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## ORIGINAL ARTICLE

# Intraspecific chemical variability of the essential oils of Moroccan endemic *Origanum elongatum* L. (Lamiaceae) from its whole natural habitats

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## KEYWORDS

*Origanum elongatum*;  
Morocco;  
Essentials oils;  
Chemical composition;  
GC/MS

**Abstract** In this study, the chemical variation of the essential oils of the endemic species *Origanum elongatum* has been studied in its biogeographical context. Essential oils of 168 individual plants collected from 30 populations growing wild in two Moroccan mountains: Rif and Middle Atlas, were analyzed by GC-FID (Gas Chromatography with Flame Ionization Detector), GC/MS (Gas Chromatography/Mass Spectrometry) and <sup>13</sup>C NMR (Nuclear Magnetic Resonance). *Origanum elongatum* produces an EOs yielding after hydrodistillation from 0.81% to 3.12% based on the dry weight of the original biomass. 28 compounds were identified, with a majority of oxygenated monoterpenes among them carvacrol, thymol and *p*-cymene constitute the most represented compounds. Moreover, a great amount of  $\alpha$ -terpinene, limonene, thymoquinone and thymohydroquinone were reported in some samples. Four chemical groups have been identified, namely; carvacrol, carvacrol/thymol, carvacrol/*p*-cymene and thymol. The geographic distributions of these chemotypes appear to vary since the carvacrol chemotype was the most distributed, while the thymol was found more abundantly in populations from Rif. However, the carvacrol/*p*-cymene

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chemotype was revealed only in Taza region. For the first time the whole chemical picture of the EOs of *O. elongatum* is presented in relation to their geographical distribution such knowledge is primordial in developing a breeding program in order to protect this species widely and intensively used for both medicinal and food uses but also supply the continuous market needs by producing high quality and stable raw material.

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## 1. Introduction

Various essential oils (EOs) of medicinal and aromatic plants constitute great alternatives in many medicinal, agriculture and food product applications (Bnouham et al., 2006; Ju et al., 2018; Zoubiri and Baaliouamer, 2011). In most cases, EOs are characterized by great variability in their components. This chemical polymorphism results from complex factors, including genetic and environmental effects, which influence, in combination or independently, the EOs quantitative and qualitative composition and properties (Crocoll et al., 2010; Kofidis et al., 2003). In fact, the chemical composition of OEs could help to define the value of each medicinal and aromatic plant, besides the fact that it could be used in addition to the morphological and genetic data, as interspecific taxonomic traits, especially for many complex taxonomic groups such as *Origanum* (Edris 2007; Skoula et al., 1999).

*Origanum* L. is one of the most popular aromatic herbs of Lamiaceae family and is characterized by high EOs chemical variability. Carvacrol represents the major compounds in most species (Aboukhalid et al., 2016; De Mastro et al., 2017; Skoula et al., 1999) but many other typical chemotypes has been reported such as thymol in *O. compactum* (Aboukhalid et al., 2016), *p*-cymene in *O. vulgare* subsp. *glandulosum* (Mechergui et al., 2016) and linalool in *O. vulgare* subsp. *virens* (Figu  r  do et al., 2006b). Furthermore, plants of the same species growing in different environmental conditions may differ in their EO compositions, and in some cases different chemotypes could be found within the same population (Aboukhalid et al., 2016; Lukas et al., 2013; Toncer et al., 2010).

Oregano was found to be a very useful medicinal plant due to its strong and diverse biological activities as antibacterial, antifungal, antioxidant and/or anti-inflammatory (Bouhdid et al., 2008; Bouyahya et al., 2017; Han and Parker, 2017; Khan et al., 2018). In addition, at culinary scale, oregano is characterized by digestive, carminative and stimulant properties (Guarrera 2003). Besides, it is used to prepare numerous local and Mediterranean traditional dishes of which the pizza remains the most well-known. For these reasons, all wild populations of *Origanum* are unfortunately subjected to massive and intensive over-exploitation which have greatly increased the vulnerability of many *Origanum* species and threatened some of them to extinction (Aboukhalid et al., 2017b). Therefore, the protection of *Origanum* populations should be a high priority now and domestication is considered as a great alternative to supply the continuous market needs by producing high quality and stable raw material, and at the same time, alleviate the pressure on natural resources from over-harvesting.

The flora of Morocco comprises five *Origanum* taxa, of which *O. compactum* and *O. vulgare* subsp. *virens* are

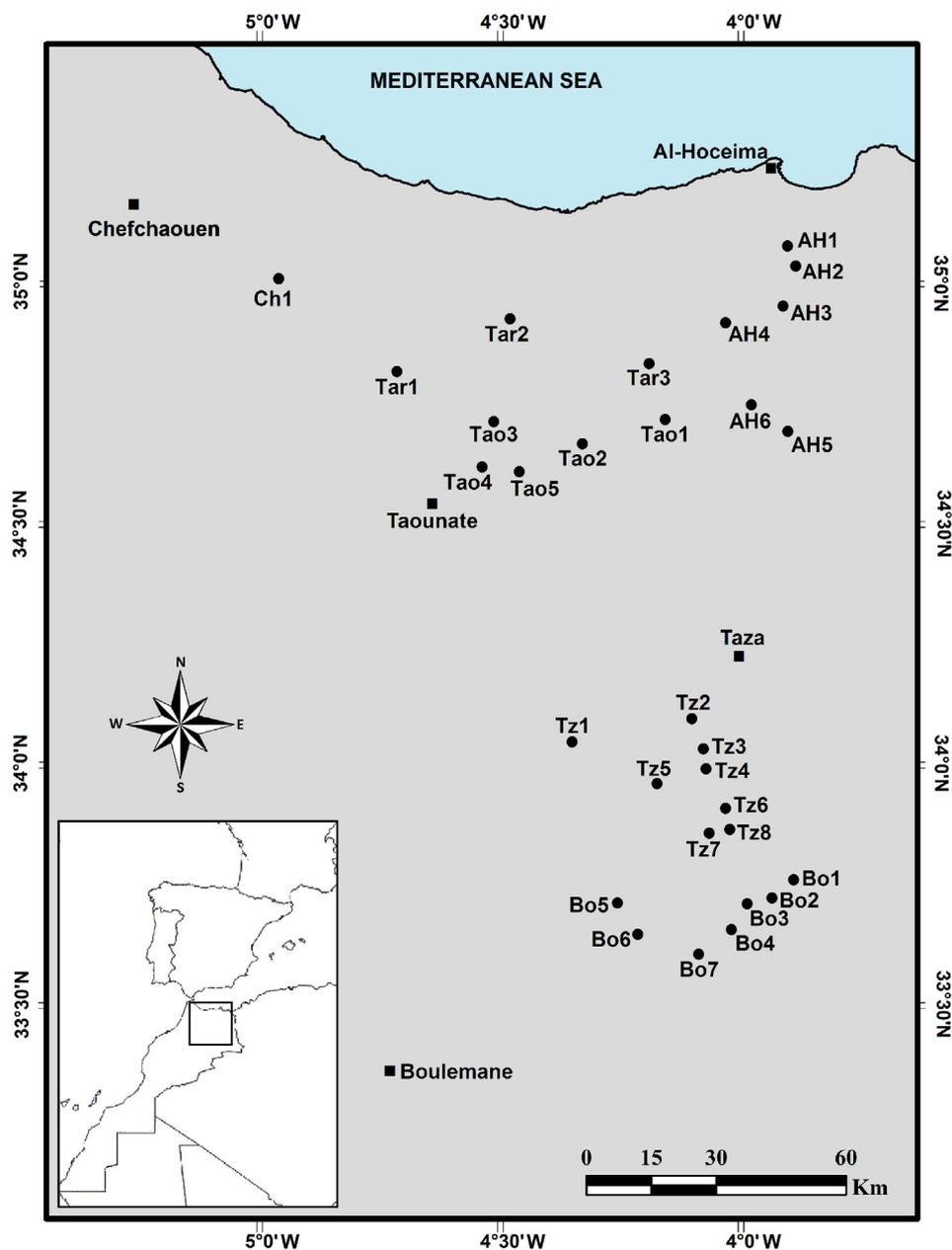
considered as Iberian-Moroccan taxa; while the three others taxa, *O. elongatum*, *O. grosii* and *O. × font-queri* are endemic to Morocco (Bakha et al., 2017). The endemic species *O. elongatum* (Bonnet) Emb. & Maire belongs to the *Elongatispica* section, and is recognized in Morocco by its Arabic common name ‘‘Zaatar’’. The geographic distribution of wild *O. elongatum* is limited to North East (NE) of Morocco and extends from the Middle Atlas to the Rif Mountain ranges, mostly in high elevation. Morphologically, many specific botanical characters such as long branches, very loose spikes and glabrous leaves and stems are distinctive (Ietswaart 1980).

The chemical variability of EOs of Moroccan *Origanum* taxa has been the subject of only a few studies (Bellakhdar and El Idrissi, 1990; El Moussaoui et al., 2013; Figu  r  do et al., 2006a; Ramzi et al., 2017). These studies did not cover the entire geographical area of the species, and only some specified the geographical origin of the samples. Our previous works (Aboukhalid et al., 2016; Aboukhalid et al., 2017a) are considered as the first deep study on the Moroccan wild *O. compactum* populations that covered the total geographical distribution of the species in Morocco. In this context and to continue the chemical characterization studies of Moroccan *Origanum* taxa, this present investigation is the first one which aims to study the chemical variability at intra-population level of the EOs of the endemic species *O. elongatum*, collected from its whole geographical distribution. Consequently, this study provides useful scientific data to promote in situ conservation and to select chemotypes for eventual domestication program.

## 2. Material and methods

### 2.1. Plant material

The sampling of *O. elongatum* plants was performed from wild populations during July 2016 using individual plants sampling method (each sample represents one genotype). 168 individual plants in the flowering stage were collected from 30 populations covering most of the geographical repartition of the *O. elongatum*, endemic to Morocco. These populations were grouped within 6 regions which all belong to 2 biogeographical areas: the Rif Mountains range including Chefchaouen, Targuist, Al-Hoceima and Taounate regions; and the Middle Atlas range covering Taza and Boulmane regions (see map, Fig. 1). Names of accessions, local climate, elevations and number of samples are reported in the Table 1. M. Ibn Tattou (Scientific Institute of Rabat-Morocco) carried out botanical identification and representative Voucher specimens were deposited in the Herbarium of National Institute of Agronomic Research, Rabat (INRA).



**Fig. 1** Geographical localizations of the 30 studied populations of *O. elongatum* (noted in Table 1). The maps were generated using ArcGIS Ver. 10.1.

## 2.2. Essential oil extraction

Aerial parts from individual plants were dried in the shade and then submitted to hydrodistillation for 3 h using a Clevenger-type apparatus. The amounts of the dry biomass used for distillation ranged from 40 g to 100 g. Essential oil yields were estimated for each sample and expressed by (%); weight of the EOs (g) for 100 g of dry plant material using the following formula reported by Marion et al. (1994):

$$EO \text{ yield (\%)} = \frac{\text{weight of EO obtained by distillation (g)}}{\text{weight of dry biomass (g)}} \times 100$$

Then, the EOs were stored in a freezer in amber vials until analysis.

## 2.3. Essential oil analysis

All EO samples were analyzed by GC-FID equipped with two columns of different polarities (to correct for any component coelutions), GC/MS and  $^{13}\text{C}$  NMR. The GC-FID analysis associated with Retention Indices was used for quantification, whereas GC/MS was used for identification of components in combination with  $^{13}\text{C}$  NMR following a methodology developed by the University of Corsica (Tomi and Casanova, 2006).

**Table 1** Geographic and climatic data, sample numbers and EO yield of investigated wild populations of *O. elongatum*.

Region		Accession No	Accession location	Sample number	Samples order	Elevation	Climate	EO yield %	
Rif Mountains rang	Chefchaouen	Ch1	Bab Berred	7	Ch1.1–Ch1.7	1216	Humid	1.14	
		Targuist	Tar1	Ketama	7	Tar1.1–Tar1.7	1496	Per-humid	3
	Tar2		Imassinene	7	Tar2.1–Tar2.7	1593	Sub-humid	0.93	
	Tar3		Beni Ammart	7	Tar3.1–Tar3.7	1489	Per-humid	1.22	
	Al-Hoceima	AH1	Issoufiyène	6	AH1.1–AH1.6	805	Semi-arid	1.48	
		AH2	Ait Mechaar	6	AH2.1–AH2.6	681	Semi-arid	1.53	
		AH3	Imassinene	7	AH3.1–AH3.7	1306	Semi-arid	1.04	
		AH4	Chakran	6	AH4.1–AH4.6	1815	Sub-humid	0.81	
		AH5	Tighezratine	7	AH5.1–AH5.7	1250	Humid	1.35	
		AH6	Ajdir	7	AH6.1–AH6.7	1151	Humid	1.20	
	Taounate	Tao1	Boured	5	Tao1.1–Tao1.5	843	Humid	2.05	
		Tao2	Ain Hamra	6	Tao2.1–Tao2.6	813	Humid	1.82	
		Tao3	Tabrente	4	Tao3.1–Tao3.4	941	Humid	2.50	
		Tao4	Bouhouda	6	Tao4.1–Tao4.6	874	Humid	3.04	
		Tao5	Bni Wanj	7	Tao5.1–Tao5.7	778	Humid	2.79	
	Middle Atlas rang	Taza	Tz1	Tazeka1	7	Tz1.1–Tz1.7	1382	Sub-humid	1.80
			Tz2	Tazeka2	4	Tz2.1–Tz2.4	1344	Sub-humid	1.18
			Tz3	Chiker	4	Tz3.1–Tz3.4	1323	Sub-humid	1.13
			Tz4	Maghraoua	3	Tz4.1–Tz4.3	1379	Sub-humid	0.90
			Tz5	Bni Zlita	7	Tz5.1–Tz5.7	1263	Sub-humid	3.12
Tz6			Tamourghout	6	Tz6.1–Tz6.6	1402	Sub-humid	1.38	
Tz7			El arssa	7	Tz7.1–Tz7.7	967	Sub-humid	1.66	
Tz8			Oued Albared	4	Tz8.1–Tz8.4	1550	Sub-humid	3	
Boulmane		Bo1	Berkile	3	Bo1.1–Bo1.3	1396	Sub-humid	1.15	
		Bo2	Icholass	4	Bo2.1–Bo2.4	1726	Sub-humid	0.92	
		Bo3	Timiznay	7	Bo3.1–Bo3.7	1866	Semi-arid	2.43	
		Bo4	Sidi Belkacem	6	Bo4.1–Bo4.6	1654	Humid	0.88	
		Bo5	Bouyablanel	5	Bo5.1–Bo5.5	1627	Humid	1.43	
		Bo6	Bouyablanel2	2	Bo6.1–Bo6.2	1560	Humid	1.19	
		Bo7	Talzemt	4	Bo7.1–Bo7.4	1713	Humid	0.92	

### 2.3.1. Gas chromatography (GC-FID) analysis

Gas chromatography (GC) analyses for all EOs samples were performed with a Clarus 500 PerkinElmer (PerkinElmer, Courtaboeuf, France) equipped with a FID and two fused-silica capillary columns (50 m × 0.22 mm, film thickness 0.25 µm), an apolar BP-1 (polydimethylsiloxane) and a polar BP-20 (polyethylene glycol). The oven temperature had increased from 60 °C to 220 °C at 2 °C/min and then held isothermal at 220 °C for 20 min, the injector temperature was 250 °C and the detector temperature was 250 °C. The helium (He) (0.8 ml/min) was used as a carrier gas. 30 mg of EO was diluted in 400 µl of CHCl<sub>3</sub> and the injected volume was 1 µl using a split ratio of 1/60. The relative proportions of the EOs constituents were expressed as percentages obtained by peak-area normalization, without using correction factors. Moreover, the retention indices (RI) were determined in relation to the retention times (RT) of a series of *n*-alkanes (C7–C28) with linear interpolation (Target Compounds software from Perkin Elmer).

### 2.3.2. GC/MS analysis

The analysis of EOs were carried out using a Perkin-Elmer TurboMass detector (quadrupole), directly coupled to a

Perkin-Elmer Autosystem equipped with a fused-silica capillary column (50 m × 0.22 mm i.d., film thickness 0.25 µm), BP-1 dimethylpolysiloxane. The carrier gas used was He at 0.8 ml/min using a split ratio of 1/60. The injection volume was 1 µl and the injector temperature was 250 °C, where the oven temperature programmed from 60 °C to 220 °C at 2 °C/min and then held isothermal (20 min). The Ion source temperature was 250 °C with energy ionization of 70 eV and electron ionization mass spectra were acquired over the mass range 40–400 Da.

### 2.3.3. <sup>13</sup>C NMR analysis

The <sup>13</sup>C NMR analyses were used to identify some compounds notably thymoquinone, carvacryl methyl oxide and thymohydroquinone. These analyses were performed using a Bruker AVANCE 400 Fourier Transform spectrometer operating at 100.63 MHz for <sup>13</sup>C and equipped with a 5 mm probe, in deuterated chloroform (CDCl<sub>3</sub>), with all shifts referred to internal tetramethylsilane (TMS). 30 mg of essential oil diluted in 300 µl of CDCl<sub>3</sub>. <sup>13</sup>C NMR spectra were recorded with a pulse width (PW) of 5 µs (flip angle 45°), an acquisition time of 2.7 s for 128 K data table with a spectral width (SW) of 25,000 Hz (250 ppm) and digital resolution 0.183 Hz/pt. The

number of accumulated scans was 2500 for each sample (about 50 mg of EO in 0.5 ml of  $\text{CDCl}_3$ ).

#### 2.3.4. Identification of individual components

The identification of components was based on: (1) comparison of their retention indices (RI) on polar and apolar columns determined relative to the retention times of a homologous series of *n*-alkanes under the same operating conditions with linear interpolation to those of authentic compounds or literature data; (2) by comparing the mass spectra with those recorded in the computer matching with laboratory-made and commercial mass spectral libraries (Adams 2007; König et al., 2001); and (3) on comparison of the signals in the  $^{13}\text{C}$  NMR spectra of EO with those of reference spectra compiled in the laboratory spectral library with the help of laboratory-developed software (Ouattara et al., 2014).

#### 2.4. Statistical analysis

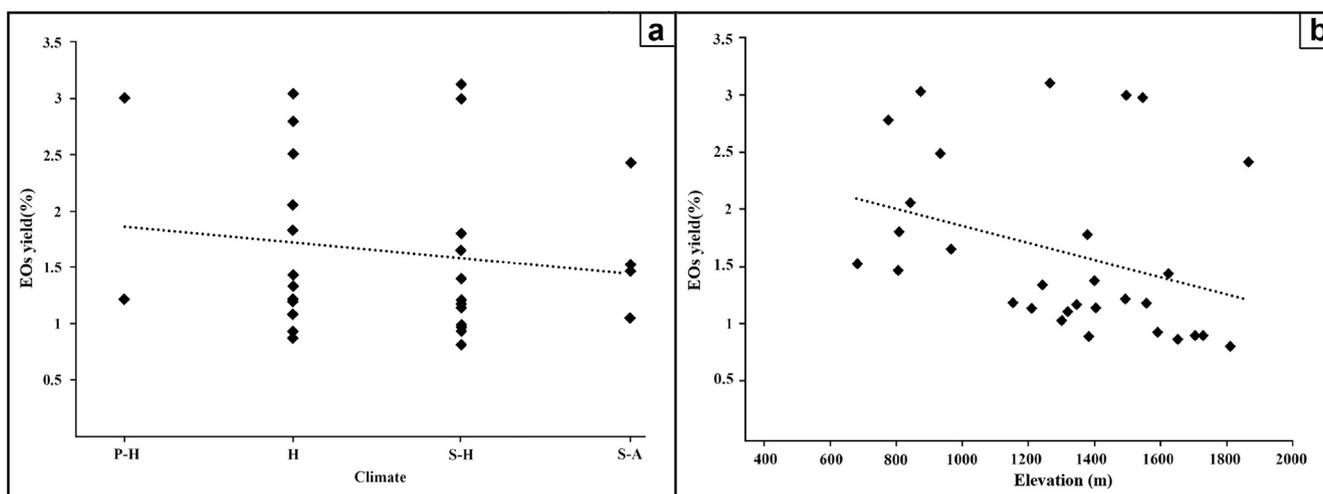
The principal component analysis (PCA) and hierarchical cluster analysis (HCA; Ward's method) were performed with PAST (Paleontological Statistics Software Package) 3.14 version (Hammer et al., 2001).

### 3. Results and discussion

The natural distribution of *O. elongatum* is limited to the NE of Morocco, where it is neighbouring with *O. compactum* in the Rif Mountains and with *O. vulgare* subsp. *virens* in the Middle Atlas. The 30 studied populations are growing under different climate conditions, including semi-arid, sub-humid, humid and per-humid, and on an altitudinal limit between 681 m and 1866 m, with the majority of the populations growing upper to 1300 m (Table 1, Fig. 1). It is very important to highlight that for the first time the EOs of wild *O. elongatum* have been studied for individual plants from a large number of accessions covering its whole distribution range.

Essential oil yields of the studied *O. elongatum* populations ranged from 0.81% to 3.12% with an average of 1.62%. These values are in accordance with those reported by Bellakhdar and Il Idrissi (1990), but lower than those found by Ramzi et al. (2017), where the yields were reported between 3.4% and 3.7%. These EOs yield values obtained in *O. elongatum* are important, although *O. elongatum* is considered as a glabrous species (Ietswaart 1980), and that could be linked to its high biomass production, since *O. elongatum* is characterized by a ratio of (leaves + flowers) / stem oscillating between 40% and 50%, where the flowers constitute up to 70% of the total biomass yield. Essential oil yields of *O. elongatum* seemed to not be influenced by climatic conditions (Fig. 2, a;  $p = 0.42$ ,  $R^2 = 0.02$ ). However, Aboukhalid et al. (2016) revealed that the EOs yields of the Moroccan *O. compactum* were considerably influenced by the climatic conditions where higher values were found in semi-arid climate. In addition, no correlation was observed between the EOs yield of *O. elongatum* and the altitudinal gradients (Fig. 2, b;  $p = 0.07$ ,  $R^2 = 0.11$ ). In general, EOs yields increase when the elevation declines as reported by several studies confirming the impact of the altitudinal gradient on the EOs production (Kofidis et al., 2003; Vokou et al., 1993), that could be due to water stress (Bahreininejad et al., 2014) and temperature (Aissi et al., 2016). Moreover, many others environmental factors could also influence the EOs yield, such as soil pH (Aboukhalid et al., 2017a).

The chemical composition of the EOs of the 168 individual plant samples of the Moroccan endemic *O. elongatum* analyzed by GC/MS revealed the presence of 28 compounds, represented by 13 oxygenated monoterpenes, 11 monoterpene hydrocarbons and 4 sesquiterpene compounds. The total identified percentages ranged from 90.79% to 99.27% with a mean percentage of 97.38%, dominated by oxygenated monoterpenes (M 82.64%, 46.15–93.15%), monoterpene hydrocarbon compounds (M 13.56%, 1.1–48.66%) while the sesquiterpene compounds were found in low concentration (M 1.18%, 0–7.01%). Interestingly, the most dominant compound was carvacrol (M 59.57%, 0.84–88.63%) followed by thymol



**Fig. 2** EOs yields of *O. elongatum* related to: (a) the climate conditions (S-A: semi-arid; S-H: sub-humid; H: humid; P-H: per-humid) and (b) the altitudinal gradient of the studies populations.

(M 16.73%, 0.04–80.79%) and *p*-cymene (M 9.04%, 0.2–43.51%). Moreover, many other particular compounds notably; limonene,  $\alpha$ -terpineol, thymoquinone, carvacryl methyl oxide and caryophyllene oxide, which are considered rare in *Origanum* taxa, were detected at a percentage reaching more than 4% in some individual plants.

The statistical analysis (PCA and hierarchical cluster analysis) displayed that the present panel of *O. elongatum* samples is characterized by a chemical polymorphism with appreciable inter and intrapopulation variability. In fact, the data related to the chemical composition of EOs were presented according to the chemical groups as well as their geographical distribution.

### 3.1. Chemical variability

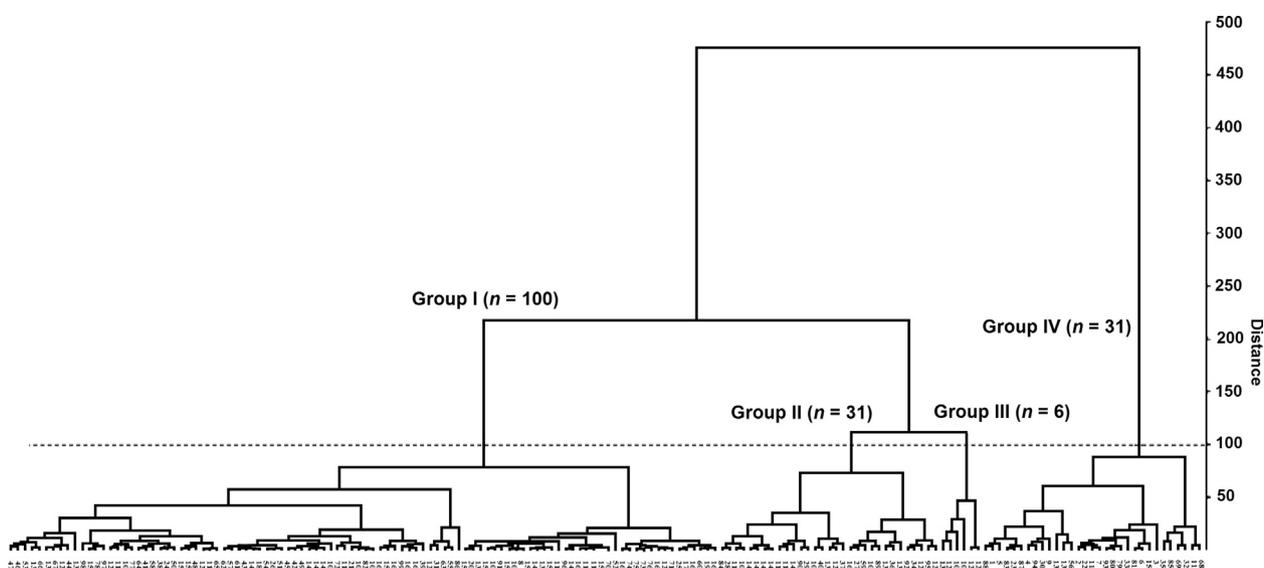
To classify the present investigated samples on chemical groups according to their major constituents, the entire data set was analysed by a Cluster Analysis (CA) using the *Ward's* method. Therefore, four different chemical groups have been distinguished. Fig. 3 represents the corresponding dendrogram, while the percentages of the chemical compounds (Mean, Min–Max) of each group are presented in Table 2.

Group I: this carvacrol-rich group (M 77.45%, 57–88.63%) is the most important one in terms of number of samples, including 100 individual plants (59.52% of samples). Several previous studies reported that the carvacrol constitutes the most dominant compound of the majority of taxa of the genus *Origanum* (Aboukhalid et al., 2016; Mechergui et al., 2016; Skoula et al., 1999). The second major compound in this group is *p*-cymene (M 7.63%, 0.20–22.95%) where 4 samples; AH1.3, AH5.2, AH6.3 and Tz5.7 presented high amounts, ranging from 16.78% to 22.95%. Moreover, many atypical compounds were observed in some samples of this group.

Interestingly, two samples exhibited noticeable amounts of terpinene derivatives:  $\alpha$ -terpineol (10.76%, Tao4.4) and  $\alpha$ -terpinene (7.87%, AH4.5). Regarding  $\alpha$ -terpineol, this compound reached higher levels in some other *Origanum* taxa

e.g. 40.3% in *O. vulgare* subsp. *vulgare* (Lukas et al., 2013), 55.7% in *O. cordifolium* (Figu  redo et al., 2005), 68.3% in *O. vulgare* subsp. *virens* (Melagari et al., 1995) and up to 73% in *O. majorana* (Novak et al., 2008). Besides, this compound reached up to 25.8% in the Moroccan *O. compactum* (Aboukhalid et al., 2016). For  $\alpha$ -terpinene (7.87%), the obtained value is the highest one detected in the genus *Origanum*, the previous studies reported values always lower than 6.6% (Skoula et al., 1999). In addition, several samples exhibited noticeable amounts of phenolic derivatives: around 5% for thymohydroquinone (AH4.6, Tz1.4 and Tz8.3), thymoquinone (6.09%, Tz8.3) and carvacryl methyl oxide (12.66%, Tao4.4). Thymoquinone and thymohydroquinone are, on that subject, very important compounds especially known for their high and strong biological activities such as anticancer, antioxidant, antidiabetic, anti-inflammatory and hypolipidemic activities (Farkhondeh et al., 2017; Taborsky et al., 2012). In fact, both compounds were rarely reported for the genus *Origanum*, of which thymoquinone is considered typical for *O. dictamnus* (22.9%) (Skoula et al., 1999), *O. syriacum* (up to 27.7%) (Toncer et al., 2010; Zgheib et al., 2016) and *O. vulgare* subsp. *hirtum* (3.56%) (Skoula et al., 1999), while thymohydroquinone was detected in lower amounts (1–2.5%) in *O. dictamnus*, *O. vulgare* subsp. *hirtum*, *O.   minoanum*, *O.   intercedens* (Skoula et al., 1999) and in *O. acutidens* (Figu  redo et al., 2006c). However, no trace of thymoquinone and thymohydroquinone has been detected in several samples of the Moroccan *O. compactum* samples, according to recent studies (Aboukhalid et al., 2016; Bakhy et al., 2014). On the other hand, carvacrol methyl oxide was usually found in low percentages in the EOs of some *Origanum* taxa, whereas, highest amounts were however detected in *O. vulgare* subsp. *glandulosum* (22.9%) (Houmani et al., 2002) and *O. compactum* (36.2%) (Aboukhalid et al., 2016).

Group II: 31 individual plant samples belonging to this group, in which carvacrol (M 55.67%, 40.31–75.57%), thymol (M 22.29%, 8.83–38.16%) and *p*-cymene (M 8.47%, 2.67–14.39%) represented the most abundant compounds.



**Fig. 3** Dendrogram obtained by cluster analysis, representing chemical composition similarity relationships among the 168 EO samples of *O. elongatum* (Group I: carvacrol, Group II: carvacrol/thymol, Group III: carvacrol/*p*-cymene and Group IV: thymol).

**Table 2** Chemical composition and chemical groups of *Origanum elongatum* EO (Content [%]: Mean (Min – Max)).

Compounds <sup>a</sup>	RI <sub>a</sub>	RI <sub>p</sub>	Total (168)	Group I (100)	Group II (31)	Group III (6)	Group IV (31)
$\alpha$ -Thujene	925	1 017	0.38 (0–1.49)	0.33 (0–1.19)	0.30 (0–0.66)	1.21 (0.83–1.49)	0.47 (0–1.39)
$\alpha$ -Pinene	932	1 014	0.28 (0–0.88)	0.24 (0–0.74)	0.24 (0–0.53)	0.69 (0.53–0.88)	0.33 (0–0.72)
Camphene	945	1 064	0.05 (0–0.5)	0.04 (0–0.19)	0.04 (0–0.33)	0.12 (0.10–0.16)	0.06 (0–0.50)
1-Octen-3-ol	962	1 446	0.43 (0–1.99)	0.39 (0–1.10)	0.39 (0–0.91)	0.62 (0.41–1.00)	0.58 (0–1.99)
3-Octanone	965	1 254	0.28 (0–1.33)	0.25 (0–1.04)	0.20 (0–0.46)	0.60 (0–1.30)	0.42 (0–1.33)
$\beta$ -Pinene	972	1 111	0.07 (0–0.33)	0.06 (0–0.18)	0.06 (0–0.12)	0.18 (0.15–0.21)	0.09 (0–0.33)
3-Octanol	979	1 389	0.05 (0–1.32)	0.05 (0–1.32)	0.03 (0–0.43)	0.05 (0–0.13)	0.07 (0–0.81)
Myrcene	983	1 159	0.38 (0–2.04)	0.32 (0–1.88)	0.31 (0–0.90)	0.55 (0–2.04)	0.64 (0–1.84)
$\alpha$ -Phellandrene	999	1 164	0.05 (0–0.93)	0.04 (0–0.21)	0.03 (0–0.09)	0.07 (0–0.15)	0.09 (0–0.93)
$\delta$ -3-Carene	1 007	1 147	0.03 (0–0.57)	0.03 (0–0.46)	0.01 (0–0.04)	0.17 (0.06–0.57)	0.03 (0–0.17)
$\alpha$ -Terpinene	1 011	1 179	0.63 (0–7.87)	0.56 (0–7.87)	0.49 (0–0.90)	1.60 (0.84–2.24)	0.79 (0–3.27)
<i>p</i> -Cymene	1 014	1 270	9.04 (0.2–43.51)	7.63 (0.20–22.95)	8.47 (2.67–14.39)	31.53 (19.00–43.51)	9.79 (1.02–21.27)
Limonene	1 023	1 200	0.28 (0–12.58)	0.18 (0–1.03)	0.19 (0–0.34)	0.57 (0.37–0.67)	0.64 (0–12.58)
$\gamma$ -Terpinene	1 050	1 243	2.37 (0–20.62)	1.72 (0.02–9.73)	1.74 (0–5.32)	3.01 (0–13.98)	4.98 (0.48–20.62)
<i>Trans</i> sabinene hydrate	1 054	1 461	0.68 (0–2.54)	0.69 (0–2.31)	0.67 (0–1.65)	0.36 (0.08–0.64)	0.72 (0.19–2.54)
Linalool	1 085	1 544	2.41 (0.17–5.87)	2.24 (0.17–5.52)	2.67 (0.63–4.16)	1.51 (0.36–2.95)	2.88 (0.38–5.87)
Borneol	1 149	1 696	0.17 (0–1.11)	0.18 (0–0.97)	0.18 (0–1.11)	0.16 (0.08–0.19)	0.14 (0–0.44)
Terpinen-4-ol	1 163	1 598	0.65 (0–1.34)	0.64 (0–1.23)	0.69 (0–0.99)	0.66 (0.35–1.07)	0.63 (0–1.34)
$\alpha$ -Terpineol	1 173	1 691	0.44 (0–10.76)	0.45 (0–10.76)	0.45 (0–3.22)	0.63 (0.16–1.39)	0.38 (0–2.19)
Thymoquinone	1 216	1571	0.36 (0–6.09)	0.41 (0–6.09)	0.36 (0–2.83)	1.05 (0–2.50)	0.07 (0–1.07)
Carvacryl methyl oxide	1 226	1601	0.16 (0–12.66)	0.25 (0–12.66)	0.03 (0–0.65)	0 (0–0.02)	0.03 (0–0.60)
Thymol	1 271	2 179	16.73 (0.04–80.79)	0.98 (0.04–4.73)	22.29 (8.83–38.16)	14.49 (0.10–25.82)	62.40 (42.13–80.79)
Carvacrol	1 279	2 206	59.57 (0.84–88.63)	77.45 (57.85–88.63)	55.67 (40.31–75.57)	35.30 (13.62–51.11)	10.52 (0.84–35.75)
( <i>E</i> )- $\beta$ -Caryophyllene	1 417	1 590	0.74 (0–2.35)	0.73 (0–1.96)	0.84 (0–1.82)	0.95 (0.21–2.35)	0.63 (0–1.42)
$\alpha$ -Humulene	1 450	1 662	0.02 (0–0.12)	0.02 (0–0.09)	0.03 (0–0.09)	0.03 (0–0.12)	0.02 (0–0.07)
$\beta$ -Bisabolene	1 501	1 720	0.03 (0–0.43)	0.04 (0–0.43)	0.04 (0–0.22)	0.11 (0–0.25)	0.01 (0–0.18)
Thymohydroquinone	1 516	2176	0.71 (0–5.02)	0.95 (0–5.02)	0.56 (0–4.15)	0.51 (0–2.29)	0.15 (0–2.12)
Caryophyllene oxide	1 569	1 974	0.38 (0–4.41)	0.39 (0–4.41)	0.39 (0–2.50)	0.36 (0.01–0.80)	0.34 (0–2.64)
Monoterpene hydrocarbons (MH)			13.56 (1.1–48.66)	11.16 (1.1–26.95)	12.59 (6.53–20.40)	39.69 (30.11–48.66)	17.89 (4.26–47.75)
Oxygenated monoterpenes (OM)			82.65 (46.15–93.15)	84.92 (69.53–93.14)	83.47 (76.85–90.25)	55.93 (46.15–54.71)	78.99 (50.47–93.15)
Sesquiterpenes hydrocarbons (SH)			0.79 (0–2.7)	0.78 (0–2.46)	1 (0.39–1.97)	1.09 (0.29–2.7)	0.66 (0–1.5)
Oxygenated sesquiterpenes (OS)			0.38 (0–4.41)	0.39 (0–4.41)	0.39 (0–2.5)	0.36 (0.01–0.8)	0.34 (0–2.64)
Total identified [%]			90.79–99.25	90.79 – 99.25	93.6 – 98.95	95.64 – 98.58	94.98 – 98.7

<sup>a</sup> Order of elution and percentages have been given on apolar column, all compounds are identified by GC-FID, GC/MS and <sup>13</sup>C NMR (for compounds over 1%), RI<sub>a</sub>, RI<sub>p</sub> retention indices on apolar and polar column.

This group is the most homogeneous since only few differences were noticed particularly in AH2.2, AH2.3, Tz7.5 and Tz8.2, which are characterized by considerable amounts of thymohydroquinone ranging from 2.93 to 4.15%.

Group III: this group is considered as *p*-cymene rich. It is the least frequent one and consists of 6 individuals (Tz2.4, Tz3.3, Tz6.1, Tz6.4, Tz6.5 and Tz7.6) all sampled from Taza region. The major compounds are carvacrol (M 35.30%, 13.62–51.11%), *p*-cymene (M 31.53%, 19–43.51%) and thymol (M 14.49%, 0.10–25.82%), while all the other compounds were found in percentage below 3%. Indeed, *p*-cymene is derived from  $\gamma$ -terpinene and constitutes the precursor to both carvacrol and thymol in their biosynthetic pathway (Crocoll et al., 2010). Previous studies reported *p*-cymene concentrations higher than those detected in the present study (i) *O. vulgare* subsp. *glandulosum* (up to 46%) (Mechergui et al., 2016), (ii) *O. vulgare* subsp. *vulgare* 49.7% (Lukas et al., 2013), (iii) *O. solymicum* 53.1% (Tümen et al., 1994), (iv) *O. compactum* 58.6% (Aboukhalid et al., 2016), (v) *O. syriacum* 62.18% (Toncer et al., 2010), (vi) in *O. calcaratum* (71.61%) (Skoula et al., 1999) and (vii) the highest values (73.6% and 83.7%) were recorded in *O. saccatum* (Figuérédo et al., 2006c).

Group IV: this group includes 31 individual plants and is considered as thymol-rich (M 62.40%, 42.13–80.79%). Three samples, Ch1.6, Tao3.3 and Bo4.5, presented the highest thymol amount (up to 75%). The thymol chemotype was detected in several *Origanum* taxa as a major compound (De Mastro et al., 2017; Mechergui et al., 2016) where the highest amounts were recorded in *O. compactum* (80.7%) (Aboukhalid et al., 2016) and in *O. vulgare* subsp. *hirtum* (90.2%) (Vokou et al., 1993). This group is also characterized by carvacrol (M 10.52%, 0.84–35.75%), *p*-cymene (M 9.79%, 1.02–21.27%),  $\gamma$ -terpinene (M 4.98%, 0.48–20.62%) and linalool (M 2.88%, 0.38–5.87%). The most dissimilar individual among this group is the individual plant Ch1.3 that reached the highest limonene amount (12.58%). This value is the second highest

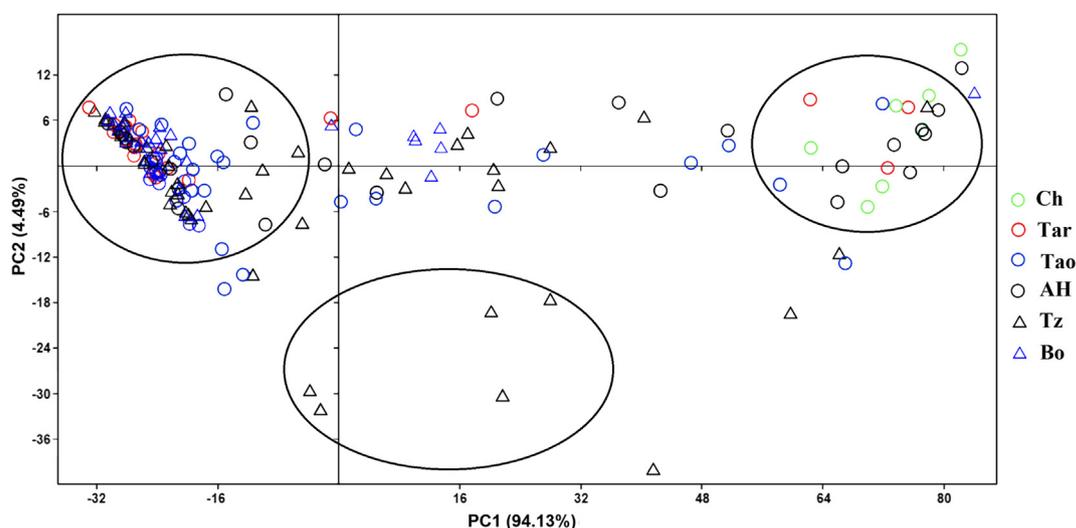
value obtained within all the investigated *Origanum* taxa after that reported in *O. majorana* var. *majorana* (14.3%) (Figuérédo et al., 2006b).

In spite of its limited and specific biogeographical conditions, it seems obvious that *O. elongatum* share the same major compounds (carvacrol, thymol and *p*-cymene) with several other *Origanum* taxa (Skoula et al., 1999), above all with the Moroccan *O. compactum* species. However, the particularity of the EO profile of this endemic species resides mainly in its minor components, notably thymoquinone and thymohydroquinone which were detected considerably in individual plants from Al-Hoceima and Taza regions.

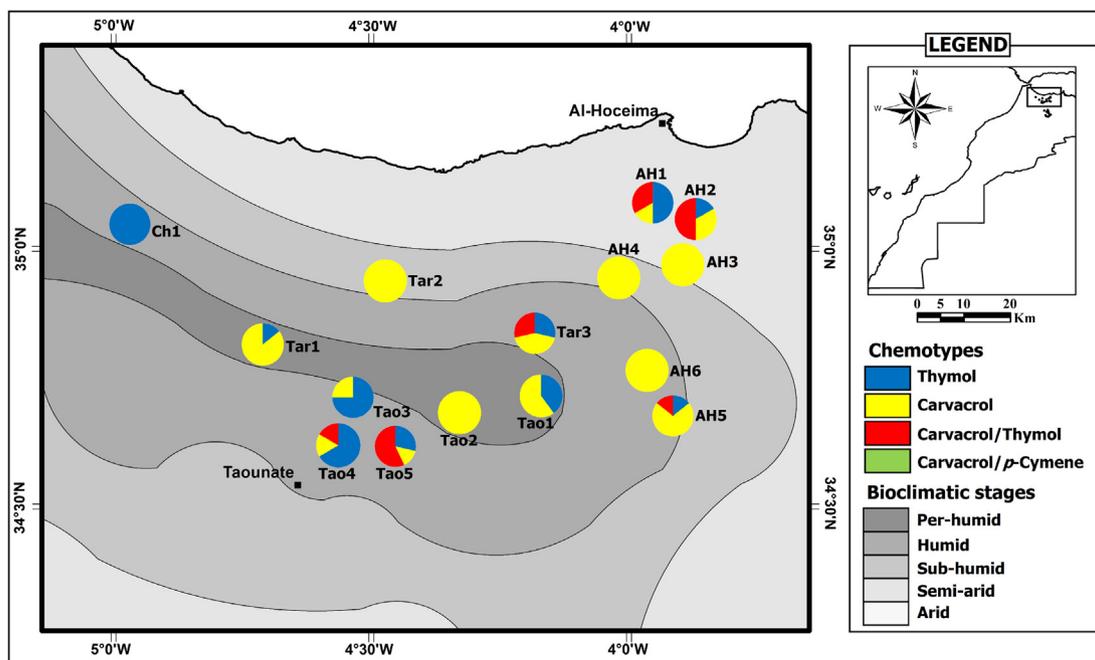
### 3.2. Geographical repartition of chemotypes

The principal component analysis (PCA) was realized based on the concentrations of the 10 major compounds of all samples presenting an average percentages higher than 0.5% ( $\alpha$ -terpinene, *p*-cymene,  $\gamma$ -terpinene, trans-sabinene hydrate, linalool, terpinen-4-ol, thymol, carvacrol, (E)- $\beta$ -caryophyllene and thymohydroquinone). The two first principal components (PC1) and (PC2) highly explained the total data variance (PC1 + PC2 = 98.62%; Fig. 4). In fact, the distribution is highly correlated to the first principal component (PC1 = 94.13%) which displayed a positive correlation with thymol and negatively with carvacrol contents. On the other hand, PC2 was positively related to carvacrol and thymol, and negatively to *p*-cymene content. Individual plants rich in thymol (right side of Fig. 4) are in majority from samples of the Rif region. Plants rich in *p*-cymene (lower part of Fig. 4) were mainly represented by samples from Taza region.

When considering the geographical distribution of the four chemotypes of *O. elongatum*, carvacrol was the most frequently chemotype, detected in 14 populations from both the Rif Mountains and the Middle Atlas. Besides, the carvacrol/thymol chemotype was detected in 8 populations belonging to the Rif Mountains and in 6 populations from the Middle



**Fig. 4** PCA of 168 oil samples from the 6 regions (Ch: Chefchaouen, Tar: Targuist, Tao: Taounate, AH: Al-Hoceima, Tz: Taza, Bo: Boulmane). The individual plants were represented according to their regions since the circles represent regions from Rif Mountains, while the triangles indicate regions from Middle Atlas range.



**Fig. 5** Geographical distribution of the chemotypes (the percentage of individuals of the described chemotypes: Group I: carvacrol, Group II: carvacrol/thymol, Group III: carvacrol/*p*-cymene and Group IV: thymol) in the populations from the Rif Mountains range related to their climatic stages (Ch: Chefchaouen, Tar: Targuist, Tao: Taounate, AH: Al-Hoceima).

Atlas. On the other hand, thymol and carvacrol/*p*-cymene chemotypes exhibited a specific geographical distribution, where thymol was more abundant in the Rif Mountains (detected in 10 of the 15 studied populations) than in the Middle Atlas region (only 4 of the 15 populations) (Figs. 5 and 6). Aboukhalid et al. (2017a) also reported that thymol chemotype is more abundant in *O. compactum* populations growing in northern Morocco. In contrast, carvacrol/*p*-cymene chemotype was detected only in 5 populations of the Middle Atlas.

Furthermore, 11 homogenous populations growing in different regions have been observed, where Ch1 population presented only thymol rich plants and Bo2 population only carvacrol/thymol rich plants, while, the remaining 9 populations, were completely dominated by the carvacrol chemotype. However, the other populations exhibited remarkable chemical diversity. In addition to the impact of the environmental factors on EOs content, the chemical variability could indicate a high genetic diversity at the (intra)population level (Thompson 2002). In the same way the homogeneous populations at the chemical scale could be a signal of low rate of genetic diversity which could result from danger genetic erosion due to the overharvesting.

**Chefchaouen region:** it is considered the most important one in terms of *Origanum* diversity in Morocco, where two others species are growing spontaneously; *O. compactum* and the endemic *O. grosii*. The studied population Ch1 was found in the neighbouring area with *O. compactum* and grows in forest at an elevation of 1216 m. At the chemical level, all the individual plants from this population were found to be rich in thymol (59.53–80.79%).

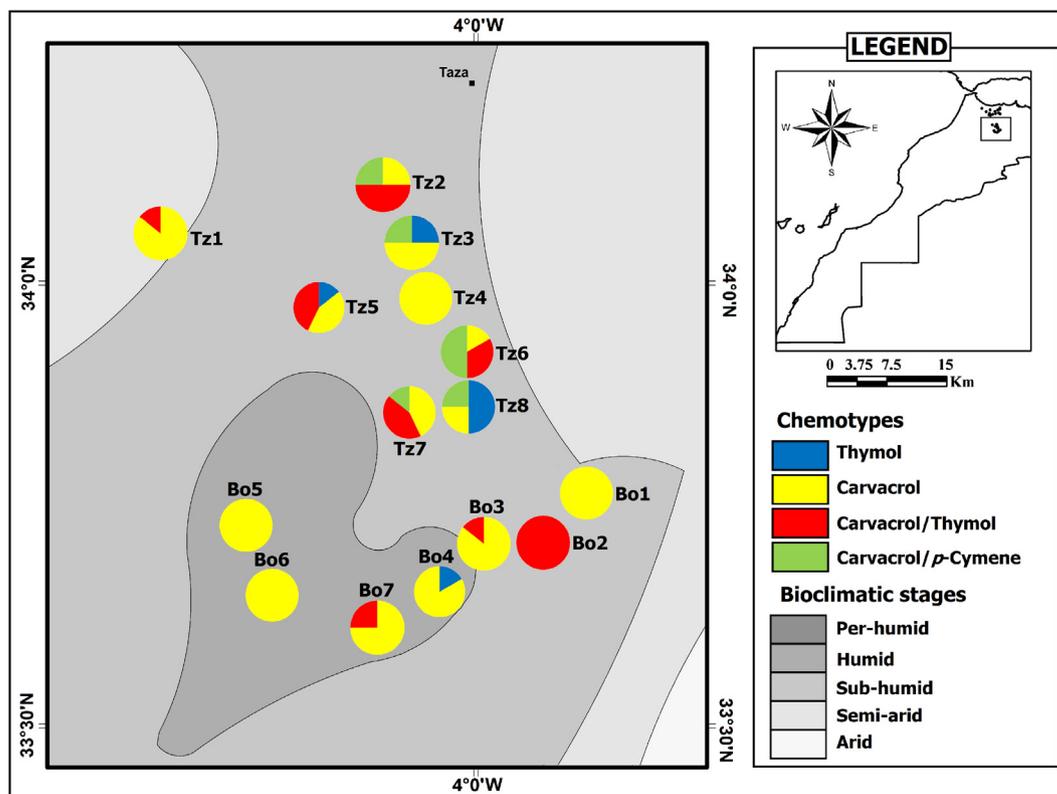
**Targuist region:** three populations growing in open areas characterized by elevations around 1500 m represented this

region. The majority of individuals harvested in Targuist region were characterized by the dominance of the carvacrol chemotype. In fact, all the analyzed samples belonging to Tar2 population consisted of the carvacrol chemotype. Moreover, almost all individuals from Tar1 population were rich in carvacrol, while the thymol chemotype was recorded only in samples from Tar1 and Tar3 populations. However, the chemotype carvacrol/thymol was exclusive to the population Tar3.

**Al-Hoceima region:** the six investigated populations were found in open areas, but at different elevations from 681 to 1815 m, among which the first three populations are growing in semi-arid climate, while the other populations were found under humid and sub-humid climates. Populations, AH3, AH4 and AH6, were found entirely rich in carvacrol, whereas populations, AH1, AH2 and AH5, are more heterogeneous and contained carvacrol, thymol and carvacrol/thymol chemotypes with no clear relation with elevation.

**Taounate region:** the five populations were established in open areas at an elevation below 1000 m. Within this region, we found one plant population (Tao2) with only the carvacrol chemotype. On the other hand, populations Tao1 and Tao3 are represented by plants characterized by the thymol and carvacrol chemotypes, whereas Tar3 and Tao5 populations were distinguished by the presence of three chemotypes, namely carvacrol, thymol and carvacrol/thymol.

**Taza region:** this region appears to be chemically the more diverse since the eight populations sampled represented all the chemotypes detected in *O. elongatum*, in addition the original carvacrol/*p*-cymene chemotype exclusive to this region. At this point, it's difficult to know if the important amounts of *p*-cymene detected in the Taza region could be related to some particular pedoclimatic factors and/or genetic peculiarities.



**Fig. 6** Geographical distribution of the chemotypes (the percentage of individuals of the described chemotypes: Group I: carvacrol, Group II: carvacrol/thymol, Group III: carvacrol/*p*-cymene and Group IV: thymol) in the populations from the Middle Atlas range related to their climatic stages (Tz: Taza, Bo: Boulmane).

Further, studies are needed to address this point. Among the 8 populations, Tz4 is considered homogeneous since all the plants represented the same carvacrol chemotype. However, the other populations are composed by individual plants from two or three chemotypes.

**Boulmane region:** the seven studied populations grew generally in the forest at elevations higher than 1400 m. The carvacrol chemotype is more dominant in this region, representing all individuals from populations Bo1, Bo5 and Bo6, and more than 75% of the samples from Bo3, Bo4 and Bo7 populations. The carvacrol/thymol chemotype was detected in all the samples from Bo2 population, and only in less than 25% of the samples from Bo3 and Bo7 populations, whereas the thymol chemotype was only found in one population of this region (Bo4).

Until now, few published papers have documented the chemical composition of the EO of *O. elongatum*. However, these studies are restricted in terms of number of samples and in terms of the explored geographical area. Five populations of *O. elongatum* originated from the Rif Mountains have been investigated with rich carvacrol (67.34–81.72%) EOs (Ramzi et al., 2017). Another study presented an EO of *O. elongatum* from the Rif region also rich in carvacrol (79.2%) and noticed that cultivated *O. elongatum* plants were in the same way dominated by carvacrol (56.1–63.9%) (Figuérado et al., 2006a). However, Bellakhdar and Idrissi (1990) characterized EOs of *O. elongatum* originated from Middle Atlas rich in thymol 60%, while El Moussaoui et al. (2013) found an EO containing mainly carvacrol, thymol, *p*-cymene and  $\gamma$ -terpinene.

#### 4. Conclusions

Our results present an important quantitative variability in the composition of the EOs of the Moroccan endemic *O. elongatum* across its entire distribution area. In fact, four chemical groups were characterized, of which the carvacrol chemotype is the most represented with a large geographical distribution, the thymol chemotype was found in abundance in the northern part (Rif Mountains), while the carvacrol/*p*-cymene chemotype was observed only in one region (Taza). Moreover, the rare chemotypes detected in this study display the large chemical diversity of *O. elongatum* and represent valuable data for valorization purposes. This chemical richness may provide multiple possibilities of applications not only for food products but also for the medicinal domain. In addition, the study of the biological activities of the chemotypes which contain noticeable amounts of thymoquinone, thymohydroquinone, carvacrol methyl oxide and  $\alpha$ -terpineol will provide valuable results for news applications of the EOs from *O. elongatum*.

Our study highlights that it can be very useful to investigate separately several individual plants from different populations of one species, in order to reveal the different chemical groups within and between populations and to detect certain rare compounds. On the other hand, the geographical and climatic data constitute important parameters for a better understanding of the biosynthesis of EOs of plants occurring under different conditions, even if it's difficult to generalize the potential effects.

This work provides also a great database concerning the chemical polymorphism of EOs of the endemic *O. elongatum*. The important chemical variability should be considered an advantage to select the best genetic material to be involved in any breeding program for this endemic species. These results will be integrated into a general study that aims to characterize and revise all Moroccan *Origanum* taxa in order to promote their in situ preservation as well as to improve their agriculture exploitation. The high ratio of biomass (leaves + flowers) and the significant yields of EOs make it possible to promote both the production of leaves and EOs; and the study of the effects of the domestication process on quantitative and qualitative chemical composition might represent a major principal prospect.

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