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Original Article

Chemical Composition and Antioxidant Activity of Essential Oil from Male and Female *Schinus terebinthifolius*

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ABSTRACT

Background: *Schinus terebinthifolius* Raddi is a medicinal plant popularly known as pink pepper tree, commonly used for wound healing, anti-inflammatory and antimicrobial purposes. Furthermore, its fruit is used in culinary. **Objectives:** In order to further understand the composition and bioactivities of *S. terebinthifolius* essential oil from vegetative and reproductive parts of the tree, we evaluated the chemical composition and the antioxidant activity of Essential Oils (EO) obtained from different aerial parts of male and female plants. **Materials and Methods:** The EO was extracted by hydrodistillation; the antioxidant activity was evaluated via two *in vitro* assays with different mechanisms of action (DPPH and $ORAC_{FL}$) and the chemical composition was determined via GC-MS. **Results:** The EO from leaf, inflorescence and fruit of *S. terebinthifolius* showed distinct chemical profiles, yields and antioxidant potentials. **Conclusion:** The aerial parts of *S. terebinthifolius* may be used as a natural source of antioxidants, since all samples showed strong antioxidant capacity, mainly in the DPPH assay.

Keywords: Antioxidant Activity, Brazilian Pepper Tree, Essential Oil, GC-MS.

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INTRODUCTION

Schinus terebinthifolius Raddi belongs to the Anacardiaceae family. It is native to South America, amply distributed in Brazil[1] and this medicinal plant is used for antiseptic and anti-inflammatory purposes.[2] It is a dioecious; broad-topped tree with evergreen leaves and white flowers arranged in clusters.[3] The ripe fruit are small red drupes known as pink peppers with an intense and appreciated flavor used in culinary around the world.[4]

The bioactive compounds from aerial parts of the tree which have therapeutic potential include volatile compounds that can be extracted, resulting in Essential Oil (EO). This product has economic relevance due the number of bioactive compounds and its EO plays an important role in the aroma of the pink pepper fruit and therefore in its culinary use. EO is known to be responsible for the antioxidant activity of several spices, such as black pepper.[5] This property of natural products has been widely investigated, mainly, to be applied in cosmetic and food industries[6,7] as an alternative to the addition of synthetic preservatives. Furthermore, EO may even be incorporated in products, such as food packing, to protect against oxidative degradation.[8]

The antioxidant and antibacterial activities of EO from leaves and fruit of *S. terebinthifolius* have been studied.[9,10] However, there are few studies about its inflorescence and the difference between male and female individuals. In this context, the yield, composition and antioxidant capacity of the EO obtained from aerial parts of male and female *S. terebinthifolius* trees was evaluated to asses which aerial parts of this plant could be used as natural antioxidants.

MATERIALS AND METHODS

Plant Materials

Two individuals of different sex were selected for this study. The first individual had female flowers (F-female) and was growing in the Experimental Field of the Institute of Biology, University of Campinas, São Paulo, Brazil (22°49'09.8"S 47°04'14.1"W), whilst the other had male flowers (M-male) and was growing one kilometer away from the first individual (22°49'29.2"S 47°04'45.9"W). Voucher specimens were deposited in the Unicamp Herbarium (UEC) under the following access numbers UEC 197984 and UEC 197985, respectively. Aerial parts from both trees were collected in 2018: unripe fruit (March), ripe fruit

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(April) and leaves (March) from F; inflorescence (January) and leaves (March) from M.

Reagents and chemicals

Ultrapure water was obtained from Milli-Q equipment, ethanol P.A was provided by Synth and Dichloromethane was supplied by Merck. The other chemicals used: 2,2-Diphenyl-1-Picrylhydrazyl Radical (DPPH), Quercetin (≥95%), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (97%), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox) (97%), randomly methylated beta-cyclodextrin (RMCD); were purchased from Sigma-Aldrich.

EO Extraction

The frozen plant material (200g) was ground in a blender and transposed to a round bottom flask with distilled water. The EO was obtained by hydrodistillation using a Clevenger-type apparatus. Leaves and inflorescence were extracted for four hours and fruit were extracted for three hours. The EO was removed from the Clevenger-type apparatus using glass Pasteur pipettes and stored at -20°C until the analyses were performed. The yield was calculated as a ratio between the mass of EO and the plant mass used in the extraction process.

GC-MS Analysis

These analyses were performed on an Agilent gas chromatographer Model 7890A (Agilent J&W, USA) fitted with a HP-5ms fused silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness) and coupled to a MS Agilent Model 5975C inert MSD with EI ionization and Triple-Axis Detector (Agilent Technologies, CA, USA), operating in full scan mode (50-450 *m/z*). The analytical method was used according to Adams[11] with modifications: injector temperature 220°C, oven temperature started at 60°C increasing to 246°C at 3°C.min^{-1}. The samples were diluted 1:10 in Dichloromethane. The injection volume was 1 µL in split mode with ratio of 1:30. Helium was used as a carrier gas at 1 mL.min^{-1}. An alkane standard solution (C_8-C_{20}, Sigma-Aldrich) was analyzed in the same conditions to calculate the retention index. The compounds were identified by comparing the retention index to those found in the literature[11] and the mass spectra with NIST Library from the equipment.

Antioxidant Activity

DPPH assay

The samples were weighed and diluted in pure ethanol and added in different concentrations (from 1 up to 200 µg.mL^{-1}) in a 96-well plate. Following,[12] a solution of DPPH at 50 mM was used. Quercetin was used as a positive control in the same concentrations as the samples while the negative control was the solvent. After 30 min, the absorbance (Abs) was determined at 517 nm in a microplate reader (Molecular Devices SpectraMax M3®). All samples were tested in triplicate. The percentage of inactivation of the radical- DPPH (%) - was calculated based on the equation DPPH (%) = (Abs control −Abs sample/Abs control) × 100. The antioxidant activities were expressed as IC_{50}, which is the concentration in µg.mL^{-1} that inhibited 50% of the DPPH radical.

Oxygen Radical Absorbance Capacity (ORAC)

The antioxidant activity was also assessed using the ORAC assay, according the literature with modifications.[13] In this assay, AAPH was used as generator of radical peroxyl. Trolox was used as a standard and fluorescein as a probe. The test was performed in a 96-well black plate. The stock solution was prepared at a concentration of 1/80 (m/v) in acetone. Subsequently, they were diluted in RMCD solution (7%) in acetone/water (50:50). RMCD is used with lipophilic samples to change this character, as the test is performed in the presence of an aqueous buffer. Pure RMCD solution was used as blank. The fluorescence decrease (excitation at 485 nm; emission at 510 nm) was measured for 80 min at 37°C in a microplate reader (SynergyHT; Biotek, Wionooski, USA). The final antioxidant capacity was calculated using the linear regression equation between the Trolox concentration and the area under the fluorescein decay curve, and the results were expressed as gram of Trolox Equivalents (TE) per gram of essential oil (g of TE.g^{-1}). The samples were tested in triplicate, and the data were analyzed using Gen5™ 2.0 software.

Statistical Analysis

The data of the antioxidant assays were submitted to Analyses of Variance (ANOVA) and Tukey's test (p<0.05) in GraphPad Prism 6 software.

RESULTS

EO yield

The ripe fruit had the highest yield of essential oil (4.12%), followed by unripe fruit (2.93%). The leaves of the Female tree (F) showed the lowest yield (0.04%). Inflorescences of Female tree (F) were not included in this study as it was impossible to fully separate flowers and unripe fruit, since the flowering and fructification events occur simultaneously on the same branches. Finally, the inflorescences from the Male tree (M) yielded more EO (0.43%) than leaves (0.24%).

EO Chemical Composition

The EO samples of pink-pepper fruit, flowers and leaves were mainly composed of monoterpenes and sesquiterpenes (Table 1), however qualitative and quantitative differences were observed in their chromatograms (Figures 1 and 2). Leaf EO from both trees had a higher percentage of sesquiterpenes, while fruit (unripe and ripe) and inflorescence showed a higher percentage of monoterpenes in their chromatograms. The Ripe Fruit (FRP)

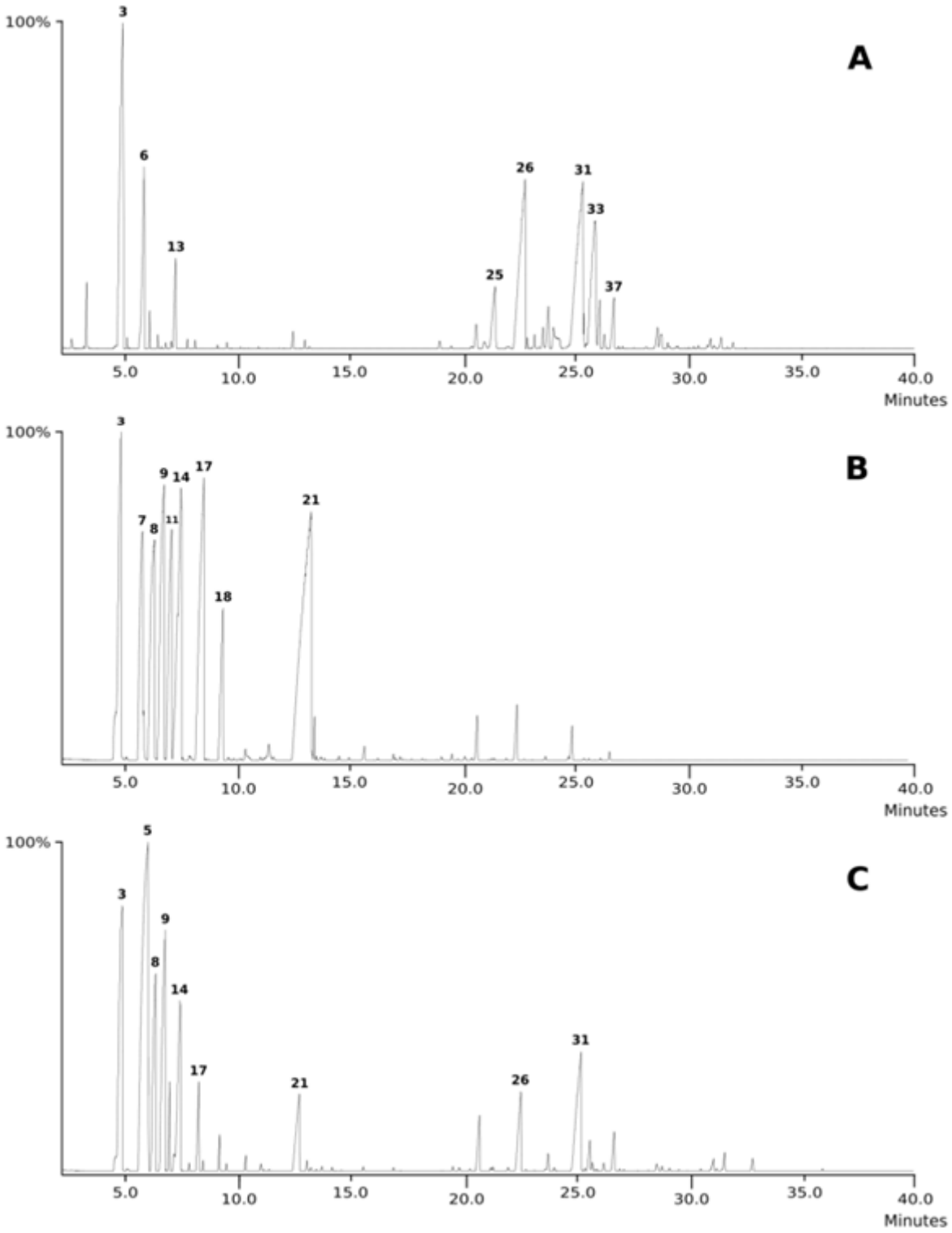

Figure 1: Essential oil chromatograms obtained by GC-MS of leaves (A), unripe fruit (B) and ripe fruit (C) of female *Schinus terebinthifolius*. Peak numbering according to Table 1.

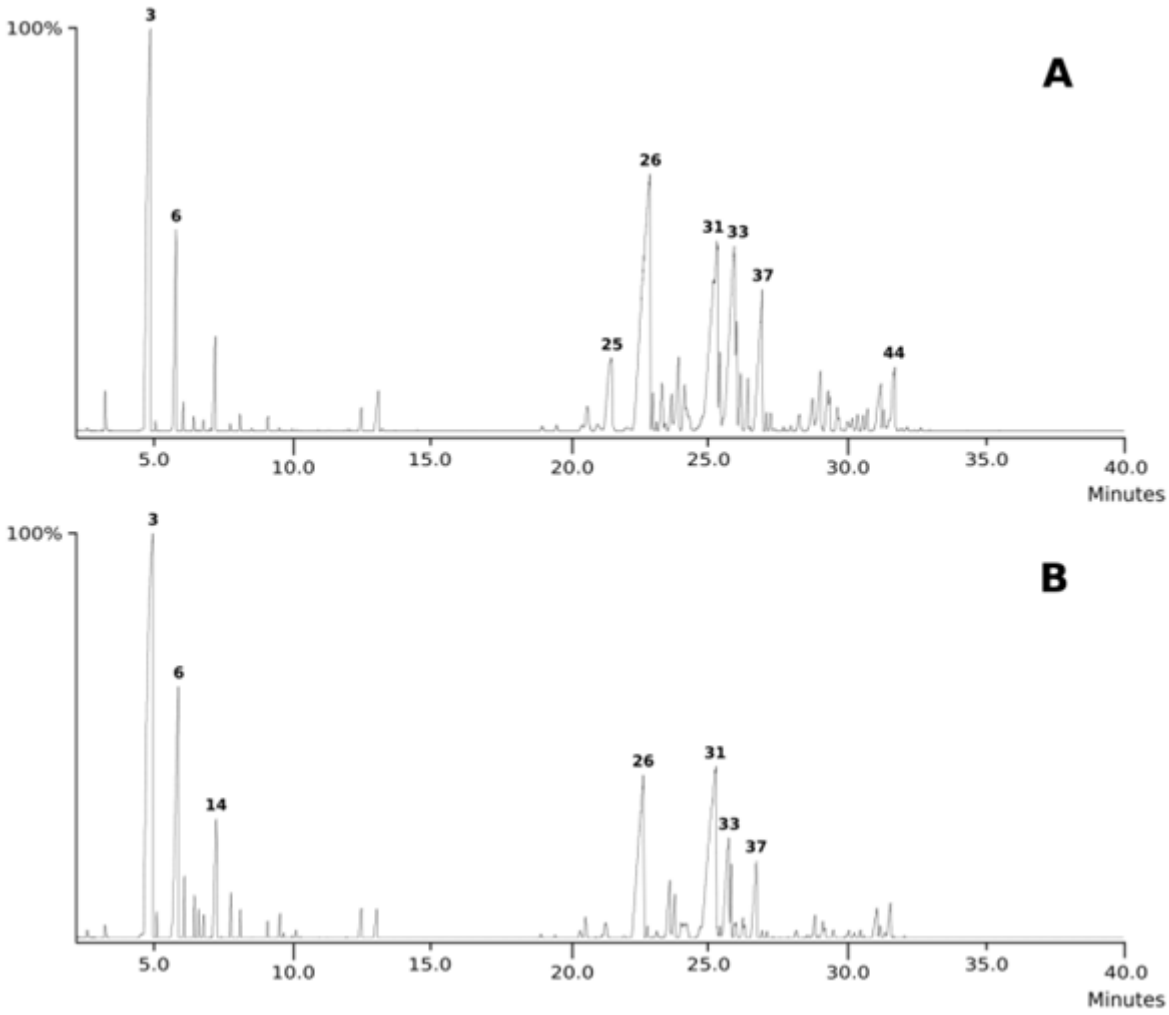

Figure 2: Essential oil chromatograms obtained by GC-MS of leaves (A) and inflorescence (B) of male *Schinus terebinthifolius*. Peak numbering according to Table 1.

EO had around 30% of sabinene, but the major compound in unripe fruit EO was terpinen-4-ol (24.1%). The compounds α-phellandrene and β-myrcene are present in both ripe and unripe fruit EO. Essential oil from leaves and inflorescences were rich in caryophyllene and germacrene D.

Antioxidant Capacity

The radical scavenging capacities of the EO were tested via two methods with different mechanisms of action (DPPH and $ORAC_{FL}$). Leaf and inflorescence EO showed no significant difference when compared to the positive control (quercetin) in the DPPH radical scavenging assay, indicating an exceptionally strong antioxidant activity (Table 2). The fruit EO had a lower antioxidant potential, nevertheless all the aerial parts demonstrated a promising antioxidant potential by DPPH. Although there are some studies about the antioxidant activities of leaf and fruit EO by DPPH,[14,15] none of them tested both samples under the same conditions. Furthermore, this is the first study of the antioxidant potential of inflorescence EO and the results indicate that this product deserves more studies.

The antioxidant activities of the *S. terebinthifolius* OE were also evaluated by $ORAC_{FL}$, but no statistical difference was observed between leaf, inflorescence and fruit EO (Table 2), with all aerial parts showing antioxidant capacity. In contrast to the DPPH results, fruit EO had the same antioxidant capacity as leaf and inflorescence EO by $ORAC_{FL}$ method.

DISCUSSION

The ripe fruit yielded the greatest amount of EO, followed by unripe fruit which was also observed by other authors.[16,17] However, fruit are present only periodically and leaves are present throughout the year, making them a promising source of EO. Furthermore, the reasonable yield of Male Inflorescences (MI) indicates a possible use for these flowers on trees that don't bear fruit; male trees are also reported to present more flowers than female individuals.[3]

Sabinene, which made up 30% of Ripe Fruit (FRP) EO, has anti-inflammatory and antioxidant activity.[18] This compound is considered important in the flavor of black pepper[19] and, therefore, is probably important for the culinary application of pink peppers as well. Conversely, the major compound in unripe fruit EO was terpinen-4-ol (24.1%). This monoterpene is commonly found in *Melaleuca alternifolia* Cheel and had been investigated due the its antitumor, anti-arthritic, anti-inflammatory and antibacterial activities.[20-23]

The compounds, α-phellandrene and β-myrcene, present in both ripe and unripe fruit EO, have been reported in ripe pink-pepper, independent of the sample origin.[24-28] Hence, they could be considered marker compounds for pink pepper fruit EO.

Caryophyllene and germacrene D, found in EO from leaves and inflorescences have important biological activities. Caryophyllene has been related to the antimicrobial activity of *Zingiber nimmonii*[29] and presents local anesthetic effects.[30] Germacrene

Table 1: Percentage area of compounds identified in *Schinus terebinthifolius* essential oil via GC-MS.

Peak Number	IR cal	IR lit	Compound	FUR	FRP	FL	ML	MI
1	804	801	Hexanal	-	-	0.18	-	0.11
2	851	855	2-Hexenal	-	-	1.32	0.47	0.18
3	940	939	Alpha-Pinene	10.04	14.22	21.25	14.37	32.49
4	951	954	Camphene	-	-	0.12	0.08	0.20
5	989	979	Beta-Pinene	-	-	7.03	3.58	10.42
6	990	975	Sabinene	-	31.39	-	-	-
7	981	987	Beta-Terpinene	7.90	-	-	-	-
8	994	990	Beta-Myrcene	9.32	7.83	0.54	0.25	0.80
9	1007	1002	Alpha-Phellandrene	10.44	11.27	0.20	0.15	0.51
10	1012	1011	3-Carene		1.41	2.81	0.12	0.31
11	1018	1017	Alpha-Terpinene	7.35	-	-	-	0.25
12	1028	1024	p-Cymene	-	0.41	0.17	-	-
13	1030	1029	Limonene	-	-	2.80	-	-
14	1031	1029	Beta-Phellandrene	11.14	7.57	0.20	-	3.44
15	1047	1037	Beta-Ocymene	-	0.09	0.14	-	0.63
16	1058	1059	Gamma-Terpinene	12.47	2.22	0.13	0.18	0.33
17	1089	1086	Terpinolene	3.54	0.59	-	0.18	0.20
18	1102	1096	Linalool	-	-	0.12	-	0.44
19	1122	1122	Trans-2-Menthenol	0.26	-	-	-	-
20	1191	1188	Alpha-Terpineol	0.40	0.17	0.19	0.92	0.72
21	1197	1177	Terpinen-4-ol	24.10	4.03	0.38	0.35	0.64
22	1348	1351	Alpha-Cubebene	0.07	0.08	-	-	0.19
23	1376	1375	Copaene	0.68	1.73	0.92	0.76	0.49
24	1392	1390	Beta-Elemene	-	-	3.77	3.61	0.61
25	1425	1419	Caryophyllene	1.00	3.51	17.82	20.64	12.01
26	1437	1441	Aromadendrene	-	-	0.32	1.15	0.15
27	1448	1451	Alpha-Himachalene	-	-	0.58	0.75	1.87
28	1453	1454	Alpha-Humulene	-	0.41	1.43	1.91	1.12
29	1459	1460	Alloaromadendrene	-	-	1.18	1.72	0.39
30	1490	1481	Germacrene D	0.53	8.62	20.84	14.47	17.38
31	1506	1496	Valencene	-	-	-	1.10	-
32	1506	1500	Bicyclogermacrene	-	0.78	11.08	12.13	4.78
33	1508	1500	Alpha-Muurolene	-	0.16	-	-	0.51

Peak Number	IR cal	IR lit	Compound	FUR	FRP	FL	ML	MI
34	1514	1513	Delta -Amorphene	-	-	-	1.20	-
35	1519	1513	Gamma-Cadinene	-	-	0.39	1.02	0.43
36	1527	1523	Delta-Cadinene	0.10	1.14	1.96	5.62	2.86
37	1532	1534	Cadin-1,4-diene	-	-	-	0.23	-
38	1540	1538	Alpha-Cadinene	-	-	-	0.29	-
39	1581	1583	Caryophyllene oxide	-	-	1.41	-	-
40	1586	1590	Globulol	-	-	-	1.74	-
41	1588	1592	Viridiflorol			0.09	1.41	0.40
42	1632	1632	Gamma-Eudesmol	-	-	-	0.40	-
43	1645	1640	Epi alpha-Cadinol	-	-	-	1.63	-
44	1658	1654	Alpha-Cadinol	-	-	-	2.15	-
			Monoterpenes	72.20	77.00	35.39	18.91	49.58
			Oxygenated monoterpenes	24.76	4.20	0.57	1.27	1.36
			Sesquiterpenes	2.38	16.43	60.29	66.60	42.79
			Oxigenated sesquiterpenes	0.00	0.00	0.09	7.33	0.40
			Others	0.00	0.00	2.64	0.47	0.29
			Total	**99.34**	**97.63**	**98.98**	**94.58**	**94.42**

RI: Retention index, lit: literature, cal: calculated

Table 2: Antioxidant potential of *Schinus terebinthifolius* essential oil determined by DPPH and $ORAC_{FL}$ assays.

Sample	IC_{50} (μg.mL^{-1})	g de TE.g^{-1} de EO
Leaf (FL)	3.5 ± 0.2	0.26±0.01
Inflorescence (MI)	2.8 ± 0.3	0.30±0.02
Ripe fruit (RP)	44.1 ± 2.6*	0.27±0.02
Quercetin	2.3 ± 0.01	nd

nd: not determined.
*Significantly different from other results in the same column, according ANOVA and Tukey test (p<0.05).

D was reported as the main component (80%) of *Kundmannia sicula* (L.) DC. EO and was correlated to its antioxidant activity.[31] Therefore, further studies of these biological activities of leaf and inflorescence EO of this species are indicated.

In contrast to the DPPH results, fruit EO had the same antioxidant capacity as leaf and inflorescence EO by $ORAC_{FL}$ method. The difference between the results of two assays could be explained by the mechanism of reaction: while the ORAC assay represents a typical HAT (hydrogen atom transfer) based method, employing a competitive reaction, the DPPH assay is based on the ET (electron transfer) reaction, wherein DPPH itself reacts with the antioxidant.[32]

No other studies of the antioxidant activity of *S. terebinthifolius* EO by $ORAC_{FL}$ were found in the literature. The values found in this study were similar to EO of basil, rosemary, citronella and dill seeds (<0.5 g de TE.g^{-1} de EO).[33]

The monoterpene, α-pinene, was found in all samples, contributing to the characteristic aroma of this species. This monoterpene has been shown to possess antioxidant capacity by the DPPH assay (IC_{50}=310 μg.mL^{-1}) and protected IEC-6 cells from oxidative stress caused by aspirin.[34] On the other hand, it presented a low antioxidant capacity by ORAC (0.08 ± 0.03 g Trolox/g pure compound).[33] Thus, α-pinene probably contributes to the strong antioxidant activity of leaves and inflorescence EO, as these EO showed higher percentage of this compound (21.25% and 32.49% respectively).

Although the antioxidant capacity of EO is related to their chemical composition, it is not a linear correlation. Inflorescence and leaf EO showed the best results via DPPH, the combined effect of many minor components with antioxidant capacity may be responsible.[35] Inflorescence and leaf EO samples also had more than 20% *α*-pinene in their composition and had oxygenated monoterpenes and sesquiterpenes, while ripe fruit EO had only oxygenated monoterpenes. This difference in chemical composition may confer greater activity to the leaves and flowers, as oxygenated compounds are more likely to donate the hydrogen electron than the hydrocarbons, necessary for the DPPH reduction mechanism.[36] In comparison, these variations in composition did not affect the results of the $ORAC_{FL}$ test, since all the aerial parts had similar antioxidant capacity. The difference

between the results of two assays could be explained by their different reaction mechanisms.[32]

CONCLUSION

According to these results, all aerial parts of *S. terebinthifolius* tree could be applied as antioxidants. This is useful information since this is species a dioecious plant; hence only female trees bear fruit and only in specific seasons. However more studies are necessary to guarantee their safety for human use. Different aerial parts of *S. terebinthifolius* showed variable chemical profiles, yields and antioxidant potentials. In spite of the fruit EO being a good source of bio-antioxidants, often evaluated in food systems, leaves and inflorescences also showed strong antioxidant activities. The expressive yield of male inflorescence EO combined with its strong antioxidant activity indicates a potential use for this native species, including the male trees. These results justify further investigation on the use of all *S. terebinthifolius* aerial parts as a natural source of antioxidants for the food and cosmetics industries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

Alexandra Christine Helena Frankland Sawaya: Conceptualization, Supervision, Resources, Writing - review and editing. Andressa Mara Baseggio: Methodology, Investigation, Data analysis, Writing – review and editing. Guilherme Perez Pinheiro: Methodology, Investigation, Data analysis, Writing – review and editing. Mara Junqueira Carneiro: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. Mário Roberto Maróstica-Júnior: Supervision, Resources, Writing - review and editing.

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