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Original article

High resolution UHPLC-MS characterization and isolation of main compounds from the antioxidant medicinal plant *Parastrephia lucida* (Meyen)

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ABSTRACT

High-resolution mass spectrometry is currently used to determine the mass of biologically active compounds in medicinal plants and food and UHPLC-Orbitrap is a relatively new technology that allows fast fingerprinting and metabolomics analysis. Forty-two metabolites including several phenolic acids, flavonoids, coumarines, tremetones and ent-clerodane diterpenes were accurately identified for the first time in the resin of the medicinal plant *Parastrephia lucida* (Asteraceae) a Chilean native species, commonly called umatola, collected in the pre-cordillera and altiplano regions of northern Chile, by means of UHPLC-PDA-HR-MS. This could be possible by the state of the art technology employed, which allowed well resolved total ion current peaks and the proposal of some biosynthetic relationships between the compounds detected. Some mayor compounds were also isolated using HSCCC. The ethanolic extract showed high total polyphenols content and significant antioxidant capacity. Furthermore, several biological assays were performed that determined the high antioxidant capacity found for the mayor compound isolated from the plant, 11- *p*-coumaroyloxyltremetone.

1. Introduction

The native plant *Parastrephia lucida* (Meyen) Cabrera is a native shrub, commonly called umatola, growing in the pre-cordillera and Altiplano in the North of Chile and Argentina (Benites et al., 2012; CONAMA, 2008; D'Almeida et al., 2012; Marticorena, 2009). This species has been historically used in traditional medicine as an anti-inflammatory agent, to treat toothache (Villagrán et al., 2003). Furthermore, this plant showed some biological activities with potential health benefits such as acaricide (Genin et al., 1995), fungicide (Sayago et al., 2006), bactericide (Zampini et al., 2009) and antioxidant activities (Rojo et al., 2009; Zampini et al., 2008). A few studies have identified different active compounds (Benites et al., 2012; D'Almeida et al., 2012), however, a comprehensive complete metabolomics analysis was not performed, letting out a number of interesting structures that may possess pharmacological interest. The related species *Parastrephia lepidophylla* showed antifungic activity and showed also 19% inhibition of cell proliferation at 200 μg/ml in antiproliferative activity tests performed in Caco-2 cells (Rodrigo et al., 2010). From this plant the analgesic compounds tremetone and methoxytremetone were isolated (Benites et al., 2012). On the other hand, the herbal teas of *P. lepidophylla* and *P. lucida* showed a protective effect against oxidative damage on human erythrocytes greater than of the standard antioxidant Trolox (Rojo et al., 2009; Zampini et al., 2008). In the present work we have performed the isolation of the main compounds plus the high resolution UHPLC orbitrap metabolomic fingerprinting analysis of the resin exudate of this plant and report several poly-methoxilated flavonoids, tremetones and terpenoids

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for the first time. The antioxidant properties of the ethanolic extract of *P. lucida* are also discussed.

2. Materials and methods

2.1. Chemicals and plant material

UHPLC-MS Solvents, LC-MS formic acid and reagent grade chloroform were from Merck (Santiago, Chile). Ultrapure water was obtained from a Millipore water purification system (Milli-Q Merck Millipore, Chile). HPLC standards, (kaempferol, quercetin, isorhamnetin, eriodictyol, luteolin, apigenin, naringenin, all standards with purity higher than 95 % by HPLC) were purchased either from Sigma Aldrich (Saint Louis, Mo, USA), ChromaDex (Santa Ana, CA, USA), or Extrasynthèse (Genay, France). Folin-Ciocalteu phenol reagent (2N), reagent grade Na_2CO_3, $AlCl_3$, HCl, $FeCl_3$, $NaNO_2$, NaOH, quercetin, trichloroacetic acid, sodium acetate, Gallic acid, Trolox, ABTS and potassium persulfate, xanthine oxidase and DPPH (1,1-diphenyl-2-picrylhydrazyl radical) were purchased from Sigma-Aldrich Chemical Co. (Santiago, Chile).

2.2. Plant material

The aerial parts of *P. lucida* (Meyen) Cabrera were collected in slopes of Chungará lake (18°23′0″ S Longitud 69°16′4″O), in March 2016 at 4524 m.a.s.l. Voucher herbarium specimens are kept in the National Herbarium of Natural History (Santiago, Chile), SGO 166498 (see Fig. 1).

2.3. Extraction

Dried and chopped aerial parts of *P. lucida* (2 g) were extracted with absolute ethanol for 30 min in the dark in an ultrasonic bath (100 mL, three times) in order to obtain an extract for UHPLC, isolation and antioxidant analyses. The extract was immediately concentrated *in vacuo* and a resulting brown gum was obtained (882 mg).

2.4. Selection of the solvent system for HSCCC

Several isocratic non aqueous solvent systems composed of *n*-hexane: ethyl acetate: methanol: water and *n*-heptane–ethyl acet ate–methanol–water in different ratios were tested. The solvent system selected was the two-phase solvent system: *n*-hexane: ethyl acetate: methanol: water 6:5:6:3 v/v/v which provided the better K values for all mayor compounds ($0.5 < K < 1.3$). A similar solvent system (*n*-heptane–ethyl acetate–methanol–water, 6:2:6:2, v/v/v/v) was previously used for the separation of terpenoids (Vieira et al., 2015).

2.5. Isolation

A portion of the extract (0.5 g) was filtered and submitted to an HSCCC centrifuge (Quattro MK-7, Bridgend, UK) for the separation of its components. After equilibration of the solvent system in a separatory funnel, the upper and lower working phases were separated and degassed in an ultrasonic bath for 15 min before use. The sample was prepared by dissolving the extract from *P. lucida* in 2.5 mL of each phase of the solvent system, filtered and loaded into an injection valve (Rheodyne model 5010A) equipped with a 5 mL loop. The preparative coil (116 ml) was filled with the upper stationary phase and the apparatus was rotated at 850 rpm. The mobile lower phase was then pumped in a Head to Tail direction (H-T) at a flow rate of 5 mL-minute. After the emersion of mobile phase and the hydrodynamic equilibrium in the column, we recorded the percentage of the retention of stationary phase (60%). The sample was injected using an injection valve at a flow rate of 6.5 mL-minute. The fractions obtained were collected with a fraction collector (1 min per tube, 6.5 ml each, 100 fractions) and monitored by an Ecom 254 nm detector (Prague, Check Republic) with Ecomac software and a chromatogram was obtained (Fig. S1, supplementary material) and fractions were further analyzed by TLC (F_{254} silica gel plates, developed with hexane:EtOAc, 1:1 v/v, while the spots were visualized by spraying using vanillin: sulfuric acid 2% in ethanol. The rotation was interrupted in tube 55 and the coil content was washed off originating 100 fractions of

Fig. 1. Photograph of aerial parts of *Parastephia lucida* (Meyen), Cabrera collected in the slopes of the Chungará lake at 4524 m.u.s.l.

6.5 mL each. The tubes were pooled into six fractions (pq-1 to pq-6) according to the HSCCC chromatogram (Fig. S1, Supplementary material) and TLC analysis. After re-purification by Sephadex LH-20 (solvent methanol), HSCCC tubes 17–23 (fraction pq-2) afforded 11-p-coumaroyloxy-tremetone (55 mg) (Bohlmann et al., 1979), whose NMR spectra and validated data is depicted in the Supplementary material, (Figs. S2–S5) and crystal structure determination was unequivocally determined by us lately (Brito et al., 2017). From tubes 24–30 (fraction pq-3) aesculetin and umbelliferone were identified by spiking experiments with authentic standards (Simirgiotis et al., 2013b), and finally, from tubes 55–63 (fraction pq-6), bacchalineol A (23 mg) was isolated, whose NMR spectra are depicted in the Supplementary material (Figs. S6–S9).

2.6. UHPLC-DAD-MS instrument

The Thermo Scientific Dionex Ultimate 3000 UHPLC system hyphenated with a Thermo Q exactive focus machine used was already reported (Simirgiotis et al., 2016b). For the analysis 5 mg of the exudate were dissolved in 2 mL of methanol, filtered (PTFE filter) and 10 μL were injected in the instrument, with all specifications set as previously reported (Simirgiotis et al., 2016b).

2.7. LC parameters and MS parameters

Liquid chromatography was performed using an UHPLC C18 column (Acclaim, 150 mm × 4.6 mm ID, 2.5 μm, Thermo Fisher Scientific, Bremen, Germany) operated at 25 °C. The detection wavelengths were 254, 280, 330 and 354 nm, and DAD was recorded from 200 to 800 nm for peak characterization. Mobile phases were 1 % formic aqueous solution (A) and acetonitrile (B). The gradient program time (min), % B) was: (0.00, 5); (5.00, 5); (10.00, 30); (15.00, 30); (20.00, 70); (25.00, 70); (35.00, 5) and 12 min for column equilibration before each injection. The flow rate was 1.00 mL min^{-1}, and the injection volume was 10 μL. Standards and the resin extract dissolved in methanol were kept at 10 °C during storage in the autosampler. The HESI II and Orbitrap spectrometer parameters were optimized as previously reported (Garneau et al., 2013; Simirgiotis et al., 2016b).

2.8. Antioxidant assays

2.8.1. DPPH free radical scavenging activity

The free radical scavenging activity of the resin was determined by the DPPH· assay as previously described (Ramirez et al., 2015). Values are reported as IC_{50}, which denotes the concentration of sample required to scavenge 50 % of DPPH free radicals.

2.8.2. ABTS assay

The activity was performed using the ABTS radical cation assay as reported (Sogi et al., 2013). Briefly, 7 mM ABTS was mixed with potassium persulfate 2.45 mM (1:1 v/v) and allowed to stand for 12 h to produce the radical cation. The obtained solution was then diluted to get and absorbance of 0.7 at 734 nm. Then the ABTS solution (3 mL) was mixed with 30 μL of sample and measured after 6 min in the spectrophotometer.

2.8.3. Superoxide anion scavenging assay

The enzyme xanthine oxidase produces superoxide anion radical (O_2^-) "*in vivo*" which in turn reduces the nitro blue tetrazolium dye (NBT), leading to a chromophore compound at 520 nm. The Superoxide anion scavenging activities (SAA) of the resin extract and standards were measured spectrophotometrically as reported previously (Simirgiotis et al., 2016a).

2.9. Polyphenol, flavonoid and anthocyanin contents

The total polyphenolic contents (TPC) were determined by the Folin-Ciocalteau method as reported (Simirgiotis et al., 2013b). Determination of total flavonoid content (TFC) of the resin was performed as previously described (Simirgiotis et al., 2013b).

2.10. Cell culture

The human breast non-tumorigenic cell line MCF10F (ATCC), considered the normal counterpart, were used to evaluate the cytotoxic effect and only MCF10F for antioxidant effect *in vitro* of ethanolic extract and pure compounds. These cells were cultured in specific media according to ATCC recommendations. The incubation conditions were established at 37 °C, complete humid atmosphere, 5% CO_2 and 95% O_2.

2.11. Reactive oxygen species (ROS) scavenging activity

MCF10F cells were seeded in 6-well plate (4×10^5 cells per well) and incubated overnight. Cells were exposed to a pretreatment of different concentrations of ethanolic extract (10–100 μg/ml) and pure compound (10–200 μM) for 1 h, and then 25 μM of pyocyanin (PCN) was added, as generator of ROS. Three controls were used: DMSO 0.5% was used as vehicle control, N-acetylcysteine 20 mM (NAC) was used for its antioxidant mechanism and pyocyanin was used has ROS producer. The effect of each treatment with PCN was assayed for 30 min. Reactive oxygen species generated by PCN treatment were detected using Muse® Oxidative Stress Kit (Merck #MCH100111) in the Muse Cell Analyzer® (Merck) according manufacture instructions. The Muse® Oxidative Stress Kit allows for the quantitative measurements of ROS in cells undergoing oxidative stress based on the intracellular detection of superoxide radicals. The assay provides relative percentage of cells that are ROS negative and positive.

2.12. Statistical analysis

The statistical analysis was carried out using the originPro 9.1 software packages (Originlab Corporation, Northampton, MA, USA). The determination was repeated at least three times for each sample solution. Analysis of variance was performed using ANOVA. Significant differences between means were determined by Tukey's comparison test (p values < 0.05 were regarded as significant). For biological assays the analysis of variance was performance using one way-ANOVA, using the Dunnett's test to determined significant difference between control group and treatments (p value < 0.05).

3. Results and discussion

The data-dependent scan experiment was very useful for the identification of unknown phenolic compounds since it provides high resolution and accurate mass product ion spectra from precursor ions that are unknown beforehand within a single run (Fig. 2). Combining data-dependent scan and MS^n experiments, phenolic compounds were tentatively identified in *P. lucida* including simple flavones, flavonols, flavanones, coumarins, tremetones, phenolic acids, fatty acids, acetophenones and clerodane terpenoids. Some of the compounds were identified by spiking experiments with available standards. As far as we know, some of the compounds are reported for the first time: aesculetin, 8-hydroxy-7-methoxy-scopoletin, 7-methoxy-8-hydroxyesculetin, luteolin-O-hexoside and its derivatives, 6-hydroxy-7-methoxytremetone, 19-hydroxy-bacchalineol A acetate and derivatives, and dehidro-19-acetyl-

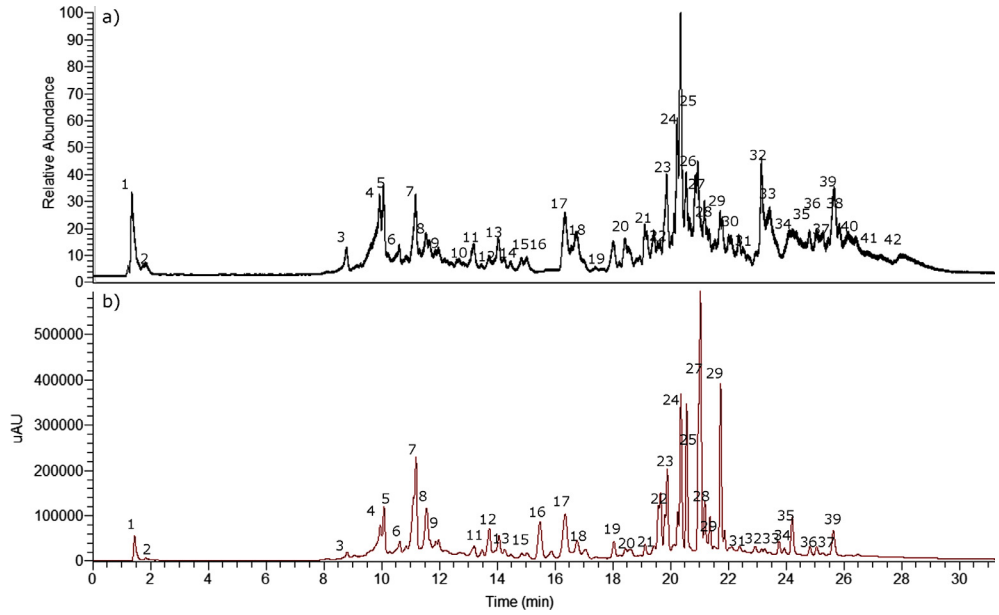

Fig. 2. UHPLC chromatograms (a) TIC (total ion current, negative mode) and (b) UV at 280 nm, of *Parastrephia lucida* ethanolic extract.

hawtriwaic acid methyl ester. The generation of molecular formulas was performed using high resolution accurate mass analysis (HRAM) and matching with the isotopic pattern. Lastly, analyzes were confirmed using MS/MS data and comparing the fragments found with the literature. Figs. S10–S12 (Supplementary material) show the full HR-MS spectra and biosynthetic relationships between the compounds under study.

3.1. Phenolics, flavonoids and antioxidant capacities of P. lucida

The ferric reducing antioxidant power (FRAP), scavenging of DPPH free radical, and superoxide anion scavenging (SAA) assays were used to evaluate antioxidant capacities of *P. lucida* (see Table 1). These methods are simple and widely used for the evaluation of antioxidant capacity (Lopez-Alarcon and Denicola, 2013). Moreover, we determined the total phenolic content (TPC) and total flavonoid content (TFC) calculated in dried weight basis of sample (see Table 1), and compared to other plants or fruit material previously analyzed by us. Thus, the TPC values of this plant, 185.12 ± 3.32 mg GAE/g dry weight, were close to those obtained for the aerial parts of *Luma apiculata* (179.83 ± 0.38 mg GAE/g), while the total flavonoid content (163.14 ± 2.84 mg Q/g), were also close than the aerial parts of *L. apiculata* (139.70 ± 1.48 mg Q/g) (Simirgiotis et al., 2013a). In the DPPH assay, *P. lucida* (4.23 ± 0.18) exhibited more DPPH scavenging capacity than the standard quercetin (9.08 ± 0.65). The DPPH values 4.23 ± 0.18 μg/mL, were higher than those obtained for three Chilean *Nolana* species (from 30 to 120 μg/mL) (Simirgiotis et al., 2015), but were close to those reported for some antioxidant Chilean berries (from 2 to 12 μg/mL) (Ramirez et al., 2015). The ABTS values (1.574 ± 11.42 μM Trolox equivalents/g dry weight) were higher to that of several parts of mango fruits, the value for several reported mango kernels were from 1110 to 1124 μM Trolox equivalents/g dry weight (Sogi et al., 2013). The superoxide anion scavenging activity (SAA) of *P. lucida* (98.12 ± 2.24%) and was higher than reported for blueberries *Vaccinium corimbosum* (72.61 ± 1.91%) (Ramirez et al., 2015) (Table 1).

3.2. Metabolomic analyses

We have determined several metabolites including: 18 flavonoids (peaks **12**, **13**, **15**, **16**, **18**–**28**, **30**, **31** and **33**), 5 coumarins (peaks **5**, **6**, **8**, **10** and **28**), 5 tremetones (peaks **29**, **32**, **35**–**37**), 5 phenolic acids (peaks **1**, **4**, **7**, **9** and **11**), 2 fatty acids (Peaks **2** and **3**), 2 acetophenones (peaks **14** and **17**), and 5 clerodane terpenoids (peaks **34**, **38**–**40** and **42**) were identified in the UHPLC chromatogram of the resin of *P. lucida* (Fig. 2). The detailed identification is explained below (see Table 2 and Figs. S11 and S12).

3.2.1. Flavonoids

These compounds are a large family with a common chemical structure: a diphenylpropane skeleton bearing two benzene rings connected by pyran ring attached to the A ring. Many biological

Table 1
Scavenging of the 1,1-diphenyl-2-picrylhydrazyl Radical (DPPH), ABTS antioxidant activity (ABTS), Superoxide anion scavenging activity (SAA), Total phenolic content (TPC), Total flavonoid content (TFC) and extraction yields of *Parastrephia lucida* from the I Region of Chile.

Species	DPPH[−a]	ABTS[b]	SAA[c]	TPC[d]	TFC[e]	Extraction Yields (%)[g]
P. lucida	4.23 ± 0.18	1.574 ± 11.42	98.12 ± 2.24[t]	185.12 ± 3.32	163.14 ± 2.84	41.2
Gallic acid*	1.47 ± 0.05	–	93.09 ± 3.27[t]	–	–	–
Quercetin*	9.08 ± 0.65	–	69.41 ± 2.92	–	–	–

[a] Antiradical DPPH activities are expressed as IC_{50} in μg/mL for extracts and compounds.
[b] Expressed as μM trolox equivalents/g dry weight.
[c] Expressed in percentage scavenging of superoxide anion at 100 μg/mL.
[d] Total phenolic content (TPC) expressed as mg gallic acid/g dry weight.
[e] Total flavonoid content (TFC) expressed as mg quercetin/g dry weight.
[g] Extraction yields expressed in percent $^{w}/_{w}$ extraction on the basis of fresh material.
* Used as standard antioxidants. Values in the same column marked with the same letter are not significantly different (at $p < 0.05$).

Table 2
Phenolic acids, tremetones, terpenoids and flavonoids identified in *Parastrephia lucida by UHPLC- HR-OT-MS.*

Peak	Tentative compound	Type of compound	t_R (min)	Theoretical mass $[M-H]^-$	Accurate mass $[M-H]^-$	Fragments (*m/z*)	Molecular formula
1	Quinic acid	Phenolic acid	1.39	191.05611	191.05578	129.01855	$C_7H_{11}O_6^-$
2	2,4,5,6,7-Pentahydroxypentanoic acid	Organic acid	1.50	209.06668	209.06636	191.05553	$C_7H_{13}O_7^-$
3	2,4,5,6-Tetrahydroxyhexanoic acid	Organic acid	8.72	195.05103	195.05061	113.02354	$C_6H_{11}O_7^-$
4	Chlorogenic acid	Phenolic acid	9.46	353.08781	353.08798	191.05562	$C_{16}H_{17}O_9^-$
5	Aesculetin	Cumarin	9.95	177.01933	177.01897	133.02879	$C_9H_5O_4^-$
6	8-Hydroxy-7-methoxy-scopoletin	Coumarin	10.53	211.04555	221.04524	119.04939	$C_{11}H_9O_5^-$
7	Caffeic acid*	Phenolic acid	11.02	179.03498	179.03456	135.0443	$C_9H_7O_4^-$
8	7-Methoxy-8-hydroxyesculetin	Cumarine	11.47	207.02990	207.02959	192.00610	$C_{10}H_7O_5^-$
9	p-Coumaric acid	Phenolic acid	11.61	163.04007	163.03954	119.04944	$C_9H_7O_3^-$
10	7-Methoxyesculetin	Coumarin	12.70	191.03498	191.03464	119.04939	$C_{10}H_7O_4^-$
11	Dicaffeoylquinic acid*	Phenolic acid	13.32	515.11950	515.11957	191.05565	$C_{25}H_{23}O_{12}^-$
12	Luteolin 7-O-glucoside	Flavone	13.71	447.09332	447.09344	285.04053	$C_{21}H_{19}O_{11}^-$
13	7-Methoxymyricetin	Flavonol	14.03	331.04594	331.04611	271.04451	$C_{16}H_{11}O_8^-$
14	4-Hydroxy-3-methoxyacetophenone	Acetophenone	14.34	165.05572	165.05524	119.04949	$C_9H_9O_3^-$
15	Kaempferol*	Flavonol	14.82	285.04046	285.04056	213.05513	$C_{15}H_9O_6^-$
16	Isorhamnetin*	Flavonol	15.00	315.05103	315.05121	192.00565	$C_{16}H_{11}O_7^-$
17	4-Hydroxy-3-methoxypropiophenone	Propiophenone	16.42	179.07137	179.07103	135.04442	$C_{10}H_{11}O_3^-$
18	Luteolin*	Flavone	16.72	285.04046	285.04050	175.03940	$C_{15}H_9O_6^-$
19	Apigenin*	Flavone	18.00	269.0455	269.04550	117.03374	$C_{15}H_9O_5^-$
20	Quercetin*	Flavonol	18.52	301.03538	301.03549	213.05513	$C_{15}H_9O_7^-$
21	3′,7-Dimethoxymyricetin	Flavonol	19.32	345.06159	345.06171	119.04948	$C_{17}H_{13}O_8^-$
22	3,7-Dimethoxymyricetin	Flavonol	19.46	345.06159	345.06183	315.01508	$C_{17}H_{13}O_8^-$
23	7-Methoxyquercetin	Flavonol	19.82	315.05103	315.05112	271.02490	$C_{16}H_{11}O_7^-$
24	7-Methoxyluteolin	Flavone	20.32	299.05611	299.05618	284.03241	$C_{16}H_{11}O_6^-$
25	Naringenin*	Flavanone	20.43	271.06120	271.06122	119.04939	$C_{15}H_{11}O_5^-$
26	3′-Methoxyquercetin	Flavonol	20.76	315.05103	315.05106	135.04439	$C_{16}H_{11}O_7^-$
27	7,3′-Dimethoxyquercetin	Flavonol	21.32	329.06668	329.06680	299.0156	$C_{17}H_{13}O_7^-$
28	7,3′,5′-Trimethoxymyricetin	Flavonol	21.86	359.07724	359.07742	117.03375	$C_{18}H_{15}O_8^-$
29	Dehydro-6,7-dimethoxytremetone	Tremetone	22.01	263.12888	263.12878	-	$C_{15}H_{19}O_9^-$
30	3′-Methoxyluteolin	Flavone	22.43	299.05611	299.05618	119.04939	$C_{16}H_{11}O_6^-$
31	7,3′-Dimethoxyluteolin	Flavone	23.54	313.07176	313.07181	119.04943	$C_{17}H_{13}O_6^-$
32	6-Hydroxy-7-methoxytremetone	Tremetone	23.86	247.09758	247.09743	135.04442	$C_{14}H_{15}O_4^-$
33	7-Methoxyapigenin	Flavone	24.45	283.06120	283.06125	268.03760	$C_{16}H_{11}O_5^-$
34	19-Acetyl-1,2-dihydroxy-hawtriwaic acid methyl ester	Diterpenoid	24.86	419.20753	419.20767	375.21777	$C_{23}H_{31}O_7^-$
35	p-Coumaroyoxyltremetone*	Tremetone	25.28	363.12380	363.12393	117.03377	$C_{22}H_{19}O_5^-$
36	Dehidro-6-hydroxy-7-methoxytremetone	Tremetone	25.83	249.11323	249.11305	161.02376	$C_{14}H_{17}O_4^-$
37	7-Methoxy-p-coumaroyltremetone	Tremetone	25.92	393.13436	393.13458	117.03377	$C_{23}H_{21}O_6^-$
38	19-Hydroxy-solidagoiol A acetate	Diterpene	26.54	359.22278	359.22293	119.04939	$C_{22}H_{31}O_5^-$
39	19-Acetyl-3-O-methoxy-hawtriwaic acid methyl ester	Diterpene	27.49	375.21770	375.21777	–	$C_{22}H_{31}O_4^-$
40	Dehidro-19-acetyl-hawtriwaic acid methyl ester	Diterpene	28.00	389.23335	389.23352	–	$C_{22}H_{31}O_4^-$
41	Umbelliferone*	Cumarin	28.43	162.0316	162.0318	–	$C_9H_6O_3^-$
42	Bacchalineol A*	Diterpene	29.12	302.2240	302.2245	–	$C_{20}H_{30}O_2^-$

* Analytes confirmed by chromatographic comparison with pure standards.

effects and health benefits have been associated with flavonoid consumption. Examples of those compounds identified are depicted in Fig. S11.

3.2.1.1. Flavones. Luteolin-7-O-glucoside (*m/z* 447.0934) was identified and confirmed through literature (Ammar et al., 2016). Its aglycone, luteolin (*m/z* 285.0405) was found and identified by co-elution with an authentic compound. The luteolin derivatives: 3′-methoxyluteolin, 7-methoxyluteolin (*m/z* 299.0562 and *m/z* 299.0562, respectively), and 7,3′-dimethoxyluteolin (*m/z* 313.0718) were also assigned. Apigenin (*m/z* 269.0455) and 7-methoxyapigenin (*m/z* 283.0613) were also identified (Table 2).

3.2.1.2. Flavonols. The flavonol isorhamnetin (*m/z* 315.0512) (Simirgiotis et al., 2016a) was identified and confirmed by co-injection with an authentic standard. An isomer of the latter was identified as 7-methoxyquercetin (*m/z*: 315.0511). Quercetin (*m/z*: 301.0355) was found and identified by co-elution with authentic compound. Some quercetin derivatives have been also identified as 3′-methoxyquercetin and 7,3′-methoxyquercetin (*m/z*: 315.0511 and 329.0668, respectively). Kaempferol (*m/z*: 285.0406) was found in *P. lucida* by analysis of the chromatograms. In addition, the examination of the chromatograms, led to identification of four myricetin derivatives: 7-methoxymyricetin (*m/z*: 331.0461), 7,3′,5′-trimethoxymyricetin (*m/z*: 359.0774) and the isomers 3′,7-dimethoxymyricetin (*m/z*: 345.0617) and 3,7-dimethoxymyricetin (*m/z*: 345.0618).

3.2.1.3. Flavanones. The flavanone naringenin have been previously reported as main component in extracts of Easter pears by us (*Pyrus communis*) (Simirgiotis et al., 2016a) and its MS and UV data matched the one obtained in our *P. lucida* chromatogram (*m/z*: 271.0612).

3.2.2. Coumarins

These compounds are classified as a member of the benzopyrone family. All of which consist of a benzene ring joined to a pyrone ring Fig. S12 (Supplementary material). It possesses immeasurable anticancer potential with minimum side effects depending on the substitutions on the basic nucleus. Coumarins have a tremendous ability to regulate diverse range of cellular pathways that can be explored for selective anticancer activity (Thakur et al., 2015). Four coumarins were identified in total ion current (TIC), in negative mode and dependent scan spectra:

esculetin (*m/z*: 177.0190), 7-methoxyesculetin (*m/z*: 191.0346), 8-hydroxy-7-methoxyesculetin (*m/z*: 207.0296), 8-hydroxy-7-methoxyscopoletin (*m/z*: 221.0452) and umbelliferone (*m/z*: 162.0318) (Simirgiotis et al., 2013b).

3.2.3. Tremetones

These core structures are considered as one of the important heterocyclic rings because of its diverse biological profiles (Kamal et al., 2011). Indeed, medicinal chemists are actively involved in the synthesis of benzofuran ring containing molecules due to its clinical importance (Keay and Hopkins, 2008; Keay et al., 2008). Many of the clinically approved drugs are synthetic and naturally occurring substituted benzofuran derivatives containing fused benzofuran rings in conjunction with other heterocycles. This heterocyclic ring was identified in 5 compounds from *P. lucida*: *p*-coumaroyloxytremetone (*m/z*: 363.1239) (Loyola et al., 1985) and its derivative 7-methoxy-*p*-coumaroyloxytremetone (*m/z*: 393.1346), dehidro-6,7-dimethoxytremetone (*m/z*: 263.1288), 6-hydroxy-7-methoxytremetone (*m/z*: 247.0974), and dehidro-6-hydroxy-7-methoxytremetone (*m/z*: 249.1131). In this work we have proposed some biosynthetic relationships for those tremetone derivatives (see Fig. 3).

3.2.4. Phenolic acids

The examination of the chromatograms revealed the presence of 5 phenolic acids: quinic acid (*m/z* 191.0558) and its derivative dicaffeoylquinic acid (*m/z* 515.1196), caffeic acid (*m/z* 179.0346) which was identified by comparison with literature (Brito et al., 2014), chlorogenic acid and *p*-coumaric acid (*m/z* 353.0880 and *m/z* 163.0395, respectively) (Simirgiotis et al., 2016a).

3.2.5. Fatty acids

Peak 2 and 3 were tentatively identified as the fatty acids 2,4,5,6,7-pentahydroxypentanoic acid (*m/z* 209.0664), and 2,4,5,6-tetrahydroxyhexanoic acid (*m/z* 195.0506).

3.2.6. Acetophenones

These types of compounds were assigned by the presence of 2 peaks on the chromatogram from *P. lucida* ethanolic extract: 4-hydroxy-3-methoxyacetophenone (*m/z* 165.0552), and 4-hydroxy-3-methoxypropiophenone (*m/z* 179.0710) (Kim et al., 2013).

3.2.7. Clerodane terpenoids

Several *ent*-clerodanes terpenoids were identified. Dehidro-19-acetyl-hawtriwaic acid methyl ester (*m/z* 389.2335) was related to the furanyl clerodane diterpene hawtriwaic acid (Simirgiotis et al., 2000) while peak 39 was identified as the related compound 19-acetyl-3-O-methoxy-hawtriwaic acid methyl ester (*m/z* 375.2178) and 19-acetyl-1,2-dihydroxy-hawtriwaic acid methyl ester (*m/z* 419.2077). Other compounds detected were related to the antibacterial compound solidagoic acid A, peak 42 (*m/z* 302.2245) (Nogueira et al., 2001; Starks et al., 2010). Thus, peak 38 was identified as 19-hydroxy-bacchalineol A acetate (*m/z* 359.2229).

3.3. Biological evaluation of p-coumaroyloxytremetone

To complement the antioxidant activity measured by chemical assays, we have also characterized the antioxidant capacity by reactive oxygen species (ROS) scavenging activity in human derived breast cell lines, induced by the ethanolic extract and the pure phenolic compound isolated from *P. lucida*.

Thus, to validate the results of antioxidant capacities measured by FRAP, scavenging DPPH and superoxide anion scavenging assays, the antioxidant capacities of the ethanolic extract and the pure isolated compound p-coumaroyloxytremetone (2) in MCF10F cell line using Muse® Oxidative Stress Kit. This assay is based on dihydroethidium (DHE) which is cell permeable; inside the cell DHE reacts principally with superoxide anions, undergoes oxidation to form the DNA-binding fluorophore ethidium bromide which intercalates with DNA resulting in red fluorescence

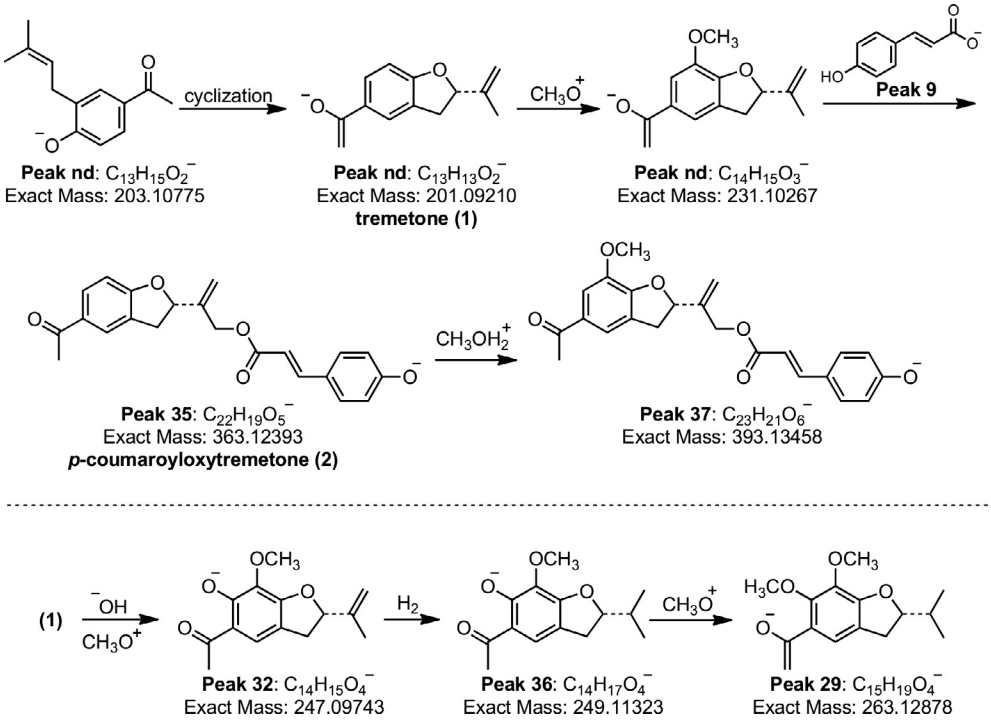

Fig. 3. Proposed biosynthetic relationships for the tremetone derivatives. nd: Peak not detected.

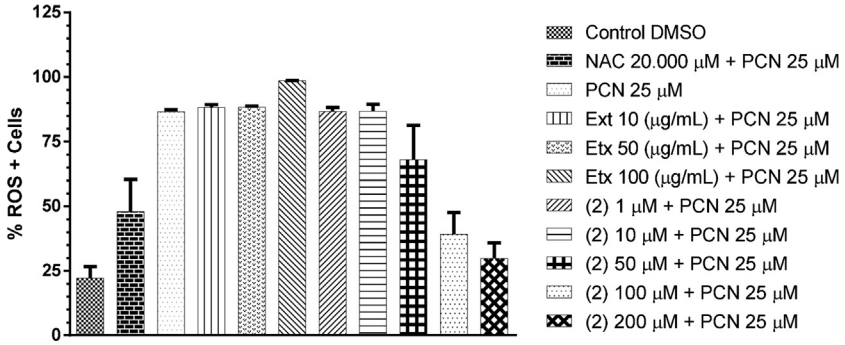

Fig. 4. Effects of ethanolic extract and *p*-coumaroyloxytremetone (2) on intracellular ROS in MCF10F cells.

(Bindokas et al., 1996). PCN was used as a ROS producer, and NAC as a positive control of antioxidant agent.

The ethanolic extract doses (10–100 μg/mL) were not able to counteract the oxidizing effect of pyocyanin in MCF10F cell line (see Fig. 4). Moreover, in the ethanol extract there are a variety of chemical compounds; these can show an antioxidant effect in chemical assays, but it should not necessarily correlate with an antioxidant activity in a vitro system, like MCF10F cells (Lopez-Alarcon and Denicola, 2013). Otherwise, the p-coumaroyloxytremetone (2) pretreatment applied on cell line MCF10F protect it against oxidant action of PCN, being evident from 50 μM, returning to ROS + control levels from 100 μM dose (no significant differences comparing with control DMSO). Cancer cells have an abnormal redox system, and it has been reported that many types of breast cancer cells have increased levels of ROS, comparing with their normal counterparts (Kobayashi and Suda, 2012). Increase ROS levels in cancer cells have been shown to correlate with the aggressiveness of tumors and poor prognosis for patients (Jezierska-Drutel et al., 2013). There are many reports including studies with phenolic compounds derived from plants of the Asteraceae family, with antioxidant properties in MCF7, HeLa, and other derived cancer cells lines (Agar et al., 2015; Kashif et al., 2015; Kumar et al., 2014), including sesquiterpenes (Al-Fatlawi et al., 2015), quinones and coumarins (Wu et al., 2015), but there are no reports with antioxidant effect of tremetones, specifically involving the derivative *p*-coumaroyloxytremetone isolated from *P. lucida*.

4. Conclusions

Using UHPLC high resolution orbitrap mass spectrometry, (UHPLC-OT-HR-MS) we have identified 42 phenolic compounds in the ethanolic extract of *Parastrephia lucida*, most of which, as far as we know, are reported for the first time. Many of these compounds are flavonoids (flavones, flavonols and flavanones), coumarins, tremetones, phenolic acids, fatty acids, acetophenones and clerodane terpenoids. Furthermore, the results obtained in this study clearly show that the ethanolic extract can be a natural source of antioxidants with potential application in food or pharmaceutical industries, given the high activity found in our evaluated models (DPPH, ABTS). Moreover, this extract showed the highest content of phenols and higher radical scavenging activity. The isolated and identified coumaric acid metabolite *p*-coumaroyoxyltremetone has antioxidant characteristics that may be associated with the radical scavenging capacity found in the crude extract. This is the first report showing the antioxidant activity of *p*-coumaroyloxytremetone on breast cell lines. This study broadens the knowledge of *P. lucida* from northern Chile. This knowledge might be helpful for further research on *P. lucida* and its applications in food industry. In conclusion, this plant is a very rich source of phenolic compounds with antioxidant activity that could be useful for the preparation of nutraceuticals or healthy foods.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsps.2017.03.001.

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