

RESEARCH ARTICLE

Extracts of *Morus nigra* L. Leaves Standardized in Chlorogenic Acid, Rutin and Isoquercitrin: Tyrosinase Inhibition and Cytotoxicity

Marcela Medeiros de Freitas^{1*}, Pedro Ribeiro Fontes¹, Paula Monteiro Souza¹, Christopher William Fagg², Eliete Neves Silva Guerra³, Yanna Karla de Medeiros Nóbrega⁴, Damaris Silveira¹, Yris Fonseca-Bazzo¹, Luiz Alberto Simeoni¹, Maurício Homem-de-Mello¹, Pérola Oliveira Magalhães¹

1 Department of Pharmacy, Health Sciences School, University of Brasília, Brasília, Distrito Federal, Brazil, **2** Department of Botany, Institute of Biological Science, School of Pharmacy, Ceilândia Campus, University of Brasília, Brasília, Distrito Federal, Brazil, **3** Department of Odontology, Health Sciences School, University of Brasília, Brasília, Distrito Federal, Brazil, **4** Immunogenetic and Chronic-degenerative Diseases Laboratory, School of Medicine, University of Brasília, Brasília, Distrito Federal, Brazil

* marcelamdf1@gmail.com



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Abstract

Melanogenesis is a process responsible for melanin production, which is stored in melanocytes containing tyrosinase. Inhibition of this enzyme is a target in the cosmetics industry, since it controls undesirable skin conditions such as hyperpigmentation due to the overproduction of melanin. Species of the *Morus* genus are known for the beneficial uses offered in different parts of its plants, including tyrosinase inhibition. Thus, this project aimed to study the inhibitory activity of tyrosinase by extracts from *Morus nigra* leaves as well as the characterization of its chromatographic profile and cytotoxicity in order to become a new therapeutic option from a natural source. *M. nigra* leaves were collected, pulverized, equally divided into five batches and the standardized extract was obtained by passive maceration. There was no significant difference between batches for total solids content, yield and moisture content, which shows good reproducibility of the extraction process. Tyrosinase enzymatic activity was determined for each batch, providing the percentage of enzyme inhibition and IC₅₀ values obtained by constructing dose-response curves and compared to kojic acid, a well-known tyrosinase inhibitor. High inhibition of tyrosinase activity was observed (above 90% at 15.625 µg/mL). The obtained IC₅₀ values ranged from 5.00 µg/mL ± 0.23 to 8.49 µg/mL ± 0.59 and were compared to kojic acid (3.37 µg/mL ± 0.65). High Performance Liquid Chromatography analysis revealed the presence of chlorogenic acid, rutin and, its major compound, isoquercitrin. The chromatographic method employed was validated according to ICH guidelines and the extract was standardized using these polyphenols as markers. Cytotoxicity, assessed by MTT assay, was not observed on murine melanomas, human keratinocytes and mouse fibroblasts in tyrosinase IC₅₀ values. This

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study demonstrated the potential of *M. nigra* leaf extract as a promising whitening agent of natural source against skin hyperpigmentation.

Introduction

Melanin is one of the most widely distributed pigments found in bacteria, fungi, plants and animals [1]. Melanogenesis is initiated with tyrosine oxidation catalyzed by tyrosinase to dopaquinone, which is converted to dopa and dopachrome through auto-oxidation. Dopa is also the substrate of tyrosinase and oxidized to dopaquinone again by the enzyme. The reaction products from dopachrome, dihydroxyindole (DHI) and dihydroxyindole-2-carboxylic acid (DHICA), suffer oxidation to form the brown-to-black eumelanin. In the presence of cysteine or glutathione, dopaquinone is converted to cysteinyl-dopa or glutathionyl-dopa subsequently forming the yellow-to-reddish-brown pheomelanin [2, 3]. The mature melanosomes located in the dendrites of melanocytes are then phagocytosed by the surrounding keratinocytes, and it is this process which is responsible for the variety of colors in human skin, hair and eyes [4]. There are two groups of pigmentary disorders: the abnormal presence of exogenous or endogenous pigments in the skin and disorders of the quantitative and qualitative distribution of normal pigment, which includes hyperpigmentation and hypopigmentation [5]. Hyperpigmentation can be induced by external factors such as UV exposure, in which melanin formation and the thickening of the epidermis are the most visible reaction to a natural defense mechanism intended to decrease further penetration to the basal layer where proliferation is taking place [6] and drugs such as certain antibiotics, oral contraceptives, certain antiepileptic agents, chloroquine, levodopa, heavy metals and chemotherapy agents. Internal factors include hormones, sometimes seen during pregnancy, and postinflammatory hyperpigmentation of the skin [7].

Changes in skin pigmentation induce significant cosmetic problems with effect on quality of life [8]. Hyperpigmentation of the skin is a common complaint among patients consulting with dermatologists [9]. The accumulation of an abnormal melanin amount in different specific parts of the skin as more pigmented patches (melasma, freckles, ephelide, senile lentiginos etc.) might become an esthetic problem [10]. Melasma has a strong impact on the emotional domain of quality of life, resulting especially from feelings about skin appearance, of which the emotional domain was particularly affected in individuals experiencing a longer duration of this disease [11]. It had long been recognized that the rate of melanin production at baseline and following UV exposure in either cultured pigment cells or intact skin depends on tyrosinase activity, rather than simply on total tyrosinase protein [12].

The enzyme tyrosinase (EC 1.14.18.1), a glycoprotein [13], which catalyzes the aerobic oxidation of tyrosine to produce the pigment melanin, is widely distributed in nature [14], found in plants, insects, mammals and marine animals [15]. Also found in bacteria and fungi, polyphenol oxidases (PPO) or tyrosinases are enzymes of dinuclear copper center active site bound by six or seven histidine residues and a single cysteine residue is highly conserved, being the most well-studied multi-copper oxidase [16, 17].

Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skin-whitening effects, but despite the extensive researches on lightening agents and hyperpigmentation, the existing agents have got limitations in term of high toxicity, low stability, poor skin-penetration, and insufficient activity [18]. Although highly effective, long-term exposure to traditional depigmenting agents, such as hydroquinone, corticosteroids, kojic acid and arbutin can raise several safety concerns with local or systemic side effects such as

pigmented contact dermatitis, poor enzyme inhibition, skin irritation and exogenous ochronosis in dark-skinned people. Many plant extracts are more potent inhibitors of melanin formation and are not associated with cytotoxicity or mutagenicity of melanocytes [19–23]. Natural tyrosinase inhibitors are generally considered to be free of harmful side effects and can be produced at reasonable low costs, especially when rich sources are identified [24, 25]. The food industry has been searching for effective antibrowning agents to control discoloration. Antibrowning substances such as cysteine, 4-hexylresorcinol, and kojic acid are well known, however, these chemicals have limited commercial use because of safety problems, cost, and government regulation. In addition, the demand for antibrowning agents from natural products is strong [26].

Moraceae is a family of a flowering plant that comprises about 40 genera and 1400 species [27]. Mulberry (*Morus* sp.) has been domesticated over thousands of years and has been adapted to a wide area of tropical, subtropical, and temperate zones of Asia, Europe, North and South America, and Africa [28]. In 2002, the mulberry (leaf and fruit) was considered not only food, but also drugs, by the Chinese Ministry of Health [29].

Morus nigra L. (Moraceae), known as “black mulberry” or “wild mulberry” belongs to the genus *Morus* and is found in Africa, South America and in Asia, possessing a wide range of medicinal uses and can be used either as single or associated drug to treat different ailments [30]. *M. nigra* is used for the treatment of diabetes, cholesterol, cardiovascular problems, obesity and gout [31], it has antimicrobial [32], anticancer [33], hepatoprotective [34, 35] and molluscicidal [30] activities, has a protective action against peroxidative damage to biomembranes and biomolecules [36] and the total flavonoids found in black mulberry fruits possess anti-inflammatory and analgesic effects [37]. In comparative studies, black mulberry fruit has proven to have the highest total phenolic and flavonoid contents when compared to white (*Morus alba* L.) and red (*Morus rubra* L.) mulberry fruits [38], a higher bioactive content due to anthocyanin concentration and antioxidant activity than purple (*M. rubra* L.) mulberry genotypes [39]. Similarly, it was demonstrated that black mulberry sugar-free extracts have greater total antioxidant activity and phenolic contents than *M. alba* extracts, stating that chlorogenic acid and rutin were found to be the dominant phenolic constituents [40]. Between black, white and Russian (*M. alba* var. *tatarica* L.) mulberry fruits, *M. nigra* showed the highest contents of reduced ascorbic acid, titratable acidity, iron, total flavonoids and total monomeric anthocyanins [41]. Due to its edible nature, easy accessibility and economical factor, *M. nigra* can be a good source of active compounds [34].

Morus species are well-known as plants rich in polyphenols and its extracts have been used as a non-toxic natural therapeutic agent, which also have high potential in applications as skin-whitening agents due to many potent tyrosinase inhibitors being isolated from different parts of the plant [42]. Tyrosinase inhibitors from *Morus* include *M. alba* roots [43], leaves [44], twigs and root bark [45]; *M. australis* roots [46], dried stems [47] and leaves [48]; *M. lhou* stem barks [49] and roots [50]; *M. multicaulis* Perr. branch bark [29]; *M. notabilis* stem [51] and *M. yunnanensis* leaves [52]. Tyrosinase inhibitors were also isolated from *M. nigra* stems [53] and roots [54] but to date no reports were found in the literature on tyrosinase inhibition by the extract of *M. nigra* leaves, which can serve as a suitable whitening agent as other species of the genus *Morus* have proven to inhibit the enzyme of interest, making this work innovative by suggesting a novel natural skin-whitening source.

Plants naturally produce phytochemicals, which are responsible for different color, flavor, smell, and its natural defense mechanism against diseases [55]. Pure phytoconstituents and plant extracts are rich in chemical diversity, an inexhaustible source for new drug development [56]. Natural products obtained from plants are important to the therapy of various disease conditions [57]. Herbal medicines are in great demand for primary healthcare because of their wide biological activities, higher safety margins and lesser costs. The lack of complete

standardization for plant formulations is a great challenge. They are prone to contamination, deterioration and variation in composition due to its natural origin. Therefore, quality control of herbal medicines is often complicated [58]. Quality control begins with the plant material, which is the most important variation factor in manufacturing herbal medicinal products. Plants are inevitably inconstant because their composition may be influenced by multiple factors, such as origin, growth, harvesting, drying, and storage conditions [59].

Due to the importance of standardization of plants to ensure quality control and the uses of *Morus* species in traditional medicine and a high source of natural compounds, the aim of this study was to evaluate the leaf extract of *M. nigra* for tyrosinase inhibitory activity, as well as the characterization of the chromatographic profile of its standardized extract identifying compounds responsible for enzyme inhibition, development and validation of a High Performance Liquid Chromatography with UV detection (HPLC-DAD) method aiming to standardize it for chlorogenic acid, rutin and isoquercitrin, and assess the viability of extract incorporation in cosmetics of topical use from its cytotoxicity in cell lines that constitute skin, such as fibroblasts and keratinocytes in order to become a new therapeutic option to whitening agents originated from natural source, which can also be used in the food industry for acting as an antibrowning agent for fruits, vegetables and juices. As far as the authors are concerned, this is the first study that evaluates tyrosinase inhibition and quantifies the presence of these three polyphenols in *M. nigra* leaf extract.

Materials and Methods

Chemicals and Reagents

Tyrosinase from mushroom (lyophilized powder, ≥ 1000 unit/mg solid), L-tyrosine ($\geq 98\%$), kojic acid, chlorogenic acid ($\geq 95\%$), rutin ($\geq 94\%$) and isoquercitrin ($\geq 90\%$) were purchased from Sigma-Aldrich (St Louis, MO, USA). Aqueous ethanol (95%) used for extraction was prepared from absolute ethanol Synth (Diadema, SP, Brazil). Methanol used to dilute samples, acetonitrile used as mobile phase for HPLC analysis and phosphoric acid 85% were purchased from Tedia (Fairfield, OH, USA). Water used to prepare the mobile phase was purified in a Milli-Q-plus System (Millipore, Bedford, MA, USA).

Plant Materials

M. nigra L. leaves were cultivated, identified and collected on March 24th 2014 in Brasilia, DF, Brazil by Professor Christopher Fagg (Botany Department, Faculty of Ceilândia, University of Brasilia). The voucher specimen was deposited in the Herbarium of the University of Brasilia, number Fagg CW 2302.

Extraction

Leaves were air-dried, then artificially dried at 40°C until humidity reached 9%. The dried material was powdered (Mesh size 230–35), divided into 5 batches of 210 g and extracted with 1050 mL aqueous ethanol 95% at room temperature for 10 days with daily manual agitation. In order to evaluate the reproducibility of the extraction process, the total solids content was measured in an infrared moisture analyzer (Gehaka model IV2000, São Paulo—SP, Brazil) in samples of 2 mL of filtered ethanol extract from each batch, in triplicate. The ethanol extracts were evaporated to dryness under reduced pressure at 40°C followed by lyophilization, thus obtaining the Standardized Extract of *M. nigra* Leaves (SEML), which were stored at -20°C. The humidity content was measured in the infrared moisture analyzer previously described with 1 g of the dried extract from each batch, in triplicate.

Assay of Tyrosinase Activity

Tyrosinase inhibition assay was performed according to Khatib et al. (2005) [60] with modifications [25]. Sodium phosphate buffer (60 μ L, 50 mM) at pH 6.5, 30 μ L tyrosinase (250 U/mL) and 10 μ L of *M. nigra* extract (1 mg/mL) were inserted into 96-well plates. Extracts from all five batches were dissolved in methanol and tested at concentrations ranging from 1000 μ g/mL to 0.49 μ g/mL. After 5 min of incubation at room temperature, 100 μ L L-tyrosine (2 mM) were added and incubated for additional 20 min. The optical density (OD) of the samples were measured in a microplate reader (Beckman Coulter, model DTX 800, Lagerhausstrasse—Austria) at 450 nm and compared to control without inhibitor, demonstrating a linear color change with time during the 20 min of the experiment. Control incubations represent 100% enzyme activity and were conducted in a similar way by replacing extracts by buffer. For blank incubation, in order to eliminate the absorbance produced by the extract, the enzyme solution was replaced by buffer. The inhibitory activity was determined by comparing the enzyme activity in the absence and presence of the evaluated inhibitor. Kojic acid was used as positive control. For negative control the solvents used in the extraction process and to dilute the crude extract, ethanol and methanol, respectively, were tested to confirm there is no interference with tyrosinase inhibitory activity.

Preparation of standard solution

Standard stock solutions (1 mg/mL) of chlorogenic acid, quercetin-3-O-rutinoside (rutin) and quercetin-3- β -D-glucoside (isoquercitrin) were prepared by solubilization of 10 mg of each reference standard with 10 mL methanol in a volumetric flask. A mixed stock solution of 100 μ g/mL was prepared from 1 mL of each reference standard (1 mg/mL) with methanol in a 10 mL volumetric flask, followed by different concentrations of mixed standard solutions in 10 mL volumetric flask (0.075–10.0 μ g/mL), for establishment of calibration curves and limits of detection and quantitation for all three polyphenols.

Sample preparation

Standardized extract of *M. nigra* leaves was weighed (10 mg, batch 4) and solubilized in methanol (5 mL) to a final concentration of 2 mg/mL. All samples were filtered in a 0.45 μ m, 13 mm Millex (Merck Millipore, Carrigtwohill Co. Cork, Ireland) and injection volume was 10 μ L.

Determination of chlorogenic acid, rutin and isoquercitrin by RP-HPLC

A High Performance Liquid Chromatography (HPLC) was used to determine chlorogenic acid, rutin and isoquercitrin levels in *M. nigra* ethanol extract. A Dionex UltiMate 3000 liquid chromatography system equipped with a Diode Array Detector DAD-3000 operating in 280, 330 and 354 nm was used. System was configured with a DGP-3600SD solvent pump unit, WPS-3000 SplitLoop injector and column oven TCC-3200 at 25°C, all from Dionex. Separation was performed in a LiChroCART Purospher STAR RP C18 column (150 x 4,6 mm, 5 μ m particle size, Merck, Germany), equipped with a pre-column of same characteristics (4 x 4; 5 μ m particle size, Merck, Germany). The mobile phase was 1% phosphoric acid and acetonitrile gradient at a flow rate of 0.5 mL/min, starting at 90% (1% phosphoric acid) decreasing to 70% until t = 40 minutes and returning to 90% until t = 45 minutes. All solvents used as mobile phase were filtered in a 0.22 μ m, 47 mm polyvinylidene fluoride (PVDF) membrane (Merck Millipore) and degas in an ultrasonic cleaner (Branson, CT, USA). Data were collected extracting chromatograms at 354 nm using the Thermo Fisher Scientific software Chromeleon, version 7.1.2.1541.

The analytical methodology was validated following the ICH guidelines [61] and the validation guide of analytical and bioanalytical methods established by the Brazilian National Agency of Sanitary Vigilance [62]. Validation was performed considering specificity, linearity, accuracy, precision, detection limit, quantitation limit and robustness.

The specificity of the method was verified by analysis of sample degradation products by acid/base hydrolysis induction. Therefore, 10 mg of SEML were weighed and solubilized in 1 mL of hydrochloric acid (1 M) for acid hydrolysis and 10 mg were solubilized in 1 mL of sodium hydroxide (1 M) for basic hydrolysis. These solutions were incubated at 60°C for 60 minutes. After cooling to room temperature, the solutions were neutralized with 1 mL of sodium hydroxide (1 M) and hydrochloric acid (1 M), respectively. After freezing, samples were lyophilized and then methanol (5 mL) was added to a final concentration of 2 mg/mL. HPLC analysis was performed in triplicate. Retention time and peak areas of chlorogenic acid, rutin and isoquercitrin on samples prepared without acid/base hydrolysis induction were compared to the results obtained from samples after acid/base hydrolysis induction.

Linearity was assessed by the linear regression method of three authentic calibration curves, through the analysis of eight different concentrations of chlorogenic acid (0.2, 0.3, 0.4, 0.5, 1.0, 2.5, 5.0, and 10.0 µg/mL) and nine different concentrations of rutin and isoquercitrin standard solutions (0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.5, 5.0, and 10.0 µg/mL). The correlation coefficient (r), y -intercept, slope of the regression line, and residual sum of squares were obtained using the software GraphPadPrism.

In intra-day precision, six determinations at 100% of the test concentration from the solubilized SEML (2 mg/mL) were analyzed by HPLC on the same day. The inter-day precision was determined in triplicate and analyzed on three consecutive days with the same analyst. The precision is expressed as the percent Relative Standard Deviation (RSD).

Accuracy was assessed using three replicates of three concentration levels through the standard addition of chlorogenic acid, rutin and isoquercitrin to the sample. In order to achieve solutions of low, normal and high concentrations (about 80%, 100% and 120%) of the solubilized SEML, mixed standard solutions (100 µg/mL) of chlorogenic acid (10 µL, 20 µL and 30 µL), rutin (30 µL, 50 µL and 70 µL) and isoquercitrin (60 µL, 100 µL and 140 µL) were prepared adjusting the final volume to 2 mL of methanol. 500 µL of each mixed solution was added to 500 µL of the solubilized SEML (2 mg/mL, 0.99 µg/mL chlorogenic acid; 2.53 µg/mL rutin and 4.84 µg/mL isoquercitrin) resulting in the theoretical final concentrations of 0.75, 1.00 and 1.25 µg/mL for chlorogenic acid, 2.02, 2.52 and 3.02 µg/mL for rutin and 3.92, 4.92 and 5.92 µg/mL for isoquercitrin. Finally, the solutions were analyzed by the developed HPLC method. Accuracy was expressed as percent of recovery, which was estimated as the relation between the experimental concentrations and the theoretical concentrations, using the formula: $\text{recovery (\%)} = 100 \times (\text{experimental concentration}/\text{theoretical concentration})$.

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined according to the method based on parameters of the calibration curve, where LOD was expressed as $(3.3 \times \sigma)/S$ and LOQ was expressed as $(10 \times \sigma)/S$, where σ is the standard deviation of the response and S is the slope of calibration curve.

The robustness of the method was determined by chromatographic analysis of samples under different conditions, such as changes in wavelength (280 and 330 nm), flow (0.3 and 0.7 mL/min) and temperature (35 and 45°C). The effects of the parameters retention time and peak area were observed.

Samples (2 mg/mL) of SEML from batches 1, 2, 3, 4 and 5 were evaluated, in triplicate, against the validated method for quantification of chlorogenic acid, rutin and isoquercitrin and analyzed statistically by ANOVA with Tukey multiple comparison method ($p < 0.05$).

Cytotoxicity Assay

The cell lines, one human keratinocyte cell line (HaCat), one fibroblast cell line (L-929) and one melanoma cell line (B16F10), were grown as monolayers in a mixture of Dulbecco's modified Eagle medium and supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin). Cells were maintained at 37°C and 5% of CO₂. For all experiments, cells were detached with trypsin (0.25%)/EDTA (1 mM) solution. All cell culture reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). The melanoma cell line was acquired by Rio de Janeiro Cell Bank—Scientific Technical Association Paul Ehrlich (Duque de Caxias—RJ, Brazil). The human keratinocyte and mouse fibroblasts cell lines used are described in the ATCC (American Type Culture Collection).

The cells were seeded at the density of 5×10^3 cells/well for HaCat and 1×10^4 for L-929 and B16F10 in a 96-well plate. After 24 hours, the cells were treated with SEML (batch 4) at concentrations ranging from 2000 µg/mL to 0.98 µg/mL. For negative control cells were treated with 5% ethanol and methanol, solvents used in the extraction process and to dissolve the plant extract, respectively. SDS 10% was used as cell death control. Following 24 hours after extracts' treatment, cell death was assessed by MTT assay using Cell Counting Kit-8 (CCK-8, Sigma) reagent and the absorbance was measured at 450 nm in the same microplate reader mentioned above. CCK-8 uses WST-8, which is bio-reduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium and is directly proportional to the number of living cells. All experiments were performed in triplicates.

Results and Discussion

Reproducibility of the extraction process

The total solids contents found for batches 1, 2, 3, 4 and 5 were $1.70\% \pm 0.26$; $1.83\% \pm 0.12$; $1.57\% \pm 0.06$; $1.53\% \pm 0.06$ and $1.50 \pm 0.10\%$, respectively. The yields of the extraction processes of *M. nigra* leaves by 95% ethanol for batches 1, 2, 4 and 5 were 7.82%; 7.35%; 8.52%; 7.68%, respectively. A sample from batch 3 was discarded due to contamination during the drying period, therefore the yield could not be calculated for this batch. The humidity contents found for batches 1, 2, 3, 4 and 5 were $0.67\% \pm 0.15$; $0.60\% \pm 0.10$; $0.70\% \pm 0.10$; $0.76\% \pm 0.19$ and $0.67\% \pm 0.06$, respectively. The data suggest that the profile of the moisture content is directly proportional to the efficiency of the extraction process. The higher the yield obtained, the greater the moisture content of the extract, as observed for batch 4 which has the largest percentage yield (8.52%) and moisture content (0.76%). In contrast, the batch with lowest yield (7.35%) had the lowest moisture content (0.60%, batch 2). The results show no significant difference between batches for total solids content and humidity content in analysis by ANOVA ($p < 0.05$) with Tukey method of multiple comparison. It was demonstrated similarity among the five batches of standardized extract of *M. nigra* leaves for the parameters evaluated, indicating that the process of obtaining the standardized extract was reproducible.

Evaluation of tyrosinase inhibition

To assess the inhibition potential of *M. nigra* leaf extract on the enzyme tyrosinase, the five batches were tested and compared against a positive control, kojic acid, a natural agent from the fungus *Aspergillus oryzae*. In order to study the inhibition profile of the extracts on tyrosinase activity the IC₅₀ values were determined by the construction of dose-response curves through serial dilution of the extracts and kojic acid at concentrations of 1000 µg/mL to 0.49 µg/mL, using the software GraphPad Prism. The percentages of tyrosinase inhibition were

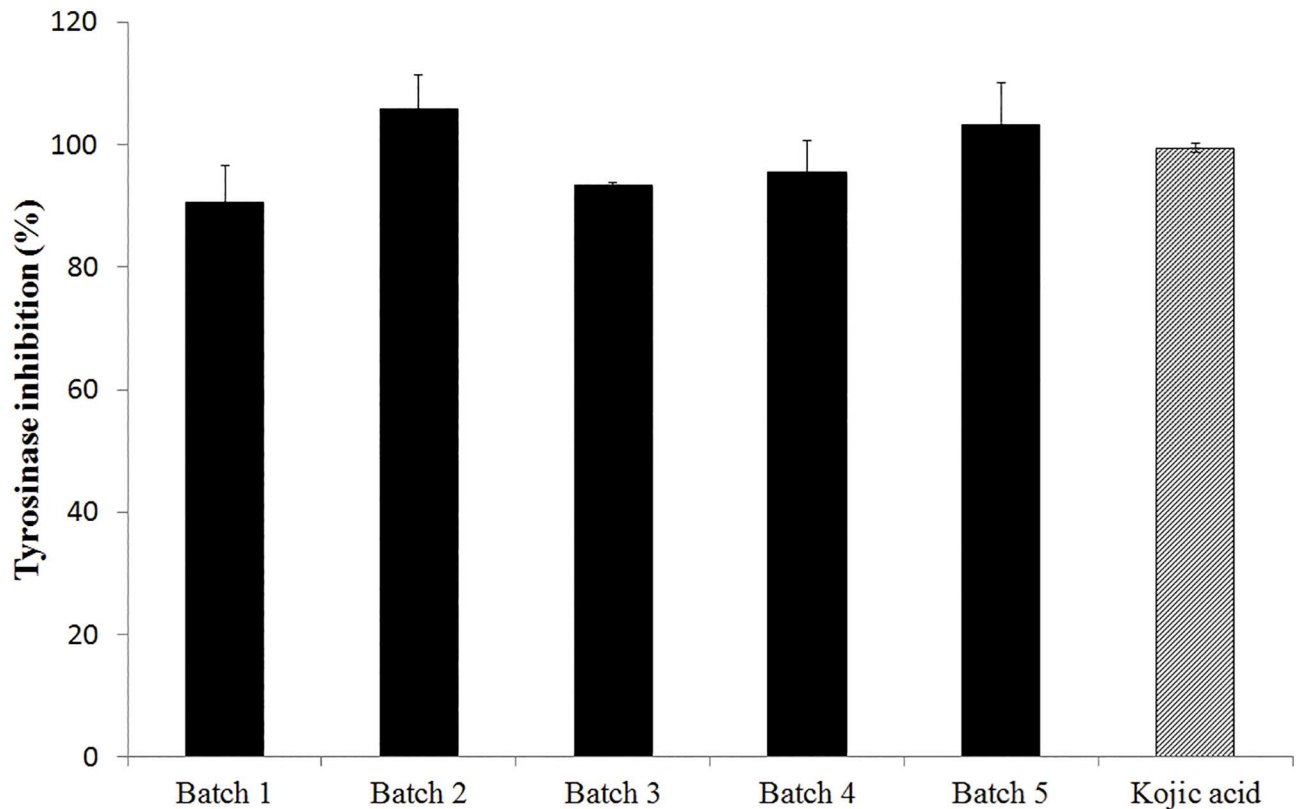


Fig 1. Tyrosinase inhibition activity profile of five standardized extracts of *M. nigra* leaves and kojic acid.

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90.48% ± 6.02; 100.00% ± 5.41; 93.45% ± 0.35; 95.59% ± 5.14 and 100.00 ± 6.70% for batches 1, 2, 3, 4 and 5, respectively. Kojic acid showed tyrosinase inhibitory activity of 99.41% ± 0.78. The data showed that the five batches of *M. nigra* were able to inhibit the enzyme tyrosinase, showing an inhibition higher than 90% until concentration of 15.625 µg/mL for all batches, similar to kojic acid (Fig 1).

The calculated IC₅₀ values were 7.75 µg/mL ± 1.55; 5.14 µg/mL ± 0.24; 5.00 µg/mL ± 0.23; 7.31 µg/mL ± 0.55 and 8.49 µg/mL ± 0.59 for batches 1, 2, 3, 4 and 5, respectively. Kojic acid obtained IC₅₀ value of 3.37 µg/mL ± 0.65. In analysis by Student's t test the five batches of standardized extract of *M. nigra* leaves showed significant difference ($p < 0.05$) compared to kojic acid (Fig 2).

The solvents used in the extraction process and solubilization of the plant extract to carry out this assay, 95% ethanol and 5% methanol, respectively, showed no tyrosinase inhibitory activity, thus ensuring non-interference with the results in enzyme inhibition. When leaving the microplate to rest for 24 hours a color change was observed from orange to black in wells where there was no enzyme inhibition. The enzyme reaction between tyrosinase and L-tyrosine generates an orange dopachrome, which leads to black melanin (eumelanin) synthesis in melanogenesis pathway [4]. Therefore, it is suggested that the color change is related to eumelanin synthesis. It was observed that kojic acid showed color change in wells of concentrations higher than the IC₅₀ values calculated in 30 minutes incubation period (solution in wells turned black in long-term incubation from 0.49 to 62.5 µg/mL), whereas all *M. nigra* extracts showed color change in the same wells of concentrations below its IC₅₀ values (solution in wells turned black

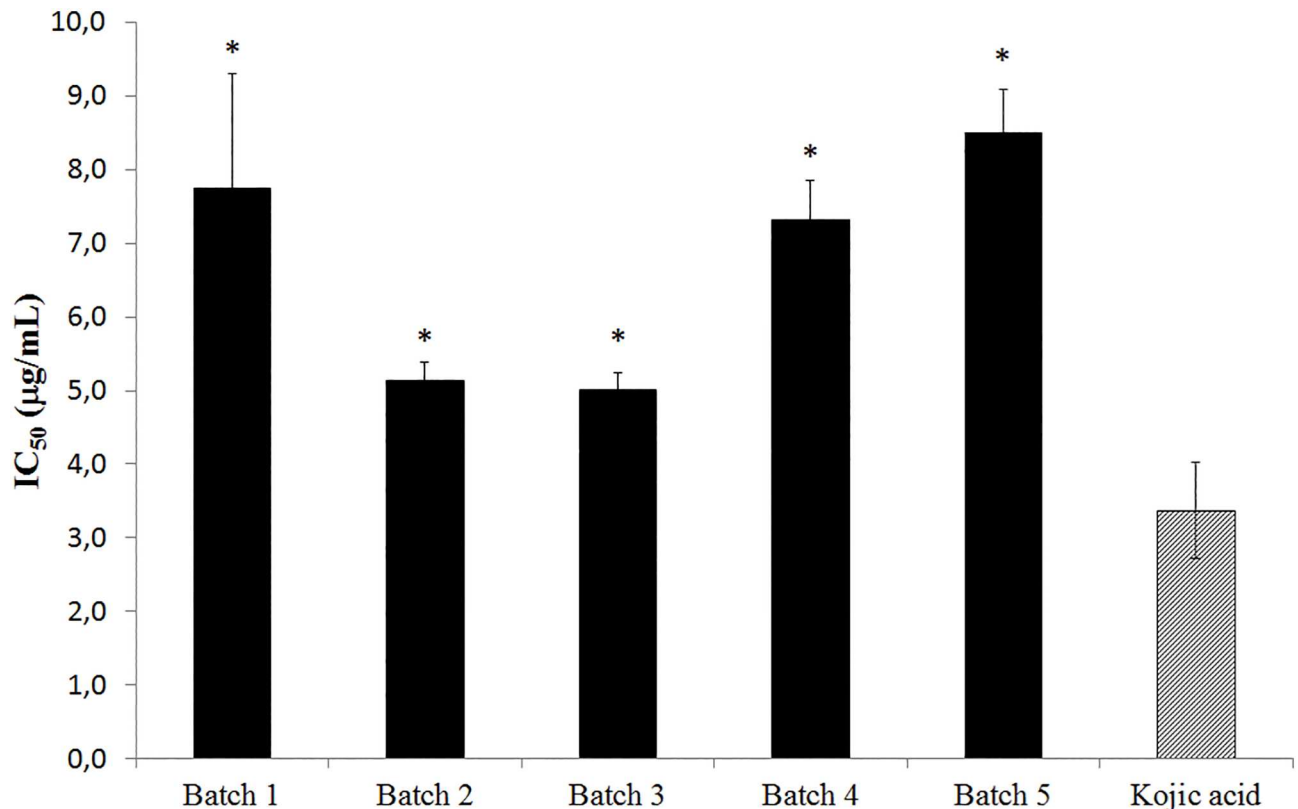


Fig 2. IC₅₀ values of five standardized extracts of *M. nigra* leaves compared to kojic acid. The significant differences related to the IC₅₀ values on tyrosinase inhibition activity (* $p < 0.05$) were calculated by Student's t test.

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in long-term incubation from 0.49 to 7.81 µg/mL in all batches), indicating that *M. nigra* extract is more stable than kojic acid at tyrosinase inhibition, since the positive control was not able to maintain its inhibitory activity over time.

Chang et al. (2011) found that the ethanolic extract of *M. alba* L. twigs and ethanolic extract of *M. alba* L. root bark exhibited 0–78% and 0–62% inhibitory effects on tyrosinase activity in the range of 0–60 µg/mL [45], while the standardized extract of *M. nigra* leaves showed tyrosinase inhibition above 90% at 60 µg/mL concentration. Zheng et al. (2012) found IC₅₀ values of 22.53±0.08 µg/mL and 27.88±0.11 µg/mL for *M. australis* root and twig, respectively [46], while the standardized extract of *M. nigra* leaves showed IC₅₀ values from 5.0±0.23 µg/mL to 8.49±0.59 µg/mL.

HPLC analysis

In the present work, HPLC with UV detection and gradient mobile phase mode was proposed as a suitable method for quantitative determination of chlorogenic acid, rutin and isoquercitrin in standardized extract of *M. nigra* leaves (Fig 3).

Peak areas were used in order to quantify the contents of these polyphenols in SEM. Three linear equations obtained by the linear regression of peak areas versus concentrations of the standards were employed, resulting in 0.50, 1.27 and 2.42 mg/g for chlorogenic acid, rutin and isoquercitrin in SEM, respectively. Sánchez-Salcedo et al. (2016) investigated the fingerprint of polyphenolic compounds in leaves of mulberry clones from *M. alba* and *M. nigra* which revealed

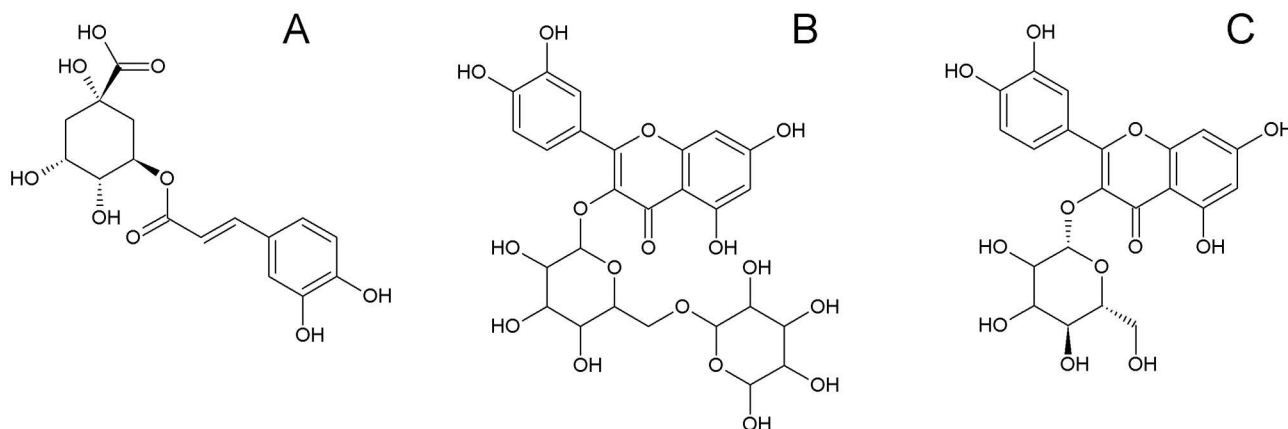


Fig 3. Identified compounds in standardized extract of *M. nigra* leaves. (A) Chlorogenic acid. (B) Rutin. (C) Isoquercitrin.

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that flavonols were the most relevant and caffeoylquinic acids are the phenolic compounds present in larger quantities in mulberry leaves, being chlorogenic acid (5-caffeoylquinic acid) the predominant one [63]. In a previous study, Sánchez-Salcedo et al. (2015) found that the predominant flavonols in mulberry leaves of *M. alba* and *M. nigra* species were quercetin-3-O-glucoside (isoquercitrin), followed by quercetin-3-O-(6-malonyl)- β -glucopyranoside = quercetin-rutinoside (rutin) and kaempferol-3-O-(6-malonyl) glucoside. In particular, they ranged from 0.92 to 3.73 mg/g dw for quercetin-3-glucoside, from 0.35 to 1.84 mg/g dw for quercetin-3-O-(6-malonyl)- β -glucopyranoside, between 0.58 to 1.80 mg/g dw for rutin, and from 0.11 to 0.80 mg/g dw for kaempferol-3-O-(6-malonyl)glucoside [64]. Isoquercitrin was found to be the major compound followed by rutin, as shown on Fig 4. In *M. alba* 70% ethanol leaf extract, Hunyadi et al. (2012) [65] found $3.58 \pm 0.06\%$ of chlorogenic acid, $1.96 \pm 0.03\%$ of rutin and $1.20 \pm 0.02\%$ of isoquercitrin, chlorogenic acid being the major compound.

When undergoing acid hydrolysis (Fig 5A) chlorogenic acid, rutin and isoquercitrin decreased by 80%, 88% and 91% from the average of the measurements taken of their peak areas, respectively, but chlorogenic acid and rutin did not elute in the same retention time of the degradation products generated by acid hydrolysis. Basic hydrolysis (Fig 5B) degraded chlorogenic acid. Flavonoids rutin and isoquercitrin decreased by 54% and 48% from the average of the measurements taken of their peak areas, respectively, but did not elute in the same retention time of the degradation products generated by basic hydrolysis, demonstrating method specificity.

The linear equation was calculated from the average absorbance values obtained in the studied concentration range (0.2–10.0 $\mu\text{g/mL}$ for chlorogenic acid and 0.1–10.0 $\mu\text{g/mL}$ for rutin and isoquercitrin). Linear regression was built from three independent calibration curves for chlorogenic acid, rutin and isoquercitrin (Fig 6).

The correlation coefficient (r), intercepts, the slope and the residual sum of squares are shown on Table 1. All calibration curves showed satisfying linear regressions within testing ranges, since all correlation coefficients are above 0.99.

In intra-day precision the mean relative standard deviation values found in the analysis of solubilized SEML (2 mg/mL) were of 10.20%, 11.73% and 8.33% for chlorogenic acid, rutin and isoquercitrin, respectively. In the inter-day analysis, variations of 6.99%, 3.59% and 3.99% were found for chlorogenic acid, rutin and isoquercitrin, respectively, as shown on Table 2. The method is precise for all three polyphenols studied once it is in agreement with the

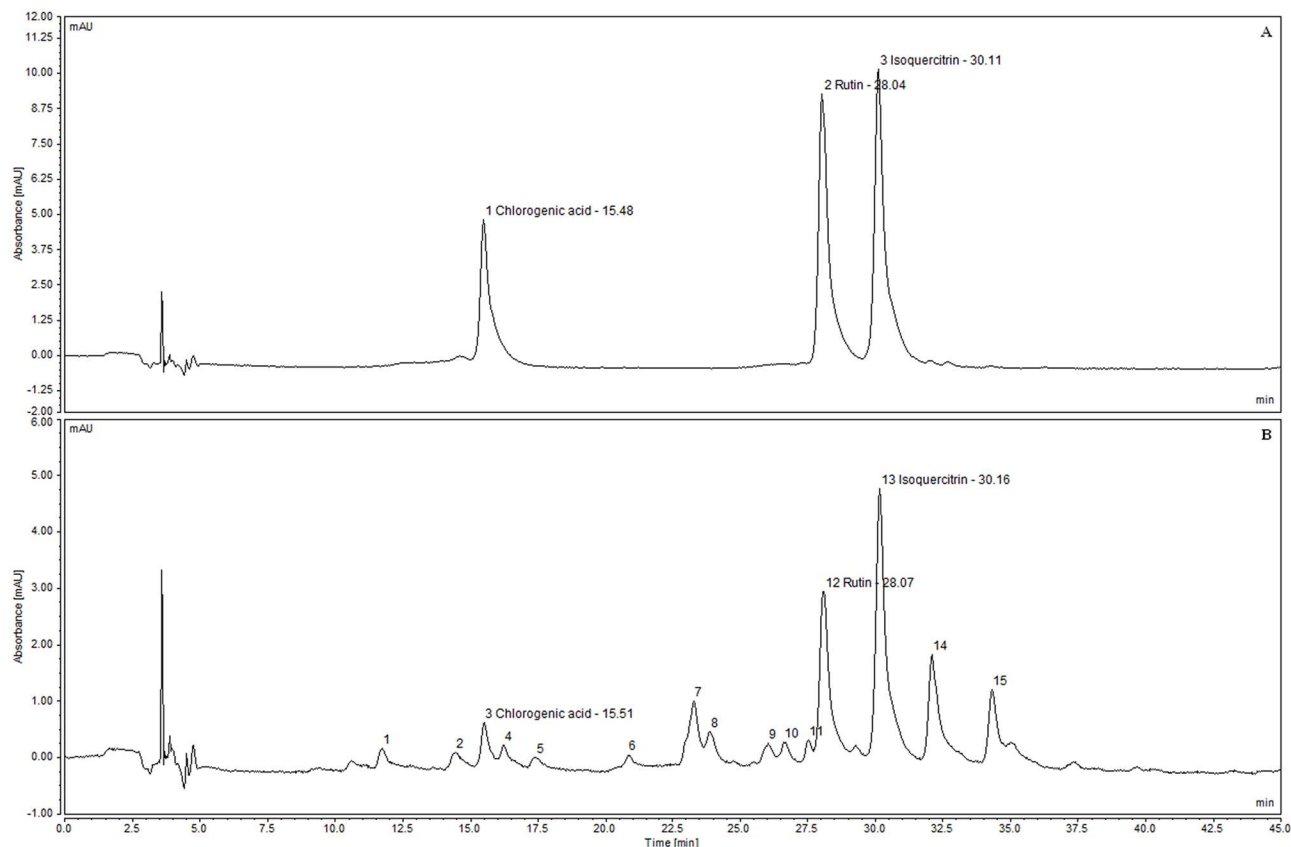


Fig 4. HPLC chromatograms. (A) Reference standards (10 µg/mL). (B) Diluted standardized extract of *M. nigra* leaves (2 mg/mL) $\lambda = 354$ nm.

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minimum requirements stated in the orientation guide for the registration of herbal drugs [66]. According to these rules, values of Relative Standard Deviation (RSD) greater than 15% are not admitted.

The mean values (\pm standard deviation) of the percentage analytical recoveries for the experimental concentration over the theoretical concentration for chlorogenic acid, rutin and isoquercitrin are presented on Table 3. These results indicate that the chromatographic conditions used are reliable to quantify these polyphenols in the range evaluated.

The detection and quantitation limits found for chlorogenic acid, rutin and isoquercitrin were determined based on the parameters of the calibration curve, which contains the compounds of interest at a range of concentrations near the detection limit (0.2–1.0 µg/mL for chlorogenic acid and 0.1–0.5 µg/mL for rutin and isoquercitrin). The estimated concentration values calculated using the standard deviation (σ) of the intercept for limit of detection were 0.11, 0.12 and 0.10 µg/mL for chlorogenic acid, rutin and isoquercitrin, respectively. The calculated quantitation limits were 0.34, 0.35 and 0.30 µg/mL, respectively. Further evaluation, using the visual method, investigated seven injections of the analyzed polyphenols at lower concentrations of 0.1 and 0.075 µg/mL. It was shown that although chlorogenic acid may not be quantified in these concentrations at a wavelength of 354 nm, the polyphenol can be quantified with precision at 330 nm, wavelength in which it has higher absorption, in the lowest concentration evaluated of 0.075 µg/mL. Flavonoids rutin and isoquercitrin were detected in 0.075 µg/mL

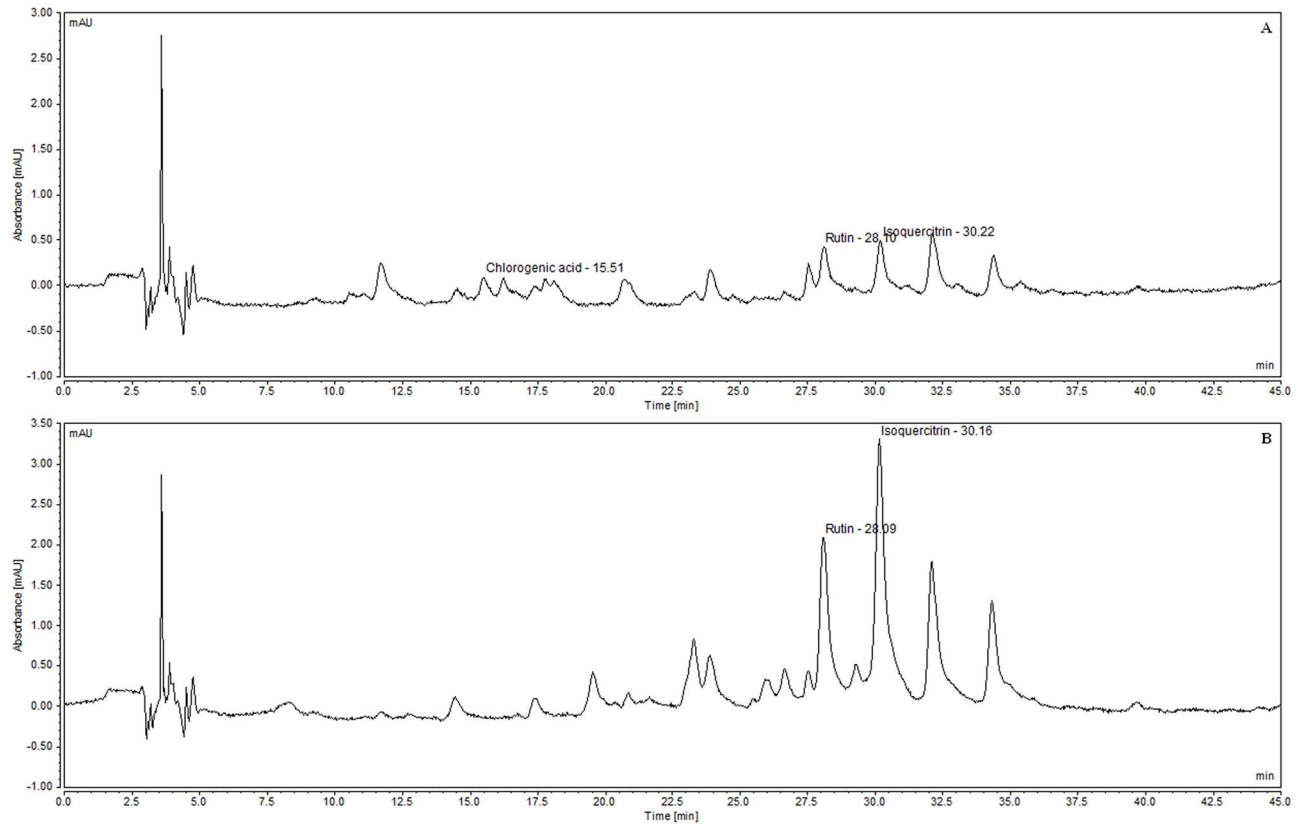


Fig 5. Specificity of the proposed method. (A) Acid hydrolysis and (B) Basic hydrolysis of diluted standardized extract of *M. nigra* leaves (2 mg/mL) $\lambda = 354\text{nm}$.

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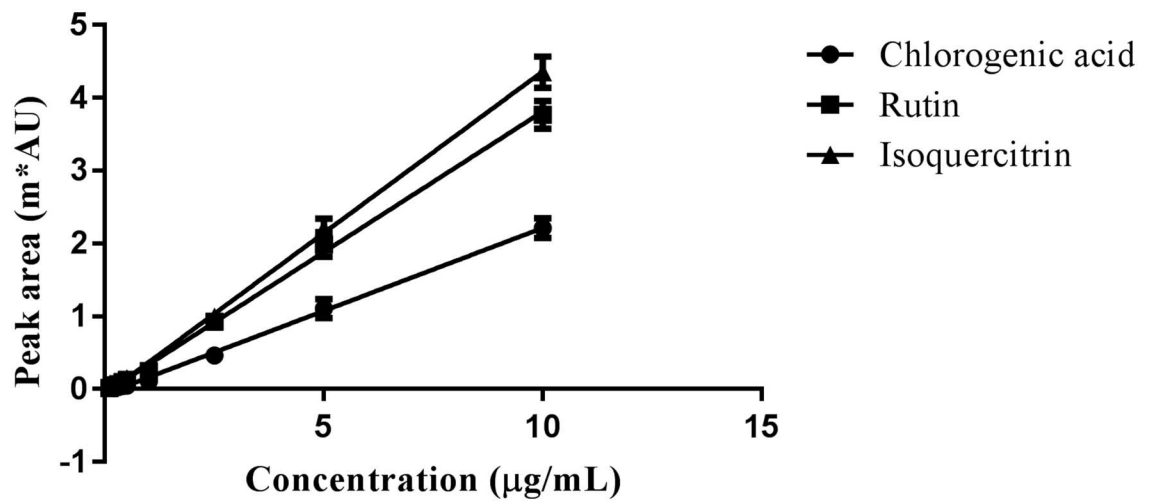


Fig 6. Calibration curves for chlorogenic acid, rutin and isoquercitrin.

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Table 1. Linearity of chlorogenic acid, rutin and isoquercitrin.

Polyphenol	Concentration (µg/mL)	Correlation coefficient (r)	Intercept	Slope	Residual sum of squares
Chlorogenic Acid	0.2–10.0	0.996	-0.0647 ± 0.0176	0.228 ± 0.00431	0.0682
Rutin	0.1–10.0	0.997	-0.0483 ± 0.0215	0.386 ± 0.00560	0.0912
Isoquercitrin		0.998	-0.0746 ± 0.0224	0.444 ± 0.00584	0.0951

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Table 2. Precision of chlorogenic acid, rutin and isoquercitrin.

Precision	Concentration (µg/mL)	RSD (%)
Chlorogenic acid		
Intra-day ^a	1.15 ± 0.12	10.20
Inter-day ^b	0.99 ± 0.07	6.99
Rutin		
Intra-day ^a	2.89 ± 0.34	11.73
Inter-day ^b	2.85 ± 0.10	3.59
Isoquercitrin		
Intra-day ^a	4.99 ± 0.42	8.33
Inter-day ^b	5.13 ± 0.20	3.99

^a6 replicates were assayed on the same day;

^b3 replicates were assayed on different days (RSD = Relative Standard Deviation). The results are mean ± standard deviation.

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and quantified with good precision at 0.1 µg/mL. The distinction between noise and analytical signal becomes less precise in concentrations below 0.1 µg/mL, therefore the limits of detection and quantitation determined for rutin and isoquercitrin are 0.075 and 0.1 µg/mL, respectively, shown on Table 4.

Other LOD and LOQ values found in the literature include 0.36 and 1.08 µg/mL [67], 14.95479 and 45.31756 µg/mL [68], respectively, for chlorogenic acid. Rutin values include

Table 3. Accuracy of chlorogenic acid, rutin and isoquercitrin.

Concentration (%)	Theoretical concentration (µg/mL)	Experimental concentration (µg/mL)	Recovery (%)
Chlorogenic Acid			
72 ^a	0.75	0.73 ± 0.06	97.49 ± 7.74
95 ^b	1.00	0.91 ± 0.06	91.40 ± 5.69
117 ^c	1.25	1.08 ± 0.06	86.49 ± 4.50
Rutin			
80 ^a	2.02	1.98 ± 0.05	98.44 ± 2.33
99 ^b	2.52	2.44 ± 0.15	97.07 ± 6.03
119 ^c	3.02	2.86 ± 0.11	95.01 ± 3.81
Isoquercitrin			
81 ^a	3.92	4.00 ± 0.07	102.06 ± 1.85
102 ^b	4.92	5.06 ± 0.23	102.92 ± 4.64
122 ^c	5.92	6.10 ± 0.35	103.09 ± 5.89

^aLow concentration;

^bIntermediate concentration;

^cHigh concentration. The results are mean ± standard deviation of 3 experiments.

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Table 4. Limits of detection (LOD) and quantitation (LOQ) for chlorogenic acid, rutin and isoquercitrin.

Polyphenol	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Chlorogenic acid	0.11	0.34
Rutin	0.075	0.1
Isoquercitrin	0.075	0.1

LOD = Limit of Detection; LOQ = Limit of Quantitation.

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LOD and LOQ of 0.09 and 0.29 $\mu\text{g/mL}$ [69], and 0.19 and 0.60 $\mu\text{g/mL}$ [70], respectively. Isoquercitrin LOD and LOQ values include 0.57 and 1.88 $\mu\text{g/mL}$ [71], respectively. The limits of detection and quantitation found in the present study are lower than above cited, showing good sensitivity to the method in detecting and quantifying all three polyphenols.

Wavelength changes did not lead to changes in the retention times for the three studied polyphenols. There was an already expected change in the area obtained for chlorogenic acid since it has higher absorption at 330 nm. The flavonoids rutin and isoquercitrin have higher absorption at 354 nm than at other wavelengths (280 nm and 330 nm), thereby obtaining a higher peak area in this wavelength (Fig 7). An increase in peak base width is also observed in this case, thus diminishing its resolution.

Increase in the column oven temperature caused displacement of the peaks, thereby altering the retention times and areas (Fig 8). Higher temperatures caused a faster elution of

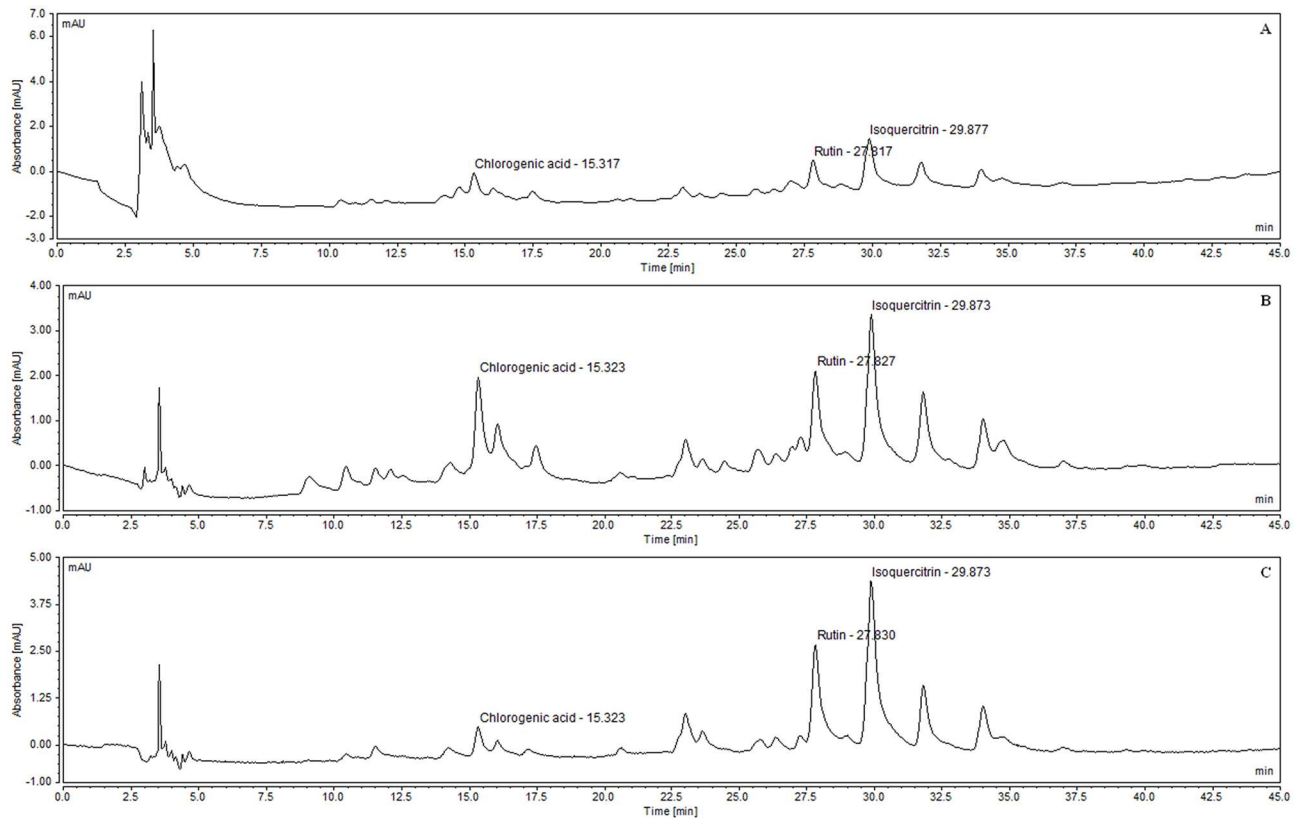


Fig 7. Wavelength variation on robustness. (A) 330 nm (B) 280 nm (C) 354 nm.

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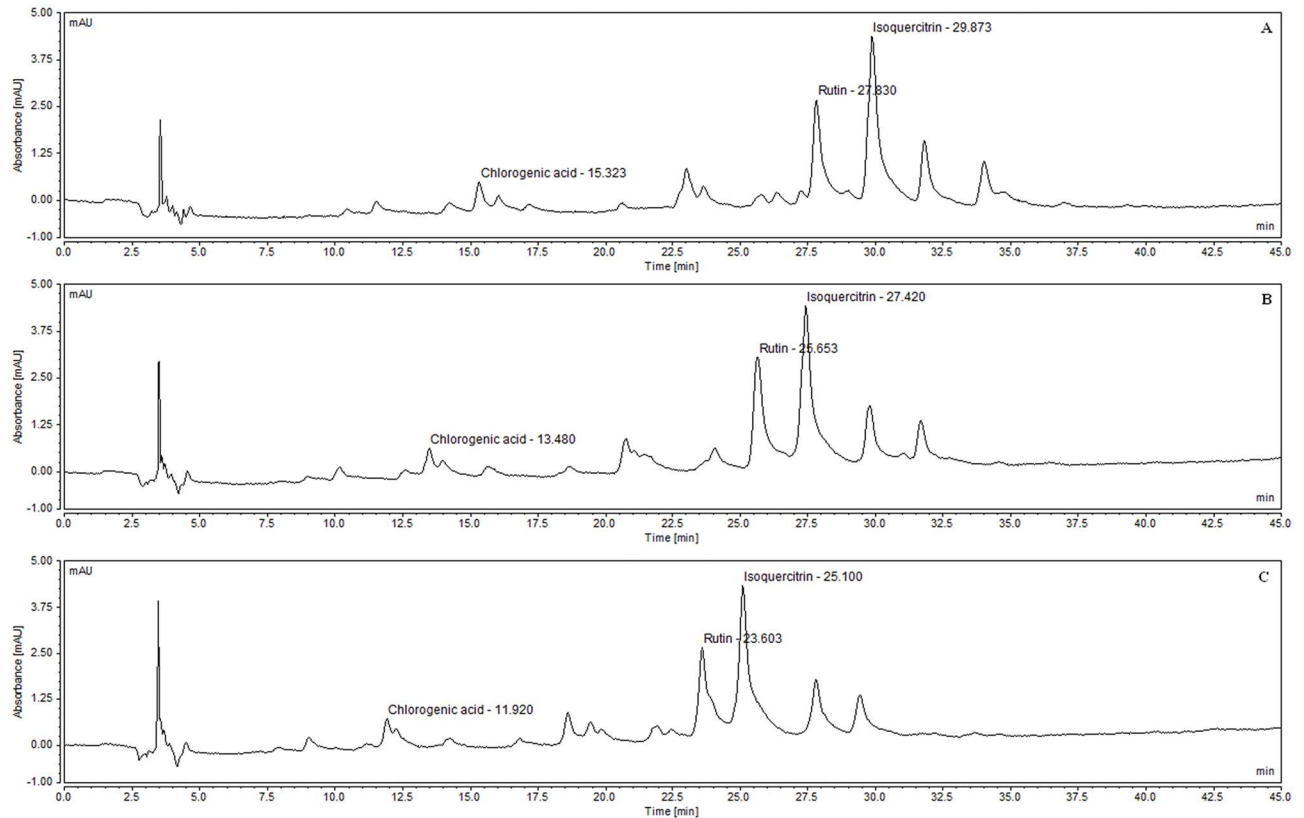


Fig 8. Temperature variation on robustness. (A) 25°C (B) 35°C (C) 45°C.

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compounds, as expected. It was observed an increase in rutin peak area at 35°C, which can be explained by the elution of another compound in the same retention time. There was a decrease of peak area for all polyphenols when column oven temperature was 45°C.

Flow change caused displacement of the peaks, thereby altering the retention times and areas (Fig 9). Higher flows caused a faster elution of compounds, as expected. Chlorogenic acid was not detected in the 0.3 mL/min flow, which makes this method less robust for this particular polyphenol, but still maintains robustness for the other parameters analyzed. It was observed an increase in peak area for all compounds when flow was slowed down to 0.3 mL/min, without altering peak purity. On the other hand, a reduction in peak area was observed for of all compounds when flow was sped up to 0.7 mL/min. The increase and decrease of peak area observed with flow reduction and elevation, respectively, are explained by the relationship between the flow rate and the area, in which the peak width will increase as the flow rate is reduced and consequently so will the peak area. Thus increasing the flow rate will result in a decrease in the response from the detector [72].

The method was robust for all three polyphenols and results are shown on Table 5.

The quantitation of chlorogenic acid, rutin and isoquercitrin in all batches showed highest polyphenol content in batch 2, followed by batch 3, batch 1, batch 4 and batch 5. The polyphenols content in the extract is inversely proportional to the IC₅₀ values calculated for tyrosinase inhibition. Batches with the highest concentrations of polyphenols in the extract showed the lowest IC₅₀ values, as seen with batch 2 (5.14 µg/mL ± 0.24) and batch 3 (5.00 µg/mL ± 0.23). Given that the major compounds in the standardized extract of *M. nigra* leaves, isoquercitrin

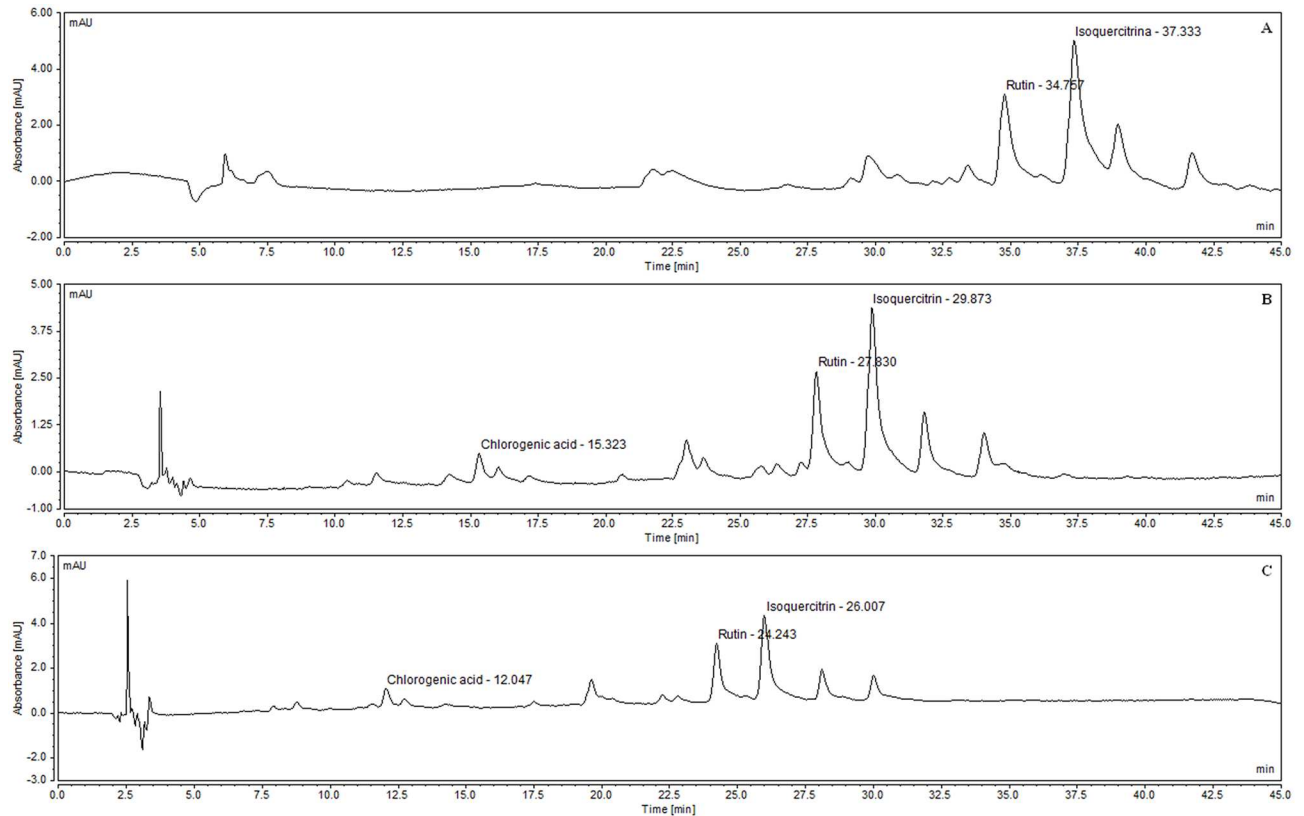


Fig 9. Flow variation on robustness. (A) 0.3 mL/min (B) 0.5 mL/min (C) 0.7 mL/min.

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and rutin, have inhibitory activities on tyrosinase due to their metal-chelating capacities [45, 73, 74] since copper is an essential cofactor for tyrosinase activity [75], it is possible to conclude that the higher the concentration of polyphenols in the extract, greater biological activity on tyrosinase inhibition, and thus, the lower the IC₅₀. According to Li et al. (2014) chlorogenic acid is probably a substrate of melanin, but their metabolic products can suppress

Table 5. Robustness of chlorogenic acid, rutin and isoquercitrin.

Parameters		Chlorogenic acid		Rutin		Isoquercitrin	
		Retention time (min)	Area (mAU*min)	Retention time (min)	Area (mAU*min)	Retention time (min)	Area (mAU*min)
Wavelength (nm)	280	15.29 ± 0.02	0.26 ± 0.01	27.80 ± 0.02	0.42 ± 0.00	29.86 ± 0.02	0.95 ± 0.01
	330	15.30 ± 0.02	0.57 ± 0.00	27.80 ± 0.03	0.64 ± 0.01	29.85 ± 0.02	1.44 ± 0.02
	354	15.29 ± 0.03	0.17 ± 0.01	27.81 ± 0.03	0.92 ± 0.01	29.85 ± 0.02	1.98 ± 0.02
Temperature (°C)	25	15.30 ± 0.02	0.17 ± 0.01	27.81 ± 0.03	0.92 ± 0.01	29.85 ± 0.02	1.98 ± 0.02
	35	13.47 ± 0.01	0.14 ± 0.00	25.65 ± 0.01	1.03 ± 0.04	27.42 ± 0.00	1.74 ± 0.04
	45	11.90 ± 0.02	0.12 ± 0.00	23.59 ± 0.01	0.44 ± 0.01	25.09 ± 0.01	1.85 ± 0.03
Flow (mL/min)	0.3	not detected	not detected	34.75 ± 0.02	1.52 ± 0.03	37.33 ± 0.01	2.78 ± 0.03
	0.5	15.30 ± 0.02	0.17 ± 0.01	27.81 ± 0.03	0.92 ± 0.01	29.85 ± 0.02	1.98 ± 0.02
	0.7	12.05 ± 0.01	0.15 ± 0.00	24.23 ± 0.02	0.72 ± 0.01	25.98 ± 0.02	1.40 ± 0.03

The results are mean ± standard deviation of 3 experiments.

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melanogenesis in B16 melanoma cells by inhibiting tyrosinase activity [76]. Furthermore, in a study conducted by Drewa et al. (1998) mice were injected with B16 melanoma cells and treated rutin solution, wherein the administration of 10 mg of rutin inhibited melanin formation by approximately 43% [77].

The RSD for the analysis of each batch performed in triplicate was less than 15%, which indicates good accuracy as the literature does not admit values greater than 15% [66].

It was possible to observe similar chromatographic profiles on all batches, wherein the major compound is isoquercitrin, followed by rutin. The ratios of polyphenol concentrations present in the extract were calculated. The concentration of isoquercitrin in SEML is, on average, 2.34 times greater than chlorogenic acid and, on average, 1.54 times greater than rutin in all batches (Fig 10).

The quantification of the polyphenols in each batch was analyzed statistically by one-way ANOVA with Tukey test ($p < 0.05$). There was a significant difference in the chlorogenic acid content between batch 2 with batches 4 and 5, and also significant difference between batches 3 and 5. For rutin and isoquercitrin there was significant difference between batches 2 and 3 with batches 1, 4 and 5 (Fig 11).

Cytotoxicity analysis

The cytotoxic effect of ethanol extract from leaves of *M. nigra* were evaluated in three cell lines, fibroblast, keratinocyte and melanoma, which take part of skin components. Treatment with SEML at the approximate tyrosinase inhibition IC_{50} value concentration (7.81 $\mu\text{g}/\text{mL}$) did not result in cell death in any cell lines, in comparison to control, after 24 hours of treatment. In all cell lines cytotoxicity was induced at the concentration of 1000 $\mu\text{g}/\text{mL}$ after 24 hours of treatment, as shown in Fig 12. The solvents used in the extraction process and solubilization of the plant extract to carry out this assay, 95% ethanol and 5% methanol, respectively, showed no cytotoxic effects, thus ensuring non-interference with the cytotoxicity results. There was no interaction between SEML and WST-8 that could lead to false positive results when testing natural compounds with reducing potential inherent on the MTT tetrazolium assay. The IC_{50} values calculated for cytotoxicity were 107.2 $\mu\text{g}/\text{mL}$ for murine melanoma, 324.2 $\mu\text{g}/\text{mL}$ for human keratinocyte and 116.3 $\mu\text{g}/\text{mL}$ for mouse fibroblast, where at these concentration values *M. nigra* extract showed tyrosinase inhibiting activity above 90%.

SEML was more toxic to tumor cell line of murine melanoma, suggesting that the extract has anti-tumor activity as already described in the literature by Qadir et al. (2014) in *M. nigra* leaf extract against human cervical cancer cell line (HeLa) [33], by Eo et al. (2014) in the study of anti-cancer activity by *M. alba* root bark extract on human colorectal cancer cell line (SW480) [78] and Turan et al. (2016) in the antiproliferative and apoptotic effect of *M. nigra* fruit extract on human prostate cancer cells (PC-3) [79].

Conclusions

Five batches of standardized extract of *M. nigra* leaves were obtained, which showed reproducibility of its extraction process for total solids content, yield and moisture content. A novel source of tyrosinase inhibitor was found, which activity was also reproducible between batches. The present work developed an innovative standardization of *M. nigra* leaves extract using an HPLC-DAD technique for determination of polyphenols chlorogenic acid, rutin and isoquercitrin. The method validation data indicate that the method is reliable and important to the development of an herbal medicine, which serves as a tool for quality control of *M. nigra* extracts. The cytotoxicity results indicate that this extract is promising as a topic cosmetic due

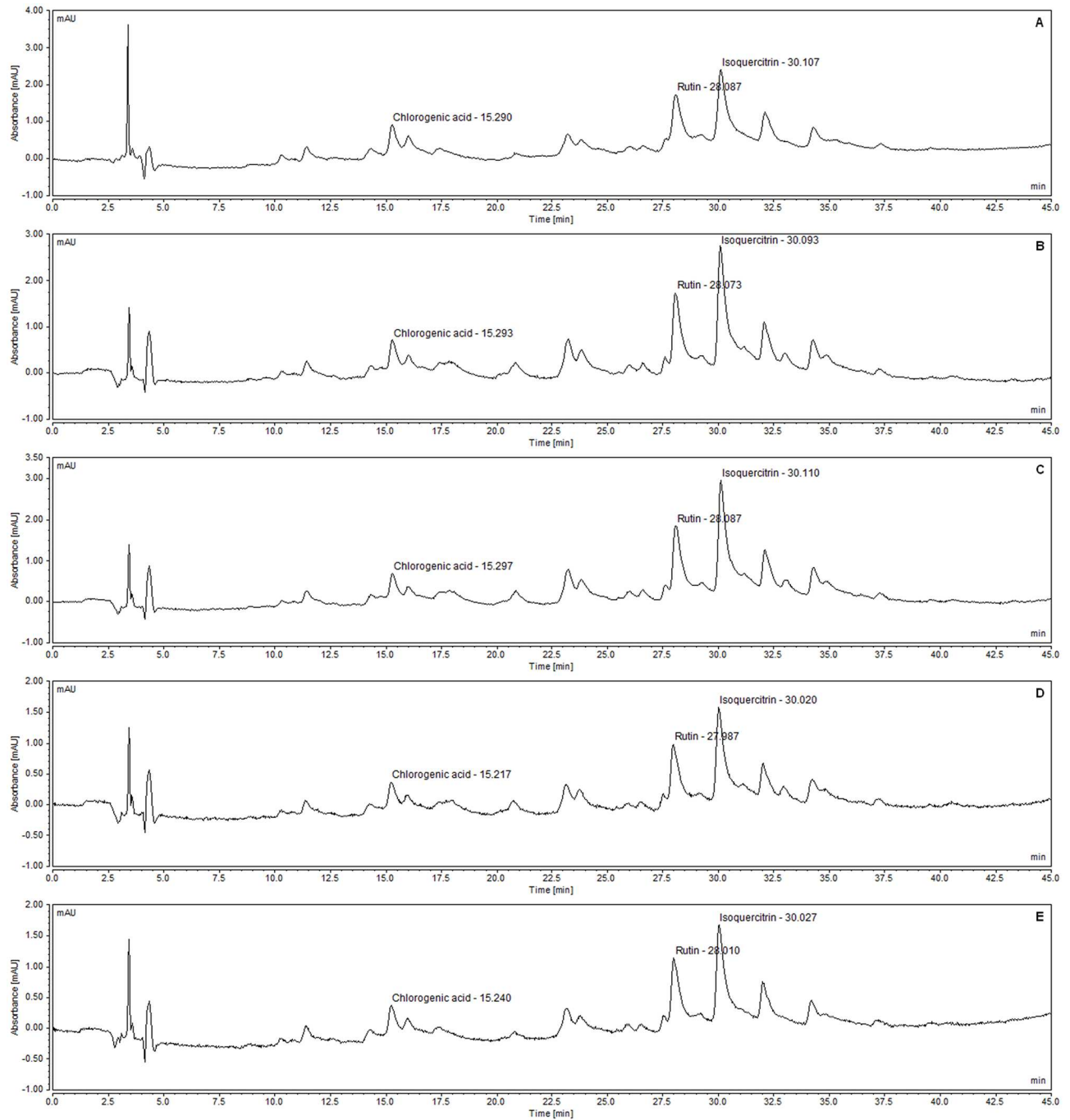


Fig 10. HPLC chromatogram of standardized extract of *M. nigra* leaves (2 mg/mL). (A) Batch 1. (B) Batch 2. (C) Batch 3. (D) Batch 4. (E) Batch 5.

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to low toxicity to cell lines that constitute the skin with high tyrosinase inhibition. The standardized extract of *M. nigra* leaves can be used as a potential skin depigmentation agent in the pharmaceutical and cosmetic industries and as an antibrowning agent of fruits, vegetables and beverages in the food industry.

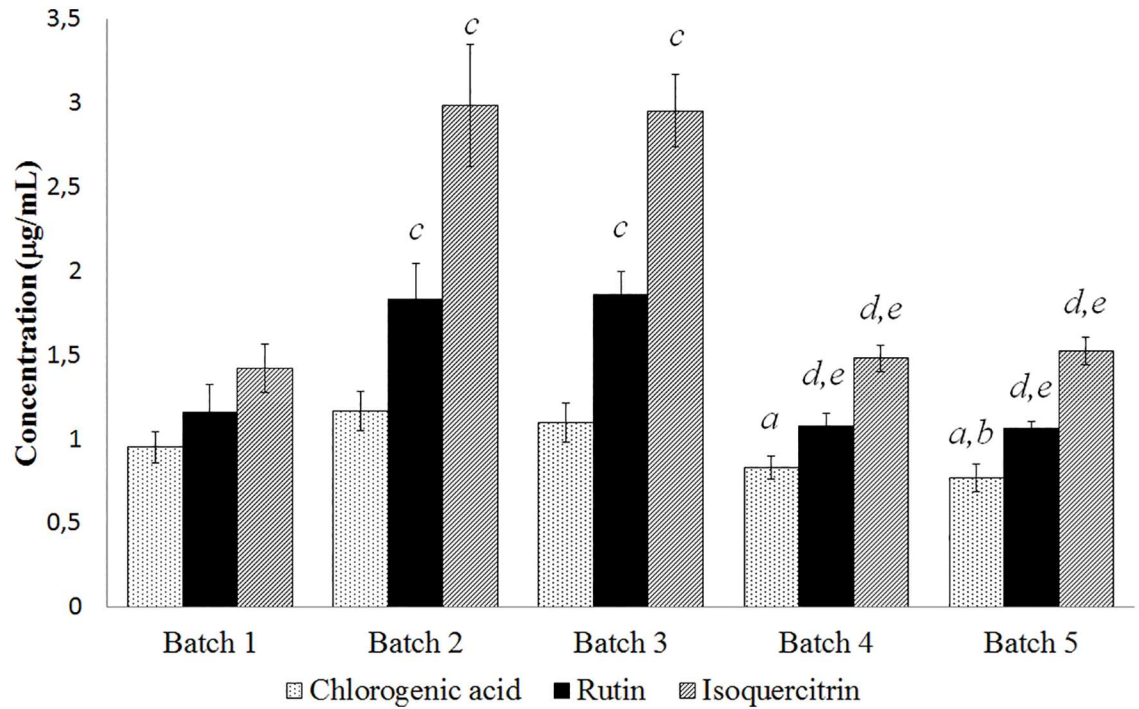


Fig 11. Quantification of chlorogenic acid, rutin and isoquercitrin of standardized extract of *M. nigra* leaves. The significant differences related to the concentration values of five batches in three experiments were calculated by one-way ANOVA with Tukey test (^a $p < 0.05$ vs Batch 2. ^b $p < 0.05$ vs Batch 3. ^c $p < 0.05$ vs Batch 1. ^d $p < 0.05$ vs Batch 2. ^e $p < 0.05$ vs Batch 3).

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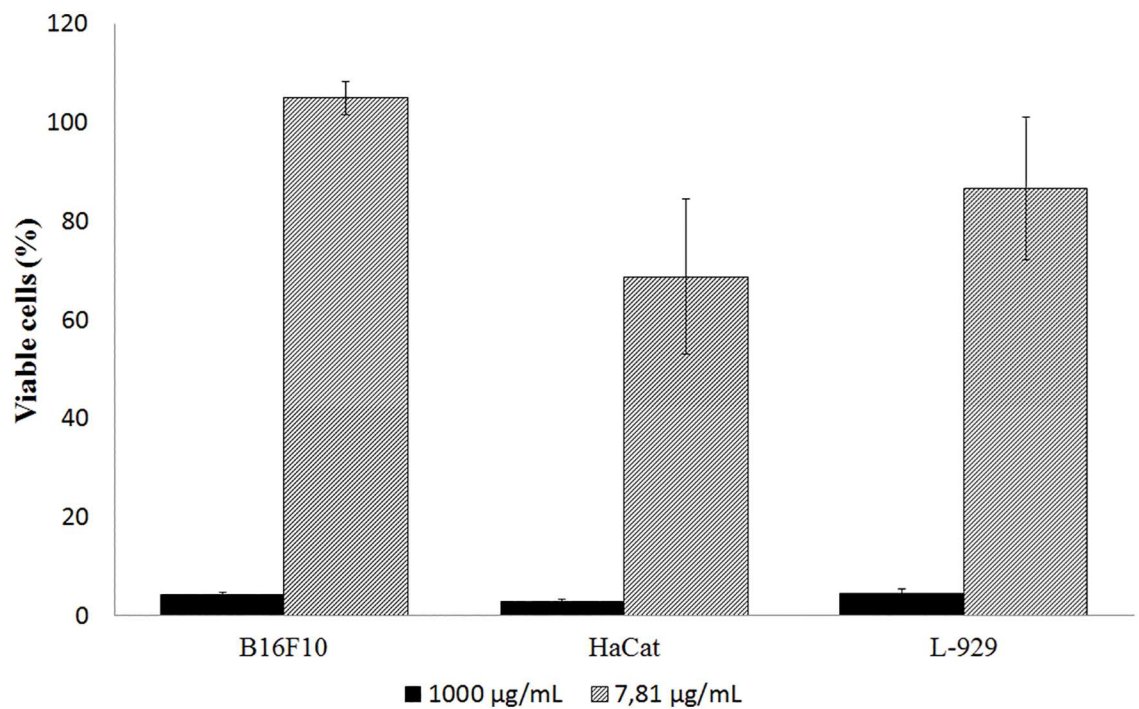


Fig 12. Cytotoxicity assay of melanoma (B16F10), keratinocyte (HaCat) and fibroblast (L-929) cell lines.

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Author Contributions

Conceptualization: POM.

Data curation: MMF PRF MHM PMS YFB.

Formal analysis: MMF DS ENSG YKMN.

Funding acquisition: POM.

Investigation: MMF.

Methodology: MMF MHM PRF PMS YFB.

Project administration: POM.

Resources: POM ENSG YKMN CWF LAS.

Software: MMF PRF MHM PMS.

Supervision: POM MHM.

Validation: MMF PRF MHM.

Visualization: POM MHM DS YFB.

Writing – original draft: MMF PRF PMS MHM YFB.

Writing – review & editing: POM MHM MMF PMS YFB DS.

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