Nor. Afr. J. Food Nutr. Res. 2024; 8 (18): 118 – 129

https://doi.org/10.51745/najfnr.8.18.118-129

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ORIGINAL ARTICLE

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Statistical optimization of microwave-assisted extraction of phytochemicals from *Retama raetam* (white weeping broom) twigs and their biological properties

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ABSTRACT

Background: Several phytochemicals derived from the genus *Retama* reported to possess diverse biological activities, including antioxidant, anti-inflammatory, and antibacterial properties. **Aims**: The aim of this study was to optimize microwave-assisted extraction (MAE) of polyphenols from *Retama raetam* twigs using response surface methodology. **Methods:** A Box-Behnken design was utilized for determining the effect of MAE factors on total polyphenol content (TPC), including ethanol concentration (50 – 70%), irradiation time (4 – 6 min), power (400 – 600 W), and solvent-to-sample ratio (15 – 25 mL/g). The optimal extract (OE) was further analyzed for total flavonoid content (TFC), total tannin content (TTC), and antioxidant activity (DPPH• scavenging and FRAP) and *in vitro* anti-inflammatory activity assessment of the OE was evaluated using two complementary assays (albumin denaturation and membrane stabilization). **Results**: The following conditions: ethanol concentration of 64.73%, irradiation time of 5.57 min, power of 569.16 W, and solvent-to-sample ratio of 22.91 mL/g, resulted in the highest TPC (181.48 ± 1.59 mg GAE/g DR). The effectiveness and statistical validity of the derived quadratic model indicated no significant discrepancies between experimental and predicted results, demonstrating its high degree of accuracy. The obtained OE demonstrated a TFC of 31.25 ± 1.5 mg EC/g DR and a TTC of 15.17 ± 1.56 mg EC/g DR. The OE showed a significant capacity to scavenge DPPH• and an appreciable ferric-reducing power, where the IC_{50} and EC_{50} values were respectively 0.44 ± 0.08 and 0.61 ± 0.03 mg/mL. At a concentration of 1.5 mg/mL, the OE displayed moderate anti-inflammatory activity by red blood cell membrane stabilization (72.72 ± 0.73%) and reduction of heat-induced albumin denaturation (50.89 ± 0.66%). **Conclusion**: The MAE of TPC from *Retama raetam* twigs was primarily influenced by EtOH concentration, irradiation time, and power. The OE exhibited moderate antioxidant and anti-inflammatory properties, suggesting its potential as a source of phytopharmaceuticals.

Keywords: *Retama raetam*, microwave-assisted extraction, optimization, antioxidant, anti-inflammatory.

ARTICLE INFORMATION

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Received: June 06, 2024
Revised: September 15, 2024
Accepted: September 29, 2024
Published: October 18, 2024

Article edited by:
Pr. Khaled Méghit Boumédiène

Article reviewed by:
Pr. Omar Aoun
Pr. Farid Dahmoune
Dr. Lamia Medouni-Haroune

Cite this article as Zaoui, O., Oughlissi-Dehak, K., Bouziane, M. (2024). Statistical optimization of microwave-assisted extraction of phytochemicals from *Retama raetam* (white weeping broom) twigs and their biological properties. (2024). *The North African Journal of Food and Nutrition Research, 8* (18): 118 – 129. https://doi.org/10.51745/najfnr.8.18.118-129

1 Introduction

The pharmacological properties and biological activities of medicinal plants are due to the presence of various chemical compounds such as polyphenols, phytosterols, and alkaloids (de Elguea-Culebras et al., 2022). Among these compounds, phenolic derivatives constitute a major group of secondary plant metabolites, exhibiting a wide variety of biological properties, such as antidepressant, antiviral, and antitumor (de Elguea-Culebras et al., 2022). Furthermore, phenolic compounds are commonly used as antioxidants, playing a crucial role in reducing oxidative stress with cardiovascular and neurodegenerative diseases. The extraction of polyphenolic compounds from medicinal plants has garnered significant attention in recent years (Čanadanović-Brunet et al., 2006). Conventional extraction techniques, including maceration, reflux, and Soxhlet, often suffer from drawbacks including low recovery rate, a large amount of plant material,

and high solvent consumption. These methods also require extended extraction time at elevated temperatures, which can lead to the thermal degradation of bioactive compounds (Paz et al., 2015). In contrast, eco-friendly extraction techniques align with six core principles that mainly aim at innovation through renewable resource utilization, byproduct production, energy conservation, and the use of green solvents (Herrero, 2023). Microwave-assisted extraction (MAE) is a green technology that utilizes microwaves to rapidly and selectively heat the solvent and the plant matrix, thus promoting more efficient mass transfer and faster extraction of phytochemicals (Boateng, 2024; Herrero, 2023). MAE offers several advantages, including reduced extraction time, selectivity, lower solvent consumption, and the preservation of thermolabile compounds (Herrero, 2023). These advantages make MAE a promising approach for developing sustainable and eco-friendly industrial processes (Boateng, 2024). Numerous recent studies have highlighted the effectiveness of MAE for the extraction of phenolic compounds from various plant matrices. Additionally, MAE has proven to be more efficient than traditional methods, providing the highest content of phytochemicals from *Retam raetam* (*R. raetam*) twigs (Zaoui et al., 2023). MAE is highly influenced by various factors such as microwave power, solvent-to-sample ratio, solvent concentration, and time. To optimize the extraction process, several studies have been employed response surface methodology (RSM) (Dahmoune et al., 2013; Medouni-Adrar et al., 2015; Nabet et al., 2019; Dahmoune et al., 2021). The Box-Behnken design (BBD) is a particularly suitable experimental design for RSM (Dahmoune et al., 2021; Nikolić et al., 2023). BBD offers several advantages, including ease of performance and interpretation, simultaneous analysis of multiple variables, high accuracy in the regression equation, and avoidance of extreme experimental conditions. (Dahmoune et al., 2021; Nabet et al., 2019; Nikolić et al., 2023).

Fabaceae plants have been traditionally employed in medicine for the treatment of various diseases such as sore throat, diabetes, hepatitis, rheumatism, fever, inflammation, eczema, and microbial infections (Mariem et al., 2014; Saada et al., 2018; Zaoui et al., 2023). The genus *Retama,* representative member of the *Fabaceae* family, is a Mediterranean shrub with a distribution area extending from Morocco to Syria. In Algeria, Retama species are prevalent in the southern regions of Djelfa, Touggourt, Ain Safra, east of Biskra, and Ouargla (Awen et al., 2011; Fdil et al., 2012; Djeddi et al., 2013; Mariem et al., 2014). The name Retama is derived from the biblical word (ROTEM), which was shortened by Arabs to "*R'tem*" or "*Retam*" (Fdil et al., 2012; Hayet et al., 2008). While the genus *Retama* is known to contain relatively a low amount of essential oils, GC/MS analysis of the aerial parts of Retama species revealed the presence of linalool (51%), 2-decen-1-ol (6.6%), and limonene (7.4%) as the major components (Awen et al., 2011; Edziri et al., 2010).

As a member of the *Fabaceae* family, Retama is characterized by its significant alkaloid profile, especially its richness in quinolizidine and dipiperidine alkaloids such as ammodendrine, dehydroammodendrine, and N-formylammodendrine (Djeddi et al., 2013). Additionally, the aerial parts of *Retama monosperma* have been shown to contain various molecules, including sparteine, retamine, lupanine, N-methylcytisine, and cytisine (Fdil et al., 2012).

Polyphenols have been isolated from the aqueous extract of the aerial parts of *R. raetam*, including notable compounds such as genistein, 6-hydroxygenistein, 3'-O-methylorobol, prunetin, biochanin A, 6-hydroxyapigenin, luteolin, kaempferol, and p-coumaric acid (Djeddi et al., 2013). The methanolic extract of *R. raetam* flowers revealed the isolation of two flavonoids: licoflavone and derrone (Edziri et al., 2010). Additionally, the 70% hydroethanolic extract of the aerial parts has been found to contain two other flavonoids: luteolin-4'-O-neohesperidoside and 5,4'-dihydroxy-(3'',4''-dihydro-3'',4''-dihydroxy)-2'',2''-dimethylpyrano - (5'', 6 '': 7, 8) - flavone (Kassem et al., 2000). Mariem et al. (2014) identified a diverse phenolic profile in the ethyl acetate extract of the aerial parts using, including gallic acid, 3,4-dihydroxybenzoic acid, catechin hydrate, resorcinol, caffeic acid, syringic acid, vanillic acid, 2,5-dihydroxybenzoic acid, naringin, and quercetin-3-rhamnoside.

Numerous studies have demonstrated the biological activities of extracts of *R. raetam*, including antioxidant, antibacterial, and cytotoxic properties. For instance, the hydro-methanolic extract has exhibited significant radical scavenging (DPPH• and ABTS•+) and reducing power (Saada et al., 2018). Extracts obtained from the aerial part of *R. raetam* using hexane and acetone have demonstrated considerable antibacterial properties against human pathogens, including *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* NCIMB 8166, and *Aeromonas hydrophila* (Mariem et al., 2014; Saada et al., 2018). Furthermore, the methanolic leaf extracts demonstrated noteworthy cytotoxic activity against the COR-L23 cell line and Hep-2 cells (Conforti et al., 2004; Edziri et al., 2010; Fdil et al., 2012).

To the best of our knowledge, no studies have been carried out to optimize the recovery of phenolic compounds from the *R. raetam* plant. The aim of this study was to develop a model for the MAE of phenolic compounds from *R. raetam* twigs, considering the influence of four important factors: X_1 (solvent concentration), X_2 (time), X_3 (power), and X_4 (solvent-to-sample ratio). The significance of this research extends beyond process optimization, as it also evaluates the antioxidant and anti-inflammatory activities of the optimal extract (OE). The novel application of MAE to extract

phytochemicals from *R. raetam*, a relatively understudied plant, contributes to the growing body of knowledge in this field. The findings of this research have potential applications in the pharmaceutical, cosmetic, and food industries by offering a more sustainable and environmentally friendly alternative to traditional extraction methods. Furthermore, this study establishes the optimal conditions for researchers seeking to maximize the recovery of polyphenols from *Retama raetam*.

2 Material and Methods

2.1 Plant material

Twig samples of *R. raetam* were collected from Bousemghoun region (El-Bayadh, Algeria) during the flowering phase in February 2019 (Saada et al., 2018). The twigs were dried at room temperature (21.0 ± 1.2 °C) in a ventilated dark room for 21 days in order to preserve compound content against photo-oxidation. Subsequently, the dried twigs were ground using an electric grinder at low speed to minimize the temperature and then sieved to retain particles smaller than 125 µm. The powdered plant material was stored in a tightly sealed container at 4 °C for further use.

2.2 Chemicals and reagents

Analytical grade ethanol 99.8% (EtOH), 3,4,5-trihydroxybenzoic acid (gallic acid; GA), 3-methoxy-4-hydroxybenzaldehyde (Vanillin), 2-hydroxybenzoic acid (salicylic acid), ascorbic acid, 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol ((+)-catechin; C), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), aluminum chloride ($AlCl_3$), Folin-Ciocalteu phenol reagent, hydrochloric acid (HCl), iron chloride ($FeCl_3$), trichloroacetic acid (TCA), potassium ferricyanide [$K_3Fe(CN)_6$], sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), albumin, albumin bovine serum (BSA), and sulfuric acid (H_2SO_4) were obtained from Sigma-Aldrich (Madrid, Spain) or Merck (Darmstadt, Germany).

2.3 Microwave-assisted extraction process

Polyphenol extraction was carried out following the methodology described by Olalere & Gan (2021) utilizing a modified household microwave oven (CW-20W, Condor, Algeria) equipped with inverter technology and a magnetron operating at 2450 MHz, providing a maximum power of 1050 W. The oven cavity dimensions were 440×358×259 mm. One gram of the plant sample was treated with an appropriate volume of aqueous EtOH under different experimental conditions. After cooling, the mixture was allowed to stand at room temperature (21.0 ± 1.2 °C) for 20 minutes. The solution was then filtered using Whatman paper No. 1. The resulting filtrate was subsequently centrifuged at 3000 rpm (EBA 200 HETTICH) for 20 min and concentrated using a rotary evaporator (HEIDOLPH LABOROTA 4000). The crude extracts were stored at 4 °C and diluted for the quantification of Total Polyphenol Content (TPC), Total Flavonoid Content (TFC) and Total Tannin Content (TTC).

2.4 Statistical modeling and optimization of the MAE process

To optimize the TPC from *R. raetam* twigs using the MAE method, the influence of each extraction parameter was individually investigated in single-factor experiments. In the first step, four solvents (EtOH 50%, methanol 50%, acetone 50%, and water) were employed to evaluate the effect of solvent type, while the remaining parameters were maintained constant at their median values. Subsequently, the optimal solvent selected in the first step was used to select the most appropriate interval (-1; 0; +1) for each independent parameter of this optimization study. This process facilitated the identification of the optimal combination of factors, including solvent concentration (X_1), extraction time (X_2), power (X_3), and solvent-to-sample ratio (X_4) (Table 1).

Table 1. Actual and coded values of independent variables

Independent variables	Symbols	Coded levels		
		Low level (-1)	Centre level (0)	High level (+1)
EtOH concentration (%)	X_1	50	60	70
Irradiation time (min)	X_2	4	5	6
Microwave power (w)	X_3	400	500	600
Solvent/sample ratio (mL/g)	X_4	15	20	25

For the statistical modeling, a RSM was applied using a BBD with four factors and three levels. This experimental design included 27 experimental runs with three replications at the central point to evaluate experimental error measurement. A total of 24 different combinations of parameters were evaluated. The following equations (1 – 2) were utilized to determine the coded levels of the specified variables and the number of trials (Manga et al., 2020):

$$Xi = (x_i - x_o)/\Delta xi = 1, 2, 3 \ldots\ldots\ldots\ldots \quad (1)$$

X: the coded value of an independent variable; x_i: the actual value of an independent variable; x_o: the actual value of an independent variable.

$$N = 2k\,(k-1) + C_0 \ldots\ldots\ldots\ldots \quad (2)$$

Where "k" is the number of independent variables and "C_0"is the number of center points.

A quadratic model was performed to determine the optimum operating conditions and to evaluate the method's performance, as shown in the following equation (3) (Dahmoun et al., 2021).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X^2{}_i + \sum_{i>1}^{k} \beta_{ij} X_i X_j + \varepsilon \quad \ldots\ldots\ldots\ldots \quad (3)$$

K: the number of variables; βo: constant-coefficient; βi: the linear coefficient for the main factor; βii: the quadratic coefficient for the main factors; βij: the second order interaction coefficient; Ɛ: the random error; Xi and Xj: the actual independent factors; Y: the predicted response.

The reliability of the developed model and its statistical significance were validated by an analysis of variance (ANOVA). Additionally, the validity of the established models was evaluated through an additional extraction experiment carried out under perfect conditions.

2.5 Phytochemical assessment

The TPC estimation of different crude extracts was determined by Folin-Ciocalteu method, as described by Serairi-Beji et al. (2017). TPC value were expressed as milligrams of gallic acid equivalents per gram of dry residue (mg GAE/g DR). TFC content was measured using the $NaNO_2$-$Al(NO_3)_3$-NaOH system, following the method of Dewanto et al. (2002) with minor modifications. TFC values were expressed as milligrams of (+)-catechin equivalent per gram of dry residue (mg CE/g DR). TTC values were assayed using the method of Serairi-Beji et al. (2017) and were also expressed as milligrams of catechin equivalents per gram of dry residue (mg CE/g DR).

2.6 Assessment of *in vitro* antioxidant activities

The DPPH• scavenging assay for the OE was performed using the procedure described by Brand-Williams et al. (1995) with slight modifications based on the decrease in absorbance of the DPPH• solution at 517 nm. The percentage of DPPH• scavenging was calculated using the following equation (4):

$$\text{DPPH} \bullet \text{ scavenging (\%)} = \frac{(\text{absorption of control negative} - \text{absorption with sample})}{(\text{absorption of control negative})} \times 100 \ldots\ldots (4)$$

The Ferric Reducing Antioxidant Power (FRAP) assay was performed using the potassium ferricyanide-ferric chloride method reported by Wu et al. (2014). The reducing power is proportional to the absorbance of the reaction mixtures. The ascorbic acid solution was the positive control in both previous assays.

2.7 Assessment of *in vitro* anti-inflammatory activity

The anti-inflammatory activity of the OE was carried out using an inhibition of albumin denaturation assay and red blood cell membrane stabilization. For the first test, a minor modified method of Mizushima et al. (1968) was followed. The percentage inhibition of protein denaturation was calculated by the following equation (5):

$$\text{Inhibition (\%)} = \frac{(Abs\ Control - Abs\ Sample)}{Abs\ Control} \times 100 \ldots\ldots\ldots (5)$$

The methodology of Shinde et al. (1999) was followed with a slight modification for the heat-induced hemolysis; the erythrocyte suspension was prepared as previously reported by Loef et al. (2020). The percentage inhibition of hemolysis was calculated using equation (6).

$$\text{Inhibition (\%)} = \frac{(Abs\ Control - Abs\ Sample)}{Abs\ Control} \times 100 \ldots\ldots\ldots (6)$$

Salicylic acid and distilled water were taken as positive and negative controls, respectively, in both previous assays.

2.8 Statistical analysis

All experiments were conducted in triplicate, and all data are presented as the standard error of the mean (± SEM) of three replicates. Analyses were performed using the Sigma-Plots statistical software (version 5). IC_{50} and EC_{50} values were determined using Origin-Pro (version 2016), through a nonlinear modeling between approach that analyzed the relationship between X concentrations and Y responses. The adequacy of the regression models generated by the factorial design was examined using a one-way ANOVA test (Tukey's S) ($p < 0.05$). Expert Design (version 11) software was used to construct BBD, analyze all results, and generate the 3D contour plots of responses.

3 Results and discussion

3.1 Characteristics of the study participants

The MAE of secondary metabolites is significantly influenced by several factors such as method employed, solvent type, solvent concentration, irradiation time, power, and solvent-to-sample ratio (Dahmoune et al., 2013; Dahmoune et al., 2021; Nabet et al., 2019; Yang et al., 2010). In order to optimize the MAE conditions of TPC from *R. raetam* twigs, traditional optimization was avoided in this study because of its several drawbacks. It is based on the study of each factor

Table 2. Results of single-factor experiments for MAE optimization

Solvent		EtOH concentration		Time		Power		Liquid/Solid ratio	
Type	TPC mg GAE/g DR	(v/v) %	TPC mg GAE/g DR	Min	TPC mg GAE/g DR	W	TPC mg GAE/g DR	mL/g	TPC mg GAE/g DR
Water	160.75 ± 1.90^{c}	40%	162.75 ± 1.23^{c}	3	153.00 ± 0.70^{e}	200W	119.75 ± 0.88^{e}	10	117.50 ± 0.35^{e}
Acetone	164.50 ± 0.70^{b}	50%	171.50 ± 1.06^{b}	4	171.50 ± 1.06^{b}	300W	150.00 ± 0.70^{d}	15	132.25 ± 1.23^{d}
Methanol	161.00 ± 0.70^{c}	60%	180.50 ± 1.06^{a}	5	187.25 ± 0.53^{a}	400W	171.50 ± 1.06^{b}	20	171.50 ± 1.06^{a}
EtOH	171.50 ± 1.06^{a}	70%	169.75 ± 1.29^{b}	6	168.50 ± 0.70^{c}	500W	182.00 ± 0.70^{a}	25	163.00 ± 2.82^{b}
		80%	140.00 ± 0.70^{d}	7	164.50 ± 2.82^{d}	600W	166.50 ± 0.70^{c}	30	154.25 ± 1.23^{c}
		100%	142.50 ± 0.70^{d}	8	162.75 ± 0.17^{d}			35	123.25 ± 0.88^{e}

Data is shown as mean ± SEM. Tukey's test was used to make comparisons between groups. Columns not sharing a common letter (a–e) differed significantly at $p < 0.05$.

Table 3. BBD with the observed and predicted value for TPC of *R. raetam* twigs using MAE

Run	EtOH (%)	Time (min)	Power (W)	Solvent/sample ratio (ml/g)	TPC (mg GAE/g DR)	
					Observed	Predicted
1	50	5	500	25	164.15 ± 1.04	163.23
2	60	4	500	25	169.48 ± 1.36	172.32
3	60	6	500	25	183.15 ± 0.5	181.004
4	70	6	500	20	170.31 ± 0.72	171.51
5	70	5	400	20	174.48 ± 1.16	173.55
6	70	5	600	20	179.98 ± 1.47	179.69
7	50	5	500	15	176.15 ± 0.5	175.87
8	50	4	500	20	170.15 ± 0.86	167.76
9	60	6	500	15	169.15 ± 0.76	167.05
10	60	5	400	25	173.65 ± 0.86	173.12
11	50	6	500	20	168.31 ± 0.92	169.21
12	60	4	500	15	180.15 ± 0.76	183.03
13	60	5	600	25	181.65 ± 1.04	181.68
14	50	5	600	20	170.31 ± 0.6	171.97
15	60	5	600	15	177.98 ± 0.72	177.32
16	60	4	400	20	180.15 ± 0.86	180.12
17	70	5	500	15	167.65 ± 0.57	169.01
18	70	4	500	20	182.31 ± 1.42	180.24
19	60	4	600	20	175.81 ± 0.88	174.59
20	60	5	400	15	175.48 ± 1.36	174.26
21	60	6	400	20	163.48 ± 0.92	165.15
22	60	5	500	20	183.15 ± 0.86	182.81
23	70	5	500	25	184.15 ± 1.6	184.87
24	50	5	400	20	165.48 ± 0.6	166.5
25	60	6	600	20	181.81 ± 0.72	182.28
26	60	5	500	20	181.31 ± 0.44	182.81
27	60	5	500	20	183.98 ± 1.09	182.81

independently; consequently, the interactions between the factors are ignored, and the chance of approaching a true optimum is very improbable (Melgar et al., 2019). Therefore, the RSM was applied for the optimization investigated.

To establish appropriate experimental levels (-1, 0, +1) for each independent parameter, preliminary single-factor experiments were conducted. Table 2 presents the experimental conditions and results obtained. Statistical analysis revealed a significant difference (p-value ≤ 0.05) in TPC among the various experiments. The type of solvent and its concentration significantly influenced TPC in *R. raetam* twigs. A hydro-ethanolic mixture was found to be the most

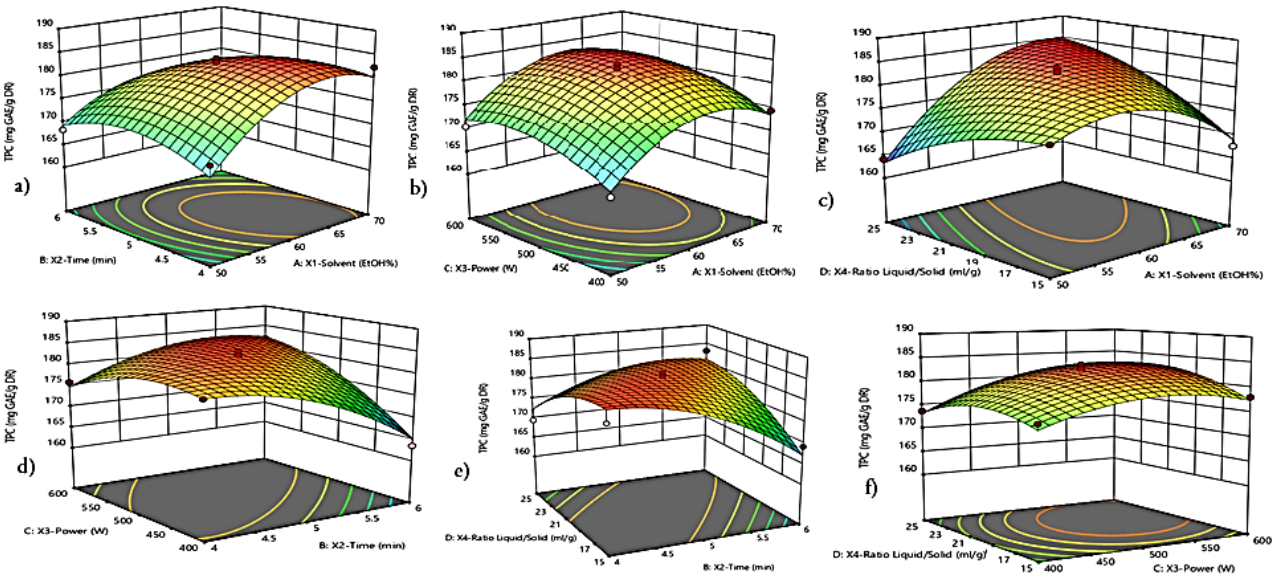

Figure. 1. Response surface plots for the TPC from *R. raetam* twigs by MAE with respect to (a): ethanol concentration and irradiation time; (b): ethanol concentration and microwave power; (c): ethanol concentration and solvent/sample ratio; (d): microwave power and irradiation time; (e): solvent/sample ratio and irradiation time; (f): microwave power and solvent/sample ratio

effective solvent, with TPC increasing as ethanol concentration rose from 40% to 70% (v/v). However, further increases in ethanol concentration led to a decline in TPC. With the increase in extraction time ranging from 3 to 6 minutes, the TPC increased, and then it subsequently stabilized without significant changes. This behavior can be attributed to the solvent's high dielectric constant and dipolar moment (Dahmoune et al., 2013). Water, EtOH, and their mixtures are recognized as alternative and environmentally friendly extraction solvents based on the theory of similarity and intermiscibility, facilitating the extraction of bioactive compounds soluble in water and/or EtOH (Cavalloro et al., 2021). Prolonged contact time between the sample and the solvent, however, can lead to the oxidation of phenolic compounds, potentially diminishing TPC (Sai-Ut et al., 2024).

Selecting the appropriate microwave power interval is a critical step in optimizing MAE (Dahmoune et al., 2021; Nabet et al., 2019). Reported results indicate that TPC increased with increasing microwave power from 200 to 500 W, followed by a moderate decrease at higher power levels. The increase in power enhances the MAE efficiency by making the cell membranes more permeable and increasing the diffusion coefficient of extractable compounds, thus facilitating solvent penetration into cells (Dahmoun et al., 2013). Conversely, the decrease in TPC yield obtained at high power could be explained by the thermal degradation of bioactive compounds (Sai-Ut et al., 2024). A relatively unfavorable TPC was observed at high solvent-to-sample ratios. Contrary to conventional techniques, MAE with a higher solvent volume may not always improve extraction performance, potentially leading to reduced efficiency (Chandrasekar et al., 2014; Medouni-Adrar et al., 2015; Sai-Ut et al., 2024).

3.2 RSM analysis

Based on the results of the single-factor preliminary study, four independent factors with three levels were selected to evaluate their effects on YTPC contained in R. raetam twigs: (X_1; solvent concentration (50%; 60%; 70%), X_2; time (4; 5; 6 min), X_3; power (400; 500; 600 W), and X_4; solvent-to-sample ratio (15; 20; 25 mL)). Table 3 summarizes the experimental conditions, results obtained, and predicted values of the investigated response Y_{TPC}. The software generated a quadratic equation (7) of polynomial regression representing the empirical relationship between the response values Y_{TPC} and the parameters (X_1; X_2; X_3; X_4) in their coded values. The model was simplified by neglecting statistically insignificant terms.

$$Y_{TPC}\ (\text{mg GAE/g DR}) = 76.84 + 6.648X_1 + 0.56X_2 - 11.20 X_4 - 0.25 X_1X_2 + 0.14 X_1 X_4 + 0.05 X_2 X_3 + 1.23X_2 X_4 - 0.06X_1^2 - 4.01X_2^2 - 0.11 X_4^2 \ldots (7)$$

The ANOVA results for the previously fitted model, including the coefficient significance of individual factors, and their interactions are summarized in Table 4. The quadratic regression model demonstrated a highly significant R^2 (0.95), and the adjusted R^2 (R^2_{Adj}) was closely aligned with R^2. Furthermore, the difference between R_{adj} and predicted R^2

(R_{pred}) was significantly less than 0.2, and the lack of fit was highly insignificant (0.2913). The coefficient of variation was relatively low (CV = 1.23%), indicating improved precision, reliability, and reproducibility at the predicted optimal values. The appropriate precision value was notably higher than four (4), suggesting an adequate signal to navigate the design space (Wu et al., 2015).

The ANOVA results presented in Table 4 demonstrate that the effects of EtOH concentration (X_1), time (X_2), and power (X_3) on Y_{TPC} had highly significant effects on TPC, with p-values of 0.0001, 0.012, and 0.0006, respectively. Additionally, the interactive effects of X_1X_2 and X_2X_3 were found to be significant, while X_1X_3 exhibited no significant interaction. These results are consistent with previous studies that have highlighted the significant influence of these three parameters on TPC (Chandrasekar et al., 2015; Dahmoune et al., 2013; Dahmoune et al., 2015; Medouni-Adrar et al., 2015; Nabet et al., 2019).

Whereas the sample-to-solvent ratio (X_4) on Y_{TPC} did not have a significant effect on TPC which is consistent with the result reported by Chandrasekar et al. (2014) but contradicts those of Dahmoune et al. (2013) and Dahmoune et al. (2015). However, the quadratic effect (X_4^2) and its interaction with other factors were found to be highly significant. Despite the insignificant effect of X_4 in the single-factor experiments, the quadratic effect suggests that the optimal level of X_4 may lie within the selected range for RSM (15 – 25 mL/g). In fact, an important increase in TPC was recorded only in the interval of 15 – 20 mL/g, but beyond that, a slight decrease was noticed. On the other hand, the ratio of 10 mL/g was avoided because it was not a sufficient volume to immerse the entire plant mass; therefore, we were obliged to work in this range. This observation was also mentioned in previous studies (Dahmoune et al., 2013; Dahmoune et al., 2021). In addition, all quadratic effects (X_1^2, X_2^2, X_3^2, and X_4^2) were found to have a significant influence on Y_{TPC}.

The previously derived equation (7) was used to generate three-dimensional RSM plots (Figure 1). These plots depict the response plotted on the Z-axis against two independent variables on the X-axis and Y-axis, while the other two independent variables are maintained at zero level.

As illustrated in Figure 1a, for the plot of the variance of TPC as a function of X_1 (EtOH concentration) and X_2 (irradiation time), while X_3 (power) and X_4 (solvent-to-sample ratio) remain at level 0. The response increased with increasing EtOH concentration and irradiation time, reaching its maximum at 176.69 GAE/g DR, while at higher EtOH concentration (61.2 – 70%) and time (4.2 – 6 min). However, the interactive effect of X_1 and X_2 appears to be less pronounced at these higher levels. Figure 1b demonstrates a positive linear relationship between Y_{TPC} and both power and EtOH concentration. With increasing X_1 and X_3, the studied response sharply increased until reaching a saturated value when X_1 and X_3 were conducted at 58% and 480 W, respectively. The strong mutual effect between EtOH concentration and solvent-to-sample ratio (X_1X_4) on TPC level is depicted in Figure 1c, assuming constant time and power. The 3D plot indicates a gradual increase in TPC production with increasing X_1X_4 interactions. These results are in agreement with previous literature studies that underline the influence of the interactive effect of EtOH concentration with irradiation time, power, and solvent-to-sample ratio on the extraction of polyphenols from vegetable tissues (Chandrasekar et al., 2014; Dahmoune et al. 2015; Sai-Ut et al., 2024).

As shown in Figure 1d, the interactive effect of power and time (X_2X_3) considerably enhanced polyphenol recovery, particularly at higher X_2 and X_3. Notably, the TPC response was slightly decreased within the X_2X_3 range of 5.2 – 6 min and 530 – 600 w, respectively, for power and time. The relationship between time and solvent-to-sample ratio (X_2X_4) exhibited a remarkably positive effect on TPC when the other two factors were held constant. According to the 3D graph in Figure 1e, TPC improved with increasing solvent-to-sample ratio and EtOH concentration, but subsequently declined with increasing the X_2X_4 parameter beyond 4.7 min and 19 mg/mL, respectively. Similar results reported that the level of TPC from different plant materials decreased after a relatively long period, confirming the explanation offered in Section 3.1 for single-factor analysis (Chandrasekar et al., 2014; Medouni-Adrar et al., 2015). The decrease noticed at high power could be explained by the thermal degradation of bioactive compounds (Chandrasekar et al., 2014; Sai-Ut et al., 2024).

As displayed in Figure 1f, the interactive effect of power and solvent-to-sample ratio interaction (X_3X_4) on Y_{TPC} was limited when EtOH concentration and time were held constant at their intermediate levels (0). Increasing power and solvent-to-sample ratio resulted in a higher content. The maximum content of 182.3 GAE/g DR can be found for X_3 and X_4 at 465 W and 21.1 mL/g, respectively. Nevertheless, when these two parameters were maintained at the highest level, they did not show any significance as the value continuously decreased. Previous studies have reported that the recovery of polyphenols increase up to a certain level and then declined at high solvent-to-sample ratios (Dahmoune et al. 2015; Sai-Ut et al., 2024).

3.3 Validation of the developed model

Numerical optimization of the data was employed to determine the optimal extraction conditions for the investigated response for maximizing TPC. The optimal conditions were found to be X_1 = 64.73%, X_2 = 5.57 min, X_3 = 569.16 W, and X_4 = 22.91

Table 5. Residual standard deviation between predicted and observed TPC of the OE

Investigated response	Predicted Value	Experimental Value	RSD%
TPC (mg GAE/g DR)	186.83	181.48 ± 1.59	2.05

mL/g, with a predicted response value of 186.83 mg GAE/g DR (Table 5). To validate and test the reliability of the mathematical model (8), we conducted a supplementary MAE experiment under the optimal conditions. According to the RSD% value (considering as observed results the average of three independent extracts obtained in the same conditions), the experimental result was perfectly coherent with the predicted value, confirming the validity and reliability of the RSM model. This indicates that the model accurately reflects the expected optimization.

3.4 Phytochemical assessment

Additional quantifications were performed for the OE to assess its TPC, TFC, TTC, and its antioxidant activity (DPPH• scavenging and FRAP). The results for this section are shown in Table 6.

Table 6. Residual standard deviation between predicted and observed TPC of the OE

	Phytochemical content			Antioxidant activity	
	TPC (mg GAE/g DR)	TFC (mg CE /g DR)	TTC (mg CE /g DR)	DPPH• IC_{50} (mg/mL)	FRAP EC_{50} (mg/mL)
OE	181.48 ± 1.59	31.25 ± 1.5	15.17 ± 1.56	0.44 ± 0.08^{a}	0.61 ± 0.03^{a}
Ascorbic acid				0.03 ± 0.02^{b}	0.22 ± 0.02^{b}

Data are shown as mean ± SEM. Tukey's test was used to make comparisons between groups. Columns not sharing a common letter (a–b) differed significantly at $p < 0.05$

The Y_{TPC} obtained through optimization in this study was higher than that those reported by Mariem et al. (2014) and Zaoui et al. (2023) using a conventional extraction method for the aerial part of the same plant in water (137 GAE/g DR and 155.13 ± 1.7 GAE/g DR, respectively). Saada et al. (2021) reported a TPC of 148.2 ± 0.74 mg GAE/g DR in the methanolic extract of the same species. It should be noted that several researchers have pointed out the limitations of the Folin-Ciocalteu phenol reagent which is to not exclusively specific to polyphenols but can also react with other oxidizable substances (Wong et al., 2006).

According to the reported outcomes, the assays revealed that the OE contained a high level of TFC (31.25 ± 1.5 mg EC/g DR) and TTC (15.17 ± 1.56 mg EC/g DR). Comparatively, Mariem et al. (2014) have revealed that the same species contains TFC values of 5.1, 10.16, and 9.23 mg EC/g DR for aqueous, acetone, and hexane extracts, respectively, after maceration of the aerial part. The TTC assessment of the OE did not deviate strongly from the study conducted by Mariem et al. (2014), who reported TFC levels of 10.43, 16.2, and 33.21 mg EC/g DR for the aqueous, acetone, and ethyl acetate extracts, respectively. From the results of secondary metabolite quantifications performed in this study, it can be concluded that the OE is extremely rich in bioactive compounds. This is mainly attributable to the conditions adopted for MAE and the high correlation between TPC, TFC, and TTC (Khiya et al., 2021).

3.5 Assessment of in vitro antioxidant activities

The OE exhibited moderate antioxidant power with a maximum inhibition of 85.33 ± 0.99% at a concentration of 1 mg/mL and a low IC_{50} value of 0.44 ± 0.08 mg/mL. However, this antioxidant activity was relatively weaker compared to that of ascorbic acid, which demonstrated an IC_{50} value of 0.03 ± 0.02 mg/mL. These findings align with previous studies by Saada et al. (2018) (IC_{50} = 0.16 ± 0.01 mg/mL), Zaoui et al. (2023) (IC_{50} = 0.34 ± 0.031 mg/mL), and Hayet et al. (2008) (IC_{50} = 0.450 mg/mL). Our results are comparable to those reported on another species (*Retama monosperma*), where Belmokhtar and Harche (2014) found that this specimen has a significant ability to scavenge the

Table 7. Effect of OE and salicylic acid on inhibition of albumin denaturation and membrane stabilization

Concentration [mg/mL]	Inhibition of albumin denaturation (%)		Inhibition of membrane stabilization (%)	
	OE	salicylic acid	OE	salicylic acid
2.50	72.72±0.73^{a}		50.89±0.66^{a}	
1.25	68.25±0.72^{b}		43.77±0.71^{b}	
0.63	57.03±0.64^{c}	85.07±0.45^{a}	25.99±0.77^{c}	93.61±0.4^{a}
0.31	51.47±0.84^{d}	83.22±0.46^{b}	13.33±0.72^{d}	83.28±0.61^{b}
0.16	34.67±0.84^{e}	73.88±0.66^{c}	3.91±0.43^{e}	67.48±0.70^{c}

Data is shown as mean ± SEM. Tukey's test was used to make comparisons between groups. Columns not sharing a common letter (a–e) differed significantly at $p < 0.05$

DPPH• (IC_{50} = 0.15 mg/mL). Our results are lower than those previously reported by Zaoui et al. (2023) for the aqueous extract of the aerial part (IC_{50} = 0.043 mg/mL). Saada et al. (2021) reported that IC_{50} values of DPPH• ranged from 0.037 ± 1.85 to 0.280 ± 1.4 mg/mL for different fractions of the methanolic extract of this species.

The OE also exhibited a strong reduction of ferric ions, with an EC_{50} value of 0.61 ± 0.03, but still lower than that of ascorbic acid (EC_{50} = 0.22 ± 0.02 mg/mL). The EC_{50} recorded is close to that found by Saada et al. (2021) (EC_{50} = 0.410 ± 0.01 mg/mL) and Zaoui et al. (2023) (EC_{50} = 0.28 ± 0.01

mg/mL), but superior to that found by Mariem et al. (2014) for the hydro-methanolic and aqueous extracts.

The antioxidant activity of the OE is mainly due to its richness in phenolic compounds. This has been confirmed by the strong correlation between phenolic compounds and antioxidant activity (Pliszka, 2020). Phenolic compounds, flavonoids, and tannins are responsible for the DPPH• scavenging activity due to their ability to release hydrogen (Chiorcea-Paquim et al., 2020). Several authors have suggested that the polar molecules present in plant extracts contribute considerably to the enhancement of their antioxidant activity, such as Niroula et al. (2021).

3.6 Assessment of anti-inflammatory activity

Several non-steroidal anti-inflammatory drugs, including diclofenac sodium, ibuprofen, and salicylic acid, have been demonstrated to suppress thermally mediated protein denaturation. However, these drugs are associated with serious side effects. Consequently, there has been a growing interest in discovering novel anti-inflammatory drugs from natural sources (Truong et al., 2019). Table 7 illustrates the *in vitro* anti-inflammatory activity assessment of the OE using two complementary assays (albumin denaturation and membrane stabilization at different concentrations). The OE was able to prevent heat-induced albumin denaturation and exhibited the highest inhibition of 72.72 ± 0.73 and 68.25 ± 0.72% at 2.50 and 1.25 g/mL, respectively. Salicylic acid exhibited the strongest inhibition of 85.07 ± 0.45% at a concentration of 0.63 mg/mL. This difference is also reflected in the IC_{50} values. The IC_{50} value of the OE was 0.45 mg/mL, compared to 0.09 mg/mL for salicylic acid. The correlation coefficient values for the extract and salicylic acid were 0.97 and 0.89, respectively. The results show that OE at concentrations of 2.50 and 1.25 g/mL significantly protected the hemolysis with inhibition of 50.89 ± 0.66 and 43.77 ± 0.71%, respectively, whereas salicylic acid at a concentration of 0.63 mg/mL exhibited inhibition of 93.61 ± 0.4%. However, it was found to be significantly lower compared to the standard anti-inflammatory drugs. The potent activity of the OE may be explained by the strong presence of polyphenolic compounds, such as phenols, flavonoids, and tannins, which are known to have anti-inflammatory abilities (Derouich et al., 2020). These findings provide scientific support for the traditional therapeutic uses of *Retama raetam* and suggest its potential as a source of novel anti-inflammatory agents (Mariem et al., 2014; Saada et al., 2018; Saada et al., 2021).

4 Conclusion

This study represents the first application of RSM to optimize the MAE of TPC from *Retama raetam* twigs based on BBD. The results demonstrate that ethanol concentration, irradiation time, and power are the primary factors influencing TPC extraction. The optimum MAE conditions identified in this study were an ethanol concentration of 64.73%, an irradiation time of 5.57 min, a power of 569.16 W, and a solvent-to-sample ratio of 22.91 mL/g. Under these conditions, the predicted optimal conditions produced an experimental TPC value of 181.48 ± 1.59 mg GAE/g DR, which was quite close to the predicted value of 186.83 with a low RSD value (2.5%). Additionally, the TPC value obtained in this study was considerably higher than the levels found in previous studies of *R. raetam*, minimizing the extraction time significantly. Moreover, the OE also exhibited moderate antioxidant and anti-inflammatory properties, suggesting that the investigated method could provide an attractive alternative approach to conventional extraction techniques for the production of phytochemicals. The OE of *R. raetam* deserves to be valorized in the agro-alimentary and pharmaceutical industries since it could be a promising source of phytopharmaceutical compounds such as natural antioxidants and anti-inflammatory drugs. However, further research focusing on the separation and purification of polyphenols from *R. raetam* twigs is essential to advance the utilization and development of this natural resource.

Source of funding: Laboratory of Biogeochemistry of Desert Environments, University of Kasdi Merbah -Ouargla,30000.

Acknowledgments: We wish to acknowledge anyone who contributed to the achievement of this study.

Previous submissions: None.

Authors' Contribution: O. Z: literature search, methodology, experimental/clinical studies data curation, formal analysis, software, manuscript preparation. O. D. K and B. M: methodology, experimental/clinical studies, investigation, data analysis, supervision, validation, review & editing.

Conflicts of Interest: We wish to confirm that there are no conflicts of interest associated with this publication, and there has been no significant financial support for this study that could have influenced its outcome.

Preprint deposit: Authors did not share this manuscript as a preprint deposit.

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