel extract is rich in polyphenols, tannins, anthocyanins, and flavonoids. Therefore, utilizing this waste source for producing bioactive compounds can not only reduce agricultural waste but also create added value and reduce environmental pollution [1, 2].

In Iran, millions of dollars are spent annually on importing substances like essential oils, extracts, and colorants, with an increasing trend. Agricultural waste from fields is the primary source of these imported materials in other countries for export to Iran and similar nations. Meanwhile, the volume of agricultural waste in Iran's farms and food processing industries is considerable. This issue becomes especially significant when we consider that in developed countries, such residues are collected and processed to transform inexpensive materials into high-value products. Residues from juice production facilities are among the main sources for producing food additives, antioxidants, and synthetic antimicrobial compounds, playing a key role in extending food shelf life and reducing waste. Efficient extraction of active compounds from residues is thus critical [3-5]. Pomegranate (*Punica granatum*), from the family *Punicaceae*, is widely cultivated in many tropical and subtropical countries [3, 6]. Iran is one of the centers of genetic diversity and a leading producer and exporter of pomegranate globally. According to the Agricultural Statistical Yearbook of Iran's Ministry of Agriculture Jihad, the pomegranate orchards covered about 97,000 hectares (92,000 hectares of fruit-bearing and the rest non-bearing) with approximately 56,000 scattered trees in 2023. The average yield per hectare was reported as 9 tons. Pomegranate peel is one of the most valuable byproducts of pomegranate juice production facilities, comprising about 30-40% of the fruit's weight [7]. Given the rising demand for pomegranate, due to its health benefits, industrial and optimal utilization of this large volume of waste is necessary. Table 1 presents the composition of pomegranate peel [8]. The fruit contains a variety of flavonoids, comprising 0.2-1% of the fruit's weight. The peel contains the highest level of phenolic compounds compared to other structural parts of the fruit, specifically anthocyanidins (such as delphinidin, cyanidin, pelargonidin 3-glucoside, and pelargonidin 3,5-diglucoside), which accumulate in the peel based on the type and developmental stage of the fruit, causing changes in peel color [9].[۸

Table 1. Approximate composition of pomegranate peel

Compositions	Amount
Moisture (% of dry matter)	69.93

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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 in pomegranate peel are diverse, comprising low- to medium-molecular-weight phenolics such as anthocyanins, gallotannins, hydroxycinnamic acid, and hydroxybenzoic acid, as well as high-molecular-weight phenolics like ellagitannins, gallagyl esters, and proanthocyanidins. Due to their unique chemical structures, phenolic compounds possess antioxidant and antimicrobial properties [10]. Although the phenolic compounds in pomegranate peel are primarily recognized for their antioxidant activity, they also demonstrate significant biological activities in organisms, showing beneficial effects in fighting diseases related to the overproduction of oxygen radicals, which can exceed the body's antioxidant defense capacity, along with antimicrobial effects [11].

Pomegranate peel is a byproduct of pomegranate juice processing. Today, pomegranate peel and its extracts have been tested in various products, including fish [12], bread [13], juice [14], yogurt powder [15], and more. This growing interest in using pomegranate peel is mainly due to its diverse properties, especially its antioxidant effects. It has been well established that pomegranate peel extract exhibits strong antioxidant activity [16]. The total phenolic content of pomegranate peel extract is reported to be approximately ten times higher than that of the pulp extract [17]. Studies have shown that the antioxidant activity of pomegranate peel is closely linked to its phenolic content. Polyphenols are the most abundant and widespread group of plant metabolites and form an integral part of the diet for both humans and animals. Extraction technology is a crucial component in the sustainable development of the food and agricultural industries [18].

Traditionally, polyphenols are extracted from plant materials using organic solvents. However, optimizing the extraction method before any qualitative or quantitative study ensures the accuracy of results. Among the extraction variables, solvent-to-sample ratio, solvent type, extraction time, and extraction temperature play an essential role in ensuring extraction efficiency [19]. The choice of solvent is critical for obtaining extracts with acceptable yields and strong antioxidant activity [20]. The objective of this study was to extract active compounds from pomegranate peel using various solvents and extraction times to determine the best solvent in terms of extraction yield, phenolic content, iron-reducing power, and antioxidant activities. Additionally, the antifungal effects of methanolic pomegranate peel extract were

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evaluated as a potential natural alternative to the chemical preservative potassium sorbate.

2- Materials and Methods

2.1 Materials

Pomegranate peel was sourced from the Mallas Pomegranate Juice Company located in Mashhad, Iran, in a quantity of 50 kg. After removing any waste materials, the peel was packed in low-density polyethylene film with a thickness of 140 microns and stored at -18°C until testing. The chemicals used included 70% ethanol, methanol, Folin-Ciocalteu reagent, DPPH reagent, gallic acid, sodium carbonate, TPTZ, iron (II) sulfate, and iron chloride, which were obtained from Merck, Sigma-Aldrich, Sharlo, and Caldon.

2.2 Methods

2.2.1 Extraction of Active Compounds from Pomegranate Peel

Active compounds were extracted from the pomegranate peel using 70% ethanol, 70% methanol, and water as solvents. For each experiment, 100 grams of pomegranate peel were accurately weighed in a beaker, and 400 mL of each solvent was added. The mixture was stirred at room temperature for 1, 12, and 24 hours. The solution was then filtered under vacuum and concentrated to remove moisture using a rotary evaporator under vacuum (Laborota 4000 efficient, Germany) at 45°C until complete dryness was achieved [21].

2.2.2 Measurement of Total Phenolic Content

To determine the total phenolic content, 100 µL of the methanolic extract was mixed with 6 mL of double-distilled water and 500 µL of Folin-Ciocalteu reagent. After 8 minutes at room temperature, 1.55 mL of 20% (w/v) sodium carbonate was added, and the mixture was thoroughly mixed. The solution was then incubated for 30 minutes at 40°C, and its absorbance was measured at a wavelength of 765 nm. Standard solutions with concentrations ranging from 100 to 950 ppm of gallic acid were prepared to create a standard curve, following the same procedure as for the sample. Total phenolic content in the samples was calculated from the standard curve and reported in mg of gallic acid per gram of dry sample [22].

2.2.3 Measurement of Iron Reducing Power (FRAP)

First, 100 mg of the extracted sample was dissolved in 2 mL of methanol. Then, 30 µL of the extract solution was mixed with 900 µL of FRAP reagent and 90 µL of distilled water in a test tube. After mixing, the tube was placed in a water bath at 37°C, and the absorbance was measured at 595 nm against a blank sample. For the standard curve, solutions with concentrations from 200 to 2000 µM of iron (II) sulfate were prepared, and their absorbance was recorded at 595 nm. The amount of iron (II) in the samples was calculated based on the standard curve [22].

2.2.4 Measurement of Free Radical Scavenging Activity (DPPH)

A 0.006% DPPH radical solution was prepared in methanol. Then, 1 mL of the methanolic extract with varying concentrations (based on the scavenging ability of the free radical) was added to 1 mL of the DPPH solution in test tubes. After thorough mixing, the tubes were kept in a dark place for 1 hour, and the absorbance was measured at 517 nm against a blank [23].

2.2.5 Determination of Minimum Inhibitory Concentration (MIC) of Mold Growth Using the Disk Diffusion Method

After determining the optimal concentration of pomegranate peel extract, filter paper disks (Whatman No. 7) were prepared by punching uniform holes, with each disk having an average diameter of 7 mm. The disks were sterilized in an autoclave and dried. They were then placed on a glass plate and coated with different volumes (5 to 25 µL) of pomegranate peel extract at a concentration of 3000 mg/mL and potassium sorbate at a

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concentration of 1000 mg/mL. The disks were dried under sterile conditions.

The *Aspergillus niger* (ATCC 16404) mold was cultured in a liquid medium containing 1% glucose, 0.5% yeast extract, and 0.5% tryptone for 48 hours. The liquid culture was evenly spread on a solid mold-specific medium (chloramphenicol dextrose agar). This medium was placed in the refrigerator for 10 minutes to allow the liquid culture to be absorbed into the solid medium. The prepared disks with different volumes were placed on the solid medium and incubated at 37°C for 24 hours. The diameter of the inhibition zones was measured using calipers, and the average value was reported [2].

2.3 Statistical Analysis

The experiments were conducted in a completely randomized factorial design with three replications. The factors studied included solvent type (water, 70% methanol, and 70% ethanol) and extraction time (1, 12, and 24 hours). The means from the tests were compared using Duncan's test at the 5% significance level ($P < 0.05$) with SPSS version 18 software. Microsoft Excel 2013 was used for drawing the graphs.

3- Results and Discussion

3.1 Effect of Solvent Type and Extraction Time on Phenolic Compounds

According to the results of the analysis of variance, the independent effects of solvent type and extraction time, as well as their interaction, significantly affected the phenolic content of the extracts obtained from pomegranate peel waste ($P < 0.05$). As shown in Figure 1, increasing the extraction time significantly increased the polyphenolic content across all solvents used. This increase was highest in water as a solvent, while methanol yielded the lowest polyphenolic content.

Comparing the mean values, the phenolic content using 70% methanol (53.05 mg/g) and 70% ethanol (36.11 mg/g) increased approximately 2.96 times and 2 times, respectively, compared to water (17.87 mg/g). Additionally, the phenolic content with 70% methanol was about 1.5 times higher than that of 70% ethanol (36.11 mg/g). This can be attributed to the fact that ethanol and methanol have lower polarity than water, and methanol has lower polarity than ethanol, leading to greater cell wall disruption and enhancing the extraction of polyphenols and anthocyanins [24]. Methanol, due to its suitable polarity, has a high efficiency in extracting flavonoids and other phenolic compounds from pomegranate peel [25].

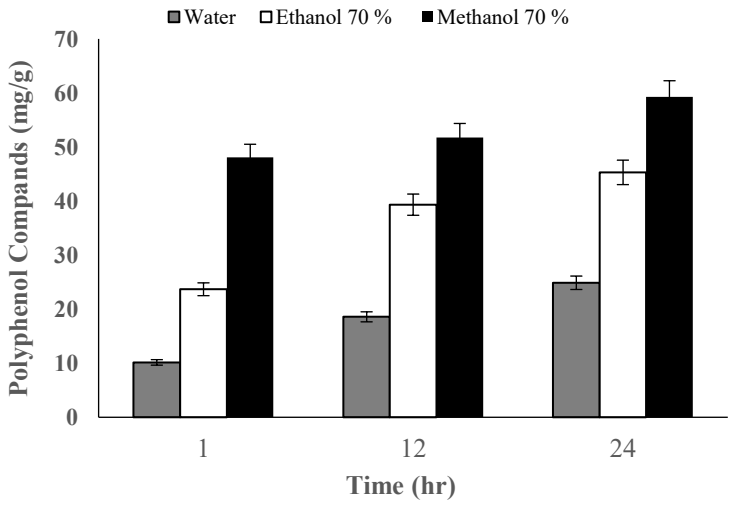

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Figure 1. The interaction effect of solvent type and extraction time on the amount of phenolic compounds from pomegranate peel extract

Comparing the mean values showed that the phenolic content using 70% methanol and 70% ethanol was approximately 2.25 and 1.23 times higher, respectively, than with water as a solvent. Additionally, the phenolic content extracted with 70% methanol was about 1.83 times higher than that with 70% ethanol. Extraction with methanol for 24 hours provided the highest iron-reducing power among the extracts obtained from pomegranate peel waste. These findings are consistent with the report by Setlodi et al. [26].

3.2. The effect of solvent type and extraction time on the antioxidant activity of pomegranate peel extract

Determination of iron reducing power (FRAP) is a rapid and convenient method for measuring the reducing power of chemical compounds and can be used as an indicator of antioxidant power. In this method, the ability of extracts to reduce trivalent iron and convert it to divalent iron is measured. The presence of reducing agents (antioxidants) leads to the reduction of ferricyanide complexes and their conversion to the ferric iron form, which is accompanied by a color change from blue to varying degrees of green and yellow, depending on the reducing power of the extracts under study. Antioxidants with higher iron reducing power have a greater ability to terminate destructive radical chain reactions. Many antioxidants prevent lipid oxidation by inactivating free radicals. According to the results of the analysis of variance, the independent effects of solvent and extraction time and their interaction effects on the FRAP in the extracts obtained from pomegranate peel waste were significant ($P<0.05$). According to Figure 2, with increasing extraction time in all solvents, the FRAP value increased significantly, which was the highest in the use of water solvent but the lowest in methanol solvent.

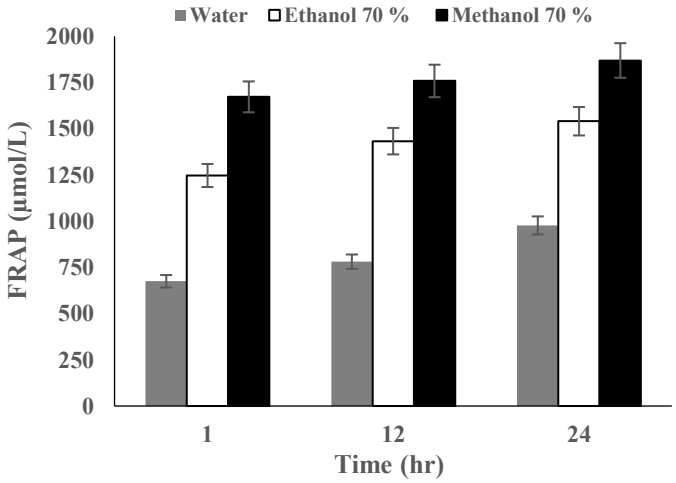

Figure 2. The interaction effect of solvent type and extraction time on the amount of Ferric reducing activity of plasma (RRAP) from pomegranate peel extract

To determine the free radical scavenging activity of antioxidants, various free radicals, such as DPPH, peroxy, hydroxyl, and superoxide radicals, are used. The DPPH free radical scavenging activity test is a common

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method for evaluating antioxidant activity. In this method, the purple color of DPPH free radicals is neutralized and becomes colorless in the presence of antioxidants in the extract. Thus, the degree of color change indicates the free radical scavenging ability of the antioxidants present [27]. The results of the analysis of variance showed that the independent effects of solvent type and extraction time, as well as their interaction, significantly affected the free radical scavenging power of the antioxidants in the extracts obtained from pomegranate peel waste ($P < 0.05$). As shown in Figure 3, increasing the extraction time significantly increased the DPPH scavenging ability across all solvents. This increase was highest with ethanol as the solvent, while methanol showed the lowest scavenging activity.

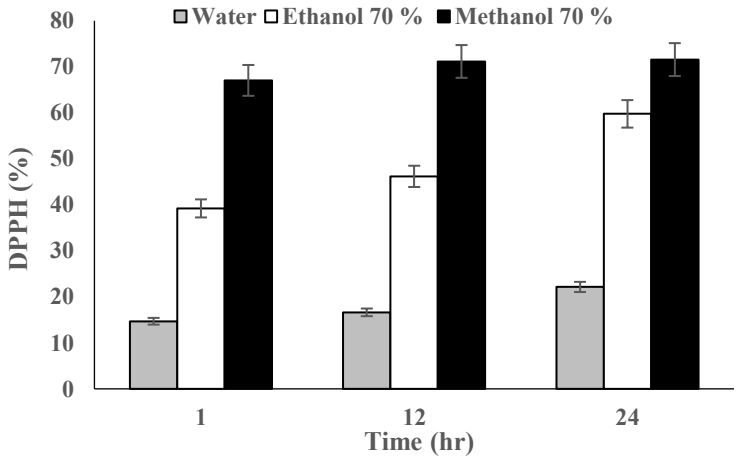

Figure 3. The interaction effect of solvent type and extraction time on the amount of DPPH free radical-scavenging from pomegranate peel extract

The mean comparisons showed that the phenolic content using 70% methanol and 70% ethanol was approximately 3.93 and 1.45 times higher, respectively, than with water as the solvent. Additionally, the phenolic content extracted with 70% methanol was about 2.72 times higher than that with 70% ethanol. These findings are consistent with the report by Singh et al. [28]. Extraction with methanol for 24 hours yielded the highest iron-reducing power in the extracts obtained from pomegranate peel waste.

3.3 Evaluation of the Effect of Methanolic Pomegranate Peel Extract (Extracted Under Optimal Conditions) on the Inhibition of *Aspergillus niger* Growth

Controlling microbial growth is one of the most critical aspects of food preservation. Additives are intentionally added during food production to prevent undesirable changes and spoilage caused by microorganisms, thereby extending the shelf life of food products. Potassium sorbate is a preservative that inhibits microbial spoilage and prolongs storage life. It is commonly used in bread and other baked goods, dairy products, jams and syrups, pickles, juices, and dried fruits. Potassium sorbate can withstand high processing temperatures, does not affect the aroma and taste of food, and does not react with vitamins, minerals, or enzymes [29].

The use of sorbic acid and its salts as food preservatives is regulated by international and national regulatory organizations, with their consumption recommended at very low

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concentrations. Adverse effects of these preservatives have been reported as skin reactions such as rashes, hives, and contact dermatitis. Concerns over the safety of chemical additives, usage limitations, and potential health risks have increased interest in replacing these additives with natural alternatives. Therefore, this study investigated the antifungal effects of methanolic pomegranate peel extract as a natural alternative to the synthetic preservative potassium sorbate.

The effects of different volumes (5 to 25 µL) of 70% methanolic pomegranate peel extract (at a concentration of 3000 mg/mL) were compared with the synthetic preservative potassium sorbate for inhibiting the growth of *Aspergillus niger* mold after 24 hr of incubation at 37°C. Figure 4 illustrates the results, and Table 2 presents the inhibition zone diameter of the methanolic pomegranate peel extract.

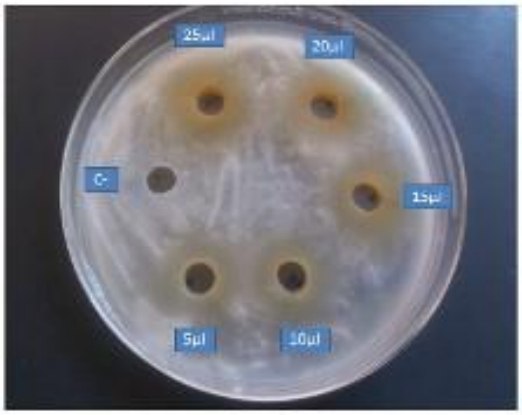

Figure 4- The effect of different amounts of 70% methanol extract of pomegranate peel (concentration 0.3%) on the growth and inhibition of *Aspergillus niger* after 24 hr at 37 °C

As shown in Table 2, the mean inhibition zone diameter for *Aspergillus niger* was 17.7 mm with the methanolic pomegranate peel extract and 18.3 mm with the synthetic preservative potassium sorbate. Therefore, the methanolic pomegranate peel extract at a concentration of 0.3% exhibited antifungal activity nearly equivalent to that of potassium sorbate at a concentration of 0.1%. Similar findings 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.

Potassium sorbate is effective against a wide range of molds and yeasts. This effect is due to the inhibition of dehydrogenase enzymes in fatty acid oxidation, inhibition of sulfhydryl-containing enzymes, uncoupling

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of oxidative phosphorylation, inhibition of catalase, and consequently, an increase in hydrogen peroxide within the cell [32]. The antifungal effect of methanolic pomegranate peel extract is likely due to its phenolic compounds (flavonoids and tannins). Phenolic compounds play an important role in preventing the growth of bacteria and fungi. Their antimicrobial activity is achieved through disruption of the bacterial membrane by altering membrane composition and permeability, oxidative stress, inhibition of respiration, and interference with ion transport processes. The effectiveness of these compounds varies depending on the type and concentration of phenolic compounds, extraction method, solvent used, and other factors [33].

4-Conclusions

This study examined the properties of pomegranate, the extraction of active compounds from pomegranate peel, and the effect of different solvents and extraction times on phenolic content, antioxidant activity, and antifungal efficacy. The results indicated that the total polyphenolic content, iron-reducing power, and DPPH free radical scavenging capacity were higher when extracted with ethanol and methanol compared to water. Due to their lower polarity, ethanol and methanol solvents disrupt the cell wall more effectively, resulting in greater extraction of polyphenols and anthocyanins. Furthermore, increasing extraction time enhanced the phenolic content, iron-reducing power, and DPPH radical scavenging capacity across all solvents used.

Additionally, methanolic pomegranate peel extract at a concentration of 0.3% exhibited antifungal activity nearly equivalent to that of potassium sorbate at a concentration of 0.1%, with an average inhibition zone diameter of 17.7 mm for *Aspergillus niger* mold compared to 18.3 mm for potassium sorbate. Therefore, pomegranate peel extract can serve as a natural and effective alternative to chemical preservatives like potassium sorbate in food products.

Acknowledgment

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مقاله علمی_پژوهشی

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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