Genotoxicity investigation of araticum(*Annona crassiflora* Mart., 1841, Annonaceae) using SOS-Inductest and Ames test

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Abstract

Although the use of medicinal plants or natural products has increased in recent decades all over the world, little information is available on their potential risk to health. *Annona crassiflora* Mart., a plant commonly known as araticum in Brazil, has been widely used in folk medicine for a long time since its seeds and leaves are often utilised in the treatment of cancer, snake bites, and venereal diseases, its fruits are consumed as tonic and astringent, and its bark powder has anti-fungal and anti-rheumatic properties. To evaluate the genotoxic and mutagenic properties induced by the ethanolic extract of araticum leaves, we performed the prophage λ induction (Inductest) and bacterial mutagenicity assays. We used *Escherichia coli* WP2s(λ) and RJF013 strains in the lysogenic induction test, whereas the mutagenic studies were carried out using *Salmonella typhimurium* histidine auxotroph strains TA97a, TA98, TA100, and TA102. Each experiment was performed three times in duplicate and included positive and negative controls. No statistically significant ($p > 0.05$) positive results were obtained for any of the strains tested, which suggests that the ethanolic extract of araticum leaves did not exhibit direct mechanisms of genotoxicity or mutagenicity that could be detected by the tests used in the present work.

Keywords: Annona crassiflora, araticum, genotoxicity, mutagenicity, Inductest, Ames test.

Investigação sobre a genotoxicidade de araticum (*Annona crassiflora* Mart. Annonaceae) usando SOS-Induteste e Teste de Ames

Resumo

Embora o uso de plantas medicinais ou de produtos naturais venha aumentando nas últimas décadas no mundo todo, existem poucas informações acerca de seu risco potencial para a saúde. *Annona crassiflora* Mart., uma planta comumente conhecida como araticum no Brasil, tem tido amplo uso em medicina popular há muito tempo, uma vez que suas sementes e folhas são frequentemente empregadas no tratamento de câncer, picadas de cobras e doenças venéreas, seus frutos são consumidos como tônico e adstringente, e o pó da casca de seu tronco apresenta propriedades antifúngicas e antirreumáticas. Para avaliar as propriedades genotóxica e mutagênica induzidas pelo extrato etanólico das folhas de araticum, utilizaram-se os testes de indução do profago λ (Induteste) e de mutagenicidade bacteriana. Foram empregadas as linhagens WP2s(λ) e RJF013 de *Escherichia coli* no teste de indução lisogênica, enquanto os estudos sobre mutagenicidade foram conduzidos utilizando as linhagens auxotróficas para histidina TA97a, TA98, TA100 e TA102 de *Salmonella typhimurium.* Cada experimento foi executado três vezes em duplicata, incluindo controles positivo e negativo. Não foram obtidos resultados positivos estatisticamente significativos ($p > 0,05$) para quaisquer das linhagens testadas, o que sugere que o extrato etanólico das folhas de araticum não apresentou mecanismos diretos de genotoxicidade ou mutagenicidade que pudessem ser detectados pelos testes usados no presente estudo.

Palavras-chave: Annona crassiflora, araticum, genotoxicidade, mutagenicidade, Induteste, Teste de Ames.

1. Introduction

Approximately 60% of the world's population relies almost entirely on plants for medication and natural products have long been recognised as an important source of therapeutically effective medicines (Harvey, 2000). A lot of what is considered alternative medicine nowadays, not too long ago constituted the only available treatment for many diseases. This could be one of the reasons why people actually believe that natural products are "good" and synthetic ones are "dangerous" (Schmitt et al., 2003). However, the indiscriminate use of plants can pose risks to human health, since many of them possess toxic compounds that are able to induce mutational events in somatic or germ cells (Almeida, 1993). The frequent use of medicinal plants has been correlated with a high incidence of tumors in the population. In fact, the proportion of tumors attributed to the use of dietetic compounds, infections, and natural carcinogens is much higher than that caused by environmental and chemical agents (Fonseca et al., 1994).

The plant *Annona crassiflora* Mart., 1841 (Annonaceae), popularly known as araticum in Brazil, is originally from the Brazilian Cerrado area and presents various applications in folk medicine. Its leaves, bark, fruits, and seeds have long been used as invigorating, astringent, antisyphilitic, and antidiarrheal agents, and also against snake bites (Corrêa, 1926; Cruz, 1979; Suleiman et al., 2008). Moreover, many other annonaceous species, such as *Cyathostemma argentium* J. Sinel, *Annona cherimola* Mill., *Annona senegalensis* Pers., *Annona glabra* L., as well as *A. crassiflora* Mart. have been traditionally used ethnomedically against cancer (Graham et al., 2000; Cochrane et al., 2008).

Several studies report that species of the family Annonaceae have important pharmacological constituents, such as: benzilate flavanones, which present cytotoxic properties; diterpenes, with antitumor activity; oliverine, with antiparkinsonian properties; and liriodenine, with antitumor, bactericidal, and antifungal activities (Leboeuf et al., 1982; Zhang et al., 2004). In addition to these properties, a class of molecules recently found in the family Annonaceae, the acetogenins, has been proven to present not only significant cytotoxic, antimitotic, and antimicrobial activities, but also a potent antitumor activity (Rupprechet et al., 1990; Zhu et al., 2001; Yuan et al., 2006). It is also believed that these molecules are potential antineoplastic agents (Alali et al., 1999; Tormo et al., 1999; Yuan et al., 2003).

Although the literature on annonaceous is vast, only a few studies are focused on *A. crassiflora*. Roesler et al. (2006) demonstrated that the pulp, peel, and seed extracts of araticum exert excellent antioxidant effects, as high as other known antioxidant plant products, such as ascorbic acid. The ethanolic extract of *A. crassiflora* also presented, along with the ethanolic extract of *A. glabra*, the highest larvicidal activity against *Aedes aegypti* when compared to other 50 Brazilian medicinal plants (Omena et al., 2007). The acetogenin isolated from the ethanolic extract of araticum, named araticulin, has shown to be identical to cherimolin-2 or bullatanocin, and also exhibits important cytotoxic activity (Santos et al., 1996; Zafra-Polo et al., 1998).

Due to the large utilisation of *A. crassiflora* Mart. and the relevant pharmacological properties it has demonstrated until now, the objective of the current study was to evaluate the genotoxic potential of multiple doses of its ethanolic extract. Because no single test can predict the potential genotoxicity of a compound, we aimed to shed light on this issue using two bacterial standard assays: SOS-Inductest (prophage λ induction) and Ames test (reverse mutation).

2. Material and Methods

2.1. Plant material

Leaves of *Annona crassiflora* Mart. (Annonaceae) were collected in Paraúna (16° 56' 52'' S and 50° 26' 55'' W), a town in the state of Goiás, Brazil. The air-dried and powdered leaves were exhaustively extracted with 95% aqueous ethanol at room temperature for three days, and the resultant alcohol solution was filtered and then concentrated to dryness under reduced pressure at 40 ºC. The crude ethanolic extract was transferred to glass flasks filled to the top and kept at 5 °C until the moment of use. The powder of ethanolic extract of araticum (EEA) used in the experiment was dissolved in 20% aqueous ethanol solution.

2.2. Strains

Escherichia coli SOS-Inductest tester strains WP2s(λ) and RJF013 and *Salmonella typhimurium* strains TA97a, TA98, TA100, and TA102 were kindly supplied by the Laboratório de Radiobiologia Molecular of the Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

2.3. SOS-Inductest – Prophage λ induction

The SOS-Inductest was performed according to Moreau (1981). An exponential-phase culture of *E. coli* WP2s(λ), grown in LB (1% bacto tryptone Difco, 0.5% bacto yeast extract Difco, and 1% NaCl) was centrifuged, resuspended and incubated at 37 °C for 25 minutes with 50 µg, 100 µg, 200 µg, 500 µg, 1000 µg, and 2000 µg of EEA. The mixture was conveniently diluted in M9 buffer (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 1% NH_4Cl, and 0.05% NaCl) and 0.1 mL of the lysogenic culture [WP2s(λ)] was added to 0.3 mL of the indicator strain (RJF013). We added 2.0 mL of top agar at 45 °C (0.6% agar Difco, 0.5% NaCl) to the mixture and poured it into Petri dishes with LB. Each assay was performed three times in duplicate and included negative (20% aqueous ethanol) and positive (1 µg mitomycin C - MMC) controls. After incubation at 37 °C for 24 hours, the plaques were counted and a bacterial survival test for virtual comparison was performed simultaneously.

2.4. Ames Test – Salmonella mutagenicity assay

The *Salmonella* histidine point mutation assay proposed by Maron and Ames (1983) was followed. We incubated 0.1 mL of bacterial suspension (1-2 x 10^9 cells/mL) of

each strain (TA97a, TA98, TA100, and TA102) with 20 µg, 40 µg, 200 µg, 400 µg, 2000 µg, and 4000 µg of EEA at 37 °C for 25 minutes. Then, 2.0 mL of top agar (0.6% agar Difco, 0.5% NaCl, 50 µM L-histidine, 50 µM biotin, 45 °C) were added to the test tubes and poured into Petri dishes containing minimal agar medium (1.5% agar, 2% glucose, and Vogel-Bonner E medium). Each assay was performed three times in duplicate and included negative (20% aqueous ethanol) and positive [5 µg and 1µg of 4-nitroquinoline 1-oxide (4-NQO) per plate for TA97a and TA98, respectively; 10 µg of sodium azide for TA100; and 5 µg MMC for TA102] controls. After incubation at 37 °C for 48 hours, the colonies (His^+ revertants) were counted.

2.5. Statistical analysis

All the results were tabulated and the experimental values were expressed as mean ± standard deviation. The data obtained in the genotoxicity experiments using the SOS-Inductest were primarily transformed into $\log_{10}$ scale and the difference among the treatments was evaluated by ANOVA and Tukey's test when necessary. The results obtained from the experiments of mutagenicity were directly evaluated by ANOVA and Tukey's test for difference of means. The dose-response relationship was analysed, for both cases, by linear regression (Vieira, 2004).

The magnitude of the lysogenic induction was measured using the induction factor (IF), calculated as the ratio between the number of plaques of each test group and the number of plaques of the negative control group. In parallel, the survival fraction (SF) as a function of the EEA doses was calculated as the ratio between the number of colonies in the test groups and the number of colonies in the negative control group. The greatness of the mutagenicity induction was measured by the mutagenic index (MI), calculated as the ratio between the number of colonies in the test treatment and the number of colonies in the negative control treatment. We used the software Excel and/or SAS in all analyses.

3. Results

The genotoxic analyses are shown in Table 1. We did not observed a statistically significant augmentation ($p > 0.05$) on prophage λ induction in any tested doses compared to the negative control group. However, we detected a significant ($p < 0.05$) difference between the negative control group and the tested doses of araticum against the positive control group (MMC). The regression analysis revealed the existence of a statistically significant ($p < 0.05$) linear relationship between EEA doses and frequency of plaques. The SF showed a potent action of EEA doses on cellular inactivation, starting at 500 µg/plate (Table 1), with an average survival of 64%, which decreased to 56% and 17% at 1000 µg/plate and 2000 µg/plate, respectively.

The results of the mutagenic analysis can be observed in Table 2. The data obtained for the positive and negative control groups indicated that the strains were in agreement with the guidance established both by Maron and Ames (1983) and Mortelmans and Zeiger (2000). We observed a similar profile in relation to the frequency of His^+ revertants for all tested strains (TA97a, TA98, TA100, and TA102) since no statistically significant difference was found between each negative control group and any doses of EEA ($p > 0.05$); however, we detected a significant difference ($p < 0.05$) between the negative control groups and the treatment doses of EEA in contrast with each positive control, as expected. As to the dose-response evaluation, strains TA97a and TA102 did not present significant linear relationship ($p > 0.05$), whereas strains TA98 and TA100 showed significant dose-responses. Nevertheless, none of the strains tested reached MI ≥ 2 with the EEA treatment, the highest induction occurring for strain TA100, which reached MI = 1.39 at the dose of 4000 µg/plate.

4. Discussion

According to Dearfield et al. (2002), there is no single test to detect the full spectrum of the different endpoints encompassed by induced genotoxicity. Because of this, in the present study we used two test systems: the prophage

Table 1. Means and standard deviation (sd) transformed into $\log_{10}$ scale (obtained from three independent experiments carried out in duplicate), Induction Factor (IF) and Survival Fraction (FS), respectively showing genotoxic and cytotoxic actions after treatment with different doses of ethanolic extract of araticum (EEA).

Treatment	Means ± sd	IF	SF
Negative control[1]	4.6 ± 0.3^A	1	1
Positive control[2]	6.3 ± 0.4^B	1.4	-
EEA 50 µg/plate	4.5 ± 0.3^A	0.80	1.11
EEA 100 µg/plate	4.4 ± 0.2^A	0.70	1.27
EEA 200 µg/plate	4.4 ± 0.2^A	0.72	1.48
EEA 500 µg/plate	4.4 ± 0.3^A	0.64	0.64
EEA 1000 µg/plate	4.3 ± 0.1^A	0.48	0.56
EEA 2000 µg/plate	4.1 ± 0.1^A	0.34	0.17

[1]20% aqueous ethanol; [2]1 µg MMC. Values followed by the same letter did not present significant differences ($p > 0.05$); $R^2_{Plaques} = 17.08$ % ($p < 0.05$).

Table 2. Means and standard deviation (sd) of histidine revertants (obtained from three independent experiments carried out in duplicate) and Mutagenic Index (MI) for four strains of *Salmonella typhimurium* after treatment with different doses of ethanolic extract of araticum (EEA).

Treatment	TA97a		TA98		TA100		TA102	
	Means ± sd	MI	Means ± sd	MI	Means ± sd	MI	Means ± sd	MI
Negative control[1]	148 ± 22^{A}	1.00	40 ± 6^{A}	1.00	128 ± 14^{A}	1.00	131 ± 15^{A}	1.00
Positive control[2]	784 ± 15^{B}	5.44	338 ± 25^{B}	15.00	1100 ± 18^{B}	6.18	1752 ± 98^{B}	13.07
EEA 20 µg/plate	132 ± 24^{A}	0.89	43 ± 7^{A}	1.09	152 ± 10^{A}	1.18	120 ± 34^{A}	0.91
EEA 40 µg/plate	186 ± 68^{A}	1.29	51 ± 4^{A}	1.29	157 ± 19^{A}	1.22	132 ± 48^{A}	1.01
EEA 200 µg/plate	176 ± 38^{A}	1.21	38 ± 2^{A}	0.94	161 ± 21^{A}	1.25	138 ± 58^{A}	1.34
EEA 400 µg/plate	150 ± 24^{A}	1.02	43 ± 4^{A}	1.11	176 ± 11^{A}	1.37	133 ± 46^{A}	1.01
EEA 2000 µg/plate	151 ± 24^{A}	1.09	40 ± 3^{A}	0.99	177 ± 9^{A}	1.38	135 ± 43^{A}	1.03
EEA 4000 µg/plate	172 ± 37^{A}	1.15	36 ± 10^{A}	0.93	177 ± 9^{A}	1.39	115 ± 46^{A}	0.88

[1]20% aqueous ethanol; [2]5 µg 4-NQO for TA97a; 1 µg 4-NQO for TA98; 10 µg for TA100; and 5 µg MMC for TA102. Values followed by the same letter did not present significant differences ($p > 0.05$), for each strain independently. $R^2_{TA97a} = 1.36\%$ ($p > 0.05$); $R^2_{TA98} = 13.24\%$ ($p < 0.05$); $R^2_{TA100} = 24.78\%$ ($p < 0.05$); $R^2_{TA102} = 0.95\%$ ($p < 0.05$).

λ induction (SOS-Inductest) and the bacterial reversion assay (Ames test).

It is presumed that the signal for the induction of SOS functions triggered by a genotoxic agent is the generation of single-stranded DNA in an attempt to replicate damaged templates or interrupt normal replication. These events follow the activation of the RecA protein. The phenomenon of phage induction is one of the SOS functions and occurs due to the structural similarity between the prophage repressor cI and the bacterial LexA repressor, which enables their interaction with activated RecA. After cI cleavage, the bacterial genes responsible for the lytic cycle are expressed (Moreau, 1981; Leitão and Alcantara-Gomes, 1994; Lewin, 2001).

On the other hand, the phenomenon of reversion to prototrophy occurs as a consequence of new mutations at the site of the preexisting ones or near the mutated genes, which restore the gene's function and allow the cells to synthesise the required amino acid histidine (Mortelmans and Zeiger, 2000). Thus, the SOS-Inductest detects agents that inhibit DNA replication (genotoxic agents), while the Ames test is effective to identify genetic damage that leads to gene mutations (mutagenic compounds).

Plant extracts do not have obvious ways of action. Ribeiro et al. (2009) showed that although the chloroform fraction of *A. populnea* (Reiss) Lundell shows no genotoxic or clastogenic/aneugenic effects in the bone marrow cells of Wistar rats, its crude bark wood extract shows a slight but significant increase in clastogenicity in those cells. On the other hand, Nikaidou et al. (2005) showed that the catechins, important constituents of green tea, are able to reduce the mutagenic potential of hydroxyl radical. And Andrade et al. (2008) showed *C. regium* (Mart. and Schr Pilger) 1924 does not present antimutagenic profile.

Our results demonstrated that the EEA was able to induce cellular inactivation through a decrease in the SF of *E. coli*. (Table 1), mainly at higher doses. In fact, the cytotoxic potential of the family Annonaceae constituents (even *A. crassiflora* itself) is widely known (Corrêa, 1926; Cruz, 1979; Leboeuf et al., 1982; Santos et al., 1996; Zafra-Polo et al., 1998; Alali et al., 1999; Tormo et al., 1999; Yuan et al., 2003).

Cytotoxic agents can induce a great variety of events in the cell: DNA or protein damage, inhibition of growth, apoptosis, etc. The nature of the interaction between a drug and its target is of critical importance in determining the cell fate. Hickman et al. (1992) affirm that the biological outcome of that interaction will also be determined by the nature of cellular events "downstream" of the initial interactions. In this way, many cytotoxic drugs employed in chemoterapy, such as MMC and cyclophosphamide, present mechanisms of action based on their genotoxic/mutagenic potential activity, which also represents a potential risk to long-term patients.

Despite the EEA cytotoxicity, the genotoxic and mutagenic evaluation of *A. crassiflora* revealed that this plant is not able to provoke sufficient genetic damage to reflect a significant augmentation in the number of plaques or colonies compared to the negative control. Using the Ames test, a compound is considered mutagenic if it reaches at least two-times the number of colonies of the spontaneous reversion. In spite of the significant dose-response of TA98 and TA100, the MIs of all strains were lower than two-fold. The low R^2 (determination coefficient) values (Tables 1 and 2) of the dose-response curves lead us to conclude that the increase in EEA dose influences very little, or not at all, the number of plaques or colonies.

These results are in agreement with our previous work (Vilar et al., 2008), in which EEA exhibited cytotoxicity due to a decrease in polychromatic/normocrhomatic erythrocyte (PCE/NCE) ratio and did not present mutagenic potential in mice bone marrow by the micronucleus test. Cell growth arrest and cell death induction are ways to avoid the proliferation of damaged genomes. Interactions between chemotherapeutic drugs and cells that could result in cytotoxicity but not in mutagenicity would be a better approach for cancer treatment, since this procedure would not induce an acquired resistance of tumor cells by mutation and would not harm normal cells. We suggest that the cytotoxic effect of EEA could be associated with its acetogenin content, because the mechanism of action of these molecules has been related to their ability to inhibit the mitochondrial complex I and, therefore, ATP synthesis (Zafra-Pólo et al., 1998).

In conclusion, the results of this investigation revealed neither direct genotoxicity nor mutagenicity of the extract, although it presented a cytotoxic profile, indicating that the ethanolic extract of *A. crassiflora* may potentially provide bioactive compounds that could be useful in primary health care.

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