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Original Article

Effects of *Acmella oleracea* methanolic extract and fractions on the tyrosinase enzyme

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ABSTRACT

The aim of the current study is to evaluate the effect of *Acmella oleracea* (L.) R.K. Jansen, Asteraceae, methanolic extract, hexane (84.28% spilanthol) and dichloromethane (approximately 100% spilanthol) fractions on the tyrosinase enzyme. The dehydrated jambu extract was obtained through maceration using methanol. The extract residue was solubilized in MeOH/H_2O (8:2) and subjected to liq.–liq. partition in organic solvents. Both the extraction and the partition procedures were conducted with three replicates. The analyses were performed using GC–MS, ^1H and ^{13}C NMR. The hexane fraction provided samples containing 84.28, 82.91 and 62.83% spilanthol in repetitions 1, 2 and 3, respectively. The dichloromethane fraction showed 88.55% spilanthol in repetition 1, and approximately 100% spilanthol in repetitions 2 and 3. The jambu extract as well as the hexane fraction (84.28% spilanthol) were able to activate the oxidizing activity of the tyrosinase enzyme for L-DOPA. The dichloromethane fraction (approximately 100% spilanthol) showed stronger inhibition effect on the tyrosinase enzyme in the first 10 min. The results raise the interest in study in spilanthol formulations for topical use, since it may prevent and/or slow skin hyperpigmentation or depigmentation processes. Furthermore, spilanthol may be used to control the enzymatic browning in fruits and vegetables.

Introduction

Acmella oleracea (L.) R.K. Jansen, Asteraceae, is a native Amazonian plant popularly known as *jambu*. It is often used as condiment in typical dishes of the Northern Brazilian cuisine, such as *tacacá* and *pato-no-tucupi* (duck intucupi sauce). It is also used in folk medicine to treat stomatitis, colds and general pain (Nascimento et al., 2013). *A. oleracea* is considered to be an important native plant in the Amazon region, and it is found in large cultivation areas (Rebello and Homma, 2005). Spilanthol has been the main metabolite often isolated from *A. oleracea*. It is an aliphatic amide described as a burning viscous oil, which produces anesthetic effect and tongue tingling (Molina-Torres et al., 1996), and it is also able to penetrate the skin (Boonen et al., 2010a,b; Spiegeleer et al., 2013). Besides Spilanthol's anti-wrinkle effect (Demarne and Passaro, 2005), it is also possible mentioning its diuretic (Ratnasooriya et al., 2004), fungistatic and bacteriostatic activities (Molina-Torres et al., 2004), sensory properties (Ley et al., 2006), antiseptic activity, immune stimulation (Rojas et al., 2006), antioxidant and anti-inflammatory properties (Dias et al., 2012), saliva-secretion induction (Ramsewak et al., 1999; Sharma et al., 2011), analgesic (Rios et al., 2007), and acaricide activity (Castro et al., 2014), as well its use against skin diseases such as eczema (Boonen et al., 2010a,b).

A. oleracea has important chemical properties that awakened the interest of the pharmaceutical industry due to its active ingredient, spilanthol (Borges et al., 2012). Currently the search for natural products with inhibitory action on melanization process has increased, focusing on the phenol oxidase tyrosinase.

Tyrosinase, also known as polyphenol oxidase (PPO), is widely distributed in microorganisms, animals and plants. It catalyzes the oxidation of monophenols, *o*-diphenols and *o*-quinones (Karioti et al., 2007). Tyrosinase is known as a key enzyme in melanin biosynthesis and it is responsible for melanization in animals and for browning in plants. Tyrosinase is responsible for enzymatic browning reactions in damaged fruits during post-harvest

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handling and processing. Thus, controlling the enzymatic browning is essential during fruit pulp manufacturing processes (Seo et al., 2003; Khan et al., 2006).

Tyrosinase synthesis occurs inside highly specialized organelles called melanosomes. Studies have shown the presence of tyrosinase in all the evaluated melanomas; fact that proves this enzyme's importance to the development of this cancer type (Figueiredo, 2003). The increased production and accumulation of melanin hyperpigmentation may lead to disorders such as melasma (Miot et al., 2009). Many chemicals and food have demonstrated inhibitory effect on melanogenesis through the inhibition of the tyrosinase enzyme activity. Such result has increased the demand for natural products. Thus, the aim of the current study is to evaluate the effect of *A. oleracea* methanol extract, hexane (84.28% spilanthol) and dichloromethane (approximately 100% spilanthol) fractions on the tyrosinase enzyme.

Materials and methods

Jambu samples

The plant material (leaves, stems and inflorescences) from the jambu samples was collected in Igarapé-açu County, which is located in Bragantina Region, in the Northeastern Pará State, Brazil, at the coordinates: 01°07′33″ S and 47°37′27″ W (Oliveira et al., 2011). The plant (MG205534) was identified as *Acmella oleracea* (L.) R.K. Jansen, Asteraceae, and it was incorporated to the herbarium of Emílio Goeldi Museum, Belém, Pará State.

The plant was initially washed in water to remove soil residues, the roots were removed using stainless knives, and the plant's torn and crumpled parts, as well as those with darkened edges were eliminated from the drying process. The raw materials were sanitized through immersion in solution containing 200 ppm (mg l^{-1}) of free residual chlorine (FRC) derived from sodium hypochlorite with 10% purity, for 10 min. The last rinse was performed through immersion in solution containing 5 ppm (mg l^{-1}) FRC, for 10 min, and subsequent water drainage.

The cold-drying process was carried out in acclimatized room using air conditioning (Midea, model MS2E-18CR, Brazil) at 25 °C, and dehumidifier (Arsec, model 160, Brazil). The room measured 4 m^2 and remained closed during the drying procedure.

Extraction procedure

The dried *A. oleracea* plant material was crushed and subjected to an exhaustive extraction process through methanol (MeOH) maceration at room temperature (Mbeunkui et al., 2011). The solvent was removedin rotary evaporator at 40 °C under reduced pressure. The methanol used showed 99.8% purity and extraction was performed for about 30 days with approximately 6 l of solvent.

The MeOH extract was solubilized in MeOH/H_2O (8:2) and the solution was subjected to successive extractions in separatory funnel with the solvents: *n*-hexane, dichloromethane (CH_2Cl_2) and ethyl acetate (AcOEt), as shown in Fig. 1. Three replications were performed to obtain the extract and the liq.–liq. extraction.

Tyrosinase enzyme activity

The reagents employed in the inhibitory investigation were obtained at Sigma–Aldrich.

Tyrosinase inhibition activity was measured by a modified Patil and Zucker (1965) UV–vis method. The modifications consisted on the use of a different concentration of EDTA. Also, L-DOPA was employed as a substrate and finally, commercial tyrosinase was used where as the authors isolated the enzyme.

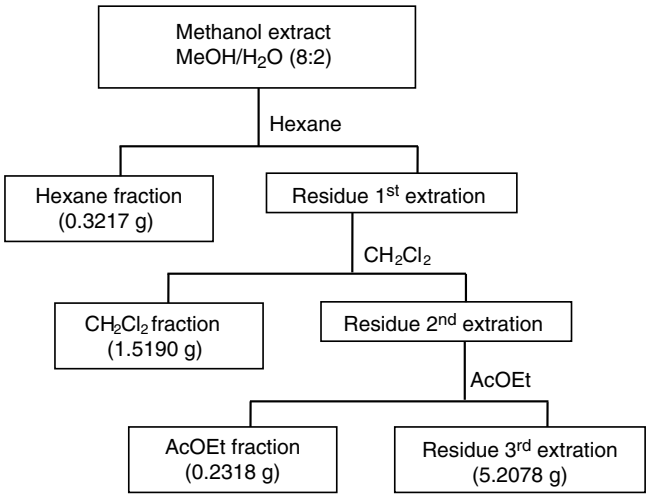

Fig. 1. Fractionation of the *Acmella oleracea* methanol extract in the second repetition.

Methanolic extract from jambu, hexane fraction (84.28% spilanthol) and dichloromethane (containing approximately 100% spilanthol) fraction were solubilized in dimethylsulfoxide (10 mg ml^{-1}) and different aliquots of the solution were added to the reaction medium containing the tyrosinase enzyme (50–100 units), EDTA (0.022 mmol l^{-1}), L-DOPA (0.17 mmol l^{-1}) in PBS (50 mmol l^{-1}, pH 8.0) in room temperature. The time for this phenolase to oxidize L-DOPA was 30 min after the reaction time readings were performed in a spectrophotometer (Shimadzu, model Mini 1240, Japan) UV–vis at 475 nm.

The other experiment evaluated the action of the methanol extracted from jambu by using the enzyme reaction medium with direct incidence of ultraviolet irradiation at 312 nm (used UV lamp) at 10 min intervals for 30 min.

The concentrations 0.66 and 0.16 mg ml^{-1} (methanolic extract); 0.51–0.05 mM (hexane fraction containing approximately 84.28% spilanthol) and 0.53 mM (dichloromethane fraction containing approximately 100% spilanthol) used in the enzyme activity evaluation test were used to determine the enzyme kinetics in the presence of sample. The evolution of the reaction was monitored by readings taken in UV–vis spectrophotometer at 475 nm for 30 min and 60 min in 10 min intervals.

The activation and inhibition values were calculated from the below equation:

$$\%\,\text{inib} = \left\{ \frac{[(B_{30} - B_0) - (A_{30} - A_0)]}{(B_{30} - B_0)} \right\} \times 100 \quad (1)$$

where B_0 = absorbance of L-DOPA + tyrosinase at t = 0 min, B_{30} = absorbance of L-DOPA + tyrosinase at time = 30 min, A_0 = absorbance of L-DOPA + tyrosinase + inhibitor/activator at time = 0 min, and A_{30} = absorbance of L-DOPA + tyrosinase + inhibitor/activator at time = 30 min.

The above equation allows the evaluation of the action of plant extracts and organic compounds on the enzyme tyrosinase, as what in t = 0 min. The possible absorption of the test samples at 475 nm (which are related to production dopacromona) is subtracted.

All experiments were performed in triplicate, and the results were expressed as means ± SD. The graphics was fit of the experimental data in Origin software (ANOVA statistical function).

Chemical analysis

The material was analyzed through a gas chromatograph coupled to a mass spectrometer – GC/MS (Shimadzu, model QP-2010 Plus, Japan) and through ^1H and ^{13}C NMR spectra (Bruker,