Rosa and Isaac. ULAKES J Med. v.1 n.2 p. 99-106, **2021**

Artigo Original

Action of the jabuticaba peel extract (*Plinia jaboticaba (Vell.) Kausel*) in *in vitro* skin irritation and cytotoxicity

Ação do extrato da casca da jabuticaba (*Plinia jabuticaba (Vell.) Kausel*) na irritação e citotoxicidade cutânea *in vitro*

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ABSTRACT
The bark of jabuticaba represent an underutilized product in the industry, since, in most cases, they are discarded after the use of the pulp. Starting from the concept that the peels are rich in several phenolic compounds, vitamins and bioactive compounds, they therefore have an active potential to be used in cosmetics. The aim of this study was to evaluate different cosmetic activities relevant to obtain a safe and effective extract. For this, the extract was obtained through remaceration. Then, using in vitro methodologies, cytotoxicity was evaluated using the MTT and NRU dyes with the cell line HaCat and the potential for skin irritation due to the degradation of ovoalbumin was evaluated. The extract of the bark of the jabuticaba showed an average cytotoxicity of 9.39 mg/mL and skin irritation rate of 0.8 to 1.6 mg/mL. Thus, our results direct the safe use of the extract of the bark of jabuticaba as a cosmetic active.
Keywords: Jabuticaba; Biodiversity; *in vitro* Skin Cytotoxicity; *in vitro* Skin Irritation.

RESUMO
A casca da jabuticaba representa um produto subutilizado na indústria, pois, na maioria das vezes, é descartada após o aproveitamento da polpa. Partindo do conceito de que as cascas são ricas em diversos compostos fenólicos, vitaminas e compostos bioativos, possuem, portanto, um potencial ativo para serem utilizados em cosméticos. O objetivo deste estudo foi avaliar atividades relevantes para a obtenção de um extrato seguro e eficaz. Para isso, o extrato foi obtido por meio de remaceração. Em seguida, usando metodologias *in vitro*, a citotoxicidade foi avaliada usando os corantes MTT e NRU com a linhagem celular HaCat e o potencial de irritação da pele devido à degradação da ovalbumina foi avaliado. O extrato da casca da jabuticaba apresentou citotoxicidade média de 9,39 mg/mL e taxa de irritação cutânea de 0,8 a 1,6 mg/mL. Assim, estes resultados direcionam o uso seguro do extrato da casca da jabuticaba como ativo cosmético.
Palavras chave: Jabuticaba; Biodiversidade; Ctotoxicidade *in vitro;* Irritação Cutânea *in vitro*.

INTRODUCTION

The jabuticabeira is a fruitful plant of the Brazilian biodiversity. They produce jabuticabas, circular fruits, with dark purple color almost black, from one to four seeds. Its shell has an astringent taste, while the pulp is very sweet. It is found in the southern and southeastern states, predominantly. It belongs to the Myrtaceae family and is divided into nine species, one considered extinct, five in research sites and three

in cultivation. The latter are: *Plinia trunciflora (Berg) Mattos* (jabuticaba-de-cabinho); *Plinia cauliflora (DC.) Berg* (jabuticaba paulista, ponhema or assu) and *Plinia jaboticaba (Vell.) Berg* (jabuticaba sabará), the best known and cultivated in the country, mainly in the states of São Paulo and Minas Gerais, which have some orchards commercial. It was first classified as *Myrciaria* in 1985 and later as *Plinia*, being nowadays considered synonymous [1,2].

The three species of jabuticaba cultivated in Brazil have very similar characteristics, which leads to a certain difficulty in botanical identification. Its high rate of perishability has been a major challenge of an industrial nature. Jabuticaba has been sold in natura, in sweets, as jellies and ice cream; drink, such as liquors and wines; bark flour, among others [3].

Jabuticaba is rich in bioactive compounds, macronutrients and micronutrients. The fruit is rich in vitamin C, tocopherol, anthocyanins, the main and in greater quantity being cyanidin-3-glycoside. It also has a large amount of phenolic compounds, flavonoids and tannins, including quercetin, gallic acid and ellagic acid. It has high values of carbohydrates (fructose, sucrose), soluble and insoluble fibers, minerals (potassium, magnesium, phosphorus, calcium) and carotenoids [4-7].

And, due to its rich composition, research that is focused on food is highlighted in the use of industrial residue from the bark of jabuticaba. This use is made in the production of cookies, cereal bars, among others. Jabuticaba residues are also studied in several areas such as: biofuel production area, pigment production, pest control in poultry production [8-13].

In the cosmetics industry, this tendency to use raw materials as natural as possible and the promotion of the use of natural assets and/or biodiversity, in their formulations has been noticed. When accompanied, this advantageous market obtained an increase in research and launch of products with the "green" label, that is, from biodiversity and also containing natural assets, which grows at a rate of almost 25%, while products with synthetic base grow around 10%. This growth is in line with the current movement of the population that seeks, every day, to grow older with health, well-being and in the most natural way possible. Thus, access to information makes consumers more aware of the consumption of their products [14].

Linked to the origin of the assets and how they are used in formulations, there is another very strong market trend: practicality. The consumer seeks cosmetic forms that combine the greatest number of functions that satisfy him. The so-called multifunctional cosmetics derive from assets with different properties [14]. Being a positive point for natural extracts, as they contain a rich and versatile composition. This is the case of the bark of jabuticaba, a waste discarded by the industry, but with great economic potential. In this article, experiments with the use of jabuticaba bark extract were presented, evaluating its potential as a new cosmetic asset,

through in vitro tests of cytotoxicity and skin irritation.

MATERIAL AND METHODS

Obtaining the extract

Five kilograms of residues from the frozen Sabará jabuticaba bark were purchased from Sitio do Belo, located in Paraibuna, in the state of São Paulo, Brazil. For the extract, alcohol 70° GL was used in the proportion 1:20 (m/v). Four extractions were performed, lasting 24 hours each. Then, the solvent was removed by rotaevaporation and, soon after, lyophilized. The extract was kept under refrigeration until freeze dried and then frozen at 4° C until use.

In vitro cytotoxicity assessment

Evaluation of cytotoxic potential by using dye *3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide* (MTT)

The assay was performed with HaCat (immortalized human keratinocytes) cell line [15,16]. HaCat were cultured in in MEM, both supplemented with 10% Bovine Fetal Serum and 1% antibiotic solution, kept at 37±2 °C in a 5% CO_2 atmosphere, in bottles suitable for cell culture. The cell density for the assays using the MTT dye was $5x10^5$ cells/mL [17]. For cell counting, trypan blue dye was used. Then, 100 µL of the solution containing the cells were transferred to each well of the 96-well plates. The plates were incubated for 24 hours. Then, the cells were treated with 100 µL of positive control (solution containing phenol), in different concentrations; negative control (culture medium with 5% serum) and different concentrations of the extract, being left in the greenhouse for 24 hours. After treatment, the treatment solutions were removed and the plates were washed gently with 150 µL PBS. After washing, 100 µL of MTT (1 mg/mL of dye in PBS) was added to each well. The cells, in the microplate, were incubated at 37±2 ºC, protected from light, for three hours, when the presence of the violet formazan crystals was observed. For the solubilization of formazan crystals, 100 µL of absolute isopropyl alcohol were used, added to each well and the spectrophotometric reading of the absorbance was performed at a wavelength of 570 nm [18].

Evaluation of cytotoxic potential by using the Neutral Red dye (NRU)

The assay used HaCat cell line. In the same parameters used previously. The cell density for the assays using the Neutral Red dye was 5×10^5 cells / mL [17]. For cell counting, trypan blue dye was used. Then, 100 µL of the solution containing the cells were transferred to each well of the 96-well plates [18]. The culture medium was replaced by serial dilutions of the sample extracts and the respective controls, with positive control, different concentrations of a stock solution of Sodium Lauryl Sulfate (LSS) and negative control, medium with 5% fetal bovine serum and antibiotic. The microplate was again incubated.

After 24 h, the treatment was removed and the plate was washed with 150 µL of PBS and 100 µL of medium containing the neutral red dye was added. The incorporation of the neutral red dye by the viable cells was verified after 3 hours. The medium was removed and the cells washed with 150 µL of PBS. Then, 150 µL of a solution containing ethanol and glacial acetic acid (50% ethanol, 1% glacial acetic acid and 49% distilled water) were added. The plate was shaken for 10 min and the reading was performed on a spectrophotometer at 540 nm [18-20].

In vitro skin irritation

The potential for skin irritation was assessed by denaturing the ovalbumin. First, a stock solution of the extract was prepared in distilled water, and a serial dilution was performed to be tested. The test consists of adding 2.5 g of the extract and 10 g of egg white, which contains freshly homogenized ovalbumin, in the magnetic stirrer and analyzing the turbidity by transmittance, in the spectrophotometry, at 660 nm. A negative control was prepared using distilled water and egg white, in the respective proportions of the test. The lower the turbidity, the greater the transmittance and the lower the irritant potential of the extract. The test was performed in triplicate [15].

RESULTS

The extract of the jabuticaba bark has a dark purple color, has a shiny appearance and is highly viscous. The results of in vitro cytotoxicity using the MTT and NRU dyes are values expressed in mg/mL and using the GraphPad Prism software was used for statistical analysis, where $p<0.05$ was obtained by ANOVA after Tukey test. Using MTT dye was obtained $9,57 \pm 0,63^{a}$ mg/mL and for NRU dye, $10,94 \pm 1,80^{b}$ mg/mL using HaCat cells. The results of the skin irritation assessment were presented in Table 1, with a 95% confidence interval. It can be observed that the lower the concentration of the extract, the greater the transmittance. The range of extract concentration that most closely matches the transmittance value of the control, that is, the potential for skin irritation from water, is contained between 0.8 to 1.6 mg/mL.

Table 1. Evaluation of skin irritation potential (Extract concentration X Transmittance).

Concentration (mg/mL)	Transmittance (nm)
Control	**0.684 ± 0,229**
100,0	0,000 ± 0,229
50,0	0,000 ± 0,229
25,0	0,000 ± 0,229
12,5	0,106 ± 0,229
6,3	0,106 ± 0,229
3,1	0,182 ± 0,229
1,6	0,476 ± 0,229
0,8	0,727 ± 0,229
0,4	0,976 ± 0,229
0,2	0,931 ± 0,229
0,1	0,954 ± 0,229

DISCUSSION

For the extraction of the extract from the bark of the jabuticaba, the concept of extraction with different polarity solvents was used. The 70 °GL alcohol presents as a non-polar solvent and, as a polar solvent, water, enabling the extraction of a greater variety of bioactive compounds, assuming that the type of polarity is capable of dragging along compounds that have similarities. Both the extraction and the removal of the solvents were carried out at room temperature or, at most, under heating at 45 °C, in order not to degrade the antioxidant compounds present in the fruit's skin[16]. The peels used were acquired from industrial waste. Knowing their nutritional composition and richness of bioactive, as peels were selected for the extract. Industrial waste from jabuticaba has been widely used, its use as biofuels, flour, beverages, among others [12,21,22] has been researched.

The results of cytotoxicity are expressed in IC50 or Inhibition Concentration of 50% of the viable cells under test, that is, it is the concentration sufficient to cause the death of 50% of the viable cells. In the initial phase of the biocompatibility tests of extracts obtained from plants, which have cosmetic potential, these extracts must not cause cell death or affect their cell functions [15,23].

The assay using the MTT dye assesses the viability of cells with active metabolism in converting the tetrazolium salt into formazan crystals, which are solubilized to be spectrophotometrically quantified; however, the tetrazolium salt may occurs some reduction and oxidation interferences, when the test substance contains phytoestrogens, polyphenols, vitamins, compounds and metal alloys in its composition. The assay using the Neutral Red dye assesses the viability of the cells' ability to accumulate the dye in lysosomes, and is also quantified by spectrophotometry. Thus, the need to use two methodologies to assess feasibility is relevant. Thus, it is possible to reduce the possibility of interference from the extract composition. Researchers reported a difference in sensitivity between cytotoxicity assays; however, methodologies using MTT and NRU dyes are still evaluated as the most sensitive [24]. Still in the study evaluating phenolic compounds, no difference was reported between results using MTT and NRU dyes [25].

The use of immortalized human keratinocytes (HaCat) are relevant in order to assess the potential for skin irritation, as they are the first cells that a topical product comes into contact with. In addition, this strain has morphology and functionalities typical of epidermal keratinocytes, being considered a robust model for studying skin toxicity tests [26].

The IC50 of the jabuticaba extract was 9.57 ± 0.63 mg/mL, while the IC50 of the positive control (phenol solution) was 2.98 mg/mL, using the experimental cell model HaCat. Taofiq (2019) reported cytotoxicity in HaCat cells with mushroom extract treatment, obtaining an IC50 of 0.1 mg/mL [26]. While Almeida-Cincotto

(2016), reported IC50 of 3.41911 mg/mL for the extract of Morus nigra L [27]. Thus, the jabuticaba extract presented a reduced irritation potential evaluated by cytotoxicity, being viable for its use in cosmetic formulations.

The IC50 of the jabuticaba extract was 10.94 ± 1.80 mg/mL, while the IC50 of the positive control (LSS solution) was 0.14 mg/mL, using the experimental cell model HaCat. Hecker (2002) evaluated the cytotoxic potential of alkyphenols from Ginkgo biloba L. using the NRU dye and obtained an IC50 of 889 mg/L, while Mostafa (2019) obtained an IC50 of 0.22457 to 0.34625 mg/ mL for Centaurea pumilio L nanoparticles, showing lower cytotoxicity in human epidermal cells [28, 29].

Considering a skin barrier in homeostasis, permeability is attributed to the stratum corneum (EC) structure. EC has keratin-rich cells added to filaggrin monomers. EC also has lipids responsible for permeability, acidification, flaking, cell signaling and water balance. Also, in skin homeostasis, the activation of the skin's innate immune response is rapid and dependent on the severity of the irritating and/or allergenic or cumulative agent. For this type of response, there is activation of interleukins-1α from the corneocytes, cells present in the EC. When an irritating agent is able to penetrate the skin, damage to keratinocytes can occur and induce pro-inflammatory stimuli [30].

In vitro skin irritation makes it possible to evaluate the potential for protein denaturation by extracting the bark of jabuticaba. This methodology has, in principle, the similarity of solubilization of albumin, compared to keratin, a protein present in large amounts in the epidermis [15,31,32].

Proteins undergo conformation changes until complete denaturation when pH, temperature, pressure are changed, among other factors [31]. The isoelectric point is one of the most important physicochemical properties of proteins, because it is the pH value where a molecule has an electrical charge equal to zero and, with higher or lower values, there is a presence of positive or negative charges. The isoelectric point of albumin is 4.7 and, for values less than 4.0, the denaturation of this protein occurs [32].

The pH of the stock solution used in this experiment was 3.10, due to the high concentration of phenolic compounds, which have an acid character. The results obtained for the skin irritation potential of jabuticaba extract (0.8-1.6 mg/mL) correspond to the denaturation of albumin by changing the pH [31,32]. The pH of the skin varies between 5.5 and 6.5. Many acidic or basic compounds promote skin peeling, which favors facial remodeling, rejuvenation and melanosis improvement [33].

CONCLUSIONS

Our results showed that the use of industrial residue from jabuticaba bark had low potential for cutaneous irritation and a high IC50 value for cutaneous cytotoxicity, both in vitro assays. Therefore, a relevant safety margin is established for its use as a new cosmetic active.

ACKNOWLEDGEMENTS

Funds for this research were also provided by CAPES 88887.334253/2019-00.

CONFLICT OF INTERESTS

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

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