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Article

Cecropia pachystachya Trécul: a promising ingredient for skin-whitening cosmetics

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Because it promotes the lightening of pigment spots, tyrosinase inhibition is one of the mechanisms of depigmenting cosmetic products. Considering the adverse effects produced by synthetic depigmenting actives, the search for new therapeutic options is desirable, and plant extracts are possible candidates for hyperpigmentation treatment. Glycolic extracts of *Cecropia pachystachya* Trécul are, therefore, the focus of this study. Its chemical characterization, antioxidant activity, tyrosinase inhibition, and cell viability were evaluated. Glycolic extracts were obtained by macerating the leaves of *C. pachystachya* in grain alcohol and glycerin or propylene glycol. Both had a similar chemical constitution, the glycerin being more efficient in concentrating phenolic compounds and flavonoids. Analyses by UHPLC-MS detected quinic acid, chlorogenic acid isomers, proanthocyanidin dimers type B and C, catechin/epicatechin, orientin/isoorientin, isoorientin 2"-*O*-xyloside, vitexin/isovitexin, and rutin. 5-*O*-caffeoylquinic acid was then quantified was then quantified, with predominance in the extract produced with propylene glycol. These extracts showed a high antioxidant capacity by the method of DPPH, β-carotene, and nitric oxide. As for depigmenting activity, both extracts were able to inhibit tyrosinase. Cell viability assay also revealed that the extracts could safely be used in concentrations of ≤ 125 μg/mL. Thus, this study demonstrated for the first time that the glycolic extracts of *C. pachystachya* have promising chemical and biological characteristics for the development of a multifunctional cosmetic with antioxidant and tyrosinase-inhibition activities.

Keywords: *Cecropia pachystachya*. Glycolic extracts. Antioxidant activity. Tyrosinase inhibition. Skin whitening.

INTRODUCTION

Tyrosinase plays a central role in catalyzing melanin pigments' synthesis, being responsible for skin color in humans. Melanin has essential physiological effects, such as protecting the skin against ultraviolet radiation, and neutralizing free radicals and reactive oxygen species (Chang, 2009). However, due to the abnormal accumulation of melanin, the formation of pigment spots can be directly related to aesthetic and emotional problems (Ikino *et al*., 2015). The depigmenting agents used clinically have several limitations in terms of high toxicity, low stability, and reduced penetration into the skin (Singh *et al*., 2016). Therefore, the search for new therapeutic options is desirable, and plant extracts are possible candidates for the treatment of hyperpigmentation (Opperman *et al*., 2020).

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Cecropia pachystachya Trécul (Urticaceae) is a plant native to Brazil and found in Central and South American countries (Aragão *et al*., 2010). It is popularly known as *embaúba*, a name derived from the indigenous word "ambaíba", which means log-hollow (Bigliani *et al*., 2010). In traditional medicine, *C. pachystachya* leaves are used in the form of teas to treat respiratory, inflammatory, endocrine, cardiovascular, renal, and gastrointestinal disorders (Yazbek *et al*., 2019). Studies have already demonstrated some of its biological activities such as hypoglycemic (Aragão *et al*., 2010), anti-inflammatory (Pacheco *et al*., 2014), wound healing (Duque *et al*., 2016), antidepressant *(*Ortmann *et al*., 2017), and anti-aging (Fernandes *et al*., 2019) effects.

Phytochemical analyses demonstrated the predominance of phenolic compounds, such as phenolic acids and flavonoids (Machado *et al*., 2021; Rivera-Mondragón *et al*., 2019). The main phenolic acids are derived from hydroxycinnamic acids, such as the isomers of chlorogenic acid, represented by 5-*O*-caffeoylquinic and 3-*O*-caffeoylquinic acids. In terms of flavonoids, *C*-glycosylated flavones, such as orientin, isoorientin, vitexin, and isovitexin, in addition to flavonols, such as isoquercitrin and rutin, were identified. Flavonoids with a flavan-3-ol skeleton, such as proanthocyanidins type B and C, in addition to catechins and epicatechins, have also been reported (da Silva Mathias, Rodrigues de Oliveira, 2019).

Due to the growing concern about the toxic or irritating effects of organic solvents, cosmetics industries have preferably opted to use glycolic extracts (Fonseca-Santos, Corrêa, Chorilli, 2015). This type of extract can be obtained through different ways, varying the proportion of solvents, temperature, and extraction time. It is generally produced from a mixture of solvents in an aqueous medium, with glycerin and propylene glycol ranking among the most commonly used (Ardisson *et al*., 2002). Both are classified as alcohol and present some advantages, such as biocompatibility, water solubility, and stability. In cosmetic formulations, glycerin and propylene glycol could act as penetration enhancers, facilitating the permeation and absorption of plant constituents (Padmawar, Bhadoriya, 2018). However, there are still no reports in the literature on preparing and obtaining glycolic extracts of *C. pachystachya*.

In this context, this work aimed at the development and chemical characterization of glycolic extracts of *C. pachystachya* and evaluation of the antioxidant, depigmenting effects, and cell viability, focusing on the search for a novel cosmetic ingredient or novel cosmetic adjuvant.

MATERIAL AND METHODS

Chemicals and cell lines

Propylene glycol, glycerin, Folin-Ciocalteu reagent, tannic acid, quercetin, ethanol, methanol, 5-*O*-caffeoylquinic acid (5-CQA), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical, linoleic acid, β-carotene, Tween 40, dichloromethane, calcium carbonate, aluminum chloride, glacial acetic acid, kojic acid, tyrosinase, tyrosine, and all the other solvents and reagents of analytical grade were acquired from Sigma-Aldrich (Saint Louis, MO, USA). The 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was acquired from Invitrogen (Carlsbad, CA, USA). Cell medium, penicillin, streptomycin, and all the other reagents for cell studies, were acquired from Gibco (Waltham, MA, USA).

Plant material

C. pachystachya leaves were collected in Juiz de Fora, State of Minas Gerais, Brazil (latitude: 21° 66'7775'' S, longitude: 43° 29'5569'' W). A voucher specimen (No. 46591) has been affixed at Leopoldo Krieger Herbarium, located at the Federal University of Juiz de Fora.

Extracts preparation

The preparation of glycolic extracts was performed according to Ardisson *et al*. (2002), with some modifications. The leaves were dried at approximately 35 °C and pulverized with the use of an industrial crusher. The extraction process was accomplished by maceration for eight days at room temperature. The two extractive conditions were: glycerin (GLY - 70 %) and grain alcohol (GA - 30 %) (CP01) or propylene glycol (PG - 70 %) and grain alcohol (GA - 30 %) (CP02). A ratio of 1 g of

powdered leaves to 10 ml of extractive solvent was used. The extracts were vacuum filtered, identified, and stored at controlled temperature (6 ± 2.0 °C).

Chemical characterization

Determination of total phenolic content

The total phenolic content was determined by Folin-Ciocalteu's method (Folin, Ciocalteu, 1927), with slight modifications. Briefly, 120 µL of Folin–Ciocalteu reagent solution (20 % v/v in water), 100 µL of calcium carbonate solution (4 % m/v in water) and 30 µL of the extract's solutions (500 µg/mL in ethanol) were added in 96-well plate. After 30 minutes, absorbance was measured at 770 nm in a microplate reader (Multiskan EX, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Tannic acid was used as standard for the calibration curve with concentrations of 0.9 to 60 µg/mL. The analyses were performed in triplicate, and results were expressed as milligrams of tannic acid equivalents per gram of extracts (mg TAE/g).

Determination of total flavonoids content

The total flavonoid content was verified by the aluminum chloride colorimetric method, previously described by Challice and Markham (1984). 250 µL of aluminum chloride solution (2 % m/v in ethanol), 10 µL of glacial acetic acid, and 250 µL of the extract's solutions (500 µg/mL in ethanol) were added in test tubes. After 30 minutes, absorbance was measured at 415 nm in a microplate reader. Quercetin was used as the reference standard for the calibration curve with concentrations of 1.25 to 125 µg/mL. The analyses were performed in triplicate, and results were expressed as milligrams of quercetin equivalents per gram of extracts (mg QE/g).

UHPLC/MS analysis

The chemical analyses were performed using ultra-high-pressure liquid chromatography (UHPLC) (Shimadzu, Kioto, Japan), coupled with a high-resolution mass spectrometer (MS) (Bruker, Bremen, Germany), a quadrupole time of flight (Q-TOF), with an electrospray ionization operated in a negative mode. The chromatographic separation was achieved in a reverse-phase C18 Kinetex (Phenomenex, Torrance, USA) analytical column (100 x 3 mm i.d., 2.6 µm particle size) and a constant flow of 400 µL/min. The injection volume was 20 µL. The mobile phase consisted of water (A) and methanol (B). Solvents were filtered and delivered as a gradient consisting of 5 % of B (0 min), 33 % (2.0 min), 37 % (10.5 to 11.5 min), and finally returned to the initial conditions to rebalance the column until the next run. The ESI parameters were a gas flow of 9 L/min, a nebulizer of 4.0 bar, and a gas temperature of 200 ºC, with nitrogen used as the drying gas.

HPLC analysis

High-performance liquid chromatography (HPLC) analysis was accomplished to quantify the chlorogenic acid 5-CQA in glycolic extracts. For such, 5-CQA was used as the reference standard compound (1 mg/mL). For the coelution, a mixture of 5-CQA and CP01 or CP02 (1:50) was injected. Furthermore, a calibration curve of 5-CQA (3 to 50 µg/mL), and the results were expressed as milligrams of 5-CQA per gram of extracts. The chromatographic separation was achieved in a high-pressure liquid Agilent 1200 HPLC Series (Agilent Technologies, Santa Clara, California, USA), with a quaternary pump, diode array detector (DAD), and automatic injector. A reverse-phase C18 Kromazil (Bohus, Sweden) analytical column (150 x 4.6 mm i.d., 5 µm particle size) was used. The mobile phase consisted of water (A) and acetonitrile (B). Solvents were filtered and delivered as a gradient that consisted of 5 % (0 to 3.0 min) and 15 % (3.01 to 20.0 min) of B. The injection volume was 20 µL, the flow was adjusted to 0.8 mL/min, and the detection wavelength was fixed at 330 nm.

Evaluation of antioxidant activity

DPPH assay

The radical scavenging activity of extracts was evaluated by DPPH assay (Brand-Williams, Cuvelier, Berset, 1995), with slight modifications. One hundred microliters of the samples diluted in ethanol at different

concentrations (0.49 to 250 µg/mL) were added to a 150 µL of DPPH solution (20 µg/mL in ethanol). After 30 minutes, the absorbance was measured at 517 nm in a microplate. Quercetin was used as the reference standard. The analyses were performed in triplicate, and the results were expressed as inhibitory concentration 50 (IC_{50}), which corresponds to the concentration of extracts required to scavenge 50 % of DPPH free radicals. Values were calculated using the software GraFit Version 7 (Horley, UK).

β-carotene bleaching assay

The capacity of glycolic extracts to reduce oxidative degradation of β-carotene was measured according to the procedure by Marco (1968), with slight modifications. One hundred microliters of β-carotene solution (10 mg/mL in dichloromethane), 30 µL of a linoleic acid, and 265 µL Tween 40 were dissolved in 500 µL of dichloromethane. After complete evaporation of the dichloromethane, 40 mL of water previously saturated with oxygen was added. 250 µL of this solution and 10 µL of the glycolic extracts (1.20 to 38.46 µg/mL in ethanol) were then added to a 96-well plate. Quercetin was used as a positive control, and the analyses were performed in triplicate. The absorbance was measured immediately (t = 0 min) and after incubation (t = 120 min) at 490 nm in a microplate reader. Results were expressed as IC_{50}, which corresponds to the concentration of extracts required to inhibit 50 % of oxidation of linoleic acid. Values were calculated using the software GraFit Version 7.

Nitric oxide radical assay

The scavenging of nitric oxide radical was measured according to Green *et al*. (1982), with slight modifications. For this, 62.5 µL of sodium nitroprusside (10 mM) in phosphate-buffered saline (PBS) were mixed with different concentrations of glycolic extracts in PBS (200, 100, and 50 µg/mL) and incubated at 25°C for 60 minutes. Next, 125 µl of Griess reagent, sulfanilamide 1 % (m/v in phosphoric acid 2.5 %) and NED 0.1 % (m/v in phosphoric acid 2.5 %) were added. The absorbance of chromophore formed was measured at 540 nm in a microplate reader. Quercetin was used as the reference standard, and the analyses were performed in triplicate. Results were expressed as inhibition percentage (I %), comparing extracts absorbance with the negative control, which considered 100 % oxidation.

Anti-tyrosinase activity

Tyrosinase inhibitory activity assay was performed according to (Khatib *et al*., 2005) spectrophotometric method, with slight modifications. Glycolic extracts (3.125 a 400 µg/mL) and the standard kojic acid (0.7812 to 100 µg/mL) were dissolved in PBS (50 mM, pH 6.5). Ten microliters of glycolic extracts or kojic acid, 60 µL of PBS, and 30 µL of the tyrosinase solution (250 U/mL in PBS) were added to a 96-well microplate. After the addition of 100 µL of L-tyrosine and incubation (30 min, 25 °C), absorbance was measured at 475 nm in a microplate reader. The analyses were performed in triplicate, and the results were expressed as IC_{50}, which corresponds to the concentration of extracts required to reduce 50 % of enzymatic activity. Values were calculated using the software GraFit Version 7.

Cell viability

Cell viability was assessed by using the MTT method (Mosmann, 1983), with some modifications. In 75 cm^3 culture bottles, the human keratinocyte cell line (HaCaT) was cultured in DMEM/F-12 medium (1:1) with 10 % fetal bovine serum and 1 % penicillin/streptomycin. The cells were maintained at 37 ºC and 5 % CO_2 until they reached 80 % confluence. Cells were seeded in a 96-well plate (1 x 10^4 cells/well). After 24 hours, 10 µL of glycolic extracts with concentrations ranging from 31.25 to 500 µg/mL were added. The DMEM/F-12 medium (negative control) and the 5 % DMSO solution (v/v) (positive control) were used as controls. The plates were incubated for 24 hours at 37ºC with 5 % CO_2. After that period, the culture medium was removed from each well, washed with PBS, and 100 µl of MTT solution (0.5 µg/mL) was added. After 3 hours, the precipitate, formazan, was dissolved in 100 µL of DMSO, and the absorbance was read at 570 nm in a microplate reader. The percentage of cell viability was calculated by comparing the absorbance of treated cells with negative control (100 % cell viability).

RESULTS AND DISCUSSION

Chemical characterization

Determination of total phenolics and flavonoids contents

Data presented in Table I show that the extraction process effectively extracts phenolic compounds and flavonoids from the leaves of *C. pachystachya*, and that glycerin was more efficient than propylene glycol in this process. These results are relevant since most cosmetic formulations present glycerin in their composition. There is a growing interest in searching for natural cosmetics containing less harmful ingredients to the human body and the environment, such as glycolic extracts (Fonseca-Santos, Corrêa, Chorilli, 2015).

TABLE I - Phenolics and flavonoids contents in the glycolic extracts of *Cecropia pachystachya*

Samples	Phenolics compounds	Flavonoids
	TAE (mg TA/g) ± SD	QE (mg Q/g) ± SD
CP01	249.16 ± 1.90	14.18 ± 0.90
CP02	189.95 ± 6.85***	8.80 ± 1.20**

Data are presented as means of values ± SD (n = 3). ANOVA followed by Tukey's test, used as post hoc. Significant values: ***p<0.001, **p<0.01, compared to CP01. CP01: glycerin (GLY - 70%) and grain alcohol (GA - 30%); CP02: propylene glycol (PG - 70%) and grain alcohol (GA - 30%); Q: Quercetin; QE: Quercetin equivalents; TA; Tannic acid; TAE: Tannic acid equivalents.

UHPLC-MS analysis

UHPLC-MS in negative ionization mode was performed to detect the metabolites present in the glycolic extracts of *C. pachystachya*. The compounds were identified by comparing those obtained with those published in the literature. As shown in Table II, three phenolic acids and nine flavonoids were identified in both extracts.

TABLE II - UHPLC-MS analysis of extracts CP01 and CP02 of *Cecropia pachystachya*

Peak	Rt (min.)	Molecular formula	MS $[M-H]^-$	MS/MS	Proposed compounds
1	1.1	$C_7H_{12}O_6$	191	-	Quinic acid
2	2.1	$C_{16}H_{18}O_9$	353	191	Caffeoylquinic acid
3	2.7	$C_{16}H_{18}O_9$	353	191	Caffeoylquinic acid
4	3.3	$C_{30}H_{26}O_{12}$	577	289; 425	B-type proanthocyanidin dimer
5	3.6	$C_{30}H_{26}O_{12}$	577	289	B-type proanthocyanidin dimer
6	3.9	$C_{45}H_{38}O_{18}$	865	576	Procyanidin C1
7	4.1	$C_{15}H_{14}O_6$	289	245	Catequin or epicatequin
8	4,9	-	451	-	Unidentified
9	6.0	$C_{21}H_{20}O_{11}$	447	327	Orientin or isoorientin
10	6.4	$C_{26}H_{28}O_{15}$	579	429	Isoorientin 2"-*O*-xyloside
11	6.7	$C_{21}H_{20}O_{11}$	447	357	Orientin or isoorientin
12	8.8	$C_{21}H_{20}O_{10}$	431	-	Vitexin or isovitexin

TABLE II - UHPLC-MS analysis of extracts CP01 and CP02 of *Cecropia pachystachya*

Peak	Rt (min.)	Molecular formula	MS $[M-H]^-$	MS/MS	Proposed compounds
13	10.2	$C_{27}H_{30}O_{16}$	609	463	Rutin
14	11.8	-	451	-	Unidentified

CP01: glycerin (GLY - 70%) and grain alcohol (GA - 30%); CP02: propylene glycol (PG - 70%) and grain alcohol (GA - 30%); Rt: retention time.

Compound 1 was identified as quinic acid based on its molecular ion at *m/z* 191 $[M-H]^-$. Although a fragment of this compound has not been observed, quinic acid has already been detected in other extracts of *C. pachystachya* (Rivera-Mondragón *et al*., 2019). Compounds 2 and 3 showed the same *m/z* 353 $[M-H]^-$ molecular ion, suggesting they are isomers of chlorogenic acid. For both signals, the fragmentation profile exhibited the ion at *m/z* 191 $[M-H]^-$, characteristic of the quinic acid, which indicated the loss of the caffeoyl group ($[M-H-162]^-$) (Machado *et al*., 2021). These data corroborate other studies that also reported the presence of isomers of chlorogenic acid in extracts of *C. pachystachya* (Aragão *et al*., 2010; Duque *et al*., 2016; Ortmann *et al*., 2017; da Silva Mathias, Rodrigues de Oliveira, 2019).

Compounds 4 and 5 were identified as type B proanthocyanidins due to the molecular ion at *m/z* de 577 $[M-H]^-$. Besides, the fragments found are characteristic of proanthocyanidins: *m/z* 425 ($[M-H-152]^-$, which arise from the retro Diels-Alder reaction, and at *m/z* 289 ($[epicatequine-H]^-$, resulting from a quinone-methide fragmentation) (Poupard *et al*., 2011). Compound 6 was identified as a trimeric form of epicatechin, called procyanidin C1. The molecular ion at *m/z* de 865 $[M-H]^-$, with a fragment of *m/z* 576 ($[M-H-289]^-$ corresponds to the quinone-methide fission in ring A of the procyanidin structure C1 (Poupard *et al*., 2011). Compound 7 showed a molecular ion at *m/z* 289 $[M-H]^-$ and was identified as a derivative of flavan-3-ol (catechin or epicatechin). The fragment at *m/z* 245 ($[M-H-44]^-$) corresponds to the loss of the enol group (da Silva Mathias, Rodrigues de Oliveira, 2019).

Compounds 9 and 11 showed a molecular ion at *m/z* 447 $[M-H]^-$, indicating that they are structural isomers of luteolin (luteolin-8-*C*-glycoside, known as orientin, and luteolin-6-*C*-glycoside, known as isoorientin). The ions at *m/z* 357 ($[M-H-90]^-$) and *m/z* 327 ($[M-H-120]^-$) correspond to collision-induced dissociation of the sugar molecule attached to the carbon atom of the flavone (Chen *et al*. 2016). Orientin and isoorientin are widely described in extracts of *C. pachystachya* (Machado *et al*., 2021; da Silva Mathias, Rodrigues de Oliveira, 2019; Rivera-Mondragón *et al*., 2019).

Compound 10 was identified as isoorientin 2"-*O*-xyloside, due to the molecular ion at *m/z* 579 $[M-H]^-$. The fragment at *m/z* 429 ($[M-H-150]^-$) corresponds to the intra-glycosidic fragmentation of the sugar molecule attached to the flavone's carbon atom. This glycosylated flavonoid has also been identified in the methanolic extract of *C. pachystachya* by da Silva Mathias, Rodrigues de Oliveira (2019). Compound 12 was assigned to vitexin (8-*C*-glycosyl-apigenin) or isovitexin (6-*C*-glycosyl-apigenin) due to the molecular ion at *m/z* 431 $[M-H]^-$. Their fragments were not identified. However, those compounds were previously detected in extracts of *C. pachystachya* (Machado *et al*., 2021; da Silva Mathias, Rodrigues de Oliveira, 2019; Rivera-Mondragón *et al*., 2019).

Finally, compound 13 was assigned to rutin (3-*O*-rutinosyl-quercetin) due to the molecular ion at *m/z* 609 $[M-H]^-$. The fragmentation profile of the molecular ion *m/z* 463 ($[M-H-146]^-$) corresponds to rhamnoside loss. This result corroborates da Silva Mathias, Rodrigues de Oliveira (2019), who reported rutin in the methanolic extract of *C. pachystachya*.

Chemical constituents for both extracts were similar, indicating that glycerin and propylene glycol could extract phenolic compounds and flavonoids from *C. pachystachya*.

HPLC analysis

The caffeoylquinic acids are a chemical and biological marker of the *C. pachystachya* species (Machado *et al*., 2021; Rivera-Mondragón *et al*., 2019). In order to quantify this phenolic acid in the extracts, the standard 5-CQA was used. As shown in Figure 1, the signals related to 5-CQA (rt = 2.49 min) and the coelution of 5-ACQ with CP01 (rt = 2.59 min) and with CP02 (rt = 2.25 min) had a similar UV profile (max UV: 325 nm). The coelution with reference substance showed a proportional increase in signal intensity in both extracts, confirming the presence of 5-CQA.

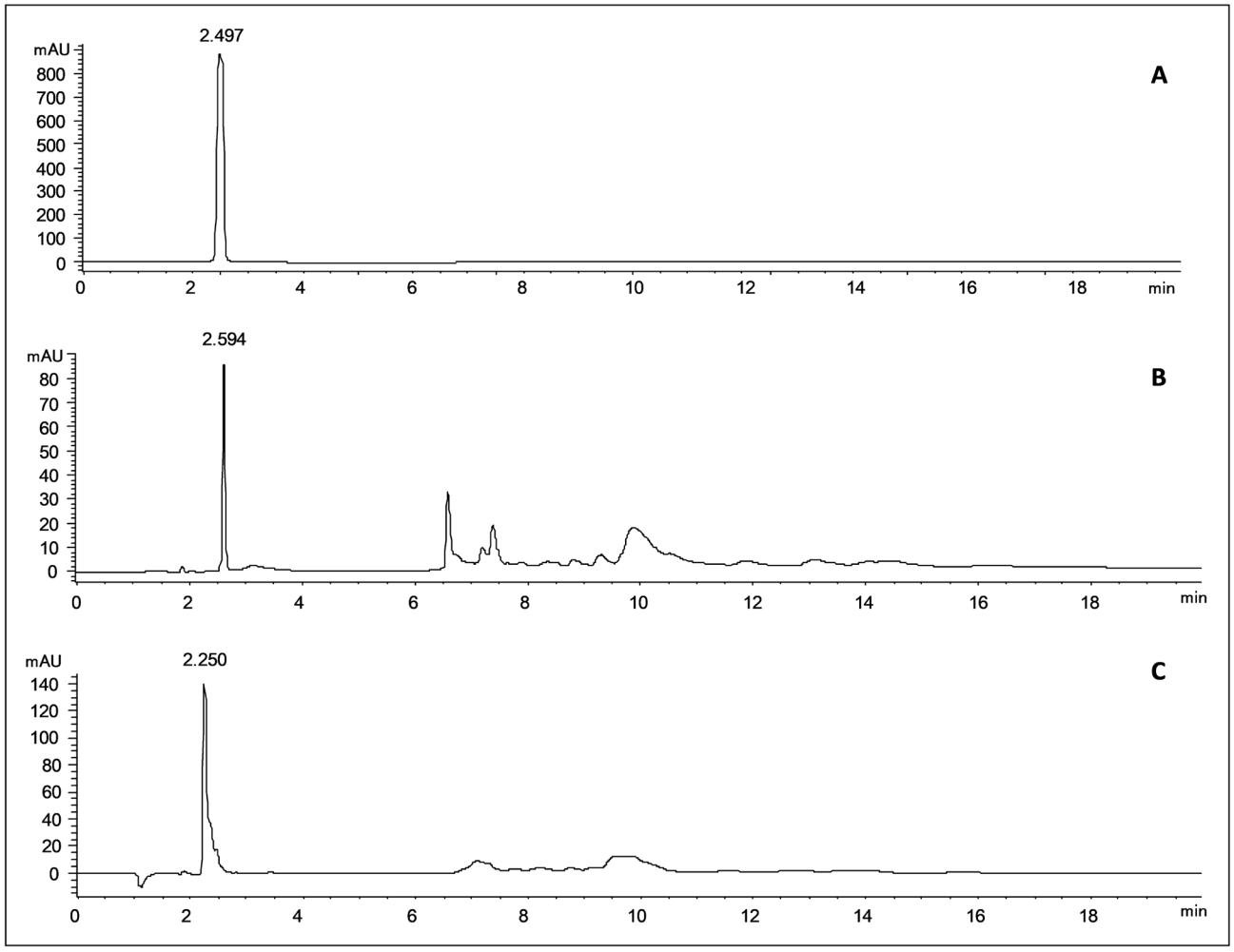

FIGURE 1 - Chromatographic profile of the 5-CQA standard (A), CP01/5-CQA coelution (B) and CP02/5-CQA coelution (C) by HPLC-UV. The detection wavelength was at 330 nm. CP01: glycerin (GLY - 70 %) and grain alcohol (GA - 30 %); CP02: propylene glycol (PG - 70 %) and grain alcohol (GA - 30 %); 5-CQA: 5-O-caffeoylquinic acid.

The contents of 5-CQA in the extracts CP01 and CP02 were 3.27 ± 0.31 and 8.45 ± 0.24 mg/g of extract, respectively. CP02 presents a higher amount of 5-CQA when compared with the extract CP01. In the literature, few studies determined the content of caffeoylquinic acid in *C. pachystachya* extracts. Costa *et al*., (2011), quantified 3-*O*-caffeoylquinic acid (3-CQA), obtaining contents of 27.2 ± 0.9 mg/g of extract. This compound

was the principal constituent in the aqueous extract of C. *pachystachya*. Pacheco *et al*. (2014) detected chlorogenic acid in the methanolic extract at 5.4 ± 0.2 mg/g of extract.

Evaluation of antioxidant activity

The antioxidant capacity of glycolic extracts was evaluated by three *in vitro* methods, as demonstrated in Table III.

TABLE III - Antioxidant capacity of glycolic extracts of *Cecropia pachystachya*

Samples	DPPH (µg/mL)	β-carotene (µg/mL)	Nitric oxide (% inhibition)		
			200 µg/mL	100 µg/mL	50 µg/mL
CP01	1.16 ± 0.15	8.25 ± 1.03***	45.58 ± 1.15	33.72 ± 2.63***	23.56 ± 5.10
CP02	1.17 ± 0.05	6.82 ± 0.57**	38.02 ± 4.81***	29.01 ± 4.92****	21.62 ± 4.54
Quercetin	1.02 ± 0.08	2.91 ± 0.60	52.26 ± 2.64	46.32 ± 1.95	28.52 ± 6.66

Data are presented as means of values ± SD (n = 3). ANOVA followed by Tukey's test, used as post hoc. Significant values: ****p<0.0001, ***p<0.001, **p<0.01, compared to quercetin. CP01: glycerin (GLY - 70%) and grain alcohol (GA - 30%); CP02: propylene glycol (PG - 70%) and grain alcohol (GA - 30%).

The DPPH reduction assay is one of the most usual methods for assessing antioxidant activity due to the speed, practicality, and efficiency in screening plant extracts. In the presence of an antioxidant substance, the DPPH radical is reduced to hydrazine through the transfer of hydrogen atoms, and a simultaneous change in color from violet to pale yellow is visualized (Brand-Williams, Cuvelier, Berset, 1995).

CP01 and CP02 showed no significant difference in respect to the quercetin pattern (Table III). The high antioxidant activity presented by the glycolic extracts could be attributed mainly to phenolic compounds, such as chlorogenic acid and the flavonoids identified by UHPLC-MS and HPLC-UV. The antioxidant action of chlorogenic acid is related to an ortho-dihydroxyl group in the aromatic ring of caffeic acid that would act as a receptor for free radicals (Palomino García, Del Bianchi, 2015).

In both extracts, CP01 and CP02, 5-CQA was quantified. The presence of another isomer of chlorogenic acid was also identified (Table II). Thus, 5-CQA and its isomer could be related to the neutralizing activity of the DPPH radical. The hydroxyl groups of the flavonoids would also function as hydrogen donors for free radicals (Lago *et al*., 2014). The structural conformation and the number of available hydroxyls would be directly related to the antioxidant capacity of these compounds as observed for orientin/isoorientin and catechin/epicatechin (Alves *et al*., 2010).

The presence of proanthocyanidins dimers (B2, B3, and, or B5) and trimers (C1) could also be related to the glycolic extract's antioxidant capacity. These compounds have several hydroxyls that act as potent hydrogen donors. Besides, conjugated double bonds allow the displacement of electrons through the molecule, stabilizing the DPPH radical (Soobrattee *et al*., 2005).

The β-carotene bleaching assay evaluated the ability of plant extracts to prevent β-carotene discoloration by donating hydrogen atoms to peroxides formed from linoleic acid. Thus, the presence of antioxidant compounds in the reaction medium minimizes the lipid substrate discoloration through the neutralization of reactive species (Alves *et al*., 2010).

As shown in Table III, extracts CP01 and CP02 were also able to protect the lipid substrate from the peroxidation process. These data suggest that the compounds of glycerin and propylene glycol extracts were efficient in inhibiting the formation of linoleic acid peroxides.

Flavonols, like rutin, are potent antioxidants in lipid systems. These compounds act by restricting the access of oxidants to the bilayer, consequently, preventing

the spread of lipid oxidation in the hydrophobic matrix of flavonol membranes, such as rutin, and are potent antioxidants in lipid systems. Reactive phenolic hydroxyls in its structure could justify the high antioxidant activity of rutin (Verstraeten *et al*., 2003). This flavonoid was identified in both glycolic extracts.

The nitric oxide radical is generated from sodium nitroprusside, which reacts with oxygen to produce nitrite. The nitrite undergoes a diazotization reaction with sulfanilamide. The product forms a complex with naphthyl ethylenediamine, which provides an absorbance maximum at 540 nm (Green *et al*., 1982).

According to Table III, CP01 exhibited a higher NO inhibition potential than CP02 at a 200 µg/mL concentration. There was no significant difference between the extracts and quercetin at a concentration of 50 µg/mL. These results correlate with phenolic and flavonoids' content, which is higher in CP01 compared to CP02.

According to Conforti *et al*., (2009), flavonoids with free hydroxyls and double bonds between C2-C3 carbons, such as orientin/isoorientin and vitexin/isovitexin, are more potent in inhibiting nitric oxide production. Also, *in vivo* studies have shown that chlorogenic acid significantly inhibits nitric oxide production, and this action is justified by the inhibition of the enzyme nitric oxide synthase (Plazas *et al*., 2014).

Anti-tyrosinase activity

Tyrosinase is a copper-containing glycoprotein that plays a central role in catalyzing the first two melanogenesis reactions. Its action lies in the conversion of L-tyrosine into L-dihydroxyphenylalanine (L-DOPA) by hydroxylation. Later, this compound is converted into L-dopaquinone by oxidation. At the end of this process, melanin pigments, responsible for skin color in humans, are formed (Chang, 2009).

In the present study, the tyrosinase inhibition profile of the glycolic extracts and the standard compound, kojic acid, is shown in Figure 2. CP01 showed an IC_{50} of 55.19 ± 4.44 and CP02 of 57.92 ± 0.11 µg/mL, both of which are equally effective in inhibiting tyrosinase. Both extracts had a lower effect than kojic acid, which exhibited a tyrosinase inhibition of 19.90 ± 4.41 µg/mL. However, kojic acid has some disadvantages, such as cytotoxicity and storage instability (Singh *et al*., 2016).

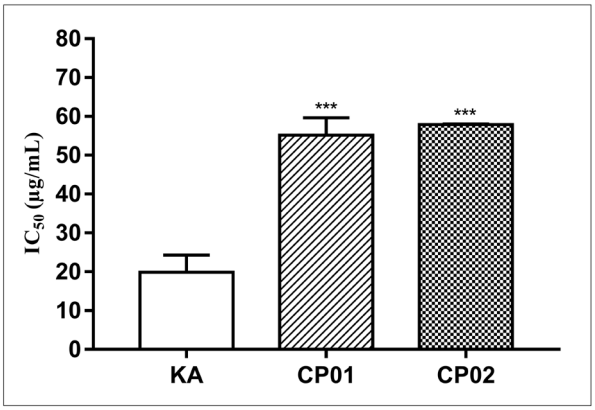

FIGURE 2 - Anti-tyrosinase activities of glycolic extracts of *C. pachystachya*. Data are presented as means of values ± SD (n = 3). ANOVA followed by Tukey's test, used as post hoc. Significant values: $^{***}p<0.001$, compared to kojic acid. CP01: glycerin (GLY - 70%) and grain alcohol (GA - 30%); CP02: propylene glycol (PG - 70%) and grain alcohol (GA - 30%); KA: kojic acid.

In general, the mechanism of tyrosinase inhibition *in vitro* assays is related to the competitive inhibition of L-DOPA by phenolic compounds present in plant extracts. These compounds present a chemical structure like L-DOPA, catalyzed by tyrosinase, preventing melanin pigment formation (Xie *et al*., 2018).

According to Chang (2009), the position and number of hydroxyls linked to the flavonoid skeleton would be directly related to tyrosinase inhibitory activity. Among the classes of flavonoids that have been described as tyrosinase inhibitors, there are flavonols, such as rutin and quercetin. These compounds have an action mechanism based on the competitive inhibition of L-DOPA oxidation by tyrosinase and chelation of copper ions due to the 3-hydroxy-4-one portion (Kubo *et al*., 2000). Flavones and flavonols have demonstrated a potent competitive inhibition against tyrosinase activity, such as orientin/isoorientin, vitexin/isovitexin, and catechin/epicatechin isomers (Itoh *et al*., 2009).

Due to the phenolic acid binding with the tyrosinase enzyme, phenolic acids, such as chlorogenic acid isomers, have a strong electron sequestration capacity to inhibit

the substitution reaction between the oxygen atom of tyrosinase and the aromatic ring of L-DOPA (Xie *et al*., 2018). Therefore, due to the variety of chemical composition, one must consider the synergistic effect of tyrosinase inhibition by the compounds present in glycolic extracts, since CP01 showed a higher dosage of phenolic compounds and flavonoids. However, the amount of chlorogenic acid was lower than that presented in CP02.

Thus, both glycolic extracts were able to inhibit tyrosinase activity at low concentrations. These data are promising for the use of *C. pachystachya* plant extracts as depigmenting agents.

Cell viability

The human keratinocyte cell line (HaCaT) was used to evaluate the cell viability of glycolic extracts. These epithelial cells are a good model for testing cytotoxicity of compounds or products for dermatological use as they are the first contact to cosmetical formulations (Taofiq *et al*., 2019).

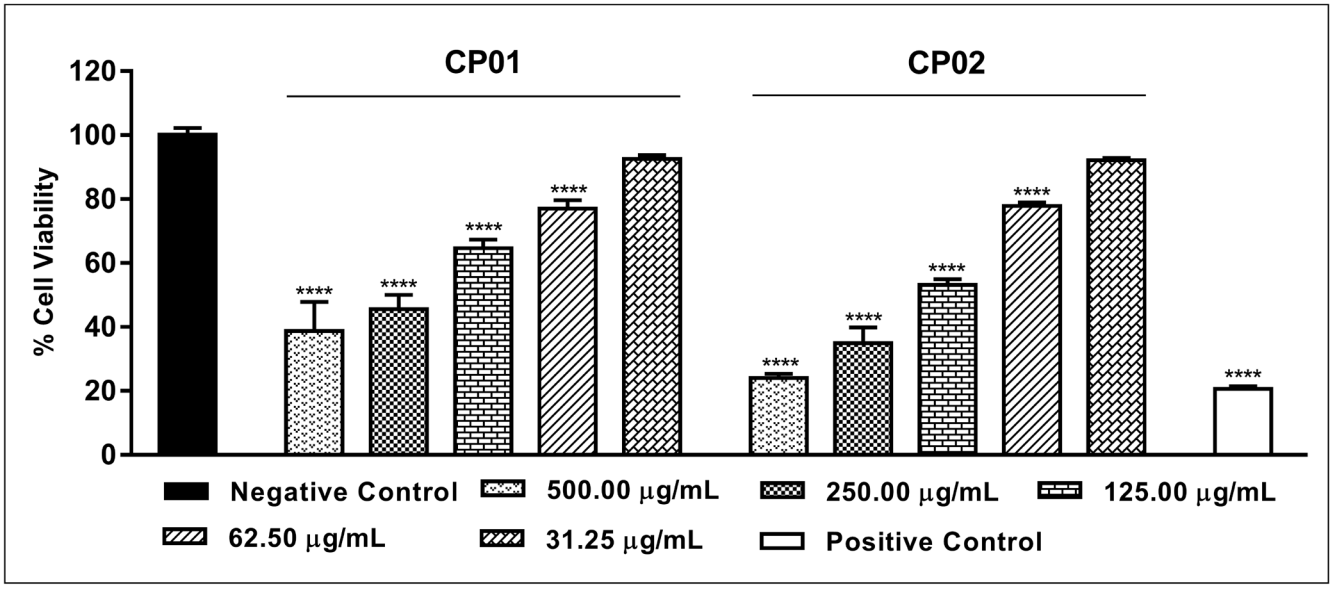

FIGURE 3 - Cytotoxicity assessment by MTT assay in HaCaT cells following the exposure of glycolic extracts for 24 hours. The concentration range was 500 to 31.25 μg/mL. Data are presented as means of values ± SD (n = 4). ANOVA followed by Tukey's test, used as post hoc. Significant values: ****p<0.0001, compared to negative control. CP01: glycerin (GLY - 70 %) and grain alcohol (GA - 30 %); CP02: propylene glycol (PG - 70 %) and grain alcohol (GA - 30 %).

According to the International Organization for Standardization (ISO), samples that reduce cell viability to values below 70 % can be considered cytotoxic ("ISO 10993-5: Biological Evaluation of Medical Devices - Part 5: Tests for Cytotoxicity: *In Vitro* Methods", 2009). With this information, glycolic extracts were able to maintain cell viability above 70 % at concentrations of 31.25 and 62.5 μg/mL (92 % for CP01 and CP02 and 77 % for CP01 and CP02, respectively), when compared to the negative control (Figure 3), which does not represent toxic effects to HaCaT cells. These results stand out since the extracts showed activity in concentrations below 62.5 μg/mL, as demonstrated in the antioxidant capacity and tyrosinase inhibition tests, which may indicate that the glycolic extracts are safe for topical application aiming at antioxidant and depigmenting actions.

On the other hand, at 125, 250, and 500 μg/mL, cell viability was 64, 45, and 38 % for CP01, respectively, and 53, 34, and 24 % for CP02, respectively, when compared to the negative control. According to the guidelines of the

Organization for Economic Cooperation and Development (OECD), for a chemical to be considered irritating to the human epidermis, the cell viability measured by the MTT assay must be less than or equal to 50 % ("OECD Test Guideline No. 439 - *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method", 2010). Thus, the extracts proved to be safe for topical use, even in higher concentrations (up to 125 µg/mL).

CONCLUSIONS

The process of glycolic extracts acquisition proved to be efficient, fast, and low cost. Both were able to extract bioactive substances from *C. pachystachya*, with glycerin being more efficient than propylene glycol in concentrating phenolic compounds and flavonoids. The extracts showed a high antioxidant capacity correlated with the bioactive compounds identified by UHPLC-MS and HPLC-UV. Both extracts were able to inhibit tyrosinase. According to the cell viability test, both extracts can be used safely in concentrations equal to or less than 125 µg/mL. Thus, this study demonstrated for the first time that the glycolic extracts of *C. pachystachya* have promising chemical and biological characteristics for the development of multifunctional cosmetics with antioxidant and anti-tyrosinase properties.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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