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Investigating the extraction conditions of olive kernel extract using a Ultrasonic Homogenizer and evaluating their antimicrobial properties

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ABSTRACT

The aim of the present study was to investigate the extraction conditions of olive kernel extract using an ultrasonic homogenizer and to investigate the antimicrobial properties of the extract with the highest amount of phenolic compounds. According to the results, the ethanolic sample which was extracted in 3 minutes has the highest amount of phenolic compounds. The kinds of solvent and extraction time have significant effects ($P<0.05$) on the amount of the sample phenolic compounds. The treatments extracted with solvent (water 0: 100 ethanol) in all three times (1, 2 and 3 minutes) and then the treatments extracted with solvent ethanol 80: water 20 in 2 and 3 minutes had the highest amount of phenolic compounds thus as the treatment for performing microbial tests were selected. In relation to antimicrobial properties, according to the results obtained from the well diffusion test, all three ethanolic extracts had antimicrobial properties. Also, the optical density measurement test (OD or OD600) was done to choose the optimal treatment. The results showed that the ethanolic extract in 3 minutes has the highest antimicrobial properties, thus this extract was selected as the best treatment and its phenolic compounds were measured by GC-MS. The results showed that the highest percentage of phenolic compounds in this extract is related to hydroxytyrosol and tyrosol.

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1- Introduction

The excessive use of chemical antimicrobial additives such as potassium sorbate and sodium benzoate causes numerous health problems for consumers, including toxicity, negative effects on liver, kidney, and brain cells, and in more severe cases, cancer; for this reason, in recent years, the demand for the use of natural and biological materials as an alternative to chemical preservatives has been considered [1]. Other disadvantages of chemicals include their limited availability, high cost, and the development of drug resistance in consumers [2]. On the other hand, plant extracts are more compatible with the human body than chemicals due to their natural origin. The absence of side effects, cheapness, and availability are other advantages of using plant extracts instead of chemicals [3]. The type and amount of phenolic compounds are very important in the effectiveness of natural preservatives. In different studies, different values have been presented for the amount of phenolic compounds extracted by different solvents. These different extraction amounts depend on the type of plant, the region where it is cultivated, and the extraction conditions [4]. Olives, with the scientific name Olea europaea, belong to the Oleaceae family. Olives are one of the most useful fruits in subtropical regions due to their high-quality oil [5]. Iran ranks 20th in the world with an annual production of 85,000 tons [6]. Due to the major applications of olives, including oil production and canning, great attention has been paid to their cultivation and cultivation in recent years. Food wastes generated during food production, distribution, and consumption have many nutritional properties; their disposal, on the one hand, causes a waste of nutrients and, on the other hand, is costly. Waste disposal also has a detrimental effect on the environment. For this reason, the food industry is looking for a solution to reduce this food waste and also to make optimal use of the by-products obtained during the production chain [7]. Abolfatah et al. (2024) investigated different variables on the antibacterial properties of olive leaves. The results showed that olive leaf extract had significant effects on Listeria monocytogenes [8]. The production of large amounts of olive kernels as waste in olive processing industries, and given the growth of these industries in recent years, has led to the development of solutions aimed at the optimal use of these wastes in the food and pharmaceutical industries. Olive kernels are a lignocellulosic structure consisting of cellulose, hemicellulose and lignin. Olive kernels contain a lot of protein and polyphenols such as tyrosol, hydroxytyrosol, oleuropein [9]. Olive kernels, which comprise 18-22% of the olive weight, are a very rich source of useful and valuable compounds [10]. Olive kernels contain many minerals such as magnesium, calcium, potassium, silica and sulfur. In addition, the abundance of phenolic compounds in olive kernels has led to this food industry by-product being recognized as a substance with antioxidant and antimicrobial properties [11]. Maestri et al. (2019) investigated the composition of olive kernels. These researchers stated that olive kernels contain dietary fiber, lipid, and protein of 47, 30, and 17 percent, respectively, based on dry weight. These researchers also reported the amount of phenolic compounds in olive kernel extract to be 280 mg/100 g of dry matter in terms of gallic acid [12]. So far, based on the available information, since no research has been conducted on optimizing the extraction of olive kernel extract using the homogenizer

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probe method; Therefore, in this study, it was attempted to first extract olive kernel powders (Shenge variety) using 5 solvent systems (including 2 single-component water and ethanol solvent systems and 3 two-component water: ethanol 50, water 20: ethanol 80, and water 80: ethanol 20) for three times of 1, 2, and 3 minutes using a probe homogenizer with a power of 100 watts (half a second on and half a second off). Subsequently, the resulting extracts were filtered using Whatman No. 4 filter paper and their phenolic compounds were evaluated. In the next step, 5 samples with the highest phenolic compounds were selected and their antimicrobial properties were examined. Finally, the extract with the highest antimicrobial properties was selected as the optimal sample and its phenolic compounds were measured by GC-MS.

2- Materials and Methods

2-1 Materials and Equipment:

Olive kernels (Shanghe variety) were obtained from the Spino factory located around Tehran, microbial strains were obtained from the Center for the Collection of Industrial Microorganisms, ethanol, Folin-Ciocalteau and sodium carbonate were obtained from Sigma, and putative dextrose agar and Mueller-Hinton agar culture media were obtained from Merck.

2-2- Methods:

2-2-1- Optimization of olive kernel extract extraction:

After washing, olive kernels were first dried in natural conditions using a suitable environment and then dried in an oven at 40°C for 4 hours to reach the desired level of drying (10%) and then ground using an industrial grinder. The powders were placed in a freezer at -18°C until further experiments. To extract the extracts, for each treatment, 5 grams of core powder was poured into a beaker with 100 ml of solvents (2 single-component solvent systems of water and ethanol and 3 two-component solvent systems of water 50: ethanol 50, water 80: ethanol 20 and water 20: ethanol 80) and extracted using a probe with a power of 100 for three times of 1, 2 and 3 minutes (half a second on, half a second off). Subsequently, the resulting extracts were filtered using a Buchner funnel and Whatman filter paper No. 4 and placed in a refrigerator at 4 degrees Celsius until further experiments [13].

2-2-2 Measurement of total phenolic compounds of extracts:

The measurement of total phenolic compounds in the extracts was carried out according to the Folin-Ciocalte method (Capenci et al., 2000) [14]. According to this method, first 1 ml of extract was mixed with 2.5 ml of Folin-Ciocalteau reagent diluted 1:10 with double distilled water. After 8 minutes, 5 ml of 7.5% sodium carbonate was added and the volume was made up to 50 ml with distilled water. The resulting mixture was kept in the dark for 0.5 h and the absorbance of the samples was read at a wavelength of 760 nm. Total phenol was reported in terms of gallic acid according to the linear equation obtained from the standard diagram:

$A=0.0011C+0.0196$, $R2=0$

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A is the absorbance of the sample at 760 nm and C is the equivalent concentration of gallic acid (µg/ml).

2-2-3- Evaluation of antimicrobial properties of selected samples:

To evaluate the antimicrobial properties (Aspergillus niger 5298 PTCC and Escherichia coli 1399 PTCC), the method of Fasihi et al. (2017) was used with slight modifications.[15] In both cases, a microbial suspension equivalent to 0.5 McFarland (each 1 cc is equivalent to half McFarland, there are 1.5 × 108 bacterial cells) and the well diffusion method was used. Regarding the antifungal properties, after pouring putative dextrose agar culture medium into a sterile plate and surface cultivation of Aspergillus niger mold, 3 wells with a diameter of 5 mm were created in each plate and 50 µl of extract was poured into them and incubation was performed (25 degrees Celsius). The diameter of the growth-free zone around the wells indicated the antifungal properties of the extracts. Regarding the antibacterial properties, after pouring Mueller Hinton agar culture medium into a sterile plate and surface cultivation of Escherichia coli bacteria, 3 wells with a diameter of 5 mm were created in each plate and 50 µl of extract was poured into them and incubation was performed (37 degrees Celsius). Celsius). The diameter of the growth zone around the wells indicated the antibacterial properties of the extracts. In the following, and to ensure the accuracy of the work, the bacterial growth pattern in a liquid medium using optical absorption was used based on the method of Habibi et al. (2018) with slight modifications. [16] In this way, 100 µl of microorganisms were added to the Luria Broth (LB) culture medium containing the extract (9 cc of culture medium and 1 cc of extract), and the growth of the microorganism was evaluated for three consecutive days at temperatures (37 °C for bacteria and 25 °C for mold) by examining the absorption at a wavelength of 600 nm.

2-2-4- Identification of the optimal extract profile:

A GC-MS device was used to identify the optimal extract compounds. The length, diameter, and thickness of the inner layer of the column used were 30 m, 0.25 mm, and 25 µm, respectively. The column type was HP-5MS with a temperature program of 40-270 degrees Celsius with a temperature increase rate of 10 degrees Celsius per minute. Helium gas acted as a carrier gas with a flow rate of 1 ml per minute. The identification of phenolic compounds of the extract was carried out by comparing the mass spectrometer and their inhibition index with the spectral bank and standard inhibition index of the compounds, respectively. The amount of compounds was determined by the total area of the peaks using the device software [17].

2-2-5- Statistical analysis:

To analyze the data, a completely randomized design was used, and to determine the difference between the mean data after analysis of variance using the ANOVA method, Duncan's multiple range test was used at the 5% level. Statistical analysis of the results was performed using SPSS statistical software. Excel software was

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used to draw graphs and charts, and all tests were performed in 3 replicates.

3- Results and Discussion

3-1 Evaluation of the total amount of phenolic compounds of olive kernels extracted by different solvents:

The total amount of phenolic compounds of extracts extracted by different solvents is given in Table 1. As can be seen, the type of solvent and the extraction time have a significant effect ($P<0.05$) on the total amount of phenolic compounds of olive kernel extracts. In other words, with increasing extraction time, an increasing trend in the amount of phenolic compounds extracted was observed in all treatments; in most cases, this trend was significant ($P<0.05$). Regarding the effect of solvent type, according to the results obtained, treatments extracted with solvent (0 water: 100 ethanol) at all three times and then treatments extracted with solvent ethanol 80: 20 water at times 2 and 3 minutes had the highest levels of phenolic compounds; and were selected as the selected treatments.

Table 1: Comparison of the average amount of phenolic compounds of extracts extracted with different solvents (mg of gallic acid per 100 grams of dry matter)

run	Time (m)	Solvent type	The amount of phenolic compounds
1	1	Ethanol 0: water 100	261.72 ± 4.11^{Hb}
2	2	Ethanol 0: water 100	263.14 ± 3.21^{Hb}
3	3	Ethanol 0: water 100	270.12 ± 1.42^{Ga}
4	1	Ethanol 20: water 80	263.43 ± 2.36^{Hb}
5	2	Ethanol 20: water 80	265.16 ± 2.19^{Hb}
6	3	Ethanol 20: water 80	279.22 ± 6.87^{Fa}
7	1	Ethanol 50: water 50	269.12 ± 1.83^{Gb}
8	2	Ethanol 50: water 50	271.61 ± 3.23^{Gb}
9	3	Ethanol 50: water 50	284.23 ± 2.12^{Fa}
10	1	Ethanol 80: water 20	281.16 ± 4.22^{Fc}
11	2	Ethanol 80: water 20	290.35 ± 3.11^{Eb}
12	3	Ethanol 80: water 20	303.77 ± 2.43^{Da}
13	1	Ethanol 100: water 0	341.91 ± 2.13^{Cc}
14	2	Ethanol 100: water 0	352.02 ± 3.22^{Bb}
15	3	Ethanol 100: water 0	384.34 ± 4.12^{Aa}

Capital dissimilar letters indicate significant differences between all samples and small dissimilar letters indicate significant differences in each of the samples at different extraction times ($p < 0.05$).

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Due to the different chemical nature of phenolic compounds of plant origin, these compounds can be extracted by different methods and with different solvents. Among the factors affecting the extraction of phenolic compounds are the type, volume and saturation of the solvent, the temperature and number of times of the extraction process, as well as the type and nature of the plant [4]. The waves created in the probe homogenizer method, by forming a cavitation phenomenon, cause cell wall destruction and, as a result, the leakage of phenolic compounds by the solvent into the environment [18]. Kadir et al. (2023) concluded by examining the amount of phenolic compounds in olive fruit, leaves and kernels using the Soxhlet method that the amount of phenolic compounds in the ethanolic extract of olive fruit, olive leaves and kernels was 270, 280 and 317 micrograms in terms of gallic acid, respectively. These researchers stated that olive kernels are rich in phenolic compounds due to the presence of an oily viscous substance containing bioactive compounds [9]. The results of this study were consistent with the results obtained in this study. In another study, Dat et al. (2011) optimized the extraction conditions of olive kernel phenolics using different solvents and reported that the optimal treatment with methanolic solvent was obtained with an extraction time of 12 hours, a temperature of 70°C and 3 extraction stages. These researchers stated that increasing the extraction time increased the amount of phenolics in the extracts. On the other hand, the results obtained by these researchers showed that the alcoholic solvent had a higher extraction efficiency than water; which was consistent with the results of this study in both cases [19]. The difference in the amount of phenolics extracted by different solvents can be attributed to the difference in the polarity range of polyphenols; therefore, choosing the optimal solvent for the extraction of phenolics is a matter [20].

3-2 Results of microbial evaluations of selected samples:

Table 2 and Figures 1 and 2 show the antimicrobial properties of selected extracts from the previous steps (extracts extracted with ethanolic solvent at times of 1, 2 and 3 minutes and also extracts extracted with hydroalcoholic solvent of ethanol 80: water 20, at times of 2 and 3 minutes). According to the results obtained from the well diffusion test, three extracts extracted with ethanolic solvent with extraction times of 1, 2 and 3 minutes had antimicrobial properties. It should be noted that the hydroalcoholic samples showed negligible antimicrobial properties. On the other hand, in the optical absorption test that was performed to verify the results of the well diffusion test and select the optimal treatment (among the extracts with antimicrobial properties in the well diffusion test, namely ethanol extracts extracted at times of 1, 2 and 3 minutes), the results showed that the ethanol extracts extracted at all three times of 1, 2 and 3 minutes had antifungal and antibacterial properties. The results indicated that the ethanol extract extracted at time of 3 minutes had higher antimicrobial properties than the other samples; therefore, it was selected as the optimal treatment for subsequent experiments. Markin et al. (2003) studied the antimicrobial properties of the aqueous extract of olive leaves and stated that this extract has inhibitory power against Escherichia coli [21]. This was consistent with the results of this study. The reasons for the antimicrobial properties of this extract

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can be mentioned as the presence of phenolic compounds such as oleuropein and hydroxytyrosol [22]. Hameg et al. (2020) also investigated the antimicrobial properties of ethanolic extract of olive leaves and reported that this extract has high antimicrobial properties against Escherichia coli [23]. Which was consistent with the results of this study. Kadir et al. (2023) investigated the antimicrobial properties of ethanolic extract of olive kernels of the Halhali variety and stated that olive kernel extract has a high inhibitory ability against bacteria and fungi [9]; which was consistent with the results of this study. On the other hand, Younes et al. (2023) investigated the phenolic content and antimicrobial properties of ethanolic extract of olive kernels and stated that this extract does not have an inhibitory effect on the gram-negative bacterium Escherichia coli. These researchers attributed this to the absence of catechins in this extract [24]; which was inconsistent with the results of this study. On the other hand, the results obtained showed that increasing the amount of phenolic compounds increases the antimicrobial properties of the extracts. Phenolic compounds in extracts, by affecting the phospholipid layer of the cell wall of microorganisms, cause membrane destruction and release of contents, resulting in the death of microorganisms. On the other hand, phenolic compounds exert their antimicrobial effects by affecting the amino acid decarboxylase enzyme of microorganisms [25].

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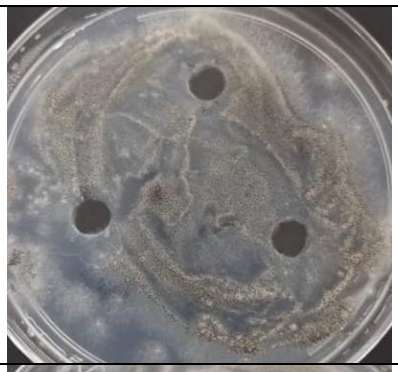
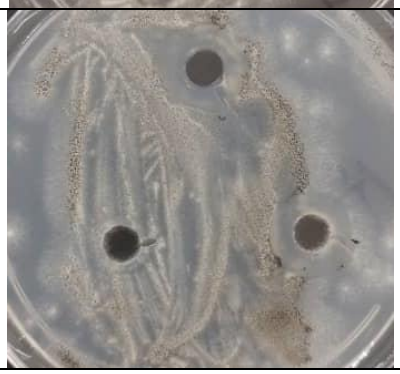
Table 2 Antimicrobial properties of selected samples

Variable / Treatment	Antifungal effects	Antibacterial effects
Ethanol 100: water 0 in 1 minute		
Ethanol 100: water 0 in 2 minutes		
Ethanol 100: water 0 in 3 minutes		

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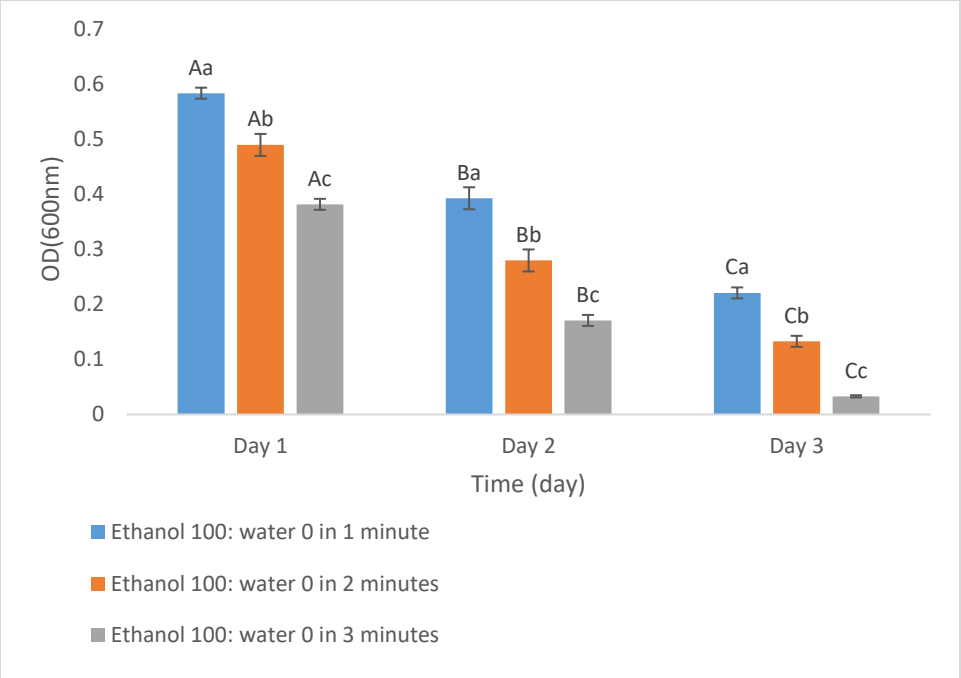

Fig 1: Antibacterial effects of ethanol extracts extracted in 1, 2 and 3 minutes. Capital dissimilar letters indicate significant differences in one sample on different days and small dissimilar letters indicate significant differences between different samples on the same day ($p < 0.05$).

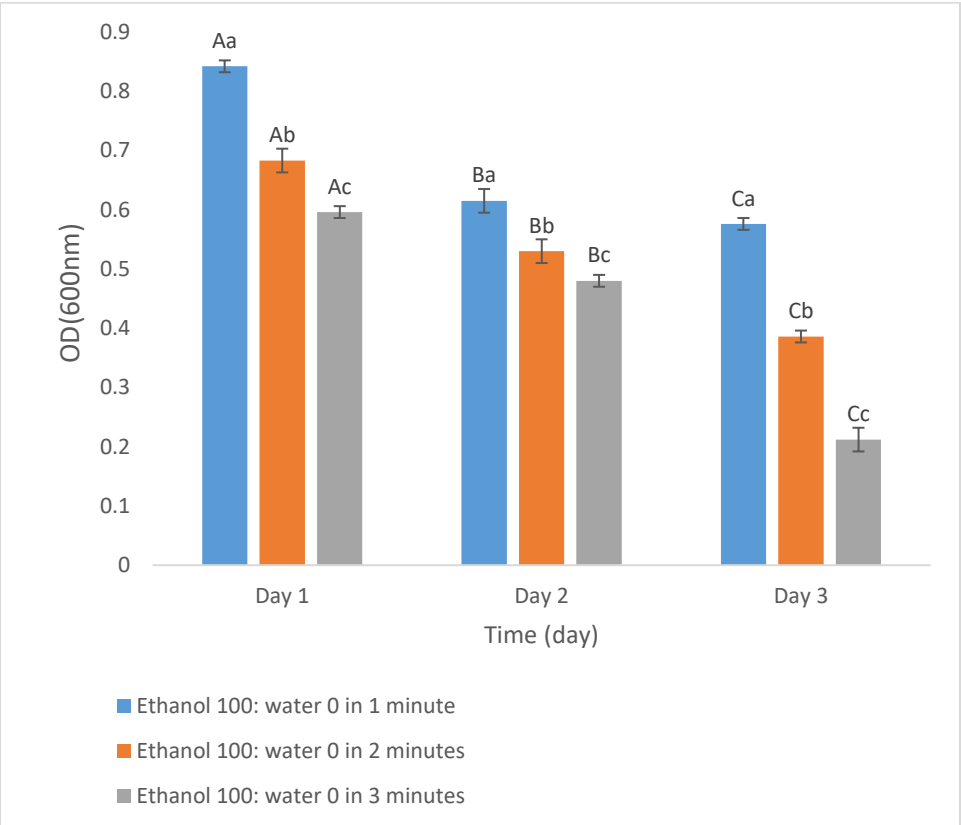

Fig 2: Antifungal effects of ethanol extracts extracted in 1, 2 and 3 minutes. (Capital dissimilar letters indicate significant differences in one sample on different days and small dissimilar letters indicate significant differences between different samples on the same day ($p < 0.05$)).

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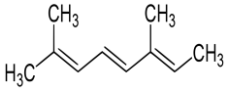
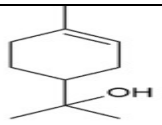
3-3 Phenolic Compound Profile of the Optimal Sample:

The type and amount of phenolic compounds present in olive kernel extract are given in Table 3. As can be seen, 13 main compounds were identified. The highest percentage of abundance was related to hydroxytyrosol and tyrosol. This was consistent with the results of Bosco (2010) who investigated the phenolic compounds of canned Greek olive fruit and kernel extracts[26]. However, the amount of these compounds was different from the present results; the reasons for this can be attributed to the olive variety. On the other hand, Malik and Bradford (2006) reported that the major complex phenolic compound in olive fruit, kernel and leaf is oleuropein; which is degraded to other phenolic compounds, including hydroxytyrosol, during maturation[27]; which was consistent with the results of this study. The results of this study, regarding the higher levels of hydroxytyrosol and tyrosol, were consistent with the results of Malamasi et al. (2021) [28] and Goncalouz et al. (2019) [29]. In another study, Ben Mansour et al. (2011) investigated the phenolic profile of olive kernels collected from Tunisian trees; the results showed that the phenolic compound nugenide had the highest level[30]; which was inconsistent with the results of the present study. The reasons for this discrepancy can be attributed to the olives selected for the experiment. In other words, unlike the present study, which used olive kernels after processing; those researchers used olive kernels on the tree and before processing for the experiment. It should be noted that the extraction process in factories destroys complex phenolic compounds. Other reasons for this include the olive variety and the type of solvent used for extraction. Because these researchers used 80% methanol solvent to extract phenolic compounds. Other phenolic compounds found in olive kernels include ligosteroids. Ligotrosides are a common phenolic compound found in all parts of the olive, such as leaves, fruit, and olive kernels; but they have been reported much less in olive kernels [31]. Regarding the presence of ligosteroids in olive kernels, the results of this study were consistent with those of Maestri et al. (2019) [9].

Table 3: The type and amount of phenolic compounds of the extracted extracts of the optimal treatment (mg of polyphenol per 100 grams of olive seed)

Run	RT(min)	Amount (%)	Phenolic compound	Chemical structure
1	6.134	0.51	Alloocimene	
2	8.53	0.23	ALPHA. TERPINEOL	

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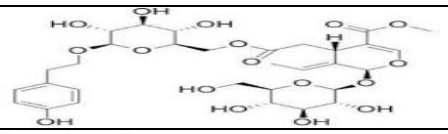
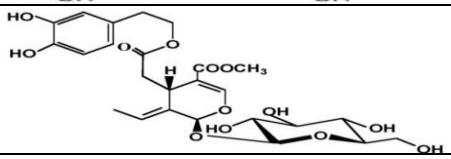
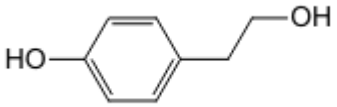
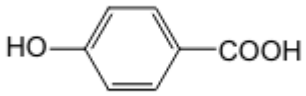
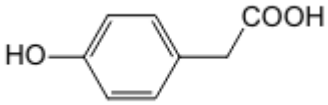
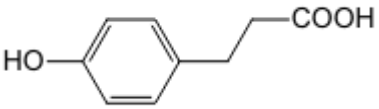
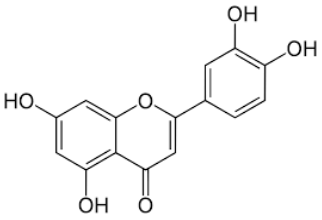
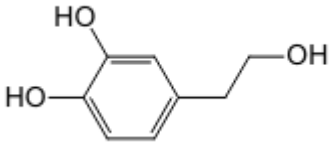
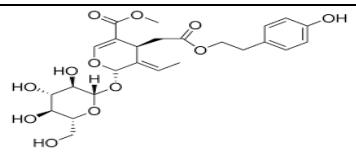
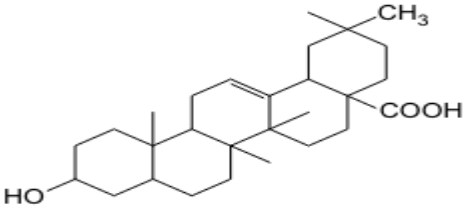
3	11.06	5.52	nuezhenide	
4	11.44	7.34	Oleuropein	
5	14.03	12.65	Tyrosol	
6	15.63	0.49	p-Hydroxy-benzoic acid	
7	17.285	0.57	p-Hydroxyphenylacetic acid	
8	18.5	11.4	Phloretic acid	
9	18.8	2.96	Luteolin	
10	18.92	28.12	Hydroxytyrosol	
11	19.36	0.46	Ligstroside	
12	22.78	8.35	Oleanolic acid	

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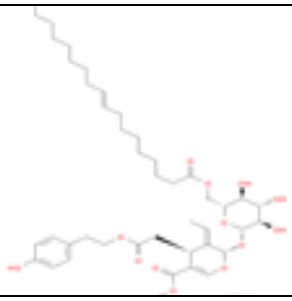
13	25.596	0.57	Jaspolyanoside	

4- Conclusion

In this study, the optimization of olive kernel extract extraction using different solvents at different times was investigated using the probe homogenizer method. The results showed that the ethanol extract extracted at 3 minutes had the highest amount of phenolic compounds. The type of solvent and the extraction time had a significant effect ($P<0.05$) on the total amount of phenolic compounds in olive kernel extracts. In other words, with increasing extraction time, an increasing trend in the amount of phenolic compounds was observed in all treatments; in most cases, this trend was significant ($P<0.05$). Regarding the effect of the solvent type, according to the results obtained, the treatments extracted with the solvent (0 water: 100 ethanol) at all three times and then the treatments extracted with the solvent 80 ethanol: 20 water at 2 and 3 minutes had the highest amount of phenolic compounds; and were selected as the selected treatments. Regarding the antimicrobial properties of the selected extracts, according to the results obtained from the well diffusion test, three extracts extracted with ethanolic solvent with extraction times of 1, 2 and 3 minutes had antimicrobial properties. It should be noted that the hydroalcoholic samples showed negligible antimicrobial properties. On the other hand, in the optical absorption test that was performed to ensure the results of the well diffusion test and to select the optimal treatment (among the extracts with antimicrobial properties in the well diffusion test, namely the ethanolic extracts extracted at times of 1, 2 and 3 minutes), the results showed that the ethanolic extracts extracted at all three times of 1, 2 and 3 minutes had antifungal and antibacterial properties. It should be noted that the ethanolic extract extracted at time of 3 minutes was selected as the best treatment and its phenolic compounds were measured by GC-MS. The results showed that the most.

5-References

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مقاله علمی_پژوهشی

بررسی شرایط استخراج عصاره هسته زیتون با استفاده از دستگاه هموژنایزر اولتراسونیک و ارزیابی خصوصیات ضد میکروبی آن‌ها

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چکیده

هدف از مطالعه حاضر بررسی شرایط استخراج عصاره هسته زیتون با استفاده از دستگاه هموژنایزر اولتراسونیک و بررسی خصوصیات ضد میکروبی عصاره‌های دارای بالاترین میزان ترکیبات فنلی بود. طبق نتایج، عصاره اتانولی استخراج شده در زمان ۳ دقیقه، دارای بالاترین میزان ترکیبات فنلی می‌باشد. نوع حلال و مدت زمان استخراج، دارای تاثیر معنی‌داری ($P<0/05$)، روی مقدار ترکیبات فنلی عصاره‌های هسته زیتون می‌باشد. همچنین، تیمارهای استخراج شده با حلال (آب ۰ : اتانول ۱۰۰) در هر سه زمان (۱، ۲ و ۳ دقیقه) و سپس تیمارهای استخراج شده با حلال اتانول ۸۰: آب ۲۰ در زمان‌های ۲ و ۳ دقیقه دارای بالاترین میزان ترکیبات فنلی بودند؛ و به عنوان تیمارهای منتخب جهت انجام آزمون‌های میکروبی، انتخاب شدند. در ارتباط با خصوصیات ضد میکروبی، طبق نتایج بدست آمده از آزمون انتشار چاهک، هر سه عصاره اتانولی، دارای خصوصیات ضد میکروبی بودند. از سوی دیگر، در آزمون اندازه گیری چگالی نوری که جهت انتخاب تیمار بهینه صورت گرفت، نتایج نشان داد که عصاره اتانولی استخراج شده در زمان ۳ دقیقه، دارای بالاترین خاصیت ضدمیکروبی می‌باشد؛ در نتیجه این عصاره به عنوان بهترین تیمار انتخاب و ترکیبات فنلی آن بوسیله دستگاه GC-MS اندازه گیری شد. نتایج نشان داد که، بیشترین درصد فراوانی ترکیب فنلی در این عصاره مربوط به هیدروکسی تایروزول و تایروزول می‌باشد.

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