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Optimizing the Method of Rosemary Essential Oils Extraction by Using Response Surface Methodology (RSM)-Characterization and Toxicological Assessment

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Abstract: Rosemary (*Rosmarinus officinalis* L.) is a plant with needle-shaped leaves. It is mainly found in Mediterranean regions (Algeria, Morocco and Tunisia). Rosemary essential oil (EO) has several therapeutic virtues that were widely studied. However, the use of this EO is restricted due to its sensitivity to oxidation. Nanoencapsulation based on EO and polymers has been developed as one of the promising techniques to overcome this limitation. In this study, the emphasis was on optimizing the extraction and formulation of a food additive based on rosemary EO. In fact, the results showed that rosemary EO extraction depended on the parameters of the extraction process, and the optimum heating temperature and extraction time were determined using an experimental design methodology. The parameters for extraction were chosen as follows: heating temperature of 250 °C and a hydrodistillation time of 180 min. This optimization revealed that the maximum oil yield can be obtained. Rosemary EO was characterized by a dominance of 1,8-cineole, camphor, α-pinene, borneol and camphene as well as by high antioxidant and antibacterial capacities with low acute toxicity. The obtained formulation of a stable rosemary EO powder can be used as a food additive in several industrial applications.

Keywords: rosemary (*Rosmarinus officinalis* L.); essential oil; antioxidant properties; antibacterial properties; toxicology; nanoencapsulation

1. Introduction

Consumer interest in natural food products is on the rise today. To limit the need for synthetic additives, natural compounds derived from aromatic and medicinal plants, herbs and spices are added to foods. Rosemary, *Rosmarinus officinalis* L., is a widely used medicinal herb all over the world. It is without a doubt one of Tunisian's most popular plants, and it exists as a single species with numerous chemotypes. Rosemary is a natural source of several metabolites with a wide range of biological activities. Rosemary has a particular essential oil that gives it its distinctive characteristics. Rosemary essential oil has anti-asthmatic [1], antidepressant [2], antinociceptive and anti-inflammatory properties [3]; and antirheumatic [4], antiseptic [5], antispasmodic [6], bronchodilator, diuretic [7], gastroprokinetics [8], hepatoprotective [9] and antioxidant [10] properties. As a result,

increasing essential oil yields has become a primary concern. In addition, the use of statistical techniques such as the design of experiments makes this improvement increasingly attainable. These methods, which allow for a small number of tests [11], allow the screening of parameters from the most important to the least important, as well as the optimization of operating conditions to achieve the desired outcome.

Several publications have highlighted the use of experimental designs in the optimization of the hydrodistillation process. Some authors have proceeded straight to optimization using response surface designs [12,13], while others have used other types of designs such as full factorial designs [14,15]. This research study is a follow-up to a study that examined the parameters influencing rosemary essential oil yields obtained through hydrodistillation [16]. It was also possible to identify a set of parameters that were thought to have an effect on the reaction in trials of response surface optimization. Response surface designs are the best advised for optimizing operational variables because this is an optimization rather than a research study of the effect of each factor [17]. The improvement of these variables has an impact on the process of rosemary EO extraction. After rosemary EO extraction, the step of enhancing rosemary EO has been well studied. Several researchers have proved the possibility of the direct use of rosemary EO in food formulation [18], other studies have proposed a change of the physical aspect of rosemary EO to widen its field of use and to reduce the risk of degradation of some molecules [19,20].

The nanoencapsulation of the active ingredient is the most popular method for preserving the characteristics of essential oils. The goal of encapsulation is to preserve the stability of bioactive compounds during processing and storage, prevent unwanted interactions and slow down degradation processes (e.g., oxidation or hydrolysis) until the product is released to the desired sites [20,21]. Encapsulation can modify the physical characteristics of the original product to facilitate its carriage, helps separate components of the mixture that otherwise react with each other and provide adequate concentration and uniform dispersion of an active agent [22].

To the best of our knowledge, there was no study focusing on optimizing the parameters for the essential oil extraction from Tunisian rosemary aerial parts using methodology design. Therefore, this study was summarized by looking for the optimal extraction method of rosemary essential oil by using the response surface methodology (RSM). The obtained essential oil was evaluated for its antioxidant, antibacterial, cytotoxicity and anti-inflammatory activities. Finally, rosemary EO was subjected to a nanoencapsulation treatment to facilitate its subsequent use in food applications.

2. Materials and Methods

2.1. Plant Material

Fresh aerial parts of wild rosemary (*Rosmarinus officinalis* var. *typicus* L.) were collected in June 2016 from Zaghouan (Northeastern Tunisia, Altitude 176 m; Latitude: 36°24′10″ North; Longitude: 10°08′34″ East). The botanist Abderrazzak Smaoui of the Biotechnology Center of Borj-Cedria (Tunisia) confirmed the herbarium specimen. A voucher specimen was deposited in the herbarium of our laboratory (RO20160025). Fresh rosemary leaves were left to dry at ambient temperature for eight days until reaching a constant mass of the plant material. Then, they were ground to a fine powder (particle size fraction smaller than 0.5 mm) using a knife mill (Grindomix GM 200, Retsch, Germany).

2.2. Optimization of Essential Oil Extraction by Using Response Surface Methodology (RSM)

The extraction temperature (X1) and the extraction duration (X2) were two experimentally accessible parameters that were optimized by this method. The entire two-level factorial plan of the two parameters selected allowed for a simultaneous change of the two parameters, allowing the collection of the most amounts of data on the system's behavior and the establishment of a mathematical model. [23]. The lower, medium and upper levels of the parameters to be optimized were placed by considering the capacity of the instal-

lation and drawing inspiration from previous studies [13,16,24,25]. The values of the real and coded variables of the two parameters are gathered in Table 1.

Table 1. Parameter levels to optimize the extraction process of rosemary EO: temperature and time.

Independent Variables	Extraction Level Parameters		
	−1 (Minimum)	0 (Medium)	+1 (Maximum)
Extraction temperature in °C (X_1)	190	225	260
Extraction time in minute (X2)	35	137.5	240

The variation in EO yield was expressed by the first-order polynomial function in X1 and X2.

Rosemary EO was extracted in triplicate from 100 g of dried rosemary leaf powder using the hydrodistillation by Clevenger apparatus (Sigma-Aldrich, L'lsle d'Abeau Chesnes, France) in 1500 mL of distilled water for 250 min. The condensed vapor obtained resulted in the essential oil, which was separated from the hydrolate (aromatic waters) by decantation after adding magnesium sulfate ($MgSO_4$, Sigma-Aldrich, Oakville, ON, Canada) to remove traces of water. The essential oils were directly collected without adding any solvent. Yield percentage was calculated as weight (*w*/*w*) of essential oil per 100 g of dry plant matter. The essential oil was stored in opaque bottles (Sigma-Aldrich, L'lsle d'Abeau Chesnes, France) at 4 °C.

2.3. Characterization of Essential Oils by Chromatographic Analysis

2.3.1. GC-FID Quantification Method

The analysis was carried out on a Hewlett-Packard 6890 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an electronic pressure control injector, a flame ionization detector and an HP Innowax (polyethylene glycol capillary, Agilent Technologies, Palo Alto, CA, USA) column (30 m × 0.25 mm; 0.25 µm) [10].

2.3.2. Identification of Volatile Compounds by GC/MS

GC/MS coupling made it possible to identify volatile compounds [14]. The released ions will be classified according to their mass/charge ratio (*m*/*z*). The analysis is carried out by a chromatograph coupled to an Agilent (Agilent Technologies, Palo Alto, CA, USA) mass spectrometer (5975C inert XL MSD) and electron impact ionization (70 eV). An HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) coated with 5% phenyl methyl silicone and 95% dimethylpolysiloxane (Agilent Technologies, Palo Alto, CA, USA) was used.

2.4. Methods for Evaluating the Activity of Essential Oils

Determination of Antioxidant Capacity

The percentage inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical degradation was determined by spectrophotometry according to Hatano et al. [26]. The lipid peroxidation inhibitory activity was performed by the measurement of the inhibitory power of β-carotene bleaching according to the method of Kaur and Kapoor [27]. The total antioxidant capacity was established by the measurement of reducing power by the Ferric Reducing Antioxidant Power assay (FRAP) method according to Benzie and Strain [28]. The measurement of the trapping capacity of the cationic radical $ABTS^{\bullet+}$ (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) was determined by spectrophotometry according to Re et al. [29]. For microcapsules, antioxidant capacity was measured in recovered hexane from the aqueous phase after the rehydration of spray-dried microcapsules [30]. All measurements of DPPH, FRAP, β-carotene bleaching and ABTS assays (Sigma-Aldrich, Oakville, ON, Canada) were carried out in triplicate.

2.5. Antibacterial Activity of Essential Oil

The antibacterial activity of rosemary EO was performed by disk diffusion [31]. The antibacterial activity of rosemary EO has been tested on Gram-negative strains such as *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter aerogenes* (ATCC 13048), *Campylobacter jejuni* (ATCC 33560) and *Salmonella enterica* (ATCC 14028) as well as Gram-positive strains such as *Bacillus subtilis* (ATCC 6051), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213). These bacterial strains were obtained from the microorganism collection of the laboratory of Aromatic and Medicinal Plants, CBBC, Tunisia.

The bacterial suspensions were prepared in NaCl 0.85% (BioMérieux, Marcy-l'Etoile, France) adjusted to a concentration of 0.5 McFarland to yield approximately 10^8 colony-forming units (CFU)/mL, and 100 µL of each suspension was inoculated on Mueller-Hinton (MH) agar. For the *C. jejuni* strain, defibrinated sheep blood 5% (BioMérieux, Marcy-l'Étoile, France) was added to the MH agar. Then, sterile filter paper discs (Sigma-Aldrich, L'lsle d'Abeau Chesnes, France) 6 mm in diameter were placed on the surface of the plate of MH agar previously inoculated and 10 µL of rosemary EO was added to the disks. The *C. jejuni* plate was incubated at 37 °C under microaerophilic conditions (85% nitrogen, 10% carbon dioxide, 5% oxygen) using GENBag microaer (BioMérieux, Marcy-l'Étoile, France). The other bacterial plates were covered and incubated for 24 at 37 °C. The diameter of the inhibition zone (mm) was measured by taking into account 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. All experiments were performed in triplicate.

The minimum inhibitory concentration (MIC) was determined by microdilution technique [32]. The bacterial suspension was prepared using a dilution of the standardized suspension (0.5 McFarland meaning 10^8 CFU/mL) for bacterial strains to yield 5×10^5 CFU/mL. Serial dilutions of rosemary EO in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Oakville, ON, Canada) were prepared to achieve various concentrations (400, 200, 100, 50, 25 and 12.5 mg/mL). In six test tubes, 100 µL of each dilution of rosemary EO, 400 µL of MH broth (BioMérieux, Marcy-l'Etoile, France) and 500 µL bacterial suspension was added, obtaining final inoculums of approximately 0.5×10^5 CFU/mL. For the *C. jejuni* strain, defibrinated sheep blood 5% (BioMérieux, Marcy-l'Étoile, France) was added to MH broth, and it was incubated at 37 °C under microaerophilic conditions (85% nitrogen, 10% carbon dioxide, 5% oxygen) using GENBag microaer (BioMérieux, Marcy-l'Étoile, France). The other bacterial tubes were covered and incubated for 24 h at 37 °C. MIC was defined as the lowest concentration of each substance that was able to inhibit bacterial growth. All experiments were performed in triplicate.

To determine the minimum bactericidal concentration (MBC), 10 µL culture from each tube, which did not show an apparent growth as confirmed by MIC determination, was taken and plated on a Columbia agar supplemented with 5% sheep's blood (BioMérieux, Marcy-l'Étoile, France). The *C. jejuni* plate was incubated at 37 °C under microaerophilic conditions (85% nitrogen, 10% carbon dioxide, 5% oxygen) using GENBag microaer (BioMérieux, Marcy-l'Étoile, France). The other bacterial plates were covered and incubated for 24 h at 37 °C. MBC was defined as the lowest essential oil concentration able to reduce and kill more than 99.9% of the initial inoculums [32]. All experiments were performed in triplicate.

2.6. Acute Toxicity of Rosemary Essential Oil

According to Ecobichon [33], acute toxicity is considered as a form of induced toxicity, which results from short-term exposure following the rapid absorption of the toxic substance by an administration of single or multiple doses not exceeding 24 h. Three groups of C57BL/6 mice (25–29 g, $n = 3$ per group) received different EO doses (10, 50 and 150 mg/kg body weight (BW)) twice daily at the same time every day for 15 days, versus a control group that received oral distilled water. The observations of toxic symptoms and mortality rate were achieved within 2 weeks. After this period, mice were sacrificed,

and the blood was subjected to biochemical analysis by assaying biochemical markers such as aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), blood uric nitrogen (BUN), total protein (TP), glucose (GLU), total bilirubin (T-BIL), creatinine (Crea) and total cholesterol (T-CHO). All experiments were performed in triplicate.

2.7. Microencapsulation Procedure

The nanoencapsulation was carried out according to the method of Hou et al. [34] using a solution of an emulsion of 1% EO with a quantity of 1% of Tween 80 (Sigma-Aldrich, Oakville, ON, Canada). Then, the mixtures were mixed with a high-speed homogenizer (Wilmington, DE, USA) at 10,000 rpm for 5 min. Then, the encapsulating agent (Maltodextrin, Sigma-Aldrich, Oakville, ON, Canada) was added at 5% (*w*/*v*) concentration. The mixture was introduced into the Buchi mini-B-290 spray dryer (Buchi, Flawil, Switzerland) to evaporate water. The inlet and outlet air temperatures were 120 and 52 °C, respectively. A feed flow of the solution to be dried at 2 mL/min, a spray air flow rate of 742 L/h and a pressure drop of 1.35 bar were used. The vacuum cleaner had a gas flow of 25 m^3/h. To maintain the homogeneity of the solution, and the suspensions were gently shaken using magnetic stirring. After spray drying, the powder was collected, weighed and stored in a closed metal bag under a vacuum [35].

2.8. Physicochemical Characterization of the Active Nanoemulsion

According to Yamamoto et al. [36], the particles sizes and zeta potential of the suspension were measured by Dynamic Light Scattering measurements using a zetasizer (Zetasizer Nano-ZS/Malvern Instruments, UK) after proper dilution. The zeta potential, defined as the charge of a particle at the level of the shear plane, is also the measure of the intensity of the electrostatic or electrical repulsion/attraction between particles. This parameter provides an essential method to predict nanoemulsion stability. This analysis is carried out with a Zetasizer Nano-ZS/Malvern Instruments (UK).

2.9. Statistical Analysis

To establish the average values and standard errors, the results were statistically analyzed using the Statistica v. 7.0 program (StatSoft Inc., Oklahoma, USA). A one-way ANOVA was utilized with Tukey's post hoc test and a significance level of $p = 0.05\%$. Significance was defined as a probability level of $p < 0.05\%$. The construction and the statistical analysis of the experimental were designed with the NemrodW (LPRAI, version 2000) software.

3. Results and Discussion

3.1. Optimization of Essential Oil Extraction by the Response Surface Methodology (RSM)

3.1.1. Design of Experiment Methodology

To determine the effect of the extraction temperature (X1) and time (X2) as well as their interaction on the extraction EO yield (Y1), the response surface methodology (RSM) by adopting the plan of the central composite type was applied. In addition, the main objective resided in the determination of the optimal conditions for EO extraction with a good yield. Based on the preliminary study, it was determined that the domain of each factor influenced the response (Y1: EO extraction efficiency) (Table 1).

Table 2 illustrates the 13 tests carried out according to the "central composite plane" model describing the combination between the factor levels. It was used extraction temperatures ranging from 200 to 250 °C, extraction time from 60 to 180 min and EO yield from 1.2 to 2.70%.

Table 2. Matrix of experiments carried out according to the two-factor model (central composite plane).

Experiments	Extraction Temperature (X1 in °C)	Extraction Time (X2 in Minutes)	Yield of EO (Y1 in %)
1	200.00	60.00	1.20
2	250.00	60.00	1.20
3	200.00	180.00	1.60
4	250.00	180.00	2.70
5	200.00	120.00	1.70
6	250.00	120.00	2.50
7	225.00	60.00	1.00
8	225.00	180.00	2.47
9	225.00	120.00	1.88
10	225.00	120.00	2.01
11	225.00	120.00	1.75
12	225.00	120.00	1.85
13	225.00	120.00	1.99

3.1.2. Factor Signification

The significance of the two factors is provided in Table 3. Results showed that the extraction temperature (X1) was expressed by the term β_1 with a significant probability with a positive coefficient (0.317). Similarly, the extraction time (X2) was expressed by the term β_2 with significant probability and positive coefficient (0.562). These positive coefficients clearly showed that efficiency depended on the heating temperature and the extraction time. The interaction between these two factors was marked by the term β_{12} possessing a positive coefficient (0.275). All those values were significant at $p < 5\%$.

Taking into account only the coefficients with significant factors, the model is written as follows.

$$\text{EO yield} = 1.932 + 0.317 \times 1 + 0.562 \times \text{X2} - 0.288 \times \text{X2} \times \text{X2} + 0.275 \times \text{X1} \times \text{X2}$$

Table 3. Coefficient significance.

Terms	Coefficients	Standard Error	T	Pr > |t|
β_0	1.932	0.071	27.22	***
β_1	0.317	0.070	4.54	**
β_2	0.562	0.070	8.05	***
β_{11}	0.077	0.103	0.74	48.6%
β_{22}	−0.288	0.103	−2.80	*
β_{12}	0.275	0.085	3.22	*

β_0, β_1, β_2, β_{11}, β_{22} and β_{12} represent the coefficients of the quadratic equation proposed by the mathematical model; T: *t*-test; Pr > |t| probability value or observed significance level. *,**,*** represent the significance levels at 95; 99; 99.5%, respectively.

3.1.3. Variance Analysis (ANOVA)

To validate the model, the analysis of variance was performed. The examination of Table 4 shows that "F-ratio" regression corresponded to the ratio between the mean square of the regression (0.606) and the residue (0.029) equal to 20.74 (F Regression), which was greater than the value-tabulated "F-ratio" tabulated (5. 7.0.05) = 3.97 < 20.740 with a probability that was less than 5%. Thus, the factor coefficients of the postulated model were significant. In addition, "F-ratio" validity corresponds to the ratio between the mean square of the validity (0.053) and the experimental error (0.011). It was equal to 4.658 (F Validity), which was less than the tabulated value "F-ratio" (3.4.0.05) = 6.59 > 4.658; ($p > 5\%$). This confirmed the validity of the postulated model.

Table 4. Analysis of variance.

Source of Variation	Sum of Squares	DDL	Average of Squares	F	Pr > F
Regression	3.030	5	0.606	20.740	***
Residue	0.204	7	0.029		
Validity	0.159	3	0.053	4.658	8.7%
Error	0.045	4	0.011		
Total	3.235	12			
R^2 = 0.937					
R^2A = 0.892					

DDL: degree of freedom; F: fisher test; Pr > F: probability value or observed significance level; R^2: determination coefficient; R^2A: adjusted regression. *** represents the significance level at 99.5%.

3.1.4. Determination of the Optimal Conditions by Iso-Response Curves

To determine the optimal conditions for extracting EOs with a good yield, the methodology of response surfaces was used. Figure 1 shows the iso-reponse curves resulting from the interaction between the two significant factors (extraction temperature (X1) and extraction time (X2)). It was found that at 225 °C and by increasing extraction times, the extraction yield of rosemary EO considerably increased, and it can reach 2 g/100 g dry weight (DW). In this context, the optimal extraction temperature was 250 °C for an extraction time of 180 min. allowing an obtained extract to be rich in EOs with a predicted yield of around 2.87 g/100 g DW.

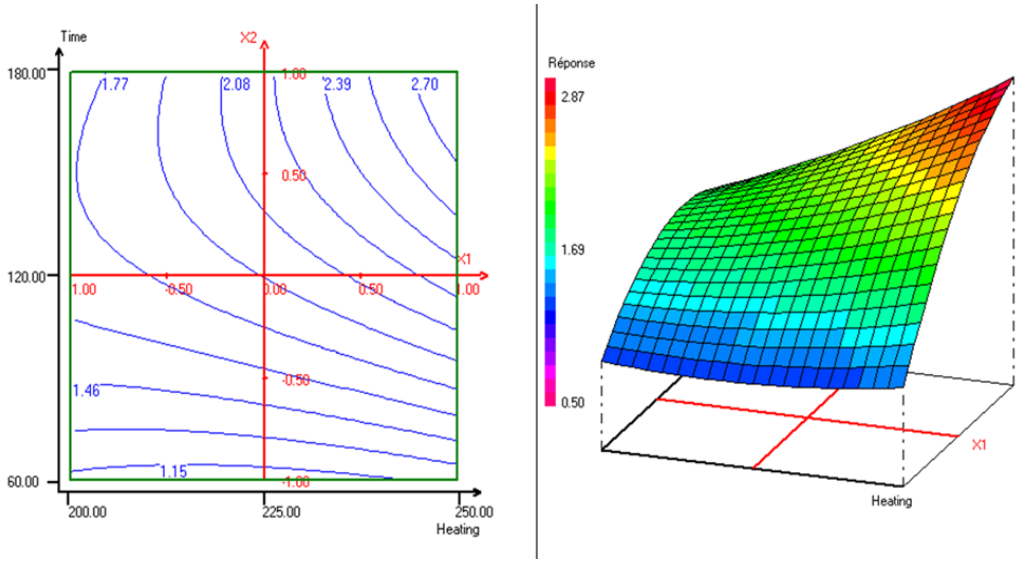

Figure 1. Estimated response surface for optimization of the extraction of rosemary essential oil by hydrodistillation.

3.1.5. Selected Extraction Parameters and Their Effects on Rosemary Essential Oil Yield

After an appropriate choice of two variables, 13 experiments resulted in a second-degree mathematical model linking the response (yield of EOs (Y1)) to the factors and allowing good control of the extraction process. After performing the tests and analyzing the data, the study determined the optimal conditions necessary for obtaining a better yield of rosemary EO. These conditions were a heating temperature of 250 °C and a time of 180 min. According to the observed results given by using the composite centred experiment design, it was shown that the ideal yield of rosemary EO obtained was between 2.24 and 2.41 g/100 g DW. To confirm the predicted responses proposed by the experiment's design model, several experiments of EO extraction were performed by the optimization of the following parameters, as indicated in Table 5. Results showed that the predicted yield

of rosemary EO was between 2.70 and 2.78 g/100 g DW. This latter was similar to that of the experimental yield with 2.85 ± 0.02 g/100 g DW. In fact, it can be mentioned that the experimental design model was valid, and the optimal extraction conditions were reached. Similar results were obtained by Fadil et al. [16] who optimized the EO extraction by a design of experiment model and achieved a reliable rosemary EO yield (2.3 ± 0.05 g/100 g DW).

Table 5. Influence of the parameters optimized by design of the experiment on EO yield.

Parameters	Fixed	Optimized	Predicted Yield (g/100 g DW)	Experimental Yield (g/100 g DW)
Leaf drying time	+			
Extraction temperature		+	2.70–2.78	2.85 ± 0.02
Extraction time		+		

3.2. Characterization of Rosemary Essential Oils by Chromatographic Analysis

The EO yield of rosemary leaves was 2.85% based on dry weight. On the basis of their mass spectra features and retention indices, 33 volatile components were identified (Table 6).

Table 6. Chemical composition of Tunisian rosemary essential oil.

No.	Volatiles Compounds	RI[a]	RI[b]	Free EO (%)	Recovered EO (%)
1	Tricyclene	919	923	0.49 ± 0.08 a	ND
2	α-Thujene	929	931	0.08 ± 0.01 a	ND
3	α-Pinene	934	938	12.34 ± 1.83 a	7.17 ± 0.83 b
4	β-Pinene	981	983	0.66 ± 0.1 b	0.76 ± 0.1 a
5	Camphene	952	953	11.63 ± 2.03 a	8.66 ± 0.03 b
6	β-Myrcene	988	991	0.3 ± 0.06 a	0.36 ± 0.06 a
8	α-Terpinene	1018	1084	0.12 ± 0.04 a	0.16 ± 0.04 a
7	α-Phellandrene	1024	1026	0.04 ± 0.01 a	ND
9	p-Cymene	1026	1024	3.04 ± 0.33 a	1.33 ± 0.33 b
10	1,8-Cineole	1033	1029	25.26 ± 7.71 b	29.15 ± 7.71 a
11	γ-Terpinene	1059	1058	0.21 ± 0.05 a	0.26 ± 0.05 a
12	α-Terpinolene	1084	1084	0.1 ± 0.05 a	0.15 ± 0.05 a
13	trans-sabinene hydrate	1089	1070	0.04 ± 0.01 a	ND
14	Linalol	1098	1089	0.1 ± 0.03 a	0.13 ± 0.03 a
15	D-Fenchyl alcoho	1112	1110	0.11 ± 0.02 a	0.13 ± 0.02 a
16	α-Campholenal	1127	1130	0.14 ± 0.02 a	0.16 ± 0.02 a
17	Borneol	1165	1169	5.05 ± 0.538 b	6.59 ± 0.538 a
18	Terpinene-4-ol	1178	1179	0.86 ± 0.19 b	1.05 ± 0.19 a
19	α-Terpineol	1185	1189	1.99 ± 0.36 b	2.35 ± 0.36 a
20	Camphor	1145	1143	29.46 ± 4.92 b	31.92 ± 4.92 a
21	Bornyl acetate	1285	1291	1.76 ± 0.37 b	2.13 ± 0.37 a
22	α-Copaene	1395	1391	0.21 ± 0.04 a	0.25 ± 0.04 a
23	Methyl-eugenol	1401	1405	0.25 ± 0.04 a	0.29 ± 0.04 a
24	α-Humulene	1440	1440	0.47 ± 0.06	0.53 ± 0.06
25	Aromandendrene	1440	1439	0.22 ± 0.03 a	0.25 ± 0.03 a
26	trans-Caryophyllene	1446	1454	1.13 ± 0.15 a	1.28 ± 0.15 a
27	α-Amorphene	1475	1478	0.09 ± 0.01 a	ND
28	α-Muurolene	1489	1487	0.11 ± 0.03 a	0.14 ± 0.03 a
29	γ-Muurolene	1502	1997	0.24 ± 0.05 a	0.29 ± 0.05 a
30	δ -Cadinene	1512	1513	0.49 ± 0.08 a	0.57 ± 0.08 a

Table 6. *Cont.*

No.	Volatiles Compounds	RI[a]	RI[b]	Free EO (%)	Recovered EO (%)
31	γ-Cadinene	1532	1530	0.24 ± 0.03 a	0.27 ± 0.03 a
32	Caryophyllene oxyde	1578	1582	0.09 ± 0.01 a	ND
33	α-Eudesmol	1652	1652	1.7 ± 0.21 a	1.91 ± 0.21 a

Elution order with nonpolar column (HP-5MS). RI[a]: retention index calculated on HP5-MS; RI[b]: retention index according to the literature. ND: non detected. The values shown in the table are given as mean ± SD (n = 3). One-way ANOVA followed by Duncan's multiple range test was used. Values with the same letters in rows did not show significant differences at $p < 0.05$.

The main components of rosemary EO were 1,8-cineole (25.26 ± 7.71%), camphor (29.46 ± 4.92%), α-pinene (12.34 ± 1.83%), camphene (11.63 ± 2.03%) and borneol (5.05 ± 0.538%). Figure 2 shows the chemical structures of some of the main components.

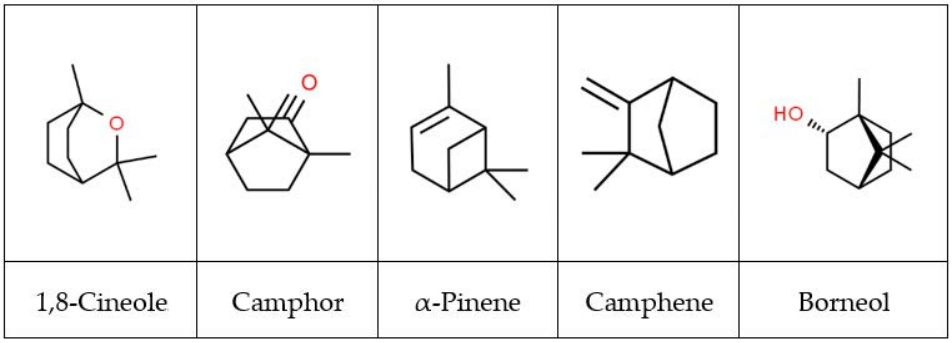

Figure 2. Chemical structures of the main compounds of rosemary essential oils.

The present research revealed that 1,8-cineole and camphor chemotype occurred in Tunisian rosemary. The chemotype of 1,8-cineole was comparable to that previously described for Tunisian rosemary [37–39]. The heterogeneity of the qualitative and quantitative composition of rosemary EO was attributed to both intrinsic (genetics and phonological stages) and extrinsic (climate, growing circumstances, extraction processes, etc.) factors [39]. The chromatographic analysis of rosemary EO extracted from nanocapsules showed that it retained the same proportions of the majority compounds. These results showed that rosemary EO encapsulation retained the biological activities of rosemary EO. Similar results were proven by Hadian et al. [40], Karadağ et al. [41] and Silva-Flores et al. [42].

3.3. Antioxidant Activity of Rosemary Essential Oil

The antioxidant activity of rosemary essential oil was evaluated using three methods, namely DPPH, FRAP and β-carotene bleaching assays (Table 7). Although the three assays produced quantitatively diverse antioxidant activity results, rosemary EO possessed a significant antioxidant activity. The IC_{50} values of DPPH were in the range of 3.87 ± 0.21 µg/mL for free EO and 4.21 ± 0.54 µg/mL for recovered EO. The total antioxidant capacity of Eos was assessed by the FRAP assay. The values did not significantly differ between free (IC_{50} = 3.88 ± 0.02 µg/mL) and recovered EO values (IC_{50} = 4.11 ± 0.02 µg/mL). The results of β-carotene bleaching activity of rosemary free (IC_{50} = 20.80 ± 1.22 µg/mL) and recovered EO (IC_{50} = 23.02 ± 1.45 µg/mL) were compatible with those of DPPH. This is in accordance with previous studies showing the potential of the combined emulsification and spray drying techniques to encapsulate rosemary leaf extract [30]. Similar results were obtained concerning the scavenging activity of rosemary EO [43–45].

Table 7. Antioxidant activity of rosemary essential oil.

Antioxidant Activity	Free EO	Recovered EO
DPPH scavenging activity (IC_{50}: µg/mL)	3.87 ± 0.21 a	4.21 ± 0.54 a
FRAP activity (FRAP IC_{50}: µg/mL)	3.88 ± 0.02 a	4.11± 0.02 a
β-Carotene bleaching activity (IC_{50}: µg/mL)	20.80 ± 1.22 a	23.02± 1.45 a

The values shown in this table were the mean of three replicates and given as mean ± SD (n = 3). One-way ANOVA followed by Duncan's multiple range test was used. Values with the same letters in rows did not show significant differences at $p < 0.05$.

3.4. Antibacterial Activity of Rosemary Essential Oil

The evaluation of the antimicrobial activity showed that rosemary EO and its main constituents (α-pinene, camphene, 1,8-cineole, borneol and camphor) possessed significant antibacterial power (Figure 3).

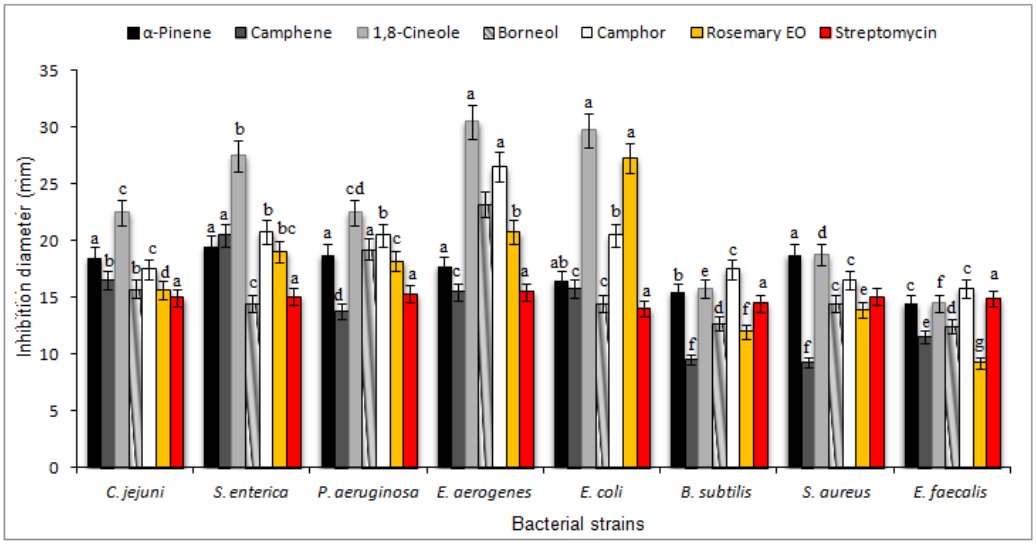

Figure 3. Inhibition diameters of rosemary essential oil and its main constituents against bacterial strains. One-way ANOVA followed by Duncan's multiple range test was used. Values with different letters (a–g) for each histogram color showed significant differences at $p < 0.05$.

Indeed, rosemary EO provoked an inhibition of the growth of Gram-negative bacteria *C. jejuni*, *S. enterica*, *P. aeruginosa*, *E. aerogenes* and *E. coli* with inhibition diameters (ID) of the order of 15.65 ± 0.36 mm; 19.05 ± 0.24 mm; 18.22 ± 0.28 mm; 20.75 ± 0.18 mm; and 27.30 ± 0.56 mm, respectively. These diameters are greater than those of the positive control streptomycin (ID = 14 ± 0.77–15.50 ± 0.75 mm). In contrast, the IDs of Gram-positive bacteria *B. subtilis* (ID = 11.99 ± 0.48 mm), *S. aureus* (ID = 13.88 ± 0.68 mm) and *E. faecalis* (ID = 9.23 ± 0.58 mm) were lower than those of streptomycin (ID = 14 ± 0.77–15.50 ± 0.75 mm). In fact, EOs act to inhibit the growth of bacterial cells and also inhibit the production of toxic bacterial metabolites. The results showed that rosemary EO had a more powerful effect on Gram-negative bacteria than Gram-positive bacteria. In fact, this effect could be due to differences in cell membrane compositions. According to Salamon et al. [46], Egyptian rosemary EO exhibited lower antibacterial activity than Tunisian EO for *E. coli* (ID = 8.50 ± 0.29 mm). Additionally, Bosnić et al. [47] reported that Bosnian rosemary EO has significant antibacterial activities against *E. coli* (ID = 13 mm) and *P. aeruginosa* (ID = 9 mm). Recent studies had shown that this antibacterial activity was mainly due to the presence of high contents of 1.8 cineole and camphor [48,49]. Generally, it is believed that the antibacterial efficacy of an essential oil is not entirely associated with a specific constituent but rather a synergistic effect of all of the constituents contained [50].

In our study, the ratio of MBC/MIC was also determined to examine the antibacterial activity of rosemary EO and its main constituents. The efficacy of an antibacterial agent is dependent on MIC and MBC values and their ratios [51]. An antibacterial agent is regarded as bactericidal if the MBC value is not more than four times the MIC value [52]. From Table 8, it can be observed that rosemary EO and its main constituents possessed MBC/MIC values inferior or equal to 2. These results revealed that rosemary EO and its main constituents had a strong bactericidal effect comparable to streptomycin. Regarding MBC/MIC values, rosemary essential oil had lower antibacterial activity compared to its main compounds. Similar results were obtained by Bernardes et al. [53], who determined the antibacterial activity of rosemary EO and its major components against oral pathogen (*E. faecalis*, *S. salivarius*, *S. mitis*, *S. mutans*, *S. sobrinus* and *S. sanguinis*).

Table 8. Antibacterial activity of rosemary essential oil and its main compounds tested by microdilution method (MIC and MBC in mg/mL).

	α-Pinene	**Camphene**	**1,8-Cineole**	**Borneol**	**Camphor**	**Rosemary EO**	**Streptomycin**
Bacterial Strains	**MBC/MIC**	**MBC/MIC**	**MBC/MIC**	**MBC/MIC**	**MBC/MIC**	**MBC/MIC**	**MBC/MIC**
Gram-negative bacteria							
C. jejuni	1.20	1.40	1.16	1.14	1.25	2	1.5
S. enterica	1.16	1.50	1.25	1.25	1.33	2	2
P. aeruginosa	1.25	1.33	1.14	1.12	1.25	2	2
E. aerogenes	1.14	1.40	1	1.20	1.33	2	2
E. coli	1.25	1.28	1.11	1.33	1.20	2	1.25
Gram-positive bacteria							
B. subtilis	1.14	1.11	1.16	1.14	1.16	1.11	1.66
S. aureus	1.25	1.12	1.20	1.16	1.16	2	1.33
E. faecalis	1.12	1.20	1.14	1.12	1.25	1	1.50

MIC: minimum inhibitory concentration. MBC: minimum bactericidal concentration.

3.5. Acute Toxicity of Rosemary Essential Oil in Mice

The acute toxicity was checked when the EO was administered twice/day at the same time every day for 15 days to batches (n = 3) of mice by gavage at three doses/day (10mg (D1), 50 mg (D2) and 150 mg/kg BW (D3)). Table 9 shows the blood analysis for the determination of biochemical markers as aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), blood uric nitrogen (BUN), total protein (TP), glucose (GLU), total bilirubin (T-BIL), creatinine (Crea) and total cholesterol (T-CHO). The analysis of the acute toxicity results showed that rosemary EO had no toxicity in the subjects treated for 15 days. There were no significant differences between the different groups compared to the control group ($p < 0.005$). In addition, no evidence of physiological deterioration or abnormal behaviour was reported for animals. Additionally, no symptoms occurred, and no signs of death were reported during treatment. Indeed, these results were in agreement with Hamed et al. [54], who found that the administration of 100 mg of Tunisian rosemary extract did not affect biochemical markers such as Crea (1.99 ± 0.3 against a control 2.31 ± 0.4 mmol/L) and BUN (0.18 ± 0.05 against a control 0.17 ± 0.03 mmol/L). These authors also reported that the oral administration of rosemary EO (500 mg/kg) showed no signs of toxicity and no mortality in any of the mice treated for 15 days during the observation period. Therefore, the oral lethality 50 (LD50) dose of rosemary EO was greater than that of 150 mg/kg.

Table 9. Acute toxicity of essential oil in mice.

Biochemical Markers	D1	D2	D3	Control
Amino transferase (U/L)	53.96 ± 0.81 a	54.15 ± 0.81a	54.23 ± 0.81 a	53.99 ± 0.81 a
Alanine amino transferase (U/L)	42.84 ± 0.25 a	42.75 ± 0.25 a	42.74 ± 0.25 a	42.60 ± 0.25 a
Alkaline phosphatase (U/L)	264.48 ± 11.24 a	263.95 ± 11.01 a	263.89 ± 19.99 a	263.98 ± 11.13 a
Blood uric nitrogen (U/L)	8.68 ± 0.06 a	8.66 ± 0.06 a	8.66 ± 0.06 a	8.595 ± 0.064 a
Total protein (mg/dL)	65.24 ± 0.55 a	64.91 ± 0.55 a	65.10 ± 0.55 a	64.99 ± 0.55 a
Glucose (mg/dL)	5.69 ± 0.02 a	5.73 ± 0.02 a	5.72 ± 0.02 a	5.70 ± 0.02 a
Total bilirubin (mg/dL)	1.97 ± 0.01 a	1.96 ± 0.007 a	1.95 ± 0.01 a	1.98 ± 0.01 a
Creatinine (mg/dL)	58.79 ± 0.03 a	58.77 ± 0.03 a	57.99 ± 0.03 a	58.86 ± 0.03 a
Total cholesterol (mg/dL)	37.52 ± 0.14 a	36.54 ± 0.14 a	37.44 ± 0.14 a	37.47 ± 0.14 a

The values shown in the table are given as mean ± SD (n = 3). One-way ANOVA followed by Duncan's multiple range test was used. Values with the same letters in rows do not show significant differences at $p < 0.05$. D1, D2 and D3 represent the three doses/day (ranging from 10mg, 50 mg and 150 mg/kg BW, respectively).

3.6. Physicochemical Characterization of the Active Nanoemulsion

Using the polysaccharide's natural shell material as maltodextrin and Tween 20, very small sized encapsulated oil particles of the order of 256.56 nm were observed. Furthermore, the data obtained from the emulsion stability diagram revealed that using maltodextrin as the coating support and Tween 20 as the surfactant allowed for the creation of an emulsion with a zeta potential of the order of −37.31 mV (Figure 4). Likewise, carvacrol encapsulated with chitosan revealed an average diameter particle size ranging between 532.5 and 716.6 nm [55]. Esmaeili and Asgari [56] had used essential oils to prepare the ionic gelation process and found that nanoparticles had an average diameter of 236–721 nm. Particle size and zeta potential were the key conditions to have a successful drug delivery [57].

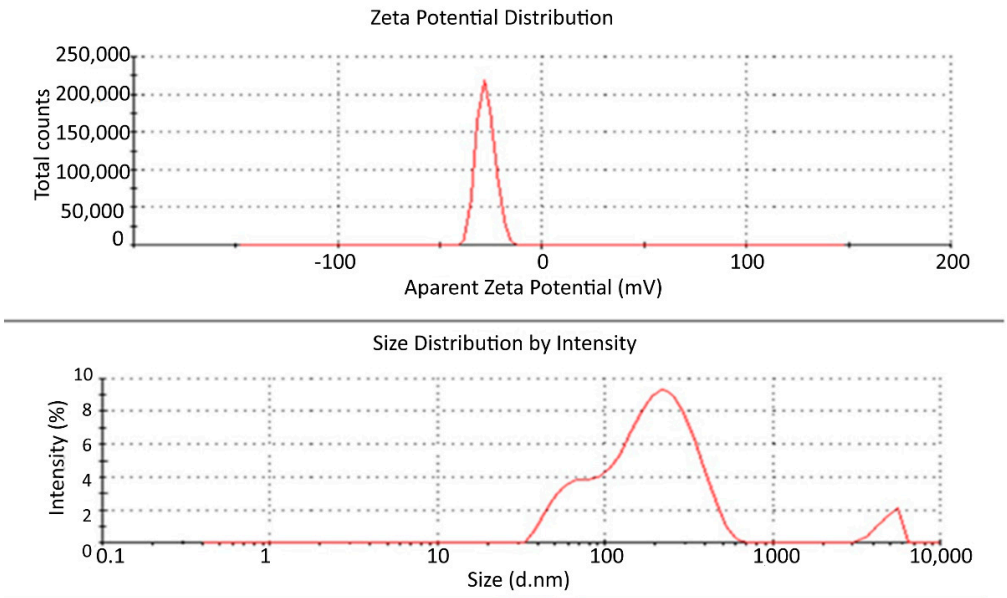

Figure 4. Particle size and zeta potential of the rosemary essential oil emulsified by Tween 20 and maltodextrin.

4. Conclusions

Extraction optimization was conducted by using the response surface methodology (RSM). It showed that the obtained rosemary EO exhibited good antioxidant and antibacterial activities with a very low toxicological profile. The antimicrobial activity and the acute toxicity of the optimized EO were evaluated before the encapsulation process, and it was demonstrated that the EO was characterized by a considerable antibacterial property against Gram-negative bacteria *C. jejuni*, *S. enterica*, *P. aeruginosa*, *E. aerogenes* and *E.*

coli. Likewise, rosemary EO had low-level toxicity, which was proved by the biochemical markers to evaluate the renal and hepatic balance. The antioxidant activity of rosemary EO was evaluated before and after the encapsulation process, and it was found that the encapsulation technique using the spray drying process can preserve the antioxidant potential of the encapsulated EO. This considerable antioxidant and antimicrobial activity of the rosemary EO can be related to the conservation of high contents of 1.8-cineole and camphor in rosemary essential oils. According to the physicochemical analysis of the nanoemulsion, it has been found that the use of maltodextrin as the coating support and Tween 20 as a surfactant allowed an improvement in stability with -37.31 mV as the potential zeta and 256.56 nm as the particle size. Finally, these results clearly show that this formula can be an important alternative for any industrial process using this particular rosemary EO.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request.

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

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