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Original Article

Optimizing Ethanol Extraction of Rosemary Leaves and Their Biological Evaluations

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Abstract

Background and objectives: Rosemary (*Rosmarinus officinalis* L.) extract is widely used in food, cosmetic and pharmaceutical industries. This study centered on the optimization of ethanolic extraction of rosemary leaves and evaluated the bioactivity of the extract.

Methods: The optimized rosemary extract was analyzed using high-performance liquid chromatography. The antioxidant activity of the rosemary extract was measured using 2,2′-Azinobis-(3-ethylBenzoThiazoline-6-Sulfonic) acid, 2,2-DiPhenyl-1-Picryl-Hydrazyl-hydrate, and ferric reducing antioxidant power assays. The antibacterial activity of the rosemary extract was tested using the disk diffusion method. Toxicity was tested using mice. Nitrite production by RAW 264.7 cells was used to determine the anti-inflammatory activity of the rosemary extract. The construction and statistical analysis of the experimental design was done using NemrodW (LPRAI, version 2000) software.

Results: We found that ethanol extraction of rosemary leaves depended on optimizing heating temperature and time. The maximum yields of carnosic and rosmarinic acids were obtained after optimizing these parameters according to the modelling software. The rosemary leaf extract had high antioxidant, anti-inflammatory, and antibacterial properties with low acute toxicity.

Conclusions: Ethanol extraction of rosemary leaves could potentially be used in several industrial applications.

Keywords: Rosemary (Rosmarinus officinalis L.) extract; Food additive; Antioxidant activity; Toxicology; Antibacterial; Anti-inflammtory activity.

Abbreviations: ABTS, 2,2′-Azinobis-(3-ethylBenzoThiazoline-6-Sulfonic) acid; ALT, alanine amino transferase; AST, aspartate amino transferase; BUN, blood uric nitrogen; CA, carnosic acid; Crea, creatinine; D1, day1; D2, day2; D3, day3; DMSO, dimethyl sulfoxide; DPPH, 2,2-DiPhenyl-1-Picryl-Hydrazyl-hydrate; EGA, equivalent gallic acid; EQ, equivalent quercetin; FRAP, ferric reducing antioxidant power; Glu, glucose; GRAS, generally recognized as safe; HPLC, high-performance liquid chromatography; IZ, inhibition zone; PAL, phosphatase alkaline; RA, rosmarinic acid; RSM, response surface methodology; T-BIL, total bilirubin; T-CHO, total cholesterol; TP, total protein; X1, extraction temperature; X2, extraction duration; Y1, carnosic acid extraction; Y2, rosmarinic acid extraction.

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Introduction

Natural food products are gaining popularity among consumers. Natural substances produced from aromatic and medicinal plants, herbs, and spices are added to foods to reduce the need for synthetic additives. Rosemary, *Rosmarinus officinalis* L., is a well-known medicinal herb that has been utilized worldwide. It is one of Tunisia's most popular plants, and it exists as a single species with a variety of chemotypes. Rosemary is a natural source of a variety of metabolites with diverse biological functions, and it has a unique set of features that distinguish it from other herbs. The rosemary plant has antioxidant,[1] antiviral,[2] antitumor,[3] antibacterial,[4] anti-hyperglycemic,[5] antidepressant,[6] neuroprotective[7] and anti-inflammatory[6] properties. Accordingly, increasing the yield of phenolic compounds from the rosemary plant has become a top priority.

Table 1. Parameter levels to optimize

Independent variables	Extraction level parameters		
	−1 (minimum)	0 (medium)	+1 (maximum)
Extraction temperature per °C (X1)	130	155	180
Extraction time per min (X2)	60	120	180

Statistical modeling has increased the feasibility of experimental designs to optimize extraction of the rosemary leaf. Specifically, these approaches allow for a limited number of tests,[8] better parameter screening (from the most critical to the least critical property), and improved operational conditions to obtain the desired results. Optimization of extraction procedures has been addressed previously. Some have used surface design experiments for optimization,[9] while others have utilized more complex designs, such as full factorial designs,[10] which can be used to determine factors that influence rosemary extract yields using solvent extraction.[11] It is also possible to identify a set of parameters that might affect the reaction in trials of response surface type optimization. Response surface designs are better for optimizing operational variables because of its optimization nature.[12] The improvement of these variables can impact the rosemary extraction process. Therefore, response surface designs are a better choice for optimizing operational variables.

Increasing extract yields after phenolic rosemary extraction has been thoroughly researched. Several authors have demonstrated that this extract may be used directly in food formulation.[13–15] Thus, our work was supported by the optimization method of rosemary phenolic extraction using response surface methodology (RSM). The obtained rosemary extract in this study was evaluated for its antioxidant, antibacterial, cytotoxicity, and anti-inflammatory activities.

Materials and methods

Plant material

Spontaneous rosemary (*Rosmarinus officinalis* var. *typicus* L.) aerial parts were gathered from Zaghouan, Tunisia's northwest governorate. The herbarium specimen was confirmed by botanist Abderrazzak Smaoui of the Biotechnology Center of Borj-Cedria (Tunisia).

Ethanol extraction of rosemary

Fresh rosemary leaves were dried at ambient temperature and ground. About 15 g dry powdered rosemary leaves were successively extracted with 1:10 w/v of absolute ethanol, which was classified as safety generally recognized as safe (GRAS) solvents, at a specific temperature using a Soxhlet type of apparatus. The extraction solvent was eliminated by vacuum evaporation using a rotary evaporator, and rosemary samples were stored in brown vials under nitrogen gas at 4°C in the dark to prevent oxidation of phenolic compounds.

Optimization of carnosic acid and rosmarinic acid extraction using RSM

This approach optimized two experimentally controllable parameters: extraction temperature (X1) and extraction duration (X2). The full two-level factorial plans of these two parameters allowed for simultaneous adjustment of both parameters in the chosen experimental area, allowing for optimal data collection and the construction of a mathematical model.[16] The parameters to be optimized were determined at the lower, basic, and upper levels, taking into account installation performance and outcomes of our prior work.[17] The values of the real and coded variables of these two parameters are presented in Table 1. The variation in yields of phenolic compounds are expressed by the first order polynomial function in X1 and X2:Carnosic acid yield = b0 + b1X1 + b2X2 + b11X1X1 + b22X2X2 + b12X1X2 Rosmarinic acid yield = d0 + d1X1 + d2X2 + d11X1X1 + d22X2X2 + d12X1X2

Quantification of main rosemary phenolic compounds

Colorimetric phenolic assays

Total phenolic compounds in the rosemary extract were quantified using the colorimetric method described by Yeddes *et al*.[18] The contents of total flavonoid compounds were measured according to Pękal and Pyrzynska,[19] and the tannin contents were realized, according to the technique proposed by Tammar *et al*.[20] The tests were carried out in triplicate.

Chromatographic analysis

The components in the rosemary extract were analyzed using high-performance liquid chromatography (HPLC) (Agilent 1260, Agilent Technologies, Germany). The carnosic and rosmarinic acids were identified and quantified using a HPLC system equipped with the DAD detector with a scanning range of 200–400 nm, a reversed phase C18 column of 4.6 × 100 mm and 3.5 µm particle size (Zorbax Eclipse XDB C18) at 25°C. The carnosic acid (≥ 95.0% (HPLC), 91209 Supelco, Germany) and rosmarinic acid (≥ 98% (HPLC), L. R4033 Sigma Aldrich, Germany) were used for the calibration curves. Two phases were used for the molecule elution: the mobile phase (A) methanol HPLC Grade (Sigma-Aldrich) and the mobile phase (B) MilliQ water consisted of 0.1% formic acid. The gradient elution was as follows: 0–5 min, 10–20% A; 5–10 min, 20–30% A; 10–15 min, 30–50% A; 15–20 min, 50–70% A; 20–25 min, 70–90% A; 25–30 min, 90–50% A; 30–35 min. The samples were filtrated through a 0.45 µm membrane and injected (2 µL) with a flow rate of 0.4 mL/min in the mobile phase. Individual components were identified using the standards. The retention time for rosmarinic acid (RA) and carnosic acid (CA) in HPLC was 21.50 min and 29.50 min, respectively (Fig. 1), and the concentration was expressed as mg CA or RA per gram of film (mg RA/g film; mg CA/g film). The CA and RA was elucidated from the calibration curve ranging from 10 to 300 µg/mL (R^2 = 0.999) with a regression equation of yCA = 114.0x + 11.52 and yRA = 7.972x + 9.618, respectively.

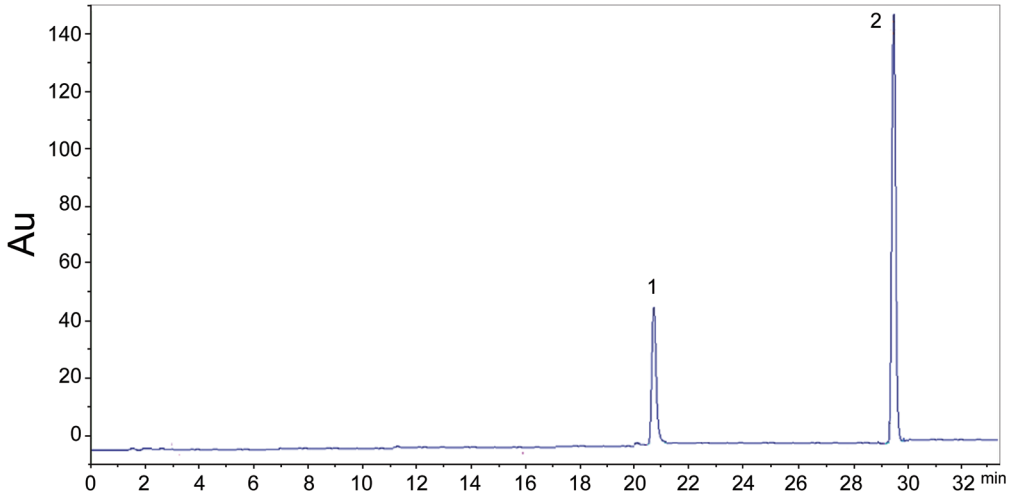

Fig. 1. HPLC chromatogram profile of rosemary leaf ethanol extract monitored at 280 nm: 1: rosmarinic acid, 2: carnosic acid.

Antioxidant activity

The percent inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical of the rosemary extract was evaluated using spectrophotometry.[21] The lipid peroxidation inhibitory activity of each sample was tested for its capacity to inhibit β-carotene bleaching.[22] The ability to reduce ferric ions was measured using the ferric reducing antioxidant power (FRAP) method.[23] Measurement of the trapping capacity of the cationic radical 2,2′-Azinobis-(3-ethylBenzoThiazoline-6-Sulfonic) acid (ABTS) was determined using spectrophotometry.[24] The reducing power of the rosemary leaf extract was evaluated.[25] The tests were carried out in triplicate.

Antibacterial activity

The antibacterial activity of the rosemary extract was tested using the disk diffusion method.[26] The tested Gram-negative bacteria included *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus aerogenes* (ATCC 13048), *Campylobacter jejuni* (ATCC 33560), and *Salmonella enterica* (ATCC 14028), as well as Gram positive *Bacillus subtilis* (ATCC 6051), *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus aureus* (ATCC 29213). The surface of Mueller-Hinton (Merck) agar plates was inoculated in triplicate with 10^5 CFU/mL (0.1 ml) of bacterial suspension of each strain tested and plated with three sterile filter paper discs (6 mm in diameter) that had been treated with 10 µL of the rosemary extract solubilized in dimethyl sulfoxide (DMSO). All plates were incubated for 24 h at 37°C. The diameters of the inhibition zone (mm) were measured after adjusting the initial diameter of the discs. The sterile disc was used as a negative control and the antibiotic streptomycin (10 µL/disc) was used as a positive control.

Toxicity of rosemary leaf extract

According to Ecobichon,[27] acute toxicity by definition is a form of induced toxicity hat results from short-term exposure following rapid absorption of the toxicant by administration of a single or multiple doses not exceeding 24 h. Three groups of C57BL/6 mice (25–29 g, n = 3 per group) were treated orally with 10, 50, or 150 mg/kg body weight twice daily for 15 consecutive days, and a control group of mice received distilled water. Behavior was monitored for potential systemic toxicity and survival was recorded within 2 weeks. Subsequently, the animals were euthanized, and their blood samples were collected for biochemical analysis of serum aspartate amino transferase (AST), alanine amino transferase (ALT), phosphatase alkaline (PAL), blood uric nitrogen (BUN), total protein (TP), glucose (GLU), total bilirubin (T-BIL), creatinine (Crea), and total cholesterol (T-CHO).

Anti-inflammatory activity

Cytotoxicity test

To evaluate the *in vitro* cytotoxicity of the rosemary leaf extract, RAW264.7 macrophages (5×10^4 cells/well) were cultured in 24-well plates overnight and treated with increasing concentrations of rosemary extract for 24 h. Cell viability was measured using the resazurin reduction assay and with an absorbance at 540 nm.[28,29]

Measurement of nitrite production

RAW 264.7 cells (2×10^5 cells/well) were cultured in 24-well plates for 24 h and treated with increasing concentrations of rosemary leaf extract in DMSO for 1 h, followed by stimulation with 100 µg/mL of lipopolysaccharide (LPS) for 24 h. The quantity of nitrite accumulated in the culture supernatant was determined using the Griess reaction assay.[30]

Statistical analysis

The data were statistically analyzed using a one-way ANOVA with the Tukey's post hoc test with the Statistica v. 7.0 program. A statistical significance was defined as a probability level of $p < 0.05$. Construction and the statistical analysis of the experimental design was carried out using NemrodW (LPRAI, version 2000) software.

Table 2. Matrix of experiments carried out according to the two-factor model

Experiments	Extraction temperature (X1 in °C)	Extraction time (X2 in minutes)	Yield of carnosic acid (Y1 in mg/g DW)	Yield of rosmarinic acid (Y2 in mg/g DW)
1	130	60	56.90	5.40
2	180	60	79.40	6.29
3	130	180	80.24	7.51
4	180	180	69.20	5.46
5	130	120	72.54	4.41
6	180	120	78.99	7.46
7	155	60	72.10	3.42
8	155	180	79.02	6.46
9	155	120	85.77	7.96
10	155	120	86.28	7.94
11	155	120	84.59	8.15
12	155	120	83.09	8.47
13	155	120	86.11	8.01

Results and discussion

Extraction optimization of rosemary leaf extract

Design of experiment methodology

To determine the effect of the extraction temperature (X1), the extraction time (X2), and their interactions on the extraction yield of carnosic and rosmarinic acids (Y1 and Y2 respectively), we adopted the experimental plan of the central composite type. The main objective was to optimize experimental conditions to extract carnosic and rosmarinic acids with a good yield. Based on the preliminary study, the domain of each factor had influenced the response. Table 2 illustrates the 13 tests carried out according to the "centered composite plane" model describing the combination between the levels of the factors.

Factor significance

The significance of two factors is given in Table 3. The results indicated that all terms were significant at $p < 0.05$. According to the coefficients of significant factors, the model was written as follows:

$$CA\ yield = 84.440 + 2.98X1 + 3.339X2 - 6.84X1X2 - 7.06X2X2 - 8.385X1X2$$

Table 3. Coefficient significance

Terms	Coefficients	Standard error	t	$p > \|t\|$
Carnosic acid				
β0	84.440	0.809	104.32	***
β1	2.987	0.796	3.75	**
β2	3.339	0.796	4.20	**
β11	−6.847	1.173	−5.84	***
β22	−7.067	1.173	−6.03	***
β12	−8.385	0.975	−8.60	***
Rosmarinic acid				
δ0	8.160	0.063	130.25	***
δ1	0.211	0.062	3.43	*
δ2	0.327	0.062	5.31	**
δ11	−0.588	0.091	−6.47	***
δ22	−1.314	0.091	−14.48	***
δ12	−1.015	0.075	−13.45	***

p: probability, t: Student's T-Test, Pr > |t|W: * significant, ** moderately significant, ***highly significant.

Table 4. Analysis of variance

Source	Sum of squares	DDL	Mean square	F	*p* > F
Carnosic acid					
Regression	833.6082	5	166.7216	43.8744	***
Residus	26.5998	7	3.8000		
Validity	19.4817	3	6.4939	3.649	12.2%
Error	7.1182	4	1.779		
Total	860.2080	12			
R^2	0.969				
R^2A	0.947				
Rosmarinic acid					
Regression	13.6274	5	2.7255	119.714	***
Residus	0.1594	7	0.0228		
Validity	0.1171	3	0.0390	3.6954	12.0%
Error	0.0423	4	0.0106		
Total	13.7867	12			
R^2	0.988				
R^2A	0.980				

p: probability, F: Fisher's F-Test, Pr > F: ***$p < 0.01$, R^2: coefficient of determination, R^2A: coefficient of determination adjusted, DDL: Degrees of freedom.

$$\text{RA yield} = 8.160 + 0.211X1 + 0.327X2 - 0.588X1X1 - 1.31X2X2 - 1.015X1X2$$

Variance analysis (ANOVA)

To validate the model, an ANOVA was performed. Table 4 shows that "F-ratio" regression corresponding to the ratio between the mean square of the regression (833.608 and 13.627) and the residue (26.560 and 0.159) was equal to 43.874 and 119.714 ($F_{Regression}$) for carnosic and rosmarinic acids, respectively. These values were greater than the values tabulated "F-ratio" $_{tabulated}$ (5. 7.0.05) = 3.97 with a *p*-value of <0.05. In addition, "F-ratio" validity corresponding to the ratio between the mean square of the validity (6.494 and 0.039) and the experimental error (7.118 and 0.042) was equal to 3.649 and 3.6954 ($F_{Validity}$) for carnosic acid and rosmarinic acid, respectively, which were less than the tabulated values "F-ratio" $_{tabulated}$ (3.4.0.05) = 6.59. Thus, the validity of the postulated model was confirmed.

Determination of optimal conditions by isoresponse curves

To determine the optimal conditions for extracting carnosic and rosmarinic acids with a high yield, we used the RSM (Fig. 2). The isoresponse curves were the results of the interaction between the two significant factors [extraction temperature (X1) and extraction time (X2)]. For carnosic acid, we found that a temperature between 140 and 165°C and increasing the extraction time from 100 to 160 m considerably increased the extraction yield of carnosic acid , reaching above 86.28 mg/g dry weight (D)W. In this context, the optimum point corresponded to the optimum extraction conditions (the optimum extraction temperature was 155°C for an extraction time of 120 m) allowing an extract rich in carnosic acid with a predicted yield of around 86.28 mg/g DW. For rosmarinic acid, heating during extraction rose from 140 and 165°C and the extraction time increased from 60 to 180 m. The extraction yield of rosmarinic acid considerably increased, reaching 8.37 mg/g DW. In this context, the optimum point corresponded to the optimum extraction conditions (the optimum extraction temperature was 155°C for an extraction time of 120 m) allowing an extract rich in rosmarinic acid with a predicted yield of around 8.37 mg/g DW. The superposition of the two results exhibited a direct correlation between the two contents of carnosic acid and rosmarinic acid.

Biochemical characterization of rosemary leaf extract

Colorimetric phenolic assays

As shown in Table 5, the ethanolic extract of rosemary leaves was rich in phenolic compounds. The colorimetric dosage of total phenolic compounds was about 44.56 mg equivalent gallic acid (EGA)/g DW. The content of flavonoids and tannins was relatively low at 3.20 mg EGA/g DW and 1.61 mg EGA/g, respectively. Rodríguez-Rojo *et al*.[31] found that the ethanolic extract of rosemary leaves had the highest total phenolic concentration at 80 mg EGA/g DW compared to our results. In comparison to ethyl acetate and acetone, Dent *et al*.[32] reported that ethanol and water were the best solvent systems for phenol extraction from rosemary and sage plants. As a result, the increased polarity of the ethanolic solvent may impact the composition of rosemary and sage extracts, which are high in physiologically active chemicals, particularly phenolic compounds. The low content of tannins in our rosmary extract was consistent with previous observations.[33–35] Chromatographic analysis of the ethanolic extract indicated carnosic acid at 101.04 mg/g DW and rosmarinic acid at 10.09 mg/g (Table 5). These contents were similar to those proposed by the mathematical model from the experimental design plan. According to Kheiria *et al*.[36]

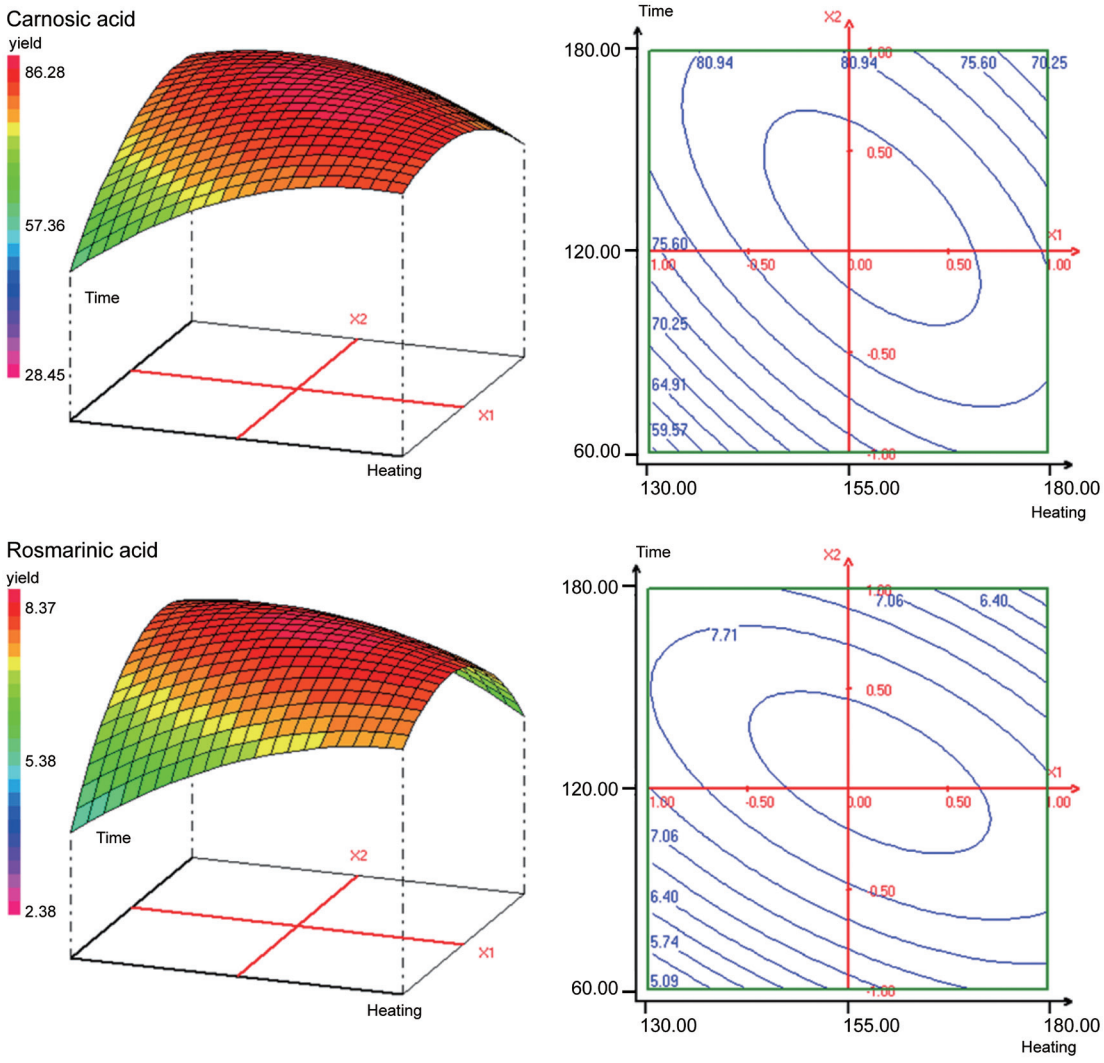

Fig. 2. Estimated response surface for optimization of the extraction of rosemary leaf extract.

carnosic acid and carnosol were the most abundant compounds in all extracts (46.3 to 76.4 mg/g and 22.4 to 43.5 mg/g, respectively).

Antioxidant activities of rosemary leaf extract

The antioxidant activity of the rosemary leaf extract was tested (Table 6). The rosemary leaf extract had stronger anti-radical activity [inhibitory concentration at 50% (IC_{50}) = 9.47 µg/ml] than the positive control Trolox (IC_{50} = 10.08 µg/ml) using the DPPH assay. However, the FRAP value of Trolox (3.86 µmol/g) was higher than the rosemary extract (2.23 µmol/g). Trolox also had the highest antioxidant activity in the ABTS (IC_{50} = 33.56 µg/ml) and β-carotene bleaching (IC_{50} = 3.03 µg/ml) assays compared to the rosemary extract (IC_{50} = 78.26 µg/ml and 50.04 µg/ml, respectively). The antioxidant potential is always associated with a high content of phenolic compounds in this extract, in particular carnosic and rosmarinic acids.[31] These results are in agreement with a previous report showing that carnosic acid was had remarkable anti-radical DPPH activity (IC_{50} = 18.7 µg/mL).[37] Rosmarinic acid was also found to have strong anti-radical activity (IC_{50} = 1.1 µg/mL) in DPPH assaay.[38]

Antibacterial activity

The antibacterial activity of the rosemary leaf extract was also measured. The rosemary extract exhibited a significant antibacterial activity against *C. jejuni* [inhibition zone (IZ) = 18.5 ± 0.21

Table 5. Phenolic contents of rosemary leaf extract

Total phenolic content	(mg GAE/g DW)	44.56 ± 0.29
Total flavonoid content	(mg QE/g DW)	3.20 ± 0.02
Condensed tannin content	(mg CE/g DW)	1.61 ±0.01
Carnosic acid content	(mg/g DW)	101.04 ± 6.25
Rosmarinic acid content	(mg/g DW)	10.09 ± 0.59

The values shown in this table were the mean of three replicates and shown as mean ± standard deviation (SD) (n = 3). Total polyphenol content is expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW). Total flavonoid content is expressed as mg of quercetine equivalents per gram of dry weight (mg QE/g DW). Amount of total condensed tannins is expressed as mg (+)-catechin equivalent per gram of dry weight (mg CE/g DW).

Table 6. Antioxidant activity of rosemary leaf extract

Antioxidant activity	Rosmary extract	Trolox	*p* value
DPPH assay IC_{50} (µg /mL)	9.47 ± 0.06	10.08 ± 0.05	0.003**
ABTS assay IC_{50} (µg /mL)	78.26 ± 0.51	33.56 ± 1.46	0.000***
β-carotene bleaching assay IC_{50} (µg/g)	50.04 ± 0.33	3.03 ± 0.03	0.000***
FRAP activity (µmol/g)	2.23 ± 0.02	3.86 ± 0.09	0.000***

The values shown in this table are the mean of three replicates and shown as mean ± standard deviation (SD) (n = 3); *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, determined by Turkey's test. Trolox: positive control.

mm], *S. enterica* (IZ = 19.50 ± 0.52mm), *B. subtilis* (IZ = 14.83 ± 0.56 mm), *S. aureus* (IZ = 15.83 ± 0.51 mm), *E. faecalis* (IZ = 14.83 ± 0.48 mm), and *E. coli* (IZ = 13.83 ± 0.29 mm). The antibacterial activity was similar to streptomycin (Fig. 3), except that the rosemary extract had antibacterial activity against *P. aeruginosa* (15.33 ± 0.53 mm) and *E. aerogenes* (14.50 ± 0.87 mm) less than that of the positive control (17.00 ± 0.20 mm and 16.33 ± 0.53 mm respectively). Hence, the ethanolic rosemary extract has considerable antibacterial activity and may be useful as a natural food additive to protect the food and prolong its shelf life.

According to Nieto *et al*.[39] and Bernardes *et al*.[40] the inhibitory activity of rosemary extract results from the bioactivity of rosmarinic acid, carnosic acid, and their derivatives, such as carnosol, rosmanol, and isorosmanol. These compounds can interact with the cell membrane of microorganisms by changing bacterial genetic material and nutrients, modifying the transport of electrons, and causing leakage of cellular components to change in the production of fatty acids, deteriorating the cell membrane. In addition, they can interact with the protein membrane, resulting in the loss of functionality and structure of the membrane. Vegara *et al*.[41] reported that the efficacy of carnosic acid against pathogenic bacteria was superior to that of other major extract components, including rosmarinic acid.

Acute toxicity of rosemary leaf extract in mice

The experimental mice were treated with increased doses of rosemary leaf extract twice daily for 15 days by gavage (n = 3), varying from 10 mg on day 1 (D1), 50 mg (D2), and 150 mg/kg BW (D3). The levels of serum AST, ALT, PAL, BUN, TP, Glu, T-BIL, Crea, and T-CHO in individual mice were measured. There were no significant differences between the healthy control and experimental groups ($p > 0.05$). Furthermore, all animals were active and survived without signs of physiological decline or inappropriate behaviors (Table 7). Similarly, during treatment, there were no clinical symptoms of relative abnormality. These findings are consistent with a previous report[42] demonstrating that treatment with 100 mg Tunisian rosemary extract did not change biochemical markers: Crea was 1.99 mmol/L compared to a control of 2.31 mmol/L, and BUN was 0.18 mmol/L compared to a control of 0.17 mmol/L.

Anti-inflammatory activity

The cytotoxicity of the rosemary leaf extract against RAW 267.4 cells was evaluated. RAW 267.4 cells were treated with increas-

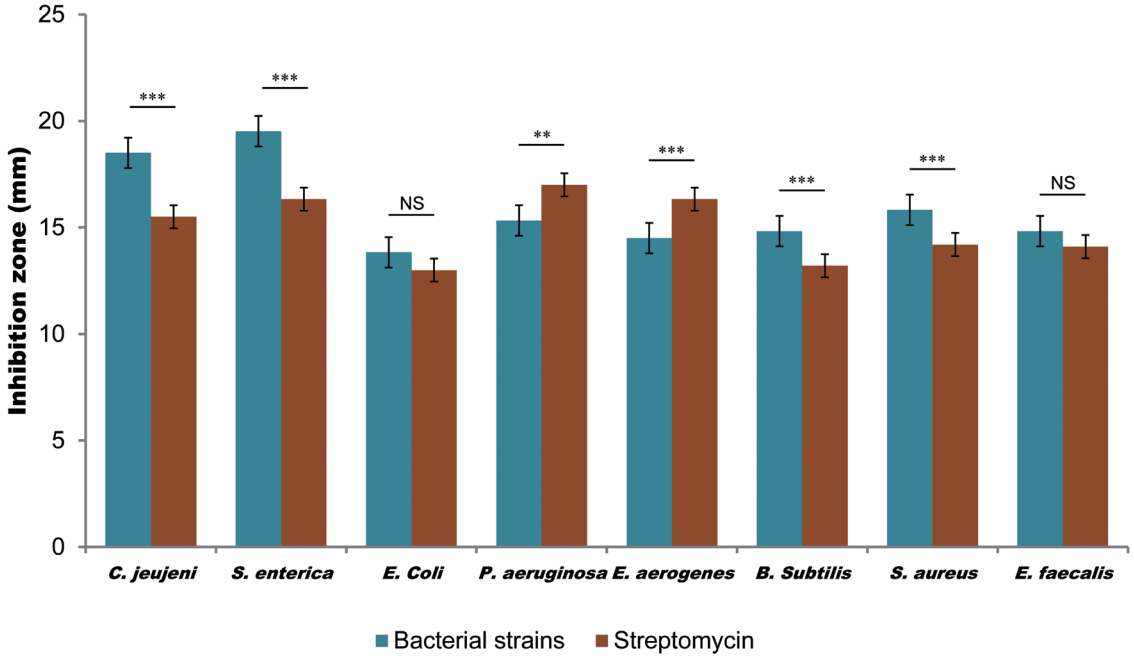

Fig. 3. Antimicrobial activity of *R. officinalis* leaf extract. Data are the mean of three replicates and shown as mean ± standard deviation (SD) (n = 3); NS: not significant at $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined using Turkey's test.

Table 7. Acute toxicity of rosemary leaf extract in mice

	D1	D2	D3	Control	*p* value
Amino transferase (AST)	54.46 ± 0.81	54.35 ± 0.81	54.34 ± 0.81	54.46 ± 0.81	0.498^{NS}
Alanine amino transferase (ALT)	42.92 ± 0.25	42.83 ± 0.25	42.83 ± 0.25	42.92 ± 0.25	0.100^{NS}
Alkaline phosphatase (ALP)	264.9 ± 11.44	264.46 ± 11.23	264.41 ± 11.21	264.9 ± 11.44	0.213^{NS}
Blood uric nitrogen (BUN)	8.70 ± 0.06	8.68 ± 0.06	8.68 ± 0.06	8.70 ± 0.06	0.321^{NS}
Total protein (TP)	65.37 ± 0.55	65.24 ± 0.55	65.23 ± 0.55	65.37 ± 0.55	0.193^{NS}
Glucose (GLU)	5.75 ± 0.02	5.74 ± 0.02	5.74 ± 0.02	5.75 ± 0.02	0.086^{NS}
Total bilirubin (T-BIL)	1.98 ± 0.01	1.98 ± 0.07	1.98 ± 0.07	1.98 ± 0.07	0.875^{NS}
Creatinine (Crea)	59.00 ± 0.033	58.88 ± 0.03	58.87 ± 0.03	59.00 ± 0.03	0.072^{NS}
Total cholesterol (T-CHO)	37.59 ± 0.14	37.52 ± 0.14	37.51 ± 0.14	37.59 ± 0.14	0.100^{NS}

The values shown in this table are the mean of three replicates and shown as mean ± standard deviation (SD) (n = 3). NS: not significant at $p > 0.05$.

ing doses of extract (50–300 µg/mL) (Fig. 4). Rosemary leaf extract at the tested doses did not have any significant cytotoxicity against RAW 267.4 macrophage cells. According to Santos *et al*.[43] rosemary extracts at ≤ 500 µg/mL had no cytotoxicity against rat pancreatic cells. Thus, our experiments were carried out at the non-toxic concentrations (50, 100, 200, and 300 µg/mL) of rosemary extract.

Excessive nitrite (NO) generation can cause inflammation. Next, we determined the effect of rosemary leaf extract on NO• production.[39] The results indicated that rosemary leaf extract decreased NO• release by RAW 267.4 cells in a dose-dependent manner. Treatment with rosemary leaf extract at 50 or 300 µg/mL decreased NO• release by 18.89% and 53.26%, respectively (Fig. 5). These data indicate that rosemary leaf extract has anti-inflammatory activity with an IC_{50} = 255 µg/mL, which may stem from high levels of rosmarinic and carnosic acids.[44] AlKahtane *et al*.[45] found that pre-treatment with carnosic acid significantly reduced Chlorpyrifos-induced increase in serum interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α concentrations in a dose-dependent manner. These anti-inflammatory activities may be due to high levels of carnosic acid that has anti-inflammatory properties both *in vivo* and *in vitro*. Likewise, rosemary leaf extract can reduce phorbol 12-myristate 13-acetate-induced inflammation in rats by downregulating IL-1 and TNF-α mRNA transcript levels in the ear. Furthermore, the extract can inhibit LPS-induced nitric oxide production in RAW 264.7 macrophages *in vitro*.[46] Osakabe *et al*.[47] demonstrated that rosmarinic acid in *Perilla frutescens* extracts inhibited carcinogenesis by two distinct mechanisms: anti-inflammatory (suppression of adhesion molecules, chemokine, and eicosanoid formation) and antioxidative (prevention of oxidative DNA injury) activities.

Future directions

The optimized rosemary extract may be an important alternative for any industrial process. Further analysis is required to evaluate the quality of foods using packaging enriched with Tunisian rosemary extract.

Conclusions

In conclusion, our data indicate that rosemary leaf extract obtained from optimization of experimental design contained high levels of carnosic and rosmarinic acids. Furthermore, rosemary extract had remarkable anti-inflammatory, antioxidant, and antibacterial activities with a very low toxicological profile.

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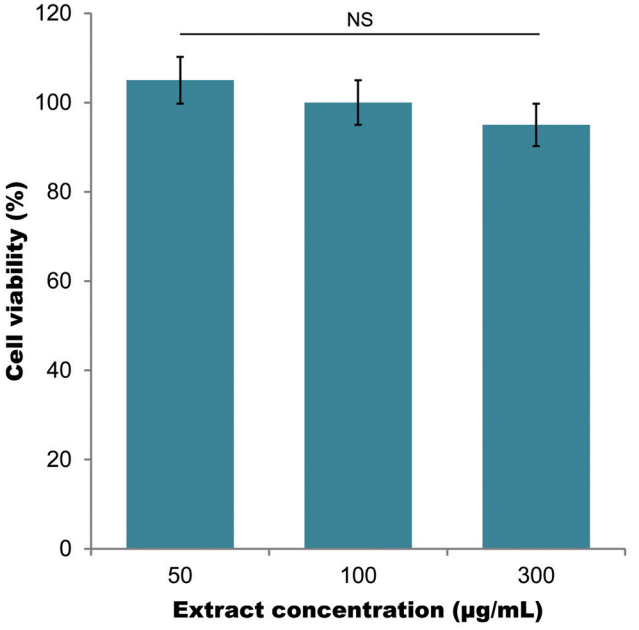

Fig. 4. Cytotoxicity of rosemary leaf ethanol extract against RAW 267.4 macrophage cells. Data are the mean of three replicates and given as mean ± standard deviation (SD) (n = 3); NS: not significant at $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, determined using Turkey's test.

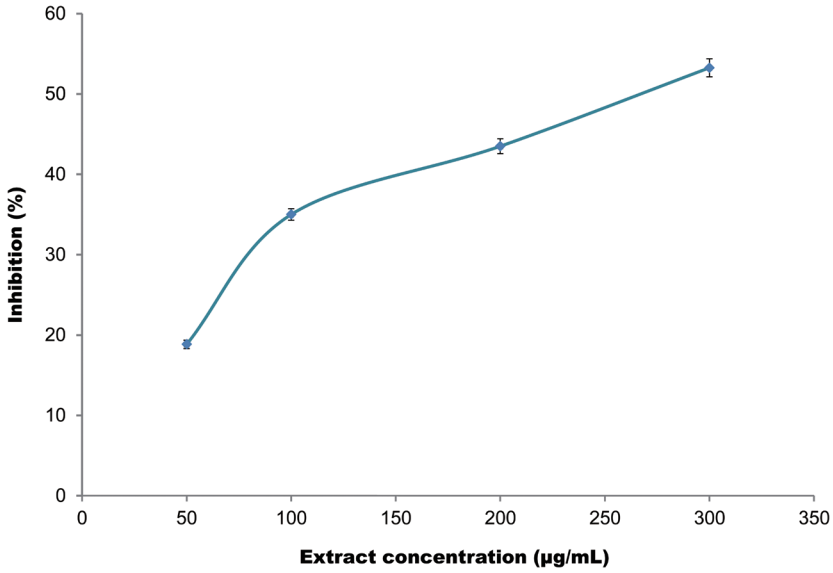

Fig. 5. Anti-inflammatory potential of rosemary leaf extract against RAW 267.4 macrophages.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization, WY; writing, WY, WAW, TGA, HG, SNM; methodology, WY, MH; investigation, WY, MST, HG; formal analysis, WY, MST; data curation, WAW, MN; resources, WY; supervision, MST, MN; writing, review, and editing, MN. All authors have read and agreed to the published version of the manuscript.

Ethical statement

This study was conducted according to the guidelines of the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. All animal protocols were approved by the Consultative Ethics Committee on Animal Experiments at the Pasteur Institute of Tunis (Pasteur Institute of Tunisia).

Data sharing statement

No additional data are available.

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