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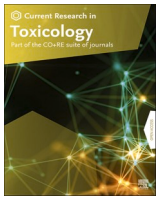

Influence of methanolic extracts from seeds and pulp of *Annona squamosa* L. on osmotic and morphological fragility in human erythrocytes

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ABSTRACT

Medicinal plants have been used for many years by communities to treat illnesses. The need for scientific proof of these vegetable's curative effects is as necessary as the proof of the inexistence of toxicity related to the use of extracts with therapeutic potential. *Annona squamosa* L. (Annonaceae), popularly known as "pinha", "ata" or "fruta do conde", has been used in traditional medicine for its analgesic and antitumor activities. The toxic effects attributed to this plant have also been explored as a pesticide and an insecticide. The aim of the present study was to investigate the toxicity of the methanolic extract of *A. squamosa* seeds and pulp against human erythrocytes. Blood samples were treated with methanolic extract at different concentrations, osmotic fragility was determined using saline tension assays and morphological analyzes were performed using optical microscopy. The extracts were analyzed using high performance liquid chromatography with diode array detection (HPLC-DAD) for phenolic quantification. The seed's methanolic extract showed toxicity above 50% from a concentration of 100 μg/mL, while also presenting echinocytes in the morphological analysis. The pulp's methanolic extract did not show toxicity to red blood cells or morphological changes at the concentrations tested. HPLC-DAD analysis revealed the presence of caffeic acid in the seed extract and gallic acid in the pulp extract. The seed's methanolic extract is toxic and the pulp's methanolic extract showed no toxicity against human erythrocytes.

Introduction

The exploitation of plant material is a common practice and finds applicability in the food industry, in health care and in the development of agribusiness (Silva et al., 2022). Medicinal plants are vegetables possessing therapeutic potential, either for the treatment or prevention of communicable and non-communicable diseases. The difficult access to Western medicine, as well as the high cost of its products, contribute to the spread of traditional medicine, especially in developing countries (Mudau et al., 2022). According to the World Health Organization (WHO) 80% of the world's population depends on traditional medicine, about 40,000 to 70,000 plant species are used and move about US$100 billion of world trade along with their by-products (Anand et al., 2022).

Herbal medicines are attractive for possibly having little to no side effects when administered correctly (Sulaiman et al., 2022). Whilst most users think that herbal drugs are safe in terms of adverse effects, this perception is extremely wrong and dangerous, and can lead to intoxication and even death of consumers. Compared to studies on the therapeutic potential of medicinal plants, there is a scarcity of studies on the toxic effects of these plants, which demonstrates the need for research related to the toxicity of phytogenetic resources (Kharchoufa et al., 2018, Pascual et al., 2022).

Erythrocytes represent an important indicator of human health. Hemolysis due to external factors, including xenobiotics, limits the application of new materials or the development of new drugs. In addition, oxidative stress contributes to biochemical changes and

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alterations in the biophysical shape of the erythrocyte membrane, limiting its lifespan due to the impossibility of producing new proteins, given the absence of nucleus and organelles (Podsiedlik et al., 2020).

The *Annona* genus comprises about 170 species distributed in tropical America. Many of these species are used in folk medicine to treat a variety of ailments, from headaches to treating tumors. Literature reports indicate that some of these activities have been proven through experimental tests. Many chemical structures have also been isolated and identified over the past few years (Leite et al., 2020). *Annona squamosa* L. popularly known as 'pinha', 'ata' or 'fruta do conde', is a plant with sweet, succulent and nutritious fruit. Different parts of the plant have been used in traditional medicine. Compounds such as alkaloids, steroids, acetogenins, terpenoids and phenols have been isolated from different extracts of *A. squamosa* and are responsible for several biological activities attributed to this plant (Nemari et al., 2022). Antioxidant, antidiabetic, hepatoprotective and antitumor activities are attributed to the crude extract and compounds isolated from *A. squamosa*, among others (Leite et al., 2021).

The present study aimed to investigate, for the first time, the cytotoxicity and osmotic fragility of methanolic extracts of the seeds and pulp of *A. squamosa*, as well as the effect of the extracts on the morphology of human erythrocytes. There are no studies of the toxic effects of *A. squamosa* extracts on human blood cells, so this is a pioneer study, which opens the way for future investigations.

Materials and methods

Equipments

Centrifuge NT 812, Novatecnica, São Paulo, Brazil; spectrophotometer T80 + UV/VIS, PG instruments LTDA, United States; HPLC 1260 infinity G1211C 1260 Quat Pump VL, Agilent technologies, United States.

Extract obtention

Annona squamosa fruits were commercially obtained in the central market of Juazeiro do Norte (Ceará, Brazil) in February 2019. The pulp was manually separated from the seed and then freeze-dried. 50 g of the lyophilized pulp was extracted with methanol in a Shaker incubator for 72 h at 50 rpm, after which the extract was concentrated, obtaining a crude yield of 18.92g (38%). The seed was sanitized with 5% sodium hypochlorite, after drying it was crushed and 50g of the resulting material was subjected to extraction in a Soxhlet system with methanol until exhaustion, after concentration the yield of the crude extract was 4g (8%).

Preparation of human erythrocytes

Samples were obtained from human blood (blood type O negative) donated by the clinical analysis laboratory of Centro Universitário Doutor Leão Sampaio, Ceará, Brazil. Initially the blood was homogenized with sodium citrate and a suspension of red blood cells from 5 to 10% was prepared in saline solution (0.9%). In a test tube, 100 mL of red blood cell suspension and 900 mL of saline were added to the test tube and centrifuged at 3500 rpm for 15 s, the supernatant was discarded, this procedure was repeated 6 times. 900 μL of saline solution (0.9%) was added to the red blood cells resulting from the wash. All salt solutions were prepared using sodium chloride PA and distilled water. The reagents used were obtained from Sigma-Aldrich.

Cytotoxicity and osmotic fragility in human erythrocytes

The cytotoxicity assay in human erythrocytes was performed according to the method of Barros et al. (2016), with adaptations. Blood samples were collected, prepared and exposed to different concentrations of the extract (10, 25, 50, 100, 250, 500, 1000 μg/mL). The solutions were kept in a water bath at 37 °C for 30 min. After this period, 2100 μL of 0.9% saline solution was added to the blood and then the samples were centrifuged, and the supernatant read in a UV–visible spectrophotometer at 540 nm. The negative control contained only red blood cells and 0.9% saline. For osmotic fragility the lowest concentration with hemolytic capacity was chosen from each extract, while testing different concentrations of NaCl (0.12, 0.24, 0.36, 0.48, 0.60, 0.72 and 0.90%). The negative control consisted of each tested concentration of NaCl without the extract. The percentage of hemolysis was calculated and "fragility curves" were plotted using the percentage of hemolysis (% hemolysis) for each NaCl concentration (relative to 100% hemolysis tube 0.12% NaCl), according to the Eq. (1):

$$\%Hemolysis = Abs_{sample} - -Abs_{blank} x100$$

$$Abs_{control} \quad (1)$$

Where, Abs_{sample} represents the sample's absorbance, Abs_{blank} represents the blank test's absorbance, and $Abs_{control}$ the control's absorbance.

Morphological analysis

Samples treated with methanolic extract from the seed and pulp of *A. squamosa* at a concentration of 250 μg/ml for the pulp and 100 μg/ml for the seed, sodium chloride (0.9% NaCl) was used as the negative control. Samples were prepared, dried and fixed for microscopic analysis (1000x) and images were obtained from erythrocyte smears using a specific programmer used to obtain qualitative images.

Quantification of phenols by HPLC-DAD

Reverse-phase chromatographic analyzes were performed under gradient conditions using a C18 column (4.0 mm × 250 mm × 5 μm). The mobile phase consisted of a mixture of solvents: A (water acidified with 0.1% formic acid) and B (Methanol) using the following linear elution gradient: 0–3.2 min: 5% B in A; 3.2–3.7 min: 10% B in A; 3.7–50 min: 50% B in A. The wavelengths used were 290 nm for gallic acid, caffeic acid, vanillin, ferulic acid and cinnamic acid, 360 nm for rutin and quercetin. The reagents used were obtained from Sigma-Aldrich. The mobile phase flow was 1.00 mL/min, and the injection volume was 20 μL. Samples and mobile phase were filtered through a 0.22 μm membrane filter (milllipore). The methanolic extract of *Annona squamosa* was dissolved in methanol HPLC (15 mg/mL). Using methanol HPLC, reference solutions were prepared from stock standards, in a concentration range of 10–50 ppm. Quantification was performed by integrating the peaks using the external standard method. The peaks were confirmed by comparing their retention time with those of the reference standards and by DAD spectra (190 to 400 nm). All chromatographic operations were performed with the column temperature set to 30 °C and in triplicate. Compound quantifications were based on analytical curves of reference norms. Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of responses and slope, using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Statistical analysis

All analyzes were performed in triplicate and data expressed as mean (n = 3) ± Standard Deviation (S.D.) using one- and two-way Analysis of Variance (ANOVA) followed by Tukey's test by multiple comparison for normally distributed data and significantly similar standard deviations with p values < 0.05; $p < 0.01$ and $p < 0.001$. Statistical analyzes and graphical presentation of the results were performed using the GraphPad

Prism program (version 6.1).

Results

Cytotoxicity and osmotic fragility in human erythrocytes

In Fig. 1 it is possible to observe high toxicity of the methanolic extract of *A. squamosa* seeds with significant differences when compared to the negative control in all concentrations, and from the concentration of 250 μg/mL the hemolysis was 100%. For the extract of *A. squamosa* pulp, there was no significant difference compared to the control in the concentrations tested, except for the concentration of 1000 μg/mL where 7.25% of hemolysis occurred. The results of the present study demonstrate that the methanolic extract of *A. squamosa* seeds is hemolytic even at lower concentrations, causing hemolysis of 50% of the erythrocytes from a concentration of 100 μg/mL, whereas the pulp's methanolic extract showed results similar to the negative control, demonstrating the absence of toxicity to red blood cells.

Fig. 2 demonstrates the protective effect of the methanolic extract of *A. squamosa* pulp against different concentrations of NaCl, decreasing hemolysis percentage when erythrocytes were submitted to hypotonic NaCl medium. The results are significantly different for the concentrations of 0.12, 0.24 and 0.36 % of NaCl, when compared to the negative control (NaCl 0.9%). For the other concentrations there was no significant difference. The methanolic extract of *A. squamosa* seed does not react positively in different NaCl concentration medium, significantly increasing the percentage of hemolysis when compared to the negative control.

Morphological analysis

In the morphological analysis shown in Fig. 3, the extract of *A. squamosa* pulp caused total hemolysis (Fig. 3b) at a concentration of 250 μg/mL. In the seed extract it is possible to observe changes in red blood cells already at a concentration of 100 μg/mL (Fig. 3c). These changes are called echinocytes, red blood cells that have lost their discoid shape and have small irregular spines, these misshapen cells can lead to impaired functionality of red blood cells resulting in diseases related to the circulatory system (Barros et al., 2016). In the smear of cells conditioned with methanolic extract of *A. squamosa* pulp, no altered red blood cells were observed, corroborating the cytotoxicity results.

The chromatograms represented in Fig. 4 revealed the presence of caffeic acid (peak 4) in the seed extract and gallic acid (peak 10) in the pulp extract (Fig. 5). The quantitative composition of caffeic acid in the seed extract was 0.17 mg/g and the quantitative composition of gallic acid in the pulp extract was 0.65 mg/g. The other tested phenols were not identified in the extracts.

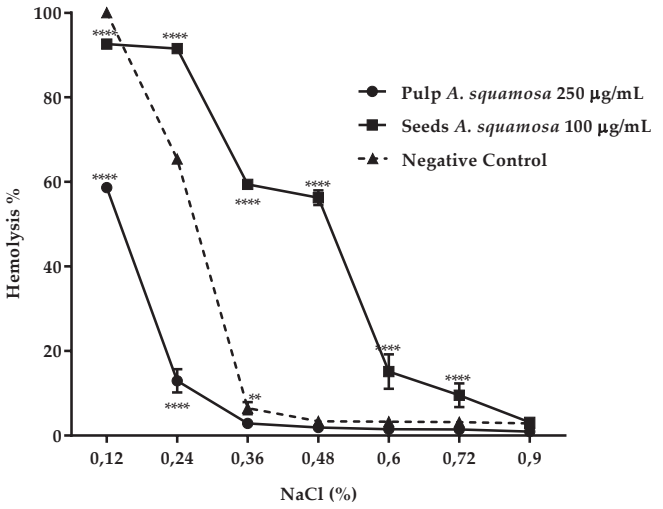

Fig. 2. Osmotic fragility of blood samples treated with 100 μg/mL of the seed extract and 250 μg/mL of the pulp extract of *Annona squamosa* at different concentrations of NaCl. Where * = $p < 0.05$, ** = $p < 0.01$ and **** = $p < 0.0001$.

Discussion

When investigating the toxicity of a natural product with pharmacological potential, it is important to observe possible interactions with the plasma membrane, since it constitutes a selective barrier for access to the interior of cells. Red blood cells represent a cellular model whose only barrier is the plasma membrane itself, being widely used to test the toxicity of synthetic drugs or natural products with pharmacological potential (Podsiedlik et al., 2020).

Situations such as variations in pH, temperature, concentration of the medium and presence of solutes influence the stability of the membrane, which is also affected by excessive fluidity (Freitas et al., 2008). Therefore, the membrane must have a degree of fluidity that allows the transport of substance into the cell without compromising its stability. The osmotic fragility assay is widely used to verify the toxicity

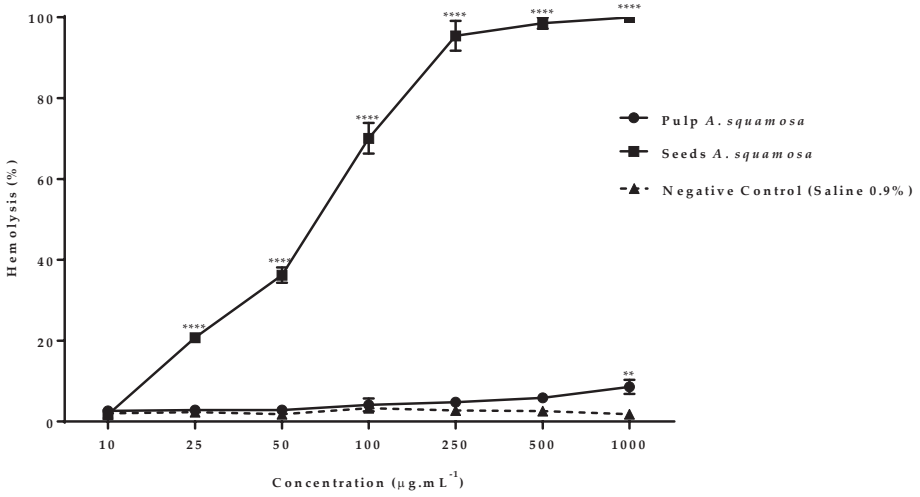

Fig. 1. Erythrotoxicity of methanolic extracts of *Annona squamosa* seed and pulp. As a negative control, a sodium chloride solution (0.9% NaCl) was used. * = $p < 0.05$, ** = $p < 0.01$ and **** = $p < 0.0001$.

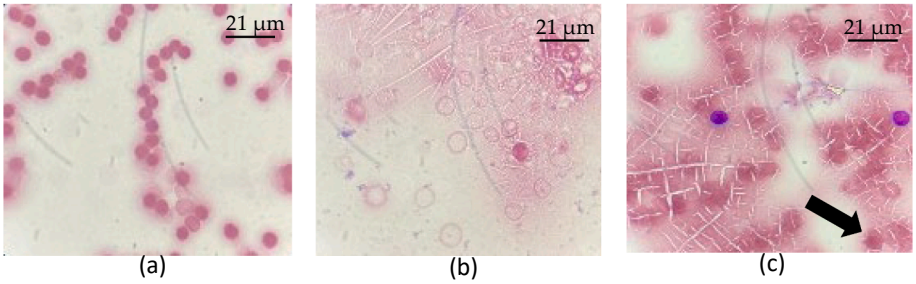

Fig. 3. (a) Morphology of red cells in the control group – 0.9% NaCl. (b) Morphology of red cells treated with methanolic extract of *A. squamosa* pulp at a concentration of 250 µg/mL. (c) Morphology of red cells treated with methanolic extract of *A. squamosa* seed at a concentration of 100 µg/mL. The arrow indicates the presence of echinocytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

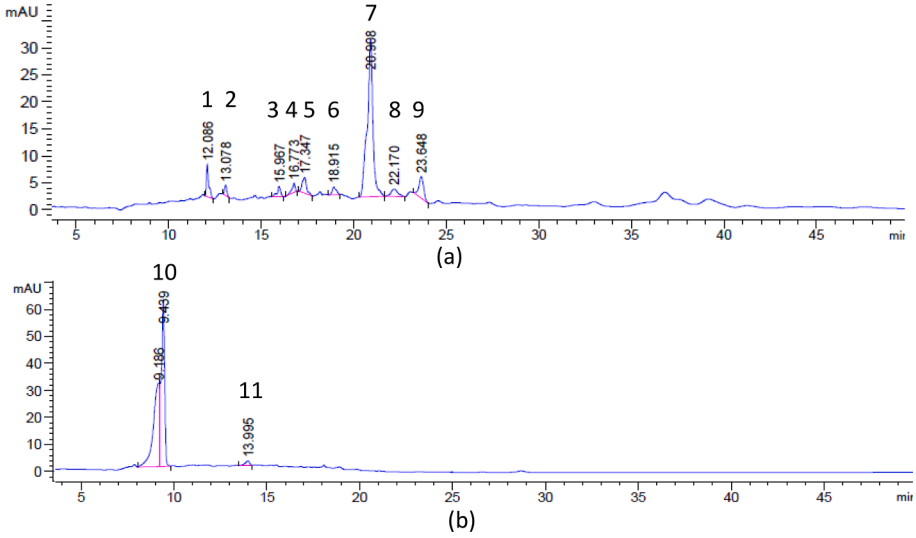

Fig. 4. Elution profiles of HPLC/DAD of *Annona squamosa* extract (a) seed and (b) pulp. Caffeic acid (peak 4, tR = 16,773); Gallic acid (peak 10, tR = 9,439); Unidentified (peaks 1, 2, 3, 5, 6, 7, 8, 9 and 11). Calibration curve for caffeic acid y = 110754x + 267.5 (r = 0.9902); gallic acid y = 13478x + 94.75 (r = 0.9911).

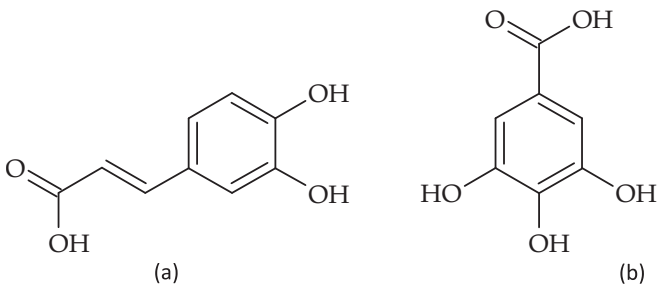

Fig. 5. Chemical structures of phenolic acids identified in *A. squamosa* extracts. (a) caffeic acid;(b) gallic acid.

of natural products since hemolysis can be monitored by changing the absorbance at 540 nm (Duarte et al., 2016).

The seeds' hemolytic property may be associated with the presence of annonaceous acetogenins in large amounts, the main compounds of their chemical constitution, which have been explored for the treatment of different types of cancers and in the fight against certain pests (Chen et al., 2017). The toxicity of acetogenins was first reported in 1991. Experiments with animal models observed reduced movement and lethargy, which was associated with decreased ATP caused by respiratory depression (Londershausen et al., 1991). In view of this evidence, the use of Anonaceae species have been widely studied in the exploitation of their biological activities, especially as pesticides and anticancers. Respiratory depression may be associated with high hemolytic capacity, decreasing the amount of red blood cells and, consequently, the availability of oxygen to the individual.

The anti-hemolytic property of the *A. squamosa* pulp extract may be associated with the presence of phenolic compounds such as gallic, protocatechoic, sinapic, ferulic, p-coumaric acids, catechin and epicatechin, as well as quinic acid, gallocatechin, gallocatechin gallate, caffeoylhexoside and dihydroxyketectin identified in the pulp extract (Baskaran et al., 2016). These compounds can relate to the erythrocyte membrane, conferring a protective effect.

Phenolic compounds are the result of secondary metabolism in plants and are largely responsible for the antioxidant characteristics of a given extract (Rodrigues et al., 2021). The presence of antioxidant compounds is important in extracts with pharmacological potential as it maintains the balance between pro and antioxidant substances. Oxidative stress can cause damage to erythrocytes in a more compromising way, given the absence of organelles for producing antioxidants of cellular origin, this in turn is manifested with biochemical and biophysical changes in the cell (Podsiedlik et al., 2020).

Caffeic acid can help in the antioxidant apparatus that protects red blood cells against oxidative damage as shown in the study by Oršolic et al (2021), however, according to the same study, a dose of 50 mg/kg administered for seven days in diabetic mice was required to observe decreased hemolysis in erythrocytes. In view of the quantification of caffeic acid in *A. squamosa* seed extract, it is likely that it exerts little influence on the control of oxidative stress. On the other hand, isopropyl caffeate, a caffeic acid derivative, showed an increase in osmotic fragility in human erythrocytes, which may be associated with the

inhibition of enzymatic activity (Lira et al., 2018).

Gallic acid is a natural polyphenolic antioxidant present in beverages and foods and has been shown to protect against osmotic fragility in red blood cells with arsenic-induced cytotoxicity, significantly decreasing the percentage of hemolysis (Panghal et al., 2020). Studies carried out by Suwalsky et al. (2016) concluded that gallic acid can block the access of oxidant species to the lipid bilayer and consequently to the interior of the red blood cell, this is due to the ability of gallic acid to bind to phospholipids present in the red blood cell plasma membrane as Dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE).

Anonaceae species have been widely studied in exploring their biological activities, especially as a pesricide and anticancer agent. Acetogenins isolated from *A squamosa* seeds demonstrated potent cytotoxic activity against human tumor cell lines such as MCF-7 human breast carcinoma and A-549 human lung carcinoma (Leite et al., 2020). The crude ethanolic extract of *A. squamosa* pulp was investigated for its toxicity on human fibroblast cells, showing positive results in comparison with 0.12% chlorhexineglocanate, representing an alternative for the treatment of oral diseases induced by bacteria (Ali and Jafar, 2022). Studies performed in TSS mice whose diet was supplemented with *A. squamosa* leaf extract showed stable leukocyte and RBC levels, attributed to its anti-inflammatory and antioxidant potential, due to the presence of flavonoids, alkaloids, saponins, tannins, glycosides, steroid-phenols and various acetogen compounds (Elghany et al., 2022). The cytotoxic effects of the leaf ex-tract macerated with methanol, acetone or water (1:10 w/v) were tested and showed potential anticancer activity against breast cancer in in vitro and in vivo experiments (Al-Nemari et al., 2022).

Conclusions

This study demonstrated that the use of *Annona squamosa* seed is not recommended at the concentrations tested, requiring studies with other tests at different concentrations and fractions of this extract in order to take advantage of its pharmacological potential. In this first report that deals with the study of the methanolic extract of the pulp of *A. squamosa*, it was determined that the pulp does not present cytotoxicity at the concentrations tested, and even though it protects human erythrocytes in hypotonic medium, investigations on the association of this protection with the fluidity of the membrane are necessary to enable the use of this extract, for therapeutic and nutritional purposes, since one of the factors that promote membrane stability is the decrease in fluidity, which would compromise its main function. Most phytotherapics and medicinal plants do not have their toxic effects well elucidated, the use of these vegetables is a common practice and often leads to intoxication of their users, which demonstrates the need for scientific investigations associated with the adverse effects of plants used by traditional communities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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