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Antioxidant activity of flavonoid rich fraction of *Ligaria cuneifolia* (Ruiz & Pav.) Tiegh (Loranthaceae)

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Cyclodextrin-Micellar Electrokinetic Chromatography

Abstract

Ligaria cuneifolia (Ruiz & Pav.) Tiegh (Loranthaceae), the "Argentine mistletoe", is a hemiparasite species largely used in folk medicine. The aim of this study was to evaluate the antioxidant activity using *in vitro, ex vivo,* and *in vivo* methods. A screening of phenolics was performed by UV spectroscopy on different fractions. The antioxidant capacity was evaluated *in vitro* by the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^\bullet$) assay on a crude extract (CE), ethyl acetate fraction (EAF), and aqueous fraction (AF). The results suggest that EAF concentrates the antioxidant capacity and was selected for further analysis. Capillary electrophoresis was employed to monitor the individual antioxidant capacity and the potential contributors to this effect. *Ex vivo* assays showed an efficient inhibition of *tert*-butyl hydroperoxide-induced rat liver phospholipid oxidation, as well as rat brain autoxidation, and H_2O_2-induced DNA damage in blood monocytes. *In vivo*, the topical application of EAF significantly decreased skin chemiluminescence in a mice model.

Introduction

Infusions from leaves and stems of several plants known as "Mistletoes" (Loranthaceae and Santalaceae families) have been widely used in traditional medicine and are extensively recognized as therapeutical herbs. [1, 2] *Ligaria cuneifolia* (Ruiz & Pav.) Tiegh (Loranthaceae), the "Argentine mistletoe", is a hemiparasite species that grows in the northwest and central regions of Argentina, [3] that has been used as a substitute of the "European mistletoe", *Viscum album* L. (Santalaceae), based on its putative activity of decreasing high blood pressure. [1, 4, 5]

Previous studies employing *L. cuneifolia* fractions in different animal models have shown a wide spectrum of beneficial biological effects, such as decreasing plasma cholesterol, [6] as well as antibacterial, [7] immunomodulatory, [8, 9] and cytostatic properties. [10] The antioxidant activity has only been preliminary explored. [11] In this work, several medicinal plants from the province of Cordoba in Argentina were screened for their antioxidant capacity by means of *in vitro* and *ex vivo* testing. An aqueous extract of *L. cuneifolia* was employed and the ferric reducing/antioxidant power (FRAP) test showed the highest values among the evaluated plant species and BHT but lower than a *Ginkgo biloba* L. (Ginkgoaceae) extract, quercetin and ascorbic acid. Thiobarbituric acid-reactive substances (TBARS) was also carried out and *L. cuneifolia* showed a "protective index" even better than quercetin. However, no data was provided on the main compounds which might account for this potential effect. Our group has also been working on *L. cuneifolia* samples from the province of San Juan and a previous report showed promising results with a flavonoid rich fraction on DPPH and ABTS tests. [12] Given that flavonoids are especially abundant in *L. cuneifolia* extracts, they may contribute to these properties.

Flavonoids are regarded as natural antioxidants since they are part of the defense mechanism that plants set in motion to counteract the toxic effects of free radicals. Their action is twofold, providing an active and passive resistance. The former refers to flavonoid continuous availability for their role

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in plant metabolism, despite the presence of stressors. The latter relates to the increase in their synthesis in response to stressful conditions. [13] Quercetin is a well-studied flavonol that has been evaluated for its potential to prevent various diseases, such as osteoporosis, some forms of cancer, tumors, and lung and cardiovascular diseases. The antioxidant effects of quercetin play a significant role in the prevention and treatment of such diseases for its ability to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical is suggested to be involved in these possible beneficial health effects. [14] From a phytochemical point of view, quercetin is an aglycon and the structural unit of the main constituents of *L. cuneifolia*. [1]

According to the World Health Organization, approximately 80% of the world's inhabitants utilize traditional medicine for their primary health care needs and, in most cases, this requires the use of herbal extracts and their active components. [15] The link between flavonoids and antioxidant activity has been a hot topic and remains to be so, given the large number of recent publications that address this subject. [15, 16, 17, 18] Reactive oxygen species are involved in the pathogenesis of various diseases and the evaluation of flavonoid rich extracts could provide a scientific support to the traditional use. There are two main strategies to assessing the oxidative/antioxidant status of an organism. The first is associated with direct determination of the content of individual antioxidants (AO). The second approach is based on the assessment of their integral content. Considering the plethora of antioxidant compounds, differences in mechanisms and the possibility of synergism of their action in the body, the second approach should be considered preferable and more informative. [19]

As a first approach, systematic flavonoid fingerprinting of *L. cuneifolia* has been recently performed by capillary electrophoresis, focusing on the analysis of specimens from different hosts and geographical regions, and subjected to various extraction procedures. Well-known antioxidant compounds, such as free and glycosylated quercetin were found, together with condensed tannins. [20] The unequivocal identification of these analytes was confirmed by chromatographic, and

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spectroscopic methods such as mass spectrometry and nuclear magnetic resonance. [21] Capillary electrophoresis is an efficient and environmental-friendly technology, with minute sample requirement. Similar compounds in a sample can be separated in a short time and the active ingredients can be screened simultaneously. The principal components include a fused silica capillary column in which separation takes place based on differences in effective electrophoretic mobility among analytes and a high voltage power supply as driving force. Compared with other modern analytical methods, capillary electrophoresis is a technique with higher efficiency, lower sample and solvent consumption, easier operation, and lower running cost. Furthermore, sensitivity can be improved from microliter to nanoliter scale by on-line enrichment. It has been widely applied to plant analysis due to its excellent efficiency, short analysis time, minute sample requirements, and use of few organic reagents. [20, 22] The biological activity screening technology is a method that combines biological activity test with separation of chemical compounds from a complex matrix and capillary electrophoresis proved to be an efficient tool for the screening of bioactive components. In this case, we applied a CD-MEKC methodology in precolumn mode where there is a reaction between DPPH and active ingredients followed by analysis and detection using capillary electrophoresis. [22]

Given the complex nature of plant extracts and the coexistence of a large number of chemicals that might exert an antioxidant effect with variable efficiency, it is critical to identify the most active ones under different experimental conditions. Moreover, instead of studying isolated antioxidant compounds, is also important to consider this effect in the context of the whole plant extract, where synergy and/or antagonism might occur. Since *in vitro* assays are generally used to confirm the antioxidant activity of plant samples within particular reaction systems, there is a need to correlate these findings with *ex vivo* and *in vivo* testing. Therefore, the aim of this work was to evaluate the antioxidant activity of *L. cuneifolia* flavonoid rich fraction using different *in vitro, ex vivo,* and *in vivo* methods.

Results and Discussion

Screening of Phenolics

The results are summarized in Table 1. In every case, the EAF showed the highest values compared to both the CE and the AF. This is expected as the ethyl acetate partition frequently generates flavonoid rich fractions from plant extracts.

Table 1: Screening of Phenolics in *L. cuneifolia* fractions. Results were expressed as mean ± SEM (n = 6)

### *In vitro antioxidant capacity assays*

	CE	EAF	AE
Estimation of Total phenolics (mg gallic acid/g dried fraction)	275.0 ± 1.5	567.1 ± 2.1	293.2 ± 1.8
Total flavonoids (mg rutin/g dried fraction)	13.1 ± 0.2	71.2 ± 0.4	ND
Hydroxycinnamic acids (mg chlorogenic acid/g dried fraction)	30.2 ± 0.5	129.0 ± 0.8	34.3 ± 0.3
Total tannins (mg gallic acid/g dried fraction)	122.1 ± 2.1	135.3 ± 2.2	99.2 ± 1.7
Condensed tannins (A550nm/mg dried fraction)	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.1

DPPH colorimetric assay

As a first approach in order to evaluate *L. cuneifolia* antioxidant activity, the total antioxidant capacity of the different plant fractions was assessed by monitoring their ability to scavenge DPPH•. Every tested sample was able to show antioxidant properties in this assay (Figure 1). However, a 53% increase was found for the antioxidant capacity of the EAF when compared to its CE (CE: 4.3 ± 0.4 mmol eq. Trolox/g dried fraction vs. EAF: 6.6 ± 0.2 mmol eq. Trolox/g dried fraction, $p<0.001$). Interestingly, total antioxidant capacity of *L. cuneifolia* EAF was similar to the one observed for a CE of a commercial sample of *G. biloba* (6.6 ± 0.9 mmol eq. Trolox/g dried fraction). *Borneo et al.* found that *L. cuneifolia* antioxidant activity was lower than *G. biloba* by the FRAP assay. Despite the methodological differences, these results could be explained by several reasons. On one hand,

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specimens belong to dissimilar phytogeographical areas: Córdoba is in the central region and San

Juan is in the Northwestern, close to the Andes mountains. On the other hand, extract preparation

is crucial. They worked with an aqueous extract whilst we tested CE, AF and EAF. Similarly, we found

that AF has lower antioxidant activity than *G. biloba* whilst EAF, a flavonoid rich fraction, performed

better. This supports the extractive power of ethyl acetate for flavonoid enrichment.

Figure 1: *In vitro* total antioxidant capacity of *L. cuneifolia* fractions assessed by the colorimetric

DPPH$^\bullet$ assay, using Trolox as standard. CE: Crude extract; EAF: Ethyl acetate fraction; AF: Aqueous

fraction. A crude extract from a commercial sample of *Ginkgo biloba* was used as a positive control.

Results were expressed as mean ± SEM (n = 6).
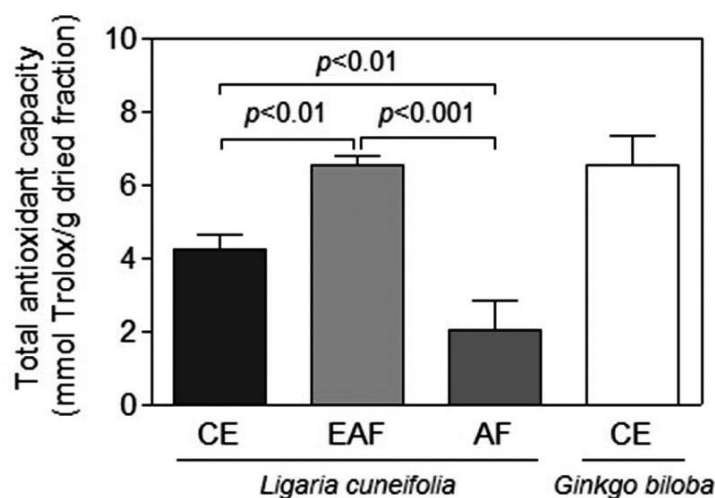
L. cuneifolia EAF flavonoid fingerprinting and DPPH CD-MEKC assay

Given that *L. cuneifolia* EAF showed the highest phenolics content and antioxidant activity *in vitro*,

this fraction was selected to further characterize its flavonoid extract composition and antioxidant

capacity.

Figure 2A depicts a typical electropherogram for the EAF, which shows the ten compounds that have

been previously identified by our group: [20] catechin (C), quercetin-3-O-glucoside (Q-3-O-G),

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quercetin-3-O-xyloside (Q-3-O-X), quercetin-3-O-arabinopyranoside (Q-3-O-AP), quercetin-3-O-rhamnoside (Q-3-O-Rh), quercetin-3-O-arabinofuranoside (Q-3-O-AF), quercetin-3-O-β-L-(2''-O-galloyl)-rhamnoside (QGG-1), quercetin-3-O-β-L-(3''-O-galloyl)-rhamnoside (QGG-2), quercetin-3-O-β-L-(2''-O-galloyl)-arabinopyranoside (QGG-3), and quercetin-3-O-β-D-(2''galloyl)-arabinofuranoside (QGG-4).

The DPPH$^\bullet$ assay combined with CD-MEKC detection is particularly suitable for the estimation of the oxidized fraction of every phenolic compound separately yet in the context of a complex mixture, such as plant extracts. [23, 24] Results are expressed as the unoxidized fraction (%) of each compound after DPPH$^\bullet$ incubation with the EAF of *L. cuneifolia*, meaning that the lower the unoxidized fraction remaining after the reaction with DPPH$^\bullet$, the better antioxidant. In this sense, the lowest values were obtained for the galloyl glycosides compounds, namely QGG-1, QGG-2, QGG-3, and QGG-4, indicating that quercetin galloyl glycosides could potentially be the main contributors to *L. cuneifolia* antioxidant effect (Figure 2B, Table 2).

Figure 2: Electropherograms of *L. cuneifolia* EAF (A), and an aliquot of this EAF after the incubation with 0.5 mM DPPH$^\bullet$ for 10 min (B). Peaks: 1 (catechin, C), 2 (quercetin-3-O-glucoside, Q-3-O-G), 3 (quercetin-3-O-xyloside, Q-3-O-X), 4 (quercetin-3-O-rhamnoside, Q-3-O-Rh), 5 (quercetin-3-O-arabinofuranoside, Q-3-O-AF), 6 (quercetin-3-O-arabinopyranoside, Q-3-O-AP), 7 (quercetin-3-O-β-L-(2''-O-galloyl)-rhamnoside, QGG-1), 8 (quercetin-3-O-β-L-(3''-O-galloyl)-rhamnoside, QGG-2), 9 (quercetin-3-O-β-L-(2''-O-galloyl)-arabinopyranoside, QGG-3), 10 (quercetin-3-O-β-D-(2''galloyl)-arabinofuranoside, QGG-4).
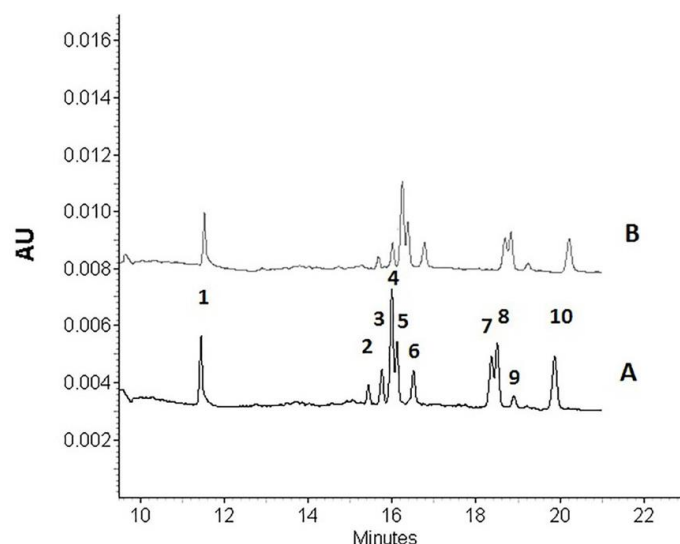
Table 2: Percentage of unoxidized fraction for every analyte in the EAF. Results were expressed as mean ± SEM (n = 6).

Compound	Migration time (min)	Unoxidized fraction (%)
C	11.500	86.5 ± 0.5
Q-3-O-G	15.517	83.7 ± 0.4
Q-3-O-X	15.833	83.4 ± 0.4
Q-3-O-Rh	16.075	82.1 ± 0.6
Q-3-O-AF	16.200	80.1 ± 0.5
Q-3-O-AP	16.604	76.2 ± 0.4
QGG-1	18.454	59.4 ± 0.4
QGG-2	18.592	56.3 ± 0.3
QGG-3	19.004	59.7 ± 0.2
QGG-4	19.996	58.3 ± 0.3

Ex vivo antioxidant capacity assays

Once the antioxidant capacity of *L. cuneifolia* EAF was characterized *in vitro,* and the potential contributors were preliminary assessed, the next step was to evaluate whether the EAF also has an antioxidant activity in biological systems.

Inhibition of phospholipid oxidation

When *tert*-Butyl hydroperoxide is added to a rat liver homogenate, it starts a characteristic lipid peroxidation process that can be followed by TBARS production. [25] The antioxidant effect of the EAF

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was evaluated in this system. When incubated with increasing concentrations of the EAF, a significant inhibition of TBARS production was observed with an IC$_{50}$ value of 14.5 μg/mL of EAF (Figure 3).

Figure 3: Inhibition of phospholipid oxidation (measured as TBARS content) in rat liver homogenates by the EAF of *L. cuneifolia*. Tissue samples were incubated with 3 mM *tert*-butyl hydroperoxide and increasing concentrations of the EAF for 1 h at 37 °C. After the incubation period, TBARS were extracted in *n*-butanol and determined using a fluorometric assay (*$p < 0.05$ vs. 0 μg/mL of EAF). Results were expressed as mean ± SEM (n = 6).
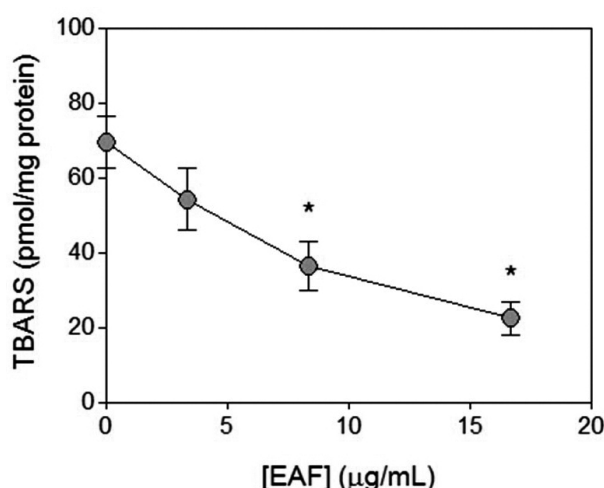
Inhibition of brain homogenate chemiluminescence (CL)

Spontaneous CL is emitted during the autoxidation of rat brain homogenates at room temperature. [26] This process can be inhibited by the addition of compounds with antioxidant properties. The incubation of rat brain homogenates with increasing concentrations of the EAF showed a significant inhibition of spontaneous brain CL (Figure 4), similar to the one observed for the inhibition of lipid peroxidation. In this case, the IC$_{50}$ value was 0.27 μg/mL of EAF.

Figure 4: Inhibition of rat brain homogenate autoxidation by the EAF of *L. cuneifolia*. CL emission of tissue samples was measured in a liquid scintillation counter after a 60 min of incubation at room

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temperature with increasing concentrations of the EAF (*$p < 0.001$ vs. 0 µg/mL of EAF). Results were expressed as mean ± SEM (n = 6).
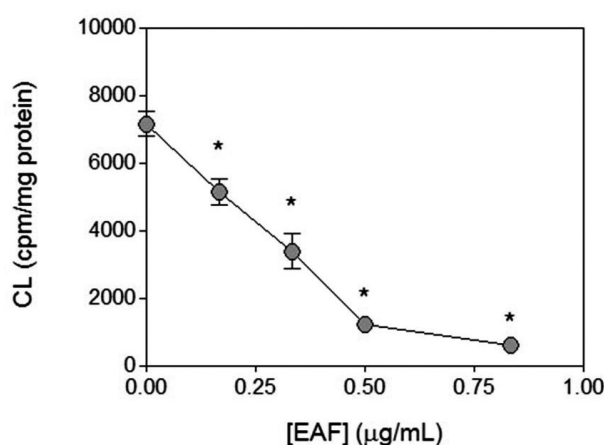

Inhibition of DNA damage induced by H_2O_2

The comet assay was used to test the ability of the EAF in inhibiting oxidative DNA damage. First, in order to discard direct DNA damage induced by incubation with the EAF, the assay was performed in the absence of H_2O_2. No significant induction of DNA damage was observed for every tested concentration of the EAF (data not shown). However, as it is shown in Figure 5, the incubation with the EAF significantly decreased H_2O_2-induced DNA damage at every tested dose in comparison with the positive control (0 ng/mL EAF).

Figure 5: Inhibition of oxidative DNA damage by the EAF of *L. cuneifolia*, evaluated by the comet assay. Mononuclear blood cells were incubated for 3 h at 37 °C with increasing concentrations of the EAF. Afterwards, samples were incubated for 20 minutes with H_2O_2 (50 µM) at 4 °C in order to induce DNA oxidative damage. Distilled water ($_dH_2O$) was used as a negative control (*$p < 0.005$ vs. 0 ng/mL of EAF). Results were expressed as mean ± SEM (n = 6).

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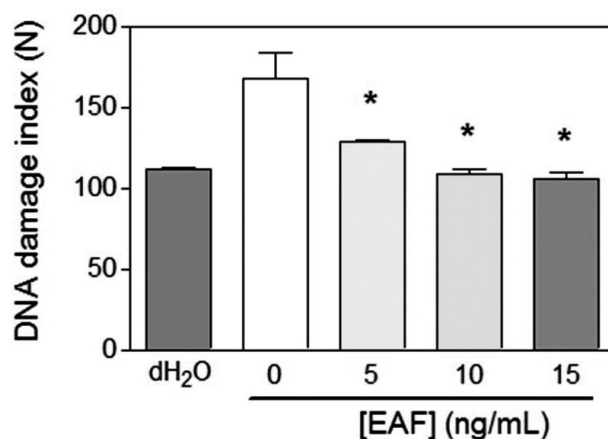
In vivo antioxidant capacity assay

Given that *L. cuneifolia* EAF showed significant antioxidant activity both *in vitro* and *ex vivo* with different biological substrates, the antioxidant activity of the EAF was tested in an *in vivo* animal model of oxidative stress.

 Inhibition of UVA-induced skin CL

Exposure of mouse skin to low dose UVA leads to an oxidative stress condition which can be followed by *in vivo* CL. [27, 28, 29] Measurements of *in vivo* CL have been used to evaluate the occurrence of oxidative stress in mice, [30] as well as the effects of antioxidant administration in several models of oxidative tissue damage. [31] As it is shown in Figure 6, the topical application with the EAF (10% w/v) produced a 38% decrease in skin CL after 30 min of irradiation (EAF: 30 ± 5 cps/cm^2 vs. vehicle: 49 ± 5 cps/cm^2, $p<0.05$). Appropriate controls with the vehicle showed no effect of the solvent on mouse skin CL, with or without irradiation (data not shown).

Figure 6: Inhibition of *in vivo* UVA-irradiated skin CL by the EAF of *L. cuneifolia*. Mice dorsal skin surfaces were irradiated with UVA light from a UVA source (maximum at 366 nm) for 30 min (total dose: 7.5 J/m^2). Determinations were performed with a Johnson Research Foundation photon counter. The effect of the topical application with the EAF (10% w/v) on the *in vivo* CL was determined using ethanol as vehicle (*$p < 0.05$ vs. vehicle). Results were expressed as mean $\pm$ SEM (n = 6).

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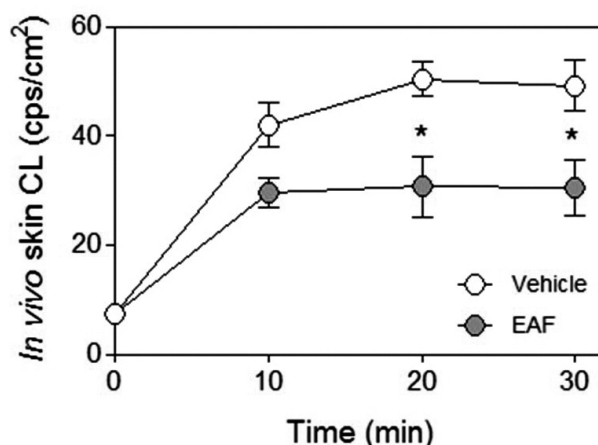
The present study shows that *L. cuneifolia* EAF exhibit antioxidant activity *in vitro*, *ex vivo*, and *in vivo*, and contain significantly more phenolics than many vegetables and fruits (nutritional plants). [32] Most interestingly, we found that galloyl quercetin glycosides could be potential contributors to this effect.

It has been extensively described that the antioxidant capacity of plant extracts is explained by their relatively high phenolic content with different antioxidant properties. [33] However, the presence of numerous antioxidant compounds in plant tissues makes it difficult to isolate and quantify each one separately. Therefore, several intermediate fractions are used to ensure an efficient extraction of the available antioxidants. [34] In this study, a crude extract (CE) of *L. cuneifolia* was employed and compared, in terms of total antioxidant capacity, to its ethyl acetate fraction (EAF) and aqueous fraction (AF). First, *in vitro* total antioxidant capacity was evaluated by a colorimetric method employing DPPH• as a source of free radicals. Assays based upon the use of DPPH• are among the most widely used spectrophotometric methods for the screening of the antioxidant capacity of natural products. DPPH• is a chromogen that can be readily reduced upon direct reaction with several antioxidants as a hydrogen atom donor, which reverses the formation of the DPPH•. The obtained results in this system are in agreement with previous studies, [11, 12] and indicate a significant

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antioxidant capacity of *L. cuneifolia* fraction, especially in the case of the EAF, even when compared with plant extracts with recognized antioxidant activity, such as those from *G. biloba*.

The observed antioxidant capacity may be attributed to the phenolic profile of the different plant fractions (Table 1). Since there is a wide degree of variation between phenolic compounds and their effectiveness as antioxidants, the relationship observed may be, at least in part, due to the estimation of total polyphenolic content. However, the structure and interactions between the different phenolics must be taken into consideration. These compounds possess ideal structural chemistry for free radical-scavenging activities and have been shown to be more effective antioxidants *in vitro* than C and E vitamins on a molar basis. This is exemplified by studies using pulse radiolysis to investigate the interactions of the hydroxyl radical (OH$^{\bullet}$), superoxide anion (O$_2^{\bullet-}$), lipid peroxyl radical (LOO$^{\bullet}$), and model *tert*-butyl alkoxyl radicals (*t*BuO$^{\bullet}$) with polyphenols, taking into account the rate constants of these reactions and the stability of the antioxidant radical generated. [35] When the content of the two fractions derived from *L. cuneifolia* whole extract were investigated, the EAF showed that it is enriched in low molecular weight condensed tannins and flavonoids.

In order to better characterize the EAF flavonoid profile, as well as to investigate which of them shows the higher antioxidant activity, a capillary electrophoretic method (CD-MEKC) was used. The CD-MEKC methodology present several advantages over traditional chromatographic approaches. [36, 37] Not only does it allow a rapid flavonoid fingerprinting of the extract, but also enables the evaluation of the antioxidant capacity of each compound upon the reaction with DPPH$^{\bullet}$ in the context of the whole plant extract, considering the potential synergy and antagonism among flavonoids. Consequently, this approach most closely resembles what actually takes place in a real sample when tested as an antioxidant.

Among the different flavonoids found in the EAF, galloyl glycosides exhibited the most relevant antioxidant capacity. Interestingly, glycosylation of flavonoids actually reduces their activity

when compared to their corresponding aglycones. Blocking the 3-hydroxyl group in the C ring of quercetin as a glycoside (while retaining the 3',4'-dihydroxy structure in the B ring) as in quercetin-3-O-glucoside, quercetin 3-O-xyloside, quercetin 3-O-rhamnoside, quercetin-3-O-arabinopyranoside, and quercetin-3-O-arabinofuranoside, significantly decreases their effectiveness as antioxidants. [38] However, the presence of a galloyl moiety counteracts this effect. Previous studies have indicated that the galloyl radical, which was formed during reaction with DPPH•, is a highly reactive species that can easily participate in a variety of reactions to form dimers through C—C and C—O coupling, thus halting the chain reaction of the radicals. [39] Flavonoid research was largely supported by the potential antioxidant capacities of these compounds based on their chemical structure. However, bioavailability should also be taken into account. There is an emerging opinion that flavonoids mostly do not act as conventional antioxidants but may exert antioxidant actions at enzymatic targets involved in multiple signaling pathways such as those involving protein kinases and redox sensitive cysteines, among other oxidant-prone moieties. [40]

Based on these results, two *ex vivo* assays were used to test the antioxidant ability of the EAF to act by a chain breaking mechanism and to inhibit lipid peroxidation: a) TBARS production induced by *tert*-butyl hidroperoxide in rat liver homogenates, and b) CL due to the autoxidation of rat brain homogenates. The TBARS assay is an indirect measurement of oxidative damage to lipids and can be employed to determine the extent of the reaction between lipids and oxidant species. [41] CL has been measured in intact and perfused organs, tissue homogenates, whole cells, and subcellular fractions, as a useful indicator of the termination steps of lipid peroxidation processes. [31] In this study, the inhibition of TBARS production in rat liver homogenates and of the autoxidation of rat brain homogenates showed IC_{50} values of 14.5 and 0.27 µg/mL of EAF, respectively. These values indicate that the EAF is an effective antioxidant, even when compared with pure polyphenolic compounds tested in similar assay conditions, [42] and suggest that the EAF could act as a chain breaking

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antioxidant and be involved in the termination steps of lipid peroxidation processes. In addition, the activity of *L. cuneifolia* EAF over H_2O_2-induced DNA damage was evaluated. Hydrogen peroxide (H_2O_2) is a genotoxic agent which is known to induce oxidative DNA damage, including DNA strand breakage and base modifications. [43] In this study, H_2O_2-induced DNA damage was quantified using the comet assay, which is a valuable method to detect cell DNA direct damage. [44] When H_2O_2 induces oxidative damage to DNA, an increase in the comet's length can be observed. When the antioxidant capacity of the EAF was tested, a decrease in the length of the comets was observed, in comparison with the positive control (Figure 4). Consequently, *L. cuneifolia* EAF could also be an effective antioxidant to prevent H_2O_2-induced DNA oxidative damage.

Finally, the antioxidant activity of the EAF of *L. cuneifolia* was evaluated in an *in vivo* mice model of oxidative stress. The UVA radiation component of sunlight (320-380 nm) has been shown to be involved in many of the consequences of the exposure to sunlight, including carcinogenesis and aging. [45] Reactive oxygen species and oxidative stress caused by UVA have been recognized to participate in these pathological conditions in the skin. [46] In previous reports, real time *in vivo* CL has been used to characterize the oxidative stress produced in the skin by the exposure to UV radiation. [29, 47] Increases in *in vivo* CL reflect an increased intracellular steady state concentration of excited species, such as excited carbonyls and singlet oxygen, and the occurrence of oxidative stress. *In vivo* CL measurements, being non-invasive, non-destructive, and specific to the organ, are particularly suitable for testing the effect of the topical application of antioxidants. The EAF was evaluated in this model and induced a significant decrease in skin *in vivo* CL after irradiation, indicating that the oxidative stress produced by UVA exposure can be controlled by the EAF. Similar effects were previously shown with well-known effective antioxidants, such as α-tocopherol and β-carotene. [29]

Conclusion

This is the first comprehensive report on *L. cuneifolia* antioxidant activity. A flavonoid rich fraction showed a significant antioxidant activity by acting as a hydrogen atom donor in *in vitro* systems. The EAF proved to be efficient as a chain breaking antioxidant during lipid peroxidation, be also involved in the termination steps of this process, in inhibiting H_2O_2-induced DNA damage, and as an *in vivo* antioxidant. CD-MEKC offered a reliable analytical advantage for flavonoid fingerprinting as well as the monitoring of the individual antioxidant capacity in the context of a real sample. Quercetin galloyl glycosides could be major contributors to the observed antioxidant properties and may be one of the central mechanisms by which *L. cuneifolia* have beneficial effects in traditional medicine.

Experimental Section

Drugs and chemicals. All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, US), except for HCl, H_2SO_4, and organic solvents, which were purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained from a Barnstead Easypure RF equipment (Thermo Fisher Scientific, Waltham, MA, US).

Plant material. Samples of *L. cuneifolia* growing on *Prosopis chilensis* (Mol) Stuntz (Fabaceae) were collected in the province of San Juan, in the west region of Argentina, during October and November (post bloom) 2018. Identification was made by Dr. Marcelo L. Wagner at the herbarium of the Museum of Pharmacobotany "Juan A. Domínguez" (School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina), where voucher specimens were kept and deposited under the accession number BAF-9018. Classification of the species was performed by means of the key according to previous reports. [48, 49]

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Preparation of plant fractions. L. cuneifolia fractions were prepared according to our earlier work. [20] Briefly, air-dried leaves (20 g) were grounded in a 5 mesh rotary blade mill (particle size < 4 mm) and successively extracted with hexane and dichloromethane for 24 h with continuous shaking, in order to eliminate chlorophylls and fats. The remaining material was submitted to consecutive extractions with methanol-water (80:20) to obtain the crude extract (CE). The organic solvent was reduced in a rotatory evaporator and the aqueous phase (AF) was collected and partitioned with ethyl acetate. This step was repeated for exhaustive extraction. The ethyl acetate fraction (EAF) was dried by evaporation under reduced pressure at 40 °C and stored in the absence of light at -20 °C for no longer than one week.

Screening of Phenolics

Estimation of Total Phenolics. Aliquots of a methanolic solution (1 mg/mL) of the different fractions were mixed with 0.25 mL of 1 N Folin-Ciocalteu reagent and 1.25 mL of 20% (w/v) Na_2CO_3. After 40 min, the absorbance of the mixture was measured at 725 nm in a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Chiyoda, Tokyo, Japan). [50] Gallic acid was used as standard and results were expressed as gallic acid equivalents (GAE) per g of dried fraction.

Total tannins. The Folin-Ciocalteu method was used as previously described, with minor modifications. [51] Briefly, 1 mL BSA buffer (0.2 M $NaCOOCH_3$, 0.17 M NaCl, and 1 mg/mL BSA fraction V, pH 5.0) was mixed with 1 mL of a methanolic solution (1 mg/mL) of the fractions, so that tannins were precipitated and the supernatant only contains simple phenolics other than tannins. The phenolic content of the supernatant was measured as described for Estimation of total phenolics. The content of total tannins was calculated by subtracting the content of simple phenolics from the content of total phenolics. Gallic acid was used as standard and results were expressed as GAE per g of dried fraction.

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Condensed tannins. The content of condensed tannins was measured using a colorimetric assay based on the hydrochloric acid catalyzed depolymerization of condensed tannin in *n*-butanol, to yield a red anthocyanin product. [52] Briefly, 0.5 mL of methanolic solutions (1 mg/mL) of the different plant fractions were mixed in a screw-capped test tube with 3 mL of butanol-HCl (95:5) and 0.1 mL of 2% (w/v) $NH_4Fe(SO_4)_2$ in 2 N HCl, and heated in a boiling water bath for 60 min. After cooling, the absorbance of the mixture was measured at 550 nm and results were expressed as A_{550nm} per mg of dried fraction.

Hydroxycinnamic acids. Aliquots of methanolic solutions (1 mg/mL) of the different plant fractions were diluted in anhydrous ethanol and the absorbance of the mixture was measured at 328 nm. Results were expressed as mg chlorogenic acid per g of dried fraction. [51, 53]

Total flavonoids. Aliquots of methanolic solutions (1 mg/mL) of the different plant fractions were diluted in 95% ethanol and incubated with with 10% (w/v) $AlCl_3$ and 1 M $NaCOOCH_3$. After 30 minutes, the absorbance of the mixture was measured at 416 nm. Results were expressed as mg rutin per g of dried fraction. [54]

Flavonoid fingerprinting. In order to further characterize the EAF, a capillary electrophoretic methodology (cyclodextrin-micellar electrokinetic chromatography, CD-MEKC) was employed based on our previous study. [20] Briefly, CE analysis was carried out with a P/ACE™ MDQ capillary electrophoresis system with diode array detector (Beckman, Fullerton, CA, USA). Uncoated fused silica capillaries (Microsolv technology, Eatontown, NJ, USA) of 60 cm (50 cm length to the detector) x 75 μm i.d. were used in all CE separations. The optimized background electrolyte (BGE) consisted of 20 mM borate buffer, 50 mM SDS, 5 mM β-Cyclodextrin (β-CD), 2% w/v sulphated β-CD, and 10% v/v methanol (pH 8.3). A stock solution of the EAF (10 mg/mL) was prepared in methanol, diluted in sample diluent (2 mM sodium tetraborate buffer in 20% (v/v) methanol, pH 8.3) to a final concentration of 0.4 mg/mL, and filtered. Samples were injected under 0.5 psi for 5 s and the

electrophoretic system was operated under positive polarity at 20 kV. Sample vials storage and the separation were carried out at 25 °C. Diode array detection at 255 nm (for quercetin glycosides) and 280 nm (for condensed tannins) were used.

In vitro antioxidant capacity assays

DPPH colorimetric assay. The antioxidant activity of the different *L. cuneifolia* extracts were evaluated on the basis of the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH•). Aliquots of methanolic solutions (1 mg/mL) of the different plant fractions were incubated with 3 mL of a methanolic solution of DPPH• (25 mg/L). After 10 min, the absorbance of the mixture was measured at 517 nm. A calibration curve was prepared using Trolox as standard and results were expressed as mmol Trolox equivalents per g of dried fraction. [55] A methanolic aliquot (1 mg/mL) of a crude extract (CE) from a commercial sample of *G. biloba* was tested as a positive control for the assay. This was used as reference material as a species with recognized antioxidant properties.

DPPH CD-MEKC assay. The antioxidant activity of the EAF was also monitored by capillary electrophoresis as early described, [23, 24] with minor modifications. Equipment conditions and BGE composition were similar to the ones described in *Flavonoid fingerprinting* and samples were injected under 0.3 psi for 5 s. Aliquots of methanolic solutions (10 mg/mL) of the EAF were incubated with 0.5 mM DPPH• to render a 1/10 final dilution. After 10 minutes, a 1/5 dilution was made in sample diluent and immediately injected (5 seg at 0.5 psi). The unoxidized fraction (%) of every compound was calculated using the original peak areas as reference.

Experimental animals

Rats (Sprague–Dawley, female, 180-200 g, 45 ± 5 days old), from the Animal Facility of the School of Pharmacy and Biochemistry of the University of Buenos Aires, were used for the ex vivo experiments. Mice (Swiss, female, 20-25 g, 60 ± 5 days old), from the Animal Facility of the School of Pharmacy and Biochemistry of the University of Buenos Aires, were used for the in vivo experiments. Every

experimental procedure was approved by the animal ethics committee of the School of Pharmacy and Biochemistry of the University of Buenos Aires (CICUAL, reference number 2346/17). Every protocol was carried out in accordance with institutional guidelines. Animals were fed ad libitum and housed under standard 12:12 light:dark cycles and controlled temperature (21 to 23 °C) and humidity (50 to 70%) conditions. For the ex vivo experiments, animals were euthanized in a CO_2 chamber and brain and liver samples were obtained by standard surgical procedures for analysis.

Ex vivo antioxidant capacity assays

Tissue homogenates. Female Sprague-Dawley rats weighing 180-200 g were used. Liver samples (1 g of wet weight) to be processed for the inhibition of the phospholipid oxidation assay were homogenized in 120 mM KCl, 30 mM phosphate buffer (pH 7.4) at 0-4 °C with a Potter Elvejhem glass homogenizer. The same procedure was performed to process brain samples (1 g of wet weight) for the inhibition of brain chemiluminescence (CL). In both cases, the obtained suspension was centrifuged at 600 *g* for 10 min at 4 °C to remove nuclei and cell debris. The pellet was discarded and the supernatant was used as homogenate. Protein concentration of tissue homogenates was determined by the method of [56] using BSA as standard.

Inhibition of phospholipid oxidation. Rat liver homogenates (1.5 mL) were incubated with 3 mM *tert*-butyl hydroperoxide and in presence of 0, 3.3, 8.3, and 16.7 µg/mL of the EAF for 60 min at 37 °C. To stop the reaction, butylated hydroxytoluene (BHT) was added to a final concentration of 0.1% (w/v). Afterwards, phospholipid oxidation was determined by the thiobarbituric reactive substances (TBARS) assay using a fluorometric technique. Briefly, 1 mL of tissue homogenates treated with *tert*-butyl hydroperoxide and the EAF was mixed with 2 mL of 0.1 N HCl, 0.3 mL of 10% (w/v) phosphotungstic acid, and 1 mL of 0.7% (w/v) 2-thiobarbituric acid, and heated in a boiling water bath for 60 min. After the incubation period, samples were cooled and TBARS were extracted in 5 mL of *n*-butanol. After a 10 min centrifugation at 800 *g*, the fluorescence of the butanolic layer was

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measured in a LS 55 luminescence spectrometer (Perkin Elmer, Waltham, MA, US) at 515 nm (excitation) and 553 nm (emission). A calibration curve was prepared using 1,1,3,3-tetramethoxypropane as standard and results were expressed as pmol of TBARS per mg of protein. [57]

Inhibition of brain homogenate CL. CL emission from rat brain homogenates in the presence of increasing concentrations of the EAF was measured in a Packard Tri-Carb liquid scintillation counter (Perkin Elmer, Waltham, MA, US), working in the out-of coincidence mode. Tissue homogenates (1 mL) were mixed with 0, 0.17, 0.33, 0.50, and 0.83 µg/mL of the EAF. Immediately, samples were placed in low potassium glass vials and protein content was adjusted to 1 mg/mL with a reaction medium containing 120 mM KCl, 30 mM phosphate buffer (pH 7.4). CL was measured after 60 min incubation at room temperature. Results were expressed as counts per minute (cpm) per mg of protein. [26]

Inhibition of DNA oxidative damage induced by H_2O_2. The extent of DNA damage was determined by alkaline micro-gel electrophoresis or "comet assay" as described by [44] with minor modifications. Heparinized venous blood was collected from male healthy donors and mononuclear cells were isolated by centrifugation in a Histopaque-1077 density gradient. The final concentration of the obtained cell suspension was adjusted to 1×10^5 cells/mL with RPMI-1640 media, and 0, 5, 10, and 15 ng/mL of the EAF was added. After 3 h at 37 °C, samples were incubated for 20 min with 50 µM H_2O_2 at 4 °C in order to induce DNA oxidative damage. Afterwards, the cell suspension was centrifuged, and the obtained pellet was resuspended in 75 µL of 0.75% (w/v) low melting point agarose, and layered over a frosted microscope slide precoated with 100 µL of 1% (w/v) normal agarose. When the low melting point agarose had solidified, a second layer of 0.75% (w/v) low melting point agarose was added. Slides were immersed in ice-cold alkaline lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10), and incubated for 60 min.

Afterwards, slides were immersed in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13.5) for 20 min to allow DNA to unwind. Electrophoresis was performed at 25 V and 300 mA for 20 min. Slides were then neutralized by three washes with 0.4 M Tris-HCl (pH 7.5) and DNA was stained with 50 μL of ethidium bromide (2 μg/mL). The whole procedure was performed in the dark at 4 °C in order to prevent additional DNA damage. The analysis of the comet length was performed by fluorescence microscopy using the semi quantitative method of visual scoring. For each sample, 100 randomly selected cells were visually classified into one of four categories (Cat) according to the length of the comet tail: Cat #1: tail < 20 μm, Cat #2: 20-40 μm, Cat #3: 40-80 μm and Cat #4: > 80 μm. A longer comet tail indicates a higher DNA damage. In order to evaluate the inhibition of DNA damage by the EAF, and to compare it with the positive (0 ng/mL of the EAF) and negative controls (dH$_2$O), DNA damage index (N) was calculated as follows:

$$N = n_1 + 2n_2 + 3n_3 + 4n_4$$

Where n_1 is the number of cells within Cat #1 of damage, n_2 is the number of cells within Cat #2 of damage, n_3 is the number of cells within Cat #3 of damage, and n_4 is the number of cells within Cat #4 of damage.

The protocol to collect the blood samples was in accordance with the policy statements of the Argentine Society of Clinical Investigation and was approved by the Ethical Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires. Informed consent was obtained from all participants.

In vivo antioxidant capacity assay

Inhibition of in vivo ultraviolet A (UVA)-irradiated skin CL. Female Swiss mice weighing 20-25 g fed a conventional diet and water *ad libitum* were used. Animals were anaesthetized (i.p.) with pentobarbital (50 mg/kg body weight), dorsal surfaces were shaved, and 50 μL of either the EAF (10% w/v) or the vehicle (ethanol) was delivered topically to the back of the animal from an automatic

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pipette. Areas of 1-3 cm^2 were irradiated with UVA light from a Mineralight UVGL-58 UVA source (Mineralight, Upland, CA, US) (maximum at 366 nm) for 30 min (total dose: 7.5 J/m^2). The percentage of UVB (290-320 nm) emitted was less than 1%. The back of the animal was placed at a distance of 15 cm from the UVA lamp. In order to measure *in vivo* CL, the whole animal was covered with aluminum foil, in which a window was cut allowing exposure of the dorsal skin only. Determinations were performed with a Johnson Research Foundation photon counter (Boston, MA, US). An EMI 9658 photomultiplier (responsive in the range 300-900 nm) cooled at -20 °C with an applied potential of -1.4 kW was used. The phototube output was connected to an amplifier-discriminator adjusted to a single photon counting which was in turn connected to both a frequency counter and a recorder. Efficient light collection and isolation from the sample were established by using a lucite rod as optical coupler placed in front of the exposed skin *in situ*. CL was measured at the beginning of the experiment and after 10, 20, and 30 min of UVA irradiation. A 1 minute delay was needed to move the mice from the UVA lamp to the photon counter. Results were expressed as counts per second (cps) per square centimeter of skin surface. [27, 28, 29]

Statistical analysis. Results were expressed as mean values ± SEM, and represent the mean of at least six independent experiments. ANOVA followed by Student-Newman-Keuls test was used to analyze differences between mean values of more than two groups. Statistical significance was considered at $p<0.05$.

Acknowledgments

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Author contribution statement

C.D. and T.M. are the main researchers in this project and wrote the manuscript. R.M. assisted in the planning of the screening of phenolics. M.G. assisted in the execution of the antioxidant capacity experiments. A.G. assisted in the execution of the comet assay. M.C. assisted in the planning and execution of the comet assay. M.W., S.L. and P. E. are research supervisors and are involved in all aspects of this work.

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