Chemical composition of essential oils from four *Piper* species, differentiation using multivariate analysis and antioxidant activity

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Abstract: Essential oils from the leaves of four species of *Piper* obtained through hydrodistillation were analyzed using GC-MS and multivariate data analysis. The chemical analysis enabled the identification of qualitative and quantitative differences among the oils. *β*-selinene (32.44 ± 1.14%), (*E*)-nerolidol (44.23 ± 2.23%), *β*-caryophyllene (19.11 ± 0.40%) and caryophyllene oxide (16.92 ± 0.21%) were identified as the major constituents of the *P. mollipilosum, P. brachypetiolatum, P. glandulosissimum* and *P. madeiranum* oils, respectively. The differences in the chemical profiles of the oils were confirmed by principal component analysis. All four species exhibited antioxidant activity. The oil from *P. brachypetiolatum* achieved the best results on the DPPH test (EC_{50} = 64.8 µg/ml) and with the ABTS radical (EC_{50} = 159.7 µg/ml).

Keywords: Essential oil; *Piper* ssp.; *P. mollipilosum*; *P. brachypetiolatum*; antioxidant activity.

Experimental

Plant material

The fresh leaves of *Piper mollipilosum* C.DC. (S 03° 11,187'; W 60° 26,481'), *Piper brachypetiolatum* Yunck. (S 03°11'05"; W 60°43'03"), *Piper glandulosissimum* Yunk. (S 03°11'07"; W 60°43'03") and *Piper madeiranum* Yunk. (S 03°11'07"; W 60°43'03") were collected in Manacapuru, metropolitan region of Manaus. The plants were identified by botanist Pereira M. R. (National Institute for Amazonian Research). Voucher of both samples were mounted and deposited in the Herbário do Instituto Nacional de Pesquisas da Amazônia (INPA), under numbers: (696) *Piper mollipilosum,* (687) *Piper brachypetiolatum,* (688) *Piper glandulosissimum* and (689) *Piper madeiranum.*

Isolation procedure of the essential oil

The essential oils from fresh leaves (100 g) were separately isolated using a modified Clevenger-type apparatus and hydrodistillation for 2h. The oil layers were separated and dried over sodium sulfate anhydrous, stored in hermetically sealed glass containers, and kept at low temperature (-5 ºC) until analysis. Total oil yields were expressed as percentages (g/100 g of fresh plant material). All experiments were carried out in triplicate.

Chemical analysis of essential oils

Quantitative GC analysis were carried out using a PerkinElmer Clarus 500 GC apparatus equipped with a flame ionization detector (FID) and a non-polar DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness) (J & W Scientific). The oven temperature was programmed from 60 to 240 °C at a rate 3 °C min^{-1}. Injector and detector temperatures were 260 °C. Hydrogen was used as the carrier gas at a flow rate of 1 mL/min in split mode (1:30). The injection volume was 0.5 µL of diluted solution (1/100) of oil in *n*-hexane. The amount of each compound was calculated from GC-FID peak areas in the order of DB-5 column elution and expressed as a relative percentage of the total area of the chromatograms. Analyses were carried out in triplicate. The qualitative Gas Chromatography-Mass Spectrometry (GC-MS) analysis were carried out using a Varian 220-MS IT GC system with a mass selective detector, mass spectrometer in EI 70 eV with a scan interval of 0.5 s and fragments from 40 to 550 Da. fitted with the same column and temperature program as that for the GC-FID experiments, with the following

parameters: carrier gas = helium; flow rate = 1 mL min^{-1}; split mode (1:30); injected volume = 1 µL of diluted solution (1/100) of oil in n-hexane.

Identification and quantification

Identification of the components was based on GC-MS retention indices with reference to a homologous series of C8-C40 n-alkanes calculated using the Van der Dool and Kratz equation (Van den Dool and Kratz 1963) and by computer matching against the mass spectral library of the GC-MS data system (NIST 14 and WILEY 11th) and co-injection with authentic standards as well as other published mass spectra (Adams 2017). Area percentages were obtained from the GC-FID response without the use of an internal standard or correction factors.

Principal component analysis

Principal component analysis (PCA) based on the complete data set (three different plant samples for each species) was conducted to evaluate the chemical variation of essential oils of *P. mollipilosum, P. brachypetiolatum, P. glandulosissimum and P. madeiranum* leaves. The GC-MS data were exported in ASCII format to Microsoft Excel to produce a data matrix of sample versus metabolite peak with associated peak areas. All the analysis were performed using the Unscrambler® software version 9.5 (CAMO Process AS, Norway, 1996-2007).

DPPH$^{\cdot}$ Radical Scavenging Activity Assay

The antioxidant activity of ethanolic extracts of the leaves from selected *Piper* species was performed against the free radical DPPH following the methodology of Silva et al. 2006. Stock solutions were prepared from the extracts and methanol fraction at several concentrations (0.10 to 5.0 mg/mL). Through preliminary analysis, appropriate quantities of stock solutions of the samples and 450 µL of the solution of DPPH$^{\cdot}$ (23.6 mg/mL in EtOH) were transferred to 0.5 mL Eppendorf tubes and the volume was completed with EtOH, following homogenization. Samples were sonicated for 30 min and the amount of DPPH$^{\cdot}$ was recorded on a UV-vis (Biochrom EZ Read 2000) device at a wavelength of 517 nm in a 96-well plate. Ascorbic acid was used as a positive control and all concentrations were tested in triplicate. The percentage scavenging activity (% SA) was calculated from the equation:

$$\%\,\mathrm{SA} = 100 \times \frac{(\mathrm{Abs}_{\mathrm{control}} - \mathrm{Abs}_{\mathrm{sample}})}{\mathrm{Abs}_{\mathrm{control}}}$$

where $Abs_{control}$ is the absorbance of the control containing only the ethanol solution of DPPH, and Abs_{sample} is the absorbance of the radical in the presence of the sample or standard ascorbic acid.

Determination of Antioxidant Activity against the Radical Cation ABTS$^{\cdot+}$

The determination of antioxidant activity from extracts of leaves from selected *Piper* species against the radical cation ABTS$^{\cdot+}$ was carried out following the methodology described by Re et al., 1999, in a UV-vis (Biochrom EZ Read 2000) device, using Trolox as the standard compound. The starting concentrations of the solutions of the samples were 0.1–1.0 mg/mL, with the addition of 450 µL of the radical ABTS$^{\cdot+}$ solution to give final concentrations of 2.5–100.0 µg/mL samples. Samples were protected from light and sonicated for 6 min. Absorbance of the samples and the positive control were measured at a wavelength at a wavelength of 734 nm using a microplate of 96 wells. Each concentration was tested in triplicate. The percentage of free radical scavenging activity

of $ABTS^{\bullet+}$ was calculated by the equation:

$$\% \, SA = 100 \times \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}}$$

where $Abs_{control}$ is the absorbance of the control containing only the ethanol solution of $ABTS^{+}$ and Abs_{sample} is the absorbance of the radical in the presence of the sample or standard ascorbic acid.

The antiradical efficiency was established using linear regression analysis and the 95% confidence interval ($p < 0.05$) obtained using the statistical program GraphPad Prism 5.0.

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Table S1. Percentage composition, yield of essential oils from leaves of *Piper* species.

Compounds	**RI[a]**	**RI[b]**	**PMOL**	**PBRA**	**PGLA**	**PMAD**	**Method of identification**
Yield (%) ± SD			**0.02 ± 0.01**	**0.06 ± 0.01**	**0.11 ± 0.01**	**0.05 ± 0.01**	
Limonene	1023	1024	0.43 ± 0.25	-	-	-	RI, MS, CI
Linalool	1096	1095	0.84 ± 0.03	-	-	-	RI, MS, CI
α-Terpineol	1188	1186	-	-	1.44 ± 0.03	-	RI, MS, CI
γ-Terpineol	1198	1199	-	-	-	1.07 ± 0.02	RI, MS, CI
α-Cubebene	1348	1345	-	-	1.65 ± 0.03	2.60 ± 0.03	RI, MS
Cyclosativene	1368	1369	-	0.56 ± 0.32	-	-	RI, MS
α-Ylangene	1373	1373	-	0.64 ± 0.37	-	-	RI, MS
α-Copaene	1376	1374	0.70 ± 0.02	-	4.83 ± 0.10	9.16 ± 0.12	RI, MS, CI
β-Cubebene	1387	1387	-	-	-	5.76 ± 0.08	RI, MS
β-Bourbonene	1388	1387	0.64 ± 0.02	-	0.43 ± 0.01	-	RI, MS
β-Elemene	1390	1389	1.89 ± 0.07	1.04 ± 0.62	-	-	RI, MS
iso-Longifolene	1391	1389	-	-	2.51 ± 0.03	-	RI, MS
β-Caryophyllene	1418	1417	-	-	19.11 ± 0.40	6.18 ± 0.08	RI, MS, CI
β-Ylangene	1420	1419	-	0.61 ± 0.05	-	-	RI, MS
β-Copaene	1432	1430	-	1.12 ± 0.06	-	-	RI, MS
α-Humulene	1454	1452	-	-	7.19 ± 0.15	2.62 ± 0.04	RI, MS, CI
4,5-di-*epi*-Aristolochene	1473	1471	-	-	0.21 ± 0.12		RI, MS
β-Chamigrene	1477	1476	2.40 ± 0.08	-	1.06 ± 0.02	1.09 ± 0.01	RI, MS
y-Muurolene	1479	1478	-	3.79 ± 0.28	0.71 ± 0.01	0.59 ± 0.01	RI, MS
Widdra-2,4(14)-diene	1482	1481	-	2.71 ± 0.02	-	-	RI, MS
Germacrene D	1485	1484	-	-	7.58 ± 0.16	-	RI, MS, CI
β-Selinene	1490	1489	32.44 ± 1.14	-	6.38 ± 0.13	8.70 ± 0.11	RI, MS
δ-Selinene	1492	1492	1.58 ± 0.06	-	-	6.44 ± 0.49	RI, MS
cis-*β*-Guaiene	1493	1492	0.36 ± 0.21	1.16 ± 0.06	-	-	RI, MS

α-Selinene	1498	1498	-	-	8.38 ± 0.17	-	RI, MS
α-Muurolene	1501	1500	-	1.40 ± 0.01	-	-	RI, MS
y-Cadinene	1512	1513	0.43 ± 0.25	2.50 ± 0.01	-	2.42 ± 0.03	RI, MS
Cubebol	1515	1514	0.49 ± 0.28		-		RI, MS
trans-Calamenene	1522	1521	0.33 ± 0.19	1.05 ± 0.06	-	1.04 ± 0.02	RI, MS
δ-Cadinene	1523	1522	-	-	3.10 ± 0.07	-	RI, MS
cis-Calamenene	1526	1528	-	-	0.94 ± 0.02	-	RI, MS
α-Cadinene	1538	1537	-	0.47 ± 0.02	-	-	RI, MS
cis-Sesquisabinene hydrate	1541	1542	-	1.02 ± 0.06	-	-	RI, MS
Elemol	1547	1548	1.11 ± 0.03	-	-	-	RI, MS
(*E*)-Nerolidol	1560	1561	1.60 ± 0.05	44.23 ± 2.23	1.30 ± 0.02	4.44 ± 0.05	RI, MS, CI
Spathulenol	1578	1577	8.21 ± 0.29	-	0.87 ± 0.02	-	RI, MS, CI
Caryophyllene oxide	1583	1582	11.7 ± 0.42	10.08 ± 0.74	4.90 ± 0.10	16.92 ± 0.21	RI, MS, CI
Gleenol	1586	1586	-	0.46 ± 0.02	-	-	RI, MS
Globulol	1591	1590	0.59 ± 0.02	-	-	-	RI, MS, CI
Viridiflorol	1592	1592	0.52 ± 0.02	-	-	-	RI, MS
n-Hexadecane	1601	1600	0.63 ± 0.02	-	-	-	RI, MS
Geranyl isovalerate	1605	1606	-	-	1.56 ± 0.03	-	RI, MS
Humulene epoxide II	1607	1608	1.94 ± 0.07	1.46 ± 0.11	-	3.59 ± 0.04	RI, MS
cis-Isolongifolanone	1613	1612	0.54 ± 0.02	-	-	-	RI, MS
1,10-di-*epi*-Cubenol	1618	1618	-	1.74 ± 0.13	-	-	RI, MS
Junenol	1617	1618	0.33 ± 0.19	0.42 ± 0.24	-	-	RI, MS
Isolongifolan-7*α*-ol	1618	1618	0.89 ± 0.03	-	1.46 ± 0.03	-	RI, MS
1-*epi*-Cubenol	1628	1627	-	1.78 ± 0.13	1.74 ± 0.03	2.40 ± 0.03	RI, MS
Eremoligenol	1629	1629	1.01 ± 0.03	-	-	-	RI, MS
γ-Eudesmol	1630	1630	0.63 ± 0.02	-	-	-	RI, MS
cis-Cadin-4-en-7-ol	1634	1635	-	1.34 ± 0.10	-	-	RI, MS

epi-*α*-Cadinol	1638	1638	-	1.58 ± 0.11	-	-	RI, MS
Caryophylla-4(12),8(13)-dien-5*β*-ol	1638	1639	-	-	1.51 ± 0.04	-	RI, MS
epi-*α*-Muurolol	1641	1640	0.69 ± 0.02	1.50 ± 0.11	-	-	RI, MS
α-Muurolol	1643	1644	-	1.13 ± 0.06	3.89 ± 0.19	3.51 ± 0.04	RI, MS
Cubenol	1644	1645	1.32 ± 0.05	-	-	-	RI, MS
(*Z*)-Methyl jasmonate	1647	1648	0.44 ± 0.25	-	-	-	RI, MS
α-Cadinol	1653	1652	-	8.97 ± 0.65	4.01 ± 0.09	-	RI, MS
Selin-11-en-4*α*-ol	1658	1658	6.81 ± 0.02	-	2.89 ± 0.06	9.26 ± 0.12	RI, MS
cis-Calamenen-10-ol	1661	1660	-	-	-	0.56 ± 0.01	RI, MS
14-hydroxy-(*Z*)-Caryophyllene	1667	1666	0.57 ± 0.02	-	-	-	RI, MS
trans-Calamenen-10-ol	1668	1668	-	0.80 ± 0.04	-	-	RI, MS
14-hydroxy-9-*epi*-(*E*)-Caryophyllene	1669	1668	5.06 ± 0.18	-	-	-	RI, MS
Andro encecalinol	1674	1675	-	-	1.61 ± 0.17	0.82 ± 0.01	RI, MS
Occidentalol acetate	1680	1681	-	-	-	1.49 ± 0.02	RI, MS
epi-*α*-Bisabolol	1682	1683	1.21 ± 0.04	-	-	-	RI, MS
cis-14-*nor*-Muurol-5-en-4-one	1687	1688	-	0.73 ± 0.02	-	-	RI, MS
Junicedranol	1690	1692	0.54 ± 0.02	-	-	-	RI, MS
10-*nor*-Calamenen-10-one	1701	1702	-	0.54 ± 0.03	-	-	RI, MS
Khusimol	1742	1741	1.95 ± 0.07		-	-	RI, MS
Eupatoriochromene	1760	1759	-	0.41 ± 0.02	-	-	RI, MS
Cyclocolorenone	1761	1759	0.60 ± 0.02	-	-	-	RI, MS
β-Costol	1765	1766	0.62 ± 0.36	-	-	-	RI, MS
14-oxy-*α*-Muurolene	1768	1767	0.36 ± 0.01	-	-	-	RI, MS
Eudesm-11-en-4*α*,6-*α*-diol	1809	1808	0.58 ± 0.02	-	-	-	RI, MS
11,12-dihydroxy-Valencene	1915	1914	0.78 ± 0.02	-	-	-	RI, MS

Monoterpene hydrocarbons	0.43 ± 0.25	-	-	-
Oxygenated monoterpenes	0.84 ± 0.03	-	1.44 ± 0.03	1.07 ± 0.02
Sesquiterpene hydrocarbons	40.58 ± 0.20	21.99 ± 0.06	64.08 ± 0.04	46.60 ± 0.03
Oxygenated sesquiterpenes	51.91 ± 0.50	77.46 ± 0.20	25.74 ± 0.10	42.99 ± 0.15
Total	93.76 ± 0.72	99.45 ± 0.51	91.26 ± 0.06	90.66 ± 0.56

RI^a = Retention indices calculated from retention times in relation to those of a series C8-C40 of n-alkanes on a 30m DB-5 capillary column. RI^b = Retention indices from the literature. PMOL = *P. mollipilosum*, PBRA = *P. brachypetiolatum*, PGLA = *P. glandulosissimum*, PMAD = *P. madeiranum*. SD = Standard deviation. RI = retention indices, MS = mass spectroscopy and CI = Co-injection with authentic compounds.

Table S2. Antioxidant activity of the essential oil of *Piper* species.

Plants	DPPH	ABTS
	EC_{50} µg/ml (Confidence interval)	**EC_{50} µg/ml (Confidence interval)**
P. brachypetiolatum	64.8 ± 3.8	159.7 ± 8.3
P. gladulosissimum	104.4 ± 6.4	200.9 ± 6.4
P. madeiranum	66.8 ± 5.2	242.6 ± 6.8
P. mollipilosum	79.0 ± 4.9	280.5 ± 6.6
Ascorbic acid	1.6 ± 0.2	-
TROLOX	-	4.1 ± 1.1

EX = Ethanol extract; EO = Essential Oil; Ascorbic acid and TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as reference antioxidants.

Figure Captions

Figure S1: Principal component analysis scores (PC1 and PC2) of the GC-MS of essential oil of leaves of *P. mollipilosum* (PMOL), *P. brachypetiolatum* (PBRA), *P. glandulosissimum* (PGLA) and *P. madeiranum* (PMAD).

Figure S2: Principal component analysis loadings (PC1 and PC2) of the GC-MS of essential oil of leaves of *P. mollipilosum*, *P. brachypetiolatum*, *P. glandulosissimum* and *P. madeiranum*.

Figure S3: GC-MS chromatogram of the essential oil from leaves of *Piper brachypetiolatum.*

Figure S4: GC-MS chromatogram of the essential oil from leaves of *Piper glandulosissimum.*

Figure S5: GC-MS chromatogram of the essential oil from leaves of *Piper madeiranum.*

Figure S6: GC-MS chromatogram of the essential oil from leaves of *Piper mollipilosum.*

Figure S7: Calibration curve of Ascorbic acid used as positive control in the DPPH test.

Figure S8: Calibration curve of Trolox used as positive control in the ABTS test.

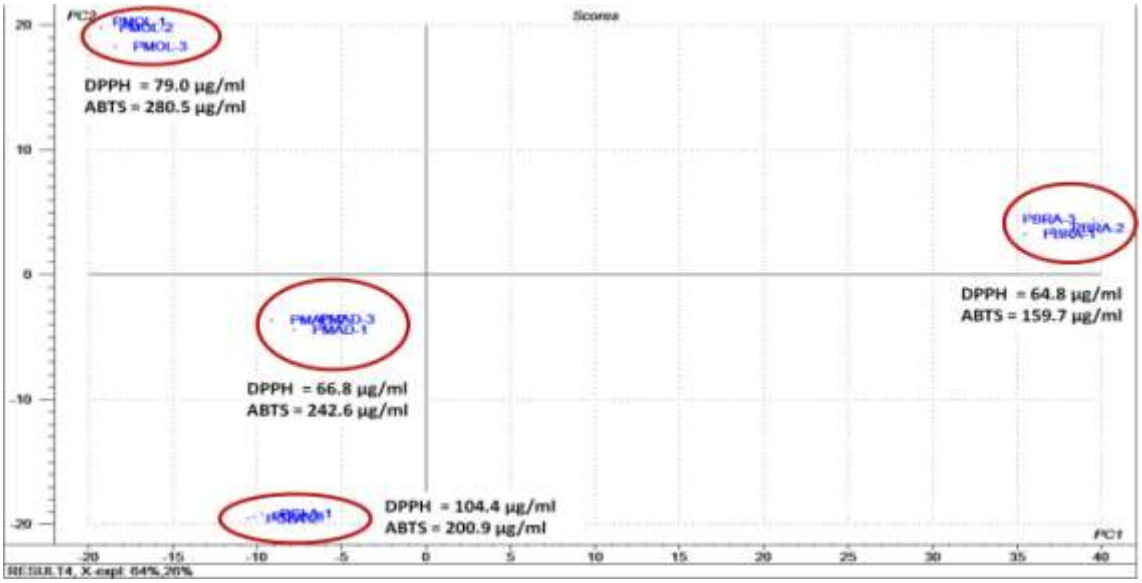

Figure S1: Principal component analysis scores (PC1 and PC2) of the GC-MS of essential oil of leaves of *P. mollipilosum* (PMOL), *P. brachypetiolatum* (PBRA), *P. glandulosissimum* (PGLA) and *P. madeiranum* (PMAD).

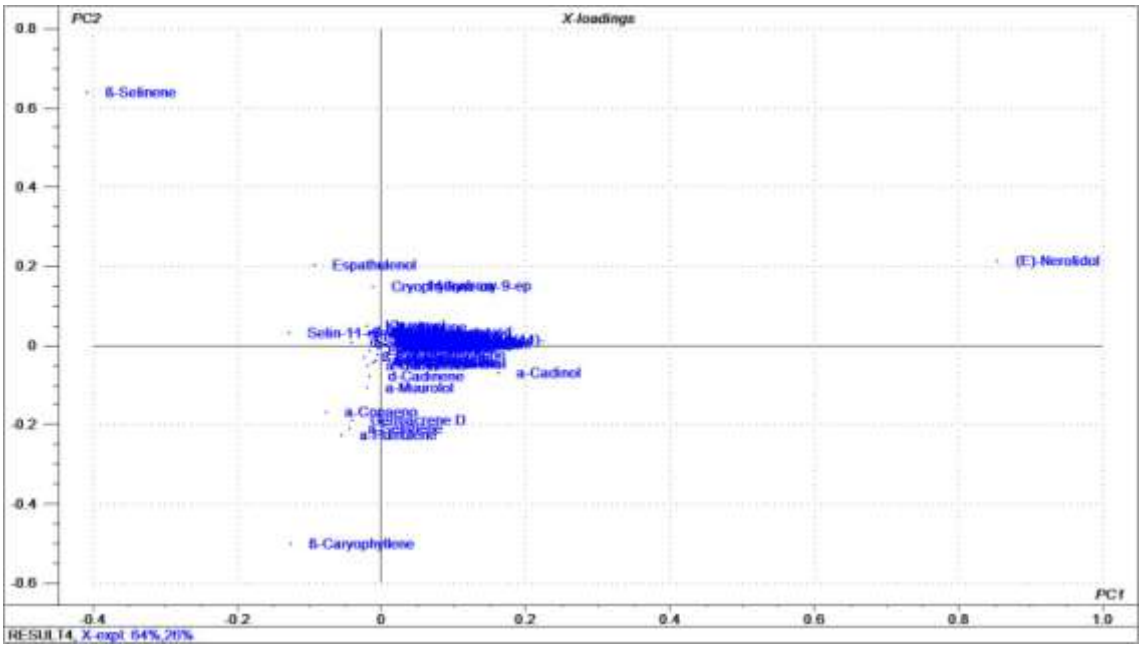

Figure S2: Principal component analysis loadings (PC1 and PC2) of the GC-MS of essential oil of leaves of *P. mollipilosum*, *P. brachypetiolatum*, *P. glandulosissimum* and *P. madeiranum*.

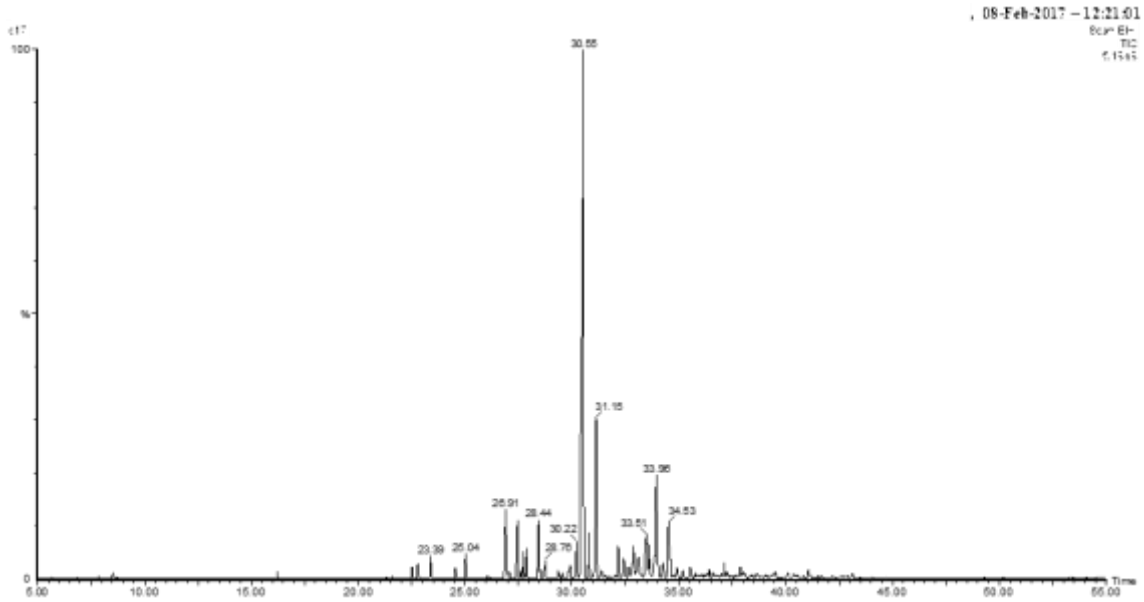

Figure S3: GC-MS chromatogram of the essential oil from leaves of *Piper brachypetiolatum.*

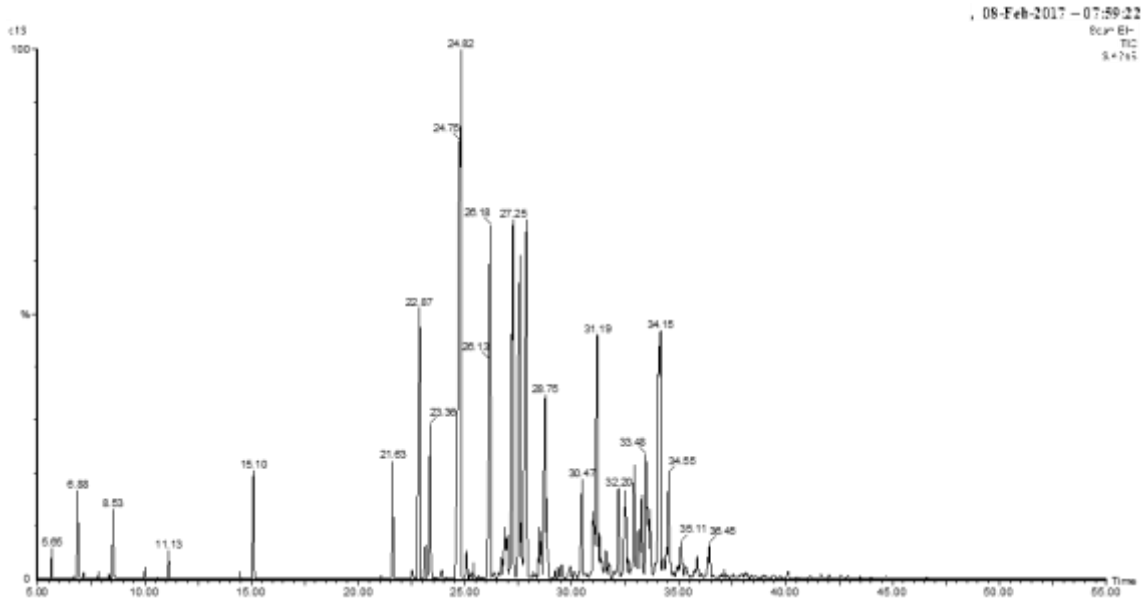

Figure S4: GC-MS chromatogram of the essential oil from leaves of *Piper glandulosissimum.*

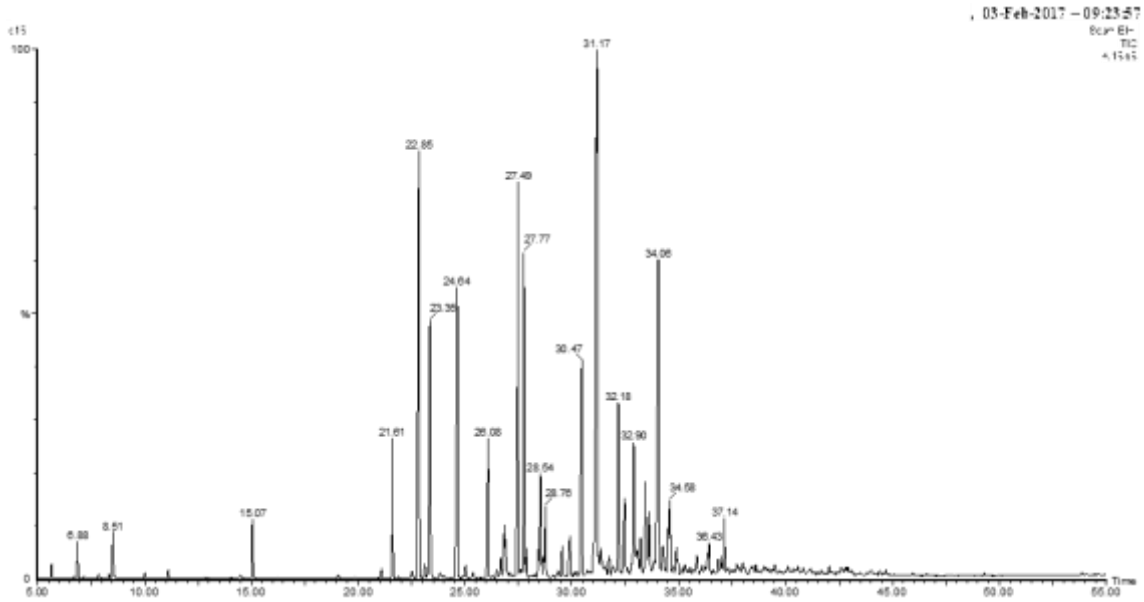

Figure S5: GC-MS chromatogram of the essential oil from leaves of *Piper madeiranum.*

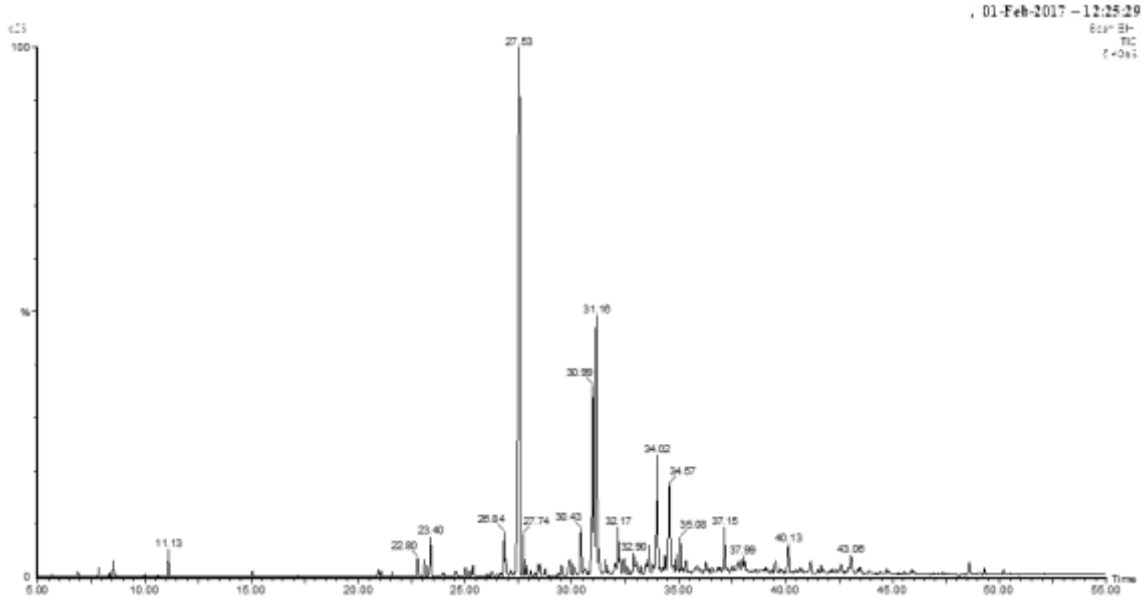

Figure S6: GC-MS chromatogram of the essential oil from leaves of *Piper mollipilosum.*

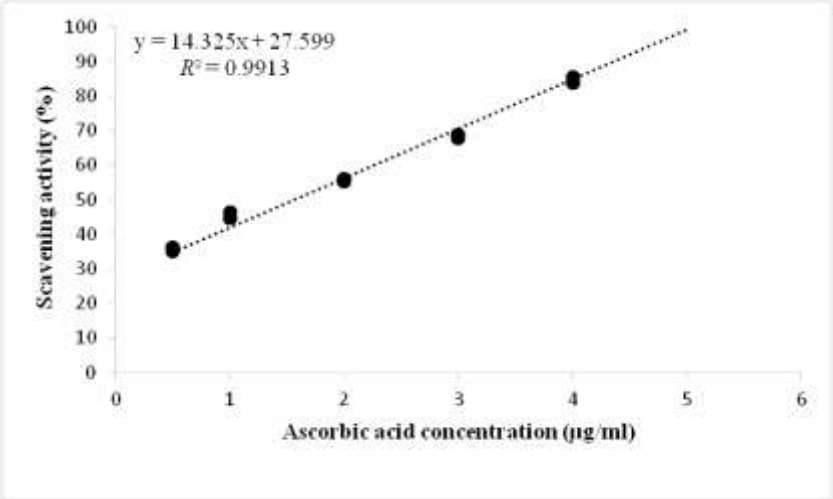

Figure S7: Calibration curve of Ascorbic acid used as positive control in the DPPH test.

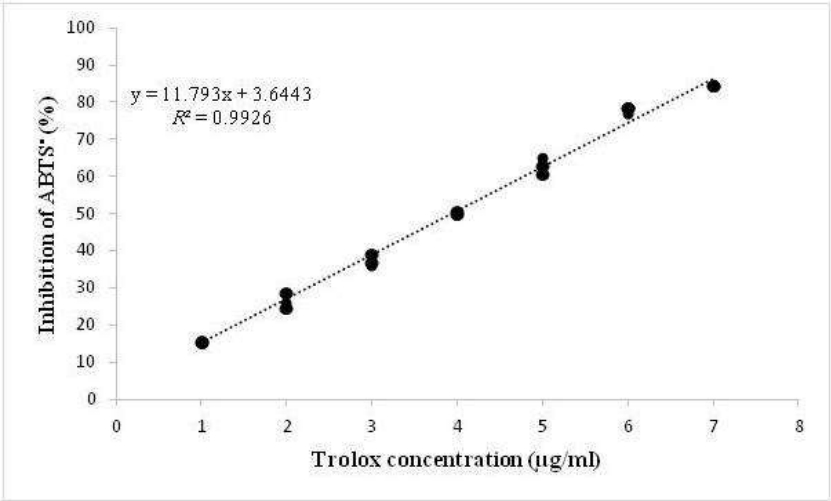

Figure S8: Calibration curve of Trolox used as positive control in the ABTS test.