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Microwave-assisted extraction of bioactive compounds from bitter orange seed cotyledon and evaluating their antioxidant properties

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ABSTRACT

In the present study, bioactive compounds were extracted from cotyledon of bitter orange (*Citrus aurantium*) seed, as a waste of citrus processing, using a microwave-assisted extraction method. The effects of four independent variables including microwave power (100-300 W), extraction time (5-15 min), sample weight (5-15 g), and solvent volume (100-200 mL) on responses of extraction yield, total phenolic content, total flavonoid content, free radical scavenging activity (IC_{50}), ferric ion reducing antioxidant power (FRAP), cupric ion reducing antioxidant capacity (CUPRAC), and chelating capacity of extracts were investigated. Response surface methodology based on the central composite design was employed to investigate the effects of independent variables on the responses and also to optimize the extraction conditions. The optimum extraction condition included microwave power of 300 W, extraction time of 15 min, sample weight of 5 g, and solvent volume of 200 mL. Regarding the extraction yield, its amount increased significantly by increasing microwave power, extraction time, and sample weight, while it decreased significantly by increasing solvent volume. Also, the highest total phenolic content in the extract was observed at the lowest levels of microwave power and extraction time. Concerning the total flavonoid content in the extract, its amount increased significantly by increasing extraction time and solvent volume, while it decreased significantly by increasing sample weight. In addition, the CUPRAC of the extract increased significantly by increasing microwave power, extraction time, and solvent volume, as opposed to sample weight. In conclusion, microwave-assisted extraction can be suggested as a suitable method for extracting bioactive compounds from the bitter orange seed cotyledon.

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1. Introduction

Food wastes, encompassing edible and inedible components of various food items, are systematically removed from the food supply chain, with the result being either recycling or disposal. The phenomenon of food waste can unfold across the entire supply chain, spanning from the farm to the processing plant and even to the retail markets. In the context of fruit and vegetable processing industries, waste includes residual parts of seeds, leaves, skin, stem, pulp, pomace, and bagasse. Ninety-three percent of the overall food waste is comprised of fruits and vegetables. Based on the data obtained from the Food and Agriculture Organization (FAO), the annual waste of fruits and vegetables reached levels of 40-50% for tubers, fruits, and legumes by 2016 [1, 2]. Through the processing of fruits and vegetables, valuable by-products emerge, unveiling a diverse array of bioactive compounds such as flavonoids, carotenoids, phytosterols, tocopherols, saponins, and organic acids. These compounds exhibit a spectrum of biological activities like antioxidant, anti-diabetic, anti-inflammatory, and anti-cancer activities, and cardiovascular protective effect. Incorporating bioactive compounds from these by-products can enhance the nutritional value and oxidative stability of foods, given their role as significant sources. Additionally, they can open avenues for the development of novel functional foods [3-5]. Extraction methods offer a means to obtain bioactive compounds from agricultural and industrial wastes [5].

The citrus industry plays a considerable role in food waste generation [6]. Annually, citrus processing yields about 10 million tons of waste [7]. Despite about one-third of citrus fruits being utilized for juice processing, substantial amounts of underutilized residues are generated during this process [6]. In the year 2020, the global citrus production reached about 158.49 million tons. Bitter oranges (*Citrus aurantium*) and sweet oranges (*Citrus sinensis*) are among the foremost citrus fruits, collectively contributing to a total production of approximately 75.41 million tons [2, 7, 8]. Bitter orange, scientifically known as "*Citrus aurantium* L.", is classified under the citrus family (Rutaceae) according to the Institute of Standards and Industrial Research of Iran No. 177 (July 2001) [9]. In Iran, the production of oranges in 2021 amounted to 2.14 million tons, as per the latest statistics from FAOSTAT [2]. The remnants, related to the post-juice extraction process, are commonly recognized as by-products of citrus. While labeled as citrus waste, citrus by-products entail substantial contents of valuable compounds such as fiber, protein, pectin, polyphenols, and essential oils. Processing citrus waste holds the opportunity to generate even more valuable products [10]. Citrus waste typically comprises 65% peel (flavedo and albedo) and 30-35% pulp and seeds. Citrus seeds, known for their abundance of natural antioxidants, present potential applications across various areas such as food, medicine, and cosmetics. Their composition includes carbohydrates (69%), moisture (6%), crude protein (6%), crude fiber (5%), lipid (12%), and ash (2%) [6, 7, 11]. Motivated by rising consumer demand, the global market for functional ingredients is anticipated to reach 96.1 billion dollars by 2026. The markets for natural antioxidants are estimated to hit 4.14 billion dollars, underling the bigger need for these compounds in the food, cosmetic, and medical industries. Consequently, managing and enhancing the value of citrus by-products can be achieved through the extraction of value-added bioactive compounds [6].

The extraction process serves as the primary step in gaining value-added compounds from citrus wastes and by-products. Innovative technologies, such as microwave-assisted extraction (MAE), have been

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implemented and studied to optimize the extraction of value-added bioactive compounds from food by-products [10, 12]. The MAE method presents distinct advantages in comparison with conventional extraction methods. By utilizing non-ionizing high-frequency radiations, the microwave creates highly localized temperature and pressure conditions that aid the extraction process. Microwaves utilize both electric and magnetic fields. The electric field generates heat through dipole rotation and ionic conduction. In contrast to conventional methods, microwave heats the entire sample at the same time. This heating breaks the weak hydrogen bonds during the dipole rotation of the molecules, while the migration of dissolved ions promotes solvent penetration into sample. The MAE mechanism involves three successive steps: 1) the separation of solutes from the sample matrix through the application of heat and pressure, 2) the diffusion of the solvent in the sample matrix, and 3) the release of the solutes by the solvent from the sample matrix. As a result, microwave achieves rapid and highly efficient heating [4, 5, 13, 14].

It is noteworthy to highlight that MAE is typically conducted through two approaches: 1) Microwave-assisted solvent extraction and 2) Microwave-assisted solvent-free extraction. In the former, the sample is combined with solvents like ethanol, methanol, water, etc. [15]. The extraction outcomes in terms of both quality (characteristics) and quantity of compounds obtained via microwave are dependent on several factors including extraction time, microwave power, and solvent-to-sample ratio. Thoughtful adjustment of extraction parameters allows for the creation of conditions, favoring a higher yield of phenolic compounds [16].

MAE has been employed to extract bioactive compounds and examine antioxidant properties across various agricultural residues, including pineapple peel waste [17], black jamun pulp [18], pomegranate peel [19], chestnut processing waste [20], grape skin [21], sour cherry peel [22], solid wastes of cauliflower, celery, chicory, and asparagus [23], lemon waste [24] and mandarin peel [25]. A study on MAE from pineapple peel underscored its effectiveness in extracting phenolic and flavonoid compounds along with their ability to scavenge DPPH free radicals. The research concluded that the MAE stands out as an advantageous method for extracting bioactive compounds, providing a higher yield [17]. Likewise, in the exploration of bioactive compounds extraction from black jamun pulp, MAE proved to be an effective method for extracting phenolic and anthocyanin compounds. The study suggested the scalability of the MAE process, moving from a laboratory-scale to a pilot-scale application [18].

Application of the response surface methodology enables the identification of variables influencing the MAE process and the determination of the optimum extraction parameters using experimental design [13]. The primary goal of this study was to optimize the extraction conditions for bioactive compounds from bitter orange seed cotyledon, a by-product (waste) of bitter orange processing, using a microwave-assisted solvent extraction method. This study also includes the assessment of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of extracts. The validation of the optimum conditions, as predicted by the software, was assessed through experimental testing conducted under these conditions. Furthermore, the contents of bioactive compounds and the antioxidant activity of the extract obtained via MAE under optimum conditions were compared with those of the extract obtained via an electromantle (served as a conventional method).

2- Materials and methods

2.1. Chemicals and reagents

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Methanol, Folin-Ciocalteu reagent, sodium carbonate, gallic acid, aluminum chloride, potassium acetate, quercetin, potassium ferricyanide, phosphate salts, iron chloride, trichloroacetic acid, ascorbic acid, diphenyl picrylhydrazyl (DPPH) reagent, copper chloride, ammonium acetate, neocuproine reagent, ethylenediamine tetra-acetic acid (EDTA), and ferrozine reagent were purchased from Sigma-Aldrich (St. Louis, MO) and Merck company (Darmstadt, Germany).

2.2. Bitter orange seed cotyledon

Bitter orange seeds were sourced as waste from Limondis Company (Tall-e Beyza Industrial Park, Beyza, Fars Province). Then, they underwent a manual separation of their cotyledons from the outer coats. The chemical composition of the resulting bitter orange seed cotyledon was analyzed using the approved methods of the American Association of Cereal Chemists [26]. Bitter orange seed cotyledon comprised of 10.72% moisture, 21.00% fat, 2.65% protein, 2.55% ash, and 63.08% carbohydrates.

2.3. Microwave-assisted extraction from bitter orange seed cotyledon

A schematic overview of the current study is illustrated in Fig. 1. A modified household microwave setup was employed for conducting the extraction, wherein a condenser was affixed to the microwave' top (frequency 2450 MHz, ME3410W, Samsung, Malaysia). The cotyledons were subjected to an extraction process using methanol as the solvent, with consideration of four independent variables —microwave power, extraction time, sample weight, and solvent volume— each set at three levels, as per the experimental design (detailed in Table 1). Following extraction, the extracts were filtered and preserved in opaque containers at 4 °C until subsequent testing. Assessment of extraction yield, TPC, TFC, antioxidant activity, and physical properties (as responses in the experimental design) was conducted. The effects of independent variables on diverse responses were thoroughly explored using response surface methodology based on the central composite design (CCD). The experimental design matrix, comprising 30 runs, is provided in Table 1.

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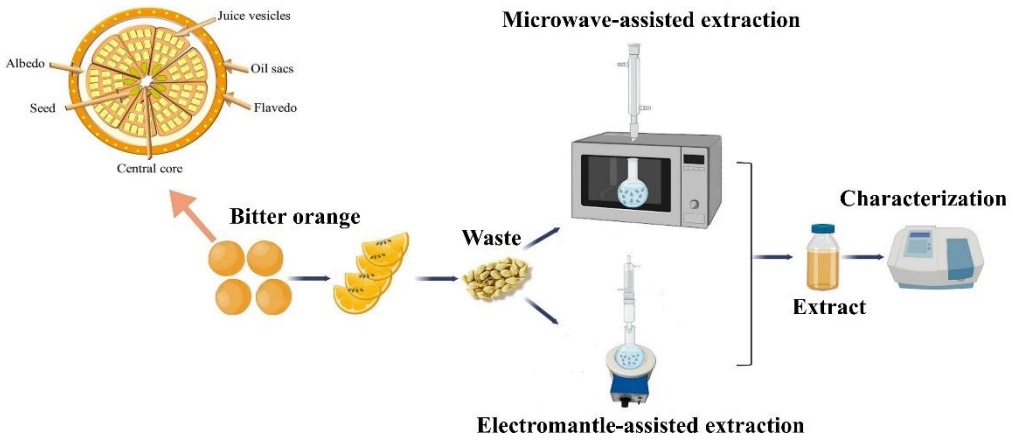

Fig. 1. Schematic of microwave-assisted extraction of bioactive compounds from bitter orange seed cotyledon and in comparison with electromantle-assisted extraction method.

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Table 1. Experimental design matrix of response surface methodology based on the central composite design as well as physical properties of extracts of bitter orange seed cotyledon, extracted using a microwave.

Run	Microwave Power (W; A)	Extraction time (min; B)	Sample weight (g; C)	Solvent volume (mL; D)	Refractive index	pH	Density (g/cm^3)	Color parameters		
								L^*	a^*	b^*
1	100	5	5	100	1.3333	7.74	0.800	66.00	-2.00	6.00
2	100	5	5	200	1.3312	7.79	0.802	68.00	0.00	4.00
3	100	5	15	100	1.3356	7.61	0.750	65.00	0.00	8.00
4	100	5	15	200	1.3333	7.78	0.788	59.00	0.00	4.00
5	100	10	10	150	1.3320	7.70	0.750	66.00	-3.00	8.00
6	100	15	5	100	1.3344	7.75	0.810	53.00	-4.00	12.00
7	100	15	5	200	1.3315	7.72	0.782	62.00	-3.00	9.00
8	100	15	15	100	1.3355	7.65	0.750	60.00	-3.00	11.00
9	100	15	15	200	1.3332	7.74	0.773	64.00	-3.00	9.00
10	200	5	10	150	1.3310	7.67	0.820	67.00	-2.00	6.00
11	200	10	5	150	1.3334	7.78	0.819	65.00	-2.00	5.00
12	200	10	10	100	1.3349	7.69	0.825	66.00	-2.00	9.00
13	200	10	10	150	1.3331	7.69	0.790	66.00	-4.00	10.00
14	200	10	10	150	1.3304	7.73	0.792	69.00	-3.00	8.00
15	200	10	10	150	1.3306	7.66	0.786	66.00	-3.00	8.00
16	200	10	10	150	1.3333	7.75	0.790	69.00	-3.00	9.00
17	200	10	10	150	1.3328	7.78	0.787	70.00	-4.00	10.00
18	200	10	10	150	1.3307	7.68	0.786	69.00	-4.00	10.00
19	200	10	10	200	1.3317	7.75	0.806	73.00	-3.00	7.00
20	200	10	15	150	1.3330	7.73	0.810	69.00	-3.00	8.00
21	200	15	10	150	1.3308	7.71	0.799	67.00	-3.00	10.00
22	300	5	5	100	1.3308	7.80	0.784	73.00	-3.00	8.00
23	300	5	5	200	1.3299	7.81	0.748	71.00	0.00	5.00
24	300	5	15	100	1.3319	7.72	0.740	72.00	-2.00	8.00
25	300	5	15	200	1.3307	7.82	0.751	69.00	-3.00	8.00
26	300	10	10	150	1.3306	7.82	0.708	72.00	-3.00	8.00
27	300	15	5	100	1.3307	7.81	0.770	73.00	-2.00	7.00
28	300	15	5	200	1.3400	7.79	0.890	64.00	0.00	4.00
29	300	15	15	100	1.3317	7.68	0.729	64.00	-4.00	12.00
30	300	15	15	200	1.3329	7.75	0.713	63.00	-3.00	10.00

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2.4. Extraction yield

To ascertain the extraction yield, the solvent was initially evaporated and separated via a vacuum rotary evaporator (T63AL, Buchi Company, Switzerland). Following this, the extraction yield was computed by dividing the weight of the dried extract by the volume of the extract, and the result was expressed as a percentage.

2.5. Total phenolic content

TPC in the extracts was assessed using the Folin-Ciocalteu reagent, following the method outlined by Habibi et al. [27]. The resulting TPC data were quantified and expressed as µg gallic acid equivalent (GAE)/g.

2.6. Total flavonoid content

TFC in the extracts was determined using the aluminum chloride colorimetric method, as the procedure described by Habibi et al. [27]. The resulting data of TFC were reported in µg quercetin equivalent (QE)/g.

2.7. Antioxidant properties

2.7.1. DPPH free radical scavenging

Antioxidant activity was evaluated by assessing the extracts' capability to scavenge DPPH free radicals, following the method detailed by Habibi et al. [27]. In conducting the test, a 1:3 ratio of sample (at concentrations ranging from 0.01 to 1.00 mg/mL) to DPPH solution was employed. Subsequently, the antioxidant activity was reported as IC_{50}, representing the concentration needed for scavenging 50% of DPPH free radicals. The results were reported in mg/mL.

2.7.2. Ferric ion reducing antioxidant power

The extracts' ferric ion reducing antioxidant power (FRAP) was determined following the procedure described by Rekha et al. [28]. The resulting FRAP values were reported in terms of mg ascorbic acid/g.

2.7.3. Cupric ion reducing antioxidant capacity

The cupric ion reducing antioxidant capacity (CUPRAC) of the extracts was assessed following the procedure described by Pascu et al. [29], utilizing a neocuproine reagent. The CUPRAC values were expressed as mg ascorbic acid/g.

2.7.4. Chelating capacity

The extracts were evaluated for their chelating capacity of ferrous ions using a ferrozine reagent, following the method outlined by Oyetayo et al. [30]. The measured chelating capacity was reported in mg EDTA/g.

2.8. Physical properties

The pH of the extracts was gauged via a pH meter (Starter 3000, Ohaus, New Jersey). The refractive index of the extracts was ascertained employing an Abbe refractometer (Carl Zeiss, Abbe, Germany) at 18 °C. The density of the extracts was determined by weighing 1000 microliters of the sample at 18 °C and expressed as g/cm^3. The color of the extracts was evaluated through the measurement of L^*, a^*, and b^* parameters, indicating brightness, redness-greenness, and yellowness-blueness, respectively. This assessment was conducted using Adobe Photoshop software (Version 8.0, Adobe system, California).

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2.9. Optimization and validation of microwave-assisted extraction

Predicted optimum MAE conditions for bitter orange seed cotyledon were determined by maximizing TPC, TFC, FRAP, CUPRAC, and chelation capacity while minimizing IC_{50}, using dedicated software. Subsequently, to verify the models, the extract was obtained via microwave at the predicted independent variable levels (an experimental test). The resulting extract was also evaluated for its characteristics. The predicted response values were then compared with the outcomes of the experimental test.

2.10. Comparison of microwave-assisted extraction method with electromantle-assisted extraction method

Under the optimum conditions predicted and selected by the software, the extract of bitter orange seed cotyledon was also obtained using the electromantle-assisted extraction method (served as a conventional method). Subsequently, the characteristics of the extract obtained via the electromantle-assisted method were compared with those of the extract obtained via the MAE method.

2.11. Experimental design and statistical analysis

Table 1 outlines the experimental design matrix, encompassing 30 runs. The effects of four independent variables namely microwave power (A), extraction time (B), sample weight (C), and solvent volume (D) on a range of responses, including extraction yield, TPC, TFC, IC_{50}, FRAP, CUPRAC, chelating capacity, and physical properties were systematically examined. By employing the response surface methodology based on CCD, the effects of individual and interactive independent variables on the responses were assessed, and the extraction conditions were optimized. Statistical significance was confirmed through the application of analysis of variance (ANOVA). A probability level of $P < 0.01$ was considered as the significant level. The experimental design matrix, data analysis, and the independent variable effects were designed and conducted using Design Expert software (Version 10, State-Ease Inc., Minneapolis).

3-Results and discussion

3.1. Experimental design

The experimental design employed response surface methodology based on CCD to explore the effects of independent variables on various responses. Table 2 presents the results of ANOVA of the models. The coefficient of determination (R^2), adjusted coefficient of determination (Adjusted R^2), and predicted coefficient of determination (Predicted R^2) values exceeded 0.97, 0.94, and 0.85, respectively. The close alignment of the coefficient of determination with the predicted values suggests a robust correlation between predicted and experimental data. Furthermore, the adjusted coefficient of determination should demonstrate comparability with the predicted values, emphasizing a good model. In this regard, the predicted coefficient of determination of the models exhibited reasonable agreement with the adjusted coefficient of determination (a difference of less than 0.2). Adequate Precision, reflecting the

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signal-to-noise ratio, is desirable for ratios greater than 4. This ratio surpassed 4 for all response models, signifying an adequate signal and a desirable model. The low coefficient of variation underscores the high precision and reliability of the experimental values [31]. Notably, the lack of fit for extraction yield, TPC, TFC, free radical scavenging, and chelating capacity was found to be insignificant.

Table 2. Analysis of variance of quadratic models applied for different responses of extracts of bitter orange seed cotyledon, extracted using a microwave.

Property (response)	Model		R^2 (coefficient of determination)	Adjusted R^2	Predicted R^2	Coefficient of variation (%)	Adequate precision
	P	*F*-value					
Extraction yield (%)	<0.0001	178.94	0.9940	0.9885	0.9949	3.38	58.44
TPC[1] (µg GAE/g)	<0.0001	82.51	0.9872	0.9752	0.9082	9.04	39.78
TFC[2] (µg QE/g)	<0.0001	38.79	0.9731	0.9480	0.9101	10.55	29.59
IC_{50} (mg/mL)	<0.0001	117.28	0.9909	0.9825	0.9585	5.31	53.73
FRAP[3] (mg ascorbic acid/g)	<0.0001	807.82	0.9987	0.9974	0.9809	8.01	151.90
CUPRAC[4] (mg ascorbic acid/g)	<0.0001	51.69	0.9797	0.9607	0.8562	7.51	29.67
Chelating capacity (mg EDTA/g)	<0.0001	63.92	0.9835	0.9681	0.9355	7.64	37.81

[1]Total phenolic content; [2]Total flavonoid content; [3]Ferric ion reducing antioxidant power; [4]Cupric ion reducing antioxidant capacity.

3.2. Extraction yield

The effect of microwave power, extraction time, sample weight, and solvent volume on extraction yield is illustrated in Fig. 2 (a-b). All variables demonstrated significant effects on the yields. Elevating microwave power from 100 to 300 W resulted in a corresponding increase in extraction yield. Likewise, an extension of extraction time (from 5 to 15 min) led to an increased extraction yield. Increasing sample weight from 5 to 15 g exhibited an increase in extraction yield. Conversely, a rise in solvent volume was associated with a decrease in extraction yield. The maximum and minimum extraction yields were 1.377% and 0.373%, respectively. It was observed that elevated microwave power increased the extraction yield. Microwaves induce volumetric heating of materials. Dipole rotation in the electric field caused hydrogen bonds to be disrupted, and the migration of ions facilitated enhanced solvent penetration into sample. Subsequently, this resulted in superior dissolution of extractable components and, ultimately, improved extraction of components [15]. In simpler terms, the temperature rise during the extraction process reduces the surface tension and viscosity of the solvent, enhancing its penetration into sample matrix [13]. In line with these findings, Prakash Maran et al. observed that an increase in the microwave power from 160 to 480 W enhanced the extraction yield of pectin from the fruit rinds of watermelon (*Citrullus lanatus*). They stated that the increase of microwave energy can heighten penetration of the solvent into the plant matrix, enabling the efficient dissolution of the components through the rapid energy transfer to the solvent and matrix [32].

Given that extraction operates as a mass transfer phenomenon, it is inherently anticipated that the extraction time will persist until reaching yield equilibrium [20]. Within the microwave extraction process, the solutes remain in contact with the

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solvent. Consequently, prolonging the extraction time can enhance the mass transfer of active compounds [33], enhance the solubility of compounds, and facilitate their diffusion from the cell to the solvent [34]. Nevertheless, it is important to note that while the extraction yield tends to rise with increased extraction time, there exists the potential for bioactive compound degradation [24].

As noted earlier, a smaller volume of solvent is associated with a higher yield. This phenomenon arises from the fact that such a volume of solvent adequately facilitates the mass transfer of compounds. Moreover, the heightened absorption of energy by the solvent, stemming from its larger volume, diminishes the absorption of microwave energy by the sample [35]. Nonetheless, it is essential to consider that, throughout the extraction process, the solvent volume should be sufficient to immerse the sample and induce swelling in the entire sample. In the current study, it was found that the interactive effects of power–time, power–sample weight, power–solvent volume, time–sample weight, time–solvent volume, and sample weight–solvent volume significantly influenced the yield of extracts.

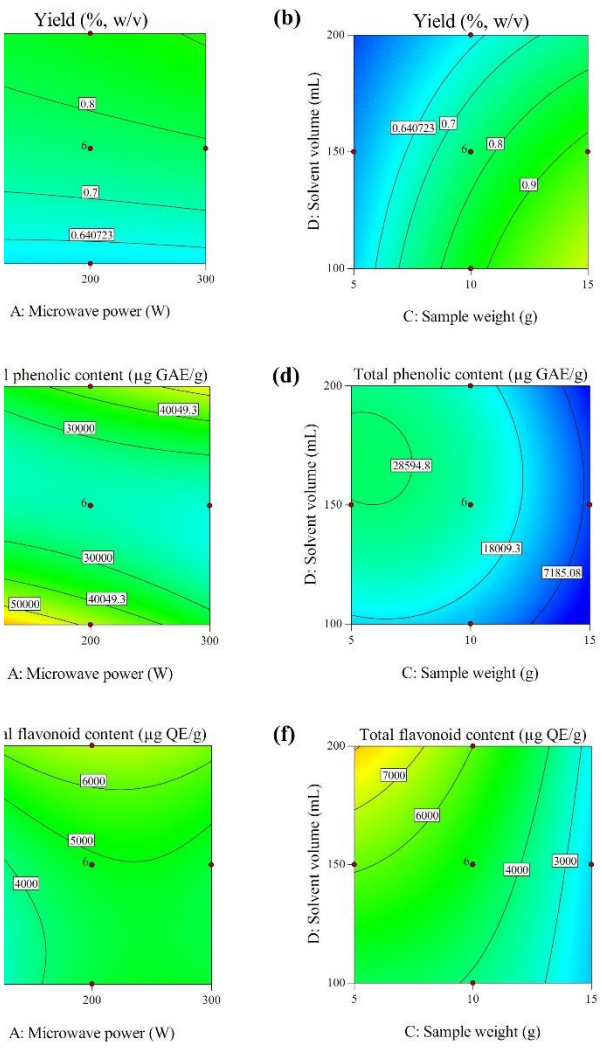

Fig. 2. Effect of different microwave-assisted extraction conditions on extraction yield, total phenolic content, and total flavonoid content of extracts of bitter orange seed cotyledon.

3.3. Total phenolic content

An elevated TPC is often indicative of increased antioxidant ability. Therefore, this measurement serves as an important metric for assessing the total antioxidant capacity. In this context, plant polyphenols exhibit multifunctionality by acting as reducing agents, hydrogen atom donors, and singlet oxygen scavengers, and some of them display the additional ability to chelate transitional metal ions [36].

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The phenolic compounds in bitter orange seeds were found to encompass hydroxybenzoic acids, hydroxycinnamic acids, flavanones, flavanols, flavonols, flavones, simple phenols, and coumarin. Notably, rosmarinic acid, gallic acid, and *p*-coumaric acid emerged as the predominant phenolic acids within bitter orange seeds [37]. The effect of microwave power, extraction time, sample weight, and solvent volume on TPC in the extracts is graphically depicted in Fig. 2 (c-d). Extraction time, sample weight, and solvent volume significantly affected TPC levels. Remarkably, the lowest microwave power level yielded the highest TPC. The maximum TPC corresponded to the shortest extraction time. An increase in the sample weight resulted in a decrease in TPC. Also, TPC increased up to 150 mL of solvent volume, beyond which a further increase in solvent volume led to a subsequent decline in TPC. The maximum and minimum TPC values were 70826.00 and 1713.38 µg GAE/g, respectively. The maximum TPC was achieved with low microwave power applied during a short extraction time. Lower microwave power and shorter extraction time were favored to minimize thermal damage to bioactive compounds. In this regard, Hayat et al. reported a significant effect of extraction time on the levels of phenolic acids (both free and bound) in mandarin peel during MAE. The sum of individual phenolic acids increased with the extension of extraction time from 5 to 15 min, in contrast to the levels of bound phenolic acids (ester-bound and glycoside-bound). The authors explained that the distinct properties of microwave absorption, permittivity, and loss factor could have led to selective or differential heating within the material. The reduction in bound phenolic acids was attributed to the heating effect induced by electromagnetic radiations. Furthermore, in cases where phenolic acids did not absorb microwave energy, the physical attractive forces among phenolic acids, organic compounds, inorganic compounds, and water in the plant matrix, coupled with thermal instability, were proposed as factors contributing to the decrease in bound phenolic acids. Also, Hayat et al. attributed the reduction in TPC (sum of free and bound phenolic acids) with increasing power and time to the microwave-induced destruction of certain phenolic acids [25]. Moreover, Dahmoune et al. observed that an increment of microwave power from 300 to 400 W could elevate the TPC in lemon residue extract, but at higher power levels, there was a decline in TPC due to the thermal degradation of bioactive compounds [24]. However, Sharma and Dash, in their investigation on the MAE of phenolic compounds from black jamun pulp over a 4-min period, found an initial rapid extraction rate that decelerated over time. The swift initial rate was attributed to the extraction of phenolic compounds primarily from the outer surface of the sample, while a subsequent decrease in the extraction rate, as the extraction process advanced, was linked to the diffusion-controlled nature of the extraction process [18].

The findings from this study reveal a rise in TPC corresponding to an increase in the solvent volume up to a specific limit (150 mL). Elevating the solvent volume enhances the swelling of plant cells, resulting in a more efficient extraction of phenolic compounds. Nevertheless, a surplus increase in the solvent volume leads to more energy absorption by the solvent. Consequently, the absorption of microwaves by the plant material decreases, leading to a decline in TPC [31]. In a related context, Mali and Kumar noted a positive effect on TPC extraction from pomegranate peel when increasing solvent-to-sample ratio from 15:1 to 20:1 mL/g (among the ratios of 10:1, 15:1, 20:1, 25:1, and 30 1 mL/g). This enhancement stems from the expanded contact surface area between the

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solvent and the sample due to the increased solvent volume, thereby enhancing the potential for extracting more compounds [19]. To elaborate, the liquid-to-solid ratio emerges as an important factor influencing the extraction process. A higher TPC is achieved due to the availability of a larger solvent volume for compound solubilization (governed by the principle of equilibrium) [20]. The current study revealed significant interactive effects of power–time and time–sample weight on TPC in the extracted samples.

3.4. Total flavonoid content

In bitter orange seeds, the flavonoid compounds, naringin and neohesperidin, stand out as the most abundant, along with noteworthy amounts of flavonols (quercetin, rutin, and kaempferol) and flavanols (catechin and epigallocatechin) [37]. Fig. 2 (e-f) illustrates the effect of microwave power, extraction time, sample weight, and solvent volume on TFC in the extracts. Each variable exhibited a significant effect on TFC. Elevating microwave power from 100 to 200 W increased TFC, followed by a subsequent decrease in TFC with a further microwave power increase. Extended extraction time led to increased TFC. As the sample weight increased, TFC decreased. The TFC increased with increasing solvent volume. The maximum and minimum TFC values were 9976.69 and 251.48 µg QE/g, respectively. It was noted that elevating microwave power from 100 to 200 W increased TFC, but a further increase in the power level resulted in a reduction in TFC. Mali and Kumar reported a significant effect of microwave power at 300 W (among the levels of 150, 300, 450, 600, and 750 W) on the TFC of pomegranate peel. Beyond 150 W (i.e. 300 W), the mixture heats up, facilitating the transfer of flavonoid compounds from the cell to the solvent, while the exposure to power levels exceeding 300 W leads to the loss of heat-sensitive compounds, such as flavonoids, due to sample overheating [19]. In addition, Singh et al.'s observations suggested an increase in TFC in the extraction of bioactive compounds from the walnut hull by elevating the microwave power up to 320 W (within the range of 160 to 480 W), attributed to localized heating and pressure generated by microwave [38].

The findings from this study demonstrated that the TFC of extracts increases with prolonged extraction time and greater solvent volume. In a related context, Ašperger et al., in their investigation on MAE of flavonoid compounds from grape skin, found that the extraction time of 5 to 15 minutes was optimal, but an extended extraction time led to a decrease in flavonols and flavan-3-ols due to thermal instability during prolonged extractions [21]. Also, Bansod et al. explored the MAE of bioactive compounds from pineapple peel waste and observed that an increase in the solvent-to-sample ratio (from 10:1 to 20:1 mL/g) created a concentration difference between plant cells and the external environment (solvent). This difference increases the rate of mass transfer, resulting in enhanced extraction of flavonoid compounds [17]. Moreover, a higher liquid-to-solid ratio promotes uniform mixing and facilitates solvent penetration into internal parts of sample [35]. The present study identified the significant interactive effects of time–solvent volume and sample weight–solvent volume on the TFC of the extracts.

3.5. Antioxidant properties

Various methods have been developed to assess antioxidant activity, each grounded in distinct mechanisms of action. These methods are mechanistically categorized into two primary

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groups: 1) single electron transfer reaction and 2) hydrogen atom transfer reaction [39]. Methods relying on single electron transfer gauge the ability of an antioxidant by transferring an electron to reduce diverse compounds, such as metal ions, carbonyl groups, and radicals. Notably, both single electron transfer and hydrogen atom transfer mechanisms typically appear simultaneously in all samples. The methods reliant on single electron transfer encompass several assays as follows: TPC measured with the Folin-Ciocalteu reagent, FRAP, DPPH free radical scavenging, and CUPRAC [36].

3.5.1. DPPH free radical scavenging

Nowadays, the DPPH assay stands out as a widely employed method to assess free radical scavenging. The IC_{50} value denotes the antioxidant concentration required to reduce the initial DPPH free radical concentration by 50%. A lower IC_{50} value indicates a greater antioxidant capacity [36].

Fig. 3 (a-b) depicts the effect of microwave power, extraction time, sample weight, and solvent volume on the IC_{50} values of the extracts. Extraction time, sample weight, and solvent volume exerted significant effects on the IC_{50} of the extracts. The lowest IC_{50} value was recorded at the minimum microwave power (100 W). With an increase in extraction time, the IC_{50} decreased. Higher sample weight led to an increased IC_{50}. An increase in solvent volume resulted in a decrease in the IC_{50} value. The IC_{50} values ranged from 11.10 to 75.30 mg/mL. As greater amounts of phenolic compounds were extracted at lower microwave power, and an extended extraction time facilitated the extraction of additional flavonoid compounds, the observed increase in radical scavenging activity can be attributed to the involvement of these compounds. A pertinent study has established a correlation between the antioxidant activity of bitter orange seeds (assessed through DPPH free radical scavenging) and the contents of phenolic and flavonoid compounds [37]. Kurtulbaş et al., in their investigation on MAE from the sour cherry peel, observed that an increase in the extraction time (at a maximum time of 90 s at power levels ranging from 350 to 500 W) resulted in heightened antioxidant activity, aligning with the content of extracted phenolic compounds [22]. Baiano et al. also found the highest antioxidant activity (DPPH free radicals scavenging) at an extraction duration of 4 min (the study encompassed the extraction times of 2 and 4 min) and a solid-to-liquid ratio of 1:2 (among ratios of 1:1, 1:2, and 1:4) for the extracts derived from the solid wastes of cauliflower, celery, and chicory, along with an elevation in the TPC. The authors attributed the increase in the TPC to alterations in the activity coefficient -describing the intermolecular interaction between the solute and the solvent- and consequently, alterations in solubility of antioxidant compounds occurred with the changes in the solid-to-liquid ratio and to the increase in contact time between the sample and the solvent in tandem with the changes in extraction time [23].

The findings of this study revealed a reduction in IC_{50} with higher solvent volumes. Inadequate solvent volume hampers the complete extraction of potent antioxidant compounds due to the diminished contact surface. Essentially, an optimal solvent volume can enhance material swelling, fostering increased contact surface between the phases, which is favorable [40]. In this current study, significant interactive effects were observed between power–time, power–solvent volume, time–sample weight, time–solvent volume, and sample weight–solvent volume on the IC_{50} value of the extracts.

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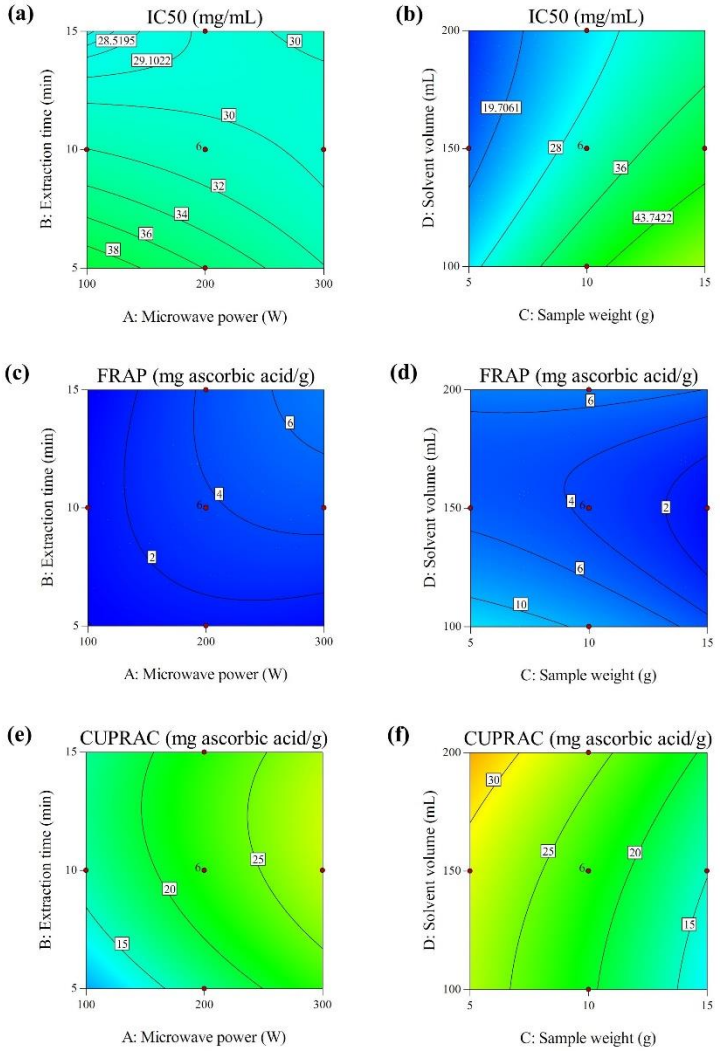

Fig. 3. Effect of different microwave-assisted extraction conditions on IC_{50}, ferric ion reducing antioxidant power (FRAP), and cupric ion reducing antioxidant capacity (CUPRAC) of extracts of bitter orange seed cotyledon.

3.5.2. Ferric ion reducing antioxidant power

The electron-donating capacity of bioactive compounds is evidenced by their ability to reduce ferric ions, and this is indicative of their antioxidant activity. Bioactive compounds, possessing antioxidant properties, can act as both reductants and inactive oxidants. The capacity of a compound to undergo reduction is viewed as a noteworthy indicator of its potential antioxidant activity [36]. Despite not directly engaging free radicals, the conversion of Fe (III) to Fe (II) contributes to the *in vivo* process of lipid oxidation [39].

The effect of microwave power, extraction time, sample weight, and solvent volume on the FRAP of the extracts is graphically depicted in Fig. 3 (c-d). All variables exerted significant effects on FRAP. Elevating microwave power resulted in a corresponding increase in FRAP. An extension of the extraction time from 5 to 15 min led to an increase in FRAP. Conversely, an increase in sample weight was associated with a decrease in FRAP. As the solvent volume increased to 150 mL, there was

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a decline in FRAP, followed by a subsequent increase. The highest recorded FRAP was 55.86, while the minimum was 0.28 mg ascorbic acid/g. The observed increase in FRAP at higher levels of microwave power and prolonged extraction times suggests the presence of co-extracted antioxidants, such as α-tocopherol, alongside phenolic compounds, contributing to the enhanced FRAP of the extracts. As it was determined in the study conducted by Mellinas et al., pH, extraction time, and solid-to-liquid ratio affected the FRAP of extracts from the cocoa bean shell waste. Additionally, the higher FRAP at increased pH levels was attributed to the presence of co-extracted antioxidants (such as polysaccharides), alongside polyphenols [33]. In our study, the interactive effects were noted as significant for power–time, power–sample weight, power–solvent volume, time–sample weight, time–solvent volume, and sample weight–solvent volume on the FRAP of the extracts.

3.5.3. Cupric ion reducing antioxidant capacity

This approach relies on its capacity to convert Cu (II) to Cu (I) in the presence of a selective Cu (I) stabilizing ligand, neocuproine (2,9-dimethyl-1,10-phenanthroline) [39]. The CUPRAC method operates on a non-radical redox potential. The redox potential of the Cu^{2+} ion (0.16 V) is lower than that of than the Fe^{3+} ion, rendering the CUPRAC reaction with greater selectivity compared to the FRAP reaction [41].

The effect of microwave power, extraction time, sample weight, and solvent volume on CUPRAC is presented in Fig. 3 (e-f), revealing their significant effects on the CUPRAC of the extracts. As microwave power increased, so did the CUPRAC. A similar trend was observed with an increase in the extraction time. Conversely, an increase in sample weight resulted in a decrease in CUPRAC. Additionally, the CUPRAC showed an increase with the rise in solvent volume. The recorded CUPRAC values ranged from a maximum of 37.86 to a minimum of 5.02 mg ascorbic acid/g. Consequently, employing higher microwave power along with extended extraction times proved to be more efficient in extracting compounds effective in copper ion reduction. Notably, the interactive effect of power–sample weight on CUPRAC of the extracts was found to be significant.

3.5.4. Chelating capacity

The determination of metal chelation capacity involves assessing the chelation effect of antioxidants with metal ions, such as ferrous ions (Fe^{2+}). This evaluation is commonly utilized as an indicator of antioxidant activity, often in conjunction with other antioxidant assays. Elemental species have the potential to generate reactive oxygen species in animal and human systems. Therefore, the capability of substances to chelate iron emerges as a valuable antioxidant property. Iron stands out as the most significant lipid pro-oxidant, among transitional metals, due to its pronounced reactivity [36]. Flavonoids and phenolic acids, renowned for their robust antioxidant power, typically feature a carboxylic acid group (in acids) or a closely situated hydroxyl group and an oxo group (in flavonoids) within their structures. These compounds can coordinate with metal centers to form stable complexes. Many flavonoids and phenolic acids engage in metal binding through the coordination of oxygen atoms, forming chelates [41].

Fig. 4 illustrates the effect of microwave power, extraction time, sample weight, and solvent volume

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on the chelating capacity of the extracts. Extraction time, sample weight, and solvent volume exhibited significant effects on the chelating capacity of the extracts. The highest chelating capacity was observed at the lowest microwave power (100 W). The highest chelating capacity corresponded to the longest extraction time. While an initial increase in the sample weight led to an increase in chelating capacity, a subsequent increase in the sample weight resulted in a decline in chelating capacity. Moreover, an increase in solvent volume increased the chelating capacity. The recorded maximum and minimum chelating capacity values were 1.69 and 0.094 mg EDTA/g, respectively. Importantly, the interactive effects of power–solvent volume and time-solvent volume on the chelating capacity of the extracts were found to be significant.

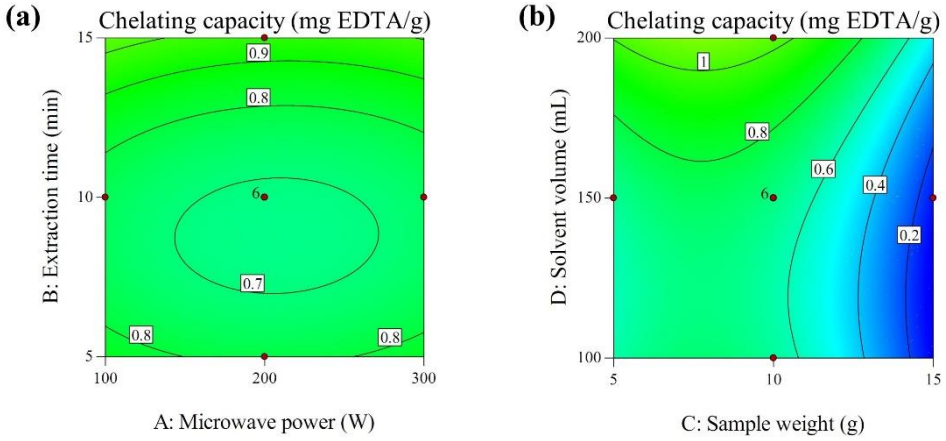

Fig. 4. Effect of different microwave-assisted extraction conditions on the chelating capacity of extracts of bitter orange seed cotyledon

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3.6. Physical properties

Table 1 presents the responses regarding the physical properties investigated in this study. The brightness (L^*) values ranged from 53.00 to 73.00, redness-greenness (a^*) values varied from -4.00 to 0.00, and yellowness-blueness (b^*) values covered a range of 4.00 to 12.00. Additionally, the extracts exhibited pH, refractive index, and density values within the ranges of 7.61–7.82, 1.3299–1.3400, and 0.708–0.890 g/cm^3, respectively.

3.7. Optimization and validation of microwave-assisted extraction

The response surface methodology based on CCD was used to optimize the conditions for MAE. The optimum MAE conditions were determined by simultaneously maximizing TPC, TFC, FRAP, CUPRAC, and chelating activity while minimizing IC_{50}. According to the software predictions, the optimum conditions were microwave power of 300 W, extraction time of 15 min, sample weight of 5 g, and solvent volume of 200 mL. A verification experiment was then conducted under these optimized settings. The experimental values closely aligned with the predicted values, as outlined in Table 3.

In practical terms, it is important to attend to both the quantity and quality indicators when optimizing the extraction process of extracts. The optimization goal extends beyond only maximizing certain compounds like TPC. In essence, achieving an

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acceptable TPC level can be considered. Given that the studied factors may yield conflicting responses, where maximizing one index might result in a decrease in TPC or contrariwise, it is recommended to consider all desired responses in an integrated manner to optimize the overall extraction process [20].

Table 3. Comparison between the optimum condition of microwave-assisted extraction from bitter orange seed cotyledon with the model as well as the electromantle.

Response	Experimental (Microwave)	Predicted (model)	Error (%)	Electromantle	Error (%)
TPC[1] (µg GAE/g)	51268.00	52544.20	2.49	53088.84	3.55
TFC[2] (µg QE/g)	9651.80	9959.33	3.19	9938.28	2.97
IC_{50} (mg/mL)	28.13	26.81	-4.69	27.56	-2.03
FRAP[3] (mg ascorbic acid/g)	9.84	10.34	5.08	10.08	2.44
CUPRAC[4] (mg ascorbic acid/g)	37.91	40.58	7.04	40.02	5.57
Chelating capacity (mg EDTA/g)	1.30	1.38	6.15	1.39	6.92

[1]Total phenolic content; [2]Total flavonoid content; [3]Ferric ion reducing antioxidant power; [4]Cupric ion reducing antioxidant capacity.

3.8. Comparison of microwave-assisted extraction method with electromantle method

Initially, the experimental test took place under the optimum MAE conditions to assess the model's appropriateness. Subsequently, a thorough comparison was made with the characteristics of the extract obtained via the electromantle-assisted extraction method. The results from the MAE method closely paralleled those of the electromantle-assisted method, as indicated in Table 3. It should be highlighted, however, that the mechanism for extracting compounds using microwave differs from the conventional electromantle method (Fig. 5). In the conventional extraction method, the plant cell wall undergoes a softening and breaking process, assisting the release of phytochemical compounds. In this method, heat is supplied through conduction and convection, creating a concentration gradient in the course of the transition of target compounds from solid to liquid phase. MAE method employs microwave energy to heat sample components through ionic conduction and dipole rotation. Therefore, the extraction is directly corresponding to the dielectric sensitivity of the solvent and sample matrix. Unlike the conventional extraction process, where heat transfers from the source to the interior of the sample, MAE generates heat within the sample in a volumetric manner [5, 12, 14].

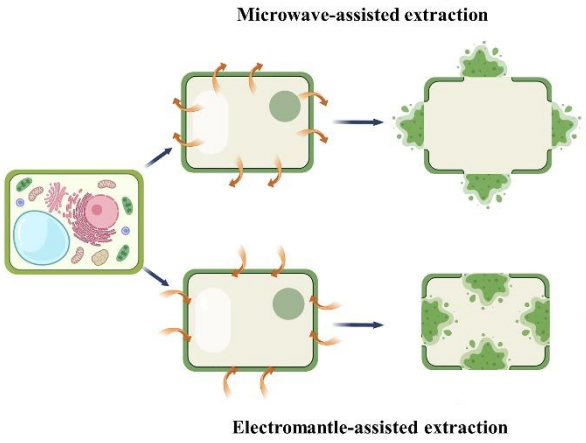

Fig. 5. Mechanism of action for compound extraction via microwave- and electromantle-assisted extraction methods.

4- Conclusion

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In the present study, the extraction of bioactive compounds and antioxidant activity in bitter orange seed cotyledon extract obtained through the MAE method were investigated. The experimental design involved microwave power, extraction time, sample weight, and solvent volume as independent variables, while experimental responses included extraction yield, TPC, TFC, IC_{50} value, FRAP, CUPRAC, chelating capacity, and physical properties. The results revealed that the optimum extraction conditions were identified as a microwave power of 300 W, an extraction time of 15 min, a sample weight of 5 g, and a solvent volume of 200 mL. Verification experiment showed no significant difference between the predicted data obtained from the software and the experimental results. The MAE method can be used as a rapid and efficient approach to extract bioactive compounds from bitter orange seed waste.

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استخراج ترکیبات زیست‌فعال مغز دانه نارنج به کمک مایکروویو و تعیین ویژگی‌های پاداکسندگی آن‌ها

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چکیده

در مطالعه‌ی حاضر، ترکیبات زیست‌فعال از مغز دانه نارنج (*Citrus aurantium*)، به‌عنوان یکی از پسماندهای فرآوری مرکبات با استفاده از روش مایکروویو استخراج شد. اثر چهار متغیر مستقل شامل توان مایکروویو (۳۰۰– ۱۰۰ وات)، زمان استخراج (۱۵– ۵ دقیقه)، وزن نمونه (۱۵– ۵ گرم) و حجم حلال (۲۰۰– ۱۰۰ میلی لیتر) بر پاسخ‌های بازدهی استخراج، میزان فنل کل، میزان فلاونوئید کل، فعالیت مهار رادیکال آزاد (IC_{50})، قدرت احیای یون فریک، ظرفیت احیای یون مس و ظرفیت کلاته‌کنندگی بررسی شد. روش سطح پاسخ مبتنی بر طرح مرکب مرکزی به‌منظور بررسی اثر متغیرهای مستقل بر پاسخ‌ها و هم‌چنین به منظور بهینه‌سازی شرایط استخراج استفاده شد. شرایط بهینه استخراج شامل توان مایکروویو ۳۰۰ وات، زمان استخراج ۱۵ دقیقه، وزن نمونه ۵ گرم و حجم حلال ۲۰۰ میلی لیتر بود. در خصوص بازدهی استخراج، با افزایش توان مایکروویو، زمان استخراج و وزن نمونه، میزان آن به‌شکل معنی‌داری افزایش یافت درحالی‌که با افزایش حجم حلال، میزان آن به‌شکل معنی‌داری کاهش یافت. هم‌چنین، بیشترین میزان فنل کل عصاره در کمترین سطوح توان مایکروویو و زمان استخراج مشاهده شد. در رابطه با میزان فلاونوئید کل عصاره، با افزایش زمان استخراج و افزایش حجم حلال، میزان آن به‌شکل معنی‌داری افزایش یافت درحالی‌که با افزایش وزن نمونه، میزان آن به‌شکل معنی‌داری کاهش یافت. علاوه بر این، ظرفیت احیای یون مس عصاره با افزایش توان مایکروویو، زمان استخراج و حجم حلال، برخلاف وزن نمونه، به‌شکل معنی‌داری افزایش یافت. در مجموع، روش استخراج به کمک مایکروویو را می‌توان به‌عنوان روشی مناسب برای استخراج ترکیبات زیست‌فعال از مغز دانه نارنج پیشنهاد داد.

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