

Antioxidant Activity of the Essential Oil and its Major Terpenes of *Satureja macrostema* (Moc. and Sessé ex Benth.) Briq.

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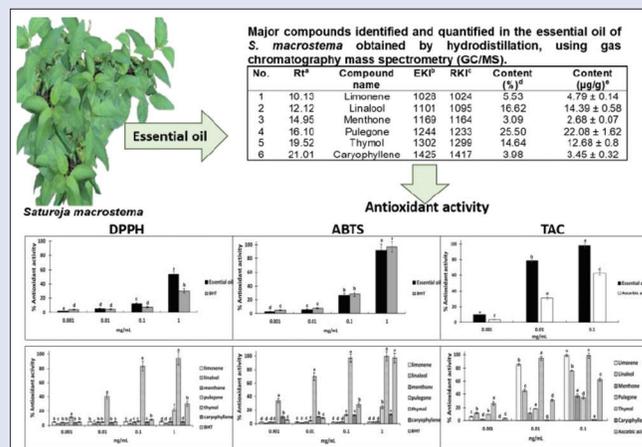
ABSTRACT

Background: The aim of this study was to investigate the *in vitro* antioxidant activity of *Satureja macrostema* (Moc. and Sessé ex Benth.) Briq. (Lamiaceae) essential oil, a Mexican medicinal plant known as nurite. **Materials and Methods:** Fresh aerial parts of *S. macrostema* plants cultivated in greenhouse for 3 months were subjected to hydrodistillation in a Clevenger apparatus to obtain essential oil. Volatile compounds were identified by gas chromatography (GC) and GC/mass spectrometry. Antioxidant effectiveness of essential oil and its major terpenes of *S. macrostema* was examined by three different radical scavenging methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and total antioxidant capacity (TAC). The concentrations tested were 0.001, 0.01, 0.1, and 1 mg/mL. **Results:** The major volatile compounds were caryophyllene, limonene, linalool, pulegone, menthone, and thymol. *S. macrostema* essential oil showed the highest free radical scavenging activity with DPPH and ABTS methods (53.10% and 92.12%, respectively) at 1 mg/mL and 98% with TAC method at 0.1 mg/mL. Thymol exerted the highest antioxidant capacity with 0.1 mg/mL, reaching 83.38%, 96.96%, and 98.57% by DPPH, ABTS, and TAC methods. Caryophyllene, limonene, linalool, pulegone, and menthone exhibited an antioxidant capacity <25% with the DPPH and ABTS methods; however, limonene showed a TAC of 85.41% with 0.01 mg/mL. **Conclusion:** The essential oil of *S. macrostema* and thymol showed a free radical scavenging activity close to that of the synthetic butylated hydroxytoluene.

Key words: Free radicals, hydrodistillation, medicinal plant, nurite, volatile compounds

SUMMARY

- The major volatile compounds of essential oil of *Satureja macrostema* were caryophyllene, limonene, linalool, pulegone, menthone and thymol
- The essential oil of *S. macrostema* showed a high free radical scavenging
- Thymol exerted the highest antioxidant capacity by DPPH, ABTS and TAC methods.



Abbreviations used: GC: Gas Chromatography; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); TAC: Total antioxidant capacity.

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INTRODUCTION

In recent years, the efficacy of herbal medicines in inflammatory and oxidant-related diseases has been reported.^[1,2] Oxidative stress plays a leading role in the pathogenesis of aging and degenerative diseases such as atherosclerosis, cardiovascular diseases, diabetes, and cancer.^[3] Free radicals are degraded to nonreactive forms by enzymatic and nonenzymatic antioxidant defenses produced in the body and others supplied by the diet. Among these, essential oils of plants have been studied for their potential antioxidant capacities,^[4-6] which can be attributed to the presence of terpenes, besides the phenolic compounds that contribute to the free radical scavenging activity.^[7]

The terpenes are the main components of the essential oils from medicinal plants, mainly from the aromatic species of the Lamiaceae family; these have been considered as natural antioxidants with high potential, which could be used as additives in food supplements to prevent the oxidative stress that contributes to the appearance of

degenerative diseases.^[6,7] This family is one of the larger families of plants with distinctive flowers, with about 236 genera and approximately 7200 species around the world.^[8]

The essential oil of basil, cinnamon, clove, nutmeg, oregano, and thyme possesses antioxidant properties due to its major terpenes.^[9] Thymol and carvacrol are responsible for the antioxidant activity of essential oils of

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Thymus spathulifolius and *Origanum vulgare* ssp. *hirtum*;^[10,11] essential oil of *Melissa officinalis* (its main constituents are neral, geranial, citronellal, isomenthone, and menthone) shows free radical scavenging activity.^[12] In addition, isomenthone and menthone are also the terpenes of higher capacity antioxidant of the essential oil of *Mentha longifolia* and *Mentha piperita*, while in the essential oil of *Mentha aquatica*, the 1,8-cineole is the responsible of this activity.^[13] Antioxidant capacity in the essential oil of *Melaleuca alternifolia* is due to α -terpinene, γ -terpinene, and α -terpinolene compounds;^[14] and the β -caryophyllene in the essential oil of *Marrubium peregrinum* is the one which presents the greater antioxidant activity.^[15]

To this family belongs the genus *Satureja* L., which includes about 200 species of aromatic plants that contain >0.5% of essential oil,^[16] its main constituents are carvacrol, thymol, phenols, and flavonoids,^[17] responsible compounds of the *in vitro* antioxidant properties of *Satureja hortensis*, *Satureja spicigera*, *Satureja cuneifolia*, and *Satureja cilicica*.^[18-20] Species from this genus have been used in traditional medicine as analgesic, tonic, or carminative for the treatment of gut disorders.^[21]

In México, a species from *Satureja* (*Satureja macrostema* “Moc. and Sessé ex Benth.” Briq.) is used as a medicinal plant in the traditional medicine and is known as nurite. It is employed in decoctions and infusions for the treatment of various diseases, including stomach pain and liver and gut diseases.^[22] The essential oil obtained by hexane extraction from the aerial parts of this plant contains mainly terpenes as limonene, pulegone, and thymol, suggesting that some of its pharmacological effects could be attributed to the presence of these valuable constituents.^[23] Limonene and thymol are some of the terpenes with higher antioxidant activity in plants.^[24]

In spite of the high content of terpenes in the essential oil from *S. macrostema*, its antioxidant activity has not been analyzed. This work was conducted to the study of the antioxidant activity of essential oil from *S. macrostema* obtained by hydrodistillation and evaluated individually its major terpenes to elucidate the compound(s) responsible for free radical scavenging activity.

MATERIALS AND METHODS

Plant material

Plants of *S. macrostema* (Moc. and Sessé ex Benth.) Briq. were obtained by micropropagation (data not shown). The seeds for *in vitro* culture were collected from plantations established in the experimental area of Nuevo San Juan Parangaricutiro, Michoacán, Mexico (19°25'23"N, 102°07'47"W), and the species was identified by Miguel Angel Bello-González PhD (Faculty of Agrobiology, Universidad Michoacana de San Nicolás de Hidalgo). Plants were grown in pots of 1.5 kg containing a mix of peat moss and perlite (1:1), under conditions of 50%–60% of relative humidity without light and temperature control, and were irrigated every 5 days. The plants were fertilized in the substrate once per month with 1 g/pot of Nutrigarden Excelso® (N-P-K, 17-17-17).^[23,25] The aerial parts (leaves and stems) of *S. macrostema* plants of 6 months old were collected to obtain the essential oil.

Sample preparation

200 g of washed and fresh aerial part (leaves and stems) of *S. macrostema* plants were subjected to hydrodistillation in a Clevenger-type apparatus, mixing together with 1000 mL of distilled water in a round flask. The operating temperature was 100°C and the extraction was done between 2 and 4 h. The essential oil was separated from the hydrolyte by liquid–liquid partitioning in separating fennel and removed with a micropipette. This was suspended in methanol at a final concentration of 1.0 mg/mL and stored at 4°C in the dark until gas

chromatography-mass spectrometry (GC-MS) and antioxidant activity analysis.

Gas chromatography-mass spectrometry

The chemical composition of the essential oil and major terpene quantification were realized using GC and GC/MS data techniques reported by Torres-Martínez *et al.*^[23] 1 μ L of the sample was injected into an Agilent Technologies (7890A) GC equipped with a mass detector (Agilent 5975C), which operated using helium as a carrying gas, with a flow of 1 mL/min, with a split injection (split 50:1) at a temperature of 250°C in HP 5MS nonpolar capillary column (30 m \times 0.25 mm internal diameter \times 0.25 μ m film), under the following conditions: initial temperature of 50°C, followed by a 5°C/min ramp to attain a temperature of 280°C during 1 min; another 25°C/min ramp to raise the temperature to 380°C, during up to 3 min. The runtime was 50 min. The MS operated at a flow speed of 1 mL/min, with an ionization voltage of 70 eV, at an interface temperature of 250°C, in a SCAN mode, and at a mass interval of 50–500 m/z.

The percentage of essential oil constituents was determined by integration of peak areas, the values shown correspond to the average value of three injections. The compounds were identified by comparison of their retention indices, relative to those of n-alkanes C8–C20, and by comparison with a library of mass spectra with the NIST02 mass spectral library (National Institute of Standards and Technology), as well as by comparison of their retention indices with those described by Adams.^[26] Quantitative determination was based on the total ion count detected by the GC-MS.

2,2-Diphenyl-1-picrylhydrazyl free radical-scavenging capacity

Measurement of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Mexico) radical scavenging capacity was carried out according to Karamac *et al.*^[27] Briefly, two mL of 0.5 mmol/L DPPH in methanol (Meyer, México) was mixed with 100 μ L of different concentrations of essential oil of *S. macrostema* and using major pure terpenes in it (limonene, linalool, pulegone, menthone, thymol, and caryophyllene; Sigma-Aldrich, Mexico) (0.001, 0.01, 0.1, and 1.0 mg/mL). After 20 min incubation, the absorbance was measured at 517 nm with ultraviolet–visible (UV/VIS) spectrophotometer (Genesys 10UV, Thermo Scientific). The percentage of free radical-scavenging capacity was calculated by the following equation:

$$\text{Radical scavenging capacity (\%)} = (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100,$$

Where A_{sample} is the absorbance of DPPH mixed with essential oil or terpenes and A_{blank} is the absorbance of DPPH in which sample has been replaced with methanol. All measurements were performed in triplicate and reported as the average value. Butylated hydroxytoluene (BHT, 1.0 mg/mL) (Sigma-Aldrich, Mexico) was used as positive control.

2,2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid cation radical-scavenging capacity

The radical scavenging capacity of the essential oil and major terpenes of *S. macrostema* were assayed with an 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Sigma-Aldrich, Mexico) assay according to the protocol of Rufino *et al.*^[28] with some modifications. The ABTS radical solution was prepared mixing 7.4 mmol/L ABTS and 2.6 mmol/L potassium persulfate (Meyer, México). Samples of 100 μ L were subsequently mixed with 1900 μ L ABTS radical solution, and the absorbance of the resulting mixtures was measured after 7 min at 737 nm with UV/VIS spectrophotometer. The free radical-scavenging capacity was calculated by the following equation:

Radical scavenging (%) = $100 - ([A_{\text{sample}} - A_{\text{blank}}] / A_{\text{control}} \times 100)$,

Where A_{sample} is the absorbance of the ABTS mixed with the sample, A_{control} is the absorbance of the ABTS mixed with deionized water, and A_{blank} is the absorbance of the sample mixed with deionized water. BHT (1.0 mg/mL) (Sigma-Aldrich, Mexico) was used as positive control.

Total antioxidant activity by phosphomolybdenum method

The total antioxidant capacity (TAC) of the essential oil and terpenes was evaluated according to the method described by Prieto *et al.*^[29] An aliquot of 100 μL of sample solution was combined with 900 μL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) (Sigma-Aldrich, Mexico). For the blank, 100 μL of deionized water was used in place of the sample. The tubes were incubated in a boiling water bath at 95°C for 90 min. After the samples were cooled at room temperature, the absorbance of the aqueous solution of each sample was measured at 695 nm in the spectrophotometer (Genesys 10 UV, Thermo Scientific). The total antioxidant activity was calculated by the following equation:

TAC (%) = $([A_{\text{sample}} - A_{\text{control}}] / A_{\text{blank}}) \times 100$,

Where A_{sample} is the absorbance of the sample mixed with the reagent solution, A_{control} is the absorbance of deionized water mixed with the sample, and A_{blank} is the absorbance of the reagent solution mixed with water. Ascorbic acid (0.001, 0.01, and 0.1 mg/mL) (Sigma-Aldrich, Mexico) was used as positive control.

Statistical analysis

Data were expressed as means \pm standard deviations. Differences in DPPH and ABTS radical scavenging capacities among the essential oil and major terpenes were analyzed by one-way analysis of variance and Tukey's test (JMP8). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Chemical composition of the *Satureja macrostema* essential oil

The essential oil of the aerial part from *S. macrostema* obtained by hydrodistillation showed amber color with a mild aromatic odor. The average yield was 0.35% on fresh weight basis. The chemical composition of the oil is presented in Table 1, in which the major terpenes are listed in order of their elution. A total of six constituents, representing 69.40% from the total oil, were identified by GC/MS. Results showed that the major compound was the monoterpene ketone pulegone, followed by linalool, thymol, limonene, caryophyllene, and menthone.

Table 1: Major compounds identified and quantified in the essential oil of *Satureja macrostema* obtained by hydrodistillation using gas chromatography-mass spectrometry

Retention time (min)	Compound name	EKI	RKI	Content (%) ^a	Content ($\mu\text{g/g}$) ^b
10.13	Limonene	1028	1024	5.53	4.79 \pm 0.14
12.12	Linalool	1101	1095	16.62	14.39 \pm 0.58
14.95	Menthone	1169	1164	3.09	2.68 \pm 0.07
16.10	Pulegone	1244	1233	25.50	22.08 \pm 1.62
19.52	Thymol	1302	1299	14.64	12.68 \pm 0.8
21.01	Caryophyllene	1425	1417	3.98	3.45 \pm 0.32

^aContent is the peak volume percentage of compounds in the essential oil sample;

^bContent is fresh weight. EK: Experimental Kovat's retention index; RK: Reference Kovat's retention index

Antioxidant activity of essential oil

Employing the DPPH method, the essential oil of *S. macrostema* showed antioxidant activity from 0.1 mg/mL, with 12.43% of free radical-scavenging [Figure 1a] and 25.95% by ABTS method [Figure 1b]. The highest antioxidant activities (53.1%) were obtained with 1 mg/mL by DPPH method [Figure 1a], 92.12% with the ABTS method [1 mg/mL, Figure 1b], and 98% of TAC with 0.1 mg/mL [Figure 1c]. The antioxidant activity of essential oil from *S. macrostema* by DPPH method was higher than BHT and similar with ABTS method. The essential oil showed an antioxidant activity more efficient than ascorbic acid with TAC assay [Figure 1c].

Antioxidant activity of the major terpenes

The results related to the antioxidant capacity assays of the major terpenes from *S. macrostema* essential oil show that thymol has the highest activity, reaching 83.38%, 98.29%, and 98.57% with 0.1 mg/mL by DPPH, ABTS, and TAC methods, respectively [Figure 2]. Thymol was most efficient by DPPH method than BHT and showed a similar scavenging free radical activity determined by ABTS [Figure 2a and 2b]. On the other hand, limonene, linalool, menthone, and pulegone exhibited low or almost null antioxidant activity determined by DPPH and ABTS methods, and this activity did not exceed the 25% at 1 mg/mL [Figure 2a and 2b]. However, limonene presented the higher antioxidant activity (98.74%), followed by thymol (98.57%), linalool (75.88%), and ascorbic acid (62.43%) [Figure 2c].

These results indicate that the terpenes present in the essential oil of *S. macrostema*, obtained by hydrodistillation, exert a high free radical scavenging capacity, being the thymol the main responsible of this effect.

DISCUSSION

The chemical analysis by GC/MS of essential oil from aerial parts (stems and leaves) of *S. macrostema* obtained from hydrodistillation indicated that the essential oil mainly contains pulegone, a monoterpene ketone, with a content of 22.08 $\mu\text{g/g}$ weight fresh; which coincides with reports of other medicinal plants from Lamiaceae. Pulegone is the major terpene in *Mentha pulegium*,^[30] *M. piperita*,^[31] and *Satureja* species as *Satureja parvifolia* and *Satureja odora*.^[32] However, linalool and thymol were also found at high contents with 14.39 and 12.68 $\mu\text{g/g}$ weight fresh, respectively. In addition, major volatiles were accompanied by less abundant terpenes as limonene (4.79 $\mu\text{g/g}$ weight fresh), caryophyllene (3.45 $\mu\text{g/g}$ weight fresh), and menthone (2.68 $\mu\text{g/g}$ weight fresh). These are constituents from essential oils of medicinal plants from Lamiaceae such as *M. piperita*,^[33] *M. longifolia*,^[34] *Minthostachys verticillata*,^[35] *Schizonepeta tenuifolia*,^[36] and *Agastache rugosa*.^[37]

Generally, the major components determine the biological properties of the essential oils of medicinal plants. Depending on the type and concentration of terpenes, they can exhibit different biological activities such as antimicrobial, anticancer, and antidiabetic; they have also been associated with hepatoprotective, cardiovascular diseases, spasmolytic, and carminative activities. Recent reports suggest that, at least in part, the encountered beneficial effects of essential oils are due to the pro-oxidant effects at cellular level.^[7,38]

The essential oil from *S. macrostema* showed high antioxidant activity *in vitro* (>50%) at 1 mg/mL with DPPH (53.11%) and ABTS (92.12%), activities higher or similar that those ejected by 1 mg/mL of BHT showing 30.39% and 97.23% of activity, respectively. However, at 0.1 mg/mL, the essential oil reached at 98.25% of TAC, one value greater than the effect observed with ascorbic acid (62.43%).

The antioxidant activity produced at 0.1 or 1 mg/mL of the essential oil of *S. macrostema* is greater that the activity reported for the essential

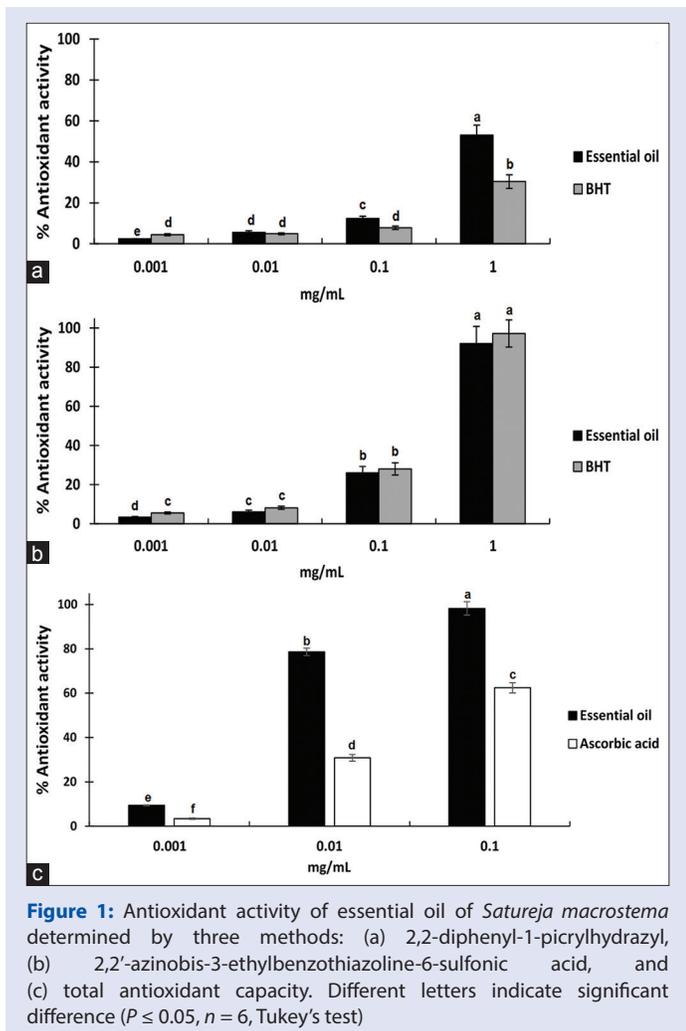


Figure 1: Antioxidant activity of essential oil of *Satureja macrostema* determined by three methods: (a) 2,2-diphenyl-1-picrylhydrazyl, (b) 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, and (c) total antioxidant capacity. Different letters indicate significant difference ($P \leq 0.05$, $n = 6$, Tukey's test)

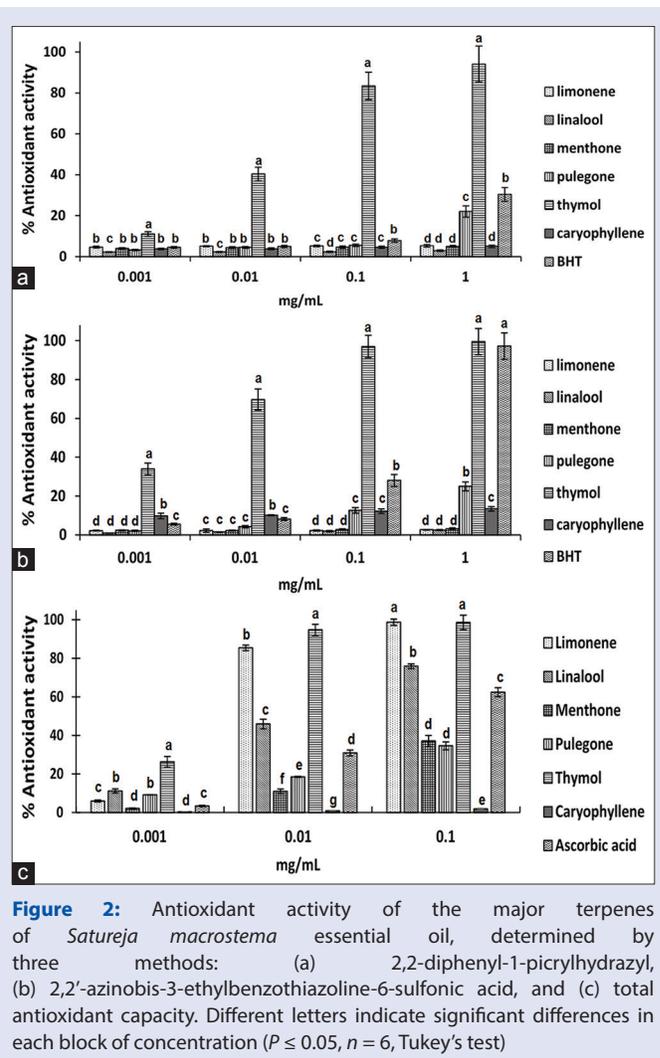


Figure 2: Antioxidant activity of the major terpenes of *Satureja macrostema* essential oil, determined by three methods: (a) 2,2-diphenyl-1-picrylhydrazyl, (b) 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, and (c) total antioxidant capacity. Different letters indicate significant differences in each block of concentration ($P \leq 0.05$, $n = 6$, Tukey's test)

oil of diverse medicinal plants from Lamiaceae (*Thymus vulgaris*, *M. officinalis*, *Pogostemon cablin* and *Rosmarinus officinalis*), which showed antioxidant activity $>50\%$ at 3 mg/mL.^[39-41]

The antioxidant activity of essential oil from *S. macrostema*, demonstrated by the three methods of free radical scavenging used in the present research, was attributed to the high content of terpenes. The most powerful scavenging constituent by DPPH and ABTS was found to be thymol showing 94.07% and 99.52% of activity, respectively, percentages of antioxidant activity higher than BHT (30.39% and 97.23%, respectively) at 1 mg/mL. Thymol and limonene shown the higher antioxidant activity determined by TAC method with 98.57% and 98.74%, respectively, percentages higher than ascorbic acid (62.43%) at 0.1 mg/mL.

The synergistic effect between terpenes of *S. macrostema* essential oil can be due to the interaction of monoterpenes with hydroxyl substituents, such as thymol and linalool. In addition, the combination of limonene and caryophyllene enhances this effect as has been reported for essential oil from many medicinal plants.^[42,43]

The high content of thymol (36.5%) is responsible of the antioxidant activity ($>80\%$) of *T. spathulifolius*,^[10] *T. vulgaris*,^[44] and oregano essential oil (*O. vulgare* ssp. *hirtum*).^[9] In fact, thymol is responsible for the antioxidant activity of many essential oils where it is present.^[44,45] However, in the essential oil of *Thymus caespititius*, *Thymus camphoratus*, and *Thymus mastichina*, the antioxidant activity is related to the high

contents of linalool, while thymol is almost absent;^[7,46] neral/geranial, citronellal, isomenthone, and menthone in *M. officinalis*^[11] and menthone and isomenthone in *M. longifolia* and *M. piperita*^[11] are related to the antioxidant activity. Some alkene terpenes such as terpinene and caryophyllene also show antioxidant capacity by retarding the peroxidation of linoleic acid.^[47]

Pulegone is an oxygenated monoterpene with a ketone group which gives a low reactivity. The essential oil of *M. pulegium* and *Mentha suaveolens* shown a low free radical scavenging activity due to the high content of pulegone.^[48,49] In agreement with this, in the present work, we detected that although pulegone is the major terpene of the essential oil of *S. macrostema*, it shows a lower antioxidant activity. Thus, this activity could be attributed to thymol content, which is in agreement with other reports.^[9,10,44,45]

With these results, it is possible to establish that the pharmacological effects attributed to *S. macrostema* in Mexican folk medicine are due in part to the antioxidant activity, which has also been linked to other species of *Satureja*.^[18-20,50] The essential oil of *S. macrostema* may be considered as potential natural antioxidants and used to prevent oxidative stress that contributes to many degenerative diseases.

CONCLUSION

The main component found in the essential oil of the aerial part of *S. macrostema* was pulegone, followed by linalool, thymol,

limonene, caryophyllene, and menthone. The essential oil of *S. macrostema* (1 mg/mL) showed a free radical scavenging activity close to that from the synthetic BHT or ascorbic acid. The thymol presented greatest antioxidant activity at 0.1 mg/mL and limonene showed a TAC at 0.01 mg/mL, while caryophyllene, linalool, pulegone, and menthone showed a lower antioxidant activity.

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Conflicts of interest

There are no conflicts of interest.

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