"Science Stays True Here"
Biological and Chemical Research (ISSN 2312-0088), Vol.9, 19-34 | Science Signpost Publishing

Intensification of phenolic compounds extraction from *Nitraria retusa* leaves by Ultrasound-Assisted System Using Box–Behnken Design and Evaluation of Biological Activities

Feten Zar Kalai[1.], Khaoula Mkadmini[1], Majdi Hammami[1], Hiroko Isoda[2.3] and Riadh Ksouri[1]

1. Laboratory of Aromatic and Medicinal Plants, Center of Biotechnology, Technopark of Borj Cedria, BP 901, 2050 Hammam-Lif, Tunisia.

2. Alliance for Research On the Mediterranean and North Africa (ARENA), University of Tsukuba, Tsukuba, Japan.

3. Faculty of Life and Environmental Sciences, University of Tsukuba, 1 1 1 Tennodai, Tsukuba, Ibaraki 305 8572, Japan.

Received: February 28, 2022 / Accepted: March 27, 2022 / Published: June 25, 2022

Abstract: The aim of this work is to optimize the antioxidant extraction conditions from leaves of *N. retusa* with an ultrasound-assisted system using response surface methodology. The Box-Behnken design was employed for optimization of extraction parameters in terms of antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and total phenolic content. The optimum operating conditions for extraction were as following: ethanol concentration, 75.58 %v/v; extraction time, 5.99 min; extraction temperature, 42.15°C. Under these conditions, the obtained extract exhibited a high content of phenolic compounds (24 mg EAG/g DR) with potent antioxidant activity (DPPH: CI_{50}= 7 µg/ml; FRAP: EC_{50}=55 µg/ml). The optimum extract exhibited high anti-inflammatory activity with no cytotoxicity which could be attributed to the synergistic action of the identified glycosylated flavonoids (Luteolin 7-O-glucoside, Rutin, Kaempferol 3-O-rutinoside, Isorhamnetin 3-O-rutinoside).

Keywords: *Nitraria retusa*, ultrasound-assisted system, Box-Behnken, antioxidant activities, biological activities.

Introduction

In recent years, researchers have taken an interest in the heritage of medicinal plant species to better understand their mode of use, their indications in various pathologies and to identify the natural substances derived from plants which are responsible for several therapeutic effects as part of the promotion of traditional medicine [1, 2]. The extraction of natural compounds from herbs in a safe, effective, and energy-efficient manner has always been a difficult issue for researchers [3]. The constraints of conventional methods, such as high solvent requirements, poor extract quality, lengthy processing times, and the possibility of heat destruction of target molecules, have paved the way for the development of novel herbal extraction techniques [4]. To overcome these restrictions, several techniques have been evolved like microwave [5], ultrasound [6], supercritical CO_2 [7], pulsed electric field [8] and enzymatic assisted extraction [9]. Furthermore, these approaches can be considered a

Corresponding author: Dr. Feten Zar Kalai, Laboratory of Aromatic and Medicinal Plants, Center of Biotechnology, Technopark of Borj Cedria, BP 901, 2050 Hammam-Lif, Tunisia. E-Mail: zarfeten@gmail.com.

"green approach" to plant extraction. The ultrasound assisted extraction (UAE) technology is now commonly recognised as a potent alternative for conventional plant extraction methods for extracting bioactive substances [10]. Ultrasonic assisted extraction is a simple, efficient and inexpensive method. In addition, the mechanical effect of ultrasound on the plant matrix induces better penetration of the solvent into the cells, thereby improving mass transfer and increasing the extraction yield and extraction kinetics [11]. In our study, we are interested in the study of *Nitraria retusa* Asch. which is a thorny shrub of the *Nitrariaceae* family which is an integral part of the diet and of the range of medicinal plants of populations of the Mediterranean basin and the Near East [12]. Numerous phytochemical studies on this plant show its diversity in flavonoids. *N. retusa* exhibits a huge number of pharmacological properties such as antiproliferative [13], antimicrobial [14], antioxidant and antigenotoxic [15], antimutagenic [16], anti-α-amylase, anti-inflammatory, antinociceptive [17] and antiobesity [18] activities. The diverse extraction parameters for medicinal plants could be adjusted utilizing various designs for scale-up and industrial purposes of plant actives. In the present study, Box-Behnken design was designated to optimize the extraction conditions for *N. retusa* using ultrasound assisted extraction (UAE) technology. The obtained extracts were comparatively evaluated for the total phenolic content and anti-free radical activity. After setting the optimal parameters, the extract obtained is characterized by the chromatographic technique and then evaluated for its anti-inflammatory activity.

1. Materials and methods

1.1. Plant sample and reagents

The whole plant of *N. retusa* was collected in the month of April from the sebkha ‘Elkelbia’located in Kairouan, Tunisia. Before processing, the plant specimen was confirmed by botanist Abderrazzak Smaoui of the Biotechnology Center of Borj-Cedria (Tunisia). The references HPLC compounds and other reagents were purchased from Sigma Aldrich (Geramny). All the solvents used in the study were of HPLC grade.

1.2. Extraction of plant sample

First, *N. retusa* leaves were lyophilised using a laboratory freeze-dryer (Christ, Alpha 2-4 LSCplusto, Germany) to prevent the degradation of natural heat-labile substances. They were then ground using a mechanical grinder until they form a fine powder. As an innovative extraction method, we adopted ultrasonic assisted extraction (Sonorex Digital 10 P, Bandelin, Germany) of the antioxidants using a hydroalcoholic solvent as the green solvent. The extraction optimizing was realised by varying the following parameters: the extraction time from 2 to 60 min, the temperature from 25 to 80 ° C, the ratio (volume of solvent / mass of dry matter) from 5 to 80 mL / g DW), the hydroalcoholic percentage from 0 to 100% v / v and the power of the ultrasonic waves from 14 to 140 W.

1.3. Experimental design

The effect of three process variables, extraction time (X1), temperature time (X2), and volume of solvent per gram of dry matter (X3), on two responses, total phenolic content (Y1) and anti-free radical activity (Y2), was investigated using a Box-Behnken design (BBD). With the use of response surface method (RSM), the optimal conditions for ultrasound assisted extraction of a specific plant

were also established. For validation and appropriateness of the model, seventeen tests were conducted, representing eleven axial points and five central points, as indicated by the design. Table 1 shows the actual and coded values of independent factors. A second-order polynomial for coded independent variables Xi and Xj, Equation (1) was used to determine the linear I quadratic (βii), and interaction terms (βij), where β0 represents the coefficient of interception [19].

1.4. Identification of natural products by liquid chromatography with diode array detector (LC-DAD)

A high-performance liquid chromatography system (consisting of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 400 bar; Agilent 1260, Agilent technologies, Germany) with a reversed phase C18 analytical column of 4.6 *100 mm and 3.5 µm particle size was used to identify natural products (Zorbax Eclipse XDB C18). The scanning range of the diode array detector was tuned to 200–400 nm. The temperature in the column was kept at 25 °C. The mobile phase flow rate was 0.4 ml/min and the injected sample volume was 2 µl. A combination of solvent A (methanol) and solvent B made up the mobile phase (milli-Q water with 0.1 percent formic acid). The retention times obtained from the extracts at 254 nm were compared to those obtained from the references standard identification analysis. Each compound's concentration was measured in micrograms per gram of DW (µg/g DW).

1.5. Total phenolic compounds assay

According to earlier research [20], total polyphenols were measured using the Folin–Ciocalteu reagent. At 760 nm, the absorbance was measured. The calibration curve with gallic acid, ranging from 0 to 500 µg/ml, was used to quantify the contents to mg gallic acid equivalent per gram of dried residue (mg GAE/g DR). All samples were examined in in triplicate.

1.6. Total flavonoids content assay

Total flavonoids were measured according to Boulaaba, Mkadmini [21]. The mixture's absorbance was measured at 510 nm against a blank in which the material was not present. Through the calibration curve of (+)-catechin, ranging from 0 to 500 µg/ml, the contents were represented as mg catechin equivalent per gram of DW (mg CE/g DW). All samples were examined in triplicate.

1.7. Radical scavenging action of DPPH assay

The ability of extracts to reduce the free DPPH radical (1,1-diphenyl-2-picrylhydrazyl) was measured using the CI50 (µg/ml) method [20], which is defined as the antiradical dose required to achieve a 50% inhibition. To do so, samples of various concentrations were introduced to a 0.2 mM DPPH methanolic solution. At 517 nm, the absorbance was measured in triplicate.

1.8. Total antioxidant capacity

The assay of the green phosphate/Mo^{5+} complex was used to determine the total antioxidant capacity of *nitraria* extracts, as described before [20]. The absorbance was measured against a blank at 695 nm. The total antioxidant activity was measured in milligrams of GAE per gram of DW. All samples were examined in triplicate.

1.9. Iron reducing power

The reduction of trivalent iron generated by $FeCl_3$: Iron(III) chloride anhydrous was the focus of this antioxidant action [20]. The intensity of the appearing blue-green color was measured at 700 nm. The *nitraria* extract concentration at which the absorbance was 0.5 was utilized as the EC50 value (µg/ml) for the reducing power, and ascorbic acid was employed as a positive control. All samples were examined in in triplicate.

1.10. Anti-inflammatory activity

According to Boulaaba, Kalai [20] the accumulation of nitrite inhibition was used to evaluate the anti-inflammatory impact of the *N. retusa* extract on the murine macrophage RAW 264.7 cell line (American Type Culture Collection, ATCC) (NO). Cells were grown in 24-well plates at a concentration of 2×10^5 cells/ml during 24 h after which *nitraria* extracts at 10, 25, 50 and 100 µg/mL were added for 1 h of treatment. The treatment group of plates received 1 µg/ml of lipopolysaccharide (LPS), while the control group received medium or only LPS. The cell-free supernatants were collected and Griess's reagent was used to measure nitric oxide (NO) levels after a 24-hour LPS stimulation. The resazurin test was used to determine whether samples were cytotoxic to cells. The nitrite concentration in the samples was assessed using a standard curve of sodium nitrite at 10, 20, and 50 M, and the absorbance was measured at 540 nm.

1.11. Statistical analysis

The results were statistically studied using Statistica v. 7.0 tool to determine the average values and standard errors. A one-way ANOVA was used with the Tukey's post hoc test and a significance level of 0.05 percent. A probability threshold of $p < 0.05$ was considered significant. The NemrodW (LPRAI, version 2000) program was used to create and analyse the experimental design. Pareto chart and residue analysis were performed by using Statistica v. 7.0 tool.

2. Results and discussion

2.1. Experimental design

Based on the preliminary study we concluded there are three factors which can influence the quality of the extract (the content of total phenolic compounds (Y_{TPC}) and the anti-free radical activity (Y_{CI50})) from the *Nitraria retusa* leaves using the ultrasonic extraction method. These three factors are the extraction time (min) (X1), the extraction temperature (°C) (X2) and the hydroalcoholic percentage (%, v / v) (X3) whose fields of study are illustrated in table 1. Then, we focused on the realization of a Box-Behnken plan which is a response surface plan in order to study the effect of each factor and their interactions on the two sought responses (Y_{CI50}, Y_{TPC}) and to determine the optimal extraction conditions of polyphenols with high yield and high antioxidant activity. Table 2 represents the 17 tests which were carried out following the combination of the levels of the three factors studied according to the Box-Behnken plan.

2.2. Coefficients significance

The coefficients significance of the second-degree polynomial model corresponding to each of the two responses studied (Y_{CI50}, Y_{TPC}) was evaluated by Student's t test, admitting a confidence

interval of 95%. Table 3 summarizes the coefficients values of the two postulated models. The results show that the two factors (X1, X2) display a significant linear effect on the two responses (Y_{TPC}, Y_{CI50}) (p <0.05). Furthermore, we note that for the two responses, the following three factors (X1, X2, X3) present a significant quadratic effect (p <0.05). This effect is in the negative trend for TPC while it is positive for DPPH CI_{50}, knowing that the higher the value of the coefficient, the more significant the factor. Concerning the effect of the interaction between the factors on the two responses studied, we note that the interaction established between the extraction time (X1) and the hydroalcoholic percentage (X3) as well as that established between the extraction temperature (X2) and the hydroalcoholic percentage (X3) are considered significant (p <0.05). In order to check the significance of the factors, we used the Pareto diagram (Figure 1) which represents the different effects in order of importance for each variable (linear effects, interactions between factors and quadratic effects).

As shown in Figure 1, the bars lengths was directly proportional to the magnitude absolute value of the coefficients estimated effects. The crossbar represents the minimum amplitude from which the effects will be considered to be statistically significant on the response variable studied, considering a confidence level of 95%. From the analysis of these diagrams and with regard to the the linear effects significance, we notice that the linear effect of extraction temperature is greater than that of extraction time for the two responses studied. The following quadratic effects: the extraction temperature (X2) 2: The hydroalcoholic percentage (X3) 2 and the extraction time (X1) 2 always have an influence on the three decreasing order responses. Regarding the effect of the interactions between the factors, we note that the interaction between the extraction temperature (X2) and the hydroalcoholic percentage (X3) shows the most important effect on the CI50 response. While for the TPC response it is the interaction between the extraction time (X1) and the hydroalcoholic percentage (X3) which has the most effect. These results are consistent with those obtained previously by the Student test.

2.3. Model fitting

Regarding the above results, the quadratic models describing the factors (linear, interactions and quadratic) significant for the responses studied (YC_{I50}, Y_{TPC}) are written as follows:

$$Y_{CI50} = 8.87 + 1.33625 * X1 + 4.04375 * X2 + 1.06373 * (X1 * X1) + 6.18375 * (X2 * X2) + 6.01125 * (X3 * X3) - 0.0905 * (X1 * X3) - 2.29 * (X2 * X3)$$

$$Y_{TPC} = 24.12 - 0.7525 * X1 - 1.78875 * X2 - 2.27875 * (X1 * X1) - 3.68125 * (X2 * X2) - 3.35125 * (X3 * X3) - 1.121 * (X1 * X3) - 1.0125 * (X2 * X3)$$

2.4. Model validity by analysis of variance (ANOVA)

This analysis makes it possible to verify that the independent variables involved in the model have an effect on the response studied. Table 3 summarizes the analysis of variance of the two models postulated to describe the phenomenon of the responses sought (Y_{CI50}, Y_{TPC}). The validity of the regression models describing the effect of significant factors on each of the two responses (CI50, TPC) was verified by Fisher's test.

Examination of this Table 3 shows that for each response studied, the value of "ratio-F" corresponding to the ratio between the mean square of the regression and the mean square of the residue is greater than the value of "ratio-F" $_{tabulated}$ at a confidence level of 95%: [("ratio-F" CI50 = 68.9880)> ("ratio-F $_{tabulated}$" CI50 (7.9.5%) = 3.29); ("Ratio-F" TPC = 15.4437)> ("ratio-F $_{tabulated}$" TPC (7.9.5%) = 3.29] ($p < 0.0001$) this means that the model coefficients are significant and therefore they have a strong effect on the response studied. In addition, for each response, the value of "ratio-F" corresponding to the ratio between the mean square of the validity and the estimate of the experimental variance is less than the value of "ratio-F" tabulated at a level of confidence. 95%: [("Ratio-F" CI50 = 4.2216) < ("Ratio-F $_{tabulated}$" CI50 (5.4.5%) = 6.26); ("Ratio-F" TPC = 5.2828) < ("Ratio-F $_{tabulated}$" TPC (5.4.5%) = 6.26) knowing that the probability is greater than 5% which confirms the validity of the models mentioned above. The coefficients of determination R^2 of the two responses are greater than 0.9 which shows the good correlation between the experimental values and those predicted by the models. The validity of the models was further confirmed by analysis of the residue for each response. Further, as shown in Figure 2, the distribution of the points of the experimental and calculated values are distributed randomly over the entire linear trajectory, which asserts a good correlation between the measured responses (Y_{exp}) and those predicted (Y_{Cal}) for each of the two postulated models.

2.5. Response surface analysis

Based on the significance of the coefficients regression and by fixing time at low duration, response surface analysis of anti-free radical activity was performed for the interaction of extraction temperature and alcohol percentage. Figure 3 shows that the CI50 for inhibition of $DPPH^{\bullet}$ radicals ranges from 4.01 to 27.68 µg/ml. The lowest values are recorded between temperatures 38 - 42°C and 73-76% alcohol. On the other hand, the higher values are associated with the increase in the extraction temperature (60 ° C). Besides, Figure 3 shows that the TPC ranges from 7 to 24.97 mg GAE/g DR. The lowest values are recorded between temperatures 35 - 47°C and 70-83% alcohol. Instead, the higher values are associated with the increase in the extraction temperature (60°C). The decrease in this activity can be explained by two propositions, the first explanation is that increasing the temperature of the solvent can broaden the spectrum of molecules extracted by this system. This proposition is supported by the publication of Che Sulaiman, Basri [22] who found that increasing the ethanolic extraction temperature of *Clinacanthus nutans* induces an increase in extraction yield as well as a decrease in anti-free radical activity. The second explanation can be explained by the denaturation of antioxidants by increasing the temperature. In a study of the thermal stability of gallic acid, catechin and vanillic acid developed by Volf, Ignat [23], it was found that phenolic degradation depends on temperature. The same observation was revealed by Ma, LIU [24] for typhaneoside and isorhamnetin-3-O-neohesperidoside.

2.6. Evaluation of the optimum point by the desirability function

The representation of the iso-response curves for the three factors clearly shows that these factors are very related, from where it is possible to find a compromise point to have a minimum of Y_{CI50} and a maximum of Y_{TPC}. This point is calculated statistically by a function proposed by the NemrodW software (LPRAI, version 2000) used later. This function, called Desirability (D) offers us the choice

of adequate experimental domains and the desired target responses. A desirability close to 100% will be the most satisfactory [25]. Taking into consideration the results of the graphic study, the desirability function was carried out by setting the following constraints: extraction time (from 5 to 6 minutes), the extraction temperature (from 35 to 47 ° C) and the percentage of alcohol (from 70 to 83%). The results shown in Table 4 show that extraction at 42.15 ° C with a 75.58% alcohol solvent system for 5.99 minutes can give a compromise point with a DPPH$^{\bullet}$ CI50 of 8.01µg / ml and a total phenolic content around 22.75 mg EGA / g DR. this point is associated with a very satisfactory desirability (D= 93.54%). The experimental values obtained for the CI_{50} and CPT responses are 6.73 ± 0.52µg/ml and 23.98 ± 0.33 mg EAG/g DR respectively. These results corroborate with those obtained by the postulated models (8.01µg / ml for the CI_{50} response and 22.75 mg EGA / g DR for the TPC response).

2.7. Identification and quantification of phenolic compounds

The quantification of the identified molecules by LC-DAD, the total flavonoids assay and other antioxidant activity assays were carried out on the hydroalcoholic extract obtained under the optimal conditions of ultrasound-assisted extraction (Table 5). The *N. retusa* extract is characterised by high total flavonoids content (9.64 ± 0.46 mg EQ/g DR) and remarkable Total phenolic compounds (23.98±0.33 mg EAG/g DR). Chromatographic analysis showed that this extract is characterized by the dominance of luteolin 7-O-glucoside with a content of 10.51 ± 0.68 mg / g DR followed by Isorhamnetin 3-O-rutinoside with a content of 7.96 ± 0.77 mg / g DR. Rutin and Kaempferol 3-O-rutinoside are considered relatively in the minority with levels below 1 mg mg / g DR. This composition is in agreement with the research work of [26-28]. These compounds have significant antioxidant activity [28, 29], which explains the antioxidant power of the *nitraria* extract obtained (Table 5).

2.8. Anti-inflammatory activity evaluation

In this study the anti-inflammatory activity of the optimum extract was evaluated by measuring its ability to inhibit the production of nitrogen monoxide (NO$^{\bullet}$) by macrophages (RAW 264.7) stimulated by an endotoxin; lipopolysaccharide (LPS). Figure 4 illustrates the variation of NO$^{\bullet}$ inhibition percentage as a function of the optimum extract concentration. The optimum extract exhibits anti-inflammatory activity with a 50% inhibitory concentration of the radical (NO$^{\bullet}$) of the order of 36.6 µg/mL. Several research studies have demonstrated the mechanisms of action of plant extract on pro-inflammatory molecules [30]. Indeed, it has been shown that flavonoids can decrease the inflammatory response by inhibiting the enzymatic activity of NO$^{\bullet}$ synthase (NOS) responsible for the production of the radical (NO •) or by inhibiting the production of pro-inflammatory cytokines: TNF-α IL-1β and IL-6 [31, 32]. Furthermore, it has been shown that these phenolic substances can inhibit enzymes involved in the inflammatory response such as phospholipase A2 which releases arachidonic acid, from which 5-lipoxygenase and cycloxygenase 2 will respectively generate leukotrienes of the series 4 and prostaglandins from series 2 which are very active pro-inflammatory molecules [33]. The hydroalcoholic extract cytotoxicity to the RAW 264.7 macrophages was analysed by the resazurin test. This molecule can be reduced by cytoplasmic enzymes during mitochondrial metabolism to a fluorescent molecule which is resorufin. An inhibition of cell growth can be explained by the decrease

in mitochondrial metabolism or enzyme activity [34]. The results revealed that no toxicity was detected for the range of concentrations used (25-100 µg/mL) of the optimum extract. Indeed, the viability percentage is greater than 97% (Figure 4). This leads us to conclude that the active concentrations tested for anti-free radical and anti-inflammatory activity are non-cytotoxic.

Conclusion

As part of the valuation of the hydroalcoholic extract from the leaves of *Nitraria retusa*, we carried out the methodology of the design of experiments (Box-Behnken plan) to optimise extraction. Thus, we succeeded in determining the optimal extraction conditions of the substances endowed with good antioxidant activities. Furthermore, we evaluated its anti-inflammatory activities and the results showed that the extract is bioactive and not cytotoxic. The potentiality of the antioxidant and anti-inflammatory activities of this extract is due to the presence of the glycosylated flavonoids (luteolin 7-O-glucoside, rutin, kaempferol 3-O-rutinoside and isorhamnetin 3-O-rutinosie) identified by liquid chromatography.

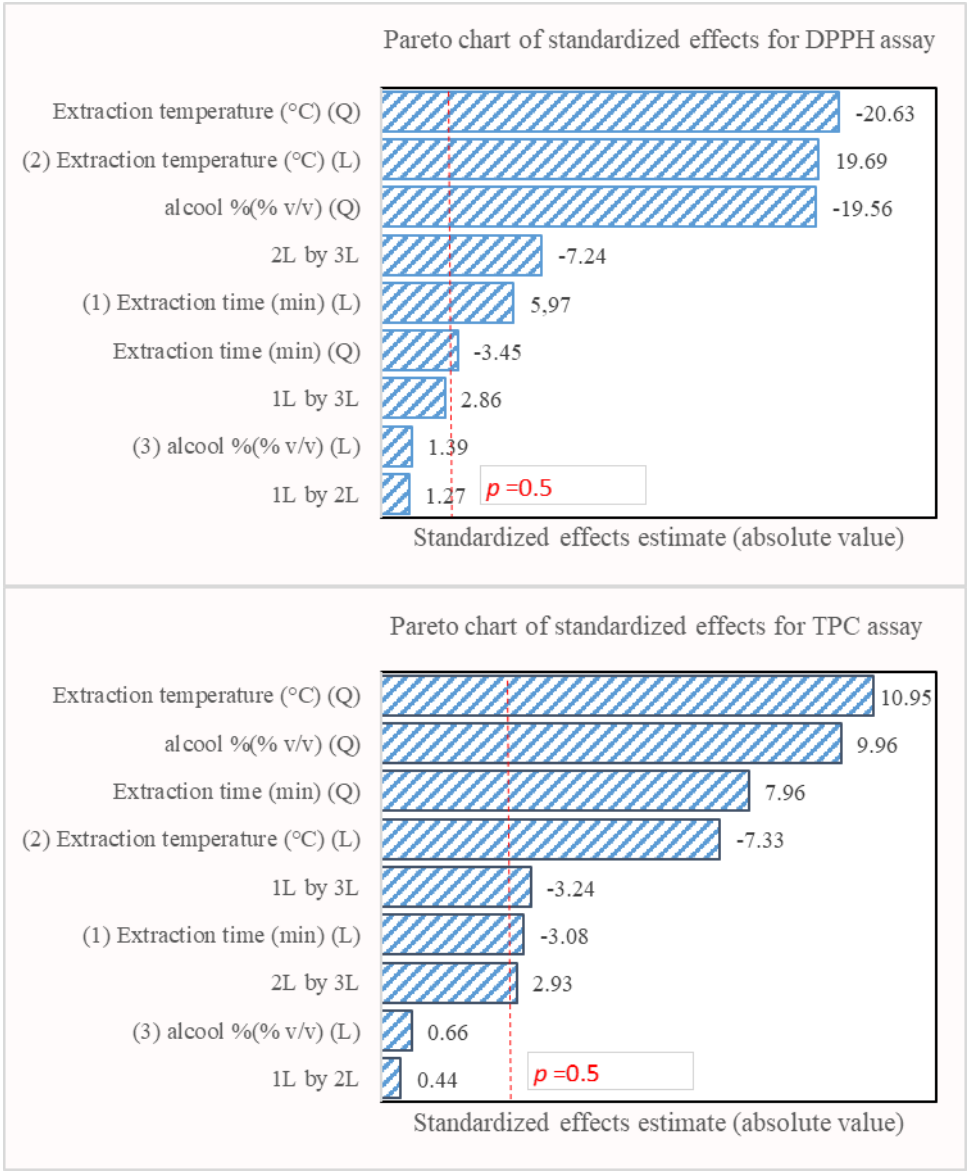

Figure 1. Pareto chart of standardized effects for DPPH and TPC assays

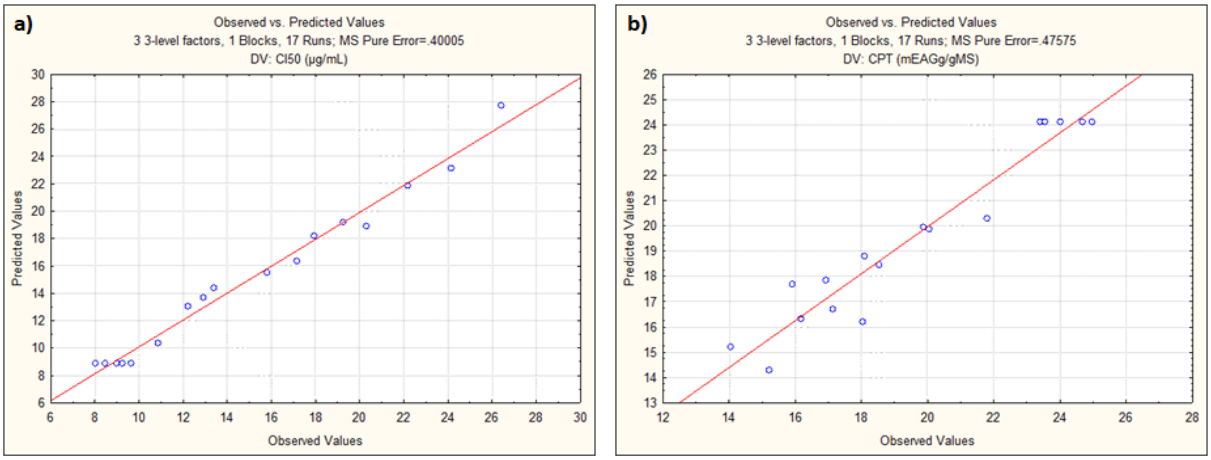

Figure 2. Graphical representation of the residue analysis (responses calculated by the model (Y_{Cal}) as a function of the measured responses (Y_{Exp}) for the two responses: a) Y_{CI50} (µg/mL); b) Y_{TPC} (mg EAG/g DW)

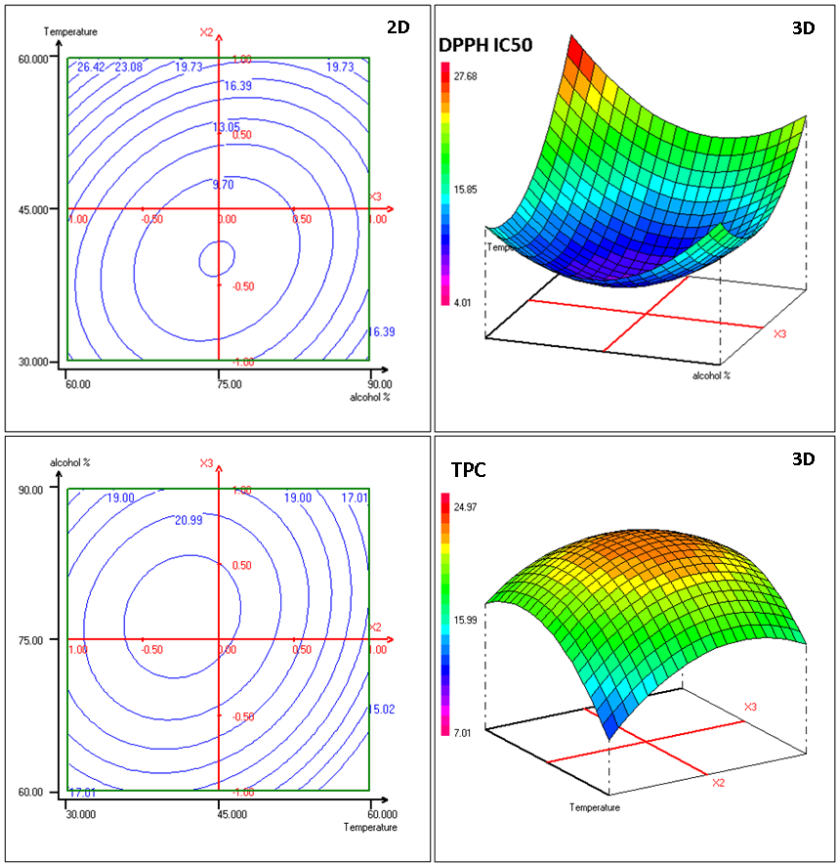

Figure 3. 2D and 3D isoresponse curves illustrating the effect of the interaction between the significant factors on Y_{CI50} and Y_{TPC} at low extraction time (5min)

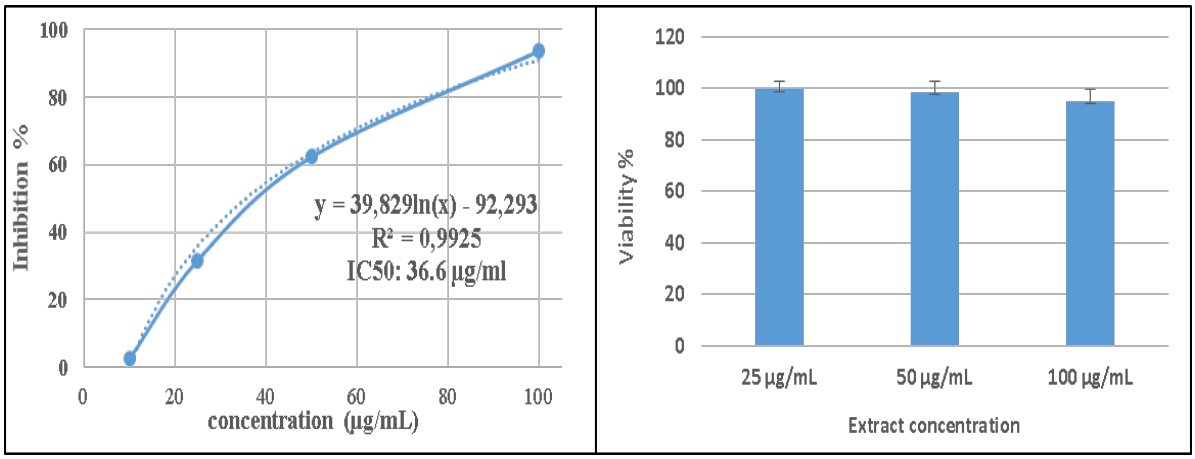

Figure 4. Nitrogen monoxide Percentage inhibition and cytotoxicity of the hydroalcoholic extract from *N. retusa*

Table 1. Limits of extraction factors

Factors	Limits		
	-1	0	+1
Extraction time (min), X_1	5	20	35
Extraction temperature (°C), X_2	30	45	60
Ethanol percentage ($\%_{V/V}$), X_3	60	75	90

Table 2. Experimental conditions and responses obtained according to the Box-Behnken plan

Exp[*]	Independent **variables**			Experimental **values (Y)**		Predicted **values (Y)**	
	X_1 Time (min)	**X_2 Temperature (°C)**	**X_3 Alcool % (%v/v)**	**Y_{CI50}[(a)] (µg/mL)**	**Y_{TPC}[(b)] (mg EAG/g DW)**	**Y_{CI50} (µg/mL)**	**Y_{TPC} (mg EAG/g DW)**
1	5(-1)	30(-1)	75(0)	10.86	21.79	10,37	20,30
2	35(1)	30(-1)	75(0)	12.20	18.10	13,05	18,79
3	5(-1)	60(1)	75(0)	19.23	17.12	19,18	16,72
4	35(1)	60(1)	75(0)	22.18	14.03	21,85	15,21
5	5(-1)	45(0)	60(-1)	15.78	15.92	15,51	17,72
6	35(1)	45(0)	60(-1)	17.17	18.54	16,37	18,45
7	5(-1)	45(0)	90(1)	12.91	19.88	13,70	19,96
8	35(1)	45(0)	90(1)	17.92	18.02	18,18	16,21
9	20(0)	30(-1)	60(-1)	13.40	20.03	14,37	19,88
10	20(0)	60(1)	60(-1)	26.42	15.22	27,75	14,28
11	20(0)	30(-1)	90(1)	20.29	16.93	18,95	17,86
12	20(0)	60(1)	90(1)	24.15	16.17	23,17	16,31
13	20(0)	45(0)	75(0)	8.03	24.97	8,87	24,12
14	20(0)	45(0)	75(0)	9.23	24.01	8,87	24,12
15	20(0)	45(0)	75(0)	8.98	23.40	8,87	24,12
16	20(0)	45(0)	75(0)	9.64	24.68	8,87	24,12
17	20(0)	45(0)	75(0)	8.47	23.54	8,87	24,12

Table 3. Analysis of variance (ANOVA) for the two responses (Y_{CI50}, Y_{TPC})

Variation Source	SC	Ddl	CM	*Ratio-F*	*Valeur-P*
CI_{50} (R^2=0,9817)					
Regression	511,5988	7	73,0855	68,9880	<0,0001
Residue	9,5346	9	1,0593		
Validity	7,9344	5	1,5868	4,2216	0,1032
Experimental error	1,6002	4	0,4000	3,9666	
Total	521,1334	16			
TPC(R^2=0,9231)					
Regression	173,8060	7	24,8294	15,4437	<0,0001
Residue	14,4696	9	1,6077		
Validity	12,5666	5	2,5133	5,2828	0,0667
	1,9030	4	0,4757		
Experimental error	188,2756	16			

Ddl: Degree of liberty; SC: Somme of square; CM: Medium squares

Table 4. Optimum point by the desirability function of *N. retusa* ultrasound extraction

Factors		Responses		D (i) %
X1:Time (min)	5.99	Y1: DPPH CI50	8.01	100.00
X2: Temperature (°C)	42.153	Y2: TPC mg EGA/ g DR	22.75	87.50
X3: Alcohol (%)	75.58			
Total desirability (D)				93.54

Table 5. Identification and quantification of phenolic compounds and their antioxidants activities

LC-DAD quantification	
Luteolin 7-O-glucoside	10.51±0.68 mg/g DR
Rutin	0.859±0.97 mg/g DR
Kaempferol 3-O-rutinoside	0.29±0.35 mg/g DR
Isorhamnetin 3-O-rutinoside	7.96±0.77 mg/g DR
Spectrometric quantification	
Total phenolic compounds	23.98±0.33 mg EAG/g DR
Total flavonoids content	9.64±0.46 mg EQ/g DR
Antioxidant activities	
DPPH assay (CI50)	6.73±0.52 µg/ml
TAC assay	48.27±0.17 mg EAG/g DR
FRAP assay (CE50)	55.493±0.05 µg/ml

References

[1]. Khan, S.R., et al., *AI-integration and plant-based traditional medicine for drug discovery.* Drug discovery today, 2021.

[2]. Sankaramourthy, D., K. Subramanian, and S.R. Sadras, *Safety and Regulatory Issues on Traditional Medicine Entrusted Drug Discovery*, in *Evidence Based Validation of Traditional Medicines*. 2021, Springer. p. 589-603.

[3]. Lu, W., et al., *Nanomedicines: Redefining traditional medicine.* Biomedicine Pharmacotherapy, 2021. **134**: p. 111103.

[4]. Picot-Allain, C., et al., *Conventional versus green extraction techniques-A comparative perspective.* Current Opinion in Food Science, 2021.

[5]. Pavlić, B., et al., *Antioxidant and enzyme-inhibitory activity of peppermint extracts and essential oils obtained by conventional and emerging extraction techniques.* Food Chemistry, 2021. **338**: p. 127724.

[6]. Kumar, K., S. Srivastav, and V.S. Sharanagat, *Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review.* Ultrasonics Sonochemistry, 2021. **70**: p. 105325.

[7]. Silva, S.G., et al., *Supercritical CO2 extraction to obtain Lippia thymoides Mart. & Schauer (Verbenaceae) essential oil rich in thymol and evaluation of its antimicrobial activity.* The Journal of Supercritical Fluids, 2021. **168**: p. 105064.

[8]. Arshad, R.N., et al., *Effective valorization of food wastes and by-products through pulsed electric field: A systematic review.* Journal of Food Process Engineering, 2021. **44**(3): p. e13629.

[9]. Catalkaya, G. and D. Kahveci, *Optimization of enzyme assisted extraction of lycopene from industrial tomato waste*. Separation Purification Technology, 2019. **219**: p. 55-63.

[10]. Wen, C., et al., *Advances in ultrasound assisted extraction of bioactive compounds from cash crops–A review*. Ultrasonics sonochemistry, 2018. **48**: p. 538-549.

[11]. Esclapez, M., et al., *Ultrasound-assisted extraction of natural products.* Food Engineering Reviews, 2011. **3**(2): p. 108-120.

[12]. Abd Elhalim, M.E., et al., *The anatomical features of the desert halophytes Zygophyllum album LF and Nitraria retusa (Forssk.) Asch.* Annals of Agricultural Sciences, 2016. **61**(1): p. 97-104.

[13]. Boubaker, J., et al., *Leaf extracts from Nitraria retusa promote cell population growth of human cancer cells by inducing apoptosis.* Cancer cell international, 2011. **11**(1): p. 1-11.

[14]. Mohamed, A.A., et al., *Chemical compositions, potential cytotoxic and antimicrobial activities of Nitraria retusa methanolic extract sub-fractions.* International Journal of Toxicological Pharmacological Research, 2015. **7**(4): p. 204-212.

[15]. Boubaker, J., et al., *Isorhamnetin 3-O-robinobioside from Nitraria retusa leaves enhance antioxidant and antigenotoxic activity in human chronic myelogenous leukemia cell line K562.* BMC Complementary Alternative Medicine, 2012. **12**(1): p. 1-9.

[16]. Boubaker, J., et al., *Mutagenic, antimutagenic and antioxidant potency of leaf extracts from Nitraria retusa.* Food Chemical Toxicology, 2010. **48**(8-9): p. 2283-2290.

[17]. Rjeibi, I., et al., *Novel antioxidant, anti-α-amylase, anti-inflammatory and antinociceptive water-soluble polysaccharides from the aerial part of Nitraria retusa.* Foods, 2020. **9**(1): p. 28.

[18]. Zar Kalai, F., et al., *Antiobesity effects of an edible halophyte Nitraria retusa Forssk in 3T3-L1 preadipocyte differentiation and in C57B6J/L mice fed a high fat diet-induced obesity.* Evidence-Based Complementary Alternative Medicine, 2013. **2013**.

[19]. Maran, J.P., et al., *Development of model for mechanical properties of tapioca starch based edible films.* Industrial Crops Products, 2013. **42**: p. 159-168.

[20]. Boulaaba, M., et al., *Antioxidant, antiproliferative and anti-inflammatory effects of Glaucium Flavum fractions enriched in phenolic compounds.* Medicinal Chemistry Research, 2019. **28**(11): p. 1995-2001.

[21]. Boulaaba, M., et al., *In vitro antiproliferative effect of Arthrocnemum indicum extracts on Caco-2 cancer cells through cell cycle control and related phenol LC-TOF-MS identification.* Evidence-Based Complementary Alternative Medicine, 2013. **2013**.

[22]. Che Sulaiman, I.S., et al., *Effects of temperature, time, and solvent ratio on the extraction of phenolic compounds and the anti-radical activity of Clinacanthus nutans Lindau leaves by response surface methodology*. Chemistry Central Journal, 2017. **11**(1): p. 1-11.

[23]. Volf, I., et al., *Thermal stability, antioxidant activity, and photo-oxidation of natural polyphenols*. Chemical Papers, 2014. **68**(1): p. 121-129.

[24]. Ma, H.-f., et al., *Studies on thermal stabilities of typhaneoside and isorhamnetin-3-O-neohesperidoside.* China Journal of Chinese Materia Medica, 2007. **32**(1): p. 36.

[25]. Candioti, L.V., et al., *Experimental design and multiple response optimization. Using the desirability function in analytical methods development.* Talanta, 2014. **124**: p. 123-138.

[26]. Kalai, F.Z., et al., *Oral administration of Nitraria retusa ethanolic extract enhances hepatic lipid metabolism in db/db mice model 'BKS. Cg-Dock7m+/+ Leprdb/J'through the modulation of lipogenesis–lipolysis balance.* Food chemical toxicology, 2014. **72**: p. 247-256.

[27]. Kalai, F.Z., et al., *Identification of Main Phenolic Compounds of Nitraria Retusa Leaf and.* biobulletin, 2021. **7(4)**(01-10).

[28]. Salem, J.H., et al., *Biological activities of flavonoids from Nitraria retusa (Forssk.) Asch. and their acylated derivatives.* Food Chemistry, 2011. **124**(2): p. 486-494.

[29]. Zuo, A., et al., *Study on the relation of structure and antioxidant activity of isorhamnetin, quercetin, phloretin, silybin and phloretin isonicotinyl hydrazone.* Free Radicals Antioxidants, 2011. **1**(4): p. 39-47.

[30]. Bremner, P., et al., *Assessing medicinal plants from South-Eastern Spain for potential anti-inflammatory effects targeting nuclear factor-Kappa B and other pro-inflammatory mediators.* Journal of Ethnopharmacology, 2009. **124**(2): p. 295-305.

[31]. Hussain, T., et al., *Oxidative stress and inflammation: what polyphenols can do for us?* Oxidative medicine cellular longevity, 2016. **2016**.

[32]. Chen, G.-L., et al., *Antioxidant and anti-inflammatory properties of flavonoids from lotus plumule.* Food chemistry, 2019. **277**: p. 706-712.

[33]. Joshi, V., et al., *Celastrol modulates inflammation through inhibition of the catalytic activity of mediators of arachidonic acid pathway: Secretory phospholipase A2 group IIA, 5-lipoxygenase and cyclooxygenase-2.* Pharmacological research, 2016. **113**: p. 265-275.

[34]. Czekanska, E.M., *Assessment of cell proliferation with resazurin-based fluorescent dye*, in *Mammalian cell viability*. 2011, Springer. p. 27-32.