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Hyperpigmentant activity of leaves and flowers extracts of *Pyrostegia venusta* on murine B16F10 melanoma

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ABSTRACT

Ethnopharmacological relevance: Pyrostegia venusta is a native Brazilian plant which has a variety of uses in traditional folk medicine including the treatment of vitiligo. However, its effectiveness on melanogenesis is not yet elucidated.
Aim of the study: This study aimed to investigate the melanogenic activity of hydroalcoholic extracts from the leaves and flowers of *P. venusta* on murine B16F10 melanoma cells.
Materials and methods: Different concentrations of the hydroalcoholic extracts of flowers and leaves of *P. venusta* were evaluated in trials of spontaneous melanin content (4 days), and cell viability by the MTT assay in murine B16F10 cells, and in the mushroom tyrosinase activity *in vitro*.
Results: Both extracts, leaves (0.1; 0.3; 1 and 3 μg/mL) and flowers (0.03 and 0.1 μg/mL) increased the melanin content in a concentration dependent manner after 4 days of incubation on melanoma cells. Leaves extract promoted enhancement of melanogenesis with maximum effect of 33.3 ± 3% (3 μg/mL), and the flower extract increased in 23.4 ± 3% (0.1 μg/mL). The cell viability test using MTT showed that in the same tested concentrations of both extracts no cell death was detected. Actually, either extract was not able to cause any change in the tyrosinase activity. HPLC analysis of *P. venusta* extracts found 0.09% and 1.08% of allantoin on leaves and flowers extracts, respectively.
Conclusions: The leaves and flowers extracts of *P. venusta* stimulates B16F10 melanogenesis at very low concentrations. These findings support the folk medicinal use of *P. venusta* on the treatment of hypopigmentation diseases, such as vitiligo.

1. Introduction

Pyrostegia venusta (Ker Gawl.) Miers (Bignoniaceae) is a native woody vine widely spread in Brazil which is specially found throughout fields, at coast, edge of the woods and along roadsides. Popularly known as "flame vine" or "cipó-de-são-joão", this species is cultivated on account of outstanding ornamental features and on its important therapeutic properties (Lorenzi and Souza, 1995). *P. venusta* leaves and stems are used in folk medicine as a tonic or antidiarrheal agent, while its flowers are used in the treatment of white patches on the body, such as leucoderma and vitiligo. Besides it is also used in cough and common diseases of the respiratory system related to infections, such as bronchitis, flu and cold (Ferreira et al., 2000; Scalon et al., 2008; Veloso et al., 2010). Roy et al. (2011) demonstrated that the extracts of flowers and roots of *P. venusta* contain significant amounts of phytochemicals with antioxidative properties that could act as inhibitors or scavengers of free radicals. Moreover, no genotoxic effect was observed for extracts of *P. venusta* on bone marrow of mice using the micronucleus and chromosome aberration assay (Magalhães et al., 2010). Therefore, *P. venusta* could be exploited as a potential source for plant-based pharmaceutical products. Despite of its known popular use for the treatment of vitiligo, there are no reports in the literature concerning this property.

Disorders of pigmentation can result from abnormalities of melanocytes (the cells responsible for skin pigmentation) migration from the neural crest to the skin during embryogenesis, as well as from immunologic or toxic destructions of melanocytes

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(Cardinali et al., 2005). More than 100 genes are involved in the process of melanogenesis, encoding important structural, enzymatic and regulatory proteins (Bennett and Lamoreux, 2003). Tyrosinase is known to play an important role in the regulation of melanogenesis (Hearing, 1999), once it is responsible by melanin synthesis which starts with the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (DOPA), which is followed by the oxidation of DOPA to DOPA quinone (Jimenez-Cervantes et al., 1993). The DOPA quinone conjugated is progressively transformed into reddish-yellow pheomelanins, or used to the synthesis of brownish-black eumelanins. Once formed, the melanin is packaged in vesicles called melanosomes and then transferred to keratinocytes through the dendritic tips of melanocytes, resulting in the distribution of melanin throughout the epidermis. Impairment of melanosome transfer to the surrounding keratinocytes, an alteration in melanin synthesis and a defective degradation or removal of melanin may lead to abnormal skin pigmentation (Cardinali et al., 2005).

The most common hypopigmentant skin disorder is vitiligo, which is an autoimmune-induced depigmentation disease (Fistarol and Itin, 2010). Vitiligo affects 1–2% of the world population and it is characterized by the destruction of melanocytes, promoting smooth formation of white patches in the midst of the normally pigmented skin which usually initiates on the hands and feet and then spreads to several parts of the body (Abu Tahir et al., 2010; Fistarol and Itin, 2010). Besides skin manifestation, vitiligo promotes profound effect on people quality of life (Radtke et al., 2010; Talsania et al., 2010). Symptoms such as depression, sleep disturbances, suicidal thoughts and anxiety are very common in people affected by this disease (Szczurko and Boon, 2008). Vitiligo is initially treated with corticosteroids, immunomodulators, ultraviolet radiation, lasers, alternate therapy, depigmentation and camouflage. When classical interventions fail, vitiligo becomes refractory and stable, surgical proceedings with melanocytes transplantation are indicated. Therefore, several alternative methods, such as topical or systemic tyrosine, cysteine, vitamins, clofazimine, chinese herbal medications, ayurvedic medicine and others, have been investigated and attempted with variable success, although sometime with undesirable side effects (Falabella and Barona, 2009).

Since ethnopharmacological information represents a great shortcut in the search for new effective therapies, the aim of this work was to investigate the activity of leaves and flowers of *P. venusta* in melanogenesis process on murine B16F10 melanoma cells, in order to support its popular use on vitiligo treatment.

2. Materials and methods

2.1. Chemicals and reagents

Allantoin (98.0%) was purchased from Sigma–Aldrich (St. Louis, MO, USA), while methanol, acetonitrile (HPLC grade) and ethanol (analytical grade) were purchased from J. T. Baker Chemicals B. V. (Deventer, the Netherlands), and potassium dihydrogen phosphate from Merck (Frankfurt, Germany). Ultrapure water was obtained using a Milli-Q purification system from Millipore (Bedford, MA, USA). Penicillin/streptomycin was purchased from Invitrogen. Mushroom tyrosinase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-tyrosine and dimethyl sulfoxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbeccoĭs modified Eagleĭs medium (DMEM) and fetal bovine serum were obtained from Cultilab (Brazil). Sodium hydroxide was purchased from Biotec and kojic acid, from Galena (Campinas, SP, Brazil).

2.2. Botanical material

Leaves and flowers of *P. venusta* were collected in July 2008 in the city of Colombo (coordinates: S 25°19′16″; W 49°09′31″), Paraná, Brazil. The specie was identified by Osmar dos Santos Ribas from Municipal Botanic Museum of Curitiba (MBM). A voucher specimen (number 338804-02) was deposited at MBM of Curitiba, Paraná, Brazil.

2.3. Extracts obtention

Leaves and flowers of *P. venusta* (100 g, each) were air dried (40 °C, forced ventilation), powdered and extracted separately with ethanol:water (70:30, v/v) by turbolysis (Ultra Turrax Biotrona BA 80, Kinematica AG) at room temperature for 2 h. Hydroalcoholic extracts (HE) were filtered through a filter paper (Adventec No. 1) followed by solvent reduction to 1/6 of the initial volume under reduced pressure, lyophilized and kept at −20 °C until further investigations. Before the pharmacological assays the lyophilized HE was dissolved in distilled water and ethanol to produce different concentrations depending upon the assay performed.

2.4. Cell culture

Murine B16F10 melanoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (10 000 U/100 μg/mL) at 37 °C with 5% CO_2 in a humidified atmosphere.

2.5. Cell viability assay

Cell viability after treatment with tested extracts was determined using 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, USA) according to the method of Tada et al. (1986). Briefly, 7×10^3 cells were added in each well of a 96-well plate. After 24 h, cells were exposed to extracts of the *P. venusta* at several concentrations for 96 h. MTT solution was added and cell viability was then assessed in a colorimetric assay through mitochondrial dehydrogenase activity in active mitochondria to form purple formazan. Absorbance of each well was read at 570 nm using a plate reader (EL808B, BioTech Instruments, Inc., Winooski, VT, USA).

2.6. Determination of melanin content in melanocytes

Extracts influence on the production of melanin in melanocytes was determined using the modified method of Tsuboi et al. (1998). Cells were added into the wells of a 24-well plate (4×10^4 cells per well). After 48 h, different concentrations (0.01–3 μg/mL) of the HE (flowers and leaves), and kojic acid (1 mM, as a reference drug) were added to the cells and incubated at 37 °C in 5% CO_2, humidified atmosphere for 4 days. Control group was incubated just with DMEM or DMEM plus extract vehicle (0.02% ethanol). Then, the medium was removed and cells were lysed with 500 μL of NaOH 1 N in 10% DMSO at 80 °C for 1 h. The relative melanin content was determined by measuring the absorbance at 490 nm in a plate reader (EL808B, BioTech Instruments, Inc., Winooski, VT, USA).

2.7. Mushroom tyrosinase activity assay

Tyrosinase activity was determined according with the method of Hyun et al. (2008) with modifications. In a 96-well microplate were added: HE (flowers and leaves) at different concentrations (0.01–3 μg/mL), 20 μL of mushroom tyrosinase (500 U/mL in phosphate buffer, pH 6.5), 170 μL of a mixture [L-tyrosine solution

1 mM, potassium phosphate buffer 50 mM/pH 6.5, and distilled water (10:10:9, v/v/v), respectively]. Microplate was incubated at 37 °C for 40 min and the absorbance of the mixture was measured at 490 nm using a microplate reader (EL808B, BioTech Instruments, Inc., Winooski, VT, USA). One unit (U) of enzymatic activity was defined as the amount of enzyme needed to increase the absorbance at 280 nm by 0.001 per min, in a 3 mL reaction mixture containing L-tyrosine at pH 6.5 and 25 °C. The value of each measurement was expressed as percentage changes from the control (reaction mixture without HE). Wells with HE diluted in buffer without tyrosinase was carried out to exclude plant extract colour interference in the absorbance measurement.

2.8. Allantoin content determination

The allantoin content on HE of *P. venusta* was determined by HPLC analysis on an Agilent 1100 LC system (Wilmington, USA), consisting of a G1311A quaternary pump, a G1379A degasser, a G1329A automatic injector, a G1315B diode array detector and a LC workstation equipped with the Chemistation A.10.02 software package for data collection and exploration. Chromatographic separations were carried out on a Zorbax NH_2 (Agilent Technologies Inc., California, USA) 250 × 4.6 mm i.d., 5 μm particle size column connected to a pre-column Zorbax NH_2 (Agilent Technologies Inc., California, USA) 12.5 × 4.6 mm i.d., 5 μm particle size, maintained at room temperature. The column was eluted with a binary gradient system consisting of acetonitrile and potassium phosphate buffer (10 mM) at flow rate of 1.0 mL/min. The elution started with 80% of acetonitrile and decreased to 50% in eight min; follow to 0% in 2 min and returning to initial conditions in 2 min. The injection volume of the sample was 10 μL. The detection wavelength was set at 220 nm. Mobile phase were filtered through a 0.45 μm PTFE membrane (Millipore, Molsheim, France).

For HPLC analysis samples of HE (1000 mg) were redissolved in 10 mL of potassium dihydrogen phosphate buffer (10 mM) and sonicated in an ultrasonic bath for 30 min. After centrifugation (3500 rpm, 10 min) samples were purified by solid phase extraction (SPE), through a SPE cartridge (LC-18 500 mg/3 mL, Macherey-Nagel) previously activated with methanol (3 mL) and then washed with water (3 mL). After addition of samples (1 mL), the cartridges were eluted with 5 mL of potassium dihydrogen phosphate buffer and transferred to a 10 mL volumetric flask that was filled with the same solution. The resulting solution was filtered through a polyvinylidene fluoride (PVDF) syringe filter (11 mm, 0.45 μm, Millipore Millex, Billerica, USA) before injection into the HPLC system.

2.9. Statistical analysis

The results are presented as mean ± SEM of three independent experiments. Statistical significance between the groups was assessed by one-way analysis of variance (ANOVA) followed by a *post hoc* Newman Keuls test. The accepted level of significance for the test was $P < 0.05$. All tests were carried out using GraphPad Software (San Diego, CA, USA).

3. Results

3.1. Determination of melanin content in melanocytes

Melanogenic activity in cultured murine B16F10 melanoma cells is directly related to the quantity of produced melanin which is estimated through the amount of melanin retained in the cells (intracellular melanin). Both HE increased melanin formation (Fig. 1A and B), since melanin content was found to be enhanced in response to several concentrations of HE from leaves (0.1, 0.3, 1 and 3 μg/mL) and flowers (0.03 and 0.1 μg/mL). As depicted in

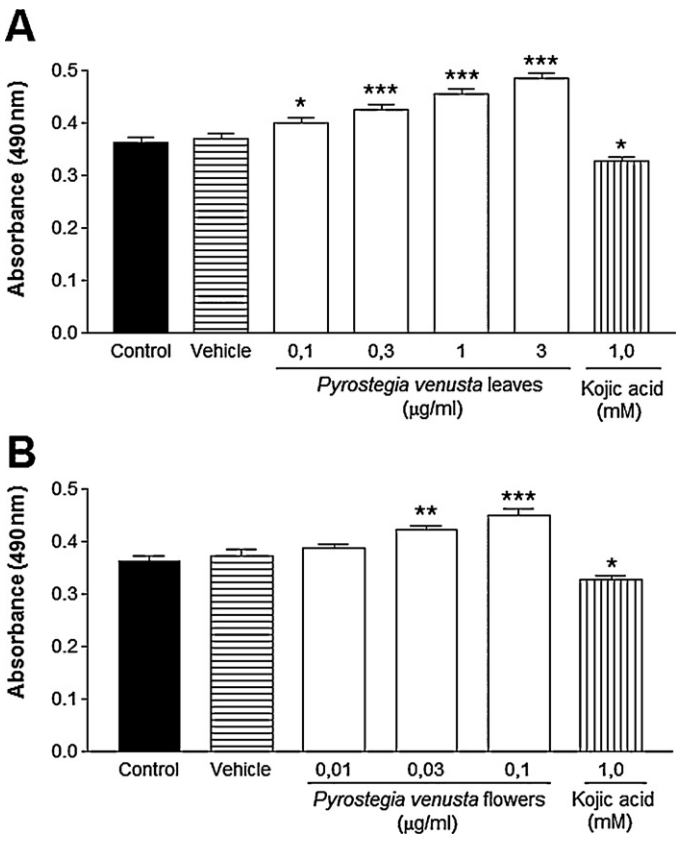

Fig. 1. Effects of leaves and flowers of *Pyrostegia venusta* on melanin content in cultured B16 murine melanoma cells. B16 cells were incubated for 96 h in the presence of leaves (A) or flowers (B) extracts at different concentrations. Kojic acid was used as a drug reference. Vehicle (0.02% ethanol). Data are mean ± SEM from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$: statistically significant *vs.* control group (DMEM).

Fig. 1A, the HE from leaves promoted enhancement of melanogenesis with maximum effect of 33.3 ± 3% (3 μg/mL). Similar result was observed with HE from flowers (Fig. 1B), which showed a maximum effect of 23.4 ± 3% (0.1 μg/mL). To exclude the possibility of interference of HE color in absorbance, all the concentrations of both HE were exposed to the used wavelength and no changes were observed when compared to the control group (phosphate saline buffer). The same procedure was done for the wavelengths used in the cell viability and tyrosinase activity assay, and no alteration was detected (data not shown).

3.2. Cell viability assay

The effect of leaves and flowers extracts from *P. venusta* on cell viability were measured by MTT assay (Fig. 2). Although the vehicle used with leaves extract promoted a significant decrease in cell viability (12.4%), the extracts did not show any additional cytotoxic effect on cells when compared with vehicle group. Besides, the cytotoxicity noted with the vehicle was not a concern since it was not able to interfere with melanin production (Fig. 1).

3.3. Mushroom tyrosinase activity assay

Once tyrosinase is the rate-limiting enzyme for melanin biosynthesis, the effect of *P. venusta* on tyrosinase activity was evaluated. However, extracts did not significantly affect the tyrosinase activity in all tested concentrations (Fig. 3). Kojic acid a known depigmenting agent used as a reference drug reduced tyrosinase activity in about 35%.

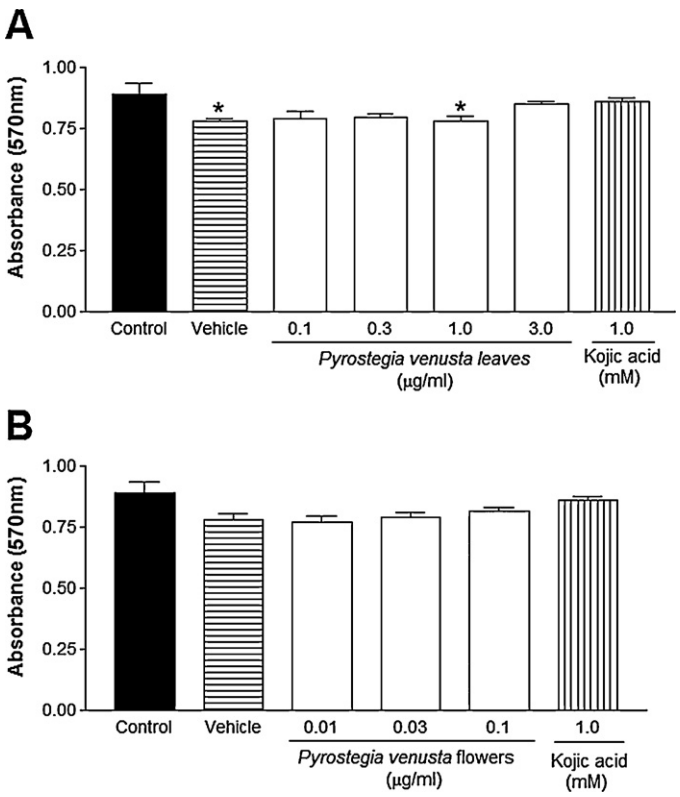

Fig. 2. Effects of leaves and flowers of *Pyrostegia venusta* on cellular viability in cultured B16 murine melanoma cells. B16 cells were incubated for 96 h in the presence of the leaves (A) or flowers (B) extract at different concentrations. Kojic acid was used as a drug reference. Vehicle (0.02% ethanol). Data are mean ± SEM from three separate experiments. $^{*}P < 0.05$: statistically significant *vs.* control group (DMEM).

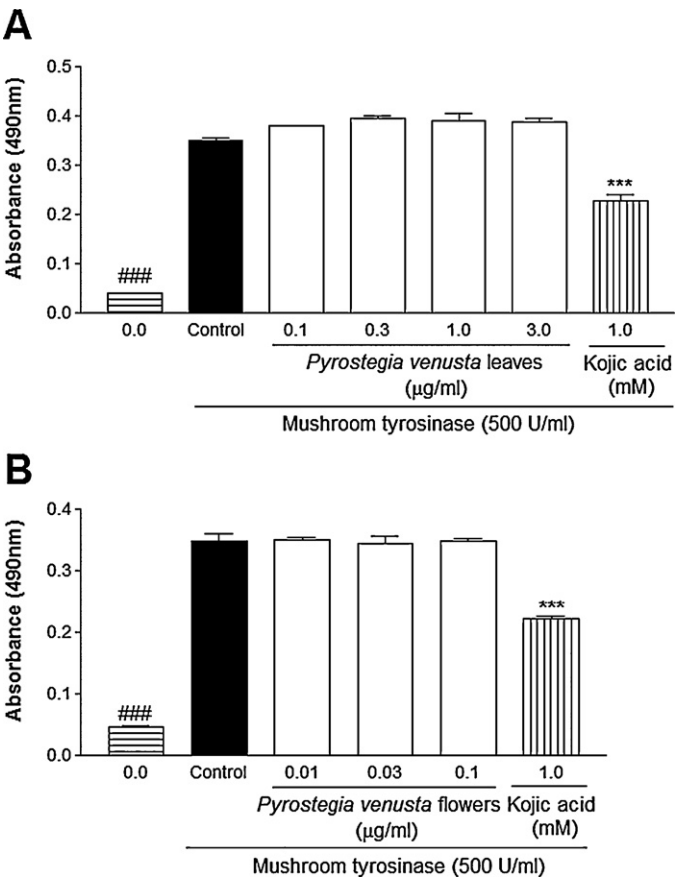

Fig. 3. Effects of leaves and flowers of *Pyrostegia venusta* on Mushroom Tyrosinase activity assay. Inhibition of mushroom tyrosinase activity by leaves (A) or flowers (B) extract at different concentrations. Tyrosinase activity was examined in the presence of L-tyrosine as substrates. It is represented as an inhibition percentage. Each value is given as mean ± SD from independent triplicate experiments.

3.4. Allantoin content determination

To determine the allantoin content in both HE of *P. venusta* an analytical method based on HPLC/DAD was performed. The identification of allantoin was done using both the spectral analysis (UV–VIS) and comparison of the RT of allantoin standard. Additionally, this compound was identified directly in the HE by 1D and 2D NMR analyses and comparison with data reported in the literature (Ferreira et al., 2000). HE of flowers and leaves of *P. venusta* presented 1.08% and 0.09%, respectively, of allantoin. To quantify allantoin in HE, a SPE clean up was carried out prior to HPLC analysis and the allantoin peak showed high purity (Fig. 4).

4. Discussion

In this study it was evaluated the melanogenic effect of two extracts (leaves and flowers) from *P. venusta* in murine B16F10 melanoma cells. This cell line has been widely used for this purpose once it is relatively easy to be cultured *in vitro*, and has similar melanogenic mechanisms to human melanocytes (An et al., 2008). When the cells were pre-treated with HE (flowers and leaves) at different concentrations, the cellular melanogenesis was significantly enhanced, although the same concentrations did not influence the activity of tyrosinase enzyme in the cell-free assay using DOPA as a substrate.

Recent advances in the knowledge of the pathogenesis of vitiligo have contributed to find better treatments. Nowadays many affected individuals have found a way for depigmented skin with medicinal or surgical therapies. However, progression of the disease, and partial or lack of complete repigmentation still occurs in a larger number of patients. Further studies, with newer and more potent therapies depend on the complete knowledge of vitiligo pathogenesis (Falabella and Barona, 2009). There are few studies with plant extracts aiming to increase melanin production. Some groups have reported the stimulation of melanogenesis by other plants, such as citrus flavonoid naringenin (Ohguchi et al., 2006), kava (*Piper methysticum*) rhizome extract and kavalactones (Matsuda et al., 2006), umberiferae plant extract and their coumarin constituents (Matsuda et al., 2005), and *Piper nigrum* leaves extract and their lignan constituents (Matsuda et al., 2004). However, all these studies suggested that the active constituents of these plants may be useful ingredients to cosmetics for prevention of white hair. Lee et al. (2007) showed that melanin content and tyrosinase expression were increased by rosmarinic acid in a concentration-dependent manner. Based on these results, the authors reported that rosmarinic acid induces melanogenesis through PKA activation signalling, and the utilization of rosmarinic acid as a pigmenting agent might be useful for treatment of hypopigmentation-related disorders (Lee et al., 2007).

Melanin synthesis is developed by a large number of effectors, but the tyrosinase is the rate-limiting enzyme. Furthermore, it has been reported other enzymes in the pathway of melanin synthesis, including tyrosinase related proteins 1 (TRP-1) and 2 (TRP-2) that also regulate melanogenesis (Tsukamoto et al., 1992). Other protein kinases such as cAMP-dependent protein kinase (Korner and Pawelek, 1977), protein kinase C-α (Gruber et al., 1992; Oka et al., 1993) and protein kinase C-β (Park et al., 1993) have been shown to participate in the regulation of melanin synthesis in pigment cells. The involvement of protein kinase C in melanogenesis

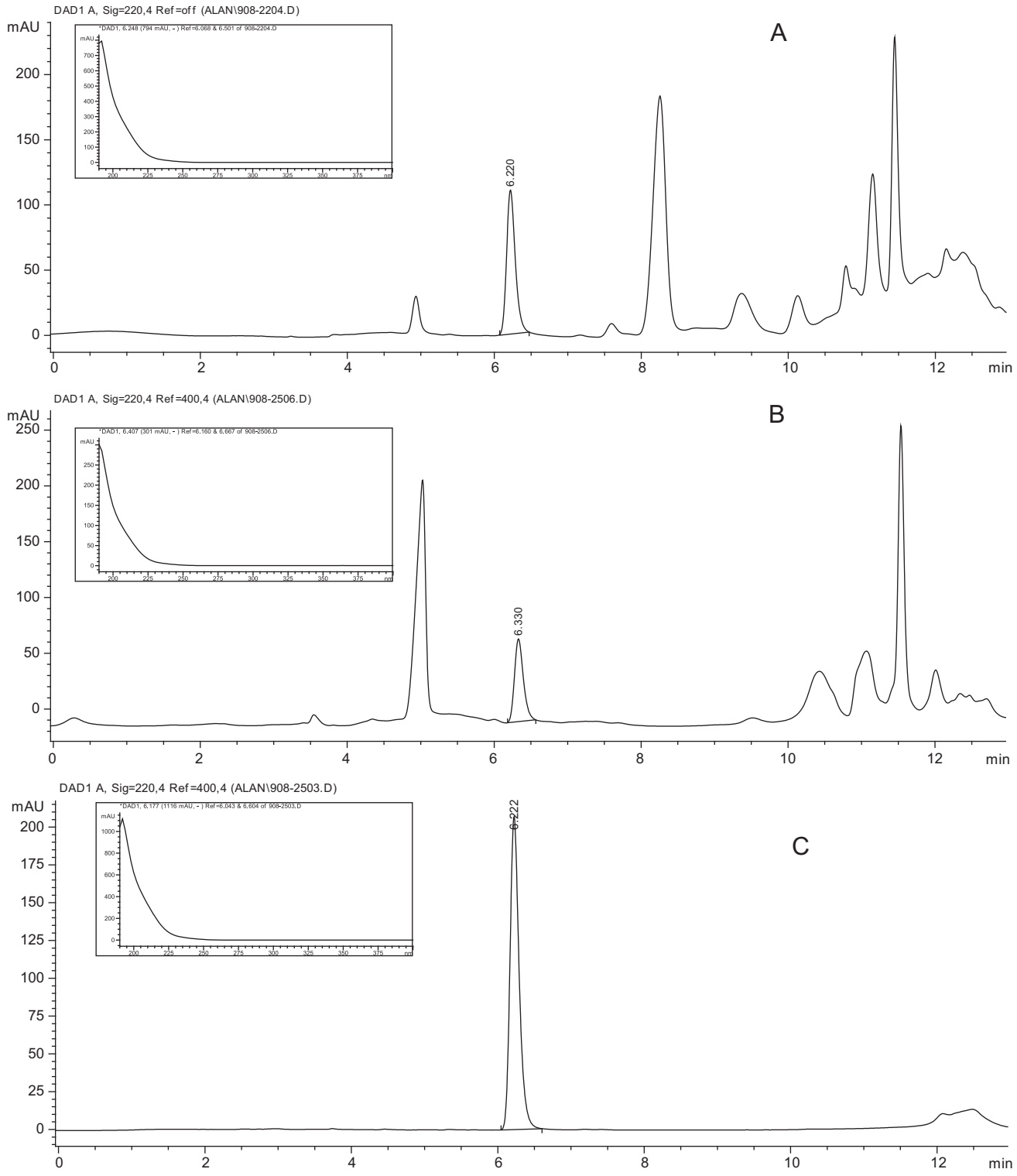

Fig. 4. HPLC chromatograms of HEs (A) flowers (allantoin RT = 6.22 min), (B) leaves of *Pyrostegia venusta* (allantoin RT = 6.33 min) and (C) allantoin standard (RT = 6.22 min). Chromatographic conditions: see Section 2.

still needs to be clarified (Carsberg et al., 1994; Oka et al., 2000). Furthermore, mitogen-activated protein kinase has been shown to be involved in cAMP-induced melanogenesis (Englaro et al., 1995) and c-kit-mediated activation of the microphthalmia-associated transcription factor (MITF), a trans-acting factor that regulates the gene transcription of tyrosinase (Hemesath et al., 1998). Thus, a probable mechanism throughout *P. venusta* stimulates melanogenesis might be related to other enzymes involved in the process of melanogenesis, other than tyrosinase. Moreover, it is also possible that the extracts of *P. venusta* could be inducing an increase in the expression of tyrosinase enzyme, without affecting its activity, which would also result in a raise in melanogenesis.

The detected compound allantoin has been widely described in the literature to have several pharmacological activities, such

as stimulation of cell mitosis (Loots et al., 1979), analgesia (Shestopalov et al., 2006), keratolytic activity (Veraldi et al., 2008), anti-inflammatory (Lee et al., 2010), antipsoriase, and immunostimulant, and it is widely employed in dermatology (Ferreira et al., 2000). Araujo et al. (2010) suggest that allantoin modulates the inflammatory response, possibly by inhibiting the chemotaxis of inflammatory cells in the site of the wound, thus preventing the release of reactive species responsible for the oxidative stress and tissue damage. Besides, allantoin treatment led to significant reduction in the levels of IgE and Th2-type cytokines, such as IL-4 and IL-5, in bronchoalveolar lavage (BAL) fluid. Since allantoin is a compound with several pharmacological properties, including in processes of skin disorders, it is possible that this compound could participate in the melanogenic action of *P. venusta*. For these reasons its content on the HE of leaves and flowers of *P. venusta* was determined, and results show low amounts in both extracts, but for the first time it is described in leaves and flowers of this plant.

Although this study clearly demonstrated the melanogenic effect of extracts of *P. venusta* in melanoma cells, important questions remain unclear, such as its *in vivo* efficacy. In fact, many other melanogenesis stimulators found in the *in vitro* studies failed to show *in vivo* efficacy, probably because they could not reach or enter skin cells because of the stratum corneum barrier. Further studies are needed to directly examine whether topically applied *P. venusta* can enhance human skin pigmentation under physiologically relevant conditions.

5. Conclusion

In summary, the present study revealed that both extracts of *P. venusta* (leaves and flowers) stimulates cellular melanogenesis at low concentrations, through a mechanism not yet elucidated. Thus, both extracts of *P. venusta* may be a potential option for the treatment of hypopigmentation diseases such as vitiligo. However, additional studies are necessary to address the mechanism of action and the safety of *P. venusta* extracts.

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