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Assessment of toxic potential of Cerrado fruit seeds using *Artemia salina* bioassay

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Abstract

Artemia salina bioassay was used to assess toxicity of seeds and kernels of Brazilian fruits from cerrado (central high plains region) and other inner regions of the country. Water extracts of the kernels were filtered and added to *Artemia* cultures containing ten individuals per mL. Dose – response curves were constructed, and LD_{50} values were calculated. Pure potassium cyanide standard was used to draw a calibration curve for comparison to detect the presence of cyanide in the samples tested. Extracts of the seeds of araticum, mangaba, cagaita, jatobá, and tucumã were found toxic to *Artemia salina*, and some of the dose – response curves were very similar in shape to those obtained with pure potassium cyanide standards, while the samples of baru, cajá-manga, siriguela, trauma, and veludo showed no toxicity at all. The Guignard test, specific for cyanide detection, showed negative results in all toxic samples, suggesting the presence of other toxic compounds rather than cyanide. The comparison of araticum dose – response curve with those of other annonaceous fruits suggests the presence acetogenins as the main toxic compounds in the seeds. These results could be useful to prevent poisoning by industrial derivatives of the fruits studied.

Keywords: cyanide; kernels; food toxicology.

1 Introduction

Brazilian central high plains region, cerrado, has a wide variety of tasty endemic fruits, which grow in its extensive biome. Most of those fruits are consumed fresh, but in the last few years several derivatives such as ice-creams, jellies, deserts, and fermented drinks have become popular and appreciated by local buyers and tourists, showing potential for production expansion and export. Many studies have reported on the nutrient content and physical and chemical properties of these fruits and their processed derivatives (SILVA et al., 2004; ROCHA et al., 2008; SILVA; GOMES; MARTINS, 2009) although other important factors such as the presence of toxic or potentially hazardous compounds have not been as commonly studied. Several toxins such as hydrocyanic acid and acetogenins and anti-nutritional factors (trypsin inhibitors, lectins, and others) have been found in different vegetable foods and even in seeds of edible fruits (ARAÚJO, 2004; MIDIO; MARTINS, 2000; CARAMORI; LIMA; FERNANDES, 2004). The risk of intoxication or poisoning is not high when fruits are consumed fresh, but it increases in industrial derivatives since seeds may be partially broken during pulp removal. Several researches have reported significant concentrations of cyanides in peach and cherry juices and wines (FERNANDEZ; NOVELLI, 1997), but to this date, there are no clinical data available about poisoning by cyanide or any other compound present in fruit derivatives in Brazil. The bioassay method using the micro-crustacean *Artemia salina* has been commonly used in ecotoxicological tests, and more recently it proved sensitive, accurate, and reliable to detect of toxic and bioactive compounds in plant extracts (PARRA et al., 2001; LACHUMY et al., 2010; POUR; SASIDHARAN, 2011) and also to detect hidrocyanic acid in cassava and kernels of cherry, plum, peach, mango, and advocado (GARCIA-RODRIGUEZ et al., 2009). Bioassay methods have the advantage of being able to detect global toxicity, *i.e.,* any toxic compound to which the bioindicator would be sensitive and not only a specific target toxin. They also allow toxin identification and quantification using dose – response curves when pure toxin standards are available (GARCIA-RODRIGUEZ et al., 2004, 2009). Bioassay is a rapid and economic alternative option, quite suitable in small laboratories with limited resources.

On the basis of the aforementioned, the aim of the present study was the detection of potentially toxic or hazardous compounds in the seeds of several fruits from the Brazilian cerrado, central high plains region, using a simple and economic bioassay method with the microcrustacean *Artemia salina*.

The results obtained in this study can be useful in the development of safety procedures during the manufacturing of commercial fruit derivatives.

2 Materials and methods

Seeds form the following fruits were tested for toxicity with *Artemia salina* bioassay: araticum (*Annona crassiflora*), baru (*Dipteryx alata*), cagaita (*Eugenia dysenterica*), cajá-manga (*Spondias cytherea Sonn*), jatobá (*Hymenaea stigonocarpa*), mangaba (*Hancornia speciosa*), siriguela (*Spondias purpurea*), tarumã (*Vitex montevidensis*), veludo (*Guettarda viburnoides*), and tucumã (*Astrocaryum aculeatum*). The seeds were broken

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with a hammer, and 5 g of the inner kernels were gently blended together with 10 mL of distilled water resulting in concentrated stock solutions (0.5 grams per milliliter), from which further serial dilutions were prepared. The raw extracts obtained were filtered through Millipore membrane (0.45 µm mesh) and frozen until use in bioassay tests. The cultures were prepared according to a method previously used to assess cyanide in seeds of commercial fruits (GARCIA-RODRIGUEZ et al., 2009) and other biological samples (GARCIA-RODRIGUEZ et al., 2004). A total of 0,2 g of *Artemia salina* eggs were placed in 200 mL of 3,5% marine salt solution under constant air bubbling and illumination for 48 hours until eclosion. New born nauplia were caught individually with a Pasteur pipette and transferred to a test tube containing 1 mL of 3.5% marine salt solution (ten *Artemia* individuals per tube). Seed extracts were added in different triplicate concentrations to test tubes containing *Artemia* cultures, in amounts no greater than 100 µL, which is equivalent to 10% of the total volume in the test tube. A larger extract volume could significantly change the salt and nutrient concentration in the culture, probably affecting the survival of *Artemia*, which would represent an additional source of error in the experiments (positive false). In each experiment, three test tubes containing 1 mL of *Artemia salina* cultures were used as control, which were treated with 100 µL of distilled water each since all seed extracts were prepared using distilled water with no further toxin purification. After 24 hours incubation, living and dead individuals were counted to calculate survival percentage. Dose – response curves were constructed and lethal dose-50 (LD_{50}) was calculated with logarithmic curve fitting using the statistical software program Origin 6.0 (Microcal™ Origin®, Microcal Software Inc., Northampton-MA, USA).

The presence of hydrocyanic acid in the seeds was assessed using the specific Guignard test based on the formation of red alkaline isopurpurate from the reaction of cyanide with sodium picrate (COSTA, 2001). A total of 2 g of blended seeds were placed in an Erlenmeyer flask containing 10 mL of distilled water. A strip of filter paper approximately 1 cm wide, previously moistened with 1% picric acid solution and then with 10% sodium carbonate solution, was placed on the border of the flask and suspended over the sample for approximately two hours. The development of a red color on the paper surface indicates the presence of cyanide in the sample. The Guignard test is a qualitative technique that allows confirming the presence or not of cyanides in the toxic extracts. If cyanides are identified in some toxic extracts, their concentration can be easily calculated using a pure potassium cyanide standard. A dose – response curve can be obtained treating *Artemia* cultures with different concentrations of pure cyanide ranging between 0.01 and 1.0 mg per mL and then, LD_{50} value can be calculated from curve parameters after logarithmic fit. Cyanide concentration in seeds is calculated from the ratio between LD_{50} values from the standard and toxic extract. Since LD_{50} values were obtained from independent samples (seed extracts), they were individually compared to each other by the Student t-test using the statistical software program Origin 6.0 (Microcal™ Origin®, Microcal Software Inc., Northampton-MA, USA).

3 Results and discussion

The kernel extracts of baru, cajá-manga, siriguela, tarumã, and veludo were not toxic to the *Artemia* cultures, which showed survival values of 100% even when treated with concentrated extracts (0.5 grams of seeds per mL). The extracts of araticum, cagaita, jatobá, mangaba, and tucumã proved toxic to *Artemia salina,* and therefore the decreasing concentrations were used to construct the dose – response curves. The concentration range was selected individually for each toxic extract after determining the minimal and maximal concentration required to obtain 100% and 0% survival, respectively, in *Artemia* cultures. Selected extract concentrations ranged from 2.0 to 20.0 $mg.mL^{-1}$ in araticum, 30.0 to 100.0 $mg.mL^{-1}$ in cagaita, 4.0-55.0 $mg.mL^{-1}$ in jatobá, 12,0 to 33.0 $mg.mL^{-1}$ in mangaba, and 10.0 to 100.0 in tucumã. Dose – response curves can provide useful information such as LD_{50} and also some ideas about the nature of the toxic compound. They can be compared to curves obtained from standard purified toxins or natural extracts containing known toxins; equal or similar slopes may suggest the presence of the same toxin or different ones with related action mechanism. Figures 1a, 2a, 3a, 4a, and 5a show dose – response curves obtained from extracts of araticum, cagaita, jatobá, mangaba, and tucumã, respectively, and their characteristic sigmoidal shape. LD_{50} values, expressed as seed milligrams per milliliter of culture are shown in Table 1 and were calculated using curve logarithmic fit. LD_{50} values are inversely proportional to toxicity and can be used to calculate toxin concentration if pure standard is available. The highest LD_{50} values (the lowest toxicity) corresponded to cagaita and tucumã seeds, while mangaba seeds showed half of those values (two times higher toxicity). Extracts of jatobá and araticum seeds were the most toxic, and the lowest LD_{50} values were found in araticum seeds.

The dose – response curve obtained with a pure potassium cyanide revealed a LD_{50} value of 0.03 $mg.mL^{-1}$, and it was similar in shape to some of toxic extract curves, mainly that of mangaba (Figures 1b, 2b, 3b, 4b, and 5b). This fact could suggest the presence of cyanides in those extracts. However, cyanide specific Guignard test displayed negative results in all toxic samples, suggesting the presence of one or more toxic compounds instead of cyanide. Several authors (COLOM et al., 2006; KIM; SON; WOO, 2001; PARDHASARADHI et al., 2005) have reported the presence of tetrahydrofuran – related acetogenins in seeds of annonaceous fruits belonging to the same taxonomic genus of araticum. Those toxins are known as strong inhibitors of mitochondrial respiration that have an action mechanism similar to that of cyanides, but not necessarily similar dose – response curves, since acetogenins selectively inhibit complex I from the electron transport chain, while cyanides inhibit only cytochrome a_3 in the complex IV. Seed extracts of other annonaceous fruits: ata (*Annona squamosa*) and atemoia (a hybrid resulting from *Annona squamosa* and *Annona cherimola* crossing-over) were prepared and added to *Artemia salina* cultures, following the same procedure described above to construct dose – response curves, which were then compared to the araticum curve, as shown in Figure 6a. The similarity between the curves of araticum and ata suggests the presence of the same toxic compound, probably acetogenins.

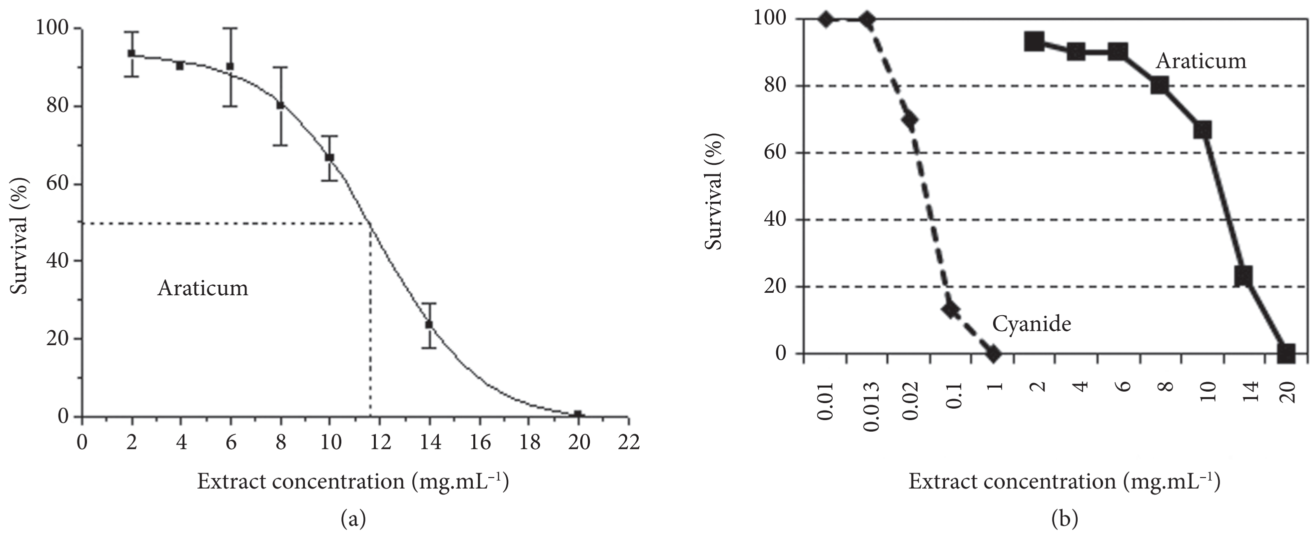

Figure 1. Toxic effect of seeds from Araticum on *Artemia salina* cultures. a) Dose – response curve with logarithmic fit. b) Comparison of curves from toxic extract and pure potassium cyanide standard.

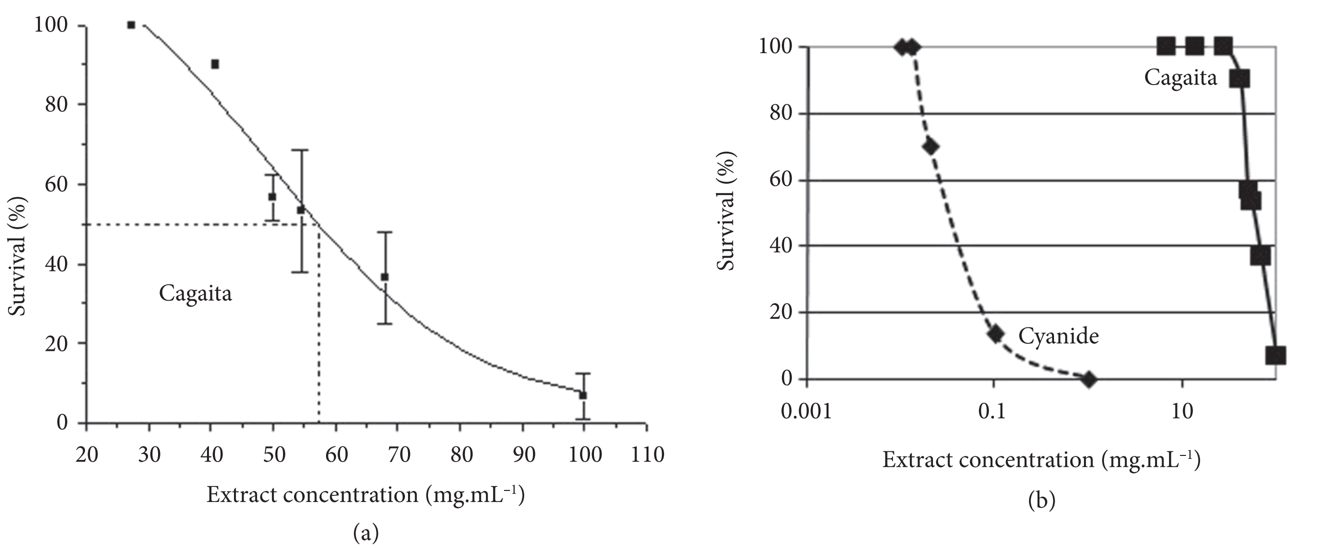

Figure 2. Toxic effect of seeds from Cagaita on *Artemia salina* cultures. a) Dose – response curve with logarithmic fit. b) Comparison of curves from toxic extract and pure potassium cyanide standard.

Table 1. Calculated LD_{50} values from seed extracts in *Artemia salina* cultures.

Seed extract	LD_{50} (mg.mL^{-1})*
Cagaita	57.0 ± 3.31
Tucumã	47.67 ± 3.68
Mangaba	24.07 ± 3.35^{a}
Jatobá	18.33 ± 1,77^{b}
Araticum	11.7 ± 1.79

*Values represent arithmetic mean and standard deviation calculated from three independent experiments. Each mean value is significantly different from the rest, except (a), as compared to (b).

The smaller LD_{50} value in ata indicates a higher toxicity, probably due to a higher toxin concentration (Figure 6b). Atemoia seeds showed the highest toxicity, with a slightly different shaped dose – response curve (Figure 6a) and a ten times smaller LD_{50} value (Figure 6b). Although it is not a natural species, atemoia results from the crossing over of two fruits that belong to genus *Annona* ; therefore, the presence of acetogenins in its seeds is expected as well as other different compound(s) that could be causing a synergic effect together with acetogenins changing the curve's shape. Caramori studied nutritional value and activity of several enzymes and antinitritional factors in some seeds from Brazilian cerrado fruits, including araticum and

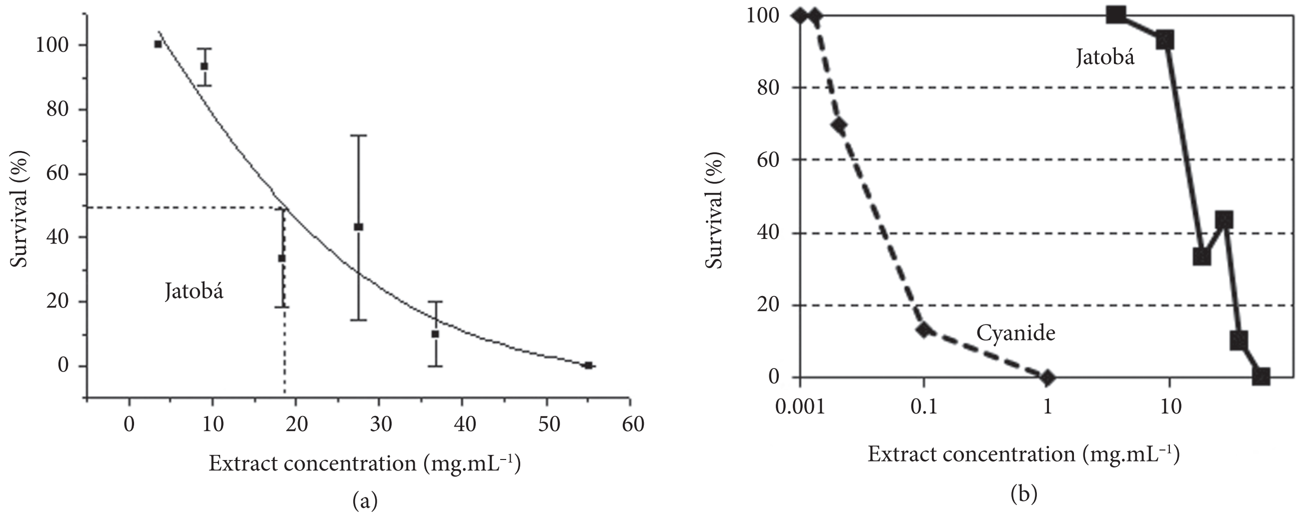

Figure 3. Toxic effect of seeds from Jatobá on *Artemia salina* cultures. a) Dose – response curve with logarithmic fit. b) Comparison of curves from toxic extract and pure potassium cyanide standard.

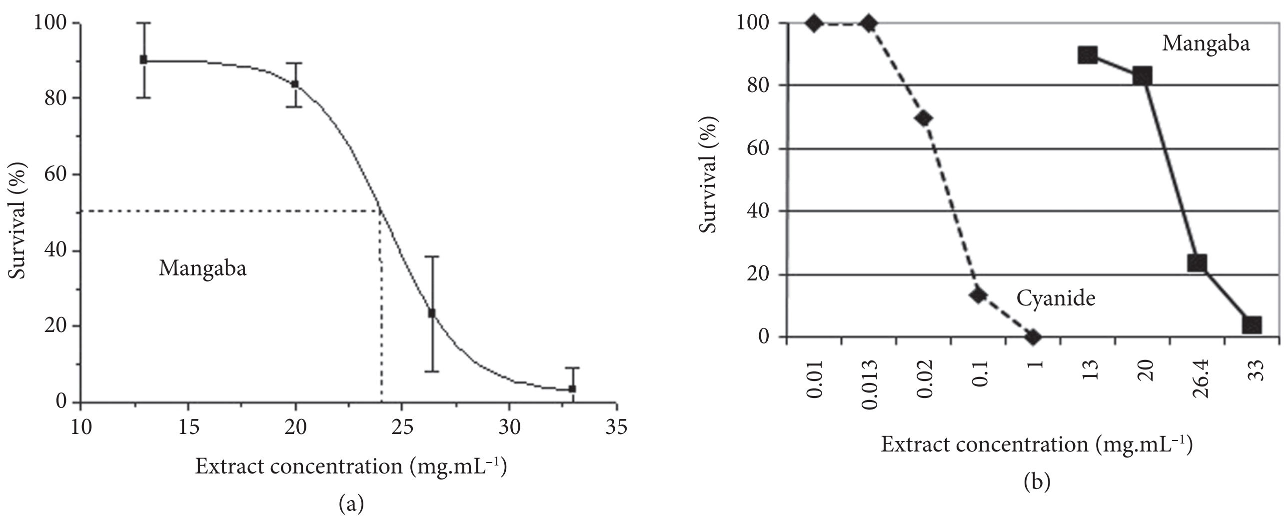

Figure 4. Toxic effect of seeds from Mangaba on *Artemia salina* cultures. a) Dose – response curve with logarithmic fit. b) Comparison of curves from toxic extract and pure potassium cyanide standard.

jatobá (CARAMORI; LIMA; FERNANDES, 2004). Although protease, peroxidase, and trypsin inhibitors activity and tannin concentration showed the lowest values in araticum seeds, they reached their highest values in jatobá seeds; and in some cases they were 100 times higher. This fact could explain the toxicity observed in seeds of jatoba, but not in araticum, in which toxicity seems to be due to acetogenins, as discussed above. Although natural toxicity in fruits from the Brazilian cerrado has been poorly studied to date, the results obtained in this study can be considered reliable since several researchers have used the *Artemia salina* cultures to successfully assess diverse toxic and bioactive compounds in both water and organic plant extracts (SANTOS et al., 2007, 2010; LACHUMY et al., 2010; POUR; SASIDHARAN, 2011; SILVA et al., 2012). Moreover, *Artemia salina* bioassay proved more accurate and sensitive to detect such compounds than the standard method using mice, previously proposed by the Association of Official Analytical Chemists for food biotoxins analysis (ASSOCIATION…, 1990; PARRA et al., 2001; GARCIA-RODRIGUEZ et al., 2004). Previously published results confirm the sensitivity and reliability of this technique for the detection of plant compounds with a wide range of action mechanisms.

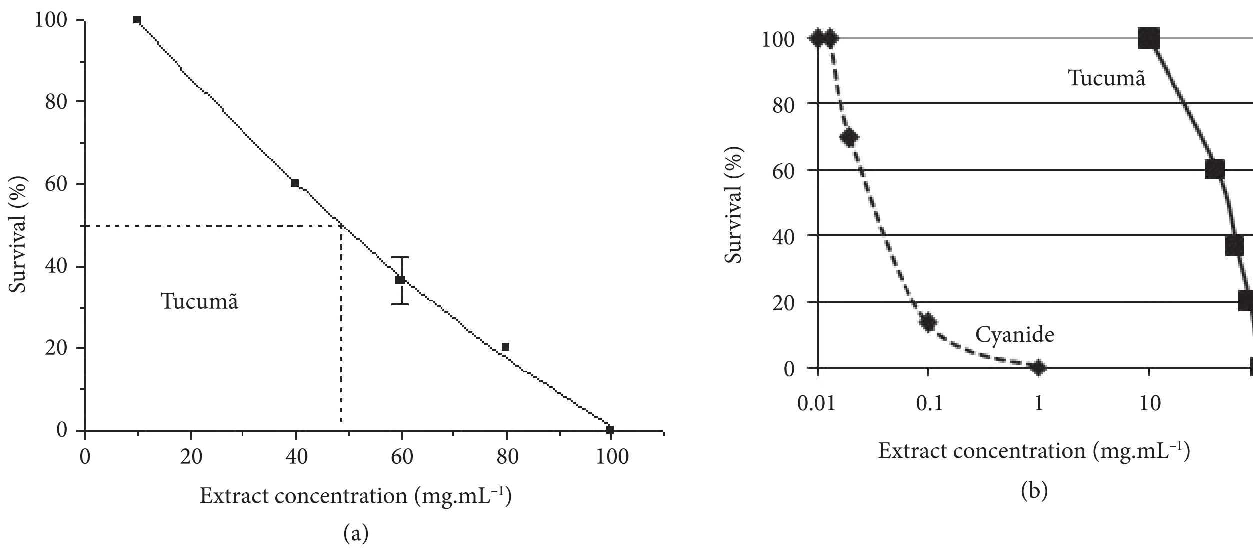

Figure 5. Toxic effect of seeds from Tucumã on *Artemia salina* cultures. a) Dose – response curve with logarithmic fit. b) Comparison of curves from toxic extract and pure potassium cyanide standard.

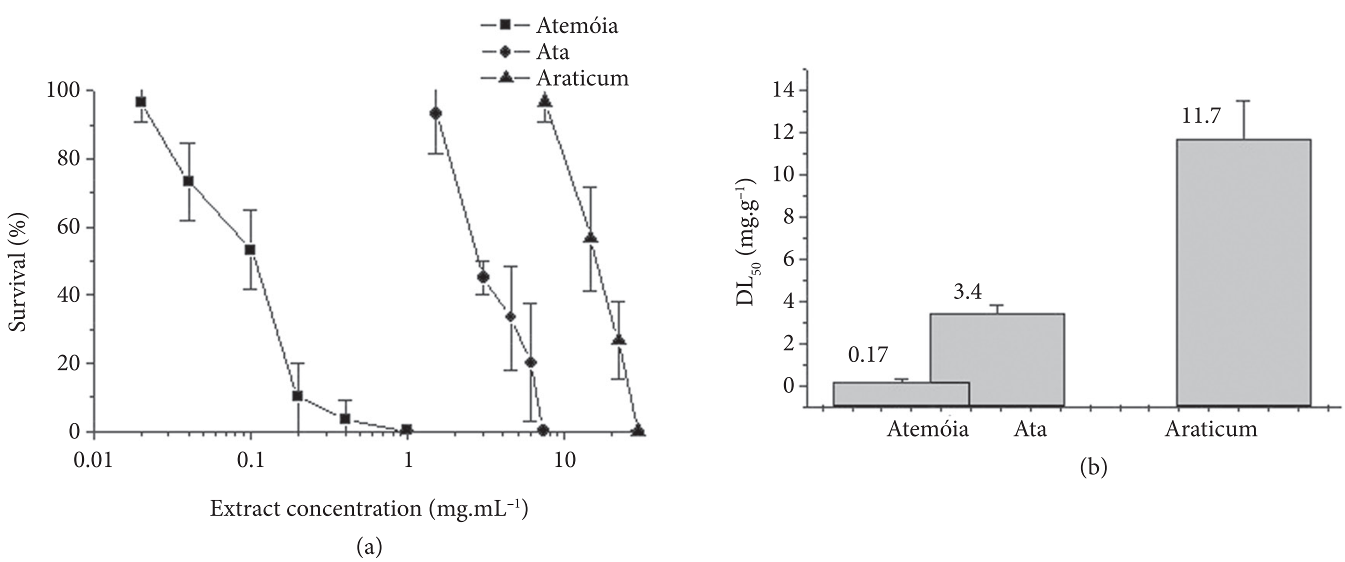

Figure 6. Comparison of toxicity from Araticum, Ata and Atemóia seed extracts. a) Dose – response curves. b) LD_{50} values represented as arithmetical mean from three independent experiments and standard deviation. Each mean value is significantly different from the other two ($p < 0.05$).

The present study is part of a research project under development, in which biochemical tests to identify and quantify other toxic compounds and antinutritional factors will be performed and thermal inactivation studies will be conducted to characterize compounds responsible for toxicity in cagaita, mangaba, and tucumã, and all seeds and kernels that are classified as toxic by the *Artemia salina* bioassay. The results obtained can be useful to propose prevention measures to be included in the manufacturing of industrial derivatives of studied fruits.

4 Conclusions

- Ten seed extracts were tested for toxicity, among which araticum, cagaita, jatobá, mangaba, and tucumã were toxic to *Artemia salina* cultures showing LD_{50} values between 11.7 $mg.mL^{-1}$ (araticum) and 57.0 $mg.mL^{-1}$ (cagaita);

- Seed extracts from baru, cajá-manga, siriguela, taruma, and veludo were not toxic at all;
- Although dose – response curves were similar to the pure potassium cyanide curve; the Guignard test showed negative results in all toxic extracts, thus excluding the possibility that cyanide is responsible for the toxic effect;
- Low LD_{50} values and the similarity between toxicity curves of ata and araticum extracts suggest the presence of acetogenins in araticum seeds. High proteolytic activity and the presence of trypsin inhibitors found in seeds of jatobá can explain their toxicity to *Artemia salina's* cultures.

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