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Pehuén (*Araucaria araucana*) seed residues are a valuable source of natural antioxidants with nutraceutical, chemoprotective and metal corrosion-inhibiting properties

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ABSTRACT

In the last decade, green chemistry has been attracting great interest in many contexts, including, among others, natural antioxidants. However, only a few works deal with natural residue extracts and biowaste, which could be an efficient, economical and environmentally friendly source for the production of useful compounds. In this study, we look for antioxidant activity in *Araucaria araucana* seeds, an iconic pine species of the Argentine and Chilean Patagonia commonly known as "pehuén". Piñones are the edible pehuén seeds, and it is estimated that approximately 40 tons of piñones are harvested annually in Argentina and Chile. The chemical composition, antioxidants, metal corrosion-inhibiting properties and biological activity of edible and discarded piñón tissues were determined. Acute toxicity was discarded by *in vitro* testing and double fluorescent staining. Biological activity was evaluated *in vivo* by determining redox markers in salivary glands from rats treated with Cyclophosphamide (an oncological drug). All piñón tissues had antioxidants and antioxidant activity, with the coats showing the highest levels (up to 404 μg ascorbic acid equivalent per mg). The coats, in particular, had high gallic acid, catechin, quercetin and tannin contents, and more antioxidant activity, polyphenols and flavonoids than berries from the region. Results by X-ray fluorescence spectrometry showed that Na, Mg, P, S, Cl, K, and Ca were majority elements in the coat, embryo and endosperm. Furthermore, coat extract also showed significant anti-corrosion activity and *in vivo* protection against oxidative damage. The results indicate that piñón biowaste is a low-cost attractive source of natural antioxidants with potential nutraceutical, medical and metal corrosion protection applications.

1. Introduction

When free radicals are increased by cellular aerobic metabolism or exogenous factors, such as solar radiation, electromagnetic fields, pharmacological drugs or cigarette smoke (among others), an increase in antioxidants is required to maintain redox balance in the human body [1–3]. Otherwise, oxidative stress is promoted, causing structural and functional alterations in essential biomolecules of living organisms. Since oxidative stress plays an important role in many human diseases, the characterization of a new source of antioxidants has attracted increasing interest. In particular, vegetable sources are being intensely studied for their preventive and therapeutic uses in cancer, Parkinson's, Alzheimer's, atherosclerosis, liver injury, rheumatoid arthritis, type 2 diabetes, neurodegeneration, kidney failure and aging, and also as chemoprotective agents in cancer therapy [4–8]. In addition, these compounds have increased industrial applications; for example, they are used as ingredients in the formulation of dietary supplements, as reservatives for food and cosmetics, and to prevent the degradation of rubber and oil-derived compounds [9,10]. Furthermore, many vegetable antioxidants are being investigated as green inhibitors for corrosion protection of metals and alloys [11,12].

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In the context of a circular economy, working with biological waste (biowaste) is crucial, because using such bioactive waste provides an efficient, economical and environmentally friendly source for the production of useful compounds. In this regard, high antioxidant content has been described in several species of the genus Araucaria [13–18]. However, only a few works deal with its residue extracts and biowaste, which are usually released into the environment [19–22].

In this work, we looked for antioxidant activity in the discarded coats of *Araucaria araucana* seeds, an iconic pine species of Patagonia. As a matter of fact, the residual coat of seeds from *Araucaria angustifolia*, a similar species present in southern Brazil and northern Argentina, has been described as rich in phenolic compounds and antioxidant capacity [17,18]. *Araucaria* is one of the three genera that belong to the Araucariaceae family, occupying a unique position among conifers, now restricted to the South American and Southwest Asia-Western Pacific region despite their extensive distribution in both hemispheres during the Mesozoic [23,24]. Therefore, most, if not all, of the current Araucarian taxa have evolved since the early Tertiary Period, suggesting that the success of these conifers may be governed by their ability to survive for long periods under adverse climatic conditions, such as drought, cold, fire, low fertility soil, or destructive volcanic disturbance. Perhaps for these reasons, *Araucaria araucana* (common name "pehuén", Fig. 1) has been successful in the volcanic environment of the Argentine and Chilean Patagonia, with trees more than 1000 years old [23–26]. Pehuén forest occupies a total area of approximately 450,000 ha, of which 263,525 ha belong to Chile and 179,289 ha to Argentina [27]. The pehuén is considered a prehistoric tree, and is protected by law in both countries. Pehuén seeds, locally called "piñones" (singular "piñón"), have been used for food and for medicinal and religious purposes since ancient times. Nowadays, piñones also have great value for gastronomic tourism in some Patagonian localities. According to published data, approximately 90 kg/ha and 273 kg/ha of piñones are harvested annually in Argentina and Chile, respectively [27,28]. Piñón pulp (mean weight 1.8 g per piñón) consists of endosperm and embryo, and is the edible part of the seed (Fig. 1). As a by-product of

Fig. 1. Photograph of *Araucaria araucana*, seeds (piñones) and its different tissues.

food preparation, piñón coat, which weighs approximately 0.7 g, is usually discarded and takes considerable time to decompose. Whole piñones are light yellow in color, covered by an adherent membrane (thin coat) and encased in a very resistant reddish-brown coat (thick coat). Piñones are fleshy and have an ovate-oblong shape, measuring 4–5 cm in length and 1.5–2.0 cm in width, and weighing approximately 2.5 g (Fig. 1). Our previous results indicate that both the edible part of piñones and the biotechnologically obtained callus of piñón embryos show antioxidant capacity [29,30]. Therefore, the piñón coat from *A. araucana* (biowaste) could be a valuable source of natural antioxidants that has not yet been explored.

Given the enormous scientific, medicinal and industrial interest surrounding natural antioxidants, the objective of this study was to analyze and compare the chemical composition and antioxidant properties of the edible and discarded parts from piñones, seeking better properties in the residues. Considering that antioxidants obtained from biowaste are environmentally and ecologically friendly, inexpensive and readily available, we advanced in the study of possible applications for piñón coat extract as a therapeutic agent and green corrosive inhibitor.

2. Material and methods

2.1. Chemical reagents

Dulbecco modified essential medium (DMEM), antibiotics, reagents, and standards for HPLC were purchased from Sigma-Aldrich. HPLC grade solvents were purchased from Fisher Scientific Co. Fetal bovine serum was purchased from Natocor (www.natocor.com.ar). The RANSOD kit (SD 125) for superoxide dismutase activity (SOD) determination was from RANDOX Laboratories Ltd. Malondialdehyde (MDA) Assay Kit (ab118970) to detect lipid peroxidation was from Abcam Laboratory. Uricostat enzimático AA (Cod. 1840107) from Wiener lab was used for uric acid determinations. MilliQ water was used in all of the experiments. PES 0.22-μm sterile filter units were from GVS, USA. Cyclophosphamide 1000 as a lyophilized powder (catalogue number: 120105-04) was from LKM Laboratory (http://www.lkmsa.com/).

2.2. Vegetable material

Pehuén seeds (piñones, Fig. 1) were collected in 2017, 2018 and 2019, during the autumn, around Villa Pehuenia, located in the midwest of the Neuquén province, at the foot of the Patagonian Andes of Argentina (38°50′02.3″S 71°12′24.1″W). Material collection was performed with the permission of the Villa Pehuenia-Moquehue authority. The collection site was located in an open forest dominated by pehuén trees. The regional climate is dry with distinct seasons, warm summers (average temperature 18.9 °C) and cold winters (average temperature −2.4 °C). Rainfall occurs mainly in the cold period, from April to September, with a snowfall of up to 792 mm (official data contributed by AIC; www.aic.gov.ar). Healthy seeds were selected, washed, and immediately transported to the laboratory to prepare the extracts. Piñón flour and piñón coffee were provided by NOUS, a food company from Villa Pehuenia, Neuquén, Argentina. The piñones were baked at 150 °C for 24 and 72 h to obtain flour and coffee, respectively.

2.3. Preparation of piñón extracts

Under normal cooking conditions (whole piñón, including coats), the seeds were divided into three groups and subjected to different treatments before the extracting procedures. The first group was untreated (fresh or control group), the second (120 g) was boiled in 1500 ml of distilled water at 100 °C for 10 min (boiled group), and the third was baked at 150 °C for 72 h (baked group). Then, each group was separated into coat and pulp. Thin and thick coats were obtained from the coat, while endosperm and embryo were obtained from the pulp. The flour and coffee contained endosperm, embryo and around 1% thin coat as contaminant. The fresh and boiled tissues were lyophilized. Water obtained from the boiled treatment was stored at −20 °C until use. The other samples were milled into a fine powder and stored at room temperature in hermetic bags. In order to obtain the extracts, an eco-friendly procedure was applied: 12 g of each dried sample were extracted with 150 ml of distilled water at 100 °C (this procedure was more efficient than extraction at room temperature, data not shown), and then kept in the dark overnight at 20 °C. Extracts were filtered through a 10 μm pore nylon filter and stored at −20 °C until use. To determine *in vivo* protective effects, extract was prepared daily as follows: 40 g of dried and milled whole fresh piñón coat [thin coat + thick coat] were extracted with 2 L of potable water at 100 °C, kept in the dark for 60 min, and then filtered through a 10 μm pore nylon filter. After that, 50 ml of extract were placed in water bottles for feeding individual rats at the laboratory.

2.4. Analysis of antioxidant properties of piñón extracts

2.4.1. Ferric reducing antioxidant power (FRAP assay)

The reducing ability of the extracts was estimated by the FRAP assay [31] according to Echeverri Del Sarto *et al.* [30], using ascorbic acid as reference.

2.4.2. Free radical scavenging capacity (DPPH assay)

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used, following the procedure of Brand-Williams *et al.* [32]. The radical scavenging activity of the extracts was monitored over 300 s. Results were compared with a reference curve of 0.1 mg/ml ascorbic acid solution. According to these results (Fig. S1 in supplementary material), the extracts' radical scavenging activity was expressed as percentage inhibition at 5 s of incubation, and calculated using the following formula: % inhibition = [(AB − AS) / AB] × 100, where (AB) = blank absorbance value (reaction mixture without sample and incubation), and (AS) = sample absorbance value at 5 s.

2.5. Chemical characterization of piñón extracts

2.5.1. Determination of total phenolic, tannin and flavonoid contents

Total phenolic content in the extracts was determined following the Folin-Ciocalteu colorimetric method [33] according to Soria *et al.* [34] using gallic acid as reference. The tannin content estimate was based on their property of co-precipitating with proteins [35]. Tannins were measured in fresh whole piñón coat extract. To do so, phenolic content was determined before and after incubation with bovine serum albumin (1 mg/ml protein). The percentage of precipitated polyphenols was considered as a percentage of total tannins. Flavonoid content was measured by the aluminum chloride colorimetric assay [36], according to Soria *et al.* [34], using quercetin as reference.

2.5.2. Analysis of phenolic compounds by high-performance liquid chromatography (HPLC)

First, fresh piñón coat extract was dried in a rotary evaporator, and then the residue was re-dissolved in ultra-pure methanol. All samples, in triplicate, were filtered through a 0.22 μm filter unit before injection. The phenolic profile was determined by an HPLC system (Agilent 1260, Quat Pump VL, ALS, TCC, DAD, and RID, with openLAB Chem Station Software) equipped with a reversed-phase ZORBAX Eclipse XDB-C18 column (4.6 × 100 mm; 3.5 μ, Agilent). Two different column temperatures (25 °C and 40 °C) were used to achieve chromatographic separation, using gradient elution with solvents, 1% formic acid in water (A) and acetonitrile (B) in both methods. Peak identification was carried out by comparison of their retention times with those

obtained by injecting the following standards in the same conditions: gallic acid, catechin, chlorogenic acid, caffeic acid, p-coumaric acid, quercetin, rutin and ferulic acid.

2.5.3. Multielemental analysis by energy-dispersive X-ray fluorescence spectrometry (EDXRF)

For the quantitative multielemental analysis of the samples, a touch-control S2 Ranger EDXRF system (Bruker AXS GmbH, Germany) with a Pd X-ray tube and XFlash® Silicon Drift Detector (SDD) was used. Piñón samples were prepared for analysis following the methodology and procedures detailed in previous work [37,38]. Spectral data were obtained at four different voltages (10, 20, 40, and 50 KV) to properly excite and determine elemental composition from sodium (n = 11) to lead (n = 82). Evaluation of EDXRF spectra and calculation of elemental concentrations were performed using the software supplied with the equipment. Instrumental setup and calibration curves were carried out using a set of 14 vegetal certified reference materials according to Gallardo *et al.* [37]. Each sample was measured three times, and mean value and standard deviation were calculated.

2.6. Analysis of biological activities of piñón extracts

2.6.1. Early effects on cell viability

The adverse effects of the coat extract on cell viability were analyzed according to a model previously developed by us to screen for toxic, cytoprotective and antioxidant plant extracts [39–42]. Accordingly, VERO cells (kidney epithelial cells from African green monkey; European Collection of Cell Cultures) were seeded onto 96-well tissue culture test plates, incubated for 24 h, and then exposed for 2 h in fresh medium containing different extract concentrations. Next, early effects on cell viability were tested by crystal violet staining or using a double-fluorescence staining technique (DMEM containing 2 μg/mL propidium iodide or 10 μg/mL fluorescein diacetate (FDA), 15 min at 37 °C in the dark).

2.6.2. Determination of the chemoprotective activity of piñón waste

The experiment included 28 12-week-old male albino Wistar rats housed in individual metabolic cages according to procedures described in Mazzeo *et al.* [43]. The proper care and use of animals followed the International Protocol for the Care and Treatment of Animals (NIH) and were approved by the Bioethics Committee of the Faculty of Medical Sciences of the National University of Córdoba, Argentina (336201801004l4CB). Each rat had free access to food and water or fresh piñón coat extract (50 ml per day per rat). Fresh whole coat extract was prepared daily as described in section 2.3. This treatment provides an antioxidant power equivalent to 40 mg of ascorbic acid per day, according to the FRAP assay. The animals were divided into four experimental groups (7 per group):

C) Control group: non-treated rats

CP) Cyclophosphamide-treated group: at the fifth and sixth day, each animal was treated with 50 mg/kg body weight of CP i.p. daily

AO) Antioxidant-treated group: the rats drank piñón coat extract throughout the experiment

CP + AO) Cyclophosphamide + Antioxidant-treated group: idem to CP, but the rats drank piñón coat extract throughout the experiment

On the seventh day, the food was removed, but the rats kept drinking water or extract, as appropriate. On the eighth day, the rats were anesthetized with a combined dose of ketamine and xylazine (80 and 12.8 mg / Kg, respectively), and the submandibular glands (SMG) were dissected. After the surgical procedure, the animals were euthanized according to approved procedures, described in Mazzeo *et al.* [43]. Immediately after extraction, 10 mg of SMG were homogenized in buffer, centrifuged at 13000 rpm for 30 min at 4°C, and then SOD (superoxide dismutase activity), MDA (Malondialdehide content) and uric acid content were determined in the supernatant following the manufacturer's instructions. All determinations were performed in triplicate.

2.7. Analysis of corrosion-inhibiting properties of piñón waste

Corrosion was evaluated by measuring weight loss following the ASTM G 31-72 (2004) method for corrosion testing of metals [44], according to Boujakhrout *et al.* [45]. Fresh piñón coat extract was dried in a rotary evaporator, and then the residue was re-dissolved in methanol. Steel flat washers of 2.45 cm^2 were polished with sandpaper, weighed accurately and immersed in a 60 ml beaker containing 20 ml of 1.0 M HCl (control) or 1.0 M HCl with different concentrations of piñón coat extract. Although 6 h were used in Boujakhrout's work, our results showed that, after 5 h of exposure, the 1 M HCl solution significantly decreased the steel washers' weight and that this time was enough to evaluate the anticorrosive properties of antioxidant piñón coat extract. Therefore, after 5 h of acid immersion, the washers were removed, washed, dried and weighed accurately. The corrosion inhibition efficiency (ηWL%) was calculated as follows: $\eta_{WL}\% = (1 - w_1/w_0) \times 100$, and the corrosion rate (C_R) as follows: $C_R = (W_b - W_a) / At$, where w_0 and w_1 are the values of corrosion weight losses of steel washers in uninhibited and inhibited (with extract) solutions, W_b and W_a are the sample weight before and after immersion in the tested solution, respectively, A is the total area of the steel washer (cm^2), and t is the exposure time (h) [45].

2.8. Statistical analysis

All the results are expressed as mean ± SD. Statistical analysis of the data was performed by Student's *t*-test, and P value < 0.05 was considered statistically significant with respect to control.

3. Results and discussion

In order to identify the contributions of separate piñón components to the overall antioxidant activity and chemical composition, Fig. 2 summarizes the results regarding antioxidant capacity, and the phenolic and flavonoid content of the different tissues from one dried fresh seed. The most relevant result was that 97% of antioxidant power, 93% of total phenols and 75% of flavonoids of the piñón were found in the coat, which represents 27% of the seed (w/w) and is discarded as waste. Therefore, the results in Fig. 2 show that pulp would provide flavonoids to the diet. The following sections describe the antioxidant, nutritional, pharmacological and metal corrosion-inhibiting properties of piñón extract from different tissues and under different treatments.

3.1. Antioxidant properties of piñón extracts

3.1.1. Ferric reducing antioxidant power (FRAP)

Fig. 3 shows antioxidant capacity per milligram of each dried piñón tissue. Under all treatments, thin coat proved to be the tissue with the highest antioxidant capacity. Although the antioxidant power of the seeds decreased after boiling (20.2 vs. 23.3 μg AAeq per mg of dried seed) and after baking (19.5 vs. 23.3 μg AAeq per mg of dried seed) with respect to control, those decreases were not statistically significant. However, a significant reduction of antioxidant capacity was observed for the boiled coat, which was accompanied by the appearance of antioxidants in water (10.8 μg AAeq per mg of dried coat, or 3.1 μg AAeq per mg of dried total seed). This result suggests a migration of antioxidants from coat to water; thus, the antioxidant power in the water shows a reduction in boiled coats (Fig. 3). Migration could be favored by the breakdown of cell wall compounds embedded into the seed and by the destruction of cell walls during heating. In addition, embryo and endosperm antioxidant power was not affected by boiling (Fig. 3). Thus, piñón pulp would not be receiving antioxidants

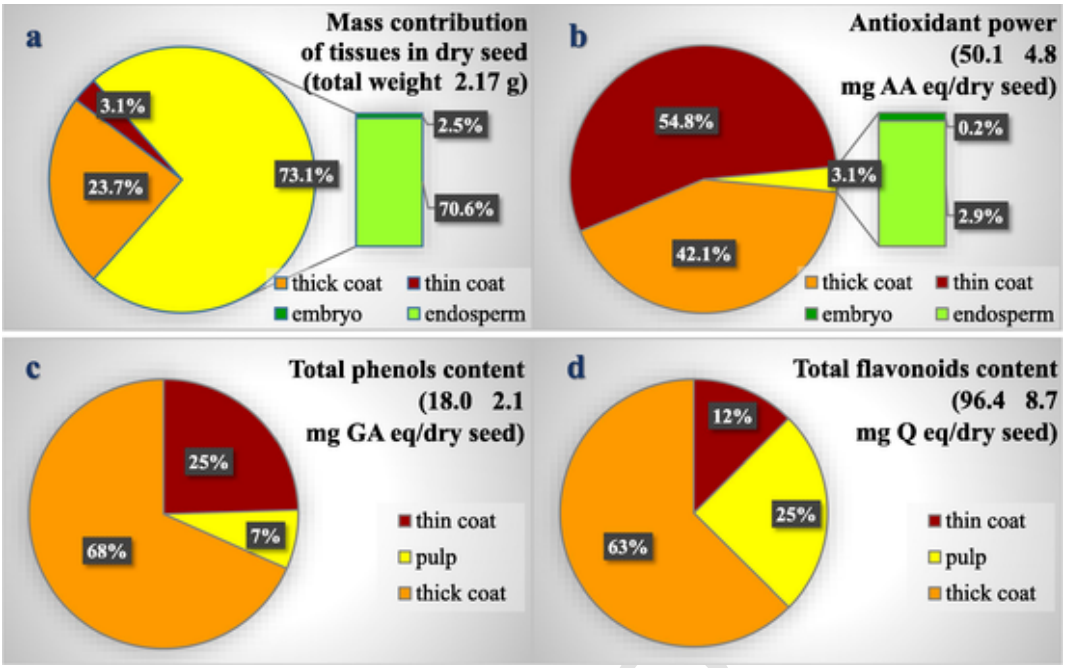

Fig. 2. Mass contribution of tissues (a), antioxidant power (b), phenolic (c) and flavonoid content (d) of the different tissues from one dried fresh seed. The values are expressed as mean ± SD of four independent experiments.

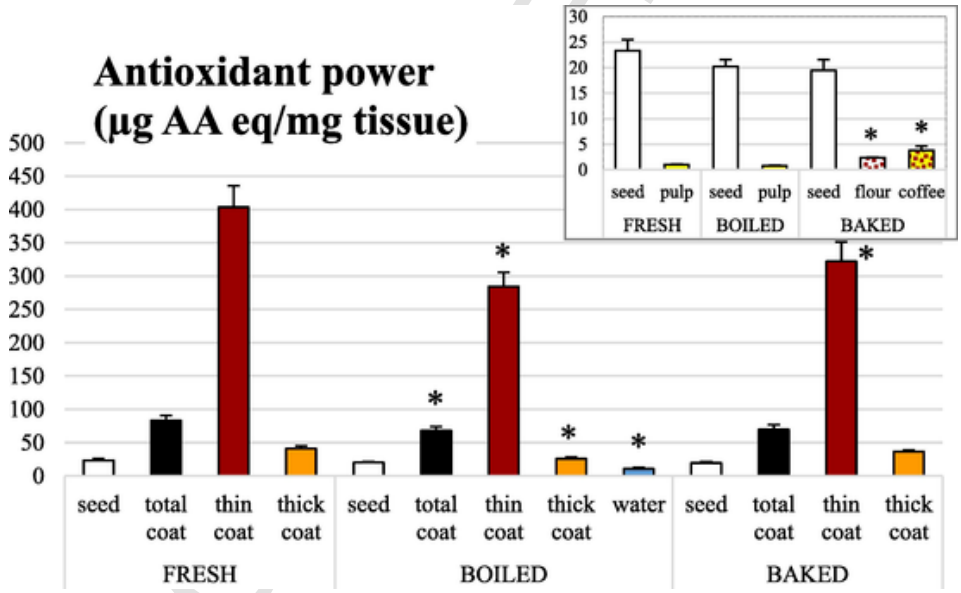

Fig. 3. Antioxidant power of extracts obtained from tissues of fresh, boiled and baked piñones, food derivatives and water obtained in boiled treatment (µg AAeq/mg tissue). The values are expressed as mean ± SD of four independent experiments. *: Significant differences with control.

from the coat during boiling either, unlike Brazilian piñones (*A. angustifolia* seeds) [17]. On the other hand, antioxidant capacity in flour and coffee (baked pulp) was higher than in the fresh pulp. These results could be explained by the presence of thin coat residues in these foods, which are difficult to remove after baking and could contribute antioxidants (Fig. 3). In short, one seed contains 50.1 mg AAeq, but only 1.5 mg are used in food form, and the rest (coat) is discarded. Thus, people are actually discarding material with more antioxidant content (83 µg AAeq per mg of dried fresh piñón coat) than regional antioxidant fruits (Argentine and Chilean berries), such as *Luma chequen* or chequén, *Ugni molinae* or murta, *Amomyrtus meli* or meli, and *Luma apiculata* or arrayán (19, 20, 22 and 23 µg AAeq per mg of dried fruit, respectively, taking into account conversion factors) [46].

3.1.2. Free radical scavenging activity

Piñón antioxidant activity was also determined by measuring the free radical scavenging activity of piñón extracts at different incubation times (curves in Fig. S1, in the supplementary material), and then 5 s of incubation were chosen to compare extract activity. Results showed that thin coat extracts were more active because they exhibited a higher inhibiting percentage of the initial DPPH radical concentration (Fig. 4). Free radical scavenging activity was significantly decreased by heating both coats at 150 °C. Decreased activity in boiled thick coat was consistent with the activity found in water, while the activity of the pulp was not altered. These results were consistent with those obtained by FRAP assay, reinforcing previous speculations.

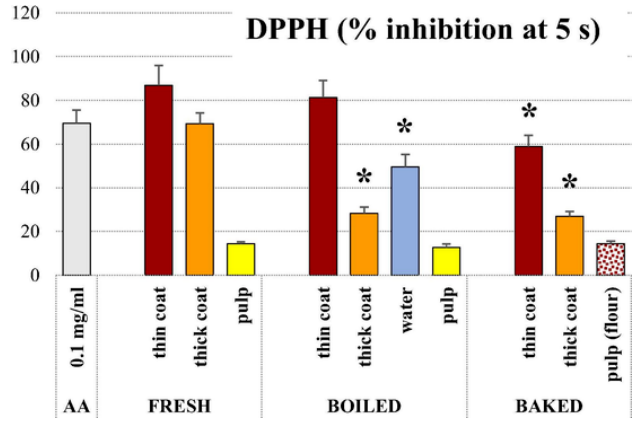

Fig. 4. Percentage inhibition of DPPH radical at 5 s by extracts obtained from tissues of fresh, boiled and baked piñones and water obtained in boiled treatment (all diluted 10 folds), compared to ascorbic acid (0.1 mg/ml). The values are expressed as mean ± SD of two independent experiments. *: Significant differences with control.

3.2. Phytochemical analysis of piñón extracts

As occurs in several vegetable foods, polyphenol content is highly related to antioxidant activity (Fig. 2). Indeed, coat provides more phenolic compounds than pulp. Additionally, the results in Fig. 5 suggest that thin coat is the tissue with most phenol content and that part of the phenolic compounds migrated from thick coat to water during boiling. Considering that several tannins are thermo-labile (hydrolysable tannins) and that small phenolic compounds can be produced by their partial degradation/hydrolysis, the migration of such compounds could explain the decreased phenolic content in thick coat and its appearance in the water. In fact, 76.5% of polyphenols in fresh coat co-precipitated with BSA (results not shown), suggesting a high proportion of tannins in this tissue (2.2% w/w). This concentration is consistent with the results described for Brazilian piñones (*A. angustifolia*) [17,47].

Although it was not statistically significant, an increase in phenolic content was found in boiled pulp with respect to control (1.14 vs. 0.81 μg GAeq per mg of dried pulp), suggesting that a minimal migration of phenols from coat to pulp could be occurring. Additionally, part of the coat phenols would be lost by heating. In this regard, phenol content (as μg GAeq per mg of dried whole coat) decreased 16% and 36% after boiling and baking, respectively, with respect to control (Fig. 5). The results shown in Fig. 5 were in agreement with the reductions in polyphenol levels and antioxidant activity observed in other vegetables after cooking [48,49]. Despite this fact, fresh piñón residues (28.7 μg GAeq per mg of dry coat) and heated residues (23.8 and 17.9 μg GAeq per mg of dried boiled and baked coat, respectively) were a rich source of phenols when compared with Argentine and Chilean berries: *Luma chequen* or chequén, *Ugni molinae* or murta, *Amomyrtus meli* or meli and *Luma apiculata* or arrayán (5.11, 9.24, 17.52 and 27.61 μg GAeq per mg of dried fruit, respectively) [46]. On the other hand, phenol content in the thin coat (around 25% of phenols in seed, Fig. 2) does not account for the high proportion of antioxidant power in this tissue (around 55% seed activity, Fig. 2). This may suggest the presence of other antioxidant compounds in piñón coat, but this is an interesting assumption that has not yet been studied.

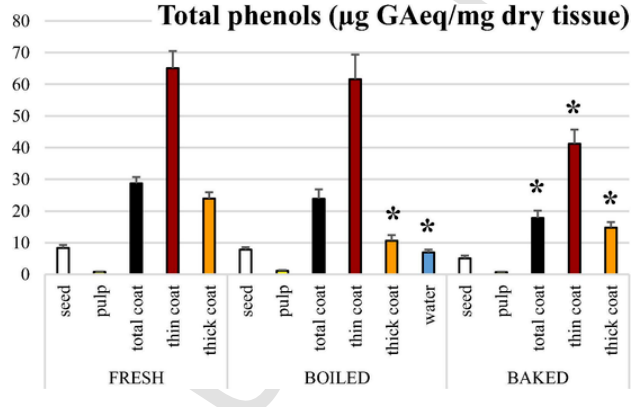

Fig. 5. Total phenolic content in extracts obtained from tissues of fresh, boiled and baked piñones, and water obtained in boiled treatment (μg GAeq/mg tissue). The values are expressed as mean ± SD of two independent experiments. *: Significant differences with control.

Similarly, the coat provided the greatest portion of seed flavonoids (Fig. 2). Again, thin coats contained more flavonoids than thick ones (Fig. 6). However, both coats lost flavonoids in a similar proportion (around 40%) after heat treatment, except for boiled piñones, which additionally lost 9% of flavonoids probably due to migration from thick coat to water (Fig. 6). As previously reported, fresh, boiled and baked piñón waste (whole coats) contained more flavonoids (123.8, 79.5 and 70.1 μg Qeq per mg of dried coat, respectively) than the Argentine and Chilean berries mentioned above: *Luma chequen* or chequén, *Ugni molinae* or murta, *Amomyrtus meli* or meli and *Luma apiculata* or arrayán (2.57, 5.54, 11.76 and 12.80 μg Qeq per mg of dried fruit, respectively) [46].

In short, the piñón coat contains more antioxidant power and higher phenolic and flavonoid content than other seed tissues, and some of those antioxidants could migrate from coat to water during boiling. Thus, all the waste obtained during cooking procedures was also rich in antioxidants.

The phenol and flavonoid content in fresh whole coat was analyzed by HPLC. Considerable peaks were observed in the analysis at 25 °C where gallic acid, with a 31.3% proportion, was the highest, while catechin represented 15.8% of the spectrogram area. Analysis at 40 °C revealed three substantial peaks, where quercetin, with a 67.6% proportion, was the main component. These results were consistent with those reported for Brazilian piñón coat, which contained mainly proanthocyanidins (chains of catechin, epicatechin, and their gallic acid esters), phenolic acids, flavonols and flavones [47,50,51]. To extend the chemical characterization of piñón tissues, including their nutritional valorization, their elemental composition was determined by EDXRF (Table 1). Piñón endosperm composition (97% w/w of edible pulp) was similar to that described for Brazilian piñones [52]. In this regard, one serving of piñones (14 pulps from fresh piñones) contains essential elements, such as P, K, Mn, Fe, Cu, and Zn, in significant nutritional concentrations, as well as Rb [53]. Furthermore, piñón coat (biowaste) contains higher concentrations of Ca, Cr, and Fe than pulp, and a sig-

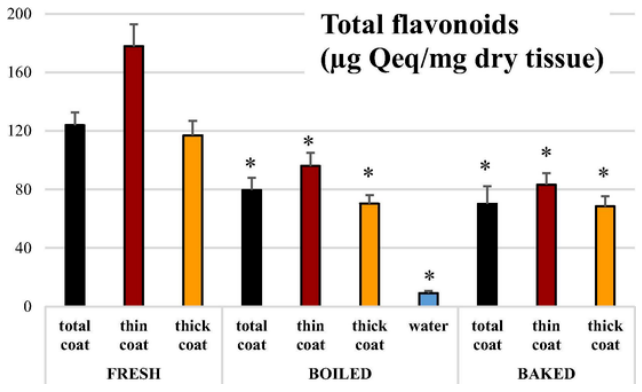

Fig. 6. Total flavonoid content in extracts obtained from tissues of fresh, boiled and baked piñones, and water obtained in boiled treatment (μg Qeq/mg tissue). The values are expressed as mean ± SD of two independent experiments. *: Significant differences with control.

Table 1
Elemental composition of tissue of piñones by EDXRF (μg/g).

	LLD	RDA	FRESH										BOILED						BAKED					
		(%)	embryo			endosperm			total coat			thin coat			thin coat			thick coat			thin coat			thick coat		
Na	152	2.7	4600	±	300	4100	±	300	4033	±	337	4800	±	600	4500	±	100	4000	±	300	6100	±	600	5000	±	400
Mg	38	1.8	555	±	40	490	±	33	540	±	200	4000	±	300	3500	±	100	3300	±	300	4000	±	200	4200	±	100
P	9	6.7	4000	±	100	2100	±	200	3000	±	200	4000	±	100	4500	±	100	3600	±	400	5100	±	200	3600	±	25
S	1.3		1415	±	60	895	±	55	820	±	96	700	±	100	600	±	100	1700	±	100	700	±	100	1400	±	25
Cl	8.6	1.6	3100	±	200	2800	±	200	2345	±	138	208	±	14	165	±	8	331	±	12	195	±	22	230	±	13
K	61	4.6	13,600	±	100	10,700	±	1100	3680	±	218	235	±	16	265	±	30	265	±	24	260	±	25	145	±	18
Ca	45	0.4	305	±	24	206	±	25	951	±	195	860	±	24	870	±	35	750	±	35	860	±	25	710	±	30
Cr	1.7	0	ND			ND			15	±	3	12	±	5	15	±	3	14	±	5	11	±	4	8	±	2
Mn	1.1	15.3	26	±	3	11	±	4	18	±	4	11	±	3	9	±	2	15	±	3	16	±	3	20	±	3
Fe	0.3	8.3	99	±	2	22	±	4	67	±	6	37	±	3	42	±	3	47	±	8	45	±	4	45	±	6
Cu	0.4	19.6	15	±	2	12	±	4	18	±	2	13	±	2	12	±	2	10	±	3	13	±	2	9	±	2
Zn	0.2	7.1	54	±	4	14	±	2	16	±	3	8	±	1	8	±	1	16	±	1	8	±	2	9	±	2
Br	0.3		6	±	1	4	±	1	4	±	1	ND			ND			ND			3	±	1	3	±	1
Rb	0.2	138	47	±	3	35	±	4	19	±	2	4	±	2	7	±	0	9	±	2	22	±	3	11	±	2
Sr	0.5		3	±	1	3	±	1	14	±	3	11	±	4	16	±	1	16	±	5	13	±	2	13	±	1

The values represent the concentration of elements as μg/g of dry tissue. LLD: Low Limit of Detection. % RDA: correspond to percentage of element provided by one serving of piñones (calculated with endosperm data, 97% w/w of edible part) with respect to recommended dietary allowances. One serving equals 25 g (14 pulps from fresh piñones) and has 16% moisture. ND: not detected.

nificant concentration of elements such as Cu, Zn, Mn, Fe, which participate in the endogenous antioxidant systems. Regarding Cr, speciation will be required to determine its toxicity. On the other hand, only Cl and K decreased significantly after heating. The results also suggest that piñón coat (fresh or cooked) could be also used for animal nutrition since it covers the mineral requirements for beef cattle [54].

In fact, the results shown in this study indicate that the piñón coat has high antioxidant power, high tannin content, quercetin, and gallic acid, as well as essential elements. Since these properties have nutritional, medical, and industrial relevance, the biological activity and corrosion-inhibiting properties of the piñón coat were studied.

3.3. Biological activities of piñón extracts

3.3.1. Early effects on cell viability

In order to rule out any toxic tissue effects, VERO cell viability was studied. It was found that aqueous extracts of embryo, endosperm and coat did not decrease cell viability at any of the concentrations used after 2 h of incubation. In addition, apoptotic phenotype was ruled out by propidium iodide exclusion, indicating plasma membrane integrity, and by intracellular green fluorescence, indicating fluorescein diacetate endocytosis followed by hydrolysis (viability) (Fig. S2 in the supplementary materials). Although the incubation time was relatively short for toxicological conclusions, these results are consistent with the historical medicinal and nutritional uses of these seeds by regional communities [55], mainly because the whole seed (pulp with coat) is processed to obtain foods and medicines. On the other hand, since the coat is usually discarded after processing, this is the first study that evaluates its effect on cell viability. That is why it is important to point out that these preliminary results did not show any cytotoxic effect of piñón waste (including water, result not shown).

3.3.2. Protective activity of piñón coat extract in chemotherapy

The cytostatic drug cyclophosphamide (CP) generates reactive oxygen species (ROS), which, among other effects, promote clinical and histological alterations in oral mucosa and periodontal tissues, as well as dysfunctions in the salivary glands and changes in saliva, in both humans and rats [56,57]. It is well known that the proper functioning of salivary glands is essential in maintaining oral health. Superoxide dismutase (SOD) and uric acid secreted by salivary glands are primarily responsible for the antioxidant potential of saliva and have displayed a positive correlation to oral health. Uric acid is a natural antioxidant that may assist in the removal of superoxide by preventing the degradation of SOD, the enzyme responsible for clearing superoxide from cells [58]. In addition, SOD and uric acid induce stress resistance and may extend lifespan [59,60]. Taking this into consideration, the present study was designed to examine the effect of piñón coat extract on SOD activity, uric acid content and lipoperoxidation (MDA) in the submandibular glands of CP-treated rats. Fig. 7 shows that the salivary glands of the control Wistar rats have a balance between antioxidant agents SOD and uric acid, and oxidative marker MAD content in a 1.1:1.9:1 ratio (units:mg:M per gram of fresh glandular tissue, respectively). In agreement with previous reports [56], a significant increase in SOD activity (235%) was observed in CP-treated animals with respect to control (100%). Since an increase in lipoperoxidation in CP-treated rats was not detected, we speculate that the SOD increase would be enough to maintain redox balance in the salivary glands. Indeed, in patients under cyclophosphamide-containing chemotherapy, salivary SOD activity increases as a defense mechanism against oxidative stress produced by the treatment [56]. A significant increment of endogenous antioxidants (605 and 436%, SOD and uric acid, respectively) was observed in the glands of rats treated with an exogenous antioxidant (piñón coat extract, AO group) with respect to control (100%); suggesting a greater free radical scavenging activity. Similar

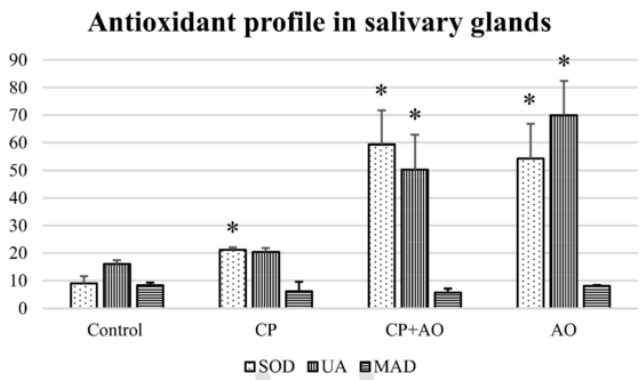

Fig. 7. Effect of fresh total coat extract (AO), cyclophosphamide (CP), and co-treatment (CP + AO) on superoxide dismutase activity (SOD; units/g), uric acid (UA; mg/g) and malondialdehyde (MDA; M/g) contents in submandibular glands of Wistar rats. Control: non-treated rats.*: Significant differences with control.

results were observed in rats under CP and antioxidant co-treatment (CP + AO group), which indicates the presence of enough endogenous antioxidants to maintain redox balance and protect against oxidative damage. In this regard, uric acid was found to provide significant protection against cyclophosphamide-induced bone marrow depression and micronucleated polychromatic erythrocytes in mice [61]. Additionally, a histological examination has shown that antioxidant vitamin E displayed salivary gland acinar protection in rats treated with CP [62]. Overall, present results indicate that piñón coat extract, which contains a wide variety of antioxidant compounds such as flavonoids and polyphenols, SOD cofactors such as Cu, Zn, Mn, Fe, and salivary antioxidant enhancers, could be an appropriate candidate for study as a chemoprotective agent against adverse effects produced on the oral cavity by oncological drugs such as cyclophosphamide.

3.4. Corrosion test

Stainless steel is widely used in various fields of modern industrial society because of its good corrosion and oxidation resistance. However, it is still susceptible to corrosion in some industrial processes, such as oil well acidizing, acid cleaning and acid descaling. The corrosion of metals and alloys, particularly in acidic media, is an important industrial problem. Based on environmental consequences, the use of non-toxic and natural products as corrosion inhibitors has become important because of their advantages: environmentally friendly and biodegradable, readily available and obtained at a relatively low cost. In this context, the corrosion-inhibiting property of piñón waste extract (coat) was evaluated. Our results show that after 5 h of exposure, piñón coat extract showed an inhibition efficiency that increased proportionally to extract concentration, up to 61% at a concentration of 400 μg dried coat per ml of HCl (Fig. 8, left). On the other hand, the corrosion rate decreased significantly by adding piñón coat extract from 100 μg/mL of HCl (Fig. 8, right). This inhibitory activity was similar to that described for solid biowaste from fresh banana leaves, sugarcane, and watermelon rind [12]. Several studies have attributed anti-corrosion capacity to the presence of molecules with polar functional groups with S, O, and N atoms, in alkaloids, polyphenols, tannins and flavonoids, which form a protective film that adheres with more resistance to the surface of the steel resulting in a barrier for the oxidizing agent such as HCl or others [11,12,63,64]. When piñón coat extract antioxidants were analyzed, a high content of tannins (2% w/w) and flavonoids (up to 124 μg Qeq/mg of dry weight) was found, with a high proportion of quercetin. This composition is consistent with others described as green corrosive inhibitors [12,63]. Overall results suggest that piñón waste (piñón coat) could be a suitable material to produce new green corrosion inhibitors.

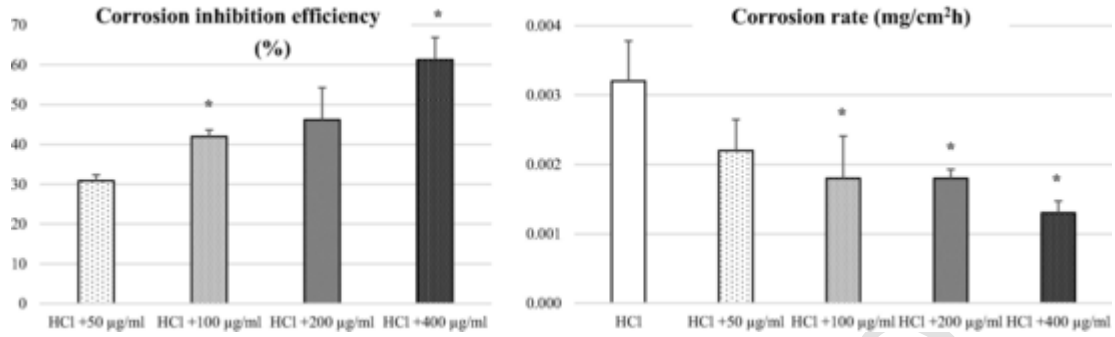

Fig. 8. Effect of fresh total coat extract at different concentrations (50, 100, 200 and 400 µg of dried coat per ml of incubation volume) on steel corrosion in HCl 1.0 M. left: corrosion inhibition efficiency (%), right: corrosion rate (mg/cm^{2}h).The values are expressed as mean ± SD of two independent experiments. *: Significant differences with control.

4. Conclusion

The results shown here indicate that the piñón coat, which is discarded as waste (biowaste), has very high antioxidant power. Hence, residues from the piñón food industry are rich in valuable phytochemicals such as tannins, quercetin and gallic acid, as well as significant nutritional elements and other not yet identified components, which contribute to their nutritive and free radical scavenging activity. Furthermore, these properties are responsible, at least in part, for inducing a significant increase in the endogenous antioxidant defenses of saliva, which may provide high protection against oxidative injury states. This activity may be particularly relevant to the development of adjunctive treatments to help prevent pain, odynodysphagia, dysgeusia, dehydration, malnutrition and systemic infections associated with treatment-related oral mucositis seen in over 500,000 cancer patients each year [65].

In addition, the antioxidant properties of piñón coat extract could be improved for use in the wide-ranging field of degradation of metallic materials in the future. Therefore, the results shown in the present study indicate that piñón coat extract would be a valuable and advantageous source of nutraceutical compounds, chemoprotective agents or green inhibitor of metal corrosion, due to its high antioxidant power, oligo-element content, non-toxic nature, low cost and environmental benefits.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of Interest statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.104175.

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