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Evaluation of *In-vivo* Antitumor Activity of *Annona crassiflora* Wood Extract

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ABSTRACT
Annona crassiflora is a native tree from Brazilian savanna area of the state of Minas Gerais. The ethanolic extract of *A. crassiflora* wood was obtained and purified, and an annonaceous acetogenins-rich fraction was obtained and characterized. The *in vivo* antitumor activity and toxicity of this fraction were evaluated in Ehrlich solid tumor-bearing Swiss mice. The annonaceous acetogenins showed a pronounced *in vivo* antitumor effect, with a reduction in the Ehrlich's tumor growth of 38% and 20% after single intratumoral and intravenous administration, respectively, at a dose of 1.25 mg/kg, as compared to the control group. Concerning toxicological studies, the absence of clinical signs and renal toxicity could be observed, and all animals survive throughout the entire experimental period (14 days). By contrast, mielotoxicity and hepatotoxicity could be detected in mice treated with the *A. crassiflora* wood extract.

Keywords: *Annona crassiflora*; antitumor activity; annonaceous acetogenins; Ehrlich solid tumor.

INTRODUCTION

The chemistry of natural products has provided many effective drugs for different diseases. As reported by Craig *et al.* [1], natural products and their derivatives represent approximately 50% of all drugs in clinical use, in which 25% of this amount are natural products from higher plants. In addition, at least 119 substances isolated from plants are considered to be important in current medical use in several countries.

Many phytochemical studies on the Annonaceae species have been reported. Over the last twenty years these studies have increased and have become associated with pharmacological studies. [2] The discovery of the annonaceous acetogenins has intensified these studies due to the several biological activities presented by this new class of natural products. The isolation of uvariamicin in 1982, the first annonaceous acetogenins reported, was achieved using the bioguided chromatography isolation process for antitumor compounds.

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The ethanolic extract from the roots of *Uvaria accuminata* exhibited an antitumor activity which acted against 3PS lymphocytic leukemia in mice and uvariamicin was detected as responsible for this activity. Since then, another 350 annonaceous acetogenins have been reported. [3] In addition to antiparasitic, pesticidal, antimicrobial and immunosuppresive activities, these are also judged as promising candidates (lead compounds) for a future generation of drugs produced to fight against resistant tumors. Although few studies have reported on *in vivo* antitumor activity using annonaceous acetogenins, they have shown promising results. Uvariamicin and asimicin have proven to act against murine lymphocytic leukemia with 157% T/C at 1.4 mg/kg and 124% T/C at 25 µg/kg, respectively, where T= average of survival time of treated rats and C= average of survival time of the control group. Bullatacin showed an antitumor efficacy in athymic mice bearing subcutaneously implanted A2780 human ovarian carcinoma xenografts. This chemical was able to inhibit 68% of the tumor growth at dose of 0.1 mg/kg per day, as compared to 78% for cisplatin at a dose of 5 mg/kg per day and was used as a standard. [4]

Annona crassiflora is a native tree from the Brazilian savanna area of the state of Minas Gerais. The crude

ethanolic extract of of *Annona crassiflora* seeds and some fractions obtained containing annonaceous acetogenins have shown great cytotoxic activity in several tumor cell lines. [5-7]

The present research consisted of a phytochemical and biological study using the ethanolic extract of *Annona crassiflora* wood. Annonaceous acetogenins, aporphine alkaloids, and steroids were isolated from this extract. The extract and some fractions presented antimalarial and antimicrobial activities. [8]

The purpose of this study was to investigate the *in vivo* antitumor activity and the toxicity of an annonaceous acetogenins-rich fraction obtained from the ethanolic extract of the tree bark.

MATERIALS AND METHODS

Plant material extration: The barks of *A. crassiflora* used in the present work were collected in Itatiaiuçu, Minas Gerais, Brazil. Voucher specimens (No. 22988) were deposited at the Instituto de Ciências Biológicas Herbarium (BHCB). Barks were dried at 40°C, weighted (4.075 kg), grounded and extracted at room temperature with ethanol/H_2O (8:2) which was removed under vacuum to produce the crude extracts (ACCF01; 684.4 g; 16.8% dry wt). Part of this extract (200.00 g) was dissolved in ethanol/water (7:3) and subsequently extracted with hexane, HCl 0.1% aqueous solution followed by chloroform. The organic phases were washed with water; the solvents were removed, in turn to producing the hexane (ACCF02; 4.5149 g) and the organic layer (ACCF03; 39.8252 g). The acid aqueous phase was alkalinized with an amonium aqueous solution until reaching pH= 9 and was then extracted with ethyl acetate, which after solvent removal yielded an alkaloidic fraction (ACC A; 0.2947 g). The ACCF03 fraction was submitted to gel filtration on Sephadex LH-20 eluted with chloroform/methanol 8:2, producing 24 fractions which were combined in 12 groups. Group **5,** involving the fractions 6-8, were characterized by a TLC silica gel with Kedd´s reagent, Dragendorff´s reagent, and phosphomolibdic acid as spray revelator. This group was also analyzed by HPLC, ^{1}H NMR, ^{13}C NMR and ESI-MS.

Antitumoral activity: The antitumor activity of ACC 6-8 was evaluated in Ehrlich solid tumor-bearing Swiss mice. Ehrlich tumors cells were transplanted subcutaneously in the right flank of female Swiss mice (2×10^6 cells/mice, 29-32g). The ACC 6-8 solution was prepared by its dissolution in a mixture of dimethylacetamide and PEG 300 (40:60, v/v) plus 5% (v/v) of Tween 80. In addition this solution was diluted in a 0.9% (w/v) NaCl solution to achieve a concentration of 0.19 mg/ml. This preparation was administered by both intratumoral (IT) and intravenous (IV) routes at doses of 1.25mg/kg (eight mice per group). Treatment was performed at single dose on day 0. Control groups were treated with the same preparation described above but without ACC 6-8. Antitumor activity was evaluated over a 27 day period by determining the tumor volume and calculating the tumor growth inhibition ratio.

Toxicity study: A toxicity study was performed on healthy female Swiss mice (27-30 g) which received ACC 6-8 by IV route at a dose of 1.25mg/kg (n = 5 animals). Over a 14-day period, behavior modifications and the weight of the mice were monitored. From the body weight data, the weight variation over time was calculated as a ratio to the initial body weight. After this time, blood samples were collected for hematological and biochemical analysis.

Statistical analysis: For antitumoral activity, Tukey's test was used to compare the mean values (tumor relative volume and tumor inhibiton growth ratio). Regression model estimates were used at time intervals for tumor growth investigations. To analyze the body weight variation parameter, the value before drug treatment was used as the covariate. Differences were considered statistically significant when P values were lower than 0.05.

Table 1: Tumor relative volume and tumor inhibiton growth ratio determined 27 days after the administration of ACC 6-8 by it or *iv* route

	Intratumoral		Intravenous	
	Control	ACC 6-8	Control	ACC 6-8
TRV[1] ± SEM (cm^3)	6.9 ± 0.8	4.3 ± 0.4[a]	4.9 ± 0.2	3.9 ± 0.1[a]
TGI[2] (%)	-	38	-	20

[1] TRV represents a tumor relative volume. [2] TGI represents tumor growth inhibition ratio. [a] represents significant difference between ACC 6-8 and the control group. *P*-values of less than 0.05 were set as the significance level (Tukey's test). The values represent the mean ± S.E.M.

Table 2: Hematological parameters of healthy female swiss mice submitted to the ACC 6-8 treatment at a dose of 1.25 mg/kg by *iv* route

Parameters	Control	ACC 6-8
Red Blood Cells (10^6/mm^3)	8.1 ± 0.2	8.7 ± 0.3
Hemoglobin (g/dl)	43.3 ± 0.5	42.7 ± 0.8
Hematocrit (%)	12.9 ± 0.3	13.0 ± 0.4
White Blood Cells (10^3/mm^3)	6.0 ± 0.7	3.7 ± 0.6 [a]
Neutrophils (10^3/mm^3)	2.0 ± 0.3	0.7± 0.1 [a]
Lymphocytes (10^3/mm^3)	3.9 ± 0.4	3.0 ± 0.5
Monocytes (10^3/mm^3)	0.07 ± 0.02	0.14 ± 0.04

[a] represents a significant difference between the ACC 6-8 treatment and the control groups. *P*-values of less than 0.05 were set as the significance level (Tukey's test). These values represent the mean ± S.E.M

Table 3: Biochemical parameters of healthy female swiss mice submitted to the ACC 6-8 treatment at a dose of 1.25mg/kg by *iv* route

Parameters	Control	ACC 6-8
ALT (IU/L)	66 ± 4	188 ± 48 [a]
AST (IU/L)	103 ± 21	300 ± 46 [a]
ALP (IU/L)	109 ± 21	242 ± 39 [a]
GGT (g/dL)	2.2 ± 0.6	2.0 ± 0.5
Urea (mg/dL)	53 ± 4	48 ± 2
Creatinine (mg/dL)	0.58 ± 0.04	0.43 ± 0.05

[a] represents significant difference between the ACC 6-8 treatment and the control groups. *P*-values of less than 0.05 were set as the significance level (Turkey's test). These values represent the mean ± S.E.M

RESULTS AND DISCUSSION

Chemical characterization: The fraction ACC 6-8 was obtained as a yellow waxy and was submitted to TLC analysis where the plates were sprayed with Kedde's reagent and phosphomolibdic acid solution. [2, 4] The plates showed a positive test, characteristic of an α,β-unsaturated-γ-lactone moiety, commonly found in annonaceous acetogenins. [4] The fraction ACC 6-8 also showed ions which are characteristic of acetogenins adduct with m/z at 677.52, 661.56, 639.40, 635.61, and 595.36 in (+) the ESI-MS spectrum. The proton NMR spectrum data (δ 7.19, 6.96, 5.04, 2.51, 2.40, and 2.24) indicated the presence of two types of α,β-unsaturated γ-lactones, whith and without an OH-group in C-4. The presence of these two types of lactones could be confirmed by ^{13}C NMR data (δ 172.23, 171.45, 150.52, 148.0, 79.37, 77.24, and 69.0). [2] NMR resonances due to oxygen-bearing carbons at δ 83.54, 82.69, 82.50, 82.32, 81.77, 79.37, 74.36, and 71.64 and the proton signals at δ 3.80, 3.56, 3.40, 3.11, 1.98, 1.65, and 1.61 were detetcted. These signals are typical of the THF rings flanked by two α-OH groups, which is characteristic of an annonaceous acetogenins structure. All

these data lead to the conclusion that this fraction consists of a mixture of annonaceous acetogenins.

Antitumoral activity: The IT treatment using ACC 6-8 as compared to control group effectively inhibited the growth of Ehrlich solid tumor (Fig. 1 A). The equations of linear regression analysis obtained for ACC 6-8 and the control groups were mean tumor volume (MTV) = 52.9x ± 334.5 and MTV = 117.2x ± 425.4, respectively, and the correlation coefficient (r) values were 0.974 and 0.984. At fifteen days post IV administration, no significant difference in the ability of ACC 6-8, as compared to the control group, to inhibit the tumor growth could be observed. However, after this time interval, ACC 6-8 proved to be efficient in controlling the tumor volume (Fig. 1 B). The equations of linear regression analysis obtained for ACC 6-8 and the control groups were MTV = 39.8x ± 364.6 and MTV = 52.1x ± 308.8, respectively, and the r values were 0.960 and 0.962. Data were presented as mean values ± the standard error of the mean (n = 8 animals for each group).

Tumor relative volume in mice that received ACC 6-8 at a dose of 1.25 mg/kg was significantly lower than that of the control group in both routes investigated. In addition, the tumor growth inhibition ratio was higher in the mice treated by IT route as compared to those treated by IV route (Table 1).

The results of this study clearly demonstrated that ACC 6-8 presents a pronounced *in vivo* antitumor effect, giving that a single administration significantly retarded the growth tumor, as compared to the control group in both evaluated routes. Previous studies with acetogenin showed that bullatacin (50 µg/kg/day) and bullatalicin (1 mg/kg/day) reduced the tumor growth of human ovarian carcinoma (A2780) cells in athymic mice by 67% and 75%, respectively. [9] Annonacin, administered in doses of 10 mg/kg over a 2 weeks period, reduced the tumor size of murine pulmonary carcinoma (LLC) cells by 59.7% in BDF-1 mice. [10] A reduction of 38% and 20% in the growth of Ehrlich tumors in mice could be observed after the IT and IV administrations of ACC 6-8 at doses of 1.25 mg/kg. The higher antitumor activity observed after IT treatment is most likely due to the higher concentration of acetogenins in the tumor as compared to the amount that actually reaches this region after IV treatment. Studies of the structure–activity relationship of acetogenins indicated that the THF system and the γ-lactone ring represent an essential factor in this antitumor activity. [2] Moreover, *bis*-adjacent-THF acetogenins proved to be the most potent whithin this family. In this study, the mass and the NMR data obtained showed the presence of bis-THF acetogenins that contain one α,β-unsaturated γ-lactone at the end of the chain, which are within the requirements for the therapeutic activity. However, further investigation into the *in vivo* antitumor activity using other dose schedules of ACC 6-8 to be administered by IV route are already underway in our laboratory.

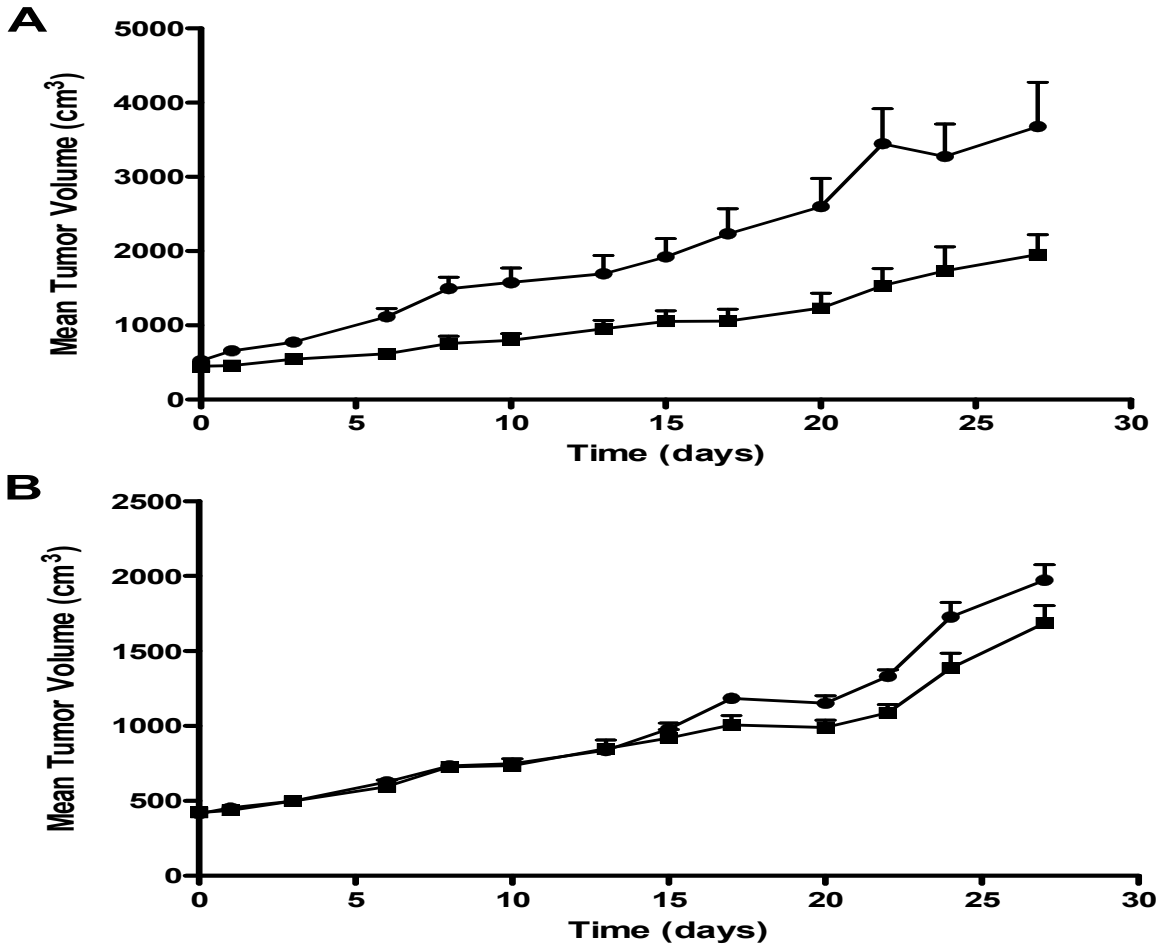

Fig. 1: Antitumor effect of the ACC 6-8 fraction (■) administered by IT (A) and IV (B) routes, at doses of 1.25 mg/kg, in Ehrlich solid tumor-bearing Swiss mice. The control group (●) was treated with a mixture of dimethylacetamide and PEG 300 (40:60, v/v) plus 5% (v/v) Tween 80 diluted tenfold in a 0.9% (w/v) NaCl solution. Data were presented as mean values ± the standard error of the mean (n = 8 animals for each group)

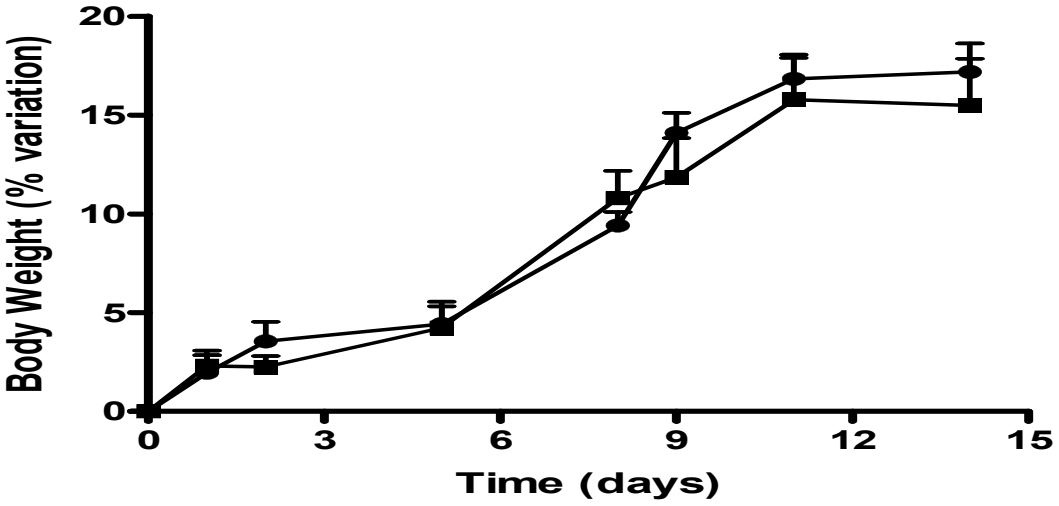

Fig. 2: Body weight variations of healthy female Swiss mice observed for 14 days after IV administration of ACC 6-8 at a dose of 1.25mg/kg (■) or vehicle (control group) (●). The values represent the mean ± S.E.M

Mice submitted to ACC 6-8 treatment showed no apparent clinical sign of toxicity throughout experimental period. In addition, no weight loss could be observed in animals treated with 1.25 mg/kg of ACC 6-8. There was also no significant difference in the body weight of the mice treated with ACC 6-8 when compared to the control group (Fig. 2).

Changes in hematological and biochemical parameters are summarized in Table 2 and 3. No significant changes in red blood cells, hematocrit and hemoglobin caused by the administration of ACC 6-8 could be observed. The total white blood cell count was significantly reduced and associated with a marked neutropenia in mice submitted to ACC 6-8 treatment (P<0.05). In addition, platelet levels increased in mice treated with ACC 6-8 when compared to the control group (Table 2). Concerning the biochemical parameters, a significant increase in ALT, AST and ALP levels were observed after ACC 6-8 treatment, indicating the appearance of hepatotoxicity. Similar results were also obtained by Kumar and coworkers [11] who evaluated the acute toxicity of oil extracted from custard apple seeds. These authors showed that this oil is able to induce hepatotoxicity and mielotoxicity. However, it is important to note that even with the appearance of hepatic and medullary toxicity after the administration of ACC 6-8, no death of the animals could be observed. Finally, no alteration could be detected in the urea and creatinine levels in mice treated with ACC 6-8 when compared to the control group (Table 3).

In conclusion, the results of the present study showed that the annonaceous acetogenins-rich ACC 6-8 fraction does in fact possess an antitumor activity, which gives grounds for the continued investigation of this fraction as a promising new anticancer agent.

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