

# Evaluation of Vegetative Growth, Chemical Composition, and Antioxidant Capacity of Essential Oil of Peppermint Under Water Regimes

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## Abstract

The purpose of the present work was to evaluate the vegetative growth of *Mentha piperita* L. cultivated under different water availability, as well its influence in content, chemical composition and *in vitro* antioxidant activity of its essential oil. Plants were propagated by mother plants microcutting and scions were transplanted to 5 L pots with soil and cattle manure. Afterward, were kept at field capacity for 30 days and under treatment for 40 days. It was treated with different levels of water deficit treatments: (T1): 100 of field capacity (FC); (T2): 80 of FC; (T3): 60 of FC; (T4) 40 of FC with 5 blocks. Vegetative growth was evaluated by dry matter contents of all part of plants and by root/aerial rate. The essential oil of the leaves was extracted by hydrodistillation, analyzed by GC-FID and GC-MS and *in vitro* antioxidant potential was evaluated. A significant decrease in the dry matter of leaves and stems accompanied with a decrease in the roots dry matter was observed with an increase in the water stress. Quantitative chemical differences were observed in the chemical composition of the essential oil, according water availability. Total antioxidant activity showed a gradual increase as water stress progressed.

**Keywords:** TAC, *Mentha piperita* L., volatile compounds, water deficit

## 1. Introduction

*Mentha piperita* (peppermint) is a sterile hybrid of *Mentha x spicata* and *Mentha x aquatic* belonging to the family Lamiaceae. It is a source of valuable essential oil used to give the mint flavor to various products, fragrances and pharmaceuticals (Lawrence, 2006). Due to its industrial use, this species is of great economic importance, and its essential oil is the fifth most commercialized in the world (Bizzo et al., 2009). The major chemical constituents of peppermint essential oil are menthol, mentone, menthofuran, mentil acetate and pulegone. However, the contents of these chemical constituents vary according to the geographic location or the result of the combination of several other factors, such as genotype, ontogeny, light, temperature, water, and nutrients (Ribeiro et al., 2018).

Water deficit is one of the most important factors limiting agricultural productivity and plays an important role in the distribution of plant species in different types of environments, besides affecting plant growth and metabolism (Xu et al., 2010). However, plants exposed to water deficit tend to increase concentrations of secondary metabolites. These variations are reported in several studies, such as *Melissa officinalis* (Meira et al., 2013), *Ocimum basilicum* L. (Radácsi et al., 2010), and *Mentha piperita* (Rahimi et al., 2018) under conditions of low water availability showed higher essential oil content.

Low water availability is often associated with increased levels of reactive oxygen species (ROS), such as superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical (HO), and singlet oxygen ( $O_2$ ) (Shao et al., 2008; Choudhury et al., 2017). Reactive oxygen species are considered unavoidable by products of aerobic metabolism being continuously produced and removed from the cells by enzymatic and non-enzymatic

antioxidative mechanisms (Zhu et al., 2009b). Plants subjected to environmental stress evolve a complex and efficient antioxidant system capable of neutralizing the harmful effects of ROS (Zhu et al., 2009a). The focus of the present work was to evaluate the effect of different water availability on the growth of *M. piperita*, as well as its influence on the content, chemical composition and *in vitro* antioxidant capacity of essential oil.

## 2. Material and Methods

### 2.1 Vegetative Growth of Peppermint Under Water Deficit

The experiment was conducted in a greenhouse with an average temperature of 26 °C and humidity of 80%. The scions were obtained by microcuttings of approximately 5 cm, from mother plants of the UFPA Medicinal Garden. These were cultivated in trays with commercial substrate Hortplant® for 15 days. After rooting they were transplanted into pots of 5 L, containing 4 kg of the soil mixture and bovine manure (3:1). The experimental period was 70 days, during the first 30 days the plants were irrigated by dripping with the volume of water related to the field capacity (FC) with interval between irrigation of 72 h. The FC was determined by the gravimetric method after 72 h of draining (Azevedo-Neto et al., 2010). At harvest at 70 days, the plants were separated into stems, leaves, and roots, and dried in an oven by forced air circulation at 40±2 °C for determination of dry matter. Four water regimes, taking into account the percentage of the soil field capacity were used: (T1) 100% of the FC; (T2) 80% of FC; (T3) 60% of the FC; (T4) 40% of the FC, with five blocks, and each experimental plot were composed of five plants.

### 2.2 Extraction and Chemical Analysis of Essential Oil

To extract the essential oil, 30 g of dried leaves of *M. piperita* from each treatment were extracted by hydrodistillation with one liter of water in a modified Clevenger apparatus for 120 minutes, 5 extractions per treatment was done. The oils were separated by decantation and stored in amber vials of 2 mL and kept under refrigeration (-4 °C). The essential oil content was determined and expressed in mg 100 g<sup>-1</sup> dry matter of the leaves and the yield in Kg Kg<sup>-1</sup> of leaf dry matter.

Quantitative chemical analyzes of the essential oil were performed using an Agilent® 7890A gas chromatography system, operated with the HP GC ChemStation Ver. A.01.14 data processing system and equipped with automatic injector/sampler, Combi PAL Autosampler System (CTC Analytic AG, Switzerland) and with a Flame Ionization Detector (FID). Samples were prepared by diluting the essential oil with ethyl acetate (1%, v/v). The injection volume was 1.0 µL, in split mode at an injection ratio of 50:1. A HP-5MS fused silica capillary column (30 m long × 250 µm internal diameter × 0.25 µm film thickness) was used for the separation of the analytes (California, USA). Helium was used as drag gas with flow of 1.0 mL/min; the injector and detector temperatures were maintained at 240 °C. The initial oven temperature was 60 °C, maintained for 1 minute, and then a 3 °C/min temperature ramp was programmed to 240 °C, followed by another 10°C/min ramp to 250 °C, keeping in isothermal condition for 1 minute. The analysis was performed in triplicate and the results expressed by mean of the percentage of normalized area relative to the chromatographic peaks ± standard deviation.

Qualitative chemical analyzes were performed on Agilent® 7890A Chromatograph coupled to an Agilent® MSD 5975C Mass Selective Detector (Agilent Technologies, California, USA), operated by 70 eV electronic impact ionization in sweep mode at a rate of 1.0 scan/s, with a mass acquisition interval of 40-400 m/z. The operating conditions were the same as those used in GC-FID analyzes. The constituents were identified by comparing their retention indices relative to the n-alkane series (C8-C20) (Sigma-Aldrich, St. Louis, USA) and by comparing the mass spectra of the NIST/EPA/NHI (NIST 2008) and literature (Adams, 2007). The retention indices were calculated using the equation proposed by Van den Dool and Kratz (1963) and for the assignments literature retention indexes were consulted (Adams 2007).

### 2.3 Total Antioxidant Capacity/Phosphor Molybdenum Assay (TAC)

The antioxidant activity (TAC) of samples was evaluated by the green phosphor molybdenum complex formation according to Prieto et al. (1999). An aliquot of 100 µl of sample solution was combined with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a 4 mL vial. The vials were capped and incubated in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The experiment was conducted in triplicates and the results reported (ascorbic acid equivalent antioxidant activity) are mean values expressed as g of ascorbic acid equivalents/100g extract. The aqueous ascorbic acid solution, calibration curve ( $y = 1.3691x - 0.1264$ ,  $R^2 = 0.99$ ) comprised concentration range from 0.08-5 mg/mL. All chemicals were of analytical grade. Microplatereader TECAN Infinity® M200 PRO operated with software I-control® version 3.37.

## 2.4 Statistical Analysis

For the statistical analysis of the data, software R (R Development Core Team, 2013) was used. The means between the treatments were submitted to analysis of variance by the F test and compared by the Tukey test, at 5% probability. Principal component analysis (PCA) was used to study the major compounds and TAC of essential oil in relation to different water regimes. Each variable (*i.e.*, percentage of an identified compound of total oil composition) was subtracted by the variable mean; this process ensured that all results would be interpretable in terms of variation from the mean. The Statistica® software, version 13.3 (StatSoft-Tulsa, USA) was used for these statistical analyses.

## 3. Results and Discussion

### 3.1 Vegetative Growth Under Water Regimes

The water availability significantly affected growth parameters, yield and the chemical composition of the essential oil ( $p < 0.05$ ). The highest leaf dry matter (LDM) and stem (SDM) production was obtained at the maximum moisture level, although it did not present significant statistical differences in relation to 60% and 80% field capacity treatments. On the other hand, the highest accumulations of root dry matter (RDM) and shoot root ratio (R/S) were obtained at the 40% field capacity level (Table 1).

Table 1. Production of plant matter of *Mentha piperita* submitted to different water regimes. Leaves dry matter (LDM); Stem dry matter (SDM); Root dry matter (RDM); Total dry matter TDM); Root/Shoot ratio (R/S)

Field Capacity	LDM (g/plant)	SDM (g/plant)	RDM (g/plant)	TDM (g)	R/S
100% (control)	11.50 a*	13.69 a	4.58b	29.77 a	0.18 b
80%	9.42 ab	11.86 ab	4.41b	25.69 b	0.20 b
60%	9.57 ab	12.26 ab	4.78b	26.61 ab	0.22 b
40%	7.93 b	11.55 b	7.60a	27.08 ab	0.38 a

Note. \*Means followed by the same letters in the column did not differ statistically from each other, according to the Tukey test at 5% significance level.

As shown in Table 1, at 100% moisture range (control treatment) the LDM was 31% higher than the 40% humidity treatment. However, conversely at 40% moisture range, the RDM was 66% higher than that of the control treatment. Similarly, an increase of up to 60% in the R/S ratio was observed plants of *Bupleurum chinense* DC under water deficit compared to treatment control (Zhu et al., 2009b). In the present study, it was observed that the plants of *M. piperita* developed an adaptation strategy to supply the lack of water in the soil, because under conditions of water deficit there was an increase in root growth in detriment to the aerial part, which was observed in the R/S ratio and in the comparison of the dry matter partition of the mints (Table 1 and Figure 1c).

A higher root/shoot ratio in the water deficit resistant also observed in *Coffea canephora* clones when compared to the less resistant ones (Pinheiro et al., 2005). Different results were reported by Arruda et al. (2018), who claimed that the R/S ratio of *Hyptis suaveolens* were greater in 100% water application. This phenomenon acts not only prioritizing the increase of water absorption, but also reduces the water losses due to transpiration, since plants under a lower humidity level have lower LDM (Figueirôa et al., 2004). Whereas, the total dry matter (TDM) in the humidity range of 40% was only 9% lower than the control treatment. Hazrati et al. (2017) reported water stress decreased plant growth and leaf yield of *Aloe vera* L. while caused an increase in biochemical compounds. Also, the results showed that different water availability effected on plant growth of *Cassia obtusifolia* L. a traditional Chinese medicinal plant (Xue et al., 2018), *Mentha piperita* (Rahimi et al., 2018) and *Hyptis suaveolens* (Arruda et al., 2018).

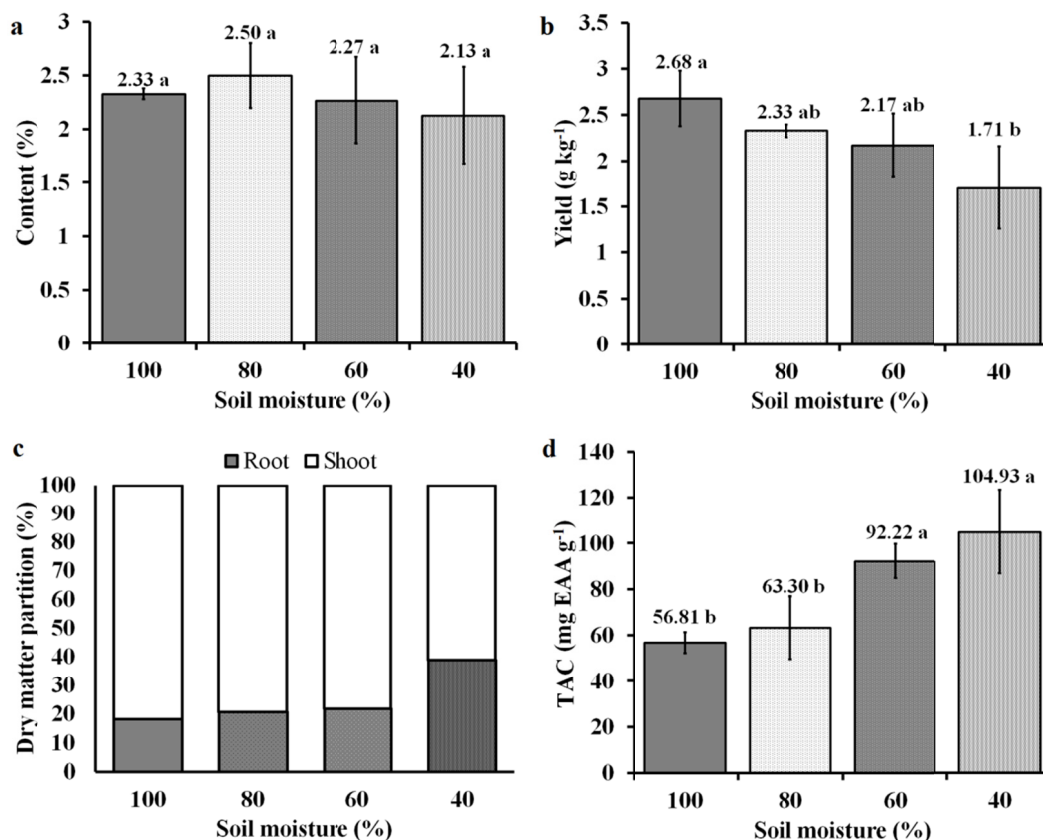


Figure 1. (a) Essential oil contents; (b) Yields of essential oils; (c) Partitions of dry matter of shoots and roots; (d) Total Antioxidant Capacity (TAC) of *Mentha piperita* grown under different water regimes. \*Means followed by the same letters did not differ statistically from each other, according to the Tukey test at 5% significance level

The maximum essential oil percentage observed in 80% FC (2.50%). However, there were no differences in the concentration of essential oil content among the treatments (Figure 1a). Whereas, the essential oil yield varied with the water deficit. The lower values being found for 40% of the field capacity, but no statistical differences were observed between the others treatments (Figure 1b). Corroborating with the results of the present study, the highest yields of essential oil were obtained with increasing soil moisture in *Lippia sidoides* (Alvarenga et al., 2012) and in *Ocimum basilicum* (Pravuschi et al., 2010). Likewise, the increase in oil yield was also demonstrated in the studies of Razmjoo et al. (2008) with *Matricaria chamomilla*. Vilanova et al. (2018) reported in *Ocimum gratissimum* that water application above field capacity (110%) with nitrogen increased dry matter and content of its essential oil and also change in the chemical constituents.

According to a study conducted in the United States by Charles et al. (1990), an increase in essential oil content was observed when *M. piperita* was submitted to one or two weeks of water deficit, although oil yield varied only after two weeks of drought. Also, Rahimi et al. (2018) reported that water deficit impacted the essential oil percentage of *Mentha piperita*. On the other hand, Ghanbari and Ariaifar (2013) observed that the yield of *M. piperita* essential oil from Iran decreased with the increase of the drought level from 70 to 30% of the field capacity. The effect of the water deficit on the essential oil depends on the plant and the genotype, which can increase, reduce or not have an effect on this parameter (Farahani et al., 2009).

More than 95% of the total chemical composition of essential oils of *M. piperita* was characterized by the presence of twenty-eight chemical constituents (Table 2). The water deficit influenced the quantitative chemical composition of several essential oils compounds. For instance, the low concentration of menthol (25.98%) was observed with 40% field capacity. In the other treatments, the average content of menthol was 29.22%. As for limonene the lowest content (1.49%) was observed in 100% of field capacity. Compared to the control treatment, menthol showed a decrease of 13% with 40% of field capacity and limonene a 27% increase in the same level of soil moisture. Khorasaninejad et al. (2011) also reported highest menthol content in *M. piperita* in 70% of field capacity, and this amount decrease under water deficit, but without variation for limonene. The other major

components did not vary significantly between treatments. In studies conducted by Charles et al. (1990) no changes were observed in menthol contents in plants of *M. piperita* under water deficit.

Table 2. The chemical composition of the essential oil of *Mentha piperita* L. grown under different water regimes

Constituents	Field Capacity			
	100%	80%	60%	40%
----- Content % ± SD -----				
<i>Monoterpenes</i>				
Menthol	29.41±1.02	28.94±2.31	29.32±1.37	25.98±1.39
Menthone	17.82±1.63	18.56±2.04	17.71±2.28	19.42±2.93
Menthofuran	15.24±1.75	17.56±0.69	17.10±1.85	16.76±1.90
neo-Menthyl acetate	8.48±1.31	3.92±0.98	8.87±0.76	8.94±1.07
1,8-Cineole	5.58±0.31	5.27±0.22	5.65±0.70	5.74±0.42
iso-Menthol	4.34±0.23	3.92±0.39	4.16±0.34	4.05±0.51
Pulegone	3.45±0.46	3.37±1.14	3.16±0.78	4.25±0.29
iso-Menthone	2.08±0.16	2.11±0.09	2.09±0.14	2.14±0.26
cis-Sabinene hydrate	1.65±0.15	1.72±0.17	1.66±0.07	1.68±0.12
Limonene	1.49±0.10	1.53±0.11	1.57±0.06	1.89±0.15
β-Myrcene	1.21±0.14	1.22±0.07	1.27±0.05	1.34±0.05
β-Pinene	0.91±0.15	0.84±0.07	0.82±0.03	0.80±0.06
β-Phellandrene	0.86±0.16	0.90±0.06	0.91±0.06	0.98±0.05
α-Terpinene	0.60±0.07	0.61±0.03	0.62±0.03	0.65±0.03
Menthyl acetate	0.58±0.12	0.54±0.09	0.57±0.05	0.59±0.11
neo-Isomenthol	0.50±0.02	0.45±0.06	0.44±0.06	0.39±0.05
iso-Menthyl acetate	0.39±0.08	0.35±0.05	0.39±0.04	0.35±0.08
Linalool	0.26±0.02	0.24±0.02	0.24±0.02	0.23±0.02
Terpinen-4-ol	0.25±0.05	0.18±0.02	0.17±0.02	0.19±0.04
Piperitone	0.25±0.05	0.26±0.01	0.23±0.07	0.24±0.05
neo-Menthol	0.21±0.03	0.18±0.03	0.18±0.02	0.17±0.04
p-Mentha-1,4-dien-7-ol	0.15±0.02	0.11±0.01	0.12±0.02	0.14±0.01
trans-β-Ocimene	0.14±0.01	0.14±0.02	0.15±0.01	0.16±0.03
γ-Terpinene	0.13±0.01	0.13±0.01	0.13±0.01	0.10±0.01
<i>Sesquiterpenes</i>				
β-Cubebene	1.35±0.17	1.53±0.21	1.18±0.37	1.20±0.36
β-Caryophyllene	0.84±0.15	1.01±0.13	0.78±0.27	0.82±0.25
(z)-β-Farnesene	0.15±0.03	0.19±0.04	0.15±0.01	0.15±0.01
<i>Aliphatic alcohol</i>				
3-Octanol	0.05±0.01	0.12±0.00	0.11±0.00	0.10±0.01
TOTAL (%)	98.37	95.90	99.75	99.45

Note. <sup>a</sup>Chemical constituents reported in order of concentration in DB-Wax column. SD: standard deviation (n = 3).

### 3.2 Total Antioxidant Capacity (TAC)

Various abiotic stresses lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress (Gill & Tuteja, 2010). The *in vitro* antioxidant activity of *M. piperita* essential oil was significantly affected by water availability (Figure 1d). The total antioxidant activity increased with the decrease of soil moisture, and plants grown at the lowest soil moisture levels (60 and 40% of field capacity) presented higher total antioxidant activity. The total antioxidant activity at the lowest field capacity level in which highest values of limonene was observed was about 54% higher when compared to the essential oil activity at the 100% field capacity level. The observed differences in the total antioxidant activity may be associated to the chemical quantitative differences of the levels of menthol and limonene between the different treatments. The essential oil of *M. piperita* L. is mainly constituted of monoterpenes. Due to the complex chemical composition, synergistic

effects between the various compounds may be determinant for the antioxidant activity of the essential oil (Amorati et al., 2013).

Drought tolerance is often correlated with a higher efficiency of the antioxidant system. The antioxidant defence of the vegetal biological system protects the plants against oxidative stress damage, by means of diverse and efficient enzymatic and non-enzymatic processes. These systems work together to control oxidative cascades and protect plant cells from oxidative damage through different chemical processes of capture of reactive oxygen species (Gill & Tuteja, 2010).

### 3.2 Comparison of Constituents of Essential Oil and TAC With PCA

The influence of field capacity (FC) on volatile compounds appears to be quite variable. In order to understand the influence of FC on volatile compounds and TAC, a PCA was used to distinguish the compounds differences occurring in the plants. The data obtained of the scores and loadings (PCA) provides a conceptual overview of the treatments by showing a total of 91.21% of the variance in FC. The level of variation associated with principal component 1 was at 53.25% whereas the principal component 2 explained 37.96% of the variation (Figure 2). The analysis of loadings permitted observing that high FC which indicates its positive influence on the menthol in this study. The plants cultivated in low FC (40%) had more concentration of limonene, menthone, and *iso*-menthone. The analysis of loadings allowed observing that limonene had negative correlation with menthol. According Pearson correlation menthol and TAC showed high negative correlation (-0.72) and limonene and TAC high positive correlation (0.84). This means that increase limonene content antioxidant capacity of *M. piperita* essential oil enhancement. These results suggest that water regimes influence pathway of monoterpenes synthesis in *M. piperita*.

## 4. Conclusions

The lower water availability (40%) influences a lower accumulation of dry matter of the aerial part demonstrating the low adaptability of the species to loss of soil moisture conditions. The water deficit influenced the quantitative chemical composition of several essential oils compounds. With higher water availability (100%) the mint presents higher yield and quality of essential oil, evidenced by a higher content of menthol. The condition of greater water deficit influences in the elevation of the total antioxidant activity of the essential oil of *Mentha piperita* L.

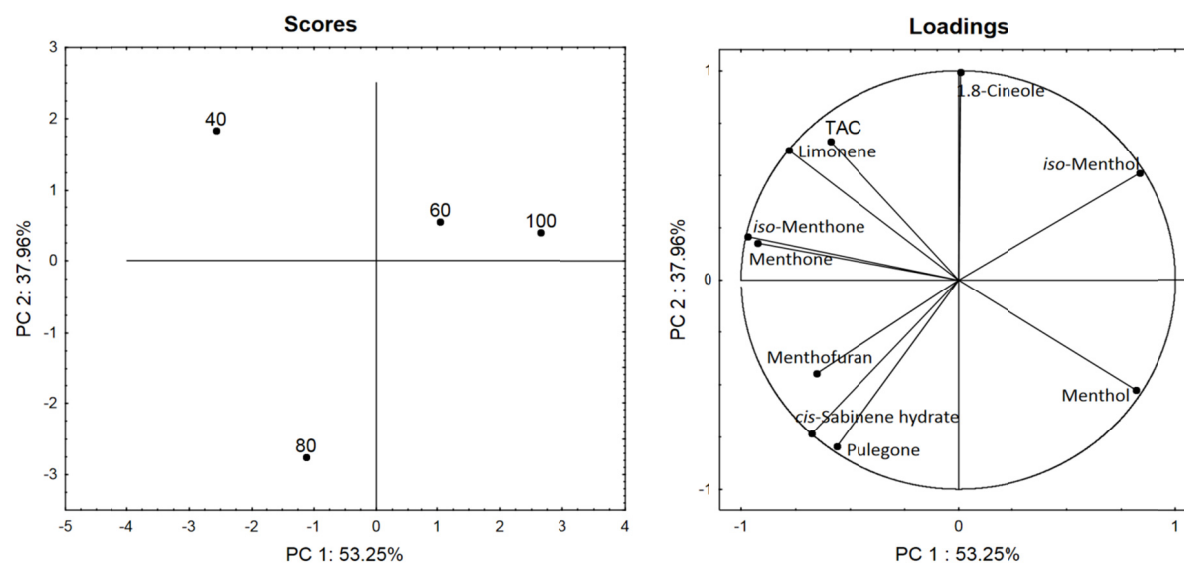


Figure 2. Principal component analysis (PCA) of the averages of compounds of the essential oil and Total Antioxidant Capacity (TAC) of *Mentha piperita* grown under different water regimes (40, 60, 80, and 100%)

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