

Optimization of hydrodistillation extraction using response surface methodology and Chemical composition of essential oil from Moroccan endemic medicinal plant *Ballota hirsuta*

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Abstract

The present study describes for the first time the optimization of the extraction by hydrodistillation and chemical composition of the essential oil (EO) of *Ballota hirsuta* aerial parts using Gas chromatography (GC/FID) and GC-mass spectrometry (GC/MS) analysis. Single-factor experiments and response surface methodology (RSM) based on a central composite design (CCD) were used to optimize the EO extraction parameters including drying period (0–21 days), extraction time (60–240 min) and water/plant ratio (5–20 ml/g). The optimum conditions for obtaining the best yield (0.91%) are a drying period of 7 days, an extraction time of 120 min and a water/plant ratio of 10 ml/g. Under these optimum condition, GC/FID and GC/MS enabled the identification of forty-three volatile components representing 82.3% of the total of EO. Germacrene-D (16%), Z-Phytol (7.7%), α -Cadinol (7.3%) and Carvacrol (6.7%) were the main constituents of this EO.

1. Introduction

Essential oils (EOs) are colorless aromatic liquids accumulated and produced in specialized secretory structures localized in the intercellular spaces of plant organs, especially flowers, leaves and seeds (Butnariu and Sarac 2018). They are volatile metabolites through which plants exhibit important ecological functions related to interactions within and between species, such as resistance to pathogens and pests and attraction to pollinators (Isah 2019; Liu and Brettell 2019). Chemically, EOs are a mixture of phytochemical components, including terpenic hydrocarbons and their oxygenated terpenoid derivatives, which may produce characteristic odors (Moghaddam and Mehdizadeh 2017). There are several well-known methods of extracting EOs, but the most common is hydrodistillation (HD) due to its simplicity of implementation and low cost; however, many factors influence the extraction yield which requires optimization of the process parameters to maximize the extraction of essential oils (Quyen et al. 2019). Indeed, to explore the optimization of the extraction parameters, two techniques namely the classical single factor experiments and the response surface methodology (RSM) have generally been used (Baş and Boyacı 2007; Montgomery 2009; Wang et al. 2014; Belhachat et al. 2018). The classical method is based on the selection of different ranges of investigated parameters by studying the effect of a single factor on the total yield while keeping the other variables constant, while RSM can be used to study the effects of several factors and their interactions with the extraction yield by predicting the optimal experimental conditions (Baş and Boyacı 2007; Montgomery 2009; Wang et al. 2014; Belhachat et al. 2018).

Botanically, *Ballota hirsuta* Benth., is a perennial subshrub characterized by hairy and woody stems 24 to 60 cm tall (Dadach and Mehdadi 2018). The leaves are opposite, oval or rounded, dentate-crenate with strong ribs; the upper leaves are sessile while the lower ones are heart-shaped or truncated at the base. The flowers are pink to purple in dense glomeruli in the leaf axils and flowering begins in May and continues until July-August. The calyx with a foliose blade is widely reticulated multifidus with 5 main teeth and a pink corolla with a bifid upper lip (Kechar et al. 2016). *B. hirsuta* is mainly distributed in most areas of the Mediterranean regions and Eurasia regions. In Morocco, this species, known locally under the

name of “Merouit” as *Marrubium vulgare*, is an endemic and spontaneous plant that is usually found in semiarid bioclimatic zones. It often grows in low and medium mountains and on limestone and siliceous substrates. In folk medicine, the leaves are the part of the plant most used for treating general health, gastrointestinal, gynecological and pediatric problems (Teixidor-Toneu et al. 2016). In Algeria, these leaves are used against bruises, injuries and rheumatic pain (Kechar et al. 2016; Bouafia et al. 2021). The phytochemical screening of various organs of *B. hirsuta* showed the existence of flavonoids, polyphenols and tannins which are thought to be responsible for its biological activities (Ferrerres et al. 1986; Kechar et al. 2015; Kechar and Hellal 2017, Kechar and Hellal 2021). However, no previous studies on the chemical composition of EO for this species have been addressed. Therefore, the present study was made to i) determine the optimal conditions leading to the highest extraction yield of *B. hirsuta* EO, using a single factor test as well as a response surface methodology (RSM) with Central Composite Design (CCD), ii) characterize its chemical composition under optimum conditions using Gas chromatography (GC) and GC-mass spectrometry (GC/MS) analysis.

2. Experimental Section

2.1. Plant material and extraction procedure

Aerial parts of *B. hirsuta* were collected wildly from Tizi n'talghamt mountain (1907 m: 32°37'48" N and 4°31'45" W) located in Errachidia region, Morocco. The herbarium sample (Voucher no°: CIM HERB # 141) was deposited at Faculty of Sciences and Techniques, Errachidia, Morocco. These harvested plants were fractionated and divided into two parts; one part was left fresh while the other was subjected to drying in the shade at room temperature in the laboratory (25°C) for three weeks. Thereafter, the dried samples were ground and passed through a 1 mm sieve to obtain a homogenous fine powder. Next, the EOs of fresh and dried samples were isolated by hydrodistillation using Clevenger-type apparatus. 100 g of each sample were placed in a 2000 mL round-bottom flask according to the designed drying period and water to raw material ratio. The flask was then heated under normal pressure for the fixed extraction time. Different ranges of drying period (0–21 days), extraction time (60–240 min) and water/plant ratio (5–20 ml/g) were selected to evaluate the effect of individual independent factors on total extraction yield.

The obtained EO was dried with anhydrous sodium sulfate and placed in an amber-colored vial for storage at 4°C until analysis. The EO yield (%) was calculated based on the weight of the dried plant.

$$\text{Yield (\%)} = \frac{\text{Amount of extracted EO (ml)}}{\text{Amount of vegetal matter (g)}} \times 100 \text{ (Eq. 1)}$$

2.2. Experimental design and statistical analysis

The RSM has been studied using CCD type to improve the extraction yield (Y) of *B. hirsuta* EO. Three factors were tested in this work: the drying period (A); the extraction time (B) and the water/plant ratio (C) as described previously (Fadil et al. 2015). with some modification. The center point and range of each

independent variable were selected from preliminary experiments. For each factor, three levels coded (−1 for the lower level, +1 for the upper level and 0 for a central level) were examined. Table 1. shows the levels used for each factor.

Table 1
Experimental variables and their levels.

Symbol	Independent variables	Coded levels		
		-1	0	+1
A	Drying period (day)	0	7	14
B	Hydrodistillation time (min)	60	120	180
C	Water/Plant ratio (ml/g)	5	10	15

According to the CCD, a series of experiments were carried out to obtain the responses of the dependent variables shown in the design diagram. A second-order polynomial equation was used to fit the experimental data of the variables studied. A quadratic design model was applied during the analysis and regression analysis was incorporated into an empirical second-order polynomial model, as shown in the following equation:

$$Y= \beta_0+ \sum_{i=1}^3 \beta_iX_i+ \sum_{i=1}^3 \beta_{ii}X_i^2+ \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij}X_iX_j+ \varepsilon \text{ (Eq. 2)}$$

Where Y is the predicted response (EO yield), β0 is the intercept of the equation, βi, βii, and βij are respectively the linear coefficients, quadratic, and interaction terms; Xi and Xj represent different independent variables, and ε is the experimental error (random).

Expert design software (Design-Expert 12.0: Stat-Ease Inc., Minneapolis, MN, USA) was used for the experimental testing and to obtain an analysis of variance (ANOVA) and regression analysis. The interactive effects of the levels of the three tested factors on the extraction procedure were carried out by the construction of the response surfaces and their respective contour curves.

2.2. Essential oil analysis

The composition of *B. hirsuta* EO was analyzed according to the same GC conditions previously described by Ansari et al. (Ansari et al. 2022) using a Perkin Elmer Autosystem GC chromatograph, equipped with two detectors (flame ionization detector (FID) and a Perkin Elmer TurboMass mass detector), a split/splitless injector and two columns (60 m x 0.22 mm i.d. thickness of the film: 0.25 μm) respectively polar (Rtx-Wax, polyethylene glycol) and apolar (Rtx-1, polydimethyl-siloxane). For GC-FID: The temperature of the injector is 250°C and that of the detector 280°C. 0.2 μl of EO was injected in split mode (1/50) using helium as carrier gas with a flow rate of 1 mL/min. The temperature programming consists of a rise from 60 to 230°C, at 2°C/min, then a 35 min level at 230°C. For GC-MS: The molecules are bombarded by electron impact (EI) ionization energy of 70 eV. The acquisitions of data is carried out

in scan mass ranged from 35 to 350 Da (scan time: 1 s). 0.2 µl of EO was injected in split mode (1/80). The identification of individual components was based by comparing their EI-mass spectra and their retention indices (RI_a) on non-polar with those authentic compound library or literature data (König et al. 2001; Adams 2007).

3. Results And Discussion

3.1. Extraction yield using single factor test

The effects of extraction factors (e.g. drying period, extraction time and water/plant ratio) on EO yield were evaluated in the range of 0–21 days, 60–240 min and 5–20 ml/g, respectively.

3.1.1. Effect of drying period

The effect of drying period ranging from 0 to 21 days was examined at a fixed hydrodistillation extraction time (120 min) and liquid/solid ratio (10 ml/g) as shown in Fig. 1a. The extraction yield increases significantly with increasing of drying period from 0 to 7 days, maximized to 0.93% at 7 days, but decreased slightly when the drying period increased from 7 to 21 days. It has been reported that the drying process can interfere with the biologically active principle of medicinal plants. According to Argyropoulos and Müller (2014), the decrease in the EO yield may be associated with moisture losses in plant material during the drying time. The moisture moves by diffusion from the interior to the surface of the plant and may carry oil with it. Prolonged drying times in species such as *Eugenia uniflora* have a high moisture loss, with a consequent increase in the rupture of the storage structures and volatilization reducing EO yield (de Assis et al. 2020).

3.1.2. Effect of extraction time

Extraction time was also an important factor that would influence the extraction efficiency of extracting EOs from aromatic plants. To obtain the optimum hydrodistillation conditions, samples treated with a drying period of 7 days and a liquid/solid ratio of 10 ml/g are subjected to hydrodistillation time for 60, 120, 180 and 240 min as shown in Figure. 1b. The results showed that the EO yield increases gradually with the extended extraction time from 60 to 180 min. This increase is most marked during the first two hours, maximized to 0.90%, whereas it becomes slower thereafter to reach 0.93% at 180 min. however, when the extraction time increases from 180 to 240 min, the yield remained almost unchanged. Hence, 60–180 min was chosen as the best range for the subsequent optimization. As reported, increasing the extraction time gives high-boiling components enough time to evacuate cells and leaf pockets, thereby dramatically increasing yield (Yingngam and Brantner 2015; Mainya et al. 2016).

3.1.3. Effect of liquid/solid ratio

The liquid/solid ratio is another important factor influencing the EO yields extracted by hydrodistillation. To obtain an appropriate value, different water/plant ratios were set at 5, 10, 15 and 20 ml/g were

investigated at a fixed extraction time (120 min) and drying period (7 days) as shown in Fig. 1c. The results showed that the EO yield increases dramatically within the ratio of 5–10 ml/g, maximized to 0.93% at 10 ml/g and gradually decreases to reach 0.86% at 15 ml/g. Hence, 10 ml/g was selected as the suitable liquid/material ratio for further optimization by RSM. Our results are in agreement with previous studies in the literature. Peng et al (Peng et al. 2012). observed that the EO yield of *Zingiber cassumunar* increased to a certain point and then started to decrease as the amount of water continued to increase. Pourmortazavi and Hajimirsadeghi (2007) reported that the low water/plant ratio, hence the low water volume, can cause overheating and burning of plant material, resulting in low yield. However, increasing this parameter ie: the whole plant is immersed in water, promotes water-matter contact and reduces resistance to mass transfer as well as the essential oils diffuse more in the water, which allows the vapor to more easily entrain them, thereby increasing the extraction yield (Kusuma and Mahfud 2017). On the other hand, increasing this ratio to a certain value resulted in a decrease in the yield of the EO because an excess of water could dissolve or emulsify the oil (Yingngam and Brantner 2015; Kusuma and Mahfud 2017).

<Figure 1>

3.2. Optimization of the extraction process using RSM

3.2.1. CCD and fitting the model

In order to determine the best conditions for an optimal EO yield of *B. hirsuta*, the RSM with CDD was carried out to evaluate the influences of three independent variables namely the drying period, the extraction time and the water/plant ratio. The designed matrix and the corresponding values were recorded in Table 2.

Table 2
CCD design matrix of three variables and the observed response for extraction of *B. hirsuta* EO.

		Factor 1	Factor 2	Factor 3	Response	
Std	Run	A: Drying period	B: Extraction time	C: Water/Plant ratio	Yield (%)	
		day	min	ml/g	Actual Value	Predicted Value
12	1	7	180	10	0.93	0.92
7	2	0	180	15	0.54	0.53
13	3	7	120	5	0.80	0.79
20	4	7	120	10	0.90	0.91
6	5	14	60	15	0.80	0.78
11	6	7	60	10	0.87	0.87
8	7	14	180	15	0.74	0.76
16	8	7	120	10	0.91	0.91
5	9	0	60	15	0.40	0.41
2	10	14	60	5	0.71	0.73
1	11	0	60	5	0.39	0.38
4	12	14	180	5	0.72	0.71
19	13	7	120	10	0.91	0.91
18	14	7	120	10	0.90	0.91
9	15	0	120	10	0.56	0.56
15	16	7	120	10	0.89	0.91
10	17	14	120	10	0.85	0.85
3	18	0	180	5	0.47	0.49
17	19	7	120	10	0.91	0.91
14	20	7	120	15	0.83	0.83

According to CCD, a total of 20 runs of experiments with 6 repetitions at the center points were carried out to obtain the responses of the dependent variables indicated in the design layout. As shown in Table 2, the EO yield (%) ranged from 0.39–0.93% with the highest EO yield found in experiment number 1, while

experiment number 11 has the lowest yield. Mathematically, the response Y can be described by the following second-order polynomial equation in coded (Eq. (3)) and acquired actual real values (Eq. (4)):

$Y = 0,905818 + 0,146 * A + 0,023 * B + 0,022 * C - 0,03375 * AB + 0,00375 * AC - 0,00125 * BC - 0,204545 * A^2 - 0,00954545 * B^2 - 0,0945455 * C^2$	(Eq. 3)
$\text{Yield} = -0,0160909 + 0,0878701 * \text{Drying period} + 0,00162386 * \text{Extraction time} + 0,0797864 * \text{Water/Plant ratio} - 8,03571e-05 * \text{Drying period} * \text{Extraction time} + 0,000107143 * \text{Drying period} * \text{Water/Plant ratio} - 4,16667e-06 * \text{Extraction time} * \text{Water/Plant ratio} - 0,0041744 * \text{Drying period}^2 - 2,65152e-06 * \text{Extraction time}^2 - 0,00378182 * \text{Water/Plant ratio}^2$	(Eq. 4)

The signal and the amplitude of the coefficients are used to explain the effects of the variables on the response. Indeed, the positive correlations were observed between the linear variables A, B and C as well as the interaction variable AC and the response, while the interaction variables AB and BC and the squared variables A², B² and C² showed negative correlations.

3.2.2. Analysis of variance (ANOVA)

The adequacy of the regression equation was analyzed by analysis of variance (ANOVA) and the statistical significance was evaluated by their corresponding by both F-test and p-value as shown in Tables 3

Tables 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of extraction yield of *B. hirsuta* EO.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.6200	9	0.0689	244.24	< 0.0001	significant
A-Drying period	0.2132	1	0.2132	755.70	< 0.0001	
B-Extraction time	0.0053	1	0.0053	18.75	0.0015	
C-Water/Plant ratio	0.0048	1	0.0048	17.16	0.0020	
AB	0.0091	1	0.0091	32.31	0.0002	
AC	0.0001	1	0.0001	0.3988	0.5419	
BC	0.0000	1	0.0000	0.0443	0.8375	
A ²	0.1151	1	0.1151	407.90	< 0.0001	
B ²	0.0003	1	0.0003	0.8883	0.3681	
C ²	0.0246	1	0.0246	87.15	< 0.0001	
Residual	0.0028	10	0.0003			
Lack of Fit	0.0025	5	0.0005	1.74	0.064	not significant
Pure Error	0.0003	5	0.0001			
Cor Total	0.6229	19				
Std. Dev.	0.0168	C.V. %	2.23			
Mean	0.7515	Adeq Precision	45.8362			
R ²	0.9955	Adjusted R ²	0.9914	Predicted R ²	0.9161	

The model terms with a lower p-value ($p < 0.05$) were considered significant, while the other terms with $p > 0.10$ were insignificant. As depicted in Table 3, linear coefficients (A, B, and C), quadratic coefficients (A² and C²), and interaction coefficients (AB) exhibited a significant influence on yield extraction, while the others, AC, BC and B² are not significant. The high F-value (244.24) suggests that the model is highly significant, and only a 0.01% chance that an F value of the model could occur due to statistical noise. It was reported that the model terms with higher F-values and lower P values have more effect on the response variable (Oza et al. 2021). Additionally, the high F-value and low p-value specified that the quadratic model used is ideal enough to reject the null hypothesis. The high "Lack of Fit F-value"

suggests that the lack-of-fit was insignificant ($p > 0.05$) and confirmed a good precision of the proposed model. Moreover, the R^2 value of 0.9955 is close to unity, indicating that the selected model was fitted sufficiently. The predicted R^2 value of 0.9161 and the adjusted R^2 of 0.9914 (Difference < 0.2) were close to their corresponding R^2 , meaning that the actual and predicted values had a high correlation (Ardestani et al. 2022). Further, adequate precision is a measure of signal/noise ratio, and a value greater than 4 is desirable. In this study, the ratio was 45.8362, which indicates a sufficient signal and the good fitness of the proposed model. In addition, the smaller value (< 5%) of the coefficient of variance (C.V) indicates the high accuracy and predictability of the model. Therefore, the C.V. value of 2.23% obtained in this work proves the better reliability of the chosen model.

In order to check whether the goodness of fit of the proposed model and demonstrate the interactions between the determined values and the predicted responses, the normal probability versus the normal external traces of residues and the actual experimental results versus the predicted value were plotted as shown in Fig. 2 and Fig. 3, respectively.

<Figure 2>

Figure 2 shows that the normal probability plot is linear, proving that the residuals were consistent with the predicted values and that the assumptions of the analysis were acceptable. Likewise, Fig. 3 shows that the extraction yield data are regularly distributed along the straight line of 45°, passing through the origin, suggesting that the predicted values of EO yield are in good agreement with the experimental values and therefore the chosen quadratic model was satisfactorily fitted (Elganidi et al. 2022).

<Figure 3>

3.2.3. Response surfaces and contour plots analysis

The 3-dimensional (3D) response surface and the 2-dimensional (2D) contour plots were constructed to visually illustrate the mutual interactions between three operational parameters on extraction yield from *B. hrisuta* EO. Graphical representations of the calculated response surface are shown in Fig. 4.

<Figure 4>

Figure 4 (a,b) showed the interactive effect of drying period (A) and the extraction time (B) on the EO yield for a fixed water/plant ratio (C) of 10 ml/g. At the start of the reaction, increasing the drying time or the extraction time leads to an increase in the EO yield. Then, the continued increase of the drying period parameter shows that the yield has not increased significantly. The maximum yield (greater than 90%) is predicted for the combination moderate drying period and highest extraction time at a water/plant ratio of 10 ml/g. Likewise, Fig. 4 (c,d) showed the effects of the drying period (A) and the water/plant ratio (C) on the extraction yield at a fixed extraction time (B) of 120 min. It was found that the EO yield increased with the increase of the two variable parameters at the beginning, but significantly decreases with a further increase of one or the other parameter. This maximum yield (90%) is achieved at moderate values of the combination drying period and water/plant ratio with an extraction time of 120 min. However,

Fig. 4 (e,f) showed the influence of the extraction time (B) and the water/plant ratio (C) on the extraction yield of rosemary EO at a drying period fixed (A) of 7 days. Clearly, an increase in the extraction time or the water/plant ratio led to an elevation in the EO yield at the first stage of the reaction but reduced at higher values of the water/plant ratio. The maximum yield ($\approx 90\%$) is reached for a maximum extraction time and the moderate water/plant ratio combination at a drying period of 7 days.

These results agreed with those obtained for classical single-factor experiments (one factor at a time). However, it should be noted that the classical single-factor design is a long and expensive process, as the response variable, depends only on the varied parameter. It excludes interactions between the various treatment parameters (Cui et al. 2018). This classic optimization also requires a large amount of material. For this, RSM appears to be a better method applied in the design for the analysis and optimization of several variables minimizing the volume of experiments, reagents, time and energy (Montgomery 2009).

3.2.4. Experimental validation

According to RSM results, the optimum predicted extraction yield of *B. hirsuta* EO (0.91%) was obtained when the drying time is 7 days, the extraction time is 120 min and the ratio water/plant is 10 ml/g. The hydrodistillation experiments were performed under these optimal conditions to validate the accuracy and acceptability of the extraction procedure. Indeed, 6 real tests were carried out and the mean value of $0.90 \pm 0.008\%$ ($n = 6$) was obtained. This finding showed a good validity of this RSM model because the difference between 0.91% and $0.90 \pm 0.008\%$ is insignificant ($p > 0.05$). Besides, the *B. hirsuta* EO was extracted under these optimal conditions and identified by gas chromatography-mass spectrometry

3.3. Essential oil composition

A total of forty-three components accounting for 82.3% of the total oil of *B. hirsuta* were identified through detailed GC and GC-MS analysis. The identified chemical composition, retention time, and percentage composition are listed in Table 4.

Table 4
Chemical constituents (%) of *B. hirsuta* EO from south-eastern Morocco.

N	Compound	RI I	RI a	RI p	%
1	1-Octen-3-ol	962	960	1447	0.2
2	Linalol	1086	1083	1535	0.3
3	Camphor	1123	1120	1499	0.4
4	Menthone	1136	1132	1438	0.2
5	Borneol	1150	1148	1679	0.2
6	α -Terpineol	1176	1171	1682	0.2
7	Decanal	1180	1183	1471	0.2
8	Pulegone	1215	1213	1623	1.0
9	Carvotanacetone	1220	1219	1657	1.0
10	Carvacrol	1278	1279	2182	6.7
11	α -Ylangene	1376	1368	1471	0.6
12	α -Copaene	1379	1373	1479	1.2
13	β -Bourbonene	1386	1380	1505	0.5
14	β -Cubebene	1390	1383	1540	0.2
15	β -Elemene	1389	1383	1575	1.0
16	Neryl acetone	1411	1408	1798	0.3
17	Trans-Caryophyllene	1421	1414	1581	4.7
18	β -Copaene	1430	1423	1572	0.3
19	Geranyl acetone	1430	1427	1835	0.3
20	α -Sesquisabinene	1446	1447	1650	0.5
21	α -Humulene	1455	1454	1652	0.6
22	Cis-Muuroloa4(14).5-diene	1465	1461	1626	1.0
23	Germacrene-D	1479	1474	1690	16
24	Trans-Muuroloa4(14).5-diene	1473	1478	1670	0.1
RI I = retention indices on the apolar column (Rtx-1) of literature [21];					
RI a = retention indices on the apolar column (Rtx-1);					
RI p = retention indices on the polar column (Rtx-Wax)					

<i>N</i>	Compound	<i>R_I</i>	<i>R_I a</i>	<i>R_I p</i>	%
25	Tridecanal	1490	1484	1808	1.2
26	Bicyclogermacrene	1494	1488	1693	1.0
27	α -Muurolene	1496	1490	1705	0.6
28	γ -Cadinene	1507	1503	1738	2.5
29	Calamenene	1517	1507	1809	0.6
30	δ -Cadinene	1520	1512	1738	2.8
31	α -Cadinene	1534	1528	1712	0.5
32	Germacrene B	1552	1556	1827	1.0
33	Spathulenol	1572	1561	2095	4.3
34	Caryophyllene oxyde	1578	1566	1955	3.8
35	Muurola-4.10-dièn-8a-ol	1594	1592	2091	1.1
36	τ-Muurolol	1633	1623	2127	3.9
37	α-Cadinol	1643	1636	2204	7.3
38	Amorpha-4.9-dien-2-ol	1679	1664	2193	3.4
39	2-Pentadecanone 6.10.14-trimethyl	1817	1826	2109	2.0
40	Z-Z-Farnesyl acetone	1840	1838	2275	0.3
41	Neophytadiene	1849	1843	1893	0.3
42	E-E-Farnesyl acetone	1887	1889	2343	0.3
43	Z-Phytol	2090	2097	2594	7.7
TOTAL					82.3
<i>R_I</i> = retention indices on the apolar column (Rtx-1) of literature [21];					
<i>R_I a</i> = retention indices on the apolar column (Rtx-1);					
<i>R_I p</i> = retention indices on the polar column (Rtx-Wax)					

As shown in Table 4, the 19 hydrocarbon sesquiterpenes content possessed the highest value content (35.7%) of the oil, among which Germacrene-D (16.0%) and trans-Caryophyllene (4.7%) were the most abundant components. Also, three cadinene (δ -Cadinene, γ -Cadinene and α -Cadinene) were found in a relatively high concentration (5.8%). The 6 oxygenated sesquiterpenes accounted for 23.8%, with α -Cadinol (7.3%), Spathulenol (4.3%), τ -Muurolol (3.9%), Caryophyllene oxide (3.8%) and Amorpha-4.9-dien-2-ol (3.4%) being the main components. The 9 oxygenated monoterpenes constituted 10.2% with

Carvacrol being the main component (6.7%). In addition, two diterpenes, Z-Phytol and Neophytadiene, were found at concentrations of 7.7% and 0.3%, respectively. GC/RI and GC/MS analysis also showed the complete absence of hydrocarbons monoterpenes and the presence of 7 compounds (4.6%) including 5 terpene ketones namely Neryl acetone, Geranyl acetone, 2-Pentadecanone 6,10,14-trimethyl, Z-Z-Farnesyl acetone and E-E-Farnesyl acetone. The chemical structures of the most abundant compounds are given in Figure 5.

<Figure 5>

To the best of our knowledge, no previous studies on the chemical composition of the EO of *B. hirsuta* have been reported in the literature to date. However, the chemical compositions of the genus *Ballota* EOs have already been investigated. Bader et al. (2003) studied the volatile components of the EO of *B. nigra* subsp. *foetida* from Jordan and they reported that β -caryophyllene (25.1%) and germacrene D (24.2%) are the main compounds. In a study by Rigano et al. (2017), the *B. nigra* ssp. *foetida* EO from Italy consisted of germacrene D (23.1%), (E)- β -caryophyllene (20.3%) and caryophyllene oxide (6.2%) as the main constituents. The same authors reported also that germacrene D (16.0%) and bicyclogermacrene (10.4%) were the main components of the *B. undulata* EO from Italy. Morteza-Semnani et al. (2007) identified caryophyllene oxide (7.9%), epi- α -muurolol (6.6%), δ -cadinene (6.5%), and α -cadinol (6.3%) as the main constituents of Iranian *B. nigra* EO. In their study, Majdi et al. (2017) showed that the main compounds the *B. nigra* subsp. *kurdica* EO from Iran were caryophyllene oxide (39.43%), trans-caryophyllene (24.88%), germacrene D (7.64%). The chemical composition of the aerial parts EO of *B. pseudodictamnus* from Greece was studied by Couladis et al (2002) which caryophyllene oxide (22.35%), phytol (11.95%) and γ -muurolene (11.35%) and (E)-caryophyllene (10.74%) were the major components.

Fraternale and Ricci (2014) reported that β -caryophyllene (22.6% and 21.8%), caryophyllene oxide (18.0% and 20.5%) and germacrene D (16.5 and 13.1%) were identified as the main components of the aerial parts EO of *B. nigra* ssp. *foetida* grown in northern Marche (central Italy) and harvested at the time of flowering and fruiting respectively. According to Vukovic et al. (2009), the major compounds of the EOs of the leaves and stems of *B. nigra* from Serbia were β -caryophyllene (39.14 vs 35.36%), germacrene D (35, 72% vs 27.36%) and α -humulene (10.37% vs 7.44%), respectively. In addition, diterpene (E)-phytol was found at a relatively high concentration in leaf EO with 3.84%. Recently, Ain Sebaa et al (2019) reported that the aerial parts EO of *B. nigra* harvested in Tlemcen (Algeria) was characterized by β -bisabolene (22.9%), caryophyllene oxide (12.5%), germacrene-D (8.5%) and β -caryophyllene (7.9%) as the main components.

According to this literature review, our results are in agreement with previous studies in the literature on the EOs of various *Ballota* species, reporting germacrene D, β -caryophyllene, caryophyllene oxide α -cadinol and phytol as the main compounds with different percentages. However, our studied sample was specifically characterized by the presence of carvacrol, spathulenol and amorpha-4.9-dien-2-ol among major components, which are scarce or absent in the oils from other species. The chemical differences observed in the composition of EOs from different *Ballota* could be related to several factors such as

environmental conditions, geographic locations, type of plant organ, stage of the vegetative cycle at harvest time and edaphic conditions. These factors can influence the biosynthetic processes of terpenes, leading to the formation of various molecules with different chemical structures (Croteau and Gershenzon 1994). In this context, Bülow and König 2000) reported that the skeletal diversity of sesquiterpenes resulted from the action of many sesquiterpene synthase enzymes. For instance, they identified that germacrene-D could be considered a precursor of many hydrocarbon sesquiterpenes.

4. Conclusion

The extraction of *B. hirsuta* EO was performed by hydrodistillation and optimized using RSM with CCD. The optimal conditions for the highest product yield (0.92%) were found to be a drying period of 7 days, an extraction time of 120 min and a water/plant ratio of 10 ml/g. Under these optimal conditions, GC/FID and GC/MS identified 43 compounds accounting for 82.3% of the total in the *B. hirsuta* EO. The hydrocarbon sesquiterpenes content possessed the highest value 35.7%, followed by the oxygenated sesquiterpenes (23.8%), the oxygenated monoterpenes (10.2%) and the diterpenes (7.7%). Germacrene-D (16%), Z-Phytol (7.7%), α -Cadinol (7.3%), Carvacrol (6.7%), trans-Caryophyllene (4.7%), Spathulenol (4.3%), τ -Muurolool (3.9%), Caryophyllene oxide (3.8%) were the major components. It should be noted that carvacrol, spathulenol and amorpho-4.9-dien-2-ol are markers of *B. hirsuta* EO differentiating it from those of other *Ballota* species.

Declarations

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Disclosure statement

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Authors' contributions

The author read and approved the final manuscript.

Availability of data and materials

All data and materials are available.

Competing interests

No competing interests.

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Figures

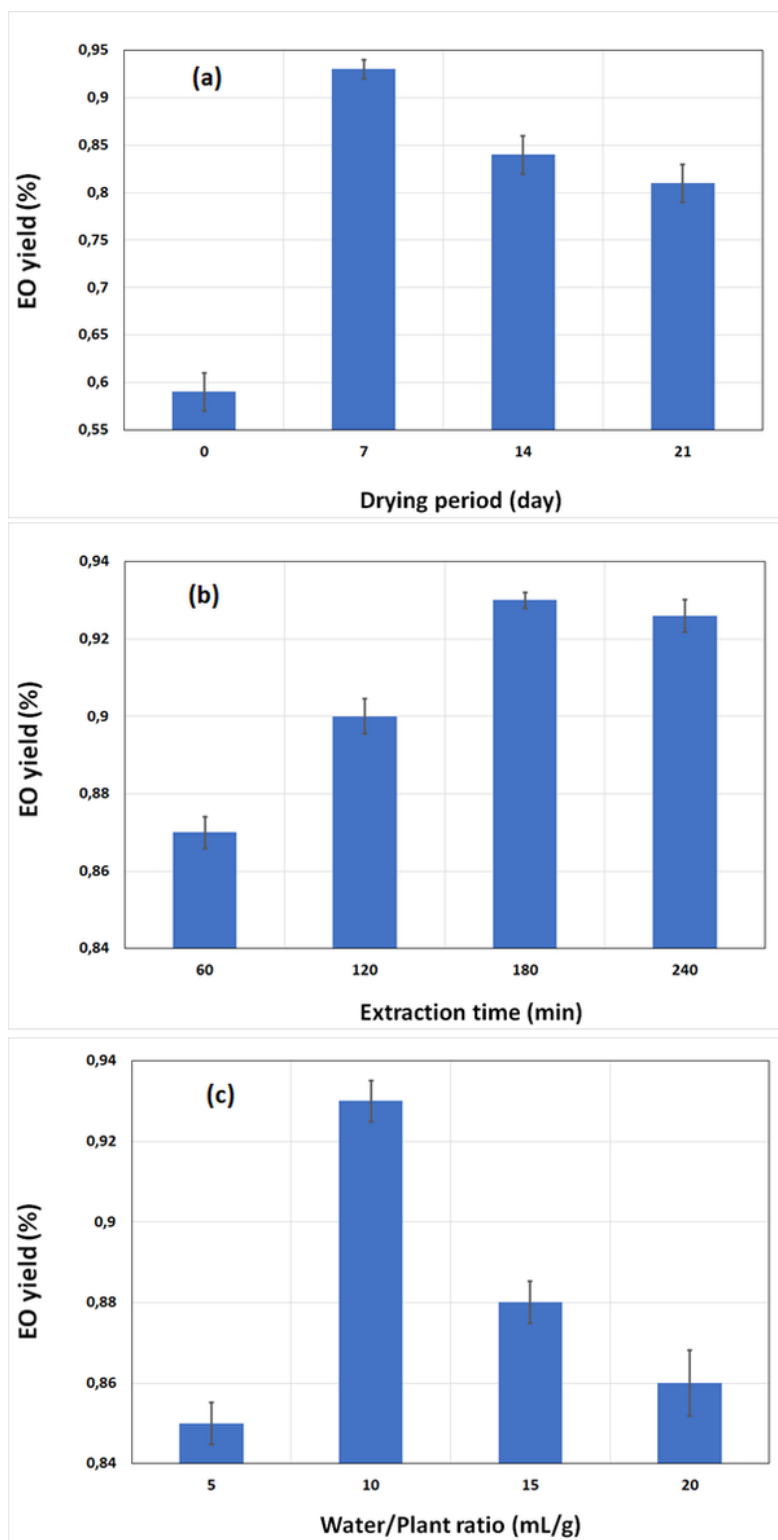


Figure 1

Effects of single factors on the yield extraction of *B. hirsuta* EO: drying period (a), extraction time (b), and liquid-to-material ratio (c). Data was shown as mean $\pm$ SD (n = 3).

YIELD

Color points by value of
YIELD:

0,39  0,93

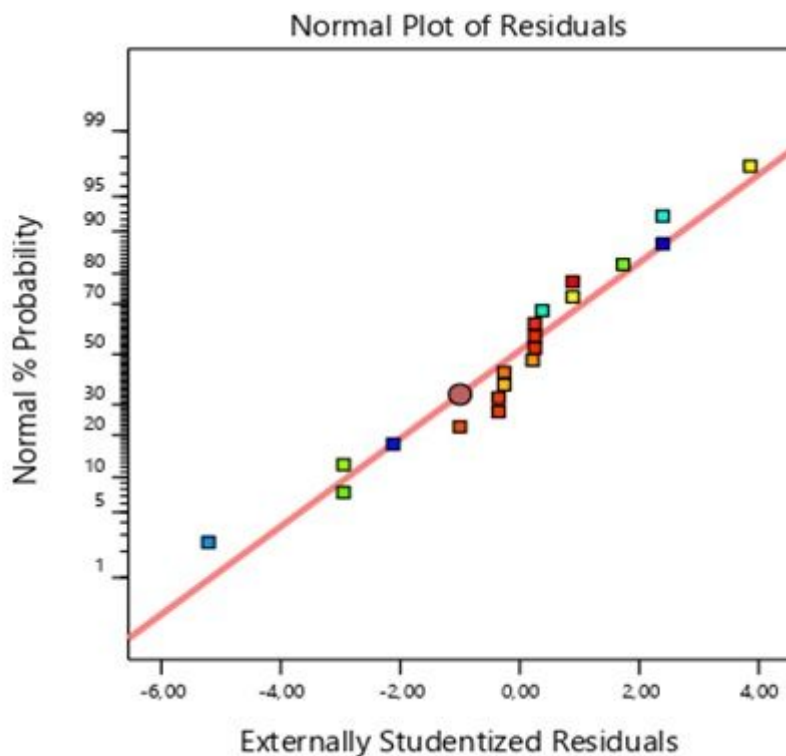


Figure 2

The Plot of the residual and predicted values of EO yield.

YIELD

Color points by value of
YIELD:

0,39  0,93

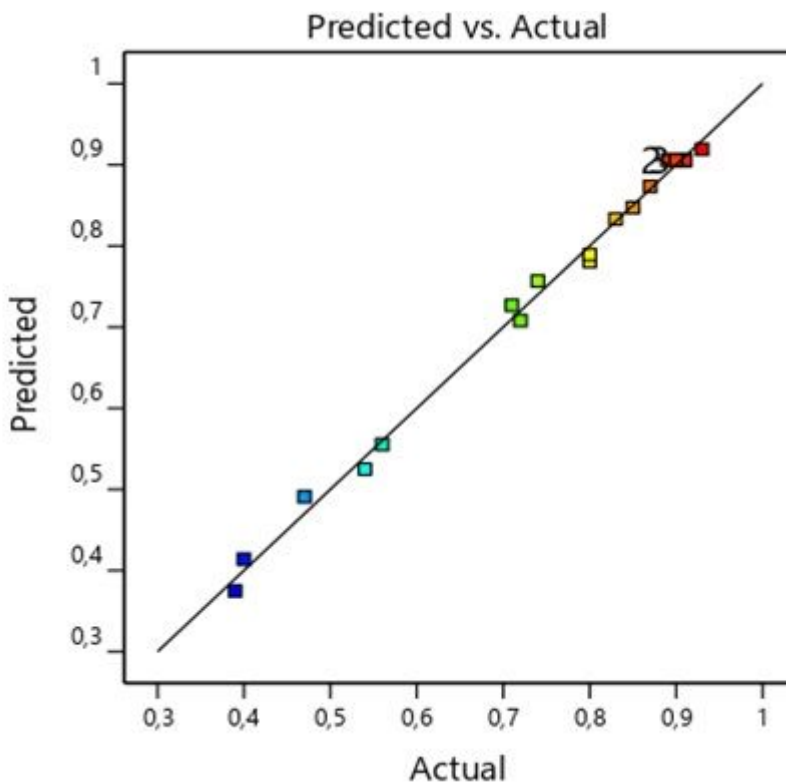


Figure 3

Experimental extraction yield vs. the predicted extraction yield under optimum extraction conditions.

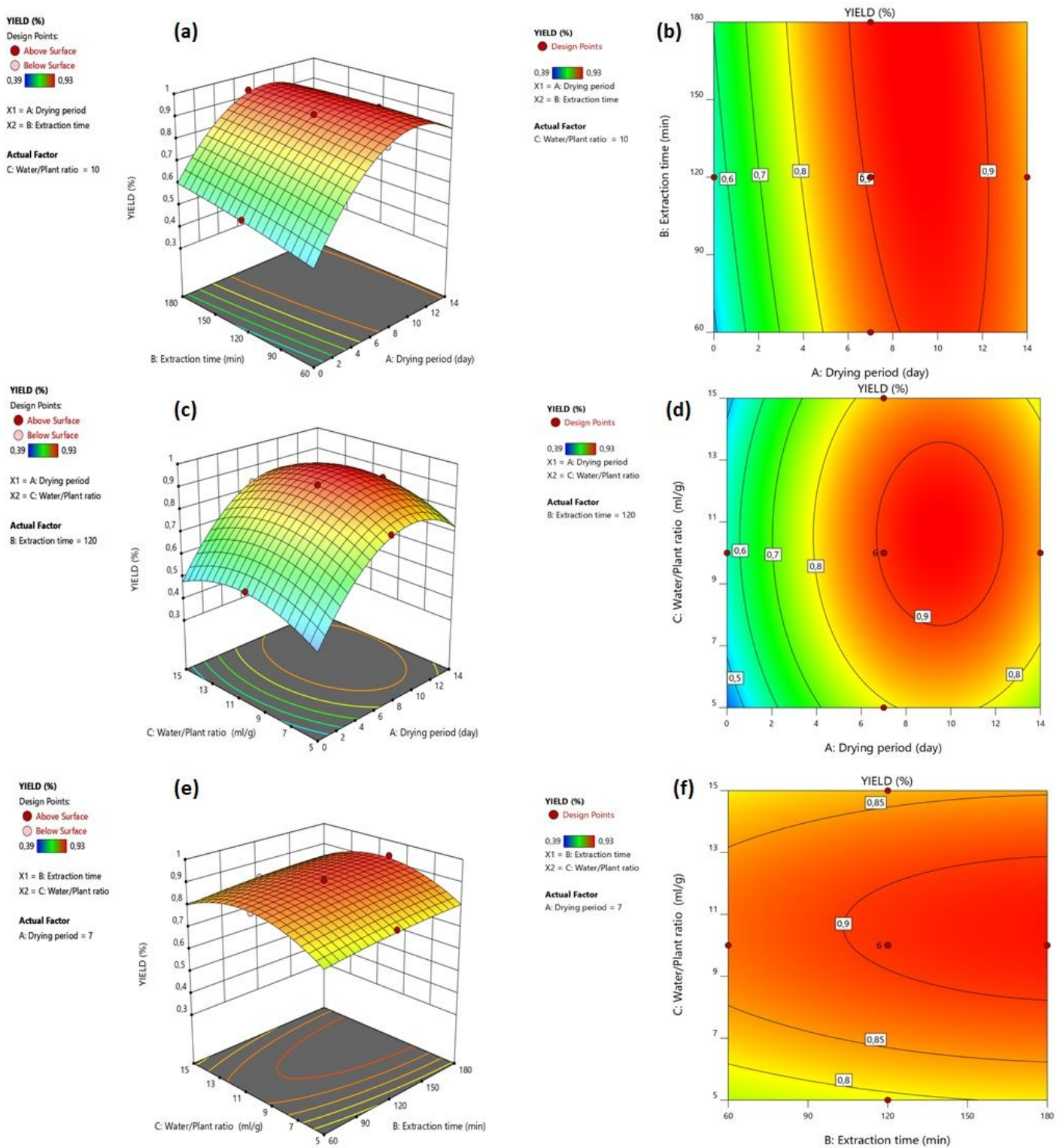


Figure 4

Response surface diagrams (a,c, and e) and contour plots (b, d, and f) of extraction yield of *B. hirsuta* EO affected by ratio of drying period (A), extraction time (B), and water/plant ratio (C).

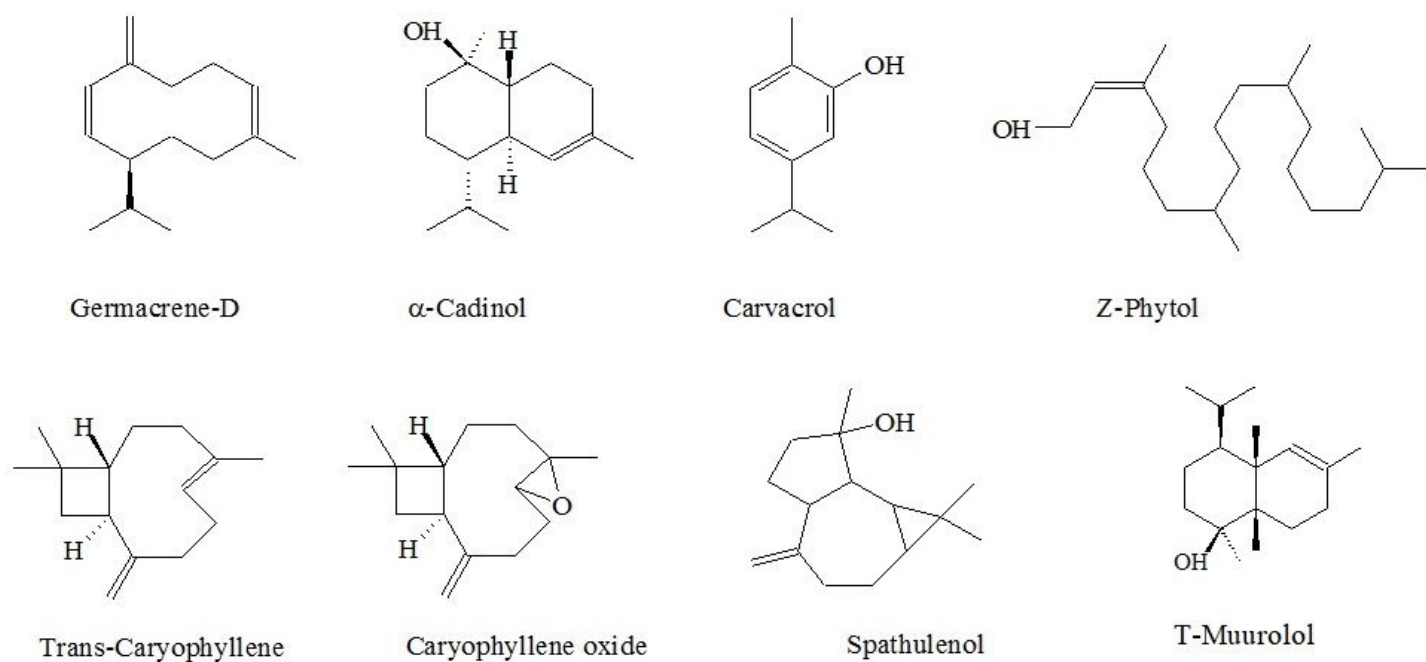


Figure 5

Chemical structures of the main compounds of *B. hirsuta* EO from south-eastern of Morocco.

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