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Inhibition of Gelatinases by Vegetable Extracts of the Species *Tapirira guianensis* (Stick Pigeon)

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Research Article

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ABSTRACT

Tapirira guianensis (Stick pigeon), a widely-used herbal medicine, has been reported to possess various biological activities. The aim of this study was the phytochemical analysis of the fractions of extracts of *T. guianensis* and the investigation of the action of these extracts on the activity of gelatinases using zymography. Matrix metalloproteinases (gelatinases) have prognostic influences in human cancers, where higher expressions of these enzymes are associated with increased aggressiveness and biological behavior of tumors. Many natural products have been tested on several stages of carcinogenesis to demonstrate their effectiveness in the inhibition or activation of molecules that are important for tumor progression. This study identified the fractions obtained from the crude extract of *T. guianensis* (Stick pigeon), which efficiently inhibited gelatinases.

Keywords: Metalloproteinases; zymography; Tapirira guianensis; cancer;

ABBREVIATIONS

AB: Activation Buffer; BM: Basement Membrane; CE: Crude Extract; DMBA: 7,12-dimethylbenz[a]anthracene; ECM: Extracellular Matrix; MMPs: Matrix Metalloproteinases MMP-2: Matrix Metalloproteinases, type 2; MMP-9: Matrix Metalloproteinases, type 9.

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1. INTRODUCTION

The matrix metalloproteinases (MMPs) constitute a family of more than 26 endopeptidases. The MMPs possess homologous protein sequences and are related to the specificity and recognition of other proteins. They differ structurally between themselves and in their ability to degrade particular groups of proteins of the extracellular matrix (ECM) (Kerlelã and Saarialho-Kere, 2003). Various studies demonstrate the prognostic influence of MMPs in human cancers, associating higher expressions of these enzymes with increased tumor aggression and biological behavior. However, this aggression may be associated with the low activity of inhibitors of these proteases in the tissue (Hofmann et al., 2000; Chaudhary et al., 2010; Qin and Tang, 2002; Luo, 2006; Vacca et al., 2001).

For the metastasis of cancer cells to occur, these cells must be able to separate from the primary tumor and adjacent structures by degrading the basement membrane (BM) and the ECM. The MMPs, especially gelatinases (MMP-2 and MMP-9), are important in this process because these as they degrade components of the ECM and BM (Kerlelã and Saarialho-Kere, 2003). Therefore, the inhibition of the proliferation and invasion that is mediated by gelatinases may be the key to preventing the metastasis of neoplastic cells.

New treatments that prevent the formation of metastases may improve the chances of survival of patients with cancer, since the process of metastasis is the greatest obstacle to the success of treatment. As such, the inhibition of the activity of MMPs may hold therapeutical potential and has become an important area in cancer research, in addition to offering excellent opportunities for the study of the mechanisms of carcinogenesis (Ding et al., 2004). Many natural products have been tested at several stages of carcinogenesis or tested to demonstrate their ability to inhibit or active molecules that are important in the tumoral progress (Tate et al., 2004; Ribeiro et al., 2010). Thus, several compounds from natural products have been proposed as alternatives to combat various stages of carcinogenesis (Seo et al., 2005; Lambert et al., 2005), either as agents to prevent cancer by inhibiting tumor proliferation and initiation or carcinogenesis (Sarfaraz et al., 2008; Vaid et al., 2010) or even by acting as inhibitors in experimentally-induced cancer (Albert-Baska and Ignacimuthu, 2010). The action of these compounds can occur by various mechanisms, such as the suppression of the expression of anti-apoptotic gene products (Aggarwal and Shishodia, 2004) and by inhibiting angiogenesis (Xiao et al., 2008).

Tapirira guianensis, popularly known as Stick pigeon, belongs to the Anacadiaceae family and is widely distributed in Brazil and South America, in several biomes and vegetation types, such as the gallery bushes that belong to the Brazilian Cerrado regions (Matos and Felfili, 2010). This species is mellifera tree that produces an aromatic oil with a lemon scent (Lorenzi, 2002). The oil is often used to treat dermatitis, syphilis (Rodrigues and Carvalho, 2001), malaria and leishmaniasis, and is still used as an antimicrobial, antifungal (Roumy et al., 2009) and depurative agent (Rodrigues and Carvalho, 2001). Correia et al (2008) identified a series of new hydrobenzofuranoids in addition to other long chain metabolites in the leaves of *T. guianensis.* Thus, the purpose of this study was to investigate the action of crude *Tapirira guianensis* and its fractions on the activity of gelatinases using zymography.

2. MATERIALS AND METHODS

2.1 Chemicals

All solvents and reagents were purchase from Sigma – Aldrich, USA

2.2 Plant Material

Samples of the T. guianensis species were collected in the Cerrado region near the city of Ituiutaba (Minas Gerais, Brazil - Latitude S 18° 58'08"and Longitude W 49º 27' 54"), and identified. Voucher specimens (number 143405) were deposited at the Herbarium of the Department of Botany, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

2.3 Extraction Procedure

Dried and powdered leaves (300.5 g) were extracted by maceration (5 L of 70% hydroalcoholic solution, 15 days) to give the Crude Extract (CE), brown residue, 6.3318 g, 2,1%, which was filtered and lyophilized afterwards. This residue was dissolved in ethanol/water (7:3) and successively extracted with hexane (C_6H_{14}), chloroform ($CHCl_3$) and ethyl acetate ($C_4H_8O_2$), resulting in 3.3174 g (52.4%), 1.1687 g (18.5%), 0,6975 g (11.0%) and 0.7065 g (11.2%) of hydroalcoholic (A), hexane (B), chloroform (C), and ethyl acetate (D) fractions, respectively. The extract and fractions were stored at -20 °C until analysis or zymography.

2.4 Phytochemical study

Phytochemical tests to detect the presence of secondary metabolites, such as tannins, flavonoids, esteroids, triterpens, coumarins, saponins and alkaloids were performed according to Matos (1988). These tests are based on the visual observation of color modification or precipitate formation after the addition of specific reagents.

2.5 Gelatin Zymography

The proteolytic enzyme activity of pro-MMP-2 was measured by gelatin zymography. MMP-2 (Sigma M9445) was used as standard and gelatinases were obtained from human saliva. This was collected by the spitting method (Navazesh, 1993). Volunteers did not ingest, or any stimulant-containing dyes, the day before the test. Oral hygiene was performed correctly and saliva was stimulated by sugar and taste-free chewing gum for 5 minutes (basal saliva) at ten minutes before the test. Saliva collected for the first 2 minutes was discarded and the remainder was placed in mini-cooled tubes, centrifuged at 14 000g, before discarding the pellet and retaining the supernatant. The total protein was measured by the Bradford method (1976) using bovine serum albumin (Sigma) as standard. Equivalent amounts (0.4 µg) of proteins from the saliva were mixed with an equal volume of non-denaturing buffer (2% SDS,125 mM Tris-HCl, pH 6.8, 10% glycerol and 0.001% bromophenol blue) and the protein samples were subjected to electrophoresis through a 7% Zymogram Ready Gel (Bio-Rad Laboratories). After eletrophoresis, the gel was incubated twice in 2.5% TritonX-100 for 20 minutes at room temperature and then incubated at 37ºC for 18 hours in activation buffer (10 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ -Tris-$CaCl_2$). Gels were stained (0.25% Comassie blue G-250, 30% ethanol, 10% acetic acid) for 1 hour and destained (30% ethanol, 10% acetic acid) for 2 hours. Gelatinolytic activity was detected as unstained bands.

Bands were quantified by scanning densitometry using software (Scion Image 2000-2001 Scion Corporation). The control for this test was the activation buffer (AB).

2.5 Effect of Plant Extracts on Gelatinolytic Activity

After gelatin gel electrophoresis, gels were washed with 2.5% Triton X-100, as described above, and then divided and incubated at 37ºC for 18 h in 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ (Tris-$CaCl_2$) in the presence of the following extracts of *T. guianensis*; crude extract (CE); hydroalcohol fraction (A); hexane fraction (B); chloroform fraction (C); ethyl acetate fraction (D). Subsequently, gels were stained and distained as described above.

3. RESULTS AND DISCUSSION

3.1 Zymography

Since the CE of *T. guianensis* inhibited the proteolytic activity of gelatinases present in the gels, fractionated extracts were then used in inhibition tests. Extracts obtained after separation had an inhibitory effect on the activity of gelatinases, as demonstrated by zymography. The proteolytic activity of gelatinases was visualized by light bands on the dark background of the gels, and the sizes of the bands were inversely proportional to the inhibitory effect of each extract. The inhibition of gelatinases was evaluated by comparative analysis of the images of bands, present in control gels, AB, with the images of the bands of the gels subjected to treatments, comparing the number of pixels in each figure (Figure 1).

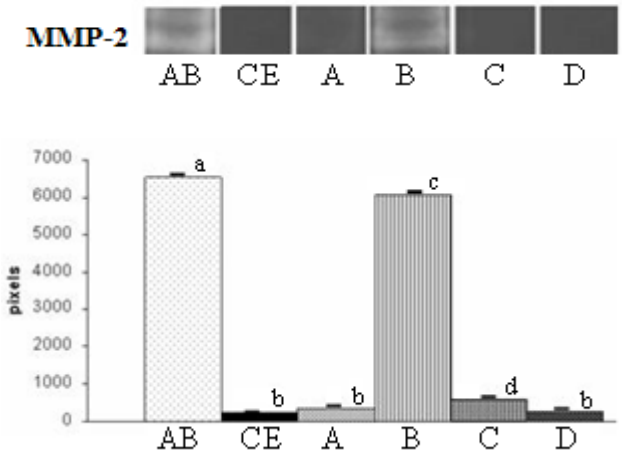

Fig. 1. Effect of different extracts on the proteolytic activity of gelatinases

(a) Zymography of fractions of T. guianensis: the proteolytic activity of gelatinases was visualized by light bands on the dark background of the gels, and the sizes of the bands were inversely proportional to the inhibitory effect of each extract. (b) Comparative analysis between the zymogram of treatments: The bands were quantified by densitometry and data represent mean ± SD. Different letters indicate p<0.001 between treatments. AB - activation buffer; CE- crude extract; A – hydroalcohol fraction; B- hexane fraction; C- chloroform fraction; D- ethyl acetate fraction.

All tests showed significant inhibition, when compared with the control gel ($p < 0.001$). However, the activity of fraction B was very small when compared to the inhibitions observed for fractions A, C and D. No difference was observed between fractions CE, A and D ($p > 0.05$).

3.2 Phytochemical Analysis of Fractions of *T. guianensis*

Considering the high inhibition of fractions A, C and D in zymography, these fractions were subjected to phytochemical analysis for the identification of its major components. After zymography, phytochemical tests were carried out with the fractions that best influence the activity of gelatinases in the gel. Table 1 shows the secondary metabolites in the crude extract (CE), hydroalcohol (A), Chloroform (C) and ethyl acetate (D) fractions of *T. guianensis*. The tannins and flavonoids were present in all extracts, whereas coumarin were not observed in extract A. Saponin, and Steroids and Triterpens were present separately in fractions C and D respectively. Alkaloids were observed only in fractions A and C.

Table 1. Phytochemical tests with the crude extract (CE), hydroalcohol (A), chloroform (C) and ethyl acetate (D) fractions of plant *T. guianensis*

Components	CE	A	C	D
Tannin	++	++	++	+++
Coumarin	+	-	++	+ +
Flavonoids	++	++	+	++
Saponin	+	-	+	-
Steroids and Triterpens	+	-	-	+
Alkaloids	+	+	++	-

Preliminary tests demonstrated that the CE of leaves of *T. guianensis* (Stick pigeon) inhibited the activity of gelatinase A. Thus, fractions were obtained with hydroalcohol, hexane, ethyl acetate and chloroform solutions, and these were used in zymographic tests to verify their potential gelatinase-inhibitory effects. Of these fractions, the A, C and D fractions were the most effective at inhibiting gelatinases by zymography. The following secondary metabolites were identified in these extracts: tannins, coumarins, flavonoids, saponins, steroids (and triterpens) and alkaloids. The C and D fractions had similar concentrations of the tannins, coumarins, flavonoids, when compared with the CE fractions. D fractions do not presented saponins and alkaloids and C fraction do not presented steroids and triterpens. Fraction A presented just tanins, flavonoids and alkaloid components.

The MMPs have a complex function, degrading ECM components, releasing ECM sequestered proangiogenic substances, and processing growth factors, integrins, and adhesion molecules (Rundhaug, 2005), in turn assisting the movement of neoplastic cells to other sites. Coumarins, isolated from plants of the *Ferula* species, have been shown to play an important role in anti-tumor promoting activities (Iranshahi et al., 2008) and diversin, another coumarin, is reported to inhibit papilloma formation in mouse skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene (DMBA), indicating that this might be valuable as a potent cancer chemopreventive agent (Iranshahi et al., 2010). This evidence may support the hypothesis that the coumarins may be responsible for the inhibition observed with the CE, C and D fractions, but does not explain the inhibition obtained for the A fraction. Since the A fraction was able to inhibit the gelatinases in a manner similar to that of the CE and the D fraction, it may be suggested that this inhibition may be related to tannin and

flavonoid, since these compounds have proven anti-tumor actions (Shih et al, 2009; Yanga et al, 2008). Moreover, the alkaloids were present in fraction C in a greater concentration, when compared to fractions CE, A and D, which showed a significant inhibitory effect on gelatinolytic activity. It is possible that the activity of fraction C is principally related to alkoloid activity with a contribution from the tannins and flavonoids. In addition to their anti-tumor promoting activities, the alkaloids may also have antimetastatic effects mediated by the inhibition of MMPs (Chang et al., 2002). A study, using glioma cells treated with berberine, showed that cancer cell metastasis can be significantly inhibited (Lin et al., 2008). Furthermore, flavonoids have demonstrated potential as chemopreventive agents, inhibiting angiogenesis and the proliferation of neoplastic cells, *in vitro*. This chemoprotective effect may also be due to the inhibitory action of gelatinases (Kim, 2003).

4. CONCLUSION

MMPs participate in fundamental neoplastic processes, such as invasion and tumor progression there is a need for further investigations to identify the molecules present in plant species that are capable of inhibiting the activities of these enzymes. So, the inhibitory effect of the extract of *Tapirira guianensis* (Stick pigeon) on gelatinolytic activities (gelatinase MMP-2) was well demonstrated in this study. Results offer important therapeutic perspectives for the treatment of cancer and suggest that several classes of secondary metabolites may be responsible for the inhibition of gelatinase. Our research group is currently concentrating on carrying out further studies of these fractions to identify the specific molecules that demonstrated these biological actions.

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