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Phenolic Profile and Antioxidant Activity of Ethanolic Extract of *Larrea cuneifolia* Cav Leaves †

Maria E. Lorenzo [1], Eugenia Sabatino [2], Patricia E. Gómez [1], Adrián F. Segovia [3], Lara C. Figueroa [3], Alejandro Quiroga [3] and María V. Baroni [2,4,*]

[1] Facultad de Ciencias Agrarias, UNCA, Maestro Quiroga 50, Catamarca 4700, Argentina, CITCA/CONICET-UNCA, Prado 366, Catamarca 4703, Argentina; ma.emilia.lor@gmail.com (M.E.L.); patriciagomezkamenopolsky@agrarias.unca.edu.ar (P.E.G.)
[2] ICYTAC-CONICET, Juan Filloy s/n, Ciudad Universitaria, Córdoba 5000, Argentina; m.eugenia_s@hotmail.com
[3] Facultad de Ciencias Agrarias, UNCA, Maestro Quiroga 50, Catamarca 4700, Argentina; fsegovia@agrarias.unca.edu.ar (A.F.S.); larafigueroa16@gmail.com (L.C.F.); quirogafcaunca@hotmail.com (A.Q.)
[4] Departamento de Química Orgánica, Facultad de Ciencias Químicas, ISIDSA—UNC ICYTAC-CONICET, Juan Filloy s/n, Ciudad Universitaria, Córdoba 5000, Argentina
* Correspondence: vbaroni@unc.edu.ar; Tel.: +54-351-5353867 (ext. 53348)
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Abstract: Genus of the Zygophyllaceae family includes evergreen shrub species. Background highlights the antioxidant and anti-tumor activity of *Larrea divaricate* and nordhydroguayaretic acid (NDGA) due to their potential as a dietary supplement and food preservative, but little is known about *Larrea cuneifolia*. The aim of this work was to determine the antioxidant characteristics of ethanolic extracts of *L. cuneifolia* leaves collected in the Central Valley of Catamarca (Argentina). Total polyphenols content (TP) was determined by Folin-Ciocalteu and the phenolic profile by HPLC-PDA-QTOF. The Antioxidant activity was measured by in vitro (FRAP, ABTS and DPPH) and cellular (HepG2 and Caco2 cells) assays. The phenolic compounds identified were mainly derivatives of NDGA and flavonols derivatives of quercetin, kaempferol, isorhamnetin, and gossipetin. TP content and antioxidant activity exceeded the values reported for *L. divaricata*. With regard to cytotoxicity, an increase in this parameter could be observed with the increase in the concentration of polyphenols in both cells types. Furthermore, in cells exposed to H_2O_2, a significant decrease in ROS concentration was observed for HepG2 cells. This effect can be used to study compounds with bioactivity on tumor cells. *L. cuneifolia* is a species rich in phenolic compounds, with antioxidant properties and is a potential source of bioactive compounds for the production of functional foods.

Keywords: antioxidant activity; polyphenols; cell lines; *Larrea cuneifolia*

1. Introduction

Larrea cuneifolia Cav. it's a specie that belongs to the Zygophyllaceae family, which includes species of evergreen shrubs distributed throughout the American continent. Larrea species are considered medicinal plants and have been used since ancient indigenous communities mainly as infusions [1]. Most of the pharmacological studies focus on the antioxidant, antimicrobial, and antitumor activity of extracts of *L. divaricata* Cav. and one of the main chemical components,

nordhydroguayaretic acid (ANHG) [2–6]. But few studies are devoted to the antioxidant properties of *L. cuneifolia* [7–9].

Polyphenols are a group of compounds that have important organoleptic and health properties, generated as a product of the secondary metabolism of plants [10] and are attributed, among other properties, the ability to be antioxidants. Some also have other associated biological activities, such as anti-microbial, anti-inflammatory and antitumor activities, and their value has been increased by demonstrating the ability to manipulate gene expression in mammalian cells [11]. That´s why there is a growing interest in characterizing the phenolic compounds present in different plant tissues [12].

The objective of this work was to determine the total polyphenols content (TPC) and the main phenolic composition of *L. cuneifolia* leaf extracts. Furthermore, the antioxidant activity was evaluated through in vitro chemical and cell culture tests (Caco-2 and HepG2).

2. Materials and Methods

2.1. Samples, Processing and Extraction of Phenolic Compounds

The plant material *Larrea cuneifolia* Cav (CORD 00097491) was collected from the central valley of Catamarca, Argentina during the fall season (April–May) in two consecutive years (2017–2018). The plant material was identified by Drs. G. Barboza and L. Ariza Espinar. A voucher specimen has been deposited in the herbarium CORD of the National University of Córdoba, Argentina. The previously conditioned plant material was grounded, lyophilized and stored in desiccators protected from light until processing. The extraction of phenolic compounds was carried out as described by Perez et al. 2014 [13] with modifications. Briefly, leaves samples were grounded with an electric grinder to obtain a fine powder. Afterwards, 0.1 g of sample was extracted using 5 mL of ethanol 50%. These were sonicated for 15 min in an ultrasonic bath (Cleanson, Villa Maipú, Argentina). Then, the extracts were centrifuged (Gelec, Buenos Aires, Argentina) for 10 min at 800× *g*, and the supernatants were collected. This process was repeated three times and all supernatants were combined, filtered and stored at −80 °C until antioxidant properties measurement and HPLC-MS/MS determinations.

2.2. Polyphenol Content and Phenolic Composition

Total polyphenol content (TPC) was measured by the Folin-Ciocalteu method according to the methodology described by Singleton et al. 2019 [14]. Samples were determined in duplicate, at 750 nm against a reagent blank. The results were expressed as μg gallic acid/g dry weight (DW).

The phenolic compounds were analyzed by HPLC-PDA-QTOF, using an Agilent 1200 LC Series system (Agilent, Santa Clara, CA, USA), coupled to a diode array UV/Vis detector (PDA) (Agilent Series 1200) in tandem with an electrospray ionization source (ESI), connected to a Micro-QTOF II high-resolution mass spectrometer (Bruker Daltonics, Billerica, MA, USA) (MS and MS/MS) according to Lingua et al. 2016 [15].

Polyphenols present in samples were tentatively identified according to their retention times, UV/Vis spectra, high resolution MS and MS/MS spectra, by comparison with pure compounds, when available, or by comparison with compounds reported in the literature [5,6,16–19].

2.3. Determination of Antioxidant Capacity by In Vitro Chemical Methods

The in vitro antioxidant activity was measured by three methodologies: free radical elimination activity assay on 1,1-diphenyl-2-picrylhydrazylradical (DPPH) following the methodology described by Brand-Williams et al. 1995 [20], ferric reduction capacity of plasma assay (FRAP) was performed according to Benzie and Strain 1996 [21] and Trolox equivalent antioxidant capacity test method (TEAC) was performed according to Re et al. 1999 [22]. For all assays, results were obtained from a calibration curve made using Trolox. Results were expressed in mmol Trolox equivalents 100 g^{-1} of dry weight (DW). All samples were analyzed in duplicate, after a 30 min reaction time.

2.4. Determination of Antioxidant Capacity by Cell Culture Assays (Caco-2 and HepG2).

The cytotoxicity induced by the ethanolic extract of *L. cuneifolia* leaf dissolved in DMSO 0.01% (vehicle) was studied. The cells were cultured for 72 h in complete DMEM (with 10% Fetal Bovine Serum) and then incubated for 24 h in complete DMEM with the polyphenol extracts (0, 0.1, 0.5 and 1 mg/mL). The cytotoxicity of the vehicle was evaluated in the same way, but replacing the extract with 0.01% DMSO in DMEM. 100% viability was determined from wells in which the cells were cultured with the culture medium (DMEM) and the vehicle. Then the antioxidant capacities of the extracts were evaluated by inducing oxidative stress for one hour with a H_2O_2 solution.

Viability was evaluated by the Trypan Blue method. The cells were dislodged from the medium by enzymatic digestion with trypsin and were resuspended in PBS buffer with Trypan Blue [23]. *Intracellular oxidation* was determined by fluorescence using the probe 2′7′-dichlorofluorescein diacetate (DCFH-DA) [24].

2.5. Statistical Analysis

The determinations by in vitro chemical methods were analyzed with INFOSTAT [25]. The ANAVA was applied to the values obtained in order to evaluate the variation between extracts ($p < 0.05$) by the method of multiple comparisons DGC. For variables with non-parametric distribution, the data were analyzed using the variance comparison procedure of General and Mixed linear Models, with a Fisher post-test ($p < 0.05$).

3. Results and Discussions

3.1. Total Polyphenols Content (TPC) and Phenolic Compounds

3.1.1. TPC by Folin-Ciocalteu

The TPC of leaf ethanolic extracts of *L. cuneifolia* from Catamarca was evaluated obtaining 228.1 ± 33.8 μg gallic acid mg-1 dry leaf sample. This value is in agreement with that reported by Rossi et al. 2008 [26] in the same species from La Rioja, Argentina and was slightly higher than those reported in other investigations for *L. divaricate* [26–28].

3.1.2. Identification of Phenolic Compounds by HPLC-ESI-MS/MS

In leaf extracts of *L. cuneifolia* a total of 18 compounds were tentatively identified. Table 1 shows the identified phenolic compounds and the spectral parameters used for their identification such as retention times (RT), exact mass and fragmentation pattern.

Table 1. Phenolic compounds and derivatives, identified with HPLC-ESI-MS/MS, in ethanolic leaf extract of *L. cuneifolia*.

N°	RT (min)	Tentatively Identified Compound	Molecular Formula	[M-H] (*m*/*z*) Theoretical	[M-H] (*m*/*z*) Experimental	Error ppm	MS/MS
1	11.4	4-caffeoylquinicacid	$C_{16}H_{17}O_9$	3.530.878	353.084	10.2	191
2	12.8	3-caffeoylquinicacid	$C_{16}H_{17}O_9$	3.530.878	353.084	9.6	191
3	18.9	Quercetin rutinoside	$C_{27}H_{29}O_{16}$	6.091.461	609.147	−0.9	301
4	19.2	Quercetin glucoside	$C_{21}H_{19}O_{12}$	4.630.882	463.089	−1.7	301
5	20.0	Kaempferolhexoside isomer II	$C_{21}H_{19}O_{11}$	4.470.933	447.094	2.5	285
6	20.2	Dihydroisorhamnetin	$C_{16}H_{13}O_7$	3.170.667	317.068	3.9	299, 289, 273, 258, 231, 207
7	21.2	Isorhamnetinrhamnosyl glucoside	$C_{28}H_{31}O_{16}$	6.231.618	623.163	−2.5	315
8	24.4	Dimethyl gossypetin	$C_{17}H_{13}O_8$	3.450.616	345.062	0.4	
9	24.7	Trimethylgossypetin	$C_{18}H_{15}O_8$	3.590.772	359.079	3.7	315, 273,
10	24.8	Naringenin	$C_{15}H_{11}O_5$	2.710.612	271.062	−1.6	177, 151, 227

11	25.5	Quercetin methylether isomer I	$C_{16}H_{11}O_7$	315.051	315.056	16.7	300
12	27.6	Kaempferol	$C_{15}H_9O_6$	2.850.405	285.041	1.7	
13	27.8	Quercetin methylether isomer II	$C_{16}H_{11}O_7$	315.051	315.053	5.8	300
14	28.1	meso-(rel7S,8S,7′R,8′R)-3,4,3′,4′-tetrahydroxy7,7′-epoxylignan	$C_{19}H_{22}O_5$	3.291.394	329.141	−3.3	177
15	28.9	Trihydroxytrimethoxy flavone	$C_{18}H_{15}O_8$	3.590.772	359.078	1.7	344, 329, 316, 301, 273
16	31.0	NDGA nordihydroguayaretic acid	$C_{18}H_{21}O_4$	3.011.445	301.146	6	273, 268, 299
17	32.0	Trimethyl quercetin	$C_{18}H_{15}O_7$	3.430.823	343.086	9.2	328, 313
18	34.2	MNDGA 3-methylnordihydroguayareticacid	$C_{19}H_{24}O_4$	3.151.602	315.162	6.7	300

Two hydroxicinnamic acids, specifically isomers of caffeoyl quinic acids, were tentatively identified (compounds 1, 2, Table 1). These acids have not been previously identified in Larrea species; however, other authors have identified different kinds of cinamic acids [6]. 12 flavonoid compounds were found, mainly glicosilated and methylated derivatives of quercetin, kaempferol, isorhamnetin and gossypetin (compounds 3–9 and 11–13). Most flavonols tentatively identified in this work have been previously described in Larrea species [16,17]. The naringenin is a flavanone (compound 10) and this was previously described in Larreas [19]. One flavone were tentatively identified in this samples (compound 15) previously describedin Larrea tridentata leaves [17]. Finally three lignans and a flavolignan (compounds 14, 16, 18 and 17) have been previously identified in other Larrea species [5,18,19].

3.2. Antioxidant Capacity

3.2.1. Antioxidant Activity by In Vitro Chemical Methods

The antioxidant activity of the ethanol extracts of L. cuneifolia determined by the DPPH test showed a mean value and SD of 94.7 ± 11.6 mmol of Trolox 100 g^{-1} sample. In another investigation [27] lower values were found for L. divaricata leaf infusions by the same method. In FRAP assay, values obtained was 77.3 ± 9.3 mmol of Trolox 100 g^{-1} sample, which is similar to the values presented for aqueous extracts of Ligaria cuneifolia (186 mmol of Trolox 100 g^{-1} sample) according to [29] and exceed the values presented by [27] for L. divaricata. Finally, in TEAC assay a mean value of 114.4 ± 23.7 was obtained, widely exceeding the values reported for L. divaricata of 70 mmol of Trolox 100 g^{-1} dry aqueous extract [27].

3.2.2. Antioxidant Activity Determined by Cell Culture Assays (Caco-2 and HepG2)

The AC or bioactivity of samples (as chemoprotectors or exogenous antioxidants) in protecting HepG2 and Caco2 cells against oxidative stress induced by H_2O_2 was studied measuring the cell viability and ROS levels and the results are shown in Figure 1a,b. First, we checked the basal effect of the polyphenols extracts on cell viability. So we evaluated the cytotoxic effects with different TP concentrations.

In HepG2 lines (Figure 1a) a cytotoxicity effect was observed when cells were exposed only tio the polyphenols extracts, increasing in a dose-response manner. On the other hand, when cells were exposed only to H_2O_2 a citotoxicity effect was also observed derived from oxidative stress sitmulus. The suplementation with polyohenols did not recover cells from this oxidative stress. However, a significant decrease in ROS levels was observed with increasing extract concentration in those cells exposed to H_2O_2, showing an antioxidant effect of *L. cuneifolia* extracts.

In Caco-2 cell line (Figure 1b) both, in control cells and in those exposed to H_2O_2, a cytotoxic effect of polyphenols was observed that rises with increasing concentration. On the other hand, as expected exposure of cells to H_2O_2 produces a cytotoxic effect increasing cell death. In this case, polyphenols are only capable of reducing the ROS level in a concentration of 0.5 ug/mL.

It should be noted that the increase in cytotoxicity is a positive outcome when the final objective is the search for treatments for tumor cells, such as the HepG2 and Caco 2 lines, probably with the use of higher doses of bioactive compounds in tests.

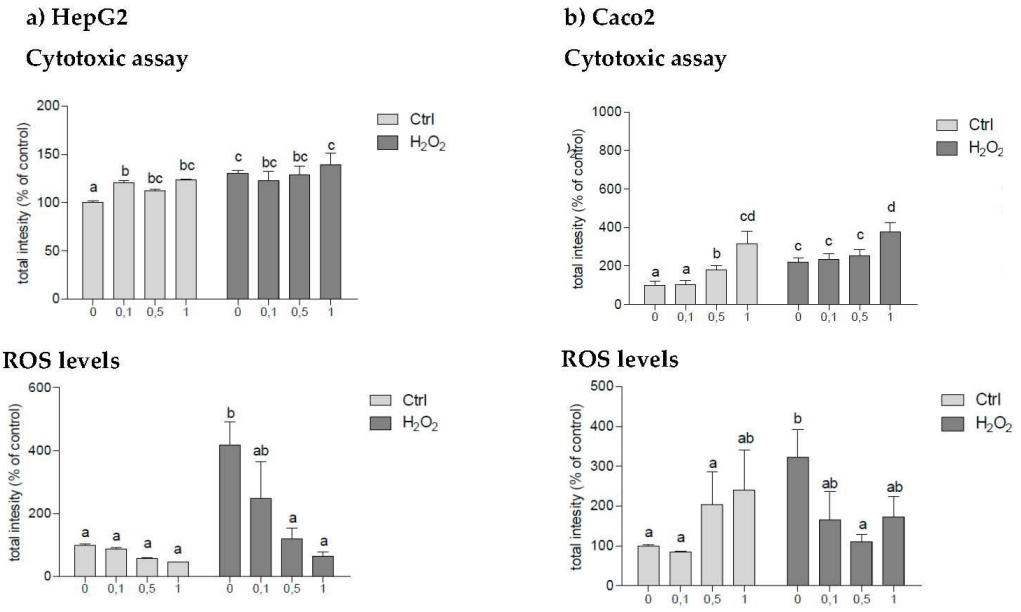

Figure 1. Cytotoxicity determined by Trypan Blue Test and Intensity of ROS levels measured by DCFH at different concentrations of the extract of *L cuneifolia* leaf (0, 0.1, 0.5 and 1 μg/mL) in HepG2 cell lines (**a**) and Caco-2 (**b**) in medium with and without H_2O_2.

4. Conclusions

L. cuneifolia Cav. is a species that grows in arid and degraded soils, is rich in phenolic compounds and possesses important antioxidant activity in vitro. The main polyphenolic components are the NDGA derivatives and flavonols (flavonols and flavones) in addition to lignans, flavolignans and cinnamic acids. In the activity on HepG2 cell culture lines, a significant decrease in ROS concentration was observed when cells are exposed to H_2O_2. On the other hand on Caco2 cells a citotoxyc effect is primarily observed. This effect can be used to study compounds with bioactivity in the search for new oncological treatments.

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