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Chemical composition, antioxidant and antiproliferative activities of essential oil from *Schinus areira* L. and *Minthostachys spicata* (Benth.) Epl. grown in Cuzco, Peru

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ABSTRACT

The chemical composition, antioxidant or anti-proliferative activities of essential oils from leaves of *Schinus areira* L. and *Minthostachys spicata* (Benth.) Epl. grown in Cuzco (Peru) was studied. Fifty-six and fifty-eight compounds were identified in the essential oils from *S. areira* and *M. spicata* leaves, respectively. In *S. areira* essential oil, monoterpene hydrocarbons were the most represented class of volatiles (58.7%), including α-phellandrene, limonene, camphene, β-phellandrene, α-pinene, *p*-cymene, and β-pinene as the major components. In *M. spicata*, oxygenated monoterpenes (87.4%) were the major class of volatiles; among them pulegone, isomenthone, and menthone were the main compounds. Antioxidant activity were examined using 1,1-diphenyl-2-picryl-hydrazyl assay. Essential oil from *S. areira* was also evaluated for its anti-proliferative activities against murine breast cancer cells (4T1). Our results justify the use of these plants in traditional medicine in Peru and open a new field of investigation in the characterization of the molecules involved in the antioxidants and anti-proliferative processes.

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Schinus areira; *Minthosthachys spicata*; essential oil; composition; antioxidant activity; anti-proliferative activity

Introduction

Molle, aguaribay, false pepper plant (Spain), pirú (Mexico), pepper tree (USA), among other, are the common names of two taxonomically related species of the genus *Schinus*: *S. molle* L. and *S. areira* L. (*S. molle* L. var. *areira*[L.] AD.). Most of the authors refer to these species identifying them as *S. molle*, but at present, it is considered that *S. areira* is distributed in the whole American continent and cultivated in other countries, while *S. molle* would have a much more enclosed distribution, being only in the Argentinean northeast, Uruguay, Paraguay and south of Brazil (1, 2). Essential oil isolated from leaves and berries has been characterized mainly by the presence of α-phellandrene, β-phellandrene and limonene (3–8). *S. areira* is used as a purgative, diuretic, parasiticide, insecticide, vulnerary, and topical disinfectant, and for the treatment of rheumatism, stomach upsets, menstrual disorders, bronchitis and conjunctivitis, while its essential oil has shown significant antibacterial, antifungal, antioxidant, and insecticidal activities (1–3, 7–15).

Minthosthachys spicata (Benth.) Epl. (Lamiaceae) is a perennial shrub 0.30–1.0 m high, mostly grows in the southern Peru, generally in dry and stony slopes (16). It is among the most intensively aromatic species in Peru. *Minthosthachys* genus is taxonomically complicated and it comprises seventeen species of scandent aromatic shrubs restricted to Andean South America (17). As in many Lamiaceae, the essential oils are the chemical components that are of commercial and pharmaceutical interest. Nevertheless, the great majority of studies are limited to *M. verticillata* and *M. mollis* (17). Other studies cannot as reliably be related to species names due to the lack of voucher specimens. Nevertheless, pulegone and menthone were the principal components of the oil in unidentified plants from Jauja in Peru (33.1 and 48.2%, respectively) (18), Lima in central Peru (47.4% and 25.3%; *M. mollis* or *M. spicata* [Benth.] Epling) (19), southern Peru (45 and 18%; probably *M. acris*) (20) and Bolivia (36–65% and 19–32%) (21).

Despite the medicinal potential of plants in Peru being considerable, knowledge of this area and studies on the biological activities of these plants remained scarce. Furthermore, as far as our literature survey could ascertain, antioxidant activities of the Peruvian aromatic plants *S. areira* L. and *M. spicata* (Benth.) Epling have

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not previously been published, although, there are many reports concerning essential oils from these species in other countries. Moreover, knowing that the chemical composition and activity of essential oils from aromatic plants depends on several factors such as the geographical origin (2), the aims of this study were (i) to determine the composition of the essential oils isolated from the leaves of *Schinusareira* L. and *Minthostachys spicata* (Benth.) Epl. grown in Cuzco (Peru) and (ii) to assess the antioxidant and anti-proliferative properties of these essential oils.

Experimental

Plant material and isolation of essential oil

Leaves of *S. areira* and *M. spicata* were collected during April 2014 in the Cuzco region, Peru (3200 m height above sea level). The species were identified, and the voucher specimens (accession numbers 11049 CUZ for *S. areira* and 24275 CUZ for *M. spicata*, respectively) were deposited at the herbarium Vargas of the National University of San Antonio Abad del Cusco. Six samples of chopped fresh leaves (ca. 400 gin each batch) from different plants were extracted using hydrodistillation for 3 hours in a Clevenger-type apparatus.

GC-FID and GC-MS

Analyses of the essential oils were performed by gas chromatography with a flame ionization detector (GC-FID) on a Konik 4000A (Konik, Barcelona) equipped with a 30 m × 0.25 mmi.d. × 0.25 mm HP-Innowax (Agilent Technologies, Santa Clara, CA, USA)or DB-5 ms fused-silica capillary columns (J & W Scientific, Folsom, CA, USA).The analyses were conducted under the following conditions for both columns: oven temperature program, 60°C (2 minutes), 60–220°C (4°C/min) and 220°C (5 minutes); carrier gas helium flow rate 1 mL/min; injector and detector temperatures 250°C, injection volume 0.2 µL and split ratio 20:1.

Essential oils were also analyzed by gas chromatography-mass spectrometry (GC–MS) using a Hewlett Packard 6890, fitted with the same columns interfaced with an Hewlett Packard mass-selective detector5973 (Agilent Technologies, PaloAlto, CA, USA). GC parameters were similar to GC-FID and interface temperature: 250°C; MSsource temperature: 230°C; MS quadrupole temperature: 150°C; ionization energy: 70eV; mass range: 35–400 *m/z*.

The different components were identified using the irretention indices and mass spectra. Retention indices, calculated using linear interpolation relative to retention times of C_8–C_{24} of *n*-alkanes, were compared with those standards and data from the literature (22, 23). Mass spectra were compared with corresponding reference standard data reported in the literature (22) and mass spectra from NIST 05, Wiley 6, NBS 75 k, and in-house Flavorlib libraries. In many cases, the essential oils were subject to co-chromatography with authentic compounds.

The quantification of compounds was performed using relative percentage abundance and normalization method with correction response factors based on grouping the essential oil components by their functional groups (24). Percentage data are the mean values of three injections per sample.

Assay of 2,2-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity

The antioxidant capacity of these essential oils was measured determining of DPPH radical scavenging ability according to the method previously described (25) with minor modifications. In the test tubes, 1500 µL of DPPH (0.075 mg/mL) in ethanol was mixture with 750 µL of five concentrations of the oil sample to evaluate in a range of concentrations between 10 and 2000 µg/mL. A control sample (absolute ethanol) and a reference sample (750 µL absolute ethanol and 1500 µg/mL of DPPH solution) were also used. The decrease in the absorbance (Abs) at 515 nm was determined in a UV spectrophotometer, until the reaction plateau step was reached. Triplicate measurements were carried out. Then, the IC_{50} values were determined by using GraphPad Prism program ver. 5 and it was defined as the total antioxidant compound necessary to decrease the initial DPPH radical concentration by 50%. Their scavenging effect of the product was calculated based on the percentage of DPPH scavenged using the followed formula:

% DPPH inhibition = (control Abs – sample Abs)/(control Abs) × 100, were control Abs represent: ethanol Abs + DPPH and sample Abs:sample Abs + DPPH.

Cytotoxicity evaluation of the essential oil in 4T1 cells

The 4T1 cells were cultured in DMEM medium supplemented with 10% newborn calf serum, penicillin 50 U/mL and streptomycin 50 µg/mL. For sub-culturing purposes, cells were detached by treatment with 0.25% trypsin/0.02% EDTA at 37°C. Cultures were used at 75% confluence. In order to study the potential toxicity of the product, cells were exposed to a wide range of concentrations of the extract (10-1000 µg/mL) for 24 hours. Control cells (only medium-treated cells) were included in the experiments.

MTT assay

Cytotoxicity induced by the essential oils from *S. areira* was measured using the MTT reduction assay with

modifications (26). MTT is a tetrazolium salt that is biotransformed into a purple formazan product after reduction by the mitochondrial enzyme succinate dehydrogenase that is only present in metabolically active live cells, not in dead cells. The formazan product can be solubilized and then photometrically quantified. Briefly, after the indicated treatments with the product, cell monolayers cultured in 96-well plates were washed with phosphate buffer saline (PBS) and 100 µL/well of MTT reagent (5 mg/mL) were added. After 4 hours in a cell incubator at 37°C, culture supernatants were discarded and cells were washed again. The dye was extracted with dimethyl sulfoxide and optical density was read at 540 nm on a microplate reader. Each experimental procedure was performed in at least two cell preparations.

The percentage of inhibition of the succinic dehydrogenase reduction of MTT was expressed as the percentage of viable cells referred to control cell culture incubated in the absence of test compounds but in presence of the vehicle (which is considered the negative control, and is considered as 100% viability value). Data were expressed as the mean ± SD. Statistical analyses of cell viability were performed by one-way ANOVA, followed by a Dunnett's test for multiple comparisons using the GraphPad Prism ver. 5 statistical software package. A $p \leq 0.05$ was considered statistically significant.

Results and discussion

The extraction yield of *S. areira* and *M. spicata* leave essential oils were 0.15% and 0.5% (v/m), respectively. Figure 1 shows the gas chromatograms of both essential oils. Quantitative data expressed as average percentage values and standard deviations, of single components in the essential oils are listed in Table 1. Fifty-six components were identified in the essential oil from *S. areira* leaves. As can be seen, monoterpene hydrocarbons were the most represented class of volatiles with 58.7%. Among their derivative, α-phellandrene, limonene, camphene, β-phellandrene, α-pinene, *p*-cymene, and β-pinene were the major compounds. Sesquiterpene hydrocarbons were found as the second major chemical class (22.9%) with (*Z*)-caryophyllene being the main component. Some studies of leaf and fruit essential oils from *S. areira* and *S. molle* showed different chemical composition. Results of this work showed a different pattern compared to those obtained from plants collected in Europe, North Africa, and American plants (3, 4, 6–8, 15, 27–35). This variability can reflect the influence of extrinsic conditions based on geographic origin (different climatic and soil-growth conditions), the effect of intra-specific differences, and perhaps an incorrect botanical identification of the vegetable material in some previous report.

To the best of our knowledge, there are only few unreliable reports on the chemical composition of the oils from *M. spicata* species, mainly due to the lack of voucher specimens. Fifty-eight constituents were identified in the essential oil from *M. spicata* leaves (Table 1). The essential oil contains basically oxygenated monoterpenes (87.4%) being the major constituents pulegone, isomenthone, and menthone. According to the most reliable report (19), the major constituents of the essential oil from this species were menthone (14.5%), 2-hydroxy-*p*-menth-1-en-3-one (12.5%), and 3,4,5-trimethoxytoluene (12.4%), but the last two ones were not found in this study.

The oxidative stress plays an important role in the initiation and progression of many diseases in the man. Although several compounds have been described in the literature with strong antioxidant properties, today the search of new products or pure compounds with significant antioxidant activity is plenty justified. The DPPH is a free radical compound has been widely used to evaluate the *in vitro* ability of compounds to free radical scavenging. At this work, we evaluated the *in vitro* antioxidant scavenging by the free radical scavenging capacity of the essential oils from *S. areira* and *M. spicata* grown in Peruvian using the DPPH assay.

Both *S. areira* and *M. spicata* essential oils exhibited a concentration dependent increase to DPPH radical scavenging effect showing a maximum effect of 71.2 ± 8.7% at the concentration of 1000 µg/mL and 76.05 ± 2.4% at 500 µg/mL, respectively, but lower than the percentage of inhibition observed for the positive control used in the study (ascorbic acid), which exhibited a percentage of inhibition of 89.0 ± 4.3% at the concentration of 100 µg/mL. On the other way, usually DPPH scavenging is also presented by IC_{50}value, the concentration of the antioxidant needed to scavenged 50% of the DPPH radical present into the test solution. In this case, the IC_{50}value observed for the products tested was 174.6 ± 12.3 µg/mL for *S. areira* and 82.19 ± 6.7 µg/mL for *M. spicata*. As these data show essential oils from *S. areira* exhibited weakest antioxidant effects than *M. spicata* and the ascorbic acid (51.2 ± 2.9 µg/mL), which agree with reports of the literature that show the low ability to some essential oils for DPPH scavenging activity (8, 15, 36–40). These studies showed some differences on the antioxidant capacity of the essential oils and it was attributed to differences in the chemical composition, but also to the low ability to essential oils with higher amounts of monoterpene hydrocarbons for DPPH scavenging activity. Although, monoterpene hydrocarbons as α-pinene, β-pinene, limonene, myrcene, sabinene and terpinolene have antioxidant properties, some of them show low activity depending on the mechanism involved in the oxidative reaction (41).

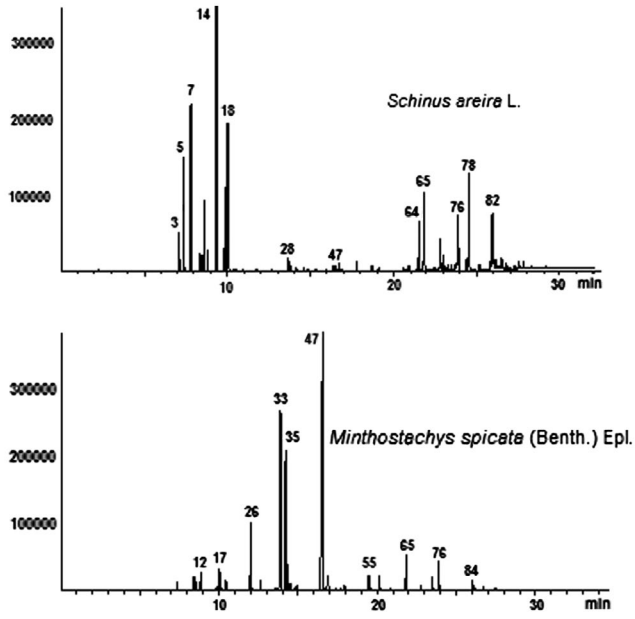

Figure 1. Chromatographic profile on DB-5 ms column of *Schinusareira* L. and *Minthosthachys spicata* (Benth.) Epl. essential oils.

Table 1. Composition of the essential oil from *Schinusareira* and *Minthostachys spicata* leaves.

Peak	Compound	RI_A[a]	RI_A[Oa]	RI_P[b]	RI_P[Ob]	*S. areira*%	*M. spicata*%
1	(*E*)-2-Hexenal	855	856	1201	1204	-	tr[c]
2	(*Z*)-3-Hexenol	859	859	1350	1353	-	tr
3	Tricyclene	929	927	1017	1015	1.7 ± 0.1	-
4	α-Thujene	930	931	1020	1019	-	tr
5	α-Pinene	940	939	1032	1033	5.2 ± 0.4	-
6	α-Fenchene*	952	953	-	-	-	0.4 ± 0.0
7	Camphene	954	956	1076	1075	8.4 ± 0.7	tr
8	Sabinene	973	972	1125	1121	0.8 ± 0.0	0.6 ± 0.0
9	β-Pinene	979	979	1114	1117	3.2 ± 0.2	-
10	3-Octanone*	983	982	-	-	-	tr
11	Myrcene	987	989	1162	1160	0.9 ± 0.1	0.3 ± 0.0
12	3-Octanol	991	992	1386	1385	tr	1.1 ± 0.1
13	(*E*)-3-Hexenyl acetate*	1002	1002	-	-	-	tr
14	α-Phellandrene	1002	1004	1168	1169	**18.2 ± 0.5**	-
15	α-Terpinene	1016	1014	1179	1180	-	tr
16	*p*-Cymene	1023	1024	1270	1268	4.4 ± 0.3	0.2 ± 0.0
17	Limonene	1027	1029	1201	1203	9.4 ± 0.8	1.0 ± 0.1
18	β-Phellandrene	1030	1031	1209	1210	6.5 ± 0.5	-
19	1,8-Cineole	1033	1033	1215	1213	tr	1.2 ± 0.1
20	(*E*)-β-Ocimene	1050	1051	1248	1246	tr	0.5 ± 0.0
21	γ-Terpinene	1060	1059	1244	1242	tr	tr
22	*cis*-Sabinene hydrate	1070	1070	1558	1560	-	0.1 ± 0.0
23	*cis*-Linalool oxide (furanoid)	1074	1075	1449	1449	-	tr
24	Methyl 2-phenethyl ether*	1083	1083	-	-	-	0.1 ± 0.0
25	Terpinolene	1088	1086	1280	1282	0.1 ± 0.0	tr
26	Linalool	1095	1097	1354	1355	tr	4.8 ± 0.2
27	Nonanal	1101	1100	1389	1390	tr	-
28	β-Thujone	1114	1110	1443	1441	0.9 ± 0.1	-
29	*cis*-2-*p*-Menthen-1-ol*	1118	1122	1553	1555	tr	-
30	3-Octyl acetate	1123	1124	1340	1338	-	0.7 ± 0.0
31	*trans*-Sabinol*	1141	1141	-	-	-	0.1 ± 0.0
32	Isopulegol	1150	1148	1569	1570	-	0.2 ± 0.0
33	Menthone	1153	1152	1478	1476	0.4 ± 0.0	**14.2 ± 0.4**
34	*iso*-Isopulegol*	1160	1160	-	-	-	0.1 ± 0.0
35	*iso*-Menthone	1161	1163	1495	1496	0.2 ± 0.0	**15.0 ± 0.3**
36	Borneol	1169	1167	1701	1700	0.1 ± 0.0	-
37	Menthol	1172	1170	1627	1628	-	1.9 ± 0.1
38	Terpinen-4-ol	1176	1174	1590	1592	0.3 ± 0.0	-
39	Cryptone*	1180	-	-	-	0.1 ± 0.0	-
40	*trans*-Isopulegone	1182	1181	1597	1599	-	0.9 ± 0.0
41	3-Decanone	1185	1184	1490	1488	-	0.1 ± 0.0
42	*neoiso*-Menthol*	1187	1186	1641	1640	-	0.2 ± 0.0

(*Continued*)

Table 1. (*Continued*).

Peak	Compound	RI_A [a]	RI_A [Oa]	RI_P [b]	RI_P [Ob]	*S. areira*%	*M. spicata*%
43	3-Decanol	1189	1190	1602	1600	-	0.2 ± 0.0
44	α-Terpineol	1191	1193	1698	1695	-	0.4 ± 0.0
45	Myrtenal*	1196	1196	-	-	-	tr
46	β-Citronellol	1225	1226	-	-	-	tr
47	Pulegone	1235	1237	1662	1663	0.4 ± 0.0	**43.2 ± 0.8**
48	Carvone	1243	1241	1725	1723	tr	-
49	Piperitone	1254	1253	1733	1735	0.1 ± 0.0	1.8 ± 0.1
50	*neo*-Menthyl acetate	1274	1274	-	-	-	0.3 ± 0.0
51	Bornyl acetate	1289	1288	1570	1567	tr	-
52	Thymol	1291	1290	2167	2169	tr	0.1 ± 0.0
53	Indole	1293	1293	2168	2172	-	tr
54	Menthyl acetate	1295	1298	1574	1573	-	0.6 ± 0.0
55	Piperitenone	1342	1340	1949	1950	-	1.2 ± 0.1
56	Thymol acetate*	1352	1352	-	-	-	0.2 ± 0.0
57	Eugenol	1357	1359	2140	2138	-	tr
58	4aα,7α,7aα-Nepetalactone*	1360	1360	-	-	-	tr
59	Neryl acetate*	1363	1362	-	-	-	tr
60	Piperitenone oxide*	1368	1369	1983	1983	-	1.2 ± 0.1
61	α-Copaene	1377	1374	1483	1485	0.2 ± 0.0	tr
62	β-Bourbonene	1388	1384	1507	1509	-	0.2 ± 0.0
63	β-Elemene	1390	1391	1576	1579	0.4 ± 0.0	-
64	α-Gurjunene	1410	1408	1538	1540	3.1 ± 0.3	-
65	(*Z*)-Caryophyllene	1419	1418	1584	1586	4.9 ± 0.4	2.2 ± 0.1
66	β-Copaene	1432	1430	1613	1610	-	tr
67	Nerylacetone*	1437	1436	-	-	-	tr
68	α-Humulene	1453	1455	1662	1665	2.2 ± 0.1	0.3 ± 0.0
69	*allo*-Aromadendrene	1460	1459	1638	1635	1.2 ± 0.1	tr
70	*trans*-Cadina-1(6),4-diene*	1476	1477	-	-	0.1 ± 0.0	-
71	γ-Muurolene	1480	1481	1682	1682	0.4 ± 0.0	-
72	Germacrene D	1485	1486	1714	1711	0.4 ± 0.0	0.8 ± 0.0
73	β-Selinene	1490	1492	1726	1723	0.1 ± 0.0	-
74	γ-Amorphene	1495	1495	1724	1726	0.4 ± 0.0	-
75	Viridiflorene	1497	1497	1716	1715	0.5 ± 0.0	-
76	Bicyclogermacrene	1505	1500	1740	1739	4.9 ± 0.3	1.9 ± 0.1
77	γ-Cadinene	1512	1514	1750	1752	0.2 ± 0.0	-
78	δ-Cadinene	1521	1523	1748	1749	3.8 ± 0.2	
79	*trans*-Cadina-1(2),4-diene*	1535	1535	-	-	0.1 ± 0.0	-
80	α-Cadinene*	1539	1539	-	-	0.1 ± 0.0	-
81	Elemol	1549	1550	2085	2085	0.5 ± 0.0	-
82	(*E*)-2-Tridecenal	1569	1566	1968	1966	1.5 ± 0.1	-
83	Germacrene D-4-ol	1575	1575	2057	2058	5.1 ± 0.4	-
84	Spathulenol	1578	1578	2130	2132	1.5 ± 0.1	0.9 ± 0.0
85	Caryophyllene oxide	1583	1584	1985	1984	-	0.2 ± 0.0
86	Gleenol	1587	1587	2051	2054	tr	-
87	Viridiflorol	1595	1593	2109	2106	1.4 ± 0.1	-
88	1,10-di-*epi*-Cubenol	1619	1620	2054	2057	0.5 ± 0.0	-
89	10-*epi*-γ-Eudesmol	1625	1624	2121	2120	0.5 ± 0.0	-
90	*epi*-α-Cadinol	1639	1640	-	-	1.8 ± 0.1	-
90	α-Muurolol	1644	1646	2193	2197	0.4 ± 0.0	-
91	α-Cadinol	1654	1654	2239	2243	2.3 ± 0.2	-
92	Monoterpene hydrocarbons					58.7	3.4
93	Oxygenated monoterpenes					2.5	87.4
94	Sesquiterpene hydrocarbons					22.9	5.4
95	Oxygenated sesquiterpenes					14.0	1.1
96	Other compounds					1.6	1.2
97	Total identified					99.7	98.5

Notes: [a]RI_A and RI_P, experimental linear retention indices on DB-5ms and HP-Innowax column, respectively; [b]RI_A^O and RI_P^O, linear retention indices from standard or literature on DB-5ms and HP-Innowax column, respectively; [c]trace (<0.1%); *tentatively identified compound by comparison with literature data.

Meanwhile, the essential oil from *M. spicata* was able to reduce DPPH into the neutral form of the radical, and this activity was concentration-dependent. According to the chemical analysis performed, the most powerful scavenging could be attributable to the presence of menthone and isomenthone in this essential oil, compounds with recognized scavenging activity (42).

Taking in mind the important role of play the oxidative stress in various human diseases, this antioxidant activities reasonably sustains the potential use of these products as a natural antioxidant with consequent health benefits. However, before final recommendations are made it would be necessary to further the relevance of such antioxidant activities on other *in vitro* battery assays and *in vivo* pharmacological models.

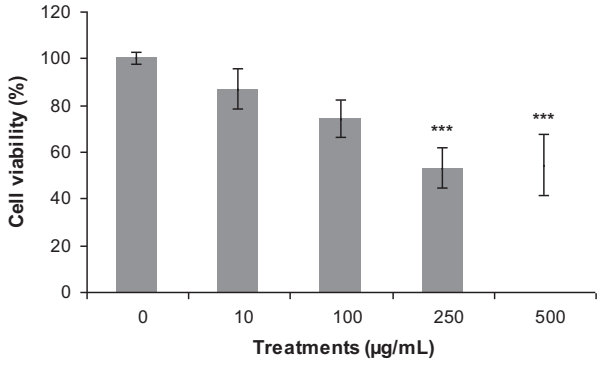

Figure 2. Cytotoxic effects of *Schinusareira* essential oil on 4T1 cells. Cells were exposed to increasing concentrations of the product and cell viability was determined 24 hours later by the MTT test. Results are expressed as the percentage of controls (untreated cells). IC_{50} = 104.6 µg/mL.Each point represents the mean ± SD of three experiments with six replicates, ***$p < 0.001$ Dunnett's test.

On the other hand, among eight direct methanolic extracts from plants used in traditional medicine, *S. molle* showed the greatest cytotoxic activity (IC_{50} = 50 ± 7 µg/mL) against a human hepatocellular carcinoma cell line, Hep G2 (10). The essential oil was cytotoxic in several cell lines, showing that it is more effective on breast carcinoma and leukemic cell lines. The IC_{50} for cytotoxicity at 48 hours in K562 corresponded to 78.7 µg/mL (15). Therefore, as shown in Figure 2, the essential oil from *S. areira* was also evaluated for its anticancer activity against breast cancer cells (4T1). Significant cytotoxic effects (IC_{50} = 104.6 µg/mL) were observed after the exposure this tumour cells to different concentrations of the essential oil from *S. areira* over a 24 hours period. The cytotoxicity exhibited by the essential oil may be related to minor constituents specifically sesquiterpenic compounds. The cytotoxic effects of sesquiterpenes have been previously described in different cellular models, especially in tumor cell lines, e.g. (*Z*)-caryophyllene and α-humulene have been shown to be active against the MCF-7 breast cancer cell lines (43, 44); α-cadinol and γ -muurolene showed anticancer activity against colon and breast cancer cell lines (45).

These results show that the leaf essential oil from *S. areira* grown in Cuzco exhibits antioxidant and induced cytotoxicity effects under our experimental conditions. In this way, future studies could be interesting to perform to elucidate the role that different components of the essential oil play in the effects observed here, since some of them could have potential anti-tumor effects, either alone or in combination.

In conclusion, *Schinus areira* and *Minthostachys spicata* essential oils showed antioxidant and anti-proliferative properties, suggesting their potential use in food, cosmetic or pharmaceutical industries.

Disclosure statement

No potential conflict of interest was reported by the authors.

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