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GC-MS identification and *in vitro* evaluation of antioxidant activity of bioactive molecules extracted from *Mentha pulegium* L. and *Thymus capitatus* native to Tunisia

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Abstract *Mentha Pulegium* L. and *Thymus capitatus* are acknowledged as an important aromatic and medicinal plants thanks to their richness in a variety of bioactive phytochemical components. The purpose of the study was to investigate the chemical composition of the essential oil and the antioxidant activity for *Mentha Pulegium* L. and *Thymus capitatus* species cultivated in Tunisia. The essential oil for both species was obtained by means of hydrodistillation method and analyzed by GC/FID and GC/MS. The essential oil yield varied considerably according to the harvest time and species processed (1.37 - 3.1 %). The *Mentha Pulegium* L. essential oil was characterized by the predominance of oxygenated monoterpenes. Pulegone (39.15 %), menthone (35.66%), piperitenone (3.98%) and piperitone (3.55%) were found to be the main constituents. Moreover, *the oil of Thymus capitatus*, gathered in two different months from Tunisia origin, was dominated by oxygenated monoterpenes. Major components were Thymol (76.50- 82.48%), with p-cymene (3.17- 4.01 %), with β- caryophyllene (1.39- 2.78%) and γ -terpinene (1.41-2.07%). Thus, *Mentha Pulegium* L. and *Thymus capitatus* species could be considered a good source of oxygenated monoterpenes. *Furthermore*, the antioxidant activity was assessed by two different methods such as $DPPH^{\cdot}$ and FRAP. *Results showed that T. capitatus* species collected in March exhibit the uppermost activity, followed by EO of the same species gathered in August. Whereas, the *M. pulegium* oil possess the lowest activity. The antioxidant activity varied according to the phytochemical constituent of the investigated essential oil.

Keyword: *Mentha Pulegium* L.; *Thymus capitatus* species; chemical composition; GC/MS; antioxidant activity

Introduction

The Lamiaceae family is considered as one of the most diverse plant families due to their ethnopharmacology importance [1]. It is highly widespread in the Mediterranean, Central Asie, America, Africa and China [2]. This plant family represented by about 230 genus and 7000 flowering species throughout the world including several aromatic and medicinal plant like Lavandula sp., Mentha sp., Marrubium sp., Hyssopus sp., Ocimum sp., Origanum sp., Rosmarinus sp., Salvia sp., Satureja sp., Thymus sp. etc [3]. These plants have a wide range of pharmacological properties such as antispasmodic, antiviral, stimulant digestive, antiseptic, antimicrobial, anti-inflammatory, antioxidant, hepatoprotective, insecticide, aromatic etc, which are mainly based on their volatile oils composition. According to their richness in bioactive molecules, numerous studies were focused on essential oils, extracts for the pharmaceutical, agri-food and cosmetics industries [4].

Mentha puleguim L., a member of the family Lamiaceae, is an aromaticherbaceous plant commonly known as "Pennyroyal" which widely grows in central, Southern and Werstern Europe, North Africa and Asia Minor [5]. The plant is traditionally used as an infusion, treating different gastric disorders and inflammations of the respiratory tract [6]. Moreover, it is widely used as a spice and flavoring agent in aliments [7]. Several reports have showed that pennyroyal oil is rich in Pulegone which is very known for their numerous therapeutic effects such as anti-feedant, antibacterial, antifungal, and insecticide activities [8,9].

Thymus capitatus is an aromatic and medicinal plant belonging to Lamiaceae family commonly acknowledged as "Zaâtr" in Tunisia, which is native to the Mediterranean region [10]. In folkmedicine, the aerial part of this plant is widely served as decoction for their antiparasitic, antitussive, tonic, anti-inflammatory and carminative properties as well as to flavor aliments. The species possess interesting biological and pharmacological activities such as antispasmodic, sedative, antioxidant [11,12], antimicrobial [10,12], antifungal [13], antibacterial [14] and antiaflatoxinogenic properties [15]. Additionally, thymus oil is considered among the world's top 10 essential oilsused to preserve food, thanks to its appreciable antioxidant activity. This is activity is most likely due to the presence of thymol and carvacrol as main constituents. Moreover, several authors have been demonstrated a different chemotype such as carvacrol and thymol, c-terpineol, thujone, geraniol, linalool and others, depending on geographic origin [16].

In the framework, the aim of this work was to determine the chemical composition and in vitro antioxidant activity via two different methods of Tunisian *Mentha Pulegium* L. and *Thymus capitatus* species.

Material and Methods

Plant Material

Aerial parts of *M. pulegium* and *T. capitatus* were gathered in a wild site at about 173 Km from Tunis city, in the mountains area of Kef (36° 10´ 56 ´´ N, 8° 42´53´´ E). *M. pulegium* species was harvested for two months such as august and March during the year 2016. Whereas, *M. pulegium* aerial parts were collected in July 2016 at the same area. After collection, herbs weredried in the shade under ambient conditions.

Essential Oil Extraction

Essential oils were extracted from plant material by hydrodistillation using a Clevenger-type apparatus. 250g of each plant were immersed in 3L water for 3 hours. The essential oil was recovered, dried with anhydrous sodium sulfate and kept at 4°C for further experiments. The extraction yield was calculated as follow:

$$\text{Yield (\%)} = \left(\frac{m}{M}\right) * 100$$

Where: m: is the weight of EO in grams, M: is the weight of raw material in grams

GC–MS Analysis

Analytical gas chromatography was applied using an Agilent 7890A GC- MSD system Agilent with a HP-5 MS capillary column (30m × 0,25mm; film thickness=0,25 µm). Nitrogen was used as carrier gas. GC oven temperature was initially kept at 50°C and programmed to 120°C for 5 min and then programmed to 280°C for 8 min and finally raised to 300°C for 2 min. Mass spectra were recorded at 70 eV. A volume of 1 µl was injected in the split mode. The analysis was lasted 40 min. The identification of the volatile molecules was realized by comparison of their retention time (RT) with those found in the literature or with those of authentic components available in the authors' laboratory. Further identification was made by matching their mass spectra with those stored in the Wiley 09 NIST 2011 mass spectral library of the GC/MS data system.

Assessment of *in vitro* antioxidant activity

DPPH. Scavenging assay

The DPPH· Radical scavenging activity was evaluated using the method described by Hanato et al **[17]**, with some modifications. Briefly, 500 µL of various dilutions of samples (EO or standard) were added to 500 µl of 0.2 mM DPPH solution. The mixture was thoroughly shaken and incubated for 30 minutes in the dark. After that, the

absorbance of each sample was measured at 517 nm using *UV-vis spectrophotometer*. Free radical inhibition by DPPH• was expressed as percent (%) and calculated according the following formula:

$$\%\ inhibition = (A\ (blank) - A(sample)/A\ (blank)) * 100$$

where A_{sample} is the absorbance of DPPH• solution with sample.

A_{blank} is the absorbance of DPPH• solution without antioxidant.

All results were expressed in terms of IC_{50} (µg/mL), which represents the EO concentration required to scavenge 50% of DPPH• radicals.BHT was used as standard. All measurements were realized in triplicate.

Ferric Reducing Antioxidant Power (FRAP) Activity

The ferric reducing ability of various EOs were assessed by the method described by Oyaizu et al [18]. This method consists of mixing 100µL of various dilutions of samples (Essential oil or standard) with 250 µL of 0,2 M sodium phosphate buffer solution (pH=6.6) and 250 µL of potassium ferricyanide (1%). After an incubation of 20 minutes at 50° C, 250 µL of trichloroacetic acid (TCA, 10 %) were added to the blending that was centrifuged at 650 g for 10 min. Then, 250 µL of upper layer fraction was mixed with 250 µL of distilled water and 50 µL of ferric chloride (0.1%) and thoroughly mixed. After that, the absorbance of each sample was measured at 700 nm using *UV-vis spectrophotometer. BHT was served as a positive control. Results were expressed in Effective concentration (EC_{50}) and was obtained through linear regression analysis.*

Results and Discussion

Yield and Chemical Composition of *M. Pulegium* and *T. capitatus* EOs

The extraction yield of *M. pulegium* and *T. capitatus* Eos obtained via hydrodistillation varies from 1.37 to 3.1 % (Table 1). The highest yield was obtained by *M. pulegium* EO gathered in July. In the other hand, the lowest yield was observed in the *T. capitatus* EO collected in March. This allows us to consider the pennyroyal oil as an interesting source of EO. Presented results demonstrated the difference in the extraction yield, which might be explained by the difference among the chemical composition.The identified compounds from the aerial parts of the two species (*M. puleguim* and *T. capitatus*) studied, their retention time and their relative content are listed in Table 1.

A total of fifty components, representing 88.76 - 97.08 % of the essential oil of *M. pulegium* and *T. capitatus* were identified. The *T. capitatus* EOs were characterized by its richness in oxygenated monoterpenes (81.99 – 85.51 %) and monoterpene hydrocarbons (6.23 – 8.76 %). The minor volatile fractions were represented by sesquiterpene hydrocarbons (1.45- 3.74 %) and oxygenated sesquiterpenes (0.80 – 1.05%). The main constituent of *T. capitatus* species was Thymol (76.50- 82.48%). Comparing the chemical content and composition of *T. capitatus* species collected in two different period, we can clearly observed that the chemical composition change according to the time of harvest. As illustrated in Table 1, it is important to point that the relative content of oxygenated monoterpenes and monoterpene hydrocarbons was found to be higher in the month of August than the EO obtained from the same species gathered in March. Whereas, the content of sesquiterpenes hydrocarbons and oxygenated sesquiterpenes reached the minimum in the month of August. *T. capitatus* EOs species was primarily dominated by Thymol (76.50- 82.48%), with p-cymene (3.17- 4.01 %), with β- caryophyllene (1.39- 2.78%) an γ –terpinene (1.41-2.07%). Thymol, γ -terpinene and p-cymene reached the highest content in the month of august. However, the content of β- caryophyllene was maximal in March. Further, the essential oil composition of *T. capitatus* harvested in August was characterized by the presence of carvacrol with the relative content equal to 3.18 %. Thus, it was obvious that harvesting time affected the essential oil yield and chemical composition of *T. capitatus* grown in Tunisia. The impact of climatic conditions the essential oil composition was confirmed by several studies. The examined, *T. capitatus* EO, displayed quite different phytochemical constituents from those reported from other area. Megdich et al reported that carvacrol (76.5%) was the major compound of collected from Ras-Jdir and Zaghouan sites (sub humid bioclimatic stage) [19]. In the other hand, other works indicated that Tunisian *T. capitatus* species was characterized by the presence of carvacrol as abundant compound [20-22]. Furthermore, Chedia et al [23] and Mkaddem et al [12] were found that the Tunisian *T. capitatus* was dominated by Thymol.

		Species		
		T. capitatus		*M. pulegium*
		August	**March**	**July**
Extraction Yield (%)		2.52	1.37	3.1
Compounds	**RT**	**%**	**%**	**%**
Sabinene	4.91	0.30	0.22	-
1R-α-pinene	5.02	-	0.16	0.20
1S-α- Pinene	5.02	0.29	-	-
3-methyl cyclohexanone	5.25	-	-	0.07
Camphene	5.25	0.11	0.09	
β-phellandrene	5.57	-	-	0.07
β -pinene	5.64	-	0.05	0.20
2(10)-pinene	5.64	0.07	-	-
β-thujene	5.76	0.68	0.44	0.12
3-octanol	5.80	-	-	0.50
α -phellandrene	6.01	0.09	0.05	-
3-carene	6.10	0.04	0.02	-
α-terpinene	6.19	0.62	0.33	-
p-cymene	6.30	4.01	3.17	0.02
p-menth-3-ene	6.37	0.34	0.18	-
γ-terpinene	6.80	2.07	1.41	-
cis-β-Terpineol	6.93	0.07	0.05	-
(+)-4-Carene	7.20	-	-	0.02
Terpinolene	7.25	0.09	0.05	-
Linalool	7.37	0.87	0.77	-
1-methyl-4-(methylethyl)-(E)-2-cyclohexenol	7.81	0.04	-	-
Menthone	8.44	-	-	35.66
Borneol	8.66	0.65	0.81	
Isopulegone	8.85	-	-	2.75
p-menth-1-en-4-ol	8.88	-	0.56	-
Terpinen-4-ol	8.88	0.69	-	-
Alpha-Terpinyl propionate	9.16	-	-	0.21
p-menth-8-en-2-one	9.32	0.07	0.08	-
Pulegone	10.43	-	-	39.15
D-carvone	10.58	0.04	-	-
Piperitone	10.79	-	-	3.55
Citral	11.29	0.05	-	-
o-cymen-5-ol	11.56	0.09	-	-
p-thymol	11.94	0.12	-	-
Thymol	12.30	82.48	76.50	-
Trans-carane	12.48	-	-	0.09
Piperitenone	13.48	-	-	3.98
Eugenol	13.87	0.10	0.19	-
Carvacrol	14.22	3.18	-	-
Alpha-gurgujene	15.21	-	0.05	-
β-cariophyllene	15.43	1.39	2.78	0.13
1,1,4,8-tetramethyl-cis,cis-4,7,10-cycloundecatriene	16.12	0.05	0.16	0.33
Germacrene D	16.63	-	-	0.09
(+)-ledene	16.88	-	0.21	-
β-bisabolene	17.05	-	0.18	-
γ-cadinene	17.20	-	0.06	-
Cadina-1(10),4-diene	17.33	-	0.12	-

Espatulenol	18.26	-	0.17	-
Caryophyllene oxide	18.36	0.80	-	-
Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (Volatile organic compounds)	24.73	0.44	-	1.62
Total		97.08	93.27	88.76
Monoterpene hydrocarbons		8.76	6.23	0.749
Oxygenated Monoterpenes		85.51	81.99	85.69
Sesquiterpenes hydrocarbons		1.45	3.74	0.55
Oxygenated Sesquiterpenes		0.80	1.05	0
Others		0.56	0.26	1.78

However, our results were in agreement with several studies performed on *T. capitatus* species collected from different area of north Africa mainly Morocco and Algeria, indicating that thymol was the principal component [24,25]. Furthermore, the chromatographic analysis of *M. pulegium* EO extracted via hydrodistillation showed the clear predominance of oxygenated monoterpenes (85.69 %). Whereas, the content of monoterpene hydrocarbons (0.74) and sesquiterpenes hydrocarbons (0.55) were found to be lower. Major components were pulegone (39.15 %), menthone (35.66%), piperitenone (3.98%) and piperitone (3.55%). The studied essential oil of *M. pulegium* growth from Tunisia displayed a similar chemical composition from those reported from other geographical origin. Only a quantitative variation was observed. The *M. pulegium oil* from Iran contained pulegone (46.18%) as main constituent [26]. Zantaret al were found that the EO obtained from Morocco was dominated by pulegeone (77.16%) and pepiritenone (6.54 %) [27].

Moreover, Ouakouak et al demonstrated that the Algerian *M. pulegium* EO was dominated by pulgeone (46.31%), piperitenone (23.3%), menthone (6.2%) and limonene (4.7%) [28]. On the other hand, Mahboubi et Haghi showed that the oils of M. pulgeium was dominated by piperitone/ piperitenone [29]. The oil of morocco region reported by El Houssine et al contained piperitone (35.56%) and piperitenone (21.18%) as major constituent [30]. Further, the essential oil obtained from Morocco region was characterized by the presence of piperitone as principal compound [31]. Whereas, the oil of *M. pulegium* of Brazil region was found to be rich in pulgeone [32]. This quantitative variation can be mainly related to their geographic appurtenances. Comparing the two different species, we can clearly observe the variation of class composition and major compound. This allowed us to conclude that the chemical composition change according several factors like climate condition, species, harvest time, extraction method and ecological factors.

In Vitro Antioxidant Activity

In this study, two assays were used to evaluate the *in vitro* antioxidant activity of *M. puleguim* and *T. capitatus* Eos using the $DPPH^{\cdot}$ and FRAP assays. All results were presented in Table 2.

Table 2: Antioxidant activity of *M. puleguim* and *T. capitatus* EO as evaluated by $DPPH^{\cdot}$ and FRAP

Species	**Months**	**Antioxidant activity**	
		$DPPH^{\cdot}$ (IC_{50},µg/mL)	**FRAP (EC_{50} ,µg/mL)**
M. puleguim	July	6800	790
T. capitatus	March	142	210
	August	180	228
BHT		18	20

The antioxidant activity evaluated of different EOs varied considerably between species and months of collect. As shown in Table 2, the highest $DPPH^{\cdot}$ activity was obtained from *T. capitatus* species collected in March, followed by EO of the same species gathered in August. Such variation can be explained by the simple fact that the chemical

composition varies according to the collection period which can generate a different antioxidant activity. Whereas, *M. pulegium* EO exhibit the lowest activity to that all EOs with an IC_{50} value equal to 6800 µg/mL. Results obtained for *T. capitatus* species were similar to the study reported by El Ouariachi et al demonstrating that the *T. capitatus* essential oil possesses a DPPH IC_{50} equal to 103 µg/mL [33]. On the other hand, results of Salah-Fatnassi et al indicated that the DPPH scavenging activity of *T. capitatus* EO ranged from 113.2 to 334.05µg/mL according to the area of collect [34]. Džamićet al showed that *T. capitatus* EO have also reduced the radical DPPH with an IC_{50} equal to 119.40 µg/mL [35]. The difference between *T. capitatus* species in the antioxydante activity can be related to the Physiological or external factors that allow each species to possess specific phytochemical compounds and therefore varying biological proprieties. Furthermore, despite the predominance of oxygenated monoterpenes in the phytochemical constituents of *M. pulegium* EO, the reducing power of this plant species is very low. As reported in the Kamkar et al study, the *M. pulegium* EO exhibit a lower antioxidant activity (IC_{50}= 1473.6 µg/mL) [36].

The $DPPH^{\cdot}$ results confirm those found for the FRAP test. Therefore, EO recovered from *T. capitatus* gathered in March exhibit the most important activity, followed by EO obtained by the same species collected in August. While, the EC_{50} found for EO of *M. pulegium* was lower than all other results with a value equal to 790µg/mL. According to the literature, we found that our results are in agreement with those found by Abou-Elella et al [37]. On the other hand, Bounatirou et al shows that the reductive potential ranged from 0.4 to 1000 µg/mL according to the flowering phase [20]. Other studied reported by Salarbashi et al on the *M. Pulegium* species demonstrated EO possess a feeble activity [38]. All results show that the antioxidant activity can be influenced by several factors such as species, collect period, flowering stage. Based in the literature, numerous studies have already demonstrated the impact of some factors likeregion of cultivation, cultural conditions and extraction methods on antioxidant activity [39,40].

Conclusion

Data of this work underline the high variation of the essential oil composition among Tunisian *M. pulegium* and *T. capitatus*. Pennyroyal and *T. capitatus* EOs prepared by hydrodistillation was characterized by a clear predominance of oxygenated monoterpenes. The chromatographic analysis showed that the oil of *M. pulegium could* be considered as a valuable source of pulgeone, a monoterpene ketone, which is very known for its therapeutic effects. Whereas, thymus oil contains a high content of thymol. Overall findings demonstrated that *T. capitatus* and *M. Pulegium* can be used as a natural source of bioactive molecules with potential application in food, cosmetic and pharmaceutical industries.

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