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Study of the effect of ultrasonic-assisted extraction based on the Box-Behnken design on the phenolic compound content of *Hammada scoparia* leaves and evaluation of their antioxidant and antibacterial activities

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ABSTRACT

Hammada scoparia is a plant of the Chenopodiaceae family. It represents a potential source of polyphenols and is used in traditional medicine in many countries. This research was carried out to evaluate the influence of ultrasound-assisted extraction parameters (ethanol concentration (30-70%), temperature (20-60 °C), time (10-30 min), and solvent-to-solid ratio (30-60 v/w) on total phenolic compound and total flavonoid contents, as well as the antioxidant capacity (DPPH assay) of *H. scoparia,* using the response surface methodology (RSM) approach and a Box-Behnken design (BBD). The obtained optimal extract of *H. scoparia* was used to evaluate ferric and molybdate reducing powers and ABTS scavenging activity as well as the antibacterial activity. Based on the BBD results, the extraction conditions, which gave the highest contents of phenolic compounds and flavonoids and the greatest DPPH activity, are: an extraction time of 30 minutes, a temperature of 20 °C, an ethanol concentration of 46.95%, and a liquid-to-solid ratio of 60 mL/g. The results of the determination of antioxidant activity revealed that *H. scoparia* extract inhibited free radicals $DPPH^{\cdot}$ and $ABTS^{\cdot}$, and reduced ferric and molybdate through different mechanisms. Concerning the antibacterial activity, the results obtained showed that *H. scoparia* extract was found to be active against all tested bacterial strains, with the exception of *Escherichia coli* ATCC 25922. *H. scoparia* optimal extract could be a promising source for the pharmaceutical industry and food applications.

KEYWORDS
Hammada scoparia; ultrasound assisted extraction; phenolic compounds; antioxidant activity; antibacterial activity; response surface methodology

KEY CONTRIBUTION
Evaluation of the effect of ultrasound-assisted extraction conditions on total phenolic content and DPPH radical scavenging activity of *Hammada scoparia.* The influence of ethanol concentration (%), time (min), solvent-to-solid ratio (mL/g), and temperature (°C) was tested. Response surface methodology (RSM) was employed to design experimental conditions. Determination of the antioxidant and antibacterial capacities of the extract obtained under optimal extraction conditions.

Introduction

Hammada scoparia (Pomel) *Iljin*, or *Haloxylon scoparium* (Pomel) is a medicinal plant commonly found in desert areas. It is considered to be a source of potentially therapeutic agents, and it exhibits biological and pharmacological activities (Bouaziz et al., 2016; Saidi et al., 2015).

Phytochemical screening of the aerial parts of *H. scoparia* revealed the presence of flavonoids, tannins, saponins, anthocyanins, alkaloids, cardinolids, and leucoanthocyanins (Benkherara et al., 2021). These compounds may have potential applications in the treatment of various diseases (Bouaziz et al., 2016; Eleroui et al., 2021; Taïr et al., 2016). *H. scoparia* has been shown to possess larvicidal, anti-cancer, and anti-plasmodial properties (Bouaziz et al., 2016). Previous research has also indicated that *H. scoparia* aqueous extract exhibits molluscicidal properties and has been used as an antileukemic agent (Eleroui et al., 2021). In traditional medicine, the leaves of *H. scoparia* are used as an antiseptic in wound healing (Bouaziz et al., 2016) and preventing liver disease, inflammation, and obesity (Bourogaa et al., 2013).

In recent years, the search for bioactive compounds, particularly phenolic compounds, from natural sources such as plants, has increased considerably (Ghafoor et al., 2009). Phenolic compounds, which are a diverse group of secondary metabolites found in a wide range of plant species, have been recognized as potent antioxidants (Plyuta et al., 2013) and crucial components in the prevention of various diseases (Gülçin et al., 2019). There is a clear correlation between the antioxidant activity and the total phenolic concentration, indicating that polyphenols play a significant role in the scavenging of free radicals (Hemmami et al., 2023). Additionally, these natural compounds have the ability to interact with microbial cells through various mechanisms, making them highly effective in combating the prevalence of drug-resistant strains (Ecevit et al., 2022).

Extraction is a crucial step in obtaining phytochemicals and has been the focus of recent research. Recently, an interest in the development of green extraction processes has been growing, which aim to reduce extraction time, minimize the use of harmful solvents, and lower energy consumption (Chupin et al., 2015). The pharmaceutical and food industries are increasingly adopting alternative extraction methods, particularly ultrasonic, to obtain bioactive molecules (Wani and Uppaluri, 2022).

Ultrasound Assisted Extraction (UAE) is an efficient extraction method characterized by its ease of use, scalability, higher reproducibility, shorter processing time, and reduced energy and solvent consumption. Due to this, UAE is widely used for extracting bioactive components in the food and

pharmaceutical industries (Wani and Uppaluri, 2022). This process has been shown to significantly enhance the extraction of phenolic compounds from various plant matrices (D'Alessandro et al., 2014).

The application of statistical techniques for the enhancement of analytical methodologies has been progressively gaining ground. These methods, collectively known as multivariate analysis, offer several advantages, including the reduction in the number of required experiments, the minimization of reagent consumption, and the decrease in laboratory workload (Ferreira et al., 2007).

Response surface methodology (RSM) is a widely used statistical tool for assessing the effects of multiple factors and their interactions on response variables. RSM requires fewer experimental trials to evaluate multiple parameters and their interactions, making it a time-efficient and cost-effective approach (Aybastıer and Demir, 2010; Aybastıer et al., 2013).

The central composite design (CCD) and the Box-Behnken design (BBD) are two of the most frequently employed response surface methodology (RSM) designs for conducting a wide range of experiments. The BBD, which is a spherical and revolving design, is often used for optimizing chemical and physical processes due to its efficient design and reliable outcomes (Aybastıer et al., 2013).

The use of ultrasound for the extraction of phenolic compounds from *H. scoparia* has not been documented in previous studies. Therefore, the objective of this research is to optimize ultrasound-assisted extraction parameters for total phenolic compounds and DPPH scavenging activity of this plant, using response surface methodology (RSM) approach and a Box-Behnken design (BBD). The extract obtained under the optimal extraction parameters has been used to assess other antioxidant activities, using various essays, and antibacterial activity against several bacterial strains.

Materials and methods

Plant material

The leaves of *H. scoparia* were collected from Oued souf region, wilaya d'El Oued (Algeria) during March 2021. *H. scoparia* plant was identified and authenticated by National higher agronomic school (NHAS) in Algiers, Algeria. The selected plant material was washed and then dried at 40 °C, and subsequently ground into a fine powder. The obtained sample was stored until further experimental analysis.

Ultrasound-assisted extraction

The extraction of phenolic compounds from *H. scoparia* was performed using an ultrasonic bath (frequency 40kHz, power generator 120 W) following the method described by Moussa et al. (2022). The ranges of the independent variables were estimated using a one-factor experiment as preliminary research. The experiments were carried out by mixing 1 g of plant powder with ethanol solvent (30 - 70%). The resulting mixture was subjected to extraction at varying temperatures (20-60 °C) and different extraction times (10-30 min), with a solvent-to-solid ratio ranging from 30 to 60 mL/g. After filtration, the liquid extracts were adjusted to the final volume using solvent extraction and stored at 4 °C until further analysis for optimization and prediction (Table 1).

Experimental design

The present study employed a response surface methodology (RSM) approach and a Box-Behnken design (BBD) to investigate the effects of four independent factors (ethanol concentration (%), extraction time (min), solvent-to-solid ratio (mL/g), and temperature (°C)) on three response variables: total phenolic content (TPC), total flavonoid content (TFC), and DPPH scavenging activity of *H. scoparia*

extract. For this, there are 27 experiments total in the BBD matrix. Table 1 presents the experimental design, the independent variables, and the related response values.

The expression of the investigated responses as a function of the independent variables tested in the present work, was carried out by applying the generalized polynomial model of the second order according to the following formula:

$$Y = \beta 0 + \sum_{i=1}^{k} \beta i\, xi + \sum_{i=1}^{k} \beta ii\, x2i + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta ij\, xixj + e \quad (1)$$

Y: represents the response variable; xi and xj: represent the independent variables; β0, βi, βii and βij: represent the regression coefficients of linear, interaction and quadratic terms respectively, and e is the error.

Table 1. Box-Behnken design (BBD) for observed and predicted values of TPC, TFC and DPPH activity of *H. scoparia*

Run	Variable levels				Observed values			Predicted values		
	x1	x2	x3	x4	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH (mg AAE/g)	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH (mg AAE/g)
1	50	20	45	40	72.37±1.51	2.65±0.09	39.62±0.76	71.23	2.56	40.01
2	30	10	45	40	67.58±0.45	2.44±0.07	33.14±0.36	68.79	2.47	32.95
3	30	20	60	40	80.18±0.43	2.79±0.09	37.94±0.52	77.63	2.84	38.19
4	50	20	45	40	70.71±0.23	2.50±0.03	39.96±0.47	71.23	2.56	40.00
5	50	30	60	40	75.96±0.35	3.13±0.12	40.86±0.28	79.02	3.23	41.09
6	50	10	60	40	68.93±0.16	3.48±0.07	38.69±1.12	69.84	3.22	38.18
7	70	20	45	60	86.89±0.19	2.88±0.14	27.92±0.20	86.09	2.90	27.98
8	70	20	45	20	69.65±1.34	2.84±0.13	28.39±0.36	70.56	2.81	28.76
9	50	20	60	60	78.17±0.56	3.38±0.11	41.52±0.80	77.96	3.44	42.01
10	50	10	45	60	77.76±0.25	3.27±0.00	36.01±0.46	78.34	3.49	37.05
11	50	20	45	40	70.60±0.39	2.53±0.06	40.42±0.90	71.23	2.56	40.00
12	50	30	30	40	63.02±0.12	2.60±0.09	28.58±0.66	63.08	2.72	28.36
13	50	10	30	40	61.48±0.91	2.80±0.11	29.63±0.06	59.38	2.57	28.67
14	70	20	30	40	67.29±0.66	2.56±0.05	22.14±0.14	67.52	2.63	21.22
15	50	30	45	20	78.42±0.32	3.77±0.08	39.99±0.27	75.52	3.68	38.28
16	70	20	60	40	83.15±0.95	2.74±0.13	30.23±0.70	80.21	2.63	29.32
17	50	20	60	20	76.56±0.21	2.91±0.03	41.56±0.08	78.28	3.07	42.01
18	70	30	45	40	78.18±0.32	2.95±0.08	26.83±0.27	78.32	2.93	28.40
19	30	20	45	20	71.96±1.42	2.79±0.16	34.54±0.57	73.73	2.64	33.75
20	30	20	45	60	76.67±0.19	2.40±0.18	35.78±0.96	76.73	2.31	34.68
21	30	30	45	40	75.40±0.54	2.49±0.05	29.19±1.11	74.29	2.41	30.75
22	70	10	45	40	68.48±0.92	2.64±0.04	23.78±0.22	70.94	2.71	23.61
23	50	10	45	20	62.85±1.23	2.58±0.13	35.24±0.33	59.79	2.75	36.01
24	30	20	30	40	63.30±0.15	1.44±0.01	23.79±0.31	63.92	1.67	24.04
25	50	20	30	60	74.72±1.02	2.53±0.01	30.02±0.02	74.35	2.37	30.96
26	50	20	30	20	53.94±0.69	3.03±0.05	29.90±0.08	55.50	2.98	30.81
27	50	30	45	60	74.76±0.23	2.74±0.10	38.81±1.08	75.50	2.70	37.38

x1: Ethanol concentration (%); x2: Time (min); x3: Liquid-to-solid ratio (mL/g); x4: Temperature (°C); TPC: Total phenolic contents (mg GAE/ g DW); TFC: Total flavonoids content (mg QE/g DW); DPPH: $DPPH^{\cdot}$ radical scavenging assay (mg AAE/g DW).

Total phenolic content (TPC)

The phenolic compound content was determined following the method described by George and Bennett (2005). An aliquot of *H. scoparia* extracts (125 µL) was mixed with 625 µL of Folin-Ciocalteu solution. After two minutes' reaction period, 500 µL of sodium carbonate (7.5%) was added. The mixture was incubated for 15 minutes at 50 °C, and the absorbance was then measured at 760 nm. The phenolic compounds content of *H. scoparia* was expressed as milligrams of gallic acid equivalent per gram of dry weight of plant (mg GAE/g DW). The calibration curve equation was y=10.125x + 0.0048, with an R^2 value of 0.9992. The gallic acid concentrations used for the calibration ranged from 0.02 to 1 mg/mL.

Total flavonoids content (TFC)

The total flavonoids content of *H. scoparia* extracts was assessed according to the method described by Serra Bonvehí et al. (2001). A volume of 1 mL of aluminum chloride (2%) was added to the same volume of *H. scoparia* extracts. After the incubation of 10 min, the absorbance was read at 430 nm. The flavonoid assay results were expressed as milligrams of quercetin equivalent per gram of dry weight of *H. scoparia* (mg QE/g DW). The calibration curve equation was Y = 35,335x + 0,0032, with an R^2 value of 0,9997.

$DPPH^{\cdot}$ radical scavenging activity

The $DPPH^{\cdot}$ radical scavenging activity of *H. scoparia* extract was assessed according to the method of Dudonne et al. (2009). To 50 µL of sample extracts were added 1.5 mL of $DPPH^{\cdot}$ solution (60 M). The absorbance was measured at 515 nm after twenty minutes of incubation at 37 °C. The DPPH scavenging activity of *H. scoparia* extracts was expressed as milligrams of ascorbic acid equivalent per gram of dry weight (mg AAE/g DW). The equation of the ascorbic acid calibration curve was y=-7.475x + 0.7978, R^2=0.9972.

Evaluation of the antioxidant potential of H. scoparia optimal extract

$ABTS^{\cdot}$ radical scavenging activity

The $ABTS^{\cdot}$ scavenging activity of *H. scoparia* optimal extract was assessed following the method of Re et al. (1999) with slight modification. The $ABTS^{\cdot}$ solution was prepared by mixing 2.45 mM of ABTS with 7 mM of potassium persulfate, followed by incubation for 16 hours in the dark. The resulting solution was then mixed with ethanol to achieve an absorbance of 0.7 ± 0.02 at 734 nm. A volume of 50 µL of *H. scoparia* extract was added to 1.5 mL of the $ABTS^{\cdot}$ solution. The absorbance was measured at 734 nm after 6 min of incubation. The percentage of $ABTS^{\cdot}$ radical inhibition was calculated using the following formula:

$$\text{ABTS}^{\cdot}\text{ radical scavenging activity} = \frac{(A_{control} - A_{extract})}{A_{control}} * 100 \quad (2)$$

$A_{control}$: is the absorbance of the control; $A_{extract}$: is the absorbance of the sample.

The IC_{50} value indicates the concentration of extract needed to reduce the absorbance by 50% at 734 nm.

Ferric reducing power

The ferric reducing power of the optimal *H. scoparia* extract was tested using the method of Oyaizu (1986). A volume of 1 mL of the sample was added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide solution (1%). The mixture was incubated at 50 °C for 20 minutes, followed by the addition of 2.5 mL of trichloroacetic acid (10%). The resulting solution was then

centrifuged at 3000 g for 10 minutes. A volume of 2.5 mL from the supernatant was diluted with 1 mL of distilled water, and 0.5 mL of ferric chloride solution (0.1%) was added. The absorbance was measured at 700 nm. The ferric reducing power was expressed as milligrams of ascorbic acid equivalent per gram of lyophilized extract (mg AAE/g dry extract) according to equation (3).

$$\text{Ferric reducing power(mg AAE/g dry extract)} = \frac{c(\text{mg AAE/mL})}{c(\text{g /mL})} \quad (3)$$

Where *c*(mg AAE/mL) and *c*(g/mL) are the antioxidant activity calculated from the standard curve and the concentration of lyophilized extract respectively. The equation of the calibration curve was y=5.0283x + 0.0159, with an R^2 value of 0.9951.

Molybdate reducing activity

The molybdate reducing activity of *H. scoparia* extract was determined by the method of Prieto et al. (1999). An aliquot of 0.3 mL of sample was added to 3 mL of a reagent mixture containing sodium phosphate (28 mM), sulfuric acid (0.6 M), and ammonium molybdate (4 mM). The reaction mixture was incubated at 95 °C for 90 minutes. The absorbance was then measured at 695 nm. The reducing activity of molybdate is expressed as equivalent milligrams of ascorbic acid per gram of lyophilized extract (mg AAE/g dry extract) (Eq. 3). The ascorbic acid calibration curve was y = 2.1339x + 0.0277, R^2=0.9988.

Antimicrobial activity

Microbial strains

In this study, the antibacterial activity of *H. scoparia* extract was evaluated against five bacterial strains, obtained from the Laboratory of Microbial Ecology (University Abderrahmane Mira Bejaia, Algeria). The bacterial strains selected for this study are *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* BAR 32, *Salmonella sp*., *Pseudomonas aeruginosa* ATCC 6633, and *Methicillin-resistant Staphylococcus aureus* ATCC 43300 (MRSA). These strains were chosen due to their clinical significance and prevalence in healthcare settings.

Well diffusion method

The well diffusion method, as described by Zeidan et al. (2013), was employed to assess the antibacterial activity of the *H. scoparia* extract obtained with the optimal extraction parameters. For this, Petri plates containing Mueller Hinton agar medium, previously inoculated with a bacterial suspension, were utilized to create wells with a diameter of 6 mm. A volume of 50 µL of the plant extract, with a concentration of 50 mg/mL was added to each well. The negative control was prepared using the solvent dimethyl sulfoxide (DMSO), while the positive controls were tetracycline (TE) and ciprofloxacin (CIP). The inoculated Petri plates were incubated at 37 °C for 24 hours. The antimicrobial activity of the *H. scoparia* extract was evaluated by measuring the zone of inhibition against the tested bacteria. The results were expressed in millimeters (mm).

Minimum inhibitory concentration (MIC)

The method used to determine the minimum inhibitory concentration (MIC) of *H. scoparia* extract consists of dilution followed by diffusion on an agar medium (Mueller Hinton) (Metrouh-Amir et al., 2015).

The minimum inhibitory concentration of *H. scoparia* extract was determined using a series of six concentrations: 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.13 mg/mL, 1.56 mg/mL and 0.78 mg/mL. Once

the plates had been seeded, the wells (6 mm) were filled with 50 µL of each dilution of the selected extract. The plates were then incubated at 37 °C for 18 to 24 hours, after which two hours of diffusion at 4 °C had taken place. The results were represented by the diameters of the zones of inhibition and expressed in millimeters (mm).

Statistical analyses

The experimental design was carried out using JMP 13 software (statistical analysis system, Inc. SAS). Each experiment was conducted in triplicate. To ascertain the significance of the model and regression coefficients, an analysis of variance (ANOVA) was performed. The significance of the regression coefficients was determined using the Student's test. The antioxidant activity graphs were created using the GraphPad Prism 8.0.2 program.

Results and discussion

Extract yields

Model parameter estimation

This study was devoted to studying the effects of ultrasound-assisted extraction parameters on total phenolic content, total flavonoid content, and DPPH activity of *H. scoparia*, using the response surface methodology. This methodology offers significant advantages over traditional single-parameter optimization, providing an effective alternative for the optimization of phenolic compound extraction with a reduced number of experiments, leading to cost savings and reduced analysis time (Böhmer-Maas et al., 2020). The extraction factors selected for this study were based on the findings of previous research, which indicated that temperature, time, solvent concentration, and solvent/solid ratio were the most effective parameters for extracting phenolic compounds (Che Sulaiman et al., 2017; Elboughdiri, 2018). The Box-Behnken design (BBD) was applied to determine the effect of solvent concentration (x_1), temperature (x_2), time (x_3), and liquid-to-solid ratio (x_4) on total phenolic compounds content (TPC), total flavonoids content (TFC), and DPPH activity (Table 1). The regression models for TPC, TFC and DPPH activity are presented in equations 4, 5 and 6.

$$\mathrm{TPC}\ (\mathrm{mg_{GAE}/g_{Dw}}) = 71.23 + 1.54\,x_1 + 3.22\,x_2 + 6.60\,x_3 + 4.63\,x_4 + 3.13\,x_1x_4 - 4.64\,x_2x_4 - 4.80\,x_3x_4 + 3.17\,x_1^2 + 2.37\,x_4^2 \quad (4)$$

$$\mathrm{FC}\ (\mathrm{mg_{QE}/g_{Dw}}) = 2.55 + 0.18\,x_1 + 0.29\,x_3 - 0.29\,x_1x_3 - 0.43\,x_2x_4 + 0.24\,x_3x_4 - 0.20\,x_1^2 + 0.28\,x_2^2 + 0.31\,x_4^2 \quad (5)$$

$$\mathrm{DPPH\ activity}\ (\mathrm{mg_{AAE}/g_{Dw}}) = 39.66 - 2.92\,x_1 + 5.56\,x_3 + 1.74\,x_1x_2 - 1.51\,x_1x_3 - 8.47\,x_1^2 - 2.59\,x_2^2 - 3.32\,x_3^2 \quad (6)$$

The analysis of the results revealed that the phenolic compound and flavonoid contents, as well as the DPPH activity, exhibited variations according to the extraction conditions tested. The phenolic compound content obtained varies from 53.94 to 86.89 mg GAE/g DW. The flavonoid content is between 1.44 and 3.77 mg EQ/g DW. In terms of DPPH activity, the values obtained range from 22.14 to 41.56 mg EAA/g DW (Table 1). The statistical analysis of the results demonstrated that the parameters tested had varying effects on the variables under study (TPC, TFC, and DPPH activity) (Table 2). In this study, all factors have a positive linear effect on total phenolic compound extraction. Regarding

flavonoids, the factors that showed a significant linear effect are the ethanol concentration and the solvent-to-solid ratio. For the antioxidant activity represented by the DPPH$^{\cdot}$ radical scavenging assay, the ethanol concentration was found to have a positive linear effect, while the liquid-to-solid ratio exhibited a negative linear effect. The results revealed that the interaction between the concentration of ethanol and the extraction temperature had a positive impact on the contents of phenolic compounds. However, this interaction did not have a notable effect on the total flavonoid content or DPPH activity. It has been demonstrated that interactions between time and extraction temperature, as well as between solvent-to-solid ratio and extraction temperature, have a negative impact on the total phenolic compound content. The interactions between time and temperature, and between solvent concentration and liquid-to-solid ratio had a negative effect on flavonoids content. Conversely, the interactions between solvent-to-solid ratio and temperature had a positive effect. The interactions between liquid-to-solid ratio and solvent concentration, and between time of extraction and solvent concentration, respectively, had a negative and positive effect on DPPH activity. Regarding the quadratic effects, the phenolic compound content was positively influenced by the ethanol concentration and the temperature. The quadratic effect of ethanol concentration was found to be negative on total flavonoid content and DPPH activity. The quadratic effects of time extraction and liquid-to-solid ratio had a negative impact on DPPH activity. However, the quadratic effects of time and temperature had a positive effect on total flavonoid content. In this work, the high values of R-squared for the tested model (0.95 and 0.81; 0.91 and 0.81; 0.97 and 0.95 for R^2 and Adj. R^2 for TPC, TFC, and DPPH respectively) indicate a remarkable correlation between the values found in the present study and those predicted. In a good statistical model, the adjusted R2 value should be comparable to the R2 value, which is the case in our results. When the coefficient of determination R^2 is equal to or higher than 80%, the model is judged good (Fadil et al., 2021). The adequacy of the model is determined essentially by the adjusted R-square ($R^2_{Adj.}$) (Zakaria et al., 2021).

Table 2. Regression results and analysis of variance for the experimental results

		TPC		TFC		DPPH	
	Source	**Estimation**	**Prob. > F**	**Estimation**	**Prob. > F**	**Estimation**	**Prob. > F**
Estimated parameters	X_1	1.5465333	0.0463*	0.1888042	0.0041*	-2.92344	<.0001*
	X_2	3.2213025	0.0006*	0.03926	0.4766	0.6489417	0.1005
	X_3	6.5992142	<.0001*	0.2901958	0.0002*	5.5612433	<.0001*
	X_4	4.63285	<.0001*	-0.06049	0.2797	0.0347583	0.9256
	$X_1 X_2$	0.4713	0.7027	0.068675	0.4724	1.749175	0.0170*
	$X_1 X_3$	-0.255625	0.8357	-0.291173	0.0084*	-1.515295	0.0336*
	$X_2 X_3$	1.3722325	0.2773	-0.036875	0.6973	0.806225	0.2260
	$X_1 X_4$	3.133225	0.0233*	0.1047	0.2801	-0.42895	0.5100
	X_2X_4	-4.642625	0.0023*	-0.43016	0.0006*	-0.483375	0.4589
	$X_3 X_4$	-4.7934	0.0018*	0.24375	0.0218*	-0.0403	0.9502
	X_1^2	3.1740029	0.0103*	-0.209327	0.0227*	-8.475332	<.0001*
	X_2^2	-1.314776	0.2319	0.2823446	0.0042*	-2.590384	0.0005*
	X_3^2	-2.081026	0.0695	0.0921233	0.2728	-3.326382	<.0001*
	X_4^2	2.3762029	0.0420*	0.3125696	0.0021*	-0.225759	0.6871
Regression results	R^2	0.950063		0.914878		0.979965	
	R^2_{Adj}	0.891803		0.815569		0.956592	
	RMSE	2.41136		0.185109		1.263332	
	C.V(%)	3.34		6.66		3.77	
	ANNOVA	<.0001*		0.0002*		<.0001*	
	Lack of fit	0.1343		0.1431		0.0813	

x1: Ethanol concentration (%); x2: Time (min); x3: Solvent-to-solid ratio (mL/g); x4: Temperature (°C); TPC: Total phenolic contents (mg GAE/ g DW); TFC: Total flavonoids content (mg QE/g DW); DPPH: DPPH$^{\cdot}$ radical scavenging assay (mg AAE/g DW).

The obtained coefficients of variation (C.V%) of TPC, TFC, and DPPH activity were 3.34, 6.66, and 3.77% respectively. If a coefficient of variation (C.V%) exceeds 10%, the average value presents significant variations and the response model developed is not sufficiently adequate (Dahmoune et al., 2015). Therefore, the reproducibility of the models in the present study was satisfied by the obtained results.
The model's fitness was examined using a lack of fit test, which indicated the ability of the model to correctly predict its variability. The lack of fit of the response surface prediction model is not significant with a p-value less than 0.05, and the model is considered fit with a p-value greater than 0.05 (Kong et al., 2010). In the present study, the lack of fit values of the phenolic compounds content, flavonoids content, and DPPH scavenging activity of *H. scoparia* are 0.1343, 0.1431, and 0.0813, respectively. The validity of the models is carried out by analyzing the lack of fit. Accordingly, the model is considered to be an accurate representation of the actual data if the p-value is determined to be insignificant and greater than 0.05 (Zakaria et al., 2021). The quadratic polynomial models produced in this study were found to be accurate and reliable in predicting the corresponding responses.

Analysis of response surfaces

In this work, in order to study the interactive effects of the independent variables tested and their mutual interaction on phenolic compound content, flavonoids content, and DPPH activity, three-dimensional response surface profiles of multiple non-linear regression models were depicted.
The effect of the interaction effects of ethanol concentration, time, liquid-to-solid ratio, and temperature extraction on the total phenolic content of *H. scoparia* was illustrated in Figure 1.
The interaction between ethanol concentration and temperature exerted an exponential effect on total phenolic content (Figure 1(a)).
Increasing extraction time improves the yield of phenolic compounds at low temperatures, the optimal value (80 mg GAE/g DW) was found at 20 °C after 30 min of extraction (Figure 1(b)). Extraction of phenolic substances is more effective with longer extraction periods (Santos-Buelga et al., 2012). This can be attributed to the fact that extending the ultrasonic extraction time allows for greater penetration of the solvent, which in turn facilitates the release and solubility of various substances from the raw materials, which favours the extraction of higher quantities of phenolic compounds (Hachani et al. 2020). The yield of phenolic compounds increases with increasing temperature after a short extraction time. An estimated 82 mg GAE/g DW was attained at 60 °C after 10 minutes. The high temperature leads to an increase in the extraction rate by promoting the rate of mass transfer by promising the solubility of the solute (Prasad et al., 2012). The obtained results show that the exposure to elevated temperatures for over ten minutes resulted in a notable decline in total phenolic content, which could be explained by the fact that these compounds are degradable at high temperatures.
Figure 1 (c) reveals that the interaction between the temperature and the liquid-to-solid ratio exerted an inversely proportional effect on total phenolic content, with approximate values of 80 mg GAE/g DW and 75 mg GAE/g DW, using a ratio of 60 mL/g at 20 °C and 30 mL/g at 60 °C, respectively. The total phenolic content increases in line with the solvent-to-solid ratio at low temperature. The content of bioactive compounds increases with the volume of extraction solvent, due to the fact that phenolic compounds are more soluble in higher volumes of solvent than in lower volumes (Silva et al., 2007). The high liquid-to-solid ratio provides high mass transfer phenomena (Al-Farsi and Lee, 2008; Chaalal et al., 2012), which confirmed the obtained results.
The effects of interactions between ethanol concentration and liquid-to-solid ratio, between time and temperature and between solvent-to-solid ratio and temperature on total flavonoids of *H. scoparia* were represented in Figure 2.

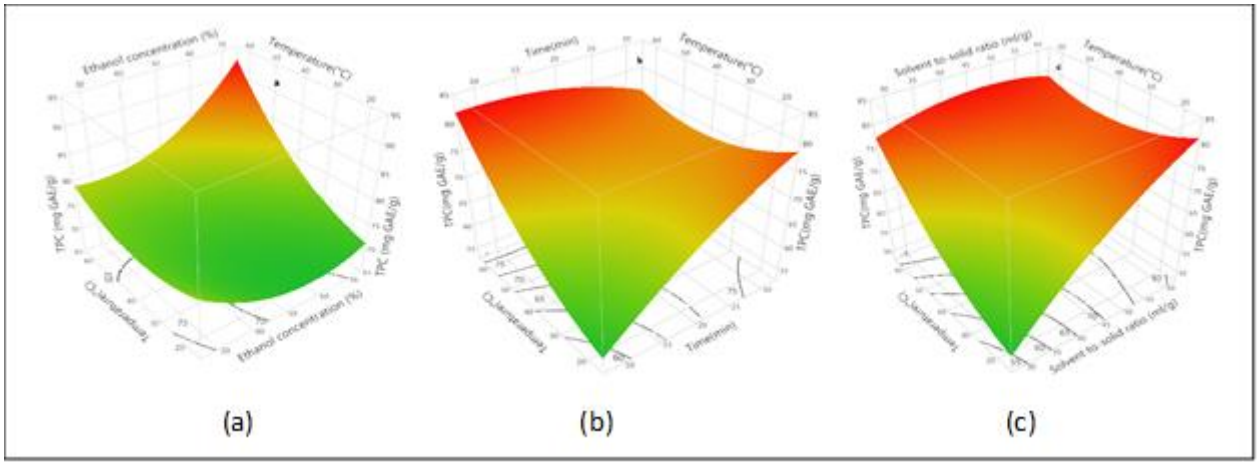

Figure 1. Response surface plots illustrating UAE factors influence the TPC of *H. scoparia*
(a) Interaction temperature - ethanol concentration; (b) Interaction temperature - time; (c) Interaction temperature - solvent-to-solid ratio

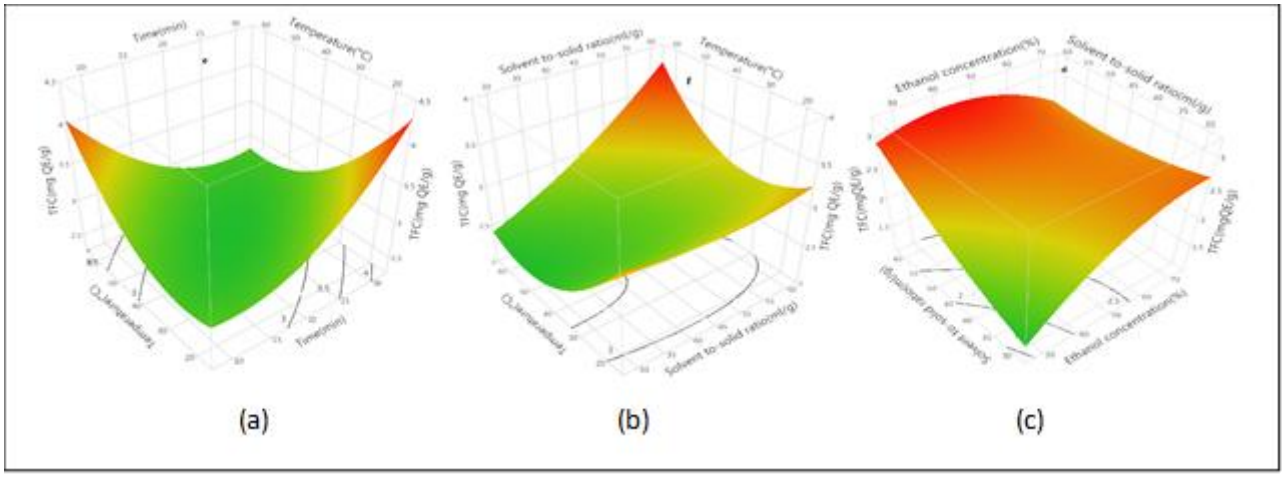

Figure 2. Response surface plots illustrating UAE factors influence the TFC of *H. scoparia*
(a) Interaction temperature - time; (b) Interaction solvent-to-solid ratio - temperature; (c) Interaction solvent-to-solid ratio - ethanol concentration

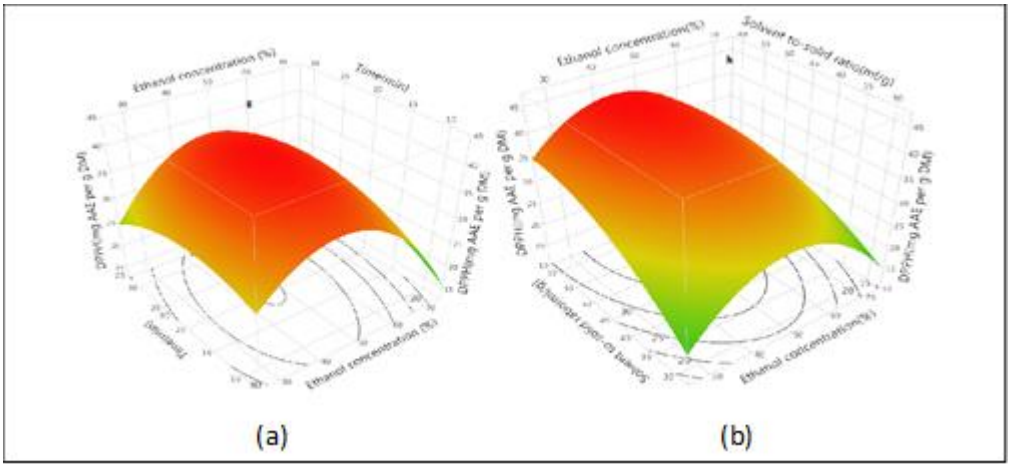

Figure 3. Response surface plots illustrating UAE factors influence the antioxidant activity of *H. scoparia*
(a) Interaction time - ethanol concentration; (b) Interaction solid-to-liquid ratio - ethanol concentration

Figure 3 illustrates the impact of ethanol concentration and solvent-to-solid ratio, solvent concentration and liquid-to-solid ratio on the DPPH activity of *H. scoparia*. The inhibition of DPPH increases with the liquid-to-solid ratio at ethanol concentrations below 50%. The results demonstrate that the DPPH activity is significantly influenced by the liquid-to-solid ratio and ethanol concentration, with highly significant linear and quadratic interaction effects ($p < 0.0001$*), as illustrated in Table 2.

The predicted values for the optimal TPC, TFC, and DPPH activity were verified experimentally. The optimal parameters were found to be a time of 30 min, a temperature of 20 °C, an ethanol concentration of 46.95%, and a liquid-to-solid ratio of 60 mL/g (Figure 4). The results demonstrate that under these optimal conditions, the values for TPC, TFC, and DPPH activity were 81.39, 3.48, and 42.44 mg EAA/g DW respectively (Table 3, Figure 4).

Table 3. Optimal conditions and appropriate response using UAE

Factors	**Optimal conditions**
Ethanol concentration (%)	46.96
Time (min)	30
Solvent-to-solid ratio (mL/g))	60
Temperature (°C)	20
Results	**Obtained Values**
TPC (mg GAE/g DW)	81.39±0.81
TFC (mg QE/g DW)	3.48±0.15
DPPH scavenging (mg AAE/g DW)	42.44±0.39

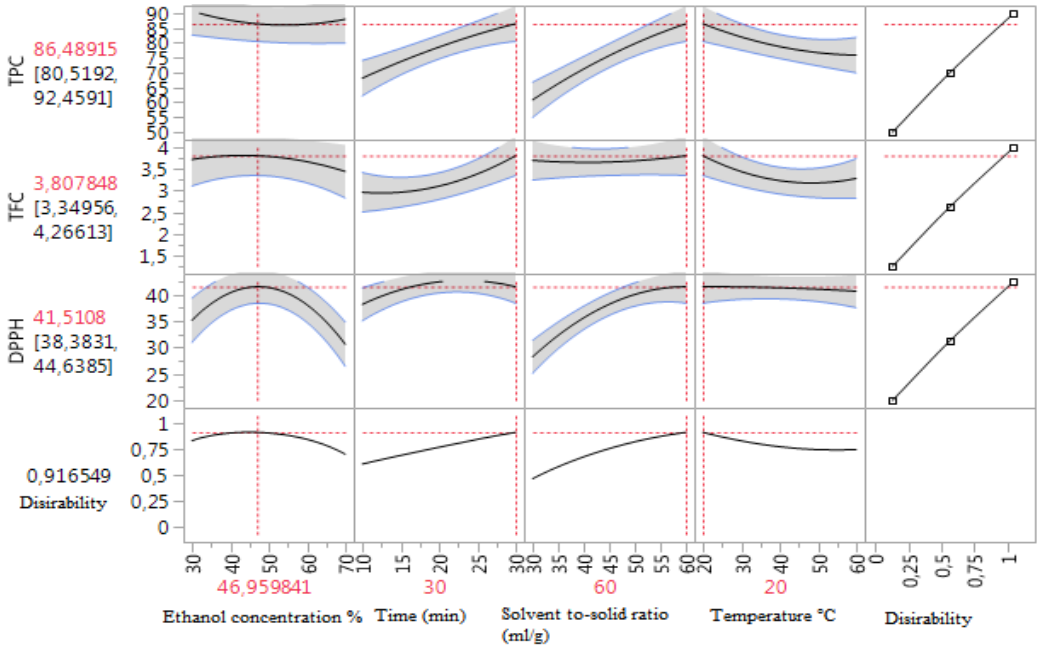

Figure 4. Prediction profile of *H. scoparia* extract using UAE

Antioxidant activity of H. scoparia

The extract obtained using the optimal extraction parameters was used to evaluate the ferric and molybdate reducing activities, and the ABTS activity.

The results of the ABTS activity of *H. scoparia* extract, showed that the percentage of the ABTS˙ radical inhibition increases with the extract concentration. The highest percentage of inhibition (93.81%) was

observed at a concentration of 1 mg/mL. The IC_{50} value for the *H. scoparia* extract was found to be 0.41 mg/mL (Figure 5).

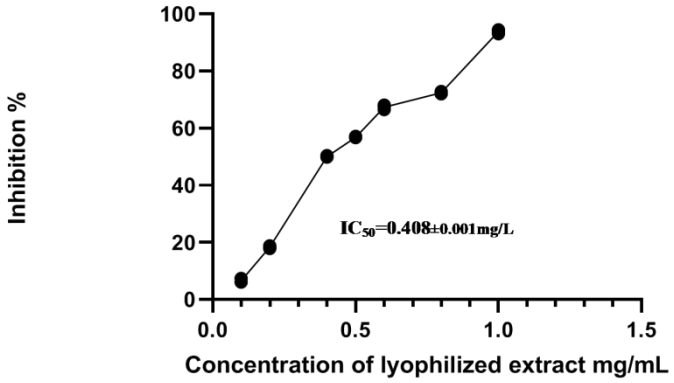

Figure 5. ABTS scavenging activity of *H. scoparia* extract

The results in Figure 6 show that the ferric and molybdate reducing activities of the studied extract increase in line with the concentration tested. The *H. scoparia* extract demonstrated a greater molybdate reduction activity than ferric at all tested concentrations. The highest levels of molybdate and ferric reducing activities were recorded at 1 mg/mL, with values of 31.46 and 16.10 mg AAE/g dry extract, respectively.

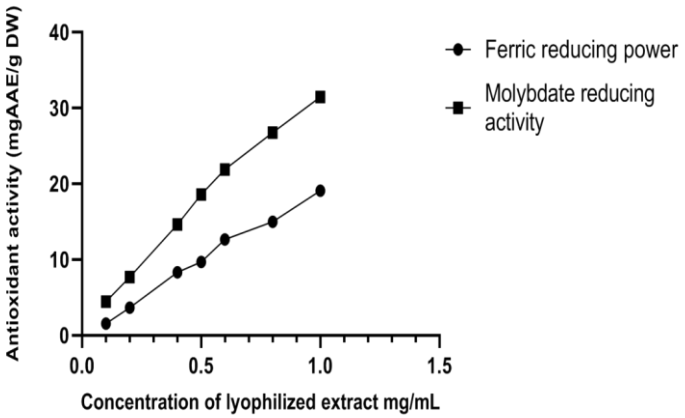

Figure 6. Ferric and molybdate reducing activities of *H. scoparia* extract.

This study revealed that *H. scoparia* extract inhibited free radicals $DPPH^{\cdot}$ and $ABTS^{\cdot}$, and reduced ferric and molybdate in different mechanisms.

Antioxidant compounds act through several chemical mechanisms: hydrogen atom transfer, single electron transfer, and the ability to chelate transition metals (Santos-Sánchez et al., 2019; Teng et al., 2014). This justifies the results of the present study.

The research conducted by Bouaziz et al. (2016) confirmed that the ethanolic extract of *H. scoparia* demonstrated both DPPH radical inhibitory activity and molybdate reducing activity. The study

conducted by Benkherara and Bordjiba (2018) demonstrated that the same plant exhibited DPPH and ABTS scavenging activities, as well as ferric reducing power, which justifies the obtained results.

Antibacterial essay

The antibacterial activity of the optimal extract was evaluated using the agar dilution method against *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* BAR 32, *Salmonella sp*., *Pseudomonas aeruginosa* ATCC 6633, and *Methicillin-resistant Staphylococcus aureus* ATCC 43300 (MRSA).

According to the findings, *H. scoparia* extract exhibited antibacterial activity against all of the tested bacterial strains, with the exception of *Escherichia coli* ATCC 25922 (Table 4). The results indicated that the optimal extract had the highest inhibitory effects against MRSA strain and *Salmonella sp*., with inhibition zone diameters of 16 mm. *Klebsiella pneumonia* BAR 32 and *Pseudomonas aeruginosa* ATCC 6633 were also inhibited by the extract, with inhibition zones of 13.66 mm and 12.33 mm, respectively. The statistical study of the antibacterial activity results revealed that no significant difference was found between the zones of inhibition of MRSA strain and salmonella sp. strain, and between the zones of inhibition of *Klebsiella pneumonia* BAR 32 and *Pseudomonas aeruginosa* ATCC 6633.

The results of this study showed that the highest antibacterial activities were exerted by the Ciproflaxin (CIP) standard, followed by those of the *H. scoparia* extract, while the lowest activities were exerted by the Tetracycline standard (TE).

Table 4. Antimicrobial activity of *H. scoparia* extract (mm)

	***Escherichia coli* ATCC 25922**	***Klebsiella pneumonia* BAR 32**	***Salmonella sp.***	***Pseudomonas aeruginosa* ATCC 6633**	**MRSA ATCC 43300**
***H. scoparia* extract**	–	13.66±0.57[b]	16±1.00[a]	12.33±1.52[b]	16±1.00[a]
Tetracycline (TE)	10	9	10	–	–
Ciproflaxin (CIP)	30	36	30	28	34

— Not detected

The larger zones of inhibition observed in these cases could be attributed to the high levels of phenolic compounds and flavonoids found in *H. scoparia* extract. Polyphenols have been recognized for their potential antibacterial properties (Bouarab Chibane et al., 2019). Among polyphenols, flavonoids have been widely studied and shown to exert antibacterial effects through multiple mechanisms. Firstly, flavonoids can directly kill bacteria by disrupting the bacterial membrane, inhibiting cellular respiration, or interfering with DNA replication. Secondly, they can also enhance the activity of antibiotics by increasing their potency or reversing antibiotic resistance in bacteria (Xie et al., 2017). These results suggest that *H. scoparia* extract has a potential as a natural antibacterial agent and that its efficacy may be related to its phenolic compound content. The role of phenolic compounds in various plant extracts has been the focus of extensive research, owing to their beneficial health properties (Kalili and de Villiers, 2011). Our study revealed the presence of polyphenols and flavonoids in the plant extract under investigation, indicating its potential antibacterial activity against pathogenic bacteria. These findings are consistent with earlier research that suggests the plant's ability to combat gram positive and gram negative organisms (Metrouh-Amir et al., 2015).

The study by Nounah et al. (2019) provides evidence that the ethanolic extract of *H. scoparia* exhibits antimicrobial activity against *Klebsiella pneumonia* BAR 32 and *Salmonella sp*. Similarly, the study conducted by Drioichea et al. (2019) reported antibacterial activity of *H. scoparia* extract against

Klebsiella pneumonia BAR 32, *Salmonella sp.* and *Pseudomonas aeruginosa.* These findings support the justification for the obtained results.

Minimum inhibitory concentration (MIC)

A low MIC signifies strong antibacterial activity (Coulidiati et al., 2009). The minimum inhibitory concentration of *H. scoparia* extract was assessed against *Klebsiella pneumonia BAR 32*, *Salmonella sp.*, *Pseudomonas aeruginosa* ATCC 6633, and *Methicillin-resistant Staphylococcus aureus* ATCC 43300 (MRSA) (Table 5). The extract showed a MIC of 12.5 mg/mL against *Klebsiella pneumonia* BAR 32, *Salmonella sp.* and MRSA. Regarding *Pseudomonas aeruginosa* ATCC 6633, the MIC was 25 mg/mL.

Table 5. Results of minimum inhibitory concentrations (MICs) in mg/mL of *H. scoparia* extract

Bacteria strains	***H. scoparia* extract**
Klebsiella pneumonia BAR 32	12.5
Salmonella sp.	12.5
Pseudomonas aeruginosa ATCC 6633	25
MRSA ATCC 43300	**12.5**

Conclusions

The Box-Behnken design (BBD) was used to optimize the experimental variables in order to enhance the extraction of phenolic compounds and antioxidant activity of *H. scoparia*. Data analysis revealed that the phenolic compound, flavonoid contents and DPPH activity of *H. scoparia* were significantly (p<0.05) affected by the extraction parameters tested (time, temperature, solvent to-solid ratio and solvent concentration). The obtained experimental results were rather similar to the anticipated results. This supports the validity of the selected experimental design. The present study revealed that *H. scoparia* extract, obtained under optimal extraction conditions, exhibited DPPH and ABTS inhibitory activities, and ferric and molybdate reducing powers. The antibacterial activity results showed that *H. scoparia* extract inhibited all bacterial strains tested except *Escherichia coli*.

Author Contributions: H.M.A. conceived the presented idea and verified the analytical methods. S.M. performed the experiment, analyzed, discussed, and interpreted the results, and wrote the manuscript. N.A. and H.M. contributed to the statistical analysis of the results. S.M. and H.M.-A reviewed the results and approved the final version of the manuscript.

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***Conflicts of Interest*:** The authors declare no conflict of interest.

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