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Evaluation of antioxidant and antimicrobial properties of ethanolic extracts extracted from Caper (*Capparis spinosa*) fruit by ultrasound method using the RSM

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ABSTRACT

Extracts and essential oils of medicinal plants have many bioactive agents, including phenolic compounds with significant antioxidant and antimicrobial properties. Choosing the suitable extraction method affects the quantity and quality of antioxidant and antimicrobial compounds. The aim of this study was to evaluate the efficiency of ultrasound waves in extracting phenolic, antioxidant and antimicrobial compounds of Caper fruit. Response surface methodology (RSM) and Box–Behnken design were used to optimize the extraction factors, including extraction time (10, 25, 40 min) and ultrasound intensity (40, 70, 100 %) with ethanolic solvent (70 %). From the results of the tests performed with the response surface methodology, ultrasound intensity was recognized as the most effective factor in extracting phenolic, antioxidant and antimicrobial compounds of Caper fruit and by increasing time and ultrasound intensity, the extraction of these compounds increased. The optimum conditions for extraction of antioxidant and antimicrobial compounds of Caper fruit were determined extraction time 36 minutes and ultrasound intensity 91 percent. In these optimum conditions, the amount of total phenolic contents and IC_{50} index of ethanolic extracts extracted from Caper fruit were obtained 23.63 mg/g and 45.30 µg/mg, respectively, the optimum amount of minimum inhibitory concentration of *coagulase positive Staphylococcus aureus* and *Bacillus cereus* were obtained 0.19 and 6.07 mg/ml, respectively, and the optimum amount of minimum batericidal concentration of *coagulase positive Staphylococcus aureus* was obtained 11.81 mg/ml. Also, the extracts extracted at time 36 minutes and ultrasound intensity 91 percent had the ability to inhibit the activity of *Escherichia coli O157: H7* and *Pseudomonas aeruginosa* at concentrations of 25 and 25 mg/ml, respectively, and had the batericidal ability of *Bacillus cereus*, *Escherichia coli O157: H7* and *Pseudomonas aeruginosa* at concentrations of 25, 50 and 50 mg/ml, respectively.

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1- Introduction

In recent years, bioactive phytochemical compounds derived from specific medicinal plants have received considerable attention for their potential to improve public health indicators, particularly in traditional medicine [1]. A significant portion of traditional medicine treatment is dedicated to essential oils and extracts extracted from medicinal plants containing a wide range of bioactive compounds [2]. Bioactive compounds are classified into two categories: molecules (such as carotenoids, polyphenols, fatty acids, peptides, proteins, and plant sterols) and living cells (probiotics) [3]. Major bioactive compounds include phenolic compounds, which have beneficial effects such as anti-allergic, anti-inflammatory, antimicrobial, and antioxidant properties [4]. Generally, compounds with antioxidant properties are divided into natural and synthetic categories. Synthetic antioxidants are phenolic compounds containing various degrees of alkyl substitutes, while natural antioxidants can be phenolic compounds such as quinones and lactones [5]. Phenolic compounds act as antioxidants through their free hydroxyl group on their aromatic ring, and their antioxidant activity depends on the number and position of hydroxyl groups they possess [6].

The extraction process is a vital stage in obtaining valuable phytochemical compounds from plant sources [7]. For the extraction of phenolic compounds from medicinal plants, traditional methods such as Soxhlet and maceration, as well as modern technologies including supercritical fluid, microwave, and ultrasound technologies, can be employed [8, 9]. Modern technologies, compared to traditional extraction methods, lead to reduced extraction time, decreased solvent consumption, increased extraction yields, and improved extract quality. Ultrasound-assisted extraction (UAE) facilitates suitable solvent penetration into plant tissues and offers better extraction speed and efficiency compared to other extraction methods [10]. The cavitational collapse temperature and pressure resulting from new ultrasound technology cause the destruction of the plant cell structure, leading to increased solubility of polyphenols and accelerated mass transfer processes [11]. The heat energy generated by ultrasound waves increases the solvent's temperature, reducing its surface tension and thus enhancing the moistening of plant materials and more efficient extraction [12]. Probe and bath systems are common methods of using ultrasound waves. The ultrasound bath is suitable for large-scale industrial applications, provides high repeatability, and significantly reduces the risk of damage to bioactive compounds, contamination, and sample loss compared to ultrasound probe systems [13, 14].

The Caper plant known as *Capparis spinosa* L. belongs to the Capparaceae family. It is a perennial herb with a woody branched root, multiple stems mostly sprawling on the ground, circular and oval leaves, white and sometimes red petals, and ovoid fruit

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containing numerous black bean-shaped seeds. Caper fruit resembles a watermelon but is about the size of a hazelnut, sometimes larger [15]. There are around 250 species of Caper plant, many of which are wild, thriving in arid and semi-arid lands in tropical and subtropical regions, with significant resilience to harsh conditions such as drought, tolerance of temperatures exceeding 40^0C in summer, down to 8^0C in winter, and suitable growth in limestone soils [16, 17].

Among the bioactive factors present in the Caper plant, mention can be made of saccharides, glycosides, flavonoids, alkaloids, indoles, phenolic acids, terpenoids, volatile oils, fatty acids, vitamin C, vitamin E, and steroids [18, 19]. The most important phytochemical compounds in the fruit of the Caper plant include Cappariside, rutin, homogalacturonan, protocatechuic aldehyde, ethyl 3 and 4-dihydroxybenzoate, syringic acid, vanillic acid, and alpha-tocopherol [20].

One of the active ingredients in Caper plant is the fruit, which has been shown to have protective effects on the liver, reduce glucose levels, and reduce serum triglycerides and cholesterol levels [21, 22, 23]. Alcoholic extracts from Caper fruit have demonstrated inhibitory antioxidant activity against the activity of gram-positive bacteria (such as *Staphylococcus aureus* and Bacillus species) and gram-negative bacteria (such as *Escherichia coli* and *Pseudomonas aeruginosa*) in biological and chemical tests [24, 25, 26]. To date, various studies have optimized the conditions for aqueous and alcoholic extraction of antioxidant and antimicrobial compounds from Caper shoot, leaves, and roots using ultrasound technology [9, 27, 28].

Given the prevalent trend of using naturally extracted compounds from medicinal plants and the application of traditional medicine in preventing and treating various diseases, this study aims to investigate optimal conditions for the alcoholic extraction of phenolic, antioxidant, and antimicrobial compounds from Caper fruit.

2. Materials and methods

1.2 Chemicals and culture media

Solutions and chemicals used in the tests were of analytical grade. The folin-ciocalteu reagent, gallic acid powder, anhydrous sodium carbonate powder, 96% ethyl alcohol (ethanol), Nutrient Agar, Tryptic Soy Agar, Muller Hinton Agar, and Muller Hinton Broth were obtained from Merck Co, Germany. Additionally, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) powder was obtained from Sigma Co, Germany.

2.2 Plant identification and preparation of plant fruit powder

In collaboration with the herbarium and systematic botanical laboratory of Islamic Azad University, Jiroft branch, the Caper plant was collected from agricultural lands in Anbarabad County, Kerman Province, and identified. The Caper fruit was harvested in the July month of 2019 year and dried completely at room temperature in a dark environment.

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Subsequently, the Caper fruit powder was prepared using a laboratory mill to achieve uniform dimensions with a 40 mesh sieve passage.

3.2 Extraction of antioxidant compounds of Caper fruit

1.3.2 Extraction of ethanolic extract using ultrasound

A mixture of 50 gr of powdered plant fruit and 200 ml of 70-degree ethanol solvent was prepared. This mixture was placed in an ultrasound bath model JK-DUC-8200LHC, manufactured in China, with a constant frequency of 35 kHz and at room temperature. The independent variables of ultrasound extraction included three levels of ultrasonication time (10, 25, and 40 min) and three levels of ultrasound wave's intensity (40, 70, and 100%). After completing the ultrasound treatment, the extracted solution was centrifuged at 7800 rpm for 30 minutes, and the upper clear part of the tubes was isolated using a pipette through a 0.45 micron diameter filter. Concentration and extraction of the solution were carried out using a rotary vacuum evaporator at 40^0C and 200 rpm to preserve phenolic and antioxidant compounds. To remove any remaining solvent, the concentrated extract was transferred to a plate and placed in a vacuum oven at 40^0C until complete dryness. The extracts were then stored in a freezer at -18^0C until testing.

4.2 Chemical tests

1.4.2 Total phenolic contents (TPC) measurement

The total phenolic content present in the plant fruit extract was evaluated using the folin-ciocalteu colorimetric method [9]. In this method, 100 µl of a 1000 ppm extract solution, transferred to a test tube using a pipette, was thoroughly mixed with 500 µl of folin-ciocalteu reagent and 1 ml of distilled water. After 1 minute, 1.5 ml of 20% sodium carbonate solution at room temperature was added to the tube, which was uniformly shaken with a tube shaker. The tube's contents were kept in a dark environment at room temperature for 2 hours, after which the absorption amount of the solution was read by an Aquarius model 7500 series spectrophotometer from CECIL Co, England, at a wavelength of 760 nanometers. The total phenolic content amount present in the plant fruit extract was reported based on the drawn line equation according to the gallic acid and in mg/g of the extract (Equation 1).

$Y=4/0458X + 0/0523$

$R^2=0/9923$ Equation 1

In this equation, X represents the absorption amount, and Y indicates the total phenolic contents according to mg gallic acid equivalents (GAE) per gram of extract.

2.4.2 Free radical scavenging activity

To determine the level of free radical scavenging activity, the extracts from the plant's fruit were subjected to the DPPH assay using 2,2-Diphenyl-1-picrylhydrazyl reagent [9]. In this assay, 5 ml of 0.004% DPPH ethanol solution were added to 50 µl of various concentrations of the prepared extracts using ethanol as a solvent. After mixing, they were held in a dark environment at room temperature for 30 minutes, and then the absorption of the samples and the control at

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517 nanometers was measured using a spectrophotometer. The DPPH free radical scavenging activity was measured using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Blank absorbance value} - \text{Sample absorbance value}}{\text{Blank absorbance value}} \times 100$$

5.2 Microbial tests

1.5.2 Preparation of a mother concentration from the alcoholic extract of plant fruit

The mother concentration of 200 mg per ml of dried alcoholic extract powder of the plant fruit was prepared using a 5% dimethyl sulfoxide solution and sterilized by passing it through a microbial filter with a pore size of 0.45 microns.

2.5.2 Preparation of a bacterial half McFarland suspension

Lyophilized ampoules of *Staphylococcus aureus coagulase positive* PTCC 1431, *Bacillus cereus* PTCC 1015, *Escherichia coli O157:H7*, PTCC 1860, and *Pseudomonas aeruginosa*, PTCC 1430 were purchased from the iranian industrial and scientific researchs organization and revived as per the manufacturer's instructions. Based on their growth, an equivalent to the standard half McFarland turbidity (1.5×10^8 cfu/ml) was prepared and their absorption at 625 nm, equivalent to 0.08-0.13 was determined using a spectrophotometer [29].

3.5.2 Determination of minimum inhibitory concentration (MIC) of growth

For the determination of the minimum inhibitory concentration of growth, the alcoholic extracts from the plant fruit were subjected to the microdilution method [30]. In this method, 95 µl of Mueller-Hinton broth sterile culture medium with double concentration was added to the microplates of 96 houses, first in 12 wells of each row. Then 100 µl of the mother concentration of 200 mg/ml alcoholic extracts were transferred to the first well and a concentration of 100 mg/ml was prepared from the extracts. Next, 100 µl were transferred from the first well to the second well and a concentration of 50 mg/ml was prepared from the extracts. This procedure was continued for all the wells except the well number 12 which was considered as positive control. Well number 11 was considered as negative control. At the end, 100 µl were removed from well number 11 and discarded. Next, 5 µl of the bacterial suspension equivalent to the standard turbidity of half McFarland was added to each of the wells except for well number 11, then the microplate was shaken for 7 seconds in the ELISA shaker and the initial turbidity of the wells was read at a wavelength of 625 nm. Next, the microplate was kept in a 37^0C incubator for 24 hours, and after this time, the amount of absorption or turbidity of the wells was read and compared again by the ELISA device. The lowest concentration of the extract that inhibited the growth of bacteria was considered as the

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minimum inhibitory concentration. The treatments were done in three replicates.

4.5.2 Determination of minimum bactericidal concentration (MBC)

The minimum bactericidal concentrations were determined based on the values of the minimum inhibitory concentrations for each extract. In this method, the microplate wells were inoculated by those wells in which bacterial growth had been stopped, and the lowest concentration at which 99.9% of the bacteria did not grow, or in other words, had no bacterial growth, was considered as the minimum bactericidal concentration. The tests were repeated three times to confirm the results [24].

6.2 Statistical method

The design expert software version 12 was used for the analysis of the independent variables' effect (time and sound intensity) on the total phenolic contents, free radical scavenging activity, and antimicrobial properties of the extracted fruit extracts from the Caper plant using the response surface statistical method. A Box-Behnken design with two variables set at three levels was chosen, and 13 different treatments were proposed to examine the trend of extraction levels and to determine the optimal conditions (Tables 1, 2, and 3).

Table 1. Treatments designed in response surface test and response values for antioxidant tests of alcholic extracts of Caper fruit

of Fruit Alcholic Extracts IC_{50} (µg/mg)	TPC of Fruit Alcholic Extracts (mg/g)	Sound Intensity (X_2) (%)	Exreaction Time (X_1) (min)	Treatment
87.92	12.82	49	14	1
90.74	12.02	40	25	2
85.22	15.10	49	36	3
81.52	16.48	70	10	4
70.10	18.90	70	25	5
62.50	19.50	70	25	6
65.41	19.32	70	25	7
68.61	19.29	70	25	8
60.21	20.10	70	25	9
54.35	21.14	70	40	10
51.62	22.16	91	14	11
46.87	24.10	100	25	12
47.67	23.22	91	36	13

Table 2. Treatments designed in response surface test and response values for antimicrobial tests of alcholic extracts of Caper fruit

MIC of *Pseudomonas aeruginosa* of Fruit	MIC of *Escherichia coli O157:H7* of	MIC of *Bacillus cereus* of Fruit	MIC of *Staphylococcus aureus* of Fruit Alcholic	Sound Intensity (X_2) (%)	Exreaction Time (X_1) (min)	Treatment

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Alcholic Extracts (mg/ml)		Fruit Alcholic Extracts (mg/ml)		Alcholic Extracts (mg/ml)		Extracts (mg/ml)						
>100	>100	50	1.56	49	14	1	100	100	12.50	0.39	70	25	8
>100	>100	50	1.56	40	25	2	100	100	12.50	0.39	70	25	9
>100	>100	25	0.78	49	36	3	100	50	12.50	0.39	70	40	10
>100	>100	25	0.78	70	10	4	50	50	6.25	0.19	91	14	11
100	100	12.50	0.39	70	25	5	25	25	6.25	0.19	100	25	12
100	100	12.50	0.39	70	25	6	25	25	6.25	0.19	91	36	13
100	100	12.50	0.39	70	25	7							

Table 3. Treatments designed in response surface test and response values for antimicrobial tests of alcholic extracts of Caper fruit

MBC of *Pseudomonas aeruginosa* of Fruit Alcholic Extracts (mg/ml)	MBC of *Escherichia coli O157:H7* of Fruit Alcholic Extracts (mg/ml)	MBC of *Bacillus cereus* of Fruit Alcholic Extracts (mg/ml)	MBC of *Staphylococcus aureus* of Fruit Alcholic Extracts (mg/ml)	Sound Intensity (X_2) (%)	Exreaction Time (X_1) (min)	Treatment
>100	>100	>100	100	49	14	1
>100	>100	>100	100	40	25	2
>100	>100	100	50	49	36	3
>100	>100	100	50	70	10	4
>100	>100	50	25	70	25	5
>100	>100	50	25	70	25	6
>100	>100	50	25	70	25	7
>100	>100	50	25	70	25	8
>100	>100	50	25	70	25	9
>100	100	50	25	70	40	10
100	100	50	25	91	14	11
50	50	25	12.50	100	25	12
50	50	25	12.50	91	36	13

3. Results and discussion

1.3 Selection of best model

The results of statistical analysis using the response surface methodology indicated that among the first-order models (Linear, 2FI, and Mean), the second-order (Quadratic), and third-order (Cubic) models, the Quadratic model showed a significant difference from other models in all the measured tests in this study, and its lack of fit was also not significant. Therefore, this model was chosen for the statistical analysis of the tests. After selecting the best model, for determination of the general equation based on the analysis of variance (ANOVA) table, the parameters for which their F-test was not significant (P>5%) were removed from the model, and other parameters with significant differences at a 95% level were retained in the model. Subsequently, the general equation was determined using the coefficients assigned to each parameter.

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2.3 Effect of time and sound intensity on the extraction of phenolic compounds from the Caper Plant Fruit

According to Figure 1, representing the simultaneous effect of time and sound intensity on the level of total phenolic contents extracted from the Caper plant fruit, it was observed that the extraction trends of phenolic compounds from the fruit increased with the increase in time and sound intensity.

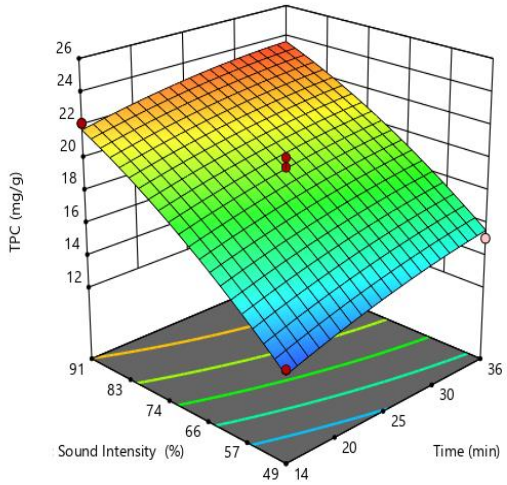

Figure 1. Three-dimensional diagram, the simultaneous effect of two extraction time and sound intensity variables on the amount of total phenolic contents of alcoholic extracts of Caper fruit

The average amount of total phenolic contents extracted from the Caper plant fruit with ethanol as a solvent using the ultrasound method was determined to be 18.78±3.72 mg/g. The high determination coefficient between the real and predicted values (0.9873 and 0.9352, respectively) indicated a very good correlation between the results obtained from experimental testing or method and the predicted amounts of total phenolic extraction by statistical methods (Table 4). Safarzaei et al. (2020) in the extraction of phenolic and antioxidant compounds from the roots of the caper plant with ethanolic and aqueous solvents by ultrasonic method reported that there is an increasing trend in the amount of extraction with the passage of time. The time factor is effective in increasing the duration of mass transfer [9]. The presence of acoustic energy due to ultrasound waves leads to physical breakdown of cell walls and membranes of plant cells and an increase in mass transfer between plant tissues and the extraction medium. Additionally, the ultrasound intensity leads to a reduction in particle size and an increase in the extraction efficiency [31]. The thermal energy generated as a result of ultrasound waves increases the extraction efficiency by disrupting the cellular structure of the plant, resulting in increased permeability of the cell membrane and decomposition of metabolites derived from matrix interactions (polyphenols with lipoproteins) and hence, an increase in the solubility and mass transfer of polyphenols. In addition, high temperature leads to the reduction of the viscosity of the extraction medium and the penetration of the solvent into the plant particles, and hence, improves and accelerates the extraction process [12].

Albu et al. (2004) in a study investigated the effect of ultrasound intensity on the extraction of antioxidant compounds from rosemary plant with butanol, ethylene acetate and ethanol solvents and reported that the effect of ultrasound on the solvent of ethanol, which is a solvent under normal conditions has reached the same level of extraction with two other solvents [32].

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Considering the significant parameters in the extraction process of phenolic compounds from the Caper plant fruit according to the analysis of variance table (Table 4), the general equation can be reported as follows:

Equation 2: General equation for determining the level of phenolic compounds extracted from the Caper plant fruit with ethanol as a solvent using the ultrasound method

$Y=19.42 + 1.24 X_1 + 4.32 X_2 - 0.71 X_2^2$

In this equation, Y represents the amount of phenolic compounds extracted from the Caper plant fruit in terms of mg/g, X_1 represents the time in terms of minutes, and X_2 represents the ultrasound intensity as a percentage. According to Equation 2, the ultrasound intensity (X_2) was identified as the most effective factor for the extraction of phenolic compounds from the Caper plant fruit with ethanol as a solvent. The optimal conditions for the extraction of total phenolic contents from the Caper plant fruit with ethanol as a solvent using the ultrasound method were determined to be 36 minutes for time and 91% for ultrasound intensity, resulting in 23.63 mg/g of extracted total phenolic contents under these optimal conditions (Table 5). Hematian et al. (2020) reported 20.01 mg/g for the level of total phenolic contents in ethanolic extracts from the Caper plant fruit using the maceration method [33]. Rashedi et al. (2015) also determined 8.14 mg/g for the level of total phenolic contents in methanol extracts from the Caper plant fruit grown in the Khuzestan province using the maceration method [34]. Bhoyar et al. (2018) also reported 7.4 mg of gallic acid equivalent per gram for the level of total phenolic contents in methanol extracts from the fruit of Caper plants in the Trans-Himalayan region using the maceration method, which is lower compared to the optimal level in the present study [25]. This difference in the level of total phenolic contents in the extracted fruit of the Caper plant in the present study compared to the above-mentioned studies can be attributed to the efficient impact of ultrasound waves in enhancing the trend of extracting phenolic compounds from the fruit of the plant compared to traditional extraction methods such as maceration. Moreover, Arrar et al. (2013) reported 58.8 mg/g for the level of total phenolic contents extracted from the Caper plant fruit with methanol as a solvent using the maceration method, which was higher compared to the optimal level in the present study [35]. Additionally, Mahboubi and Mahboubi (2014) reported 31.7 mg/g for the level of total phenolic contents extracted from the Caper plant fruit with 70% ethanol as a solvent using the maceration method [24]. In the present study, the optimal level of total phenolic contents extracted from the Caper plant fruit was lower than this level, and this difference may stem from the differences in environmental conditions and climatic factors of the plant's growth location [36]. Dehghan Tanha et al. (2019) focused on optimizing the extraction conditions of phenolic compounds in red pepper fruit using methanol as a solvent through the response surface methodology, and reported the highest amount of phenolic

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compounds in red pepper 49.6 mg/g at 49^0C, 39 minutes, and 89% ultrasonic bath intensity. They demonstrated that an increase in time and ultrasonic intensity led to an increase in the extraction of phenolic compounds in red pepper, identifying ultrasonic intensity as the most influential parameter in the extraction, which aligns with the findings of the present study [37].

Table 4. ANOVA of the quadratic model of total phenolic contents of alcholic extracts of Caper fruit

Source	Sum of Squares	df	Mean Squares	F-Value	P-Value
Model	165.76	5	33.15	109.03	<0.0001
X_1	12.33	1	12.33	40.54	0.0004
X_2	149.16	1	149.16	490.54	<0.0001
$X_1 X_2$	0.3721	1	0.3721	1.22	0.3052
X_1^2	0.7737	1	0.7737	2.54	0.1547
X_2^2	3.49	1	3.49	11.48	0.0116
Residual	2.13	7	0.3041		
Lack of fit	1.36	3	0.4541	2.37	0.2114
Pure error	0.7661	4	0.1915		
R^2	0.9873				
Adj. R^2	0.9783				
Pred. R^2	0.9352				

Table 5. Results of the optimization process for antioxidant and antimicrobial tests of alcholic extracts of Caper fruit

Desirability	MBC of *Staphyloccus aureus* (mg/ml)	MIC of *Bacillus cereus* (mg/ml)	MIC of *Staphyloccus aureus* (mg/ml)	IC_{50} (µg/mg)	TPC (mg/g)	Sound Intensity (%)	Time (min)	Optimal Points
0.979	11.81	6.07	0.19	45.30	23.63	91	36	1
0.977	12.78	6.84	0.21	45.71	23.60	91	34	2
0.974	13.50	7.36	0.22	46.07	23.57	91	33	3
0.965	14.48	8.02	0.24	46.74	23.49	91	32	4

3.3 Effect of time and sound intensity on IC_{50} value of alcoholic extracts of plant fruit

Figure 2 illustrates the effect of time and sound intensity on the IC_{50} value of extracted extracts from the Caper plant fruit. Simultaneously, an increase in the duration of ultrasound exposure and the intensity of ultrasound waves indicates a noticeable reduction in the IC_{50} value of extracted extracts from the plant fruit. The IC_{50} index refers to the concentration of an extract capable of inhibiting 50% of DPPH free radicals, effectively halving the levels of DPPH free radicals. The assessment of DPPH free radical inhibition, effectively measuring the antioxidant activity of the extracts, is a common method and is identifiable through the discoloration of the DPPH solution due to the antioxidants present in the extracts [9]. DPPH is a type of stable free radical that produces a purple-colored solution in methanol at room temperature. When free radicals react with antioxidants, their free radical property diminishes, causing their color to change from purple to pale yellow. The color change in the solution results from the transfer of a hydrogen atom from the antioxidant to the free radical. Antioxidants reduce DPPH free radicals to a more stable

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form [38]. Therefore, extracts that are able to inhibit 50% of DPPH free radicals at lower concentrations will have higher antioxidant power. The study found that the average IC_{50} value of the ethanol extracts from the Caper plant fruit using the ultrasound method were 67.13±15.16 µg/mg. Predicted IC_{50} results by the model through statistical analysis had a very strong correlation with those obtained experimentally (with coefficients of determination of 0.8671 and 0.9245, respectively) (Table 6).

Based on the significant parameters in the extraction process of antioxidant compounds from Caper plant fruit (Table 6), the general equation could be reported as follows:

Equation 3: General equation for determining the IC_{50} value of extracted extracts from the Caper plant fruit using ethanol as a solvent

$$Y=65.37 – 5.63 X_1 – 16.99 X_2$$

In this equation, Y represents the IC_{50} value of the extracted extracts from the Caper plant fruit in terms of µg/mg, X_1 represents the time in terms of minutes, and X_2 represents the ultrasound intensity as a percentage. Following equation 3, ultrasound intensity was identified as the most influential factor in the extraction of antioxidant compounds from the Caper plant fruit. The optimal conditions for IC_{50} of the extracted extracts from the plant fruit with ethanol using the ultrasound method were determined to be 36 minutes for time and 91% for ultrasound intensity, resulting in an IC_{50} value of 45.30 µg/mg under these optimal conditions. Zia-ul-Haq et al. (2011) in a study reported the antioxidant activity of methanolic extracts extracted from the fruit of Caper plant using maceration method by determining the IC_{50} index, 69.1 µg/ml [39], which compared to the optimal amount of the present study, extracts extracted from the fruit of the plant have less antioxidant activity. In a study, Bhoyar et al. (2018) also reported the IC_{50} value of methanolic extracts extracted from the fruit of Caper plant in the Trans-Himalayan region by maceration method as 0.097 mg/ml [25], which compared to optimums of the present study had less antioxidant activity. In a study, Rashedi et al. (2015) reported the IC_{50} value of the methanolic extracts of the fruit of the Caper plant in Khuzestan province to be 6.11 µg/mg [34], which have more antioxidant activity compared to the optimal amount of the present study. Aliyazicioglu et al. (2013) in a study reported the average inhibitory concentration of the compounds of the extract of the Caper plant by the ultrasonic bath method as equal to 0.32 mg/ml [36], which showed a lower antioxidant activity compared to the optimal amount of the present study. Mahboubi and Mahboubi (2014) in a study also reported the IC_{50} value of the extracts extracted from the fruit of the Caper plant with 70% ethanol solvent by maceration method as 560 µg/ml [24], which compared to the optimal level of the current study, they had less antioxidant activity. Hematian et al. (2020) also reported in a study, the IC_{50} value of ethanolic extracts extracted from the fruit of the Caper plant by maceration method was 1.48 mg/ml [33], which compared to the optimal value of the present study has less

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antioxidant activity. In general, the reason for this difference in the amount of antioxidant activity of plant fruit extracts in numerous studies is due to the effect of environmental conditions on plant growth, the difference in the species and habitats of the Caper plant, the difference in the time of harvesting the plant fruit, and also the solvent effect and type of extraction method [9].

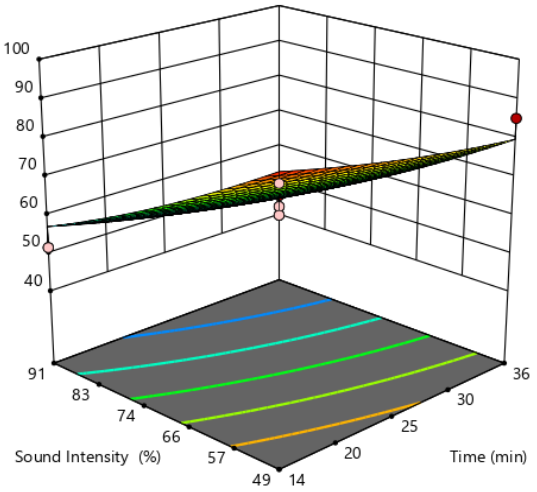

Figure 2. Three-dimensional diagram, the simultaneous effect of two extraction time and sound intensity variables on the amount of IC_{50} of alcoholic extracts of Caper fruit

Table 6. ANOVA of the quadratic model of IC_{50} of alcholic extracts of Caper fruit

Source	Sum of Squares	df	Mean Squares	F-Value	P-Value
Model	2588.81	5	517.76	17.14	0.0008
X_1	253.96	1	253.96	8.41	0.0230
X_2	2308.31	1	2308.31	76.40	<0.0001
$X_1 X_2$	0.3906	1	0.3906	0.0129	0.9127
X_1^2	10.34	1	10.34	0.3421	0.5770
X_2^2	19.03	1	19.03	0.6298	0.4535
Residual	211.50	7	30.21		
Lack of fit	143.77	3	47.92	2.83	0.1704
Pure error	67.73	4	16.93		
R^2	0.9245				
Adj. R^2	0.8705				
Pred. R^2	0.8671				

4.3 Minimum inhibitory concentration levels of growth of alcoholic extracts of Caper plant fruit

1.4.3 Minimum inhibitory concentration levels of growth of the *Staphylococcus aureus coagulase-positive* and *Bacillus cereus*

In Figure 3, with an increase in the time and sound intensity, the minimum inhibitory concentration levels of growth of *Staphylococcus aureus* and *Bacillus cereus* by ethanolic extracts ectracted from the Caper plant fruit decreased. Ethanolic extracts extracted from the plant fruit at high sound intensities (91% and 100%) showed the ability to inhibit the growth of *Staphylococcus aureus coagulase-positive* bacteria at the lowest serial concentration (0.19 mg/ml) and the ability to inhibit the growth of *Bacillus cereus* at a concentration of 6.25 mg/ml. This indicates the high sensitivity of *Staphylococcus aureus coagulase-positive* bacteria to ethanolic extracts extracted from the Caper plant fruit compared to *Bacillus cereus*.

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Based on significant parameters in the extraction process of antimicrobial compounds from the Caper plant fruit, the overall equations based on the analysis of variance tables (Tables 7 and 8) can be reported as follows:

Equation 4: General equation for determining the minimum inhibitory concentration levels of growth of *Staphylococcus aureus coagulase-positive* bacteria by the ethanol extracts from the Caper plant fruit

$$Y=0.39 - 0.17\ X_1 - 0.49\ X_2 + 0.19\ X_1X_2 + 0.08\ X_1^2 + 0.23\ X_2^2$$

Equation 5: General equation for determining the minimum inhibitory concentration levels of growth of *Bacillus cereus* by the ethanol extracts from the Caper plant fruit

$$Y=12.50 - 5.33\ X_1 - 15.55\ X_2 + 6.25\ X_1X_2 + 2.73\ X_1^2 + 7.42\ X_2^2$$

In these equations, Y represents the minimum inhibitory concentration levels of growth of the plant fruit extracts in terms of mg/ml, X_1 represents the time in terms of minutes, and X_2 represents the ultrasound intensity as a percentage. According to equations 4 and 5, ultrasound intensity (X_2) was identified as the most influential factor in the extraction of antimicrobial compounds from the Caper plant fruit. The optimal conditions for the minimum inhibitory concentration levels of growth of *Staphylococcus aureus coagulase-positive* bacteria and *Bacillus cereus* by the ethanol extracts from the Caper plant fruit using ultrasound method were determined to be 36 minutes for time and 91% for ultrasound intensity. Under these optimal conditions, the minimum inhibitory concentration levels of growth of *Staphylococcus aureus coagulase-positive* bacteria and *Bacillus cereus* were found to be 0.19 and 6.07 mg/ml, respectively. This signifies the strong antimicrobial capabilities of ethanol extracts from the Caper plant fruit in inhibiting the growth of gram-positive bacteria. Gram-positive bacteria show less resistance and higher permeability to antimicrobial compounds, including phenols with multiple hydroxyl groups, due to their single-layer cell wall structure [40]. In a study, Rouhani (2016) reported the minimum inhibitory concentration of growth of *Staphylococcus aureus* bacteria of ethanolic extracts extracted from the Caper plant fruit by maceration method as 25 mg/ml [41], which compared to the optimal level of the present study, the minimum inhibitory concentration shows more growth and lower ability to inhibit microbial activity. Mahboubi and Mahboubi (2014) in a study, reported the minimum inhibitory concentration of growth of *Staphylococcus aureus* and *Bacillus cereus* of ethanolic extracts extracted from the Caper plant fruit by maceration method as 12.8 and 6.4 mg/ml, respectively [24], which compared to the optimal values of the present study have lower antimicrobial activity. Hematian et al. (2020) in a study, reported the minimum inhibitory concentration of growth of *Staphylococcus aureus* of ethanolic extracts extracted from the Caper plant fruit by maceration method as 0.148 mg/ml [33], which compared to the optimal amount of the present study, the minimum inhibitory

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concentration of growth is almost the same and it shows closeness. In a study, Rahimifard et al. (2015) reported the minimum inhibitory concentration of growth of *Staphylococcus aureus* bacteria of the total methanolic extract obtained from the aerial parts of Cartilaginea and Mucronifolia species of Caper plant as 31.25 and 11.7 µg/ml, respectively, which in compared with the optimal amount of the present study, they show higher antimicrobial activity [42]. This difference in the minimum inhibitory concentration of bacterial growth in several studies can be caused by the difference in the species of Caper plant, the environmental conditions of plant growth, the amount of phenolic and antioxidant compounds and the type of extraction method.

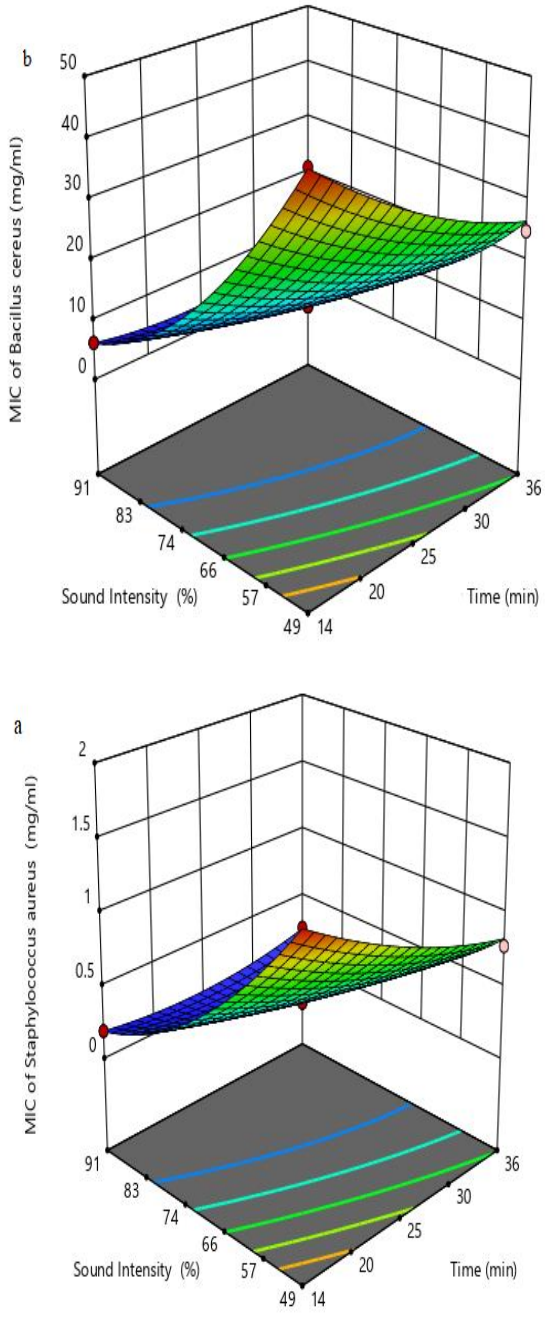

Figure 3. Three-dimensional diagram, the simultaneous effect of two extraction time and sound intensity variables on the amount of MIC of ***Staphylococcus aureus*** of alcoholic extracts of Caper fruit (a) and amount of MIC of ***Bacillus cereus*** of alcoholic extracts of Caper fruit (b)

Table 7. ANOVA of the quadratic model of MIC of ***Staphylococcus aureus*** of alcholic extracts of Caper fruit

Source	Sum of Squares	df	Mean Squares	F-Value	P-Value
Model	2.66	5	0.5324	321.61	<0.0001
X_1	0.2216	1	0.2216	133.88	<0.0001
X_2	1.90	1	1.90	1147.06	<0.0001

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$X_1 X_2$	0.1521	1	0.1521	91.88	<0.0001
X_1^2	0.0503	1	0.0503	30.36	0.0009
X_2^2	0.3680	1	0.3680	222.31	<0.0001
Residual	0.0116	7	0.0017		
Lack of fit	0.0116	3	0.0039		
Pure error	0.0000	4	0.0000		
R^2	0.9957				
Adj. R^2	0.9926				
Pred. R^2	0.9692				

Table 8. ANOVA of the quadratic model of MIC of ***Bacillus cereus*** of alcholic extracts of Caper fruit

Source	Sum of Squares	df	Mean Squares	F-Value	P-Value
Model	2722.74	5	544.55	327.64	<0.0001
X_1	227.67	1	227.67	136.98	<0.0001
X_2	1933.54	1	1933.54	1163.36	<0.0001
$X_1 X_2$	156.25	1	156.25	94.01	<0.0001
X_1^2	52.01	1	52.01	31.29	0.0008
X_2^2	383.19	1	383.19	230.56	<0.0001
Residual	11.63	7	1.66		
Lack of fit	11.63	3	3.88		
Pure error	0.0000	4	0.0000		
R^2	0.9957				
Adj. R^2	0.9927				
Pred. R^2	0.9697				

2.4.3 Minimum inhibitory concentration levels of growth of the *Escherichia coli O157: H7* and *Pseudomonas aeruginosa*

The minimum inhibitory concentration levels of growth of *Escherichia coli O157:H7* and *Pseudomonas aeruginosa* by ethanolic extracts extracted from the Caper plant fruit were found to vary from 25 to over 100 mg/ml, depending on the time and intensity of ultrasound exposure. Ethanol extracts obtained at 36 minutes and 91% ultrasound intensity, as well as 25 minutes and 100% ultrasound intensity, exhibited the lowest minimum inhibitory concentration levels of growth of *Escherichia coli O157:H7* and *Pseudomonas aeruginosa* (25 mg/ml). This indicates higher resistance of gram-negative bacteria compared to gram-positive bacteria to the antimicrobial compounds present in Caper plant fruit extracts. Gram-negative bacteria, due to their extra, multi-layered cell walls, demonstrate higher resistance and lower permeability to antimicrobial compounds [40]. Mahboubi and Mahboubi (2014) reported minimum inhibitory concentration levels of growth of *Escherichia coli* and *Pseudomonas aeruginosa* by ethanolic extracts extracted from the Caper plant fruit as 6.4 and 3.2 mg/ml, respectively, using the maceration method, showing higher antimicrobial activity and greater growth inhibition capabilities compared to the minimum levels in the present study. Gull et al. (2015) reported a minimum inhibitory concentration level of growth of *Escherichia coli* by ethanolic extracts extracted from the Caper plant fruit as 266.7 µg/ml, displaying greater growth inhibition capabilities compared to the present study's minimum inhibitory concentration levels [26]. Rouhani

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(2016) reported the minimum inhibitory concentration of growth of *Escherichia coli* of the ethanolic extract of the Caper plant fruit as 50 mg/ml [41], which compared to the minimum inhibitory concentration of growth of the present study, antimicrobial activity and ability to inhibit bacterial growth have shown less. Rahimifard et al. (2015) reported minimum inhibitory concentration levels of growth of *Escherichia coli* and *Pseudomonas aeruginosa* by methanolic extracts extracted from the Caper plant's Cartilaginea and Mucronifolia species as 41.67, 31.25, 46.87, and over 1000 µg/ml respectively, demonstrating higher antimicrobial activity compared to the minimum inhibitory concentration levels in the present study [42].

5.3 Minimum bactericidal concentration levels of alcoholic extracts of Caper plant fruit

1.5.3 Minimum bactericidal concentration levels of the *Staphylococcus aureus coagulase-positive* and *Bacillus cereus*

According to Figure 4, with the increase in time and intensity of ultrasonic waves, the minimum bactericidal concentration of *Staphylococcus aureus coagulase-positive* of ethanolic extracts of the Caper plant fruit shows a noticeable decrease.

According to the significant parameters in the process of extracting antimicrobial compounds from the Caper plant fruit based on the variance analysis table (Table 9), the general equation can be reported as follows:

Equation 6: The general equation for determining the minimum bactericidal concentration of *Staphylococcus aureus* coagulase-positive of ethanolic extract of plant fruit

$$Y=25 - 12.23\ X_1 - 29.53\ X_2 + 9.37\ X_1X_2 + 6.25\ X_1^2 + 15.62\ X_2^2$$

In this equation, Y: the minimum bactericidal concentration of ethanolic extract of Caper plant fruit in terms of mg/ml, X_1: time in terms of minutes and X_2: ultrasound intensity in terms of percent. According to equation 6, ultrasound intensity (X_2) was recognized as the most effective factor for the extraction of antimicrobial compounds from Caper plant fruit. The optimal conditions for the minimum bactericidal concentration of *Staphylococcus aureus coagulase- positive* ethanolic extracts of the plant fruit were determined by ultrasonic method, time 36 minutes and sound intensity 91%, and in these optimal conditions, the minimum bactericidal concentration of *Staphylococcus aureus coagulase-positive* was 11.81 mg/ml. The minimum bactericidal concentration of *Bacillus cereus* of the ethanolic extracts of Caper plant fruit varied from 25 to more than 100 mg/ml depending on the time and intensity of the ultrasound waves. Ethanol extracts extracted from Caper plant fruit had the lowest bactericidal concentration of *Bacillus cereus* (25 mg/ml) in 36 minutes and 91% ultrasound intensity and 25 minutes and 100% ultrasound intensity. In a study, Rouhani (2016) reported the minimum bactericidal concentration of Staphylococcus aureus of ethanolic extracts extracted from Caper plant fruit by maceration method as 50 mg/ml [41], which compared to the optimal level of the current study, the minimum bactericidal concentration more and shows

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lower antimicrobial activity. Mahboubi and Mahboubi (2014) in a study, reported the minimum bactericidal concentration of *Staphylococcus aureus* and *Bacillus cereus* of ethanolic extracts extracted from Caper plant fruit by maceration method as 12.8 and 51.2 mg/ml, respectively [24], which have lower antimicrobial activity compared to the optimal and lowest values of the minimum bactericidal concentration of the present study. Hematian et al. (2020) in a study, reported the minimum bactericidal concentration of *Staphylococcus aureus* of ethanolic extracts extracted from Caper plant fruit by maceration method as 0.283 mg/ml [33], which compared to the optimal level of the present study, They show lower minimum bactericidal concentration and higher antimicrobial ability. This difference in the minimum bactericidal concentration of *Staphylococcus aureus* and *Bacillus cereus* bacteria compared to the present study is due to the efficient effect of ultrasound waves in extracting strong antioxidant and therefore antimicrobial compounds from Caper plant fruit compared to traditional extraction methods.

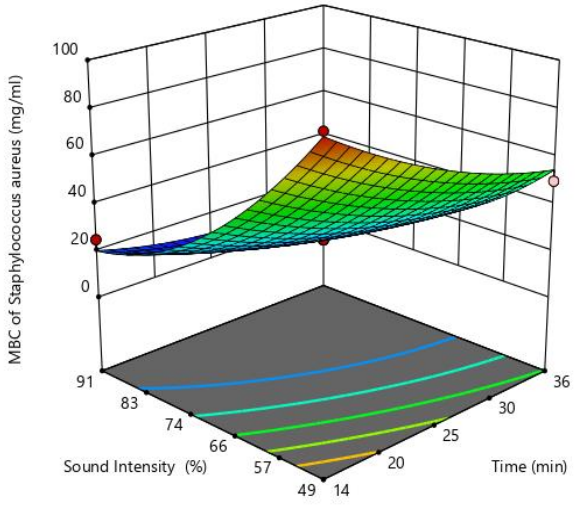

Figure 4. Three-dimensional diagram, the simultaneous effect of two extraction time and sound intensity variables on the amount of MBC of ***Staphylococcus aureus*** of alcoholic extracts of Caper fruit

Table 9. ANOVA of the quadratic model of MBC of ***Staphylococcus aureus*** of alcholic extracts of Caper fruit

Source	Sum of Squares	df	Mean Squares	F-Value	P-Value
Model	10348.82	5	2069.76	134.27	<0.0001
X_1	1196.96	1	1196.96	77.65	<0.0001
X_2	6976.38	1	6976.38	452.56	<0.0001
$X_1 X_2$	351.56	1	351.56	22.81	0.0020
X_1^2	271.74	1	271.74	17.63	0.0040
X_2^2	1698.37	1	1698.37	110.17	<0.0001
Residual	107.91	7	15.42		
Lack of fit	107.91	3	35.97		
Pure error	0.0000	4	0.0000		
R^2	0.9897				
Adj. R^2	0.9823				
Pred. R^2	0.9266				

2.5.3 Minimum bactericidal concentration levels of the *Escherichia coli O157: H7* and *Pseudomonas aeruginosa*

The minimum bactericidal concentration of *Escherichia coli O157:H7* and *Pseudomonas aeruginosa* ethanolic extracts of Caper plant fruit varied from 50 to over 100 mg/ml

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depending on the time of sonication and intensity of ultrasound waves. Ethanolic extracts extracted from plant fruit at 36 minutes and 91% ultrasound intensity, as well as at 25 minutes and 100% ultrasound intensity, had the lowest minimum bactericidal concentration of *Escherichia coli O157:H7* and *Pseudomonas aeruginosa* (50 mg/ml). Mahboubi and Mahboubi (2014) reported in a study that the minimum bactericidal concentration of *Escherichia coli* and *Pseudomonas aeruginosa* of ethanolic extracts of Caper plant fruit by maceration method were 25.6 and 6.4 mg/ml, respectively [24], which showed higher antimicrobial activity compared to the lowest minimum bactericidal concentration in the present study. Rouhani (2016) reported the minimum bactericidal concentration of *Escherichia coli* of ethanolic extract of Caper plant fruit as 50 mg/ml [41], which had similar antimicrobial activity compared to the lowest minimum bactericidal concentration in the present study.

4. Conclusion

Bioactive factors present in extracts extracted from plants, including phenolic compounds, have potential antioxidant properties, ability to inhibit free radicals activity, and strong antimicrobial properties with the ability to inhibit growth and kill both gram-positive and gram-negative bacteria. The novel ultrasound extraction method has the capability to efficiently extract bioactive factors from different parts of plants in a short period compared to traditional extraction methods such as maceration and Soxhlet. The results of this study indicated that with an increase in sonication time and intensity, the extraction process of phenolic compounds, antioxidants, and antimicrobials from Caper plant fruit increased. Sound intensity is recognized as the most effective factor. Using response surface statistical method, optimal conditions for extracting antioxidant compounds from Caper plant fruit were determined to be 36 minutes sonication time at 91% sound intensity, resulting in total phenolic content and IC_{50} index for ethanolic extracts being 23.63 mg/g and 45.30 µg/mg, respectively. The optimal minimum inhibitory concentration of growth of *Staphylococcus aureus coagulase-positive* bacteria was found to be 0.19 mg/ml and for *Bacillus cereus* bacteria was found to be 6.07 mg/ml, while optimal minimum bactericidal concentration of *Staphylococcus aureus coagulase-positive* bacteria was found to be 11.18 mg/ml. Additionally, ethanolic extracts at an ultrasound intensity of 91% for 36 minutes showed inhibitory activity against *Escherichia coli O157:H7* and *Pseudomonas aeruginosa* at concentrations of 25 and 25 mg/ml, as well as bactericidal activity against *Bacillus cereus, coli O157:H7* and *Pseudomonas aeruginosa* at concentrations of 25, 50, and 50 mg/ml.

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مقاله علمی_پژوهشی

ارزیابی ویژگی‌های آنتی‌اکسیدانی و ضدمیکروبی عصاره‌های اتانولی استخراجی از میوه گیاه کمرگل به روش فراصوت با استفاده از مدل سطح پاسخ

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چکیده

عصاره‌ها و اسانس‌های استخراجی از گیاهان دارویی دارای عوامل زیست‌فعال متعدد از جمله ترکیبات فنلی با ویژگی‌های بالقوه آنتی‌اکسیدانی و ضدمیکروبی چشمگیر می‌باشند. انتخاب روش استخراج مناسب بر کمیت و کیفیت ترکیبات آنتی‌اکسیدانی و ضدمیکروبی اثرگذار است. هدف از این مطالعه، بررسی کارایی امواج فراصوت در استخراج ترکیبات فنلی، آنتی‌اکسیدانی و ضدمیکروبی از میوه گیاه کمرگل بود. بهینه‌سازی با استفاده از روش آماری سطح پاسخ و طرح باکس بنکن با انتخاب فاکتور زمان فراصوت‌دهی در سه سطح (۱۰، ۲۵ و ۴۰ دقیقه) و شدت امواج فراصوت در سه سطح (۴۰، ۷۰ و ۱۰۰ درصد) و کاربرد حلال اتانول ۷۰ درصد انجام گردید. از نتایج آزمون‌های انجام شده با روش آماری سطح پاسخ، شدت فراصوت به عنوان موثرترین فاکتور استخراج ترکیبات فنلی، آنتی‌اکسیدانی و ضدمیکروبی از میوه گیاه کمرگل شناخته شد و با افزایش زمان و شدت امواج فراصوت میزان استخراج این ترکیبات افزایش یافت. شرایط بهینه استخراج ترکیبات آنتی‌اکسیدانی و ضدمیکروبی از میوه گیاه کمرگل، زمان ۳۶ دقیقه و شدت فراصوت ۹۱ درصد تعیین گردید و در این شرایط بهینه، میزان ترکیبات فنلی کل و شاخص IC_{50} عصاره‌های اتانولی استخراجی از میوه گیاه به ترتیب ۲۳/۶۳ میلی‌گرم بر گرم و ۴۵/۳۰ میکروگرم بر میلی‌گرم، میزان بهینه حداقل غلظت بازدارندگی از رشد باکتری‌های *استافیلوکوکوس اورئوس کوآگولاز مثبت* و *باسیلوس سرئوس* به ترتیب ۰/۱۹ و ۶/۰۷ میلی‌گرم بر میلی‌لیتر و میزان بهینه حداقل غلظت باکتری‌کشی *استافیلوکوکوس اورئوس کوآگولاز مثبت* ۱۱/۸۱ میلی‌گرم بر میلی‌لیتر حاصل گردید. همچنین عصاره‌های استخراجی در شدت فراصوت ۹۱ درصد و زمان ۳۶ دقیقه دارای قابلیت مهار فعالیت باکتری‌های *اشریشیاکلی O157 : H7* و *سودوموناس آئروژینوزا* در غلظت‌های به ترتیب ۲۵ و ۲۵ میلی‌گرم بر میلی‌لیتر و قابلیت کشندگی باکتری‌های *باسیلوس سرئوس*، *اشریشیاکلی O157 : H7* و *سودوموناس آئروژینوزا* در غلظت-های به ترتیب ۲۵، ۵۰ و ۵۰ میلی‌گرم بر میلی‌لیتر بودند.

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