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Discrimination of *Mentha* species grown in different geographical areas of Algeria using ^{1}H-NMR-based metabolomics

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ABSTRACT

^{1}H-NMR-based metabolomics have been applied to identify potential NMR-markers and biomarkers capable of distinguishing, qualifying and classifying three *Mentha* species:- *Mentha pulegium* L., *Mentha* × *rotundifolia* (L.) Huds., *Mentha spicata* L., and their ecotypes. Samples of the 3 species were collected in seven different locations in Algeria, with the aim to establish a quality control protocol based on the use of NMR fingerprint profiles of polar extracts. NMR data indicate that the identification of the *Mentha* genus can be confirmed by the presence of the doublet proton signals with identical coupling constants at δ 7.49 (d, 15.9 Hz) and δ 6.29 (d, 15.9 Hz); these correspond to the protons of the double-bond conjugated to the ester group of rosmarinic acid, a bioactive compound found in all three species. Differences in NMR proton chemical shifts and/or signal intensities were clearly demonstrated on the orthogonal projections to latent structures discriminating analysis (OPLS-DA). Several potential biomarkers discriminating the three *Mentha* species were originated using S-plots, loading score plots, NMR data analysis and literature search. These discriminating signals point to glycosylated flavonols, oxygenated terpenoids and hydrocarbon terpenoids to distinguish *M. pulegium*, *M.* × *rotundifolia* and *M. spicata*, respectively. Within the same species, Principal Component Analysis (PCA) scores clearly discriminated the metabolite content according to regions in which the plants were grown. The 6 zones in which *Mentha pulegium* samples were harvested were clearly separated along either or both PC1 and PC2; by contrast, the harvesting locations were divided into two groups along PC1 for both *M.* × *rotundifolia* and *M. spicata*. The total antioxidant activity of the *Mentha* species was impacted by the abiotic factors of the different regions.

1. Introduction

Mentha species (Lamiaceae) are widely distributed in Europe, Asia, Africa, Australia, and North America [1]. Fifteen *Mentha* species are scattered across Algeria, and *Mentha* species are some of the most popular food and medicinal herbs in this country [2]. According to Quezel and Santa [2] the Algerian flora contains five substantial species : *Mentha aquatica* L., *Mentha longifolia* (L.) L., *Mentha pulegium* L., *Mentha* × *rotundifolia* (L.) Huds., and *Mentha spicata* L. However, the last three species are the most exploited. They are commonly used for seasoning food and as herbal remedies to treat many diseases including chest pains, biliary disorders, dyspepsia, enteritis, aerophagia, and intestinal colic [1]. Different modes of preparation (decoction, infusion and hydrodistillation) of *Mentha* leaves have been used to treat digestive disorders in Algerian folk medicine [3]. In addition, *Mentha* species have a broad spectrum of medical effects, which vary between species. *M. pulegium* is used as antiemetic and antitussive [4]. *M.* × *rotundifolia* is used to treat furunculosis and abscesses, and eliminate dental pains [5]. *M. spicata* treats menstrual cramps, gingivitis, and odontalgias [5].

Hybridization is very frequent between species of the genus *Mentha* which makes them difficult to classify [6]. Besides, the distinction between species growing in different localities is arduous.

Mentha species as medicinal and edible plants have a considerable number of antioxidant biochemicals such as flavonoids,

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phenolics, and phenylpropanoids, which are proven to be advantageous for health [1]. Phenolic compounds produced in nature play an important role in host-pathogen interactions and have been shown to assist the growth of plants in diverse environmental conditions [7]. Despite the range of therapeutic and nutritional applications of *Mentha* species in Algeria, there is limited information available to discriminate between species and to determine their origin or their quality.

In our previous studies, we examined the variability in antioxidants species, mainly phenolics, among *M. pulegium* collected in two localities from Algeria (Tizi-Ouzou and Bejaia). The phenolic contents of aqueous and ethanolic extracts were significantly different [8]. These findings correlate with other data obtained in other countries [9,10] and reflect the impact of biotic and abiotic factors on the production of secondary metabolites from the same species grown in different environments [11].

The biological activity and nutritional value of plant material can be directly correlated to chemical composition. Therefore, precise and credible procedures are required to establish herbal quality and to further understand on the impact of geographical origin on their chemical and metabolomic composition. Metabolomics is an emerging field that focuses on the comprehensive analysis of metabolites in a sample. NMR-based plant metabolomics is recognized as an important tool for studying biological systems, notably the diversity of secondary metabolites. Such metabolites are major mediators of plant-environment interactions, their qualitative and quantitative profiles are influenced by environmental and agronomical conditions, and these variations impact on the biological activity of a plant [12]. Coupling ^{1}H-NMR spectroscopy and multivariate data analysis, a metabolic profile of a wide range of chemical components can be created based on the proton spectrum signals. This fast, simple and extremely robust technique has yielded a predominant profiling method that can be used for the characterization, classification and quality control of medicinal herbs [13].

There is a great interest in developing methods to rapidly identify and distinguish *Mentha* species, cultivars and ecotypes. As each species is characterized by its own applications and traditional uses [14], misidentification can indeed impact the commercial value of marketed batches, their culinary use but, most of all, the therapeutic response sought from specific *Mentha* treatments. Thus far there have been no studies using ^{1}H-NMR spectroscopy coupled with Principal Component Analysis (PCA), to produce metabolomic fingerprints for Algerian *Mentha* species, or to use this technique to differentiate the geographic origins of these species.

In the present study, NMR-based metabolomics were applied to polar extracts to determine NMR signals confirmatory of the *Mentha* genus and identify potential biomarkers capable of identifying and classifying local ecotypes of the 3 major Algerian *Mentha* species, *i.e. M. pulegium*, *M.* × *rotundifolia* and *M. spicata*.

2. Materials and methods

2.1. Plant collection

A total of 72 *Mentha* samples (24 leaves samples for each *Mentha* species, *i.e. M. pulegium*, *M.* × *rotundifolia* and *M. spicata*) were collected, before the flowering stage, in seven regions in Algeria (6 location for each species and four biological replicates for each location), as described in Table 1. Climatology data at harvesting location and period were obtained from the Algerian Meteorology Services. Samples were identified by B. Seddik, a botanist at University Abderrahmane Mira of Béjaia, Algeria, comparing with voucher specimens previously harvested and deposited in the Herbarium of the National Botanical Garden of Meise (Belgium), references BR 0,000,006,946,227 for *Mentha spicata*, BR 0,000,006,946,043 for *Mentha pulegium*, and BR 000,000 6,946,197 for *Mentha* × *rotundifolia*.

2.2. Extraction

The leaves were dried at room temperature in the shade and powdered. Four hundred mg of each sample were extracted by sonication for 10 min at room temperature in 15 mL of 50 % EtOH. The extracts were filtered through Whatman filter paper N°1 which was then rinsed to 20.0 mL in volumetric flasks [15]. Extracts were dried under vacuum and dissolved in 0.6 mL deuterated methanol-water (1:1) to record ^{1}H-NMR spectra.

2.3. NMR spectroscopy

All experiments were performed on a Bruker Advance 600 spectrometer (Bruker Biospin GmbH, Milton, Canada) operating at 800 MHz and equipped with a 5 mm TXI probe at 298 K. All ^{1}H-NMR spectra were acquired using a standard Bruker noesyprld pulse sequence and the residual water peak was irradiated during the relaxation delay of 1.0 s and during the mixing time of 100 ms [16]. A total of 256 scans were collected into 63,536 data points over a spectral width of 12,195 Hz and pulse width of 10.5 μs, with 5 s repetition time. The spectra were then collected using TopSpin 3.1 software program.

2.4. Data reduction of NMR spectra

The ^{1}H-NMR spectra were processed using MestReNova (version 6.0.1). A line broadening of 0.3 Hz was applied to all spectra prior to Fourier transformation, phasing and baseline correction. The spectra between 0 and 10 ppm were binned every 0.04 ppm and normalized to the total area of the peaks. The integral was processed in Microsoft Excel before exporting to the software.

2.5. Total antioxidant activity (TAA)

The TAA (phosphomolybdate assay),was assessed as described in our previous paper [8]. Briefly, 2 mL of each sample was added to 200 μL of reagent (phosphate buffer, 0.6 M H_2SO_4, 28 mM sodium molybdate and 4 mM ammonium molybdate). The samples were incubated at 95 °C for 90 min after that the absorbance was measured at 695 nm. The results were expressed as mg GAE/g of dry weight from a standard curve plotted using the gallic acid linear regression. Assays were conducted in triplicate.

2.6. Statistical analysis

All data were centralized and scaled to unit variance, then analyzed by PCA and OPLS-DA based on the correlation matrix, using SIMCA 13.0 multivariate data analysis software (Umetrics, Umea, Sweden). Potential markers for group separation were subsequently identified by analyzing the loading S-plots, which plot the covariance (p) against the correlation (p(corr)). For a marker, both the contribution to the model expressed in *p* and the effect and reliability of this contribution expressed in p(corr) should be high; thus, the potential markers are located on the outer ends of the S-shaped point swarm. These discriminating metabolites were further validated by independent-sample t test. One-way ANOVA analysis was done using Systat statistical program version 12.0 (Monte Carlo, Monaco).

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Table 1
Locations of collection for the leaves of 3 *Mentha* species, *M. pulegium, M.* × *rotundifolia* and *M. spicata* (Algeria, March 2012). Samples were collected following order from 1 to 6. The altitude (m) was measured by GPS and climatic variables were recorded from meteorology data at the collection time. The soils type of each region was determined according to the clay content (Spring et al., 2003).

Plant collection regions			Altitude (m)	Climate	Average temperature (°C)	Rain (mm/year)	Precipitation (mm) [a]	Type of soil	Texture of the soil
M. pulegium	*M.* × *rotundifolia*	*M. spicata*							
1-El-Ghaba	1-El-Ghaba	1-El-Ghaba	383	Humid / mild winter	11.6	855.5	113.7 (13.5)	Heavy	Clayey
2-Thybyatine	2-Thybyatine	2-Thybyatine	482	Subhumid / mild winter	11.1	671.9	79.3 (11.8)	Heavy	Clayey
3-Tizi-Ouzou	3-Tizi-Ouzou	3-Tizi-Ouzou	636	Subhumid / mild winter	10.2	630.9	74.4 (12.6)	Heavy	Clayey
4-Khemis-Meliana	4-Khemis-Meliana	4-Khemis-Meliana	330	Hot / dry in summer; cold in the winter	11.9	712.8	84.1 (11.8)	Medium	Loamy
5-Tichy		5-Tichy	6	Mild summer/ winter hot	13.7	975.1	95.7 (12.0)	Medium	Balanced
	5-Tajboujth*	6-Tajboujth*	45	Subhumid/ winter hot	13.5	802.9	94.7 (14.0)	Medium	Balanced
6-Chemini	6-Chemini		803	Subhumid/slighy mild winter	9.3	598.1	70.6 (11.8)	Heavy	Clayey

[a] For the month of harvesting; in brackets, % relative to the year. Data from the Algerian Meteorology Services.

3. Results

3.1. Collection of plants

Leaves were collected in different locations in Algeria, taking into account various ecological factors which may influence the composition of the samples, *e.g.* the climate, temperature (from 9.3–13.7 °C), pluviometry (from 598.1–975.1 mm/year), *etc.* Seven regions at an altitude increasing from 6 to 803 m, Tichy, Tajboudjth, Khemis-Meliana, Elghaba, Thybyatine, Tizi-Ouzou and Chemini were selected; 24 representative plant material could be collected for each *Mentha* (*M. pulegium*, *M.* × *rotundifolia,* and *M. spicata*) among 7 different locations (Table 1).

3.2. Visual comparison of NMR profiles within- and between-Mentha species

An average ^{1}H-NMR spectrum was calculated for each *Mentha* and harvesting locations (Fig. 1). The metabolic profiles of each *Mentha* were very similar to each other but some differences in peak intensities were obviously shown by visual inspection. These illustrated clearly in the carbohydrate (δ 5.2–3.0 ppm) and aromatic (δ 8.5–6.0 ppm) regions, allowing a clear distinction between the metabolic profiles of *M. pulegium* and the other two *Mentha* (*M.* × *rotundifolia* and *M. spicata*). The spectra of *M. spicata* indicate different shifts and intensities of peaks in the aliphatic region (δ 2.0 to 0.5 ppm) (Fig. 1).

3.3. Identification of common NMR signals confirmatory of the genus Mentha

Visual inspection of the aliphatic and carbohydrate regions of the 72 ^{1}H NMR spectra obtained from the 3 *Mentha* species could not reveal common signal(s); signals mapping did not bring useful information because of signal density and overlapping. By contrast, the aromatic region (δ 6.0–8.0 ppm) indicated (Fig. 2) the constant presence of a clear signal corresponding to coupled protons doublets at δ 7.49 (d, 15.9 Hz) and 6.29 (d, 15.9 Hz). These characteristic signals could be assigned to the protons on C7 and C8, in α and β of the ester group typical of rosmarinic acid [17].

3.4. Multivariate statistical analyses within Mentha species

Visually comparing the average ^{1}H-NMR spectra within *Mentha* species gives some limited understanding about the peak variations in the metabolic profiles but does not allow to precise the variability between samples, notably regarding the harvesting location, or to identify eventually discriminating biomarkers. Therefore, unsupervised PCA-X was used to study the fluctuation of the recorded ^{1}H-NMR spectra.

3.4.1. Mentha pulegium samples

As shown in the score plot (Fig. 3A), the first two principal components explain 91.0 % of the variability in the dataset (PC1: 66.1 % and PC2: 24.9 %) and clearly identify the 6 different clusters of samples, corresponding to the collection regions; this indicates a similarity of the polar metabolic profiles for samples harvested in the same locations. Two separate areas were seen for each principal component with locations 1 and 2 in the positive PC1 region and locations 3, 5 and 6 in the negative PC1 region. The samples collected from locations 1 and 3 and locations 2 and 5 appear well discriminated.

The loadings plot (Fig. 3A') indicates that the chemical shift variance along the first principal component PC1 and along the second principal component PC2 is mainly driven by dominant signals in the carbohydrate (δ 4.05–3.2 ppm) and aromatic (δ 7.15–6.6 ppm) regions.

3.4.2. Mentha × rotundifolia samples

The first two principal components explain 82.2 % of the variability in the dataset (PC1: 51.6 % and PC2: 31.1 %). Four *M.* × *rotundifolia* (locations 1, 2, 3, 5) were clearly separated on both PC1 and PC2 axes from locations 4 and 6 that are themselves quite different from each other (Fig. 3B). The corresponding loading plot (Fig. 3B') indicates that the dominant chemical shifts in the aromatic (δ 6.26–7.51 ppm) regions were principally found on the negative PC1. Along the positive PC2, the dominant chemical shift signals were noticed in the carbohydrate region (δ 3.45–3.85 ppm) and along the negative PC2 the aliphatic signals (δ 1.28–2.77 ppm).

3.4.3. Mentha spicata samples

The first two principal components explain 81.8 % of the variability in the dataset (PC1: 60.5 % and PC2: 21.3 %). Four clusters

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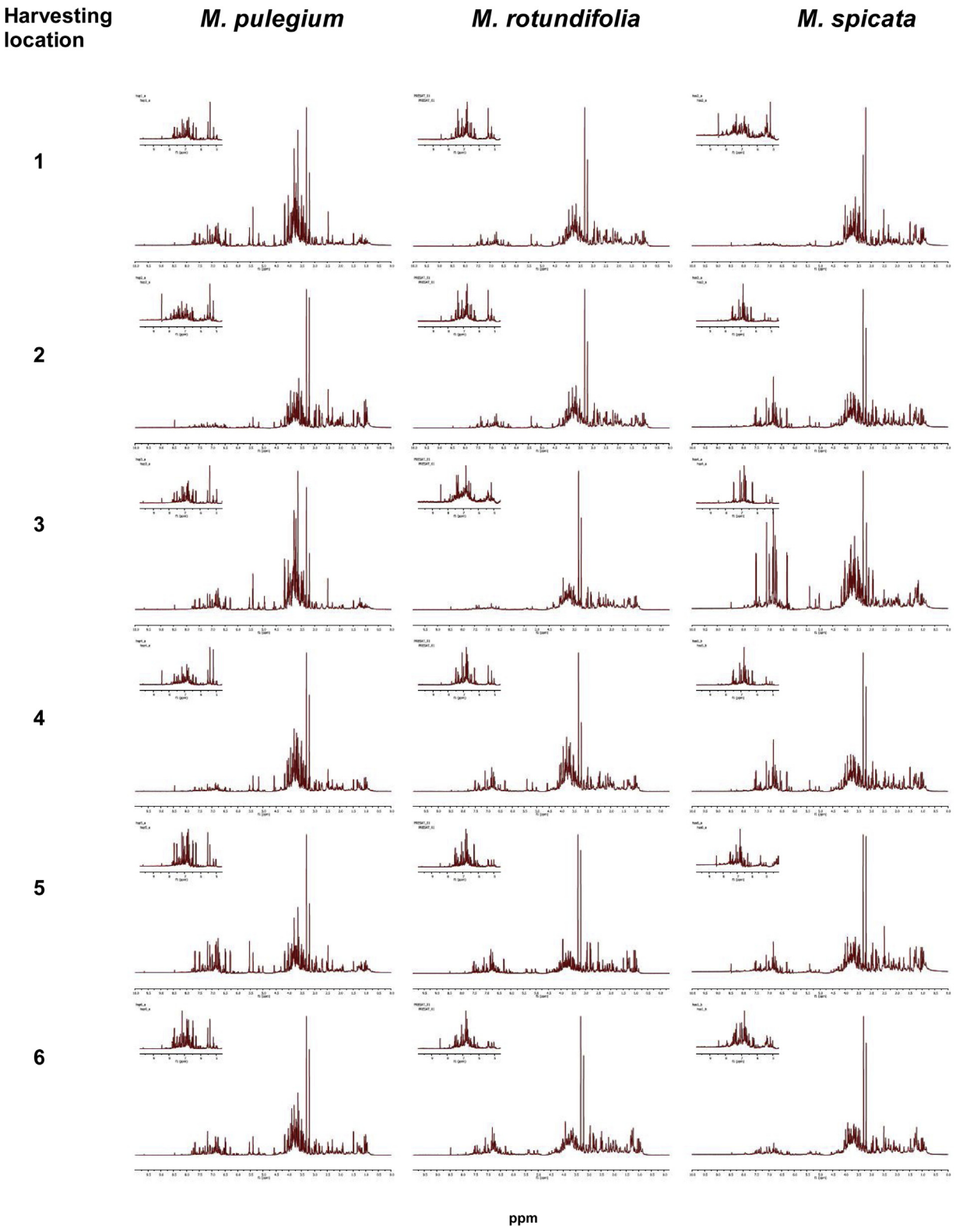

Fig. 1. Averaged ^{1}H-NMR spectra for each of the 3 *Mentha* species (left to right, *M. pulegium, M.* × *rotundifolia and M. spicata*) and harvesting location in Algeria *(ordered 1-6 as per* Table 1*)*. The NMR spectrum range of 4.5-10 ppm was zoomed in for each spectrum (*displayed on top left*).

are quite well discriminated, corresponding to locations 1, 2, 4 and 5 (Fig. 3C). Strongly positive loadings on PC1 are observed (Fig. 3C') for aliphatic signals (at δ 1.28, 2.44 and 2.48 ppm) and carbohydrate signals (at δ 3.45 and 3.61 ppm) whereas the aromatic signals (δ 6.82–7.51 ppm) exert negative PC1 loadings.

3.5. *Multivariate statistical analysis between* Mentha *species*

To provide a comparative interpretation and visualization of the metabolic differences between the 3 Algerian *Mentha* species, PCA was applied to the complete ^{1}H NMR data set. [18], the genus *Men-*

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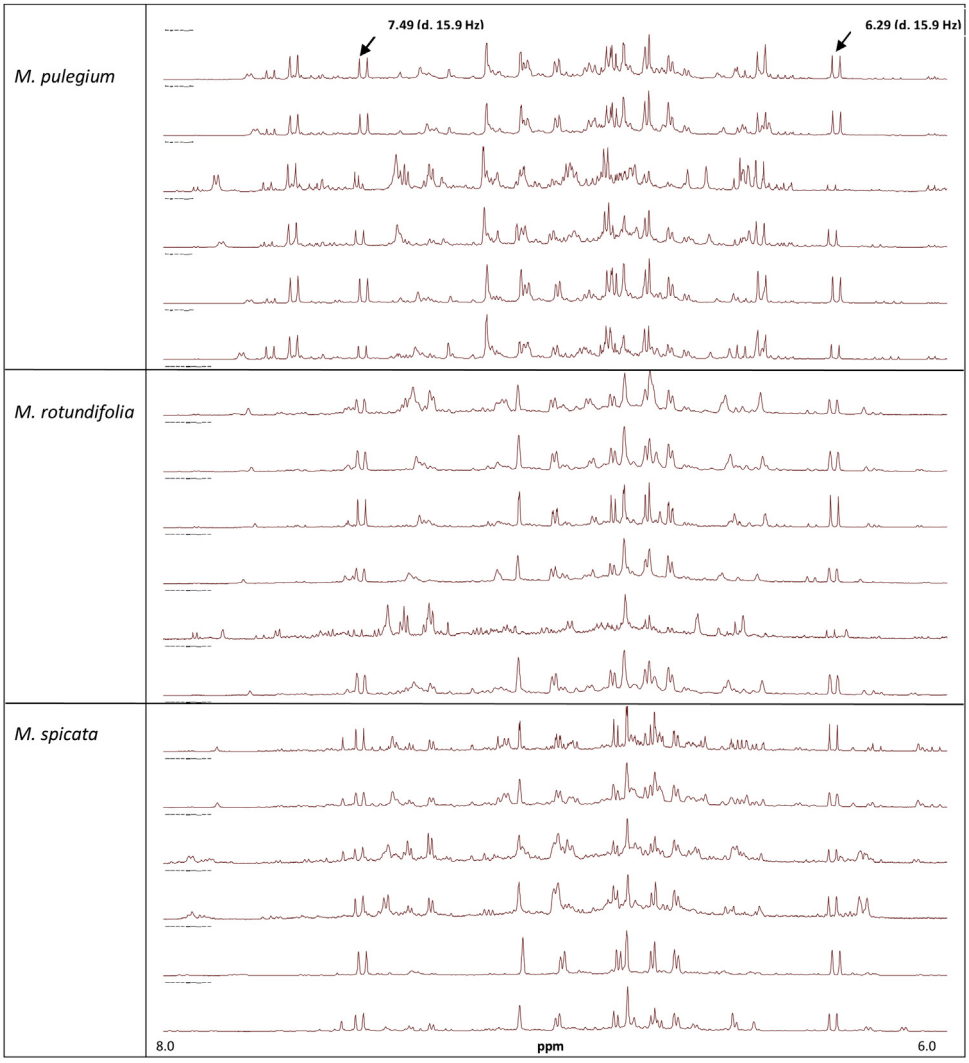

Fig. 2. Average ^{1}H-NMR spectra for each of three *Mentha* species *(ordered* up to down *1-6 as per* Table 1*)* zoomed in the range of 8.0-6.0 ppm. All spectra indicate the common presence in the three *Mentha* species of the coupled peak signals at 7.49 (d, 15.9 Hz) and 6.29 (d, 15.9 Hz) typical of rosmarinic acid.

tha is subdivided based on a phylogenetic analysis of morphology, chromosome numbers, and major essential oil constituents. And so, the genetic distance between the sections Pulegium (comprising *M. pulegium*) and Mentha (comprising *M.* × *rotundifolia* and *M. spicata*) is more important than within the section Mentha. It is interesting to remark that, despite these differences, the score plots could not identify samples clusters; PCA scores, tested by a one-way-ANOVA, confirmed non-significant difference between the profiles of *Mentha* species ($F = 1.015$; $p = 0.453$). To avoid any biased interpretation of group discrimination based on PCA analysis, the dataset was reanalyzed using OPLS-DA, a supervised method based on *a priori* knowledge of distinct samples.

For each species, the dataset homoscedasticity was tested by one-way-ANOVA demonstrating non-significant differences among sample variances ($p > 0.05$ for each group). The OPLS-DA afforded sensibly better predictions in classifying the dataset as compared to PCA analysis (predictability of the total model: Q^2 OPLS-DA > Q^2 PCA; 0.978 and 0.706, respectively). OPLS-DA facilitates the identification of the different sources of variation that contribute to the differences between the extracts, by separating inter-group variation (*i.e.*, variation that is predictive of differences within each species) from intra-group variation (*i.e.*, variation that is unrelated to group separation). As shown in Fig. 4A, a part of the total variance (predictive component, Cp 37.2 %) is predictive, being specifically related to the differences induced by *M. pulegium* and *M.* × *rotundifolia*. The first orthogonal component (Co1) describing 28.8 % of the total variation in the dataset is responsible of the high variability between *M. pulegium*/ *M. spicata* and *M.* × *rotundifolia*. The loading score plot (Fig. 4B) indicates the variables discriminating between the 3 species. The discriminating signals were interpreted through pairwise loading S-plots, *i.e. M. pulegium*/*M.* × *rotundifolia, M. pulegium*/*M. spicata* and *M.* × *rotundifolia*/*M. spicata* which allows distinguishing both the covariance (contribution of the effect) and the correlation (reliability of the predicted variables) for the identification of the major contributors in samples discrimination.

3.6. *Potential biomarkers discriminating* Mentha *species*

The variables distinguishing *M. pulegium* (mp-Biomarkers) from the other two species correspond to the signals of carbohydrates (δ 3.65–4.17) and aromatic derivatives (δ 5.38–7.67) indicating flavonoid glycosides could be discriminating biomarkers for *M. pulegium.*

The extracts of *M.* × *rotundifolia* and *M. spicata* showed similar discriminating signals and these could only be distinguished by the signal intensity. The results of S-plots analysis and loading scatter plots suggest that the aliphatic shifts δ from 1.24 to 2.93

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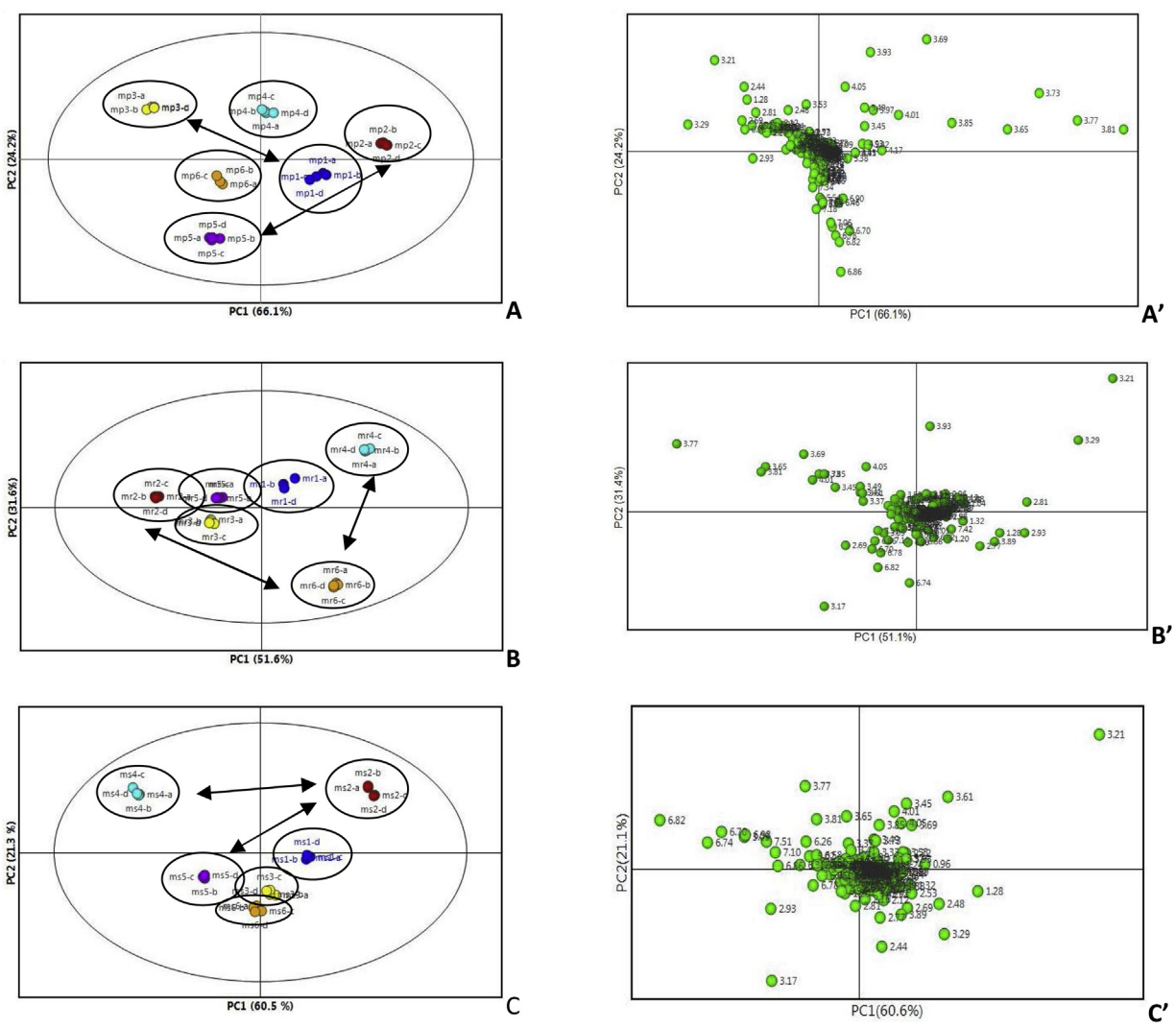

Fig. 3. Score and loading scatter plot of PCA-X performed on ^{1}H-NMR spectra of *M. pulegium* (**A & A'**), *M.* × *rotundifolia* (**B & B'**) and *M. spicata* (**C & C'**) cultivated and collected in various locations in Algeria (see Table 1). Each point in **A** (*designed by mp*), **B** (*designed by mr*), and **C** (*designed by ms*) represents a linear combination of all signals from an individual sample on PCA-X scores; each color corresponds to a harvesting location. Points in **A'**, **B'** and **C'** correspond to chemical shifts (*ppm*) arranged according to their PC1 and PC2 coordinates; the NMR signals most discriminating for harvesting location are those located the farthest from the (0,0) origin of axes.

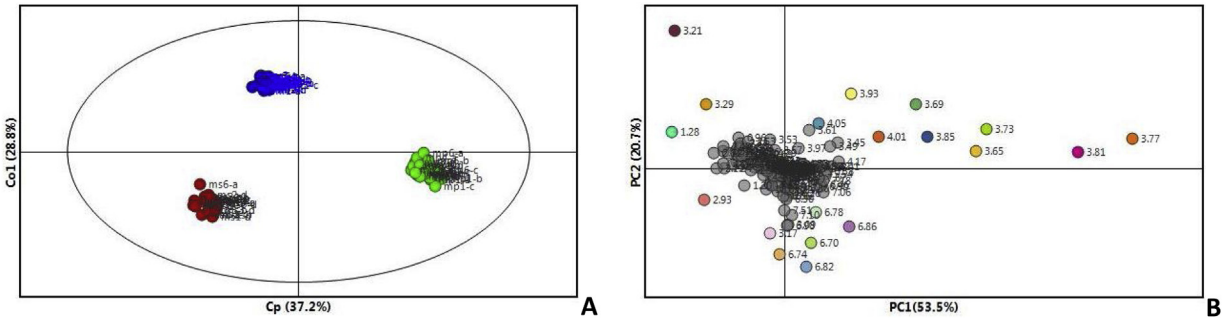

Fig. 4. A: The orthogonal projections to latent structures discriminating analysis (OPLS-DA) of ^{1}H-NMR spectra for the extracts of three Algerian *Mentha* species, *M. pulegium* (green), *M.* × *rotundifolia* (blue) and *M. spicata* (red), collected from different locations in Algeria (1 to 6 correspond to Table 1). The predictive component (Cp, X-axis) explains 37.2 % and the first orthogonal component (Co1, Y-axis) 28.8 % of the dataset variation; **B**: Loading scatter plot of PCA-X performed on the ^{1}H-NMR spectra of three Algerian *Mentha* species (*M. pulegium*, *M.* × *rotundifolia* and *M. spicata*). Points correspond to chemical shifts (*ppm*) arranged according to their PC1 and PC2 coordinates; the NMR signals most discriminating for harvesting location are those located the farthest from the (0,0) origin of axes.

(terpenoids) could be discriminating biomarkers for both species; comparing the loading scatter plots for carbohydrate and aromatic chemical shifts to those of *M. pulegium*, flavonoid glycosides could also be discriminating biomarkers for these two species (Table 2).

3.7. Total antioxidant activity (TAA)

The highest TAA was observed for the sample of *M. pulegium* from region 5 (Tichy) (1900 ± 35 mg GAE/100 g DW, followed by

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Table 2
Variables (*ppm*) discriminating between the 3*Mentha*species were deduced from pairwiseloading S-plots (p > 0.05). The symbols of mp, mr and ms design *M. pulegium*, *M.* × *rotundifolia* and *M. spicata*,respectively. Discriminating variables (mr & ms) were found in both *M.* × *rotundifolia* and *M. spicata.*

Discriminating variables (*ppm*)					
M. pulegium		*M.* × *rotundifolia* + *M. spicata*			
mp_biomarkers		mr & ms_biomarkers			
Carbohydrate signals	Aromatic signals	Aliphatic signals		Carbohydrate signals	Aromatic signals
3.65	5.38	1.24	2.12	3.25	6.90
3.69	5.54	1.32	2.20	3.85	7.14
3.73	6.46	1.72	2.48	3.93	
3.81	6.81	1.76	2.57	4.17	
3.85	7.06	1.92	2.73	4.21	
3.93	7.18	2.00	2.77	4.33	
4.01	7.67	2.04	2.81	4.57	
4.17		2.08	2.93	5.18	

Table 3
Total antioxidant activity (as GAE (mg/g of dry extract) of *Mentha* species extract growing in different regions of Algeria.

Growing regions	*M. pulegium*	*M.* × *rotundifolia*	*M. spicata*
1	1650 ± 141^{b}	1075 ± 106^{c}	425 ± 35^{c}
2	1625 ±177^{b}	1425 ± 106^{b}	125 ± 35^{d}
3	675 ± 35^{d}	1425 ± 106^{b}	600 ± 27^{b}
4	1000 ± 71^{c,e}	675 ± 35^{d}	**1025 ± 35**^{a}
5	**1900 ± 35**^{a}	**1825 ± 177**^{a}	625 ± 106^{b}
6	1025 ± 35^{c}	1500 ± 0^{b}	450 ± 18 ^{c}

GAE: gallic acid equivalents.
M. pulegium : 1-El-Ghaba ; 2-Thybyatine ; 3-Tizi-Ouzou ; 4-Khemis-Meliana ; 5-Tichy ; 6-Chemini. For *M.* × *rotundifolia* : 1-El-Ghaba ; 2-Thybyatine ; 3-Tizi-Ouzou ; 4-Khemis-Meliana ; 5-Tajboujth ; 6-Chemini. For *M. spicata* : 1-El-Ghaba ; 2-Thybyatine ; 3-Tizi-Ouzou ; 4-Khemis-Meliana ; 5-Tichy ; 6-Tajboudjth.
The values not bearing the same letters are significantly different ($P \leq 0.05$) and the results are ranked in descending order; a> b> c> d> e> f.

those harvested from regions 1, 2, 6 and 4 with values of 1650 ± 141; 1625 ± 176; 1025 ± 35; 1000 ± 70 mg GAE /100 g DW, respectively. The extract of the sample growing in region 3 demonstrates the lowest activity (625 ± 35 mg GAE / 100 g DW).

Regarding MR, the best TAA was attributed to the sample from location 5 (1825 ± 176 mg GAE/100 g DW) followed by the samples harvested in regions 6, 3, 2, and 1 with values of 1500; 1425 ± 106; 1425 ± 106 ; 1075 ± 106 mg GAE/100 g DW, respectively. According to the statistical study, we find that there is no significant difference between the regions 6, 3, and 2. The *M.* × *rotundifolia*. The sample growing in the region 4 demonstrated the lowest activity (675 ± 35 mg GAE/100 g DW).

The sample of MS from location 4 presents the highest TAA (102 ± 35 mg GAE/100 g DW) followed by the samples growing in 5, 3, 6, 1 regions whose TAA were 292 ± 45; 625 ± 106; 600 and 450 mg GAE/100 g DW, respectively. The lowest capacity was recorded for the sample from location 2 (125 ± 35 mg EAG / 100 g DM).

By comparing the studied species, it is well established that *M. pulegium* samples have a significantly higher TAA than *M.* × *rotundifolia* and *M. spicata* (Table 3).

4. Discussion

NMR is a ubiquitous and sensitive technique used to analyse the plant metabolome. It does not rely on ionization process nor on the analysis of components, a distinct advantage comparatively to chromatographic methods [19]. The comparison between metabolic profiles of the three studied plants, harvested from seven Algerian regions, indicates significant differences in the quantitative profiles of metabolites.

For the same plants, qualitatively similar in terms of their metabolic profiles, quantitative differences between the individual metabolites were observed, most significantly in the carbohydrate region of the NMR spectra. This observation is supported by literature data that reveal high levels of carbohydrates in the polar extracts of *Mentha* species with a value of 14.46 ± 0.15 %, for *M. spicata* [20]. Pérez et al. [21] have shown that ribose, arabinose, galactofuranose, xylopyranose mannopyranose, arabinopyranose and glucopyranose are the major carbohydrates of the *M. piperita* infusion. Glucose and sucrose were detected by ^{1}H NMR, in the hydroalcoholic extract of *M. spicata* from Bejaia location (Algeria) [22].

Low levels of aliphatic compounds were also detected in all spectra in the current study and may correspond to terpenoids. Riahi et al. [23] quantified many terpenoids in the aqueous methanolic extracts of *M. rotundifolia* ecotypes. The most abundant ones were *m*-cymenene, piperitenone, isopiperitenone, *o*-eugenol, *cis*-cinerolone, and carvone oxide. This aliphatic region (δ 1.5–0.5 ppm) can also correspond to aliphatic amino acid/organic acids. Low molecular weight metabolites such as amino acids (alanine, valine, isoleucine, 4-aminobutyric acid, glutamine, tyrosine, tryptophan) and organic acids (lactic, glycolic, succinic, malic, treonic and tartaric acids) were found in the metabolite profile of the infusions of mint plants [21]. Brahmi et al. [22] also identified alanine, asparagine, glutamic acid, glutamine, valine with citric acid, malic acid, and succinic acid in the hydromethanolic extract of *M. spicata*.

NMR spectra of the three *Mentha* species also showed relatively high-intensity peaks in aromatic regions. According to literature, the spectral resonances of metabolites were assigned to phenolic compounds. *Mentha* species indeed produce a variety of phenolic compounds : flavones are represented mainly by luteolin and its derivatives, flavanones such as the glycoside eriocitrin, phenolic acids including caffeic acid and its derivatives, chlorogenic and mainly rosmarinic acids [1]. The principal phenolic compound in *M. spicata* that has been detected by ^{1}H NMR was rosmarinic acid [22].

Rosmarinic acid has been also described in many Lamiaceae species. It possesses a range of biological activities, notably as antibacterial, antioxidant, anti-carcinogen, anti-inflammatory, anti-viral, immunosuppressive agent, hepato- and neuro-protector [24]. Our previous HPTLC analyses of extracts form the leaves of all 3 *Mentha* species, identified rosmarinic acid, which is a likely contributor to their high antioxidant activity [25]. The rosmarinic acid content was determined using HPTLC in samples harvested in summer 2009 (*M. spicata*, 3.6 ± 0.1 %; *M. pulegium*, 1.0 ± 0.1 %; *M.* × *rotundifolia*, 1.0 ± 0.1 %; n = 3).

Rosmarinic acid can clearly be differentiated in the 600 MHz ^{1}H NMR of all species making it a clear biomarker. No other metabolites could be discriminated with the same ease and confidence in these complex metabolomic fingerprints.

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PCA and OPLS-DA approaches for biochemometric analysis are employed as a statistical method to offer a very efficient resolution for classification, particularly, graphical presentations, namely score and loading plots [26]. According to the results of the PCA analysis used to distinguish within *Mentha* species, the ecophysiological factors, edaphic and climatic, are probably responsible for the observed separations between the samples. These most probably influence the metabolic profiles of each *Mentha*, with qualitative and quantitative variations dependent on growth conditions [27].

- For *M. pulegium*, the differences in the PCA scores leading to the separation of two regions were examined. Two of three samples located in the negative PC1 regions were collected at high altitude [636 m (3) and 803 m (6)], the altitude of region 3 (636 m) is almost double compared to region 1 (383 m). Different average temperatures and precipitations were also observed in these regions. In contrast, the two samples placed in the positive PC1 were collected at lower altitude [383 m (1), 482 m (2)]. The sample situated in the center of the plot was also taken at low altitude [330 m (4)]. There is an exception, the sample collected at the lowest altitude [6.26 m (5)] is found in the negative PC1 region. This may be the consequence of higher precipitations during the year (975 mm) in comparison to that in other locations. Temperature may also positively affect the separation of *M. pulegium* 5 and 6 and negatively affect the separation of *M. pulegium* 1 and 2. The soils of the regions 2 and 5 are also not of the same type (Table 1).
- For *M.* × *rotundifolia*, ecological factors may also explain the observed differences. Location 4 (Khemis- Meliana) is quite different from all others and characterized by strong temperature differences according to season, with a climate hot and dry in summer and cold in winter; location 6 (Chemini) is characterized by high altitude (803 m) and low precipitations. The amount of clay in the soils of the regions 4 and 6 was also different. We noted a cluster of four regions (1, 2, 3 and 5). Whereas regions 1, 2 and 3 are relatively similar in altitudes, climate and soil, there is no obvious parallelism for region 5, which may point to eventual biotic factors, so far non-identified, to explain the observed clustering.
- For *M. spicata*, the altitude and temperature showed impact on separation of the samples. Specimens collected in the regions 1 (El-Ghaba) and 2 (Thybyatine) and placed in the positive PC1 were collected at lower altitudes [383 m (1), 482 m (2)]. Besides, these regions almost have the same average temperatures [11.6 °C (1), 11.1 °C (2)]. On the other hand, the rain amounts may negatively affect *M. spicata* harvested in regions 4 (Khemis-Meliana) and 5 (Tichy). These regions have a medium soil according to their clay rate. In a further study we performed (data not shown), the extracts of *M. spicata* from these two regions showed the highest levels of total phenolics and rosmarinic acid.

These results are consistent with the few data reported in the literature for *M. pulegium*. Karray-Bouraoui et al. [28] documented that some factors like environment, development stage and their interaction have an important influence on phenolic contents. Severe environmental conditions may increase phenolic biosynthesis since these compounds preserve the plant during times of UV, salt or pathogenic stress. We previously inferred an implication of *M. pulegium* polyphenol amounts and antioxidant capacity in environmental adaptations [8]. In addition, Riahi et al. [23] demonstrated the impact of the ecotype on the phenolics content of Tunisian germplasms of *M. rotundifolia* L.

Apart these climatic factors, other non-identified factors, such as local phytopathogens and parasites, probably play a vital role in the metabolic profiles of Algerian *Mentha*.

To further extend our understanding of the dependence of the *Mentha* metabolic and biosynthetic patterns on geographical origin, the total antioxidant activity of the samples extracts was determined. The production of antioxidant molecules by plants is associated with defence against oxidative stress [21]. The total antioxidant capacity can be evaluated by measuring the amount of transition metal ion Mo (VI) that is reduced by the test samples [25]. In this study the results indicate that the activity varies within and between the three species. Samples of *M. pulegium* and *M.* × *rotundifolia*, harvested from location 5 demonstrated the strongest antioxidant activity (1900 ± 35 and 1825 ± 177 mg/g GAE of dry extract, respectively). These two regions are characterized by a hot winter with a relatively high average temperature, which may favour the biosynthesis of the antioxidant secondary metabolites such as phenolics. For *M. spicata*, samples collected in region 4 showed the highest activity, which may be due to the lack of rain in the region. Other abiotic and biotic factors such as the nature of the soil could also have an effect. In a study by Karray-Bourraoui et al. [28] looking at the antioxidant activity of *Mentha* species, *M. pulegium* samples with the highest activity were isolated from areas with the hardest climatic and edaphic conditions. The same trend was noticed by Riahi et al. [23] and Ben Haj Yahia et al. [10] who investigated the antioxidant activities of two *M.* × *rotundifolia* L. ecotypes from Tunisia.

Among the three species investigated in the current study, *M. pulegium* has the most potent antioxidant effect. Phenolic compounds are considered as the principal antioxidants in polar extracts; carbohydrates may also act as antioxidants and contribute to oxidative stress stabilization [21]. The results of this study suggest that rosmarinic acid probably plays an important role in this regard. However, further research is required in order to determine which compounds effectively explain the observed antioxidant activity of such complex extracts.

From the results of loading plots, the discriminating signals indicate that secondary metabolites, possibly flavonoid glycosides, might account as major potential biomarkers to separate growth locations of Algerian *M. pulegium*, *M.* × *rotundifolia* and *M. spicata* according to ecotypes. In addition, some terpenoids could be as contributors to the discrimination among Algerian ecotypes of *M.* × *rotundifolia*.

As regards the potential biomarkers discriminating *Mentha* species, their identity was postulated from ^{1}H NMR chemical shifts analysis and by literature analysis. According to the corresponding S-plots, flavonoids of the luteolin-type, *i.e.* luteolin 7-O-glucoside, luteolin 7-O-rutinoside and luteolin 7-O-glucuronide, could be responsible for the separation of *M. pulegium* and *M.* × *rotundifolia* from *M. spicata* [29]. Oxygenated terpenoids, such as piperitenone, pulegone, carvone, and hydrocarbon-type terpenoids, for example pinene, caryophyllene, could be biomarkers to classify *M.* × *rotundifolia* and *M. spicata*, respectively [30]. All discriminating signals of each species were validated by PCA-X indicating the discriminating shifts are capable to classify the three *Mentha* species.

5. Conclusion

This study indicates that the application of ^{1}H NMR-based metabolomics to polar extracts can be a high throughput and reproducible method for the inter and intra-species classification of *Mentha* samples collected in different locations in Algeria. Each *Mentha* extract was prepared for NMR acquisition by a quite simple extraction/drying/dissolution method and the experiment time required for each measurement was about 5 min. Several diagnostic ^{1}H NMR signals have been identified, and although not unique to *Mentha* species their intensity and could be proposed for *Mentha* identification and quality control. From these, some metabolites were proposed as likely discriminating biomarkers for each *Mentha* species.

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The genus *Mentha* is represented in the flora of Algeria by 5 major species [*Mentha rotundifolia* (L.) Huds., *Mentha pulegium* L., *Mentha spicata* L., *Mentha aquatica* L., and *Mentha longifolia* (L.) L.] (Quezel and Santa, 1963). Among these local species, the present work has investigated the three most abundant species and those most used by the population. But, despite the easiness of NMR metabolomics, other analytical methods are indeed required for full *Mentha* identification; further research is in progress with a view to confirm and complete the identification of biomarkers discriminating these *Mentha* growing in their corresponding ecotypes. This is especially important for a locally harvested and possibly cultivated plant, considering there are so many close species and difficult-to-distinguish hybrids in culture throughout the world.

Declaration of Competing Interest

Authors declare that there are no conflicts of interest.

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