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

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Abstract: The genus of the Zygophyllaceae family includes evergreen shrub species. Background highlights the antioxidant and anti-tumor activity of *Larrea divaricata* and nordihydroguayaretic 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-Ciocâlteu and the phenolic profile by HPLC-PDA-QTOF. The antioxidant activity was measured by in vitro (FRAP, TEAC 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 gossypetin. 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 cell types. Furthermore, in cells exposed to H2O2, a significant decrease in reactive oxygen species (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. is a species 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 pharmacological studies focus on the antioxidant, antimicrobial and antitumor activity of extracts of *L. divaricata* Cav. and one of the main chemical components, nordihydroguayaretic acid (ANHG) [2–6]. However, 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 increased by demonstrating the ability to manipulate gene...
expression in mammalian cells [11]. As a result, 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 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 with sample code CORD 00097491. 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 ground 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-picrylhydrazyl radical (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 Dulbecco’s Modified Eagle’s medium (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. A full 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 H2O2 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 Phosphate buffer saline (PBS) 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 analysis of variance (ANOVA) was applied to the values obtained in order to evaluate the variation between extracts (p < 0.05) by the method of multiple comparisons Di Rienzo, Guzmán and Casanoves test (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.

| № | 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_{20}H_{17}O_{9} | 3.530.878              | 353.084                 | 10.2           | 191 |
| 2 | 12.8    | 3-caffeoylquinicacid            | C_{20}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_{28}H_{31}O_{16} | 6.231.667              | 317.068                 | 3.9            | 299, 289, 273, 258, 231, 207 |
| 7 | 21.2    | Isorhamnetinhamnosyl glucoside  | C_{20}H_{31}O_{16} | 6.231.618              | 623.163                 | −2.5           | 315 |
| 8 | 24.4    | Dimethyl gossypetin             | C_{20}H_{31}O_{8}  | 3.450.616              | 345.062                 | 0.4            | 315 |
| 9 | 24.7    | Trimethylgossypetin             | C_{20}H_{31}O_{8}  | 3.590.772              | 359.079                 | 3.7            | 315 |
| 10| 24.8    | Naringenin                      | C_{20}H_{31}O_{8}  | 2.710.612              | 271.062                 | −1.6           | 177, 151, 227 |
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 cinnamic acids [6]. A total of 12 flavonoid compounds were found, mainly glycosylated 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 *Larrea* sp. [19]. One flavone was tentatively identified in this samples (compound 15) previously described in *Larrea tridentata* leaves [17]. Finally, three lignans and a flavonolignan (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, the value 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 H₂O₂ was studied, measuring the cell viability and reactive oxygen species (ROS) levels. The results are shown in Figure 1a, b. First, we checked the basal effect of the polyphenols extracts on cell viability, evaluating the cytotoxic effects with different polypheol concentrations.

In HepG2 lines (Figure 1a) a cytotoxicity effect was observed when cells were exposed only to the polyphenol extracts, increasing in a dose-response manner. On the other hand, when cells were exposed only to H₂O₂, a cytotoxicity effect was also observed derived from oxidative stress stimulus. The supplementation with polyphenols 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 H₂O₂, showing an antioxidant effect of *L. cuneifolia* extracts.

In the Caco-2 cell line (Figure 1b), in both the control cells and in those exposed to H₂O₂, a cytotoxic effect of polyphenols was observed rising with increasing concentration. On the other hand, as expected, exposure of cells to H₂O₂ 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 µg/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 2′,7′-dichlorofluorescein diacetate (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 H₂O₂.

### 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 nordihydroguayaretic acid (NDGA) derivatives and flavonols (flavonols and flavones) in addition to lignans, flavonolignans and cinnamic acids. In the activity on HepG2 cell culture lines, a significant decrease in ROS concentration was observed when cells are exposed to H₂O₂. On the other hand, a cytotoxic effect is primarily observed on Caco2 cells. This effect can be used to study compounds with bioactivity in the search for new oncological treatments.

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