Original Article

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

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**A B S T R A C T**

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/H2O (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 13C 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 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.

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**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 taccá 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 (Rebelllo 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), fungistic 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...
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 Igaraçá County, which is located in Bragança 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 Emilio 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 an acclimatized room using air conditioning (Midea, model MS2E-18CR, Brazil) at 25 °C, and dehumidifier (Arsec, model 160, Brazil). The room measured 4 m² 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 removed in 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/H₂O (8:2) and the solution was subjected to successive extractions in separatory funnel with the solvents: n-hexane, dichloromethane (CH₂Cl₂) 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.

<table>
<thead>
<tr>
<th>Methanol extract</th>
<th>MeOH/H₂O (8:2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane fraction</td>
<td>(0.3217 g)</td>
</tr>
<tr>
<td>AcOEt fraction</td>
<td>(0.2318 g)</td>
</tr>
</tbody>
</table>

**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 of 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 ¹³C NMR spectra (Bruker,
model advance III, E11A). Dichloromethane (99.9%, HPLC grade, Sigma–Aldrich Inc., EUA) was used as solvent in the GC/MS analysis. The CDCl₃ (99%, Sigma–Aldrich Inc., USA) was used as solvent in the NMR analysis. The MEOD (99%, Sigma–Aldrich Inc., USA) was used as solvent in the NMR analysis of the jambu methanol extract. The GC/MS was equipped with a Factor Four VF-5 ms fused-silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness), using helium as carrier gas at 1 ml/min. The initial oven temperature was 100 °C; it was kept constant for 40 min and increased at the rate of 10 °C min⁻¹ to 290 °C; the final isotherm (300 °C) was held for 20 min. The injection volume of the sample was 1 μl (1:50 split mode). Both the injector and the detector were set at 300 °C. The mass spectra were obtained in the range of m/z 10-300, using the electron impact technique at 70 eV. The chemical composition of the samples was analyzed in a HP 5890 Series II gas chromatograph with flame ionization detector (FID). The same operational conditions and column type were used in the GC/MS analysis, except for the temperatures of the injector and the detector, which were 250 and 300 °C, respectively.

The percentage of each component was calculated through the integral area under the respective peaks in comparison to the total area of all the components of the sample.

The major component was identified according to information from the aforementioned analysis methods and from data generated through the comparison using ¹H NMR (Bruker 500 MHz spectrometer) and carbon ¹³C NMR (Bruker 125 MHz spectrometer).

**Results and discussion**

Table 1 shows the data of the *A. oleracea* methanol extract and fractions from three replications.

The main component in the hexane (F1R1) and dichloromethane (F2R32) fractions was identified through the peaks at 12.21 and 12.09 min in the GC chromatogram (Figs. 2a and 3). Both peaks presented mass spectrum, with main peaks at m/z (%): 221 (M⁺, 2), 141 (M-C₂H₅, 50), 126 ([C₇H₁₂NO]⁺, 40), 98 ([C₆H₆NO]⁺, 35), 81 (C₆H₅⁺, 100), as it is shown in Fig. 2b, and these data meet the spilanthol structure. The spilanthol structure was also confirmed through ¹H NMR and ¹³CNMR analysis and through its comparison with data described in the literature (Nakatani and Nagashima, 1992).

It is important to emphasize the presence of N-(2-methylbutyl)-(2E,6Z,8E)-decatrienamide in the hexane fraction. This amides' chemical structure is similar to that of spilanthol. This compound is also known as homospilanthol, and it is the second most abundant N-alkylamide in the *Spilanthes* ethanolic extract, since it represents 9.04% of the total N-alkylamides amount (Boonen et al., 2010a).

Colormetry is readily adaptable to an analysis of tyrosinase substrates and inhibitors. Substrates can be determined by their enzymatic conversion to darkly pigmented melanin-like materials with absorption maxima about 570 μ. In the presence of tyrosine-tyrosinase, inhibitors of the reaction will prevent colour development (Spencer et al., 1956).

The mechanism of action of tyrosinase and its interaction with l-DOPA was described in detail by Chang (2009, 2012).

Sample analyses show that the methanolic extract from jambu and the hexane fraction (84.28% spilanthol) are able to activate the oxidizing action of the tyrosinase enzyme for l-DOPA (Table 2).

The increased dopachrome formation was assessed after the direct ultraviolet light radiation over the methanolic extract was performed at 312 nm, and it showed high power activation in the enzyme. This wavelength is part of the UV-B radiation which is partly absorbed by the ozone in the atmosphere. The portion of this radiation that actually reaches the Earth is responsible for skin injuries. This analysis was performed at two concentrations, namely: 0.66 and 0.16 mg ml⁻¹, and it resulted in 57% and 9% increase, at the end of the 30-min analysis, respectively. The direct
Table 1
Methanol extract fractions from Acmella oleracea leaves, stems and flower plant material.

<table>
<thead>
<tr>
<th>Replications (dried jambu mass)</th>
<th>Methanol extract mass</th>
<th>Fractions</th>
<th>Code</th>
<th>Fraction mass (g)</th>
<th>Spilanthol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st (66.00)</td>
<td>12.51</td>
<td>Hexane</td>
<td>F1R1</td>
<td>0.0821</td>
<td>84.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dichloromethane</td>
<td>F2R1</td>
<td>0.2264</td>
<td>88.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethylacetate</td>
<td>F3R1</td>
<td>0.2662</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residue</td>
<td>F4R1</td>
<td>4.4151</td>
<td>–</td>
</tr>
<tr>
<td>2nd (265.00)</td>
<td>48.45</td>
<td>Hexane</td>
<td>F1R2</td>
<td>0.3217</td>
<td>82.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dichloromethane</td>
<td>F2R2</td>
<td>1.5190</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethylacetate</td>
<td>F3R2</td>
<td>0.2318</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residue</td>
<td>F4R2</td>
<td>5.2078</td>
<td>–</td>
</tr>
<tr>
<td>3rd (263.90)</td>
<td>40.58</td>
<td>Hexane</td>
<td>F1R3</td>
<td>0.3819</td>
<td>62.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dichloromethane</td>
<td>F2R3</td>
<td>3.1742</td>
<td>79.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethylacetate</td>
<td>F3R3</td>
<td>0.2791</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residue</td>
<td>F4R3</td>
<td>6.9670</td>
<td>–</td>
</tr>
</tbody>
</table>

* Relative spilanthol concentration in the samples.

Table 2
Tyrosinase activation through jambu methanolic extract and hexane fraction.

<table>
<thead>
<tr>
<th>Jambu methanolic extract</th>
<th>Hexane fraction (%4.28% spilanthol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[mg ml⁻¹]</td>
<td>% of activation</td>
</tr>
<tr>
<td>0.83</td>
<td>357</td>
</tr>
<tr>
<td>0.66</td>
<td>297</td>
</tr>
<tr>
<td>0.5</td>
<td>107</td>
</tr>
<tr>
<td>0.33</td>
<td>63</td>
</tr>
<tr>
<td>0.16</td>
<td>39</td>
</tr>
</tbody>
</table>

Fig. 4. The UV (312 nm) radiation effect on the activation power in jambu methanol extract on the tyrosinase enzyme.

impact of the UV radiation at 312 nm significantly decreased the power activation in the jambu methanolic extract (Fig. 4).

Another interesting fact was observed during the kinetic experiment that used the here in adopted concentrations. The potential tyrosinase enzyme activation through hexane fraction at 475 nm containing 84.28% spilanthol reached its maximum in thirty min; however, there was no significant power activation decay up to 60 min (Fig. 5).

The dichloromethane fraction containing approximately 100% spilanthol showed tyrosinase enzyme inhibition (IC₅₀ = 0.50 mM) due to the higher enzyme inhibition power within the first 10 min. After this time, the inhibition power decayed (Figs. 6 and 7).

In these experiments we obtained the increase in the percentage of dopachrome production, percentage activation, in addition to the enzyme kinetics in the presence of these samples.

The ability of the dichloromethane fraction to inhibit the tyrosinase activity may be translated into its potential as skin whitening agent. Melanin production is reduced when the tyrosinase enzyme activity is inhibited, and it results in a fairer skin (Karim et al., 2014).

Fig. 5. The kinetic action of the tyrosinase enzyme on L-DOPA in the presence of hexane fraction.

Fig. 6. Inhibition activity of dichloromethane (containing approximately 100% spilanthol) fraction on the tyrosinase enzyme, with L-DOPA substrate.

Fig. 7. Kinetics of the tyrosinase enzyme inhibition through dichloromethane fraction containing approximately 100% spilanthol (0.53 mM).
These results indicate that spilanthes can be used to control the enzymatic browning in fruits and vegetables, since this amide inactivates the tyrosinase enzyme (polyphenol oxidase) involved with these products’ darkening process. Thus, spilanthes may increase the shelf life and the nutritional quality of processed fruits and vegetables, such as standard tyrosinase inhibitors kojic acid, IC50 value of 0.130 mM (Okinjii et al., 2007).

Tyrosinase inhibitors are chemical agents able to reduce enzymatic reactions, such as fruit and vegetable’s skin browning and human skin melanization. Therefore, these agents have good commercial potential in both the food processing and the cosmetic industries (Lim et al., 2009).

Conclusion

The activating or inhibitory actions against the tyrosinase enzyme depend on the degree of purity of jambu extracts and fractions. The jambu methanolic extract results on the tyrosinase enzyme corroborated the here incarried out analyses, which used hexane fraction of 84.28% spilanthes. Such result proved the tyrosinase enzyme activation through dopachrome increase. Therefore, jambu-based products may be used as cosmetics (creams, soap, etc.). At random, they can accelerate melanin production (which darkens the skin), as well as form melamnas. However, its use against located depigmentation processes (skin and hair) demands further studies, since the analysis using UBV irradiation showed viable formulations containing this extract in tropical countries.

Since the dichloromethane fraction (containing approximately 100% spilanthes) showed ability to inhibit the tyrosinase enzyme, spilanthes arouses interest in the study based on formulations with this amide in topical products. Surely it can to prevent and/or reduce skin hyperpigmentation processes. Another possible application of spilanthes is the control of enzymatic browning in fruits and vegetables. The most tyrosinase inhibitors are not currently commercially available, especially those from natural sources, and this limits their further evaluation in an in vivo study, where usually a large amount is needed for an inhibition test.

Thus further studies need to be performed to found inhibitors with a human clinical point of view including help and cooperation of cosmetic or biotechnology groups.

Authors’ contributions

AFB, KCS, MCCO, MGC, and AUOSR all contributed to the writing of this article. AFB, MGC, and AUOSR obtained and identified samples. KCS and MCCO performed enzymatic analyzes.

Conflicts of interest

The authors declare no conflicts of interest.

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