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Original Article

Oral supplementation of *Ilex paraguariensis* extract and eggshell membrane exhibit beneficial effects on the skin of mature male rats

[La suplementación oral de extracto de *Ilex paraguariensis* y membrana de cáscara de huevo muestran efectos beneficiosos sobre la piel de ratas macho maduras]

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Abstract

Context: *Ilex paraguariensis* (IP), a South American plant mostly consumed as an infusion, contains several phytocompounds with demonstrated positive effects on skin health and photoprotective properties. Eggshell membrane (ESM), a by-product of the poultry industry, also presents photoprotective properties. Both IP and ESM contain compounds that absorb ultraviolet radiation (UVR) and stop the mechanisms by which UVR damages the skin. The synergistic effects of these natural products on the reparation of skin structure and dermal collagen production deserves to be investigated.

Aims: To determine to what extent the oral supplementation of IP and ESM, administrated individually or in combination, promotes beneficial effects on the skin of mature Wistar rats.

Methods: The chemical composition of IP and ESM and the spectrophotometric solar protection factor (SPF) of the IP extract were characterized. *In vitro*, antioxidant and anti-inflammatory effects were assessed using erythrocytes. *In vivo* investigation consisted of macro and microscopic image analysis and diagnosis of the skin of the dorsal region of rats.

Results: The IP extract showed low SPF capacity combined with a protective effect against AAPH-induced erythrocyte haemolysis. IP extract inhibited erythrocyte haemolysis induced by both hypotonic solution and heat. The antioxidant and anti-inflammatory activity of the IP could be explained in terms of its membrane-stabilizing properties. Histologic analysis showed an increase in the collagen content of the skin of supplemented rats. A clear synergistic increase in collagen levels resulted when IP and ESM were administered together.

Conclusions: IP and ESM are beneficial for skin health, present synergistic effects, and could be used as ingredients in oral cosmetics.

Keywords: chemopreventive capacity; membrane-stabilizing property; skin structure.

Resumen

Contexto: El *Ilex paraguariensis* (IP), planta sudamericana consumida principalmente en infusión, contiene varios fitocompuestos con efectos positivos demostrados sobre la salud de la piel y propiedades fotoprotectoras. La membrana de cáscara de huevo (ESM), un subproducto de la industria avícola, también presenta propiedades fotoprotectoras. Tanto la PI como la ESM contienen compuestos que absorben la radiación ultravioleta (RUV) y detienen los mecanismos por los que la RUV daña la piel. Merece la pena investigar los efectos sinérgicos de estos productos naturales en la reparación de la estructura de la piel y la producción de colágeno dérmico.

Objetivos: Determinar en qué medida la suplementación oral de IP y ESM, administrados individualmente o en combinación, promueve efectos beneficiosos sobre la piel de ratas Wistar maduras.

Métodos: Se caracterizó la composición química de IP y ESM y el factor de protección solar (SPF) espectrofotométrico del extracto de IP. *In vitro*, se evaluaron los efectos antioxidantes y anti-inflamatorios utilizando eritrocitos. La investigación *in vivo* consistió en el análisis y diagnóstico por imagen macro y microscópica de la piel de la región dorsal de ratas.

Resultados: El extracto IP mostró una baja capacidad SPF combinada con un efecto protector contra la hemólisis eritrocitaria inducida por AAPH. El extracto IP inhibió la hemólisis eritrocitaria inducida tanto por solución hipotónica como por calor. La actividad antioxidante y anti-inflamatoria de IP podría explicarse por sus propiedades estabilizadoras de la membrana. El análisis histológico mostró un aumento del contenido de colágeno de la piel de las ratas suplementadas. Cuando se administraron conjuntamente IP y ESM se produjo un claro aumento sinérgico de los niveles de colágeno.

Conclusiones: IP y ESM son beneficiosos para la salud de la piel, presentan efectos sinérgicos y podrían utilizarse como ingredientes en cosméticos orales.

Palabras Clave: capacidad quimiopreventiva; propiedad estabilizadora de la membrana; estructura de la piel.

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INTRODUCTION

The skin is the largest organ of the human body, constitutes a protective barrier against environmental factors, and is involved in maintaining the internal homeostasis of the organism. Therefore, skin cells suffer from intrinsic chronological aging processes and extrinsic aging processes caused by external factors such as ultraviolet (UV) radiation, low humidity, and xenobiotic agents (Nur et al., 2021). Cosmetics are commonly used to disguise, minimize, and even prevent skin-degrading aging-related changes. However, these topical products do not effectively reduce damage in previously aged skin. That limitation constitutes the main driving force for researching and discovering new oral supplements with beneficial effects in reverting damage and maintaining skin health. Oral supplements based on natural antioxidants are of major interest. Natural antioxidants are commonly obtained from plant extracts. Natural antioxidants are presented as isolated or complex mixtures of phytocompounds. Natural antioxidants have the ability to boost the skin's physiological defenses (Michalak et al., 2021).

In this sense, some studies have been carried out looking for the chemopreventive capacity of plant extracts of the genus *Ilex* spp., the elucidation of their secondary metabolites, and the mechanisms of action (Ferreira Cuelho et al., 2018; Yi et al., 2019). These investigations showed that polyphenolic extract of *Ilex* spp. helps in maintaining proper skin structure by inducing hyaluronic acid and collagen synthesis. They also demonstrated the capacity of *Ilex* spp. to inhibit matrix metalloproteinases (MMP-2, MMP-9) and myeloperoxidase in rodent skins. In addition, *Ilex* spp. extracts have been reported to improve skin tissue structure by activating DNA repair mechanisms in cells damaged by external agents such as UV radiation (Barg et al., 2014). These observations suggest that the extract from *Ilex* leaves contains chemical compounds that can induce skin repair processes by enhancing cell renewal and collagen production.

Ilex spp. is a widespread genus native to South America. *Ilex paraguariensis* St. Hil. var. paraguariensis (*Aquifoliaceae*), also known as "yerba mate" (IP), is a plant of economic importance in the northeastern region of Argentina, where it is cultivated and consumed in the form of infusion (Ramirez et al., 2022). Despite the vast reported photoprotective activity of IP, there are no studies demonstrating its effects on aged skin of rats.

Eggshell membrane (ESM) is used in traditional Asian medicine to treat skin diseases (Ohto-Fujita et al., 2011). ESM has been reported to contain functional ingredients such as glycoproteins and glycosaminoglycans that could be used to produce nutraceuticals and skin care cosmetics. Pharmacological studies have shown that the topical application of ESM acts as a promoter of cellular activity and collagen synthesis. It also prevents skin aging and minimizes the damage caused by UV light and inflammation (Yoo et al., 2015). Oral administration of an ESM-based nutritional supplement in conjunction with botanical extracts was also found to alter some markers of inflammation in dogs with osteoarthritis (Muller et al., 2019).

This paper is devoted to explore the synergistic activity of oral supplementation of ESM and IP extract on the skin structure of healthy rats. The effects of ESM and IP supplements on overall skin morphology and collagen expression were investigated using histological techniques. *In vitro* anti-inflammatory and antioxidant activities of IP extract on rat blood cells (RBC) were also investigated.

MATERIAL AND METHODS

Plant material

Seven IP samples of 500 g each were withdrawn from a cultivated IP field located at the Department of San Ignacio in the Province of Misiones, Argentina. The exact geographical coordinates from where the samples were taken are: 1) 27°3'49.70"S–55°15'47.29"W; 2) 27°3'46.42"S–55°15'43.46"W; 3) 27°3'48.06"S–55°15'41.71"W; 4) 27°3'46.64"S–55°15'40.04"W; 5) 27°3'48.40"S–55°15'38.15"W; 6) 27°3'47.21"S–55°15'36.76"W and 7) 27°3'49.70"S–55°15'36.77"W. The plantation is set over clay-sandy soil, known as lateritic soil. This soil is rich in K and Fe oxides, and the local relief of the plantation permits rain waters to flow without stalling in the soil. This agro-ecological region has a subtropical climate without a dry season and is particularly conducive to the commercial exploitation of IP culture.

The collection of samples was carried out according to the following procedure. A pile of just-harvested, ripe green leaves was formed with the withdrawn samples. The pile was homogenized to obtain a representative sample of the entire pile. Then, aliquots of 500 g were withdrawn from the homogenized pile and submitted to the natural drying process at room temperature under open-air conditions for two weeks. Finally, the aliquots were grounded. The fraction trapped between the sieves of a rectangular aperture of 5 × 70 mm^2 and 1 × 20 mm^2 was recovered for subsequent studies. (Ramirez et al., 2022).

The ethanolic extract was prepared in agreement with the Argentina Pharmacopeia (2013). The ground-dried leaves (100 g) were extracted for 72 hours with ethanol (1000 mL). The extract was then macerated, filtered, and lyophilized. The dry extract (DE) was aliquoted and stored at −20°C until use. DE was re-suspended in a hydroalcoholic solution (10% of ethanol) before administration. The extraction yield using ethanol as solvent was 10%, w/w.

Total polyphenols and flavonoids measurement

Total phytocompounds were also determined by spectrophotometry. The analysis was performed according to the methodology described by Ramirez et al. (2011). The amount of total phenolic compounds was determined according to the Folin-Ciocalteu method. The determination was carried out following the method described above. For this, a calibration curve for gallic acid was prepared, and the result was expressed as µg GAE (gallic acid equivalents/100 g extract). The absorbance was read at 720 nm in a spectrophotometer after 90 minutes of incubation in the dark. The aluminium chloride colorimetric method was used to determine the flavonoid content of the *Ilex paraguariensis* extract. Absorption readings at 425 nm were taken after 30 minutes of incubation in the dark. Quercetin was used to prepare a standard curve, and the total flavonoid contents were expressed as milligram quercetin equivalent per gram of extract (mg QE/g).

Eggshell membrane (ESM)

Obtaining of natural eggshell membrane

ESM is obtained from eggshells (ES). 50000 tons of ES are produced annually as a by-product of the poultry industry World-Wide (Miksik et al., 2007). ES final disposal in sanitary landfills creates severe environmental problems and financial burdens. Alternative uses and valorization of this by-product are of obvious interest.

ES from Hy-line white eggs of *Gallus Domesticus* were provided by an egg producer located in Santa Fe city in Argentina. ESM was obtained from ES according to an efficient method developed in our laboratory. The process consisted of several stages. Firstly, ES were washed, sanitized, and ground. Secondly, the ground ES was suspended in an aqueous solution. Thirdly, the ESM part was separated from the calcium carbonate (CC) part by flotation. Flotation was induced by injecting an air-water mixture into the suspension. Fourthly, the ESM and the CC fractions were recovered and dried in a stove at 40-50°C under moderate vacuum. Optimal particle size after grinding and conditions of the flotation system are important adjusting factors necessary to ensure an effective separation. Fifthly, the separated ESM was washed with distilled water and treated with a 0.45 M sodium chloride solution at 4°C in a stirred vessel to remove non-collagen compounds. Finally, the precipitate was washed with a 0.2% NaOH solution to remove lipids, pigments, and unwanted elements. And ultimately washed with distilled water at pH = 7 and filtered (Stadelman, 2000). The obtained collagen fraction yield was 2.8%.

Characterization of ESM for bioassays

Moisture and ash content were determined using the AOAC Standard Method (2000). Official methods of analysis (Creigthon, 1989). Gaithersburg, MD, USA: Association of Official Analytical Chemists. The protein content was determined by multiplying the nitrogen content (obtained by the Kjeldahl method) by 6.25. The fat content was determined from the quantification of the ethereal extract. Ash was determined by incinerating the sample in a muffle furnace at 550°C for 12 h. Carbohydrate content was obtained by difference [100 − (moisture + protein + fat + ash)]. Microbiological characterization was carried out using a bacteriological kit to determine: total plate count, total coliforms, and *Salmonella*. The content and purity of collagen were determined by SDS polyacrylamide gel electrophoresis (Young, 1995).

Determination of the value of the Sun Protection Factor (SPF) in vitro

Extract preparation 1.0 g of *I. paraguariensis* was weighed, diluted to 100 mL with ethanol, followed by ultrasonication for 5 min, and filtered. Subsequently, a 5.0 mL aliquot was diluted to 50 mL with ethanol, and later a 5.0 mL aliquot was transferred to a volumetric flask, and the volume (25 mL) was completed with ethanol (Sayre et al., 1979). The absorption spectra of *I. paraguariensis* dry leaves in solution were obtained in the range of 290 to 320 nm (every 5 nm), comprising the entire UVB range, not being possible to calculate the UVA followed by the application of the Mansur equation (Mansur et al., 1986).

Animals and treatment

Adult male Wistar rats (8 months- weighing on average 246.21 ± 9.81 g) were fed a commercial diet (Ganave, Argentina) and water *ad libitum* throughout the experiment. All animals were maintained under standard laboratory conditions (T° at 21 ± 2°C, 12-12 h artificial light–dark cycle). Prior to use, the animals were acclimatised to these conditions for 15 days. All experiments described in this study were approved by the Ethics Committee (CEYSTE-CES 00632/2021, CCT-CONICET, Santa Fe and Res Nº 0010-CICUAL-

21, UNNE, Corrientes), and the experiments were carried out in according to the lnternational Guiding Principles for Biomedical Research lnvolving Animals (2012). For this purpose, rats were randomly assigned to one of four groups with 6 rats each. Control groups received by intragastric gavage 10% ethanol, and 90% water. Supplemented animals received 0.70 g/kg dry extract of *I. paraguariensis* (re-suspended in 10% ethanol, 90% water) daily by intragastric gavage (21 days) prior to sacrifice. The scientific literature (Barg et al., 2014) and data obtained in previous experiments were used as a reference to establish the dose. ESM-supplemented rats received 0.15 g/kg daily by feeding (21 days). The dose was established using the work of Sung and Kim (2021) as a reference, with some modifications. The fourth group received oral treatment with *I. paraguariensis* plus ESM (21 days at the previously mentioned doses). The animals were sacrificed under anaesthesia 24 h after the supplementation, following overnight fasting, and blood samples were collected. Blood samples from rats were centrifuged to obtain the plasma, which was stored at 25ºC until biochemical analysis. The skin from the back of the animals was removed and used for macroscopic and microscopic analysis.

Erythrocyte suspension

Rat erythrocytes constitute a valuable experimental model to investigate the anti-inflammatory and antioxidant potential of dietary components and botanical extracts (Shinde et al., 1999; Zou et al., 2001). Here this methodology was used to determine the ability of the extracts to inhibit haemolysis of red blood cells induced by heat and hypotonic solution. For this purpose, whole blood was collected from untreated rats under anesthesia. Blood was centrifuged in heparinized tubes at 3000 rpm for 10 minutes. The supernatant was removed, and the pellet containing red blood cells was washed three times with phosphate buffer saline pH 7.4. The volume of saline was measured and reconstituted as a 40% v/v suspension with isotonic buffer solution (pH 7.4 sodium phosphate buffer) (Shinde et al., 1999).

Heat-induced haemolysis

Portions of 5 mL of the isotonic buffer (NaCL, in sodium phosphate buffer, PH 7.4) containing 50-200 µg DE/mL of *I. paraguariensis* extract were put into two duplicate sets of centrifuge tubes. In the same amount, the vehicle was added to another tube as a control. Erythrocyte suspension of 30 µL was added to each tube and mixed by inversion. A group of the tubes was incubated at 54ºC for 20 min in a water bath. The other pair was maintained at 8ºC in an ice bath (Shinde et al., 1999). The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant (haemoglobin) was measured at 540 nm using Biotraza (model 752) spectrophotometer. Diclofenac 50-200 µg/mL, was used as a reference standard.

Hypotonic solution-induced haemolysis

Stock erythrocyte suspension 30 mL was mixed with 5 mL of the hypotonic solution containing the extract of *I. paraguariensis* at concentrations of 50-200 µg/mL, while the control sample was mixed with a drug-free solution. The experiments were carried out in duplicate pairs. The mixtures were incubated for 10 min at room temperature and centrifuged for 3 min at 1300 *g*, and the absorbance of the supernatant was measured at 540 nm. Diclofenac 50-200 µg/mL was used as a reference standard (Shinde et al., 1999).

The percentage inhibition or acceleration of haemolysis in both tests was calculated according to the equation [1].

$$\Delta H(\%) = 100 \cdot \frac{[1 - OD2 - OD1]}{OD3 - OD1} \quad [1]$$

Where ΔH(%), OD1, OD2 and OD3 are the acceleration or inhibition of haemolysis, the test sample unheated or in an isotonic solution, the test sample heated or in hypotonic solution, and the control sample heated or in hypotonic solution, respectively.

Antioxidant assays using the rat erythrocyte model

In order to induce free-radical chain oxidation in erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH (dissolved in PBS, final concentration 50 mM). To study the protective effects of *I. paraguariensis* extract against AAPH-induced haemolysis, an erythrocyte suspension (at 40 %) was pre-incubated with the extract (15 min), at final concentrations of 50-200 µg DE/mL, at 37ºC for 30 min, followed by incubation with and without AAPH, for 3 h (Zou et al., 2001). In all assays, a negative control (erythrocytes in PBS), as well as extract controls (erythrocytes in PBS with extract) were used. The reaction mixture was taken out at 3 h of incubation, diluted with saline, and centrifuged at 4000 rpm for 10 min to separate the erythrocytes. The percentage of haemolysis was determined by measuring the absorbance of the supernatant at 545 nm and compared with that of complete haemolysis. Gallic acid was used as a reference antioxidant compound (0.5-5 µg/mL). Three independent experiments were used for these calculations. The haemolysis percentage was calculated using the equation [2].

$$C(\%) = \left[\frac{A_{AAPH} - A_{sample}}{A_{AAPH}}\right] \cdot 100 \quad [2]$$

Where C%, A_{AAPH} and A_{sample} are the Clearance, the absorbance at 545 nm in tubes treated only with 50 mM of AAPH, and the absorbance at 545 nm in tubes treated with 50 mM of AAPH and extracts or standard, respectively.

Toxicity assay using the rat erythrocyte model

The haemolytic effect of the *I. paraguariensis* extract was evaluated spectrophotometrically at 550 nm according to the previously described methodology, with some modifications (Pagano and Faggio, 2015). Extract (300 to 500 µg DE/mL) or vehicle (hydroalcoholic solution, 0% haemolysis control) were put in contact with a 40% erythrocyte suspension. A 100% haemolysis control was carried out by exposing the 40% erythrocyte suspension to deionized water. The haemolysis percentage was calculated using the equation [3].

$$H\% = \left[\frac{A0 - As}{A0}\right] \cdot 100 \quad [3]$$

Where H%, A0 and As are the percentage of haemolysis, the absorbance of the control without extract and an equivalent amount of ethanol / water and the absorbance in the presence of the extract, respectively. In addition, a 100% haemolysis control was carried out, in which the saline solution was replaced by demineralized water in the presence of equivalent volumes of hydroalcoholic solution.

Serum biochemical analysis

The biochemical determinations were carried out using Commercial Wiener kits (Wiener laboratory, Rosario – Argentina). The values obtained were expressed in mg/dL or UI/L. Blood was collected in test tubes and was subsequently separated into aliquots and stored until the completion of serum analysis to determine the levels of glucose, total triglycerides, and total cholesterol as HDL cholesterol. Glutamic-oxalacetic transaminase (GOT) were also determined (Frankel, 1970; Trinder, 1969).

Histopathological study

At the end of the assay, following the sacrifice of the animals, the liver and kidneys were removed, stored in 10 % neutral formalin, and embedded in paraffin wax. The organs were sectioned and stained with hematoxylin and eosin, according to the methodology described by Culling (1965). For histological evaluation, the tissue sections were examined using an optical microscope.

Histological observations of skin tissue

The observation of epidermal tissue was performed by H & E staining. To evaluate collagen degradation, Masson's trichrome staining was performed according to conventional methodology. Epithelium thickness was evaluated using an objective 40× (4–40×) and expressed as the mean ± SD of the number of epidermal layers.

Statistical analysis

The results were expressed as the mean ± standard deviation from three separate experiments performed in triplicate. The t- Student test after the ANOVA procedure was applied to determine the difference among the means, which were considered statistically significant when $p<0.05$. Software Infostat (UNC-Argentina, version 2020).

RESULTS

Characterization of *Ilex paraguariensis* extract

The total polyphenolic content found in this work was 1811.9 mg GAE/100g (gallic acid equivalents, dry wet). The amounts of polyphenols found here are lowest than those reported by other authors (Mesquita et al., 2021). The flavonoids total of *I. paraguariensis* reported in this study was 103.35 mg/100g (quercitrin equivalents, dry wet), which is consistent with the value reported in a previous study made with a similar extraction method (Bojic et al., 2013). The chemical composition of *I. paraguariensis* depends on biological and environmental factors (light intensity, leaf age, and agronomic conditions) (Jacques et al., 2007). Furthermore, different conditions employed by *I. paraguariensis* producers, principally in temperature and time span of the diverse industrialization manners, directly contribute to these quantitative differences (Isolabella et al., 2010).

Characterization of ESM for bioassays

The proximate compositional analyses of ESM are shown in Table 1. The eggshell naturally has two internal membranes consisting of a mixture of proteins and glycoproteins, which are closely associated with the shell. In this study was detected that around 82% of the membrane structure is composed of proteins and collagen (35%). These results are in agreement with the literature (Miksık et al., 2007). Since the membrane is a rich protein material, it is a source of organic pollution if it is discarded into the environment. Therefore, the use of membranes as raw material is an example of this waste reduction and potential income generation. Table 1 also presents the results of the bacteriological analysis of ESM. All other determined values are orders of magnitude lower than the safe limits established or proposed for these products. Furthermore, the negative mycotoxin and microbiological assays constitute evidence that the agricultural and manufacturing setting for the production of ESM in our laboratory present no risk to

Table 1. Elemental content of eggshell membrane (ESM).

Chemical properties	**Total**
Moisture (%)	14.0
Protein (%)	82.0
Fats (%)	2.6
Carbohydrate (%)	0.7
Ash (%)	0.7
Collagen (in protein) (%)	35
Microbiological properties	
Mesophilic aerobic bacteria	100 CFU/g
Mold and yeast	98 CFU/g
Salmonella spp	Negative in 25 g
Escherichia coli	Negative in 1 g

Table 2. Absorbance of the extract of *Ilex paraguariensis* and their respective Sayre's constants for the calculation of the SPF.

λ	**Abs**	**EE x I**	**EE x I x Abs**
290	1.30	0.0150	0.195
295	1.01	0.0817	0.898
300	0.97	0.2874	2.586
305	0.61	0.3278	1.966
310	0.43	0.1864	0.745
315	0.33	0.0839	0.025
320	0.24	0.0180	0.036
		FPS	**6.447**

The absorption (Abs) data were obtained in the range of 290 to 320 every 5 nm, and 3 determinations were made at each point, followed by the application of the Mansur equation.

animal or human health related to contaminants from the soil or postharvest processing. Based in these results it is possible to suggest that the chemical composition of ESM presents no barrier to its use as a supplement for animal or human consumption.

Determination of the Sun Protection Factor (SPF) value *in vitro*

In this research, *I. paraguariensis* extract was evaluated by UV spectrophotometry applying Mansur mathematical equation (Mansur et al., 1986). This method is used to measure the SPF *in vitro*, which shows a good correlation in replacement of *in vivo* tests because it relates the absorbance of the extract with the erythematous effect of radiation and the intensity of light at wavelengths from 290 to 320 nm (Sayre et al., 1979). The results showed that (Table 2), the photoprotective capacity of the *Ilex paraguariensis* extract, *in vitro*, is low according to the classification of scientific literature (Mansur et al., 1986; FDA, 2012).

Haemolysis test

In this assay, haemolysis is induced with hypotonic saline solution or heat, and the ability of anti-inflammatory agents to prevent haemolysis by stabilizing the erythrocyte membrane is evaluated and correlated with a similar effect at the lysosomal membrane level. Previous *in vitro* and *in vivo* studies demonstrated the anti-inflammatory action of the *I. paraguariensis* extract (Schinella et al., 2014; Mesquita et al., 2021). The evidence presented here supports these findings, as the results indicate that the *I. paraguariensis* extract effectively prevents haemolysis of erythrocytes exposed to both hypotonic solution and heat (Table 3). The hydroalcoholic extract produced 46.84 % inhibition of haemolysis of rat's erythrocytes,

Table 3. Effect of *Ilex paraguariensis* on hypotonic saline solution or heat induced hemolysis in rat erythrocytes.

Treatment	Haemolysis inhibition (%)	
	Heat-induced	Hypotonic solution-induced
Control		
I. paraguariensis (µg/mL)		
50	13.23 ± 4.59	11.12 ± 4.71
100	31.05 ± 5.23*	26.15 ± 6.61
200	46.84 ± 6.75*	43.21 ± 5.26
Diclofenac (µg/mL)		
50	5.08 ± 1.2	10.25 ± 2.22
100	12.08 ± 1.73	23.21 ± 2.88
200	18.33 ± 2.01	45.44 ± 3.42

Values are mean ± S.D., $n = 6$; *$P < 0.05$ vs. reference standard, Student's *t*-test. Diclofenac was used as a reference standard. Control: consisted of the vehicle plus the stock red cell suspension (drug-free solution). Subsequently used to calculate the percentage inhibition or acceleration of haemolysis according to the equation described in the materials and methods section.

Table 4. Effect of *Ilex paraguariensis* on AAPH-induced haemolysis in rat erythrocytes.

Concentration (µg/mL)	Haemolysis inhibition (%)		
	Ilex paraguariensis	**Gallic acid**	**Control (AAPH 50 mM)**
50	24.15 ± 4.63*	24.17 ± 3.45*	-
100	37.52 ± 6.71*	33.44 ± 2.22*	
150	48.01 ± 5.11*	43.01 ± 4.28*	
200	52.66 ± 5.41*	48.66 ± 3.42*	

Antihemolytic effect of leaves extracts from *I. paraguariensis* at different concentrations against AAPH-induced hemolysis on rat erythrocytes. Values are mean ± S.D., *$P < 0.05$ vs. control. Student's *t*-test. Cells treated with AAPH (50 mM) alone were considered as control (100% haemolysis).

Table 5. Effect of *Ilex paraguariensis* on cytotoxicity assay of erythrocyte membranes.

Samples	Haemolysis (%)
Control (water)	100 ± 7.65
I. paraguariensis 300 µg	3.65 ± 0.81*
I. paraguariensis 400 µg	4.36 ± 0.91*
I. paraguariensis 500 µg	3.41 ± 1.02*

Hemolysis rates induced on rat red blood cells by various doses of *I. paraguariensis* leaves extract. Cells treated with demineralized water alone were considered as control (100% haemolysis). Values are mean ± S.D. Student's *t*-test. (*p<0.05).

in vitro, as compared to 45.44 % produced by diclofenac.

Oxidative haemolysis inhibition assay

Table 4 shows the anti-haemolytic effects of *I. paraguariensis* extract (50-200 µg/mL) on rat erythrocytes exposed to the water-soluble radical initiator AAPH. Erythrocytes incubated at 37°C in PBS were stable, with little haemolysis observed within 3 h (6.1 ± 0.8%). When the cells were incubated with extracts of *I. paraguariensis* or gallic acid alone, haemolysis was maintained at a background level similar to that in the control samples (AAPH untreated samples). *I. paraguariensis* extract significantly protected the erythrocyte membrane from haemolysis induced by AAPH in a concentration-dependent manner after 3 h of incubation (pretreated samples).

Toxicity assays

In Table 5, it can be seen that up to the maximum concentration evaluated, the *I. paraguariensis* extract did not affect red blood cells, showing levels of haemolysis of less than 5%. Previous research suggested a toxicity index that classifies as non-toxic those samples with haemolysis percentages lower than 9% (Pagano et al., 2015). In this study, the haemolysis percentages ranged from 4.36% to 3.41%, demonstrating that *Ilex paraguariensis* extract is not cytotoxic in the

Table 6. Variations in body weight after 21 days of supplementation.

Groups	Body weight (g)
Control	262.41 ± 10.72
Ilex paraguariensis	255.66 ± 12.56
ESM	269.26 ± 9.21
Ilex plus ESM	257.05 ± 11.05

Data are expressed as the mean ± standard deviation. (n = 6 per group). I + ESM = rats supplemented with *Ilex paraguariensis* and ESM. No statistical difference was detected between the treated groups compared to the control.

Table 7. Effect of the experimental supplementation on serum biochemical parameters after 21 days of administration.

Determinations	Control	ESM	IP	ESM + IP
Glucose (mg/dL)	123.04 ± 7.31	126.70 ± 6.72	128.01 ± 8.36	122.87 ± 6.67
Total triglycerides (mg/dL)	97.12 ± 9.51	89.51 ± 11.02	85.21 ± 13.06	90.66 ± 12.85
Total cholesterol (mg/dL)	60.21 ± 6.65	58.50 ± 7.25	50.04 ± 9.31	49.75 ± 6.49*
Cholesterol-HDL (mg/dL)	48.05 ± 4.83	45.03 ± 3.11	39.11 ± 6.65	35.51 ± 4.16*
GOT (UI/L	80.22 ± 15.02	78.32 ± 14.08	76.05 ± 16.12	78.12 ± 13.85
Alkaline phosphatase (UI/L)	360.00 ± 49.21	352.01 ± 51.73	346.02 ± 49.78	369.75 ± 55.42
Creatinine (mg/dL)	0.37 ± 0.42	0.35 ± 0. 51	0.38 ± 0.33	0.40 ± 0.46
Calcium (mg/dL)	8.6 ± 0.61	8.95 ± 0.79	8.21 ± 0.64	8.07 ± 0.83

Values are expressed as mean and standard deviation. Differences between means were statistically tested by a one-way ANOVA test, followed by Tukey's post hoc test. *P<0.05 *vs.* control. IP + ESM = rats supplemented with *Ilex paraguariensis* extract and ESM.

range of concentrations tested. In control of 0% haemolysis, the extracts were replaced by equivalent volumes of hydroalcoholic solution. In control of 100% haemolysis, the saline solution was replaced by demineralized water in the presence of equivalent volumes of hydroalcoholic solution.

Animal studies

No significant changes in food consumption were observed among the treatment groups and control group. Animals drinking water control, ESM, or *I. paraguariensis* supplemented showed no mortality during the trial. The rats in overall appeared healthy and alert with no signs of sickness or lethargy. Further, no signs of tremors or diarrhea were observed after treatment. No abnormality was observed in the gross evaluations of skin, fur, eyes, locomotor activity, and behavior pattern. Stress and inflammation can also cause a loss of body weight (BW). There were no differences in terminal body weight between the treatment groups and control group. Because the BW gain pattern in the supplemented groups did not differ significantly from the control group it can be inferred that supplementation has no tendency to produce drastic tissue destruction nor does it seem to interfere with absorption of the nutrients. The BW and relative organ weight changes are shown in Table 6. There was no change in relative organ weights suggestive of any negative or inflammatory effect of supplementation (data not shown). The sickness was also not evident from biochemistry variables such as the aspartyl aminotransferase and alanine aminotransferase values, which are linked to hepatic dysfunction (Table 7). Similarly, there was no elevation in the levels of blood calcium and creatinine that would indicate kidney dysfunction. The results also showed that the blood HDL-cholesterol levels were reduced in rats supplemented, although the mechanism for its decrease is not understood. Alkaline phosphatase is also a good indicator of liver damage and bone disorders. In this study, no significant changes in alkaline phosphatase levels were detected in the treated animals compared to the control. Considering the microscopic data and the biochemical findings, it can be suggested that ESM or *I. paraguariensis* are safe natural products.

Histopathological assessment

The histological study evidenced a control group (Fig. 1B) with an epithelium thickness of 5.5 ± 0.3 cells. The *I. paraguariensis*-treated group (Fig. 2B) showed a hypertrophic or hyperplasic epithelium thickness of 6.5 ± 0.6 cells. The ESM-treated samples (Fig. 3D) showed a reduced epithelium thickness of 5.0 ± 0.4 cells. However, The *I. paraguariensis* plus ESM-treated group (Fig. 4C) evidenced a lesser epithelium thickness of 4.3 ± 0.3 cells.

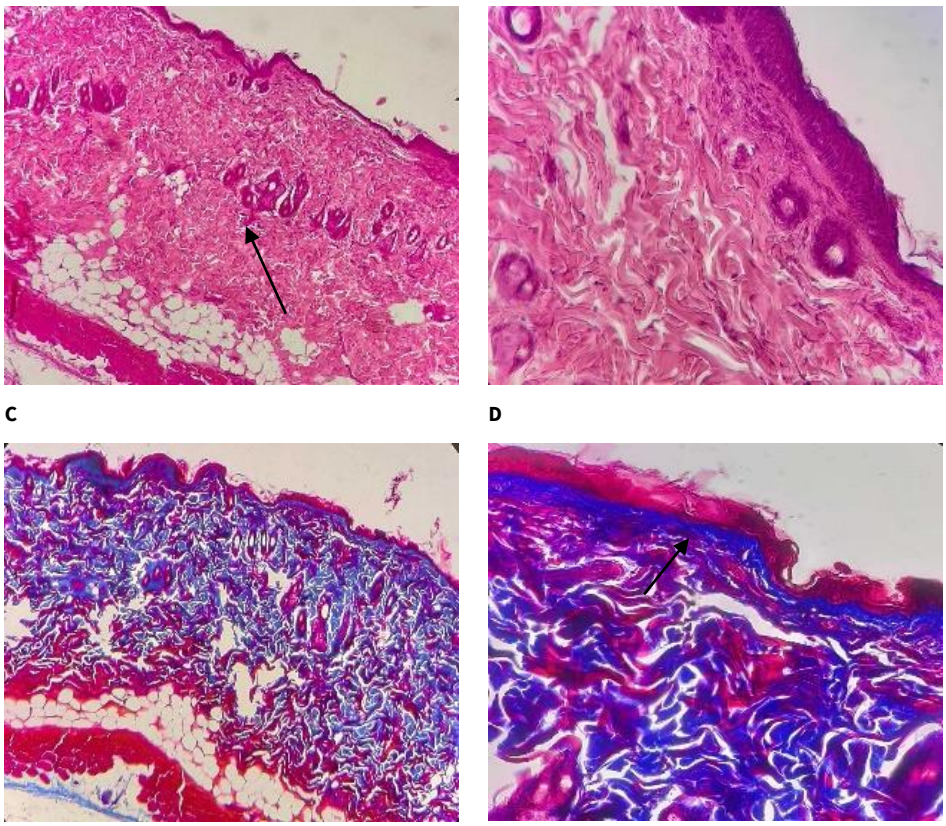

Figure 1. Histological sections of the skin of control rats.

(A) Photomicrograph of a skin slice, stained with H&E, showing a dense and irregular deposit of slightly acidophilic collagen fibers in the dermis (black arrow), with a 10× objective. **(B)** Photomicrograph of a skin slice, stained with H&E, showing an epidermis with 5-6 layers of keratinocytes, and a papillary dermis of lax connective tissue with a predominance of cells, with a 40× objective. **(C)** Photomicrograph of a skin section, stained with Masson's Trichrome, showing blue collagen fibers in the dermis, with a 10× objective. **(D)** Photomicrograph of a skin section, stained with Masson's trichrome, showing a papillary dermis denser with collagen fibers (black arrow), with a 40×objective.

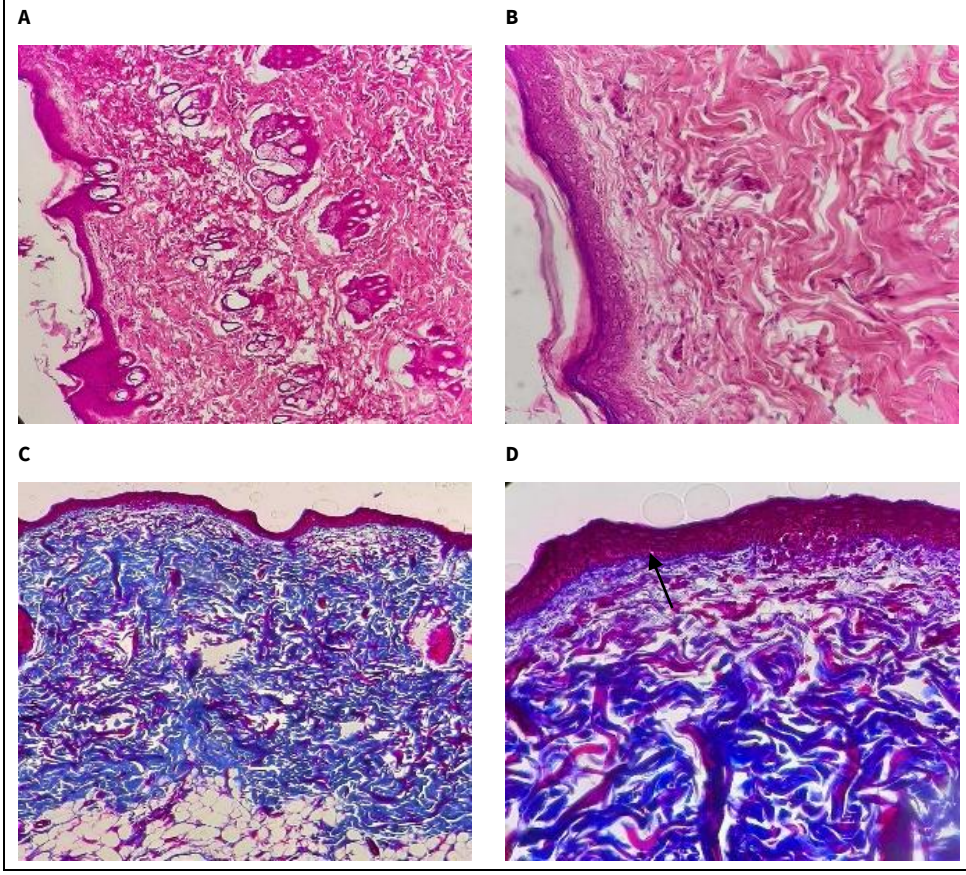

Figure 2. Effect of oral administration of *Ilex paraguariensis* extract on the skin of mature rats.

(A) Photomicrograph of a skin slice, stained with H&E, showing a thickened epidermis, and an irregular deposit of collagen fibers in the dermis, with a 10× objective. **(B)** Photomicrograph of a skin slice, stained with H&E, showing an epidermis with 6-7 layers of keratinocytes, and a papillary dermis of loose connective tissue, with a 40× objective. **(C)** Photomicrograph of a skin section, stained with Masson's Trichrome, showing blue collagen fibers in the dermis, more dispersed, with a 10× objective. **(D)** Photomicrograph of a skin section, stained with Masson's trichrome, showing a papillary dermis less dense with collagen fibers (black arrow), with a 40× objective.

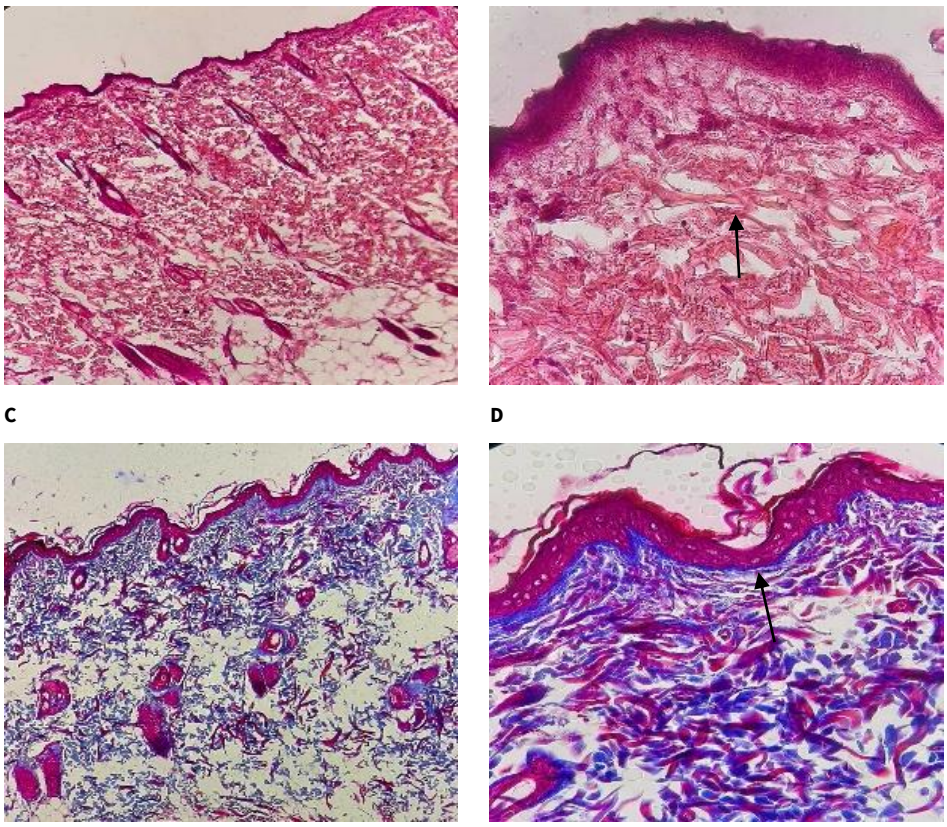

Figure 3. Effect of oral administration of ESM extract on the skin of mature rats.

(A) Photomicrograph of a skin slice, stained with H&E, showing an unpatterned dense connective tissue dermis with thin, widely dispersed collagen fibers (black arrow), with a 10× objective. **(B)** Photomicrograph of a skin slice, stained with H&E, showing a papillary dermis of loose connective tissue and slightly acid-filtered collagen fibers (black arrow), with a 40× objective. **(C)** Photomicrograph of a skin section, stained with Masson's Trichrome, showing a well-demarcated epidermis, and a connective tissue dermis with scattered collagen fibers, with abundant extracellular matrix, with a 10× objective. **(D)** Photomicrograph of a skin slice, stained with Masson's Trichrome, showing an epidermis with 5 layers of keratinocytes, a dense layer of its own (black arrow), a papillary dermis of loose connective tissue, and a 40× objective.

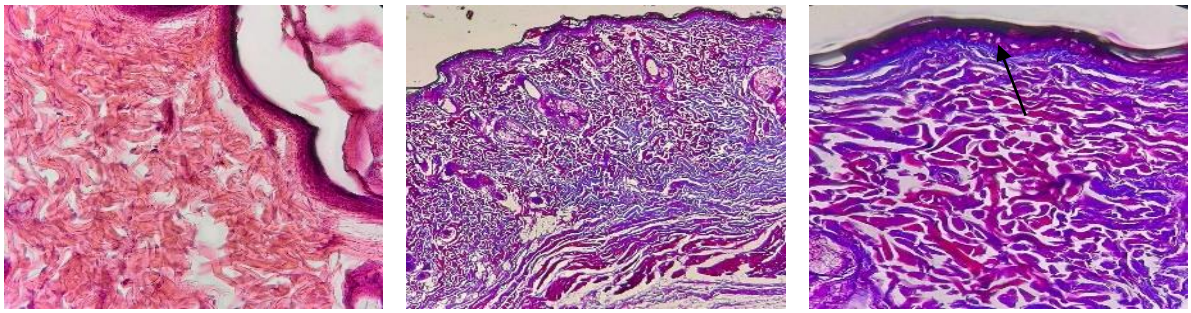

Figure 4. Effect of oral administration of *I. paraguariensis* plus ESM extract on the skin of mature rats.

(A) Photomicrograph of a skin slice, stained with H&E, showing a thin epidermis and irregularly shaped acidophilic collagen fibers in the dermis, with a 40× objective. **(B)** Photomicrograph of a skin slice, stained with Masson's Trichrome, showing a dense dermis of collagen fibers, with a little extracellular matrix, with a 10× objective. **(C)** Photomicrograph of a skin section, stained with Masson's Trichrome, showing an epidermis with 4-5 layers of keratinocytes, a blue layer of its own of connective tissue (black arrow), below the dermis, with a 40× objective.

Histological assessment of animals treated with *I. paraguariensis* extract*:* The epithelium of animals supplemented with *I. paraguariensis* showed a slight increment of its thickness and tissue cellularity. No effects were observed on the organization of cell layers and base membrane. The dermal-epidermal junction remained within normal characteristics. A greater development of the papillary dermis was observed when compared to the control group. The adipose tissue of the hypodermis also preserved its thickness in relation to the control group (Figs. 1 and 2).

Histological assessment of ESM-treated animals*:* The epidermis was observed to be preserved. The papillary dermis increased its density of collagen fibers. And the reticular dermis presented higher dispersion of collagen fibers and a greater amount of the ground substance (Fig. 3).

Histological assessment of ESM-treated animals and treated with *I. paraguariensis* extract*:* The most remarkable feature observed in the animals that received ESM and fed with *I. paraguariensis* after 21 days' post-supplementation was a reduction in the

thickness of the epidermis (Fig. 4). The base membrane retained its normal characteristics. And the dermal-epidermal border was clearly visible. The most important change in the dermis was the widening of the papillary dermis. This probably occurred due to the increment in the size of blood capillaries and an accumulation of intercellular substances. The reticular dermis showed an increase in cross-linked collagen fibers with little amorphous substance. Adipose tissue was observed in the hypodermis. Collagen fibers deposited below the hypodermis were also observed. Atrophy of hair follicles and other epidermal appendages was not observed. These results suggest that the treatment produces an increase in collagen fibers, contributes to changing the nutritional medium that feeds the epidermis, and leads to various processes that lead to tissue regeneration.

DISCUSSION

In vitro and *in vivo* studies reported that oral or topical administration of *I. paraguariensis* extracts has photoprotective properties. However, so far, no studies have determined the SPF of *I. paraguariensis, in vitro*. For this reason, the present study aimed to determine the *in vitro* photoprotective activity of the *I. paraguariensis* extract by a spectrophotometric method in the UVB region (290-320 nm). The SPF value obtained was 6.4 ± 0.35, which is an acceptable reference value when compared with other studies carried out with natural extracts and under the same conditions (Costa et al., 2015). However, according to the classification of international organizations, the *I. paraguariensis* leaf extract has a low level of photoprotection (FDA, 2012). In this extract, we also quantified the polyphenolic compounds and flavonoids, considered powerful antioxidants able to absorb UV rays. The radiation that can be absorbed by polyphenols includes the entire UVB spectrum and part of the UVC and UVA spectra. This indicates that the low SPF values detected in this study could be related to the method used to determine the photoprotective activity because this method restricts the effectiveness of the extracts to compounds that absorb radiation only at the UVB range (i.e., 290 to 320 nm), and does not include the entire UV range (i.e., from 200 to 400 nm) (Mansur et al., 1986; Sayre et al., 1979). Furthermore, we evaluated the antioxidant and anti-inflammatory activity and potential *in vitro* toxicity of the *I. paraguariensis* extract using the rat erythrocytes membrane stability model (Table 3). The results obtained indicate that *I. paraguariensis* extract by itself did not cause haemolysis to rat red blood cells (RBCs) and showed moderate protection against AAPH-induced haemolysis. On the basis of previous research, it is possible to suggest that the effect of *I. paraguariensis* in protecting red blood cells may be due to the direct chemical neutralisation of AAPH-generated peroxyl radicals or their cellular uptake. The presence of polyphenols, and flavonoids such as rutin, quercetin, and kaempherol, could be at least partly responsible for the observed antioxidant activity (Filip et al., 2000). *Ilex paraguariensis* extract also inhibited heat- as well as hypotonic solution-induced haemolysis of erythrocytes *in vitro*. This finding suggests that the membrane-stabilising activity of *Ilex paraguariensis* may be playing a significant role in its anti-inflammatory activity. Previous studies have shown that compounds with membrane-stabilising properties have the ability to interfere in the early phase of inflammatory reactions, i.e., in preventing the release of phospholipases that trigger the formation of inflammatory mediators (Aitdafoun et al., 1996). Therefore, it is possible to suggest that the anti-inflammatory activity of *I. paraguariensis* extract may be related to the inhibition of the early phase of inflammatory events (Schinella et al., 2014). Similarly, cytotoxicity tests showed that polyphenolic compounds contained in the *I. paraguariensis* extracts do not induce haemolysis, therefore, do not cause a lytic effect on rat RBCs cells in the tested range of concentrations (Table 5). These results suggest that *I. paraguariensis* extract may positively affect living organisms by protecting red blood cells against oxidation. On the other hand, histological analysis of the skin of rats supplemented with Ilex extract (21 days) showed an increase in the density of collagen fibres in the reticular dermis under basal conditions (Fig. 2). Similar results have been observed by Yi et al. (2019) in irradiated animals treated topically with *Ilex kudingcha*. These authors reported a reduction in tumor necrosis factor-alpha (TNFα), and an increase in the levels of type 1 collagen, hydroxyproline, and hyaluronic acid, demonstrating the photoprotective effect of *Ilex* spp. extract. Animals supplemented with ESM showed a moderate increase in collagen fiber density in the papillary dermis (Fig. 3), suggesting that ESM is absorbed and utilised in the rat body (Matsuoka et al., 2019). However, animals supplemented with *I. paraguariensis* extract plus ESM showed a more developed papillary dermis compared to the control (Fig. 4). In the reticular dermis, a large accumulation of cross-linked collagen fibres with little amorphous substance was detected. In the hypodermis, adipose tissue and collagen fibres deposited beneath it could be observed. This finding suggests that supplementation with ESM plus *I. paraguariensis* extract strengthens the dermis by inducing the biosynthesis of dermal matrix macromolecules (Yoo et al., 2015). As shown in Table 1, ESM is mainly composed of fibrous proteins, which are resistant to gastric proteases. It has been reported that about 40% of ESM is digested and absorbed, while the remaining 60% is excreted in the faeces, suggesting that these com-

pounds may exert a beneficial effect *in vivo* (Matsuoka et al., 2019). On the other hand, enteric flora is known to metabolise non-digestible dietary substrates or residues, and polyphenols from Ilex sp. play an important role in the structure of colonic microbiota (Xie et al., 2017). Therefore, it is possible to suggest that phytonutrients from *I. paraguariensis* favour the absorption of ESM constituents in the intestinal tract and, consequently, their bioavailability, resulting in good nutritional status and reflected in good skin health.

CONCLUSION

In this study, it has been shown that polyphenols from *Ilex paraguariensis* show a positive effect on skin structure when administered orally for 21 days. Oral ESM supplementation also exhibits similar activity. However, when both extracts were administered together, a potentiating effect on the parameters evaluated was observed. In the next stage, it is intended to evaluate the influence of supplementation on skin elasticity and skin tonus (glycosaminoglycans and collagen I, III, and IV) and the biochemical mechanism of action.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Ramirez MR	Rintoul I	Leiva M	Molli S	Yori JC
Concepts or ideas	x	x			x
Design	x	x			x
Definition of intellectual content	x	x			x
Literature search	x		x	x	x
Experimental studies	x		x	x	x
Data acquisition	x		x	x	x
Data analysis	x		x		x
Statistical analysis	x				x
Manuscript preparation	x				x
Manuscript editing	x	x			x
Manuscript review	x	x	x	x	x

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