Effect of Harvest Time on Yield, Chemical Composition, Antimicrobial and Antioxidant Activities of *Thymus vulgaris* and *Mentha pulegium* Essential Oils

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**Authors’ contributions**

This work was carried out in collaboration between all authors. All authors designed the study. Author SZ performed the experimental work. Authors AL, MC and MHZ supervised author SZ and managed the analysis of the study. Authors DG and MB realized the taxonomic identification of plants, performed the statistical analysis and chemical identification of oils components. Authors SZ, MHZ and RP managed the literature searches, wrote the first draft of the manuscript and did the final moderation of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

**Aims:** This work aims to study the effect of the harvest time (pre-flowering and full-flowering) on the yield, chemical composition, antimicrobial and antioxidant activities of *Thymus vulgaris* and *Mentha pulegium* essential oils (EOs).

**Study Design:** Leaves and flowers from each period (pre and full-flowering) were used for EOs extraction. EO yield, chemical composition, antimicrobial and antioxidant activities were determined for each plant at the two harvest times studied.

**Place and Duration of Study:** The studied plants (*T. vulgaris* and *M. pulegium*) were collected during the period of April 2011 (Pre-flowering) to June 2011 (Full-flowering). Experiments were conducted at the chemistry and microbiology laboratory of the National Institute of Agronomic Research of Tangier (Morocco).

**Methodology:** The EOs were extracted via steam distillation. Chemical composition has been determined by a GC/MS analysis. Antimicrobial activity against *Escherichia coli* (SCTC 471), *Salmonella Senftenberg* 775W (ATCC 43845), *Listeria monocytogenes* (SCTC 4031) and *Staphylococcus aureus* (SCTC 976) was determined by paper disc agar plates. Antioxidant activity were determined by the radical scavenging activity assay.

**Results:** The greatest yield for *T. vulgaris* (3.6%) and for *M. pulegium* (3.5%) EOs was obtained during the full-flowering period. The chromatographic analysis showed that the studied EOs were constituted mainly by carvacrol for *T. vulgaris* and pulegone for *M. pulegium*. Harvest time affected quantitatively but not qualitatively the chemical composition of both EOs. *T. vulgaris* EO showed a greater antimicrobial and antioxidant activity than that of *M. pulegium*. The antimicrobial and antioxidant activities were maximal during the full-flowering period for *T. vulgaris* EO whereas they were greater in the pre-flowering period for *M. pulegium* EO.

**Conclusion:** The full-flowering period would be the best time to harvest *T. vulgaris* plants to obtain EOs with better yield, antimicrobial and antioxidant activity. In contrast, the pre-flowering stage would allow producers to obtain a *M. pulegium* EO with higher antimicrobial and antioxidant activities although with a lower yield.

*Keywords:* Essential oils; harvest time; yield; chemical composition; antimicrobial activity; antioxidant activity.

1. INTRODUCTION

Essential oils of medicinal and aromatic plants are subjected to a high demand. This demand is justified by the biological effects exhibited by some active compounds contained in these oils and by their condition of natural products as an alternative for replacing chemical substances in the pharmaceutical, cosmetic and food industries [1].

Among biological properties investigated in EOs, antimicrobial and antioxidant activities have been widely demonstrated [2-6]. For this reason, they have been proposed as potential agents for food preservation.

Nowadays, the major concern for EOs producers is to obtain oils with better quality and quantity, and with known and stable biological properties. However, the quantity (yield) and the quality (chemical composition) of EOs are influenced by the interaction among several factors such as genotype, environment, plant age, season and time of harvest [7-11].

While the effect of the vegetative cycle on yield and chemical composition of EOs has been well demonstrated [7,8,12-15], its implication on biological properties has scarcely been studied. Among the few published data in this sense, it can be deduced that the plant vegetative cycle is of great importance in determining EOs biological properties [16,17].

Due to its geographical position, Morocco offers a wide range of Mediterranean bioclimates allowing the growth of rich flora, which is constituted by more than 4200 species. Aromatic and medicinal plants are estimated by 500 to 600 species, and most of them are endemic [18].

Morocco is a traditional supplier of medicinal and aromatic plants to Europe. More than twenty plants are used for EOs or other aromatic extracts production to be used in perfumery and cosmetic industry, as hygiene products, etc.
**T. vulgaris** and **M. pulegium** are two spontaneous aromatic and medicinal plants belonging to the Lamiaceae family that grow in several regions of Morocco. Both aromatic plants have an undeniable commercial interest because of the diverse biological activities of their extracts which have been widely demonstrated [19-22].

Despite the great interest of both plants, to the best of our knowledge, there are no studies about the effect of the vegetative cycle (pre-flowering and full-flowering stages) on their antioxidant or antimicrobial activities, which makes difficult the adequate exploitation of these natural resources.

This study aims to investigate the effect of the vegetative cycle (pre-flowering and full flowering stages) on the yield, chemical composition, antimicrobial and antioxidant activity of the EOs of **T. vulgaris** and **M. pulegium** harvested in the north and north east of Morocco, respectively.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

The studied plants (**T. vulgaris** and **M. pulegium**) were collected during the period of April 2011 (Pre-flowering) to June 2011 (Full-flowering). **T. vulgaris** plants were harvested in the region of Beni Idder (391m, 35°23’59”N, 5°30’35”W) in the Northeast of Morocco and **M. pulegium** plants from Bougedour in the North (35m, 35°39’35”N, 5°50’59”W). The taxonomic identification was performed following the procedure described by Fennane et al. [23]. Subsequently, plants were dried for 48 h at 40°C under ventilation. Leaves and flowers were used for the extraction of EOs.

### 2.2 Essential Oils Extraction

The EOs were extracted via steam distillation for 2 h using a Clevenger-type apparatus. The supernatant was separated by decantation after adding 50% of NaCl. The EOs were stored in sealed glass vials at 4°C prior to analysis. The yield was based on the dry weight of the samples (% w/w). EOs were collected and stored at 4°C in dark bottles in order to protect them from heat and light radiation.

### 2.3 Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

The GC/MS analyses were carried out using a gas chromatograph coupled with Mass Spectrometer (MS) (GC-MS Trace GC ultra - ITQ900, Thermo Scientific, USA) operating in the electron-impact ionization mode (70eV). Mass acquisition mode ranged from 40 to 450 m/z. The capillary column used was 1MS (30 m x 0.25 mm x 0.25 μm film thickness). Oven temperature programmed from 50 to 200°C at 10°C/min and from 200 to 290°C at 35°C/min. Helium was used as the carrier gas at a constant flow of 1 mL/min. Injector and MS transfer line temperatures were set at 250 and 300°C respectively. Diluted samples in n-hexane (v/v) of 1 µL were injected in the split mode at a ratio of 1:20. The analysis was repeated twice for each sample.

Identification of the components was made by the determination of the their retention indices (KI) relative to those of a homologous series of n-alkanes (C8-C26) (Fluka, Buchs/sq, Switzerland) and by matching their recorded mass spectra with those stored in the spectrometer data base (NIST MS Library v. 2.0) and the bibliography [24]. Quantification was computed as the percentage contribution of each compound to the total amount present.

### 2.4 Antimicrobial Activity

EO antimicrobial activity was determined by paper disc agar plates. Microbial strains were provided by the Spanish Collection of Type Cultures (CECT). Two Gram negative bacteria, *Escherichia coli* (SCTC 471) and *Salmonella Senftenberg* 775W (ATCC 43845), and two Gram-positive, *Listeria monocytogenes* (SCTC 4031) and *Staphylococcus aureus* (SCTC 976), were used. A volume of 10 mL of Muller Hinton Agar medium (Liofilchem, Roseto degli Abruzzi, Italy) (MHA) was poured into Petri dishes. One hundred μL of culture bacteria were plated at a final concentration of 10⁶ cells/mL approximately.

After 15 min, a paper disc Whatman No. 1 of 6 mm (Whatman International L7d Maidstone, England), impregnated with 10 μL of the EO, was placed on the agar surface. The plates were incubated for 24 h at 37°C (*E. coli, Salmonella Senftenberg, S. aureus*) or 48 h at 30°C (*L. monocytogenes*). The diameters of the resulting zones of inhibition were measured including the diameter of the paper disc. An inhibition zone (disk diameter included) lower than 9 mm was considered as non inhibitory. An average zone of inhibition was calculated after carrying out three independent replicates.
2.5 Antioxidant Activity

2.5.1 Radical scavenging activity assay

Following the method described by Blois [25], for each EO, 1 mL from a 1 mM methanol solution of 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich) (DPPH) was added to 3 mL of diluted EO in ethanol (1, 15, 30 uL/mL), butylated hydroxytoluene (Sigma-Aldrich) (BHT) (reference) or ethanol (control). Mixtures were shaken and incubated (room temperature/dark). After 30 min, absorbance at 517 nm was measured. Scavenging activity of DPPH radical was calculated as follows:

\[
\frac{[A_{\text{blank}} - A_{\text{sample}}]}{A_{\text{blank}}} \times 100
\]

where \( A_{\text{blank}} \) is the absorbance of the control reaction (containing all reagents except the test compound), and \( A_{\text{sample}} \) is the absorbance in presence of the test compound.

2.6 Statistical Analysis

Analyses of variance were performed by Statgraphics Plus 4.0 statistical software. All experiments were performed in triplicate and the results were presented as the mean with its standard deviation (n = 3) in each case. Differences were considered significant at \( P \leq 0.05 \).

3. RESULTS AND DISCUSSION

3.1 Yield and Chemical Composition

The yields of EO extraction for \( T. vulgaris \) and \( M. pulegium \) plants, as a function of the vegetative cycle, are illustrated in Table 1. For \( T. vulgaris \) EO, average yield at pre-flowering period was 1.9% while it was 3.6% at full-flowering stage. These percentages are relatively high when compared to some published data that found values ranging from 0.5 to 1.2% [7,18,26]. Table 1 also shows that yield values for \( M. pulegium \) EO were 0.9% and 3.5% for pre and full-flowering period respectively. These percentages were similar to those obtained by Hmiri et al. [20]. Therefore, these results demonstrate that the highest yield was obtained during full-flowering period. Therefore, it seems advisable to control the plants vegetative period in order to optimize oil production and obtain the maximum yield per plant. Similar trend has been previously reported by other authors [27,28]. Verma et al. [29] reported that EOs high yield at this stage is probably due to its ecological role in attracting pollinators and in being an antifungal defense mechanism.

The EOs obtained at pre- and full-flowering period were analyzed by GC-MS and results are included in Tables 2 and 3 for \( T. vulgaris \) and \( M. pulegium \) respectively. Chromatographic analysis highlighted the occurrence of six main components for \( T. vulgaris \) EO at higher levels than 0.5% of the content. Carvacrol was the major component with a content ranging from 70.97 to 74.10% accompanied with other components at relatively low levels: δ-terpinene (1.05 to 1.09%), p-cymene (5.17 to 8.74%), γ-terpinene (4.08 to 5.89%), β-humulene (2.40 to 3.04%). Our results are in accordance with those obtained by Boughid [30] who found that carvacrol was the major compound. However, they differ from those obtained by Imelouane et al. [26] in eastern Morocco and Jordan et al. [8] from Murcia region in Spain. For Imelouane et al. [26], camphore and camphene were the major compounds, while 1,8-cineole (29.2 to 36.5 %) and terpenyl acetate (18.2 to 25%) were identified as major compounds for Jordan et al. [8]. Such variations between countries could be due to numerous factors, such as differences in climatic conditions, geographical location, season at the time of collection, stage of development, processing of plant materials before extraction of the oils, and occurrence of different chemotypes.

Table 2 also shows that vegetative cycle affects significantly the chemical composition of \( T. vulgaris \) EO. The differences consist of the variation of carvacrol percentage that increased from 70.97 to 74.10% and the decrease in the content of p-cymene from 8.74 to 5.17 and γ-terpinene from 5.89 to 4.08%. Our results are in accordance with those obtained by Hudaib et al. [7] who found that the chemical composition of EOs varied markedly with vegetative cycle. These authors concluded that the end of vegetative cycle was the best period to obtain EOs with better quality and quantity. We also noted that when the content of carvacrol increased, the content of p-cymene and γ-terpinene decreased. This might be explained by the simultaneous bioconversion of p-cymene and γ-terpinene to carvacrol [31].

With respect to \( M. pulegium \) EO, chromatographic analysis has highlighted four major compounds (Table 3). Pulegone was the...
major constituent with a content of 77.16 to 77.90%, accompanied by small amounts of limonene (1.59 to 1.88%), menthone (1.37 to 6.25%) and pepiritenone (1.71 to 6.54%).

Our results are similar to those reported by Sutour [32] who found that pulegone was the major compound, but differ from those of Mahboubi and Haghi [33] and Verma et al. [29] who identified pipiritenone and menthol as the major constituents. As in T. vulgaris EO, mostly quantitative rather than qualitative variations were observed with respect to chemical composition as affected by vegetative cycle stages. The differences were reflected in the variation of pepiritenone percentage that increased from 1.71 to 6.54%, and the decrease of menthone from 6.25 to 1.37%. Nevertheless, the content of the major component (pulegone) was not affected. Similarly, Verma et al. [29] described considerable variation in the chemical composition of Mentha arvensis and Mentha piperita EOs at different stages of plants growth.

| Components       | Plant          | Yield (% w/w) |
|------------------|---------------|---------------|
|                  | Thymus vulgaris | Pre-flowering | Full-flowering |
| α-thujene        | 922           | 0.83±0.06     | 1.10±0.09     | 1,2 |
| δ-terpinene      | 1021          | 1.09±0.03     | 1.05±0.05     | 1,2 |
| p-cymene         | 1029          | 8.74±0.1      | 5.17±0.04     | 1,2 |
| γ-terpinene      | 1064          | 5.89±0.05     | 4.08±0.07     | 1,2 |
| carvacrol        | 1308          | 70.97±0.34    | 74.10±0.25    | 1,2 |
| β-humulene       | 1440          | 3.04±0.06     | 2.40±0.05     | 1,2 |
| Total            | 94.05         | 90.51         |               |     |
| ** means on the same file followed by the same letters are not significantly different (P>0.05). |
| *RI. retention index relative to n-alkanes on 1MS capillary column. |
| 1. Identification by comparison to retention index. |
| 2. Identification by comparison with MS data spectra |

Table 3. Effect of harvest time on chemical profile of Mentha pulegium essential oil

| Components       | RI* | Pre-flowering (% peak area) | Full-flowering (% peak area) | Identification |
|------------------|-----|----------------------------|----------------------------|----------------|
| α-pinene         | 938 | 0.40±0.01                  | 0.37±0.02                  | 1,2 |
| β-pinene         | 983 | 0.22±0.01                  | 0.20±0.02                  | 1,2 |
| 3-Octanol        | 996 | 0.45±0.06                  | 0.23±0.08                  | 1,2 |
| limonene         | 1034| 1.88±0.08                  | 1.59±0.02                  | 1,2 |
| p-mentha-3,8-diene | 1076 | 0.31±0.04                  | 0.10±0.04                  | 1,2 |
| menthone         | 1156| 6.25±0.06                  | 1.37±0.04                  | 1,2 |
| isomenthol       | 1186| 0.49±0.03                  | 0.43±0.05                  | 1,2 |
| pulegone         | 1252| 77.90±0.25                 | 77.16±0.15                 | 1,2 |
| pipertone oxide  | 1277| 0.15±0.02                  | 1.82±0.05                  | 1,2 |
| pepiritenone     | 1375| 1.71±0.02                  | 6.54±0.04                  | 1,2 |
| β-humulene       | 1440| 0.32±0.09                  | 0.25±0.05                  | 1,2 |
| germacrene D     | 1475| 0.74±0.06                  | 0.55±0.04                  | 1,2 |
| Total            | 93.21| 94.52                     |                           |     |
| ** means on the same file followed by the same letters are not significantly different (P>0.05). |
| *RI. retention index relative to n-alkanes on 1MS capillary column. |
| 1. Identification by comparison to retention index. |
| 2. Identification by comparison with MS data spectra. |
3.2 Antimicrobial Activity

The average diameters of the inhibition zone observed around the disks impregnated with the EOs of both plants studied are summarized in Table 4. According to these results, *T. vulgaris* EO showed a strong activity on all tested bacterial strains based on the inhibition diameters obtained between 30.7 and 36.7 mm. The largest antimicrobial activity was observed against *L. monocytogenes* (36.7 mm) and the weakest against *E. coli* (30.7 mm). Our results also demonstrated that Gram-positive bacteria were more sensitive to *T. vulgaris* EO than Gram-negative bacteria. Similar trend was observed by Rota et al. [3]. The resistance of Gram-negative bacteria to EOs is partly due to the complexity of the cell wall of these microorganisms which contains an outer membrane, unlike the simpler cell wall structure of the Gram-positive ones [1].

Despite the chemical variation of *T. vulgaris* EO between the two vegetative stages investigated (pre- and full-flowering period), this variation did not statistically (P <0.05) affect its antimicrobial activity against *E. coli*, *Salmonella* Senftenberg and *S. aureus*. Only *L. monocytogenes* was more sensitive to the EO from the full-flowering period. The difference in sensitivity of *L. monocytogenes* between pre- and full-flowering periods might be due to the higher concentration of carvacrol at this stage, which is a very effective antimicrobial agent, especially against Gram positive bacteria [5,34,35].

*M. pulegium* EO showed a lower antibacterial activity compared to the EO of *T. vulgaris* (Table 4); the inhibition diameters ranged from 9 mm to 12.7 mm. The largest antimicrobial activity was obtained against *E. coli* (12.7 mm) and the weakest against *Salmonella* Senftenberg (9 mm). Our results differ from those described by Ait-Ouazzou et al. [36] and Teixeira et al. [37] that demonstrated a higher antimicrobial activity of *M. pulegium* EOs.

Like in *T. vulgaris*, the chemical variation of *M. pulegium* EO during the vegetative cycle did not affect its antimicrobial activity against *E. coli*, *Salmonella* Senftenberg and *S. aureus*. In fact, the concentration of the major compound (pulegone) did not vary with the vegetative cycle. Again, only *L. monocytogenes* was more sensitive to the EO obtained during the pre-flowering period. The difference in sensitivity of *L. monocytogenes* between pre- and full-flowering periods could be due to the main differences found: the concentrations of menthone that changes from 6.25 to 1.37% and piperitenone from 1.71 to 6.54%

Therefore, these results point out the full-flowering period as the most appropriate time to harvest *T. vulgaris* EO and the pre-flowering for the *M. pulegium* EO in order to guaranty their greatest antimicrobial effect.

3.3 Antioxidant Activity

The effect of the vegetative cycle on the scavenging activity of DPPH radical of *M. pulegium* and *T. vulgaris* EOs is given in Table 5. As we can see, *T. vulgaris* EO showed a greater antioxidant activity than *M. pulegium* EO. Antioxidant activity of *T. vulgaris* increased with increasing concentration and it was comparable to the antioxidant effect of BHT at a concentration of 15 µL/mL. EOs from full-flowering period exhibited significantly higher antioxidant activity (P<0.05). This reduction capacity of free radicals of *T. vulgaris* EO is mainly due to its chemical profile, rich in carvacrol that has already been proved to possess a strong antioxidant activity [38-40].

Opposed to the results obtained with *T. vulgaris* EO, *M. pulegium* EO exhibited a higher antioxidant activity at pre-flowering than full-flowering period. Probably, the difference between the antioxidant activities from the two periods studied was related to the proportion of the present active components, especially the

| Strain               | Diameter of inhibition zone (mm) | Thymus vulgaris | Mentha pulegium |
|----------------------|---------------------------------|----------------|----------------|
|                      | Pre-flowering | Full-flowering | Pre-flowering | Full-flowering |
| Gram-                | *Escherichia coli*              | 31.7±0.6       | 34.7±1.5       | 12.7±2.52     | 9.3±0.6       |
|                      | *Salmonella* Senftenberg        | 30.7±1.2       | 34.3±3.2       | 9.7±2.08      | 9.0±1.0       |
| Gram+                | *Listeria monocytogenes*        | 32.3±0.6       | 36.7±3.2       | 12.3±0.6      | 10.3±0.6      |
|                      | *Staphylococcus aureus*         | 32.7±2.9       | 35.0±1.0       | 10.0±2.0      | 10.7±0.6      |

a,b,c,d Inhibition zones on the same row followed by the same letters are not significantly different (P>0.05)
Table 5. Effect of harvest time on antioxidant activity of *Thymus vulgaris* and *Mentha pulegium* essential oils determined by DPPH test

| Concentration in µL/mL | Positive control (BHT) | *Thymus vulgaris* | *Mentha pulegium* |
|------------------------|-------------------------|-------------------|-------------------|
|                        |                         | Pre-flowering     | Full-flowering    | Pre-flowering     | Full-flowering    |
| 1                      | 90.4±0.0                | 74.9±0.1          | 78.1±0.3          | 11.5±0.2          | 4.8±0.2           |
| 15                     | 94.5±0.6                | 94.2±0.4          | 95.2±0.1          | 71.4±0.7          | 67.7±0.9          |
| 30                     | 94.5±0.4                | 94.9±0.6          | 95.7±0.2          | 86.0±0.6          | 80.6±0.7          |

*a,b,c,d,e* Antioxidant activity on the same row followed by the same letters are not significantly different (P>0.05)

content of menthone that decreased from 6.25 (pre-flowering) to 1.37% (full-flowering). Our results are in accordance with those obtained by Zhen et al. [41] who observed that the antioxidant activity of *Citrus medica* EOs decreased with increasing maturity at harvest. Also, Hussain et al. [16] showed that the antioxidant activity of *Ocimum basilicum* EOs varied significantly with the season.

So, our results demonstrate that full-flowering is not always the best period to harvest aromatic and medicinal plants with better antioxidant activity, but that depends on the type of plant.

4. CONCLUSION

As far as we know, this is the first time that the effect of vegetative cycle on the yield, chemical composition, antimicrobial and antioxidant activity of *T. vulgaris* and *M. pulegium* EO from Morocco has been studied.

According to our results, in general, full-flowering period is the best period to harvest *T. vulgaris* plants in order to produce EOs with better yield, antimicrobial and antioxidant activity. In contrast, pre-flowering stage is recommended to obtain *M. pulegium* EO with higher antioxidant and antimicrobial activity. The optimization of the harvest time will facilitate the exploitation of these natural resources and will allow producers to obtain EOs with better quality and quantity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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