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ORIGINAL PAPER

Anti-wrinkle and anti-whitening effects of jucá (*Libidibia ferrea* Mart.) extracts

Tatiana do Nascimento Pedrosa[1,2] · Aline Oliveira Barros[1] · Jéssica Rodrigues Nogueira[1] · Andréa Costa Fruet[2] · Isis Costa Rodrigues[1] · Danielle Queiroz Calcagno[3,4] · Marilia de Arruda Cardoso Smith[4] · Tatiane Pereira de Souza[1] · Silvia Berlanga de Moraes Barros[2] · Marne Carvalho de Vasconcellos[1] · Felipe Moura Araújo da Silva[5] · Hector Henrique Ferreira Koolen[5] · Silvya Stuchi Maria-Engler[2] · Emerson Silva Lima[1]

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Abstract Skin aging is a natural process of the human body that may be accelerated due to extrinsic causes. *Libidibia ferrea*, popularly known as *jucá*, is a small tree, which possesses an abundant phenolic composition with potential antioxidant and enzymatic inhibition activities. Thus, this work aimed to investigate the anti-wrinkle and anti-whitening potentials of *jucá* trunk bark (LFB) and pod (LFP) extracts. A comprehensive analysis of LFB and LFP phenolic composition was accomplished by means of liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Effects on skin degradation were assessed by inhibitory enzymatic activity against elastase, hyaluronidase and collagenase through colorimetric assays. Cellular viability in B16F10 and primary fibroblasts were determined by Trypan Blue exclusion assay. Anti-melanogenic effects on B16F10 cells were evaluated using cellular tyrosinase, melanin content, western blot, and RT-qPCR analyses. Inhibition of matrix metalloproteinase-2 and metalloproteinase-9 (MMP-2 and MMP-9) was determined by gelatin zymography and western blot methodologies. LC–MS/MS analyses of LFB and LFP extracts allowed the characterization of 18 compounds, among them, flavonoids, phenolic acids, and secoridoids. Additionally the pod and trunk bark compositions were compared. Hyaluronidase inhibitory activity for both extracts, LFB ($IC_{50} = 8.5 \pm 0.8$ μg/mL) and LFP ($IC_{50} = 16 \pm 0.5$ μg/mL), was stronger than standard rutin ($IC_{50} = 27.6 \pm 0.06$). Pro-MMP-2 was significantly inhibited by both extracts. LFB and LFP decreased the melanin content in B16F10 due to tyrosinase inhibitory activity. *L. ferrea* extracts has high potential as a cosmetic ingredient due to its anti-wrinkle and depigmentant effects.

Keywords *Libidibia ferrea* · Skin aging · Melanogenesis · Tyrosinase · Polyphenols

Introduction

Skin aging is a natural process characterized by progressive structural and physiological changes in the skin. The main consequences are the atrophy of the skin with loss of elasticity and a slowed metabolic activity (chronologic skin aging) under genetic and hormonal factors. Striking changes occur in the dermis, such as massive deposition of abnormal elastic fibers, collagen degeneration and loss of hyaluronic acid [11].

Extrinsic factors can accelerate skin aging by increasing the production of reactive oxygen species (ROS) and promoting exacerbated physical changes in the skin such as changes in the connective tissue, formation of lipid peroxide products, alteration in cell enzyme contents [31, 34].

Excessive exposure to sunlight, environmental pollution and modern lifestyle habits, such as smoking, accelerate

✉ Emerson Silva Lima
eslima@ufam.edu.br

1 Faculty of Pharmaceutical Sciences, Federal University of Amazonas (UFAM), General Rodrigo Otávio Campos de Jordão Avenue, 6200, Coroado, Manaus, AM CEP: 69077-000, Brazil

2 School of Pharmaceutical Sciences, University of São Paulo (USP), São Paulo, Brazil

3 Research Group in Oncology, Federal University of Pará (UFPA), Belém, Brazil

4 Department of Morphology and Genetic, Federal University of São Paulo, São Paulo, Brazil

5 Dempster Mass Spectrometry Group, Amazonas State University (UEA), Manaus, Brazil

the production of enzymes in the skin that degrade the main components of the extracellular matrix of dermis, such as elastin and collagen [29]. Moreover, ROS also exacerbate enzyme activity from tyrosinase and elastase. Tyrosinase is the principal enzyme in the melanogenesis process, participating in two stages of melanin synthesis, which has an important photoprotective role; however, the excessive production of melanin results in undesired hyperpigmentation [31].

One compelling perspective of aging is that it is nothing more than an imbalance between oxidative damage and the antioxidant defense system; consequently several studies have been seeking to develop effective antioxidants. One advantage in the use of natural products is the safety and efficacy compared to synthetic antioxidants [34]. There is considerable interest in searching for new cosmetic ingredients derived from natural sources that can be used as anti-aging agents [29]. Plant extracts can protect the skin through different mechanisms, such as the removal of ROS decreasing their activity, rust inhibition, photoprotection, enzyme inhibition and prevention of skin aging. Among natural compounds, triterpenes and specially polyphenols present this potential [16].

Libidibia ferrea Mart. is a native Brazilian tree belonging to the Leguminosae-Caesalpininoideae family widely distributed in the north and northeast regions of Brazil, being popularly known as pau-ferro or jucá [12]. Studies indicated that *L. ferrea* extracts have antiulcerogenic [2], anti-inflammatory [9], cancer chemopreventive [27, 28], antioxidant and hepatoprotective activities [4]. Previous phytochemical studies revealed the presence of coumarins, flavonoids, saponins, steroids and tanins in *L. ferrea* [12], all potential antioxidant and enzyme inhibitors [34]. Thus, considering its popular use and the phytochemical properties of this plant and the lack of studies about the anti-aging activity of this species, the aim of this study was to investigate the anti-aging potential of *L. ferrea* extracts of bark and pod and its chemical composition.

Materials and methods

Plant material

The bark and pods of *L. ferrea* were supplied by the National Institute for Amazonian Research (INPA), and identified in the Herbarium of the same institution. A voucher specimen was deposited under (number 802222). The material was dried over ambient temperature (ca. 25 °C) during 7 days, and then powdered. Extractions were performed by static maceration of the plant material (48 h) with ethanol:water (1:1, 75 g/L of solvent) at 25 °C. The solvent was evaporated by spray drying yielding trunk bark (LFB) and pod (LFP) extracts (5.47 and 6.37 g, respectively). The dried extracts were stored at −20 °C until the experiments.

Characterization of *L. ferrea* compounds by LC–MS/MS

All chemical analyses were performed in a LC–MS/MS system consisting of a Surveyor liquid chromatography system (Thermo, USA) coupled to a LCQ *fleet* ion trap mass spectrometer equipped with an electrospray ionization (ESI) source. To achieve the chromatographic separation a Phenomenex (Torrance, CA, USA) Luna-C18 column was used (5.0 µm, 4.6 mm i.d. × 150 mm) using a binary mobile phase. Solvent A was water and solvent B was acetonitrile (Tedia, Mexico city, Mexico). The gradient elution at 35 °C was as follows: 0–2 min, 10 % B; 2–19 min, 10–70 % (v/v) B; 19–21 min, 70–100 % (v/v) B; and 21–24 min, 100 % B at a flow rate of 0.8 mL/min. The autosampler temperature was maintained at 15 °C and the injection volume was 10 µL. The ESI source parameters were previously optimized, in brief: spray voltage, 5 kV; sheath gas, 10 arb; auxiliary gas, 5 arb; sweep gas, 0 arb; capillary temp, 200 °C; capillary voltage, 40 V; tube lens, 120 V; microscans, 4; max, injection time (ms), 100.000. Helium (Praxair, Danbury, CT, USA) was used as collision gas, where the collision energies ranged from 15 to 35 %. Tentative identifications were performed by manual interpretation of MS/MS spectral data and comparison with previously published data [5, 6].

Elastase inhibition assay

This test was performed according to the literature [19] in 60-mM Tris–HCL (Sigma-Aldrich, St. Louis, MO, USA, at pH 7.5) and 5 %-DMSO buffer (Lichrosolv, Merk-Milipore, USA). Briefly, extracts of the bark and pods of *Libidibia ferrea*, standard and blank were incubated with porcine pancreatic elastase (PE-EC3.4.21.36, Sigma-Aldrich, St. Louis, MO, USA), with a final volume of the reaction at 0.03 units/mL), and buffer for 5 min in an oven at 37 °C using 96-well plates. After this, 25 µL of substrate, *N*-succinyl-Ala–Ala-Ala-*p*-nitroanilide (AAAPVN, Sigma-Aldrich, St. Louis, MO, USA) at 10 mM in Tricine Buffer (pH 7.5 with 400 mM NaCl and 10 mM $CaCl_2$) was added. The first measurement of the absorbance was performed holding the wave length at 405 nm. After this first reading, the plate was again incubated for 1 h in an oven at 37 °C. The second measurement was carried immediately after the end of the incubation period in the same conditions. For the all absorbance measurements, a DX800 spectrophotometer was used (Beckman Coulter, Pasadena, CA, USA).

Collagenase inhibition assay

The test was based on the spectrophotometric method described in the literature [36] with minor modifications. The test was performed in a 50-mM Tricine buffer (pH 7.5 with 400 mM NaCl and 10 mM $CaCl_2$). *Clostridium histolyticum* collagenase (ChC-EC.3.4.23.3, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in buffer for use in an initial concentration of 0.8 units/mL according to the data provider. The synthetic substrate *N*-[3-(2-furyl) acriloyl-Leu-Gly-Pro-Ala (FALGPA, Sigma-Aldrich, USA) was dissolved in Tricine buffer to 2 mM. *L. ferrea* extracts were incubated with the enzyme and the buffer for 15 min at 37 °C before adding the substrate. The end of the reaction mixture (160 µL) contained Tricine buffer (80 µL), 0.8 mM FALGPA (40 µL), 0.1 units of ChC (20 µL) and 20 µL of buffer control or the inhibitors under study. The absorbance was measured at 335 nm immediately after the addition of substrate and continuously measured every 5 min (30 min total) using a 96-well microplate reader (TP-Reader, Thermoplate, Italy).

Hyaluronidase inhibition assay

Hyaluronidase inhibitory assay was performed by a method previously described in the literature [32] with minor modifications. In brief, a test sample of 5 µl was pre-incubated with bovine hyaluronidase (1.50 units in 100 µL, Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37 °C. Subsequently, the assay was initiated by adding hyaluronic acid sodium salt from a 100-µL rooster comb at 0.03 % (in 300 mM sodium phosphate, pH 5.35) to the incubation mixture, and incubated further for 45 min at 37 °C. After incubation, 40 µL of 0.8 M potassium tetraborate was added to the reaction mixture, which was placed in a boiling bath for 3 min. Then, an aliquot of 90 µL of *p*-dimethylaminobenzaldehyde (DMAB, Sigma-Aldrich, USA) was added, and the final mixture incubated again at 37 °C for 20 min. The absorbance was measured at 560 nm. Pure water was used as a negative control and rutin (Sigma-Aldrich, St. Louis, MO, USA) as a positive control.

Cell culture

Murine B16F10 melanoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and primary cultures of skin cells (fibroblasts) were obtained from the foreskins of University Hospital from the University of São Paulo (HU–USP, São Paulo, Brazil) patients. This project was approved by the Ethics Committee of HU-USP (No. CEP Case 943/09). Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) medium (Gibco, San Jose, CA, USA) supplemented with 10 % fetal bovine serum (GIBCO, USA), 50 U/mL penicillin, and 50 µg/mL of streptomycin (Gibco, USA) in a humidified atmosphere with 5 % CO_2 at 37 °C.

Cell viability by Trypan Blue exclusion assay

Cell viability was assessed by standard Trypan Blue dye-exclusion assay using 0.4 % Trypan Blue solution in phosphate-buffered saline (PBS). To perform this test, 1.5×10^4 cells from each cell line were seeded on 24-well plates, and after 24 h post attachment, they were treated with the extracts. Following a 24–48 h incubation period, cell viability was assessed by counting live versus dead cells using standard Trypan Blue solution (0.4 % in PBS) exclusion staining [8].

Tyrosinase activity and melanin content in B16F10

Treatment and lysis of cells

B16F10 cells were stimulated with IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich, USA) at 25 µM for 24 h and then treated with both extracts at 25 µg/mL and gallic acid (GA, positive control) at 5 µg/mL for 48 h. Afterwards, cells were collected for two microtubes (one for melanin content with 1×10^6 cells/microtube) and lysed with Triton-X (Sigma-Aldrich, USA) in PBS with 1 mM PMSF (phenylmethanesulfonyl fluoride, Sigma-Aldrich, St. Louis, MO, USA). Afterwards centrifugation at 10,000 rpm at 4 °C for 10 min was carried out to obtain the supernatant for tyrosinase activity and the pellet for melanin content.

Tyrosinase activity

Tyrosinase activity was estimated by measuring the rate of L-DOPA oxidation as reported in the literature [35] with modifications. Protein concentrations were determined by the Bradford method, using bovine serum albumin (BSA, Sigma-Aldrich, USA) as standard. A total of 100 µL of supernatant containing the same 100-µg total proteins was added to each well in a 96-well plate, and then mixed with 100 µL of L-DOPA (Sigma-Aldrich, USA) 3 mg/mL. After incubation at 37 °C for 90 min, the dopachrome was monitored by measuring the absorbance at 475 nm.

Melanin content

Melanin content was measured as described in the literature [14] with modifications. The pellets of B16F10 cells (1×10^6) after lysis were mixed with 250 µL of NaOH (1 N) containing 10 % DMSO for 1 h at 95 °C. The

absorbance at 405 nm was measured using a microplate reader. The melanin content was determined from a standard curve prepared from a standard of synthetic melanin (Sigma-Aldrich, USA).

Quantitative reverse transcriptase PCR real time (qRT-PCR)

Total RNA was extracted with Qiazol (Qiagen, Germany) according to the manufacturer's instructions. RNA concentration and overall quality were determined using a NanoDrop spectrophotometer (Kisher, Germany) and 1 % agarose gel. Complementary DNA was synthesized using the kit High-Capacity cDNA Reverse Archive kit (Applied Biosystems, Poland) following the manufacturer's instructions. Real-time qRT-PCR primers and TaqMan probes targeting Tyr (Mm00495817_m1) were purchased as Assays-on-demand Products for Gene Expression (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. GAPDH was selected as internal control for RNA input and reverse transcription efficiency with the TaqMan Mouse Endogenous Control (Mm99999915_g1) (Applied Biosystems, USA). All real time qRT-PCR reactions for target gene and internal control were done in triplicate. Gene expression values were calculated using the ΔΔCt method, where for each sample, a non-treated sample correspondent was designated as a calibrator [23].

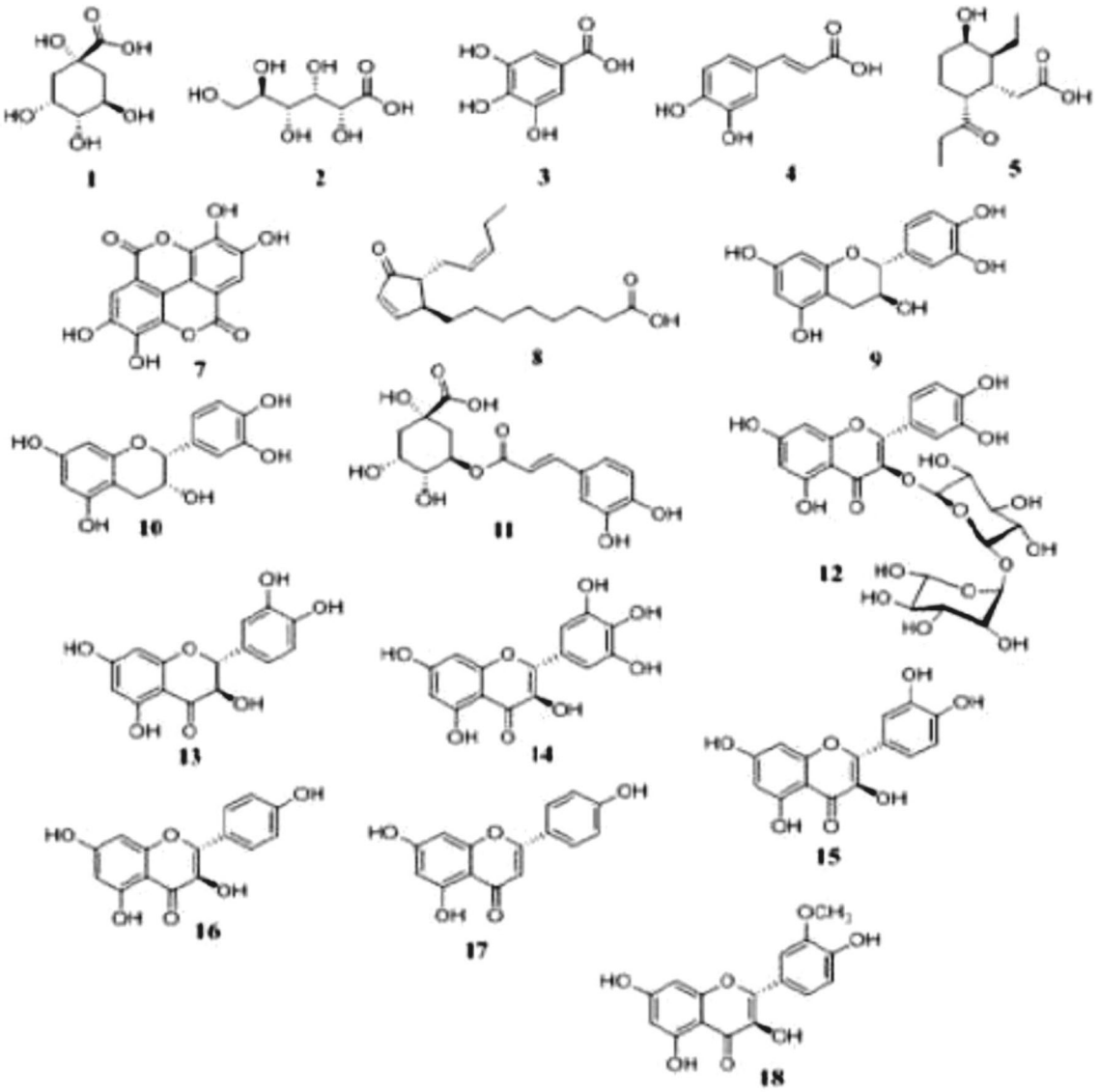


Fig. 1 Structures of the identified compounds for *Libidibia ferrea* extracts

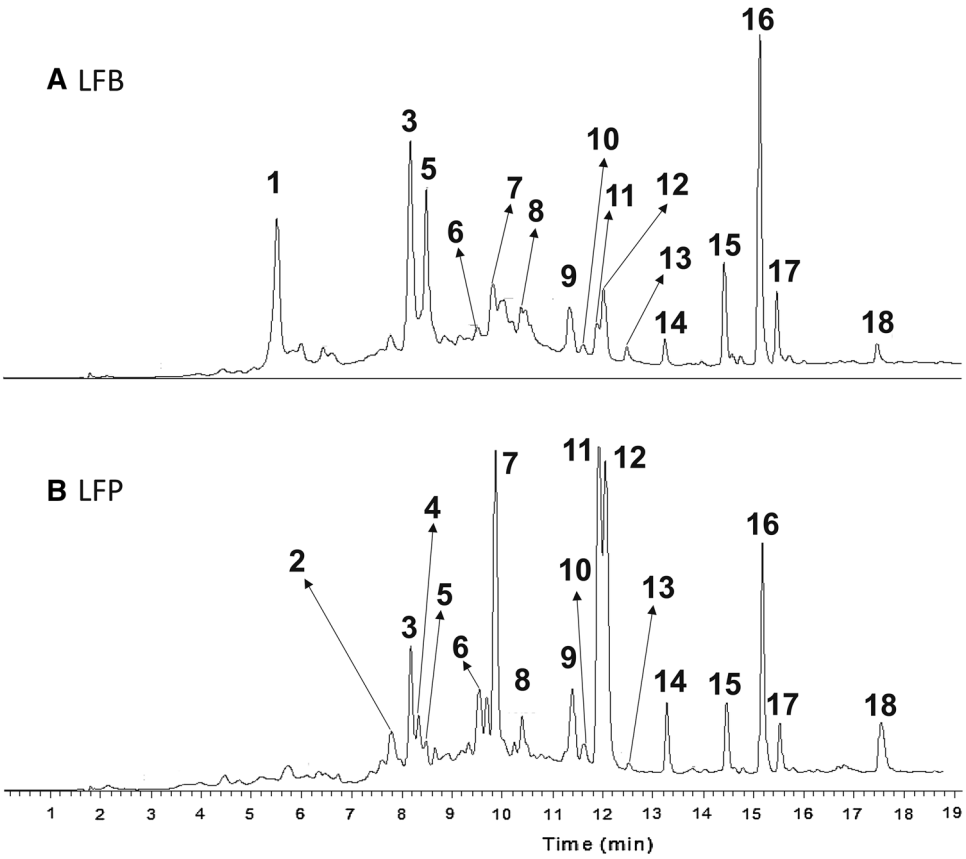


Fig. 2 LC–MS/MS chromatogram of the LFB (**a**) and LFP (**b**) extracts obtained from jucá displaying the following compounds: quinic acid (**1**), gluconic acid (**2**), gallic acid (**3**), caffeic acid (**4**), 2-(2-ethyl-3-hydroxy-6-propionylcyclohexyl)acetic acid (**5**), an unknown substance (**6**), ellagic acid (**7**), 12-oxo-phytodienoic acid (**8**), catechin (**9**), epicatechin (**10**), chlorogenic acid (**11**), rutin (**12**), taxifolin (**13**), myricetin (**14**), quercetin (**15**), kaempferol (**16**), apigenin (**17**), and isorhamnetin (**18**)

Table 1 Retention time, experimental *m/z*, MS/MS fragments, and phenolic compounds characterized by LC–MS/MS

Peak	R.T.	$[M-H]^-$	Productions	Compounds	Class	Extracts
1	5.54	191	171, 127, 111	Quinic acid	Phenolic acid	A, B
2	7.79	195	177, 159, 129	Gluconic acid	Organic acid	B
3	8.18	169	125	Gallic acid	Phenolic acid	A, B
4	8.33	179	161, 143	Caffeic acid	Phenolic acid	A, B
5	8.48	223	205, 125, 111	2-(2-Ethyl-3-hydroxy-6-propionylcyclohexyl)acetic acid	Secoridoid	A, B
6	9.53	252	234, 208, 136, 115	Unknown	–	A, B
7	9.85	301	284, 257, 229, 185	Ellagic acid	Phenolic acid	A, B
8	10.39	291	247, 165	12-Oxo-phytodienoic acid	Prostaglandin	A, B
9	11.50	289	245, 205	Catechin	Catechin	A, B
10	11.67	289	245, 205	Epicatechin	Catechin	A, B
11	11.92	353	335, 265, 247, 221	Chlorogenic acid	Phenolic acid	A, B
12	12.06	609	301	Rutin	Flavonol glucoside	A, B
13	12.52	305	269, 221	Taxifolin	Catechin	A, B
14	13.28	317	179, 151	Myricetin	Flavonol	A, B
15	14.47	301	179, 151	Quercetin	Flavonol	A, B
16	15.18	285	257, 197, 151	Kaempferol	Flavonol	A, B
17	15.50	269	225, 201, 107	Apigenin	Flavanone	A, B
18	17.51	315	300, 151, 109	Isorhamnetin	Flavonol	A, B

Gelatin zymography

Gelatin-substrate gel electrophoresis was used to measure MMP-2 and 9 activities as previously described in the literature [10] with minor modifications. In brief, to perform this assay, 1.5×10^4 of human foreskin fibroblasts cells were seeded on 24-well plates maintained in DMEM containing 10 % FBS for 24 h. After this period, the medium was removed and the cells were cultured for 24 h in the presence of extracts (LFP and LFB) in medium without FBS. The culture medium was collected after 24 h treatment and protein concentration was determined by the Folin method [24]. Supernatant protein (30 μg) from each sample was separated by electrophoresis on a 10 % acrylamide gel containing 0.5 mg/mL of gelatin for 1 h at 100 volts. The gel was washed twice with 2.5 % Triton X-100 for 15 min at 37 °C, and once with reaction buffer (0.05 M Tris–HCl (pH 8), 5 mM $CaCl_2$, 5 mM $ZnCl_2$). The gel was then incubated for 17 h in the same reaction buffer at 37 °C. Gels were stained with Coomassie solution (0.5 % Coomassie Brilliant Blue R-250 in 30 % methanol and 10 % acetic acid) for 30 min and destained in 10 % methanol and 10 % acetic acid. Clear zones of gelatin lysis against a blue background indicated the presence of the active form and pro-form of MMP-2 and MMP-9. Each clear zone within a given sample lane was analyzed by scanning densitometry (450 d.p.i. in GS-700, Bio-Rad, Hercules, CA, USA). The assay was performed in quadruplicate.

Western blot analysis

To detect the intracellular production of tyrosinase, MMP-2 and MMP-9 by B16F10 and NHF, the cells were treated with *L. ferrea* extracts at 25 μg/mL and GA at 5 μg/mL for 24 h. Proteins were extracted with RIPA buffer (Nonidet P-40, 1 mM Tris–HCl pH 7.5 (Sigma-USA), 125 mM EDTA [Sigma-USA), 5 M NaCl, (Merk, Germany)]. The total protein was determined by the Bradford method [7, 19]. Thirty micrograms of total protein was subjected to electrophoresis on 10 % gradient SDS gels under reducing conditions, and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The proteins were probed with primary antibodies to tyrosinase (AB58450, Abcam, Biotech company, Cambridge, UK), MMP-2 (SC10736 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), MMP-9 (MAB13415, Millipore). β-actin (A5441-Sigma-Aldrich, Aldrich) was used as a loading control. Peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG was used as a secondary antibody. Protein bands were detected by chemiluminescence system ECL (Amersham Pharmacia Biotech) [7].

Statistical analysis

All results were expressed as mean ± standard deviation (SD) from three independent experiments. Means and standard deviations were calculated from the data obtained and subjected to one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparison with controls. p values <0.05 were considered significant. IC_{50} values were calculated using the statistical software package, GraphPad Prism 5.0 software.

Results

Libidibia ferrea trunk bark (LFB) and pods (LFP) composition were analyzed by LC–MS/MS which evidenced the presence of phenolic acids and flavonoids as the most recurrent substances (Figs. 1, 2; Table 1). In minor proportions were organic acids, secoridoids and phytohormones. For LFB, the main component was kaempferol followed by gallic and quinic acids, respectively, while for LFP ellagic acid, catechin and epicatechin were the three main compounds (Fig. 2).

Inhibitory activity in collagenase, hyaluronidase and elastase enzymes was examined in vitro using spectrophotometric assays. The results showed that LFB and LFP have strong elastase inhibitory activity. The extracts were tested at 0–250 μg/mL, and showed 35.99 ± 2.87 % of elastase inhibition at 250 μg/mL for LFB and 19.6 ± 0.7 % for LFP; however, this activity was not higher than the activity presented by the standard, gallic acid (GA), which showed 98.9 ± 1.23 % of elastase inhibition at 25 μg/mL. For collagenase, there was no significant inhibition at different concentrations (0–100 μg/mL). LFP at 100 μg/mL inhibited the activity of collagenase only 15.2 %. The results showed that *L. ferrea* bark (LFB) and *L. ferrea* pod (LFP) have better inhibition of hyaluronidase activity compared with the standard (Rutin). The IC_{50} is shown in Table 2.

Cell viability of B16F10 cells (a murine melanoma) and normal human fibroblasts (NHF) after treatment with the extracts at concentrations in the range 0–100 μg/mL were assessed by Trypan Blue Exclusion Assay. LFP showed an

Table 2 Hyaluronidase inhibition by *L. ferrea* bark, *L. ferrea* pod and the standard rutin

Sample	Hyaluronidase inhibition IC_{50} (μg/mL)
L. ferrea bark	8.5 ± 0.8[a]
L. ferrea pod	16.0 ± 0.5[a]
Rutin	27.6 ± 0.06[a]

Data represent mean ± SD of three independent experiments

[a] $p > 0.001$ compared with the negative control

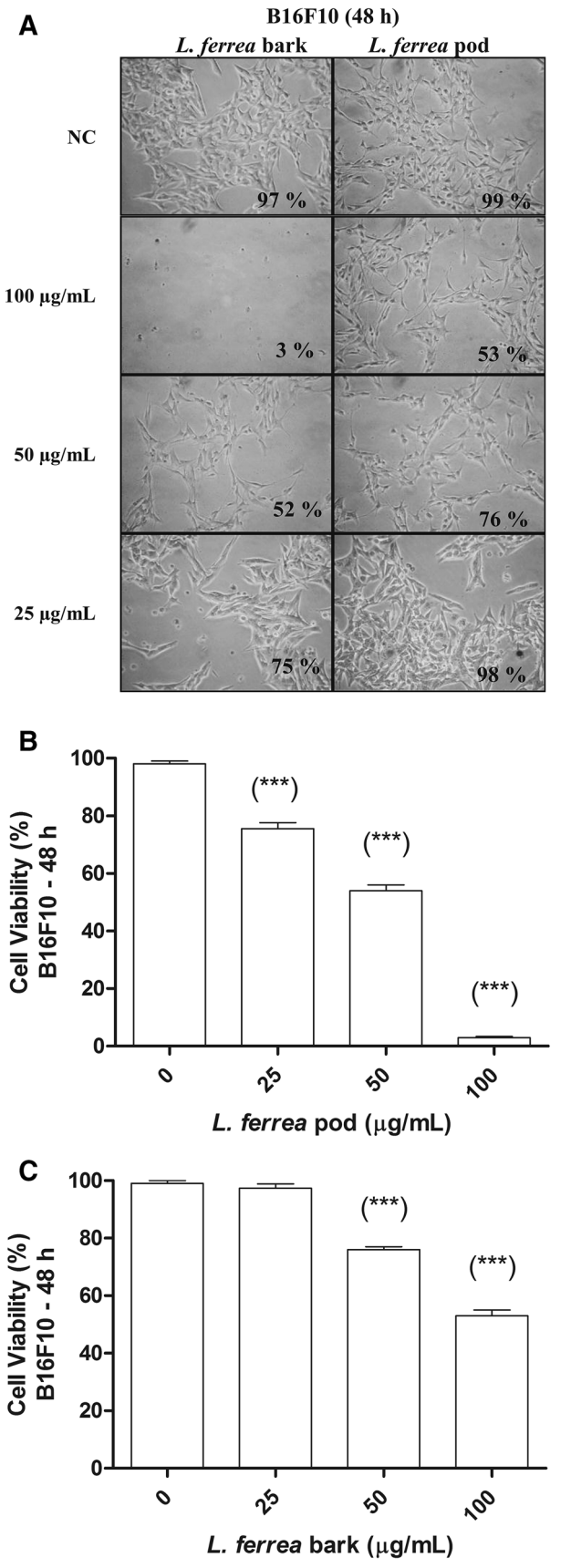


Fig. 3 Cell viability of *L. ferrea* pod and *L. ferrea* bark on murine melanoma (B16F10). The photomicrographics (**a**) and figures (**b**, **c**) indicate viability of melanoma murine cells following incubation with samples for 48 h as assessed by Trypan Blue staining (**a**). At 48 h after incubation, cell photographs were taken, and the percentage of cell death was determined by Trypan blue staining (**b**, **c**). *Each bar* represents the mean ± SD of three separate experiments. ***$p < 0.001$ compared with the control

$IC_{50} = 50.1 \pm 0.41$ µg/mL in B16F10 cells after 48 h of treatment. LFB reduced the cell viability in B16F10 cells in 47 ± 2.00 % at 100 µg/mL (IC_{50} of LFB >100 µg/mL) (Fig. 3). Both extracts showed similar levels of cytotoxicity in NHF after 24 h of treatment at 25 µg/mL and there was no observed significant cytotoxicity in NHF at this concentration. LFB and LFP at 50 µg/mL reduced NHF viability in 22.3 and 10 %, respectively (Fig. 4).

The anti-melanogenic effects of the extracts were studied on B16F10 cells. B16F10 cells were pre-treated with IBMX for 24 h and then treated with extracts at 25 µg/mL for 48 h. Cells treated with IBMX showed an increase in tyrosinase activity and when treated with the extracts of the bark and pod of *L. ferrea* these activity were significantly decreased (99.0 ± 6.34 and 96.4 ± 3.54 % of inhibition of tyrosinase activity, respectively) similar to that observed for GA at 5 µg/mL (89.5 ± 1.39 %) (Fig. 5a).

The melanin content was measured in the lysate of B16F10 cells. The levels of melanin content were satisfactorily reduced by both extracts (LFB and LFP) and the standard (gallic acid) at 42.4 ± 2.15, 40.1 ± 3.45 and 43.5 ± 2.39 %, respectively, when compared to cells treated with 25 µM IBMX (Fig. 5b).

The effects of the extracts on the expression protein and mRNA levels of tyrosinase were evaluated in B16F10 cells treated as showed before. The expression levels of tyrosinase protein were determined by Western blot in 25 µM IBMX stimulated B16F10 cells, and to determine whether the activity of these samples is involved in modulation of tyrosinase gene expression, we assessed the changes in the levels of expression by RT-qPCR and found no changes in tyrosinase protein and gene expression levels, which indicates that there is post-transcriptional control of melanogenesis by extracts and the standard (Fig. 5c, d).

The effects of the extracts in MMP expression and activity were studied in NHF cell and assessed by western blot and zymography. On gelatin zymography assay there was no inhibition of the activity of MMP-2 and MMP-9. However, there was a statistically significant inhibition of pro-MMP-2 activity. LFB and LFP reduced the activity of pro-MMP-2 (22.1 ± 1.45 and 21.9 ± 0.20 %, respectively). To evaluate extract effects on protein expression of MMP-9, pro-MMP-2 and active MMP-2 we used Western blot analysis. MMP-9 expression was not altered by GA

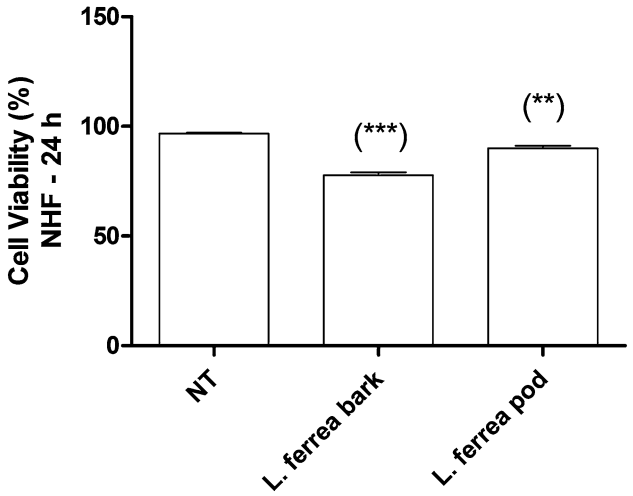


Fig. 4 Cell viability of LFB (*Libidibia ferrea* trunk bark) and LFP (*Libidibia ferrea* pod) on normal human fibroblasts (NHF). The figure indicates viability of NHF following incubation with samples at 50 μg/mL for 24 h as assessed by Trypan Blue staining. Each bar represents the mean ± SD of three separate experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the control

but was altered by *L. ferrea* extracts. LFB and LFP caused a small but significant decrease in the expression of active MMP-2 and caused a large decrease in pro-MMP-2 expression (Fig. 6).

Discussion

In this work, we evaluated the anti-wrinkle and anti-whitening potentials of *L. ferrea* extracts and a comprehensive LC–MS/MS analysis has identified eighteen substances present in this extracts such as flavonoids, organic acids, secoridoids and phytohormones. The lack of studies of chemistry and biological activities of this species encouraged us to accomplish this study. Cosmetic formulations based on natural products have been developed from ancient times and play an important role in the present cosmetic industry. However, scientific validations of the safety and efficacy of plant extracts and components of skin aging are sorely needed [19]. In a previous communication, we showed that the aqueous extracts of the trunk bark and pods of *L. ferrea* possess high antioxidant capacity [4]. In this work, we demonstrate that both extracts contain a complex composition, where polyphenols are the main compounds and play a central role in the pharmacological activities observed for LFB and LFP due to the presence of strong antioxidant compounds [18].

Antioxidants originating from natural products present as a cost effective and safe alternative to modulate the effects of oxidative stress [31], which is directly involved in the acceleration of skin aging [3, 4]. It is believed that ROS are involved in loss of skin elasticity and it is known that they are also capable of inducing the expression of proteinases responsible for reshaping the extracellular matrix (ECM), such as MMPs, elastase and hyaluronidase [29, 34]. Flavonoids derived from plants have the potential to bind metalloenzymes, thus inhibiting the metabolic pathways due to having the ability to form complexes with metal ions [34].

The *Libidibia ferrea* bark (LFB) and *L. ferrea* pod (LFP) extracts show a hyaluronidase inhibitory activity higher than rutin suggesting that antioxidant activity and presence of GA may be contributing to this high inhibition. Hyaluronidase is one of the most important enzymes involved in the ECM degradation process and plays an important role in controlling the size and concentration of hyaluronic acid strands (AH) [30]. The GA and rutin are reported in the literature as inhibitors of hyaluronidase activity [3, 22].

LFB and LFP showed low elastase inhibition. Elastase is an important enzyme that can hydrolyze almost all components of the ECM [30]. The presence of epigallocatechin, a potent elastase inhibitor [34], in hydroalcoholic extract of *L. ferrea* was reported in our previous work [4]. This result suggests that observed elastase inhibition may have occurred due to the presence of this substance in our extracts. Studies have shown that GA inhibits collagenase ($IC_{50} = 2.07$ mM) [3], while rutin has no action on this enzyme [26]. These studies suggest that only LFP inhibited collagenase in our work because of the highest GA concentration in this extract.

Both extracts inhibited pro-MMP-2 activity and expression (gelatinase). These results indicate that extracts attenuate collagen degradation in the ECM by reducing MMPs expression, thereby decreasing wrinkle formation. The extracts also reduced active MMP-2 and MMP-9; however, no direct reduction in activity was observed when the MMP activity was assessed by gelatin zymography assay.

Melanin plays an important role in protection against ultraviolet radiation (UVR), but overproduction and accumulation of this polymer can lead to problems such as freckles, age pigment and melasma. Thus, this problem has stimulated the search for cosmetic and medical treatments for hyperpigmentation [17]. In the present study, LFB and LFP showed potent antimelanogenic activity that can be attributed to excellent antioxidant activity that has been reported for these extracts [4] in addition to the high capacity of tyrosinase inhibitory activity reported for GA [28] and depigmenting activity attributed to rutin [11, 33]. The antioxidant activity is important in melanogenesis inhibitors because it prevents oxidation of essential substances for the formation of melanin [13]. It is reported that the anti-tyrosinase activity of gallic acid occurs due to their antioxidant properties.

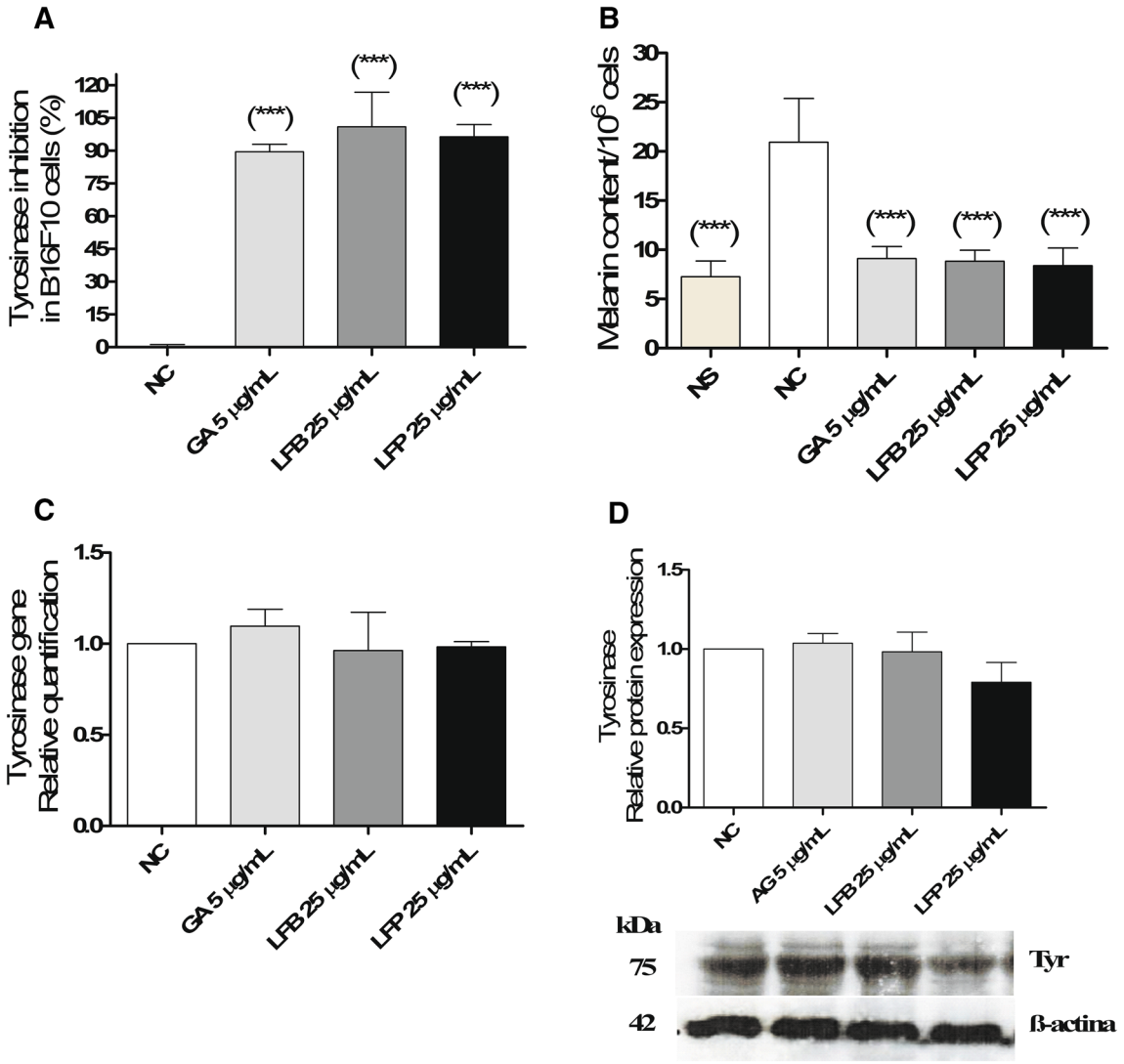
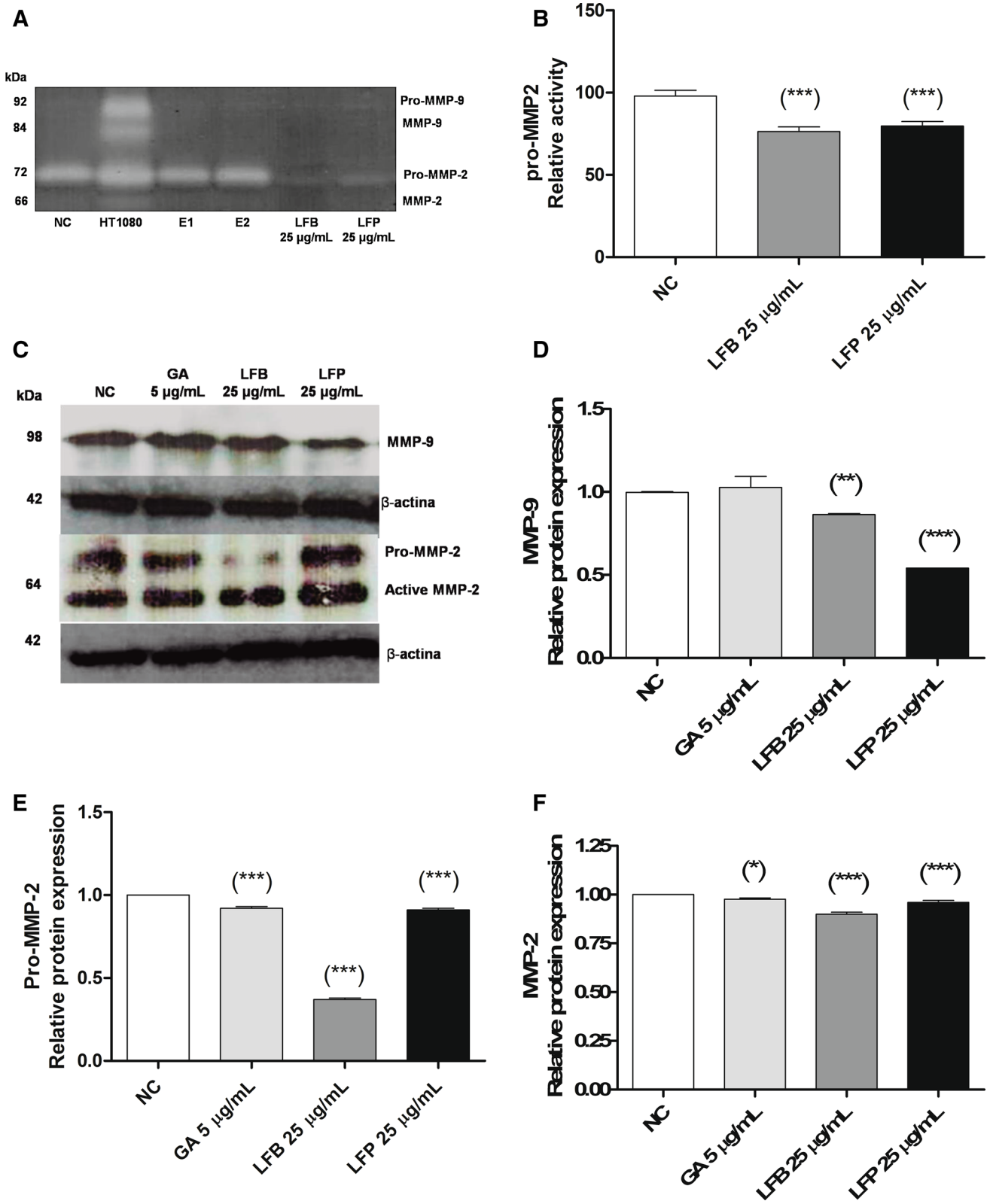


Fig. 5 **a** Inhibitory effects of gallic acid (GA), *L. ferrea* bark (LFB) and *L. ferrea* pod (LFP) extracts on tyrosinase activity in B16F10 cells. Gallic acid was used as standard. Cells were stimulated in the presence of 25 μM IBMX for 24 h, after, B16F10 cells were treated with *L. ferrea* bark (LFB) at 25 μg/mL, *L. ferrea* pod (LFP) at 25 μg/mL and gallic acid at 5 μg/mL for 48 h. *NC* negative control (cells stimulated with 25 μM IBMX). Values represent the mean ± SD ($n = 3$). ***$p < 0.001$ compared to cells treated with IBMX 25 μM (NC). **b** Inhibitory effects of extracts and gallic acid in cellular melanin content. Melanin content was measured in B16F10 cells treated for 48 h with *L. ferrea* extracts at 25 μg/mL and gallic acid at 5 μg/mL after stimulation with 3-isobutyl-1-methylxanthine (IBMX) 25 μM for 24 h. *NS* Melanin content in B16F10 cells without IBMX stimulation; *NC* negative control (cells stimulated with 25 μM IBMX). Results were expressed as mean ± SD ($n = 3$) and were calculated and compared to the negative control. ***$p < 0.001$ compared to cells treated with IBMX25 μM (NC). **c** The expression of tyrosinase was determined by RT-PCR using GAPDH as internal standard. Data were expressed as mean ± SD from three different experiments. **d** Protein relative expression of tyrosinase by western blot analysis. The protein β-actin was used as a protein quantification control ($n = 3$). $p > 0.05$

Melanogenesis requires in addition to tyrosinase activity, the action of some reactive species of oxygen and nitrogen, which are known for their role in the acceleration of aging. Thus, control of oxidative stress is important for the regulation of melanogenesis [1]. Although numerous studies have reported on anti-melanogenic agents, including kojic acid, hydroquinone and arbutin, such agents have undesirable side effects reports such as skin irritation, low photostability or cell toxicity. Therefore, there is a need for the search for new depigmenting agents that are safer and more effective and natural products are presented as an alternative [17]. Observing the IC_{50} values of the extracts on B16F10 and NHF and comparing the concentrations needed to obtain good depigmenting activity, hyaluronidase inhibition and pro-MMP-2 inhibitory activity, we found that the extracts can be considered safe for use. However, it is noted that the LFP is more cytotoxic to B16F10 cells and LFB more cytotoxic to NHF. In this work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LFB 25 µg/mL
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LFB 25 µg/mL
LFP 25 µg/mL
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GA 5 µg/mL
LFB 25 µg/mL
LFP 25 µg/mL

◀**Fig. 6** Effects of *L. ferrea* bark (LFB) and *L. ferrea* pod (LFP) extracts on gelatinase activity. **a** Gelatin zymography of normal human fibroblasts incubated for 24 h in the absence and presence of LFB and LFP at 25 μg/mL. Note that only the pro-MMP-2 was inhibited in the presence of *L. ferrea* extracts as shown by the absence of clear zones. This compound did not alter the activity of MMP-2 and MMP-9. **b** Histograms represent the percentage of pro-MMP-2 in the absence and presence of LFB and LFP at 25 μg/mL compared to controls (no treatment). *NC* negative control, *E1* and *E2* two different extracts of other native Amazonian plants. $***p < 0.001$ compared with the control. Histograms are representative of three separate experiments done in triplicate. **c** Evaluation of MMP-9, active MMP-2 and pro-MMP-2 protein expression in normal human fibroblasts following *L. ferrea* bark (LFB) and *L. ferrea* pod (LFP) extracts treatment as compared with negative control (NC). Gallic acid (5 μg/mL) was used as standard. **d–f** Protein relative expression of MMP-9, pro-MMP-2 and MMP-2 in western blot analysis, respectively ($n = 3$). The protein β-actin was used as a protein quantification control. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

we demonstrated the presence of epicatechin and catechin in the extracts. Epicatechin and catechin are polyphenols that are known to be tyrosinase inhibitors [20, 21].

The traditional use of plants against skin diseases, especially for cosmetic purposes is a common practice in the folk medicine of many cultures, and might provide clues to discover better depigmenting agents [25]. Some potent tyrosinase inhibitors such as cuminaldehide, oxyresveratrol, kaempferol, quercetin and gallic acid derivatives, have been isolated from various plants [15]. The present study demonstrates that there was no regulation in the gene and protein expression of tyrosinase enzyme by the extracts, and it suggests a post-transcriptional regulation and suggests that the mechanisms is by the inhibition of the tyrosinase enzyme, probably due the strong antioxidant activity presented by the extracts.

The extracts, LFP and LFP, are safe and have great potential as anti-aging cosmetic or just as a depigmenting agent. The high antioxidant capacity of the extracts in combination with plant metabolites [flavonoids (e.g. rutin), organic acids (e.g. gallic acid), phenolic compounds (e.g. epigallocatechin gallate), etc.] interacts to enhance the *L. ferrea* anti-aging potential.

In conclusion, the extracts of bark and pod from *L. ferrea* have shown great capacity for inhibition of hyaluronidase, matrix metalloproteinase-2 and inhibitory action on melanogenesis, mainly due to presence of active agents such as kaempferol, ellagic acid, catechin and epicatechin content and it can be a promising agent for use as anti-aging cosmetic ingredient.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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