Comparative study with dry crude extracts of *Ananas comosus* L. Merril using LC_{50} and erythrocyte osmotic fragility as toxicity parameters

Estudio comparativo con extractos crudos secos de *Ananas comosus* L. Merril utilizando CL_{50} y fragilidad osmótica eritrocitaria como parámetros de toxicidad

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Resumen. *Ananas comosus*, conocido popularmente como piña, tiene una anatomía de planta bien definida y numerosos fitoquímicos farmacológicamente activos. Algunos de éstos son responsables del potencial antimicrobiano de la especie, que ha sido ampliamente estudiado dada la resistencia bacteriana a los antibióticos actualmente utilizados. Teniendo esto en cuenta, se define que la característica principal de un medicamento es la ausencia de efectos tóxicos, por lo tanto, es necesario buscar datos sobre la toxicidad de *A. comosus*, colaborando para su posible uso como fármaco. Comparando extractos hidroalcohólicos de las hojas de la corona, cáscara y pulpa de la infrutescencia, fue posible determinar que los tres extractos probados no son tóxicos, siendo el de menor toxicidad para *Artemia salina* Leach (extracto de hojas con CL_{50} igual a 994 µg/mL) y a sangre de cordero (extracto de pulpa con porcentaje de hemólisis igual a 0,83%). Los estudios sobre principios y metodologías similares a los utilizados aquí han encontrado resultados comparables que indican la baja toxicidad de la planta. Estos resultados aportan a las investigaciónes que promueven el uso de la piña como agente fitoterapéutico y reafirman su presencia en el Sistema Único de Salud de Brasil.

Palabras clave: *Ananas comosus*; Fragilidad eritrocitaria; *Artemia salina*; Piña; Eritrocito; CL_{50}

Abstract. *Ananas comosus*, popularly known as pineapple, has well defined plant anatomy and numerous pharmacologically active phytochemicals. Some of these are responsible for the species antimicrobial potential, which has been widely studied given the bacterial resistance to the currently used antibiotics. Considering this, it is defined that the main characteristic of a drug is the absence of toxic effects, thus, there is a need to seek data regarding the toxicity of *A. comosus*, collaborating for its possible use as a drug. Comparing hydroalcoholic extracts from the crown leaves, skin and pulp of the infructescence, it was possible to determine that the three tested extracts are non-toxic, being the one with the lowest toxicity to *Artemia salina* Leach (leaves extract with LC_{50} equal to 994 µg/mL) and to lamb's blood (pulp extract with percentage of haemolysis equal to 0.83%). Studies concerning principles and methodologies similar to those used here have found comparable results stating the low toxicity of the plant. These results contribute to the research that promotes the use of pineapple as a phytotherapeutic agent and reaffirms its presence in the Brazilian Unified Health System.

Keywords: *Ananas comosus*; Fragilidad eritrocitaria; *Artemia salina*; Pineapple; Erythrocyte; LC_{50}.

Introduction

The *Ananas comosus* species (pineapple) belongs to the Bromeliaceae family, which is responsible for about 56 genera and more than 3000 species (Lopes Neto *et al*. 2015). According to Manetti *et al*. (2009) the description of the family is attributed to the French priest Charles Plumier, who, at the end of the seventeenth century, came across several different plants and decided to name them bromeliads in honour of the Swedish botanist Olaf Bromel. Belonging to this family and to the genus *Ananas*, the pineapple is popularly widespread, being considered one of the most popular tropical fruits in the world. It is also included in the group of plants with CAM metabolism (Crassulaceae Acid Metabolism), which main feature is to close their stomata during the day and open them at night to save water (Kerbauy 2004; Vieira *et al*. 2010). In Brazil, the species is found in the National Relation of Medicinal Plants of Interest to the Brazilian Unified Health System (Renisus).

It is also an herbaceous monocotyledonous (Franco 2010), perennial, with narrow, long and rigid leaves that possess serrated margins, being able to reach from 60 to 90 cm in height. It

has a tuft of leaves on its top, known as crown, and in its skin each "squama" is a true fruit that grows from a flower and merges, receiving the name of infructescence (Silva & Tassara 2001; Crestani *et al*. 2010). It is rich in vitamins A, B and C and contains potassium, sucrose, micronutrients and antioxidants, carotenoids, phenolic compounds (Chakraborty *et al*. 2015a) and saponins (Da Paixão 2016). It has several pharmacologically active phytochemicals, such as anannase beta-sitosterol, campesterol, chlorogenic acid, rutin, naringenin, bromelain and flavonoid glycosides (Parle and Goel 2010). As a chemical marker it has bromelain, that is the generic name given to the set of endopeptidases and proteases present in the members belonging to the Bromeliaceae family (Chakraborty *et al*. 2015b). This enzymatic set is present throughout the pineapple.

Among the pharmacological activities observed during the *A. comosus* research, it is worth mentioning its anti-inflammatory effect, which is associated to bromelain when used in fasting, mucolytic, antimicrobial, anti-helminthic and depolymerizing of sclerotic protein fibers of connective tissue, arranged around the cellulite nodules (Manetti *et al*. 2009; Pinto 2013; Offia-Oluan and Ekwunife 2015). In popular medicine, it has recognized medicinal properties such as stomachic, carminative, diuretic and anti-inflammatory (Manetti *et al*. 2009). Its mucolytic action, popularly identified through the various expectorants made from pineapple, which are used to eliminate respiratory secretions, tends to be the most well known pharmacological activity. Despite this, it is the antimicrobial activity that has attracted considerable attention, due to the rise of bacterial resistance to the already developed and used drugs. Observing this and considering the increased number of researches in the phytotherapy area and the market of phytotherapic medicine, the need to explore possibilities regarding this species was strongly considered. Since the main characteristic of a drug is primarily not causing any harm that can be fatal to the user, evaluating the toxicity of the chosen material for future formulations is essential. Thus, dry crude extracts of *Ananas comosus* L. Merril were produced and tested against *Artemia salina* Leach and the erythrocyte osmotic fragility was determined.

Materials and methods

The present study was carried out in the laboratories of Centro Universitário Tabosa de Almeida (Asces-Unita), in the city of Caruaru-PE. It has an experimental laboratory type design, in which the acute toxicological potential and the erythrocyte fragility of the dry crude extracts of *Ananas comosus* L. Merril were evaluated. The exsiccate was deposited in the Instituto Agronômico de Pernambuco – Dárdano de Andrade Lima, with registration number 91175, being Dr. Rita de Cássia Pereira the responsible for the taxonomic identification.

Preparation of the dry crude extracts of A. comosus L. Merril

The infructescence of the plant drug was collected between 7-9 o'clock in the morning, observing a healthy aspect of the vegetable part. It was weighed, washed with clean running water and dried with paper towels. The crown leaves of *Ananas comosus* L. Merril were shaded for a 24-hour period and afterwards placed in the botanical oven at 40 °C for drying, while the skin and pulp were manually reduced to small pieces and routed separately to the maceration stage.

Subsequently, the dried leaves were ground in an industrial mill and sent to maceration, as well as the skin and pulp were previously: with 95% v/v hydroalcoholic solution for 7 days. After this period, the three hydroalcoholic solutions were separately filtered to obtain the fluid crude extracts. All three solutions (crown leaves, skin and pulp) were extracted in a rotary evaporator at a temperature of 60 °C. After evaporation of 95% of the solution, the extracts were stored in a vacuum desiccator and in an air circulating oven (B.O.D.), achieving total dryness and obtaining the dry crude extracts of the three parts of the plant material.

Determination of the LC_{50} against Artemia salina Leach

The determination of the LC_{50} followed the methodology described by Meyer *et al*. (1982). The eggs of A. salina Leach were incubated in marine solution in a plastic container, which remained under artificial light (40 W lamp) and constant temperature of 28 °C for 48 hours. After this procedure, the metanauplius stage was obtained, standard model for toxicity tests due to its higher sensitivity. For the control group the metanauplius were submitted to only 5 mL of saline water to evaluate their motility and mortality without the addition of the extracts.

Thus, 50 mg of the dry crude extracts of *Ananas comosus* L. Merril were use, 1 mL of 5% Tween 80 being added to aid their solubilizations. The

solutions were homogenized, and the volume was completed to 5 mL with saline water at pH = 8.0. From these solutions, aliquots of 500, 375, 250, 125, 50 and 25 µL were withdrawn and transferred to test tubes previously containing 5 mL of saline, obtaining concentrations of 1000, 750, 500, 250, 100 and 50 µg/mL for each sample. The test was performed by triplicate analysis and the samples were submitted to artificial lighting for 24 hours. After this period, the number of live and dead larvae was counted and the data tabulated using the Microcal Origin® 4.1 software.

Study of erythrocyte osmotic fragility (EOF)
The erythrocyte osmotic fragility test aims to measure the resistance of red blood cells to haemolysis when submitted to plant extracts (Sant'ana 2001; Rodrigues 2009). The test was based on the technique described by Darcie & Lewi (1975), in which the lamb's blood was kept under refrigeration in a vessel with glass beads to avoid its coagulation. For the control group the blood cells were submitted to only 5 mL of 0.9% saline to evaluate haemolysis of the erythrocytes without the addition of the extracts.
Fifty milligrams of the dried crude extracts of *Ananas comosus* were diluted with 0.9% saline and homogenized, and the volume of the mixture was filled up to 5 mL with 0.9% saline. From these solutions, aliquots of 500, 375, 250, 125, 50 and 25 µL were transferred to test tubes previously containing 5 mL of 0.9% saline, obtaining the concentrations of 1000, 750, 500, 250, 100 and 50 µg/mL for each extract. Then, 25 µL of lamb's blood were added to each tube. Finally, the samples were centrifuged at 3000 rpm for 15 min at room temperature and, after this procedure; the absorbance of the supernatant of each tube was measured in a 545 nm bioPLUS spectrophotometer. The curve regarding the percentage of osmotic fragility was obtained based on the haemoglobin absorbance value of the supernatant multiplied by the total percentage and divided by the mean absorbance value of the complete haemolysis of the erythrocytes.

Statistical analysis
The obtained information was statistically treated with Microsoft® Office Excel 2013 and Microcal Origin® 4.1 software.

Results and discussion

Lethal Concentration (LC_{50}) against Artemina salina Leach

Artemia salina Leach is a microcrustacean that presents four stages of development: egg, nauplius, metanauplius and adult (Seyfried 2010). In tests performed with this microcrustacean species, it is important to analyse criteria such as behaviour and mortality. The results obtained from this *in vivo* assay show good efficiency and correlation with *in vitro* toxicity results, suggesting it as the first cytotoxic potential analysis of new samples. In the present study, bioassays were carried out on *Artemia salina* Leach for each one of the pineapple extracts. The lethal concentrations were obtained by calculating the parameters A and B, according to the linear regression equation: y = A + B * x.
In which y represents a 50% probability of lethality, A is the angular coefficient and B is the linear one. These values are automatically generated when the life and death data of the microcrustacean are plotted in Microcal Origin® 4.1 software, in accordance with each tested concentrations of the extract. The program itself, through the linear regression equation, determines the live curve for each extract according to the concentrations and conditions that were tested. In relation to this, it is taken as a parameter that the closer to zero is the LC_{50}, the more toxic the plant is; and the closer to 1000, the lower the toxicity of the plant. In this bioassay the control group did not present changes in mobility or mortality, validating the observed results. Any changes in these parameters would indicate an execution error, implying the necessity of repetition the test. The crown leaves extract presented LC_{50} of 994 µg/mL, showing to be practically non-toxic (*Figure 1*).

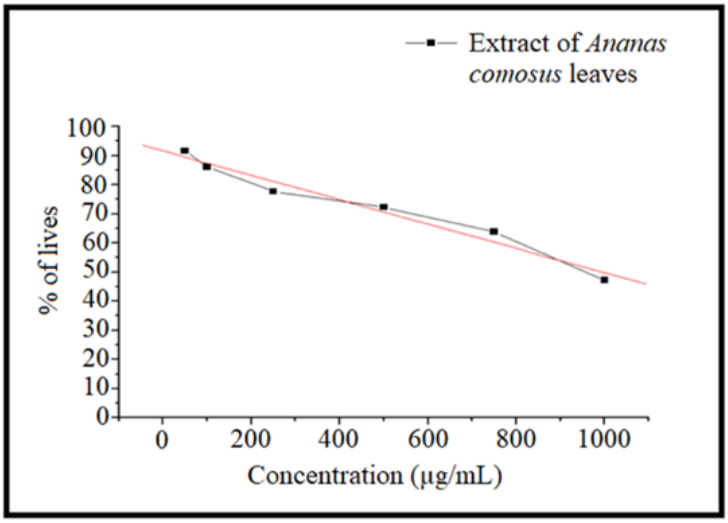

Figure 1. Graph of the percentage of live related to the concentrations of the dry crude extract of the pineapple crown leaves.

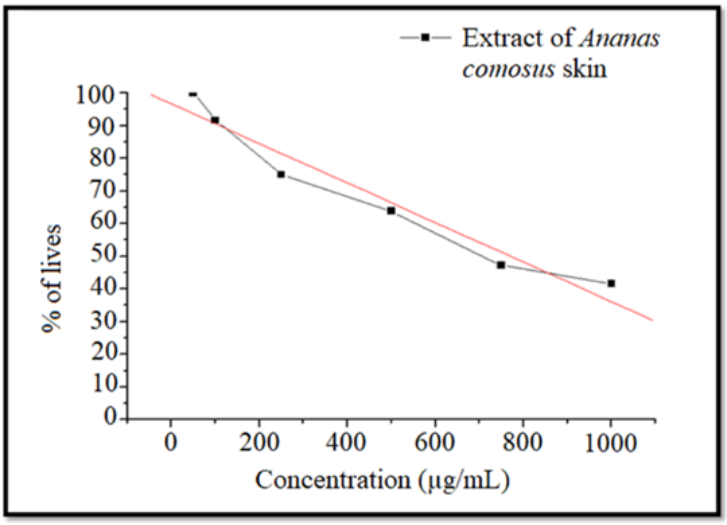

Figure 2. Graph of the determination of the LC_{50} in the tested concentrations of the pineapple skin extract.

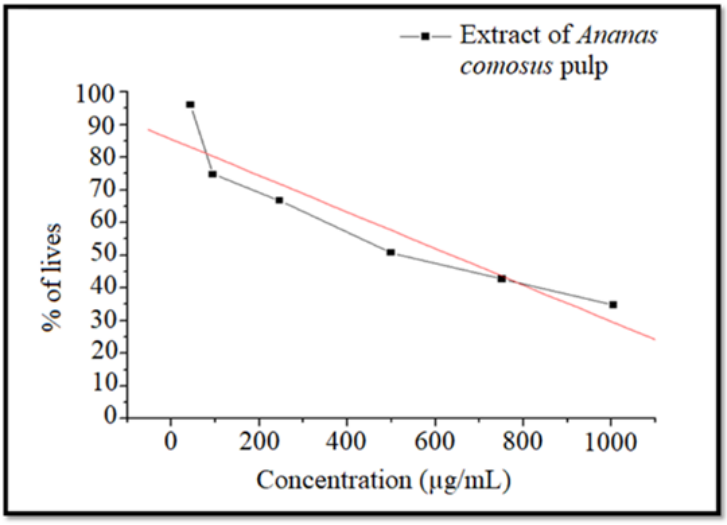

Figure 3. Percentage of live of *Artemia salina* Leach in relation to the pulp extract of *Ananas comosus* L. Merril (pineapple).

However, the skin extract showed to be slightly toxic, with a final LC_{50} of 770 µg/mL, when applied to the linear regression equation (*Figure 2*). The swim ratio of the animals was slower when submitted to the three highest concentrations (500, 700 and 1000 µg/mL).

The extract of the pineapple pulp had the lowest LC_{50}, which can be described as moderately toxic when compared to the other extracts tested in this study. With a lethal concentration of 622 µg/mL (*Figure 3*), the swim ratio of the tested animals was significantly slower when compared to the other extracts, indicating the sensitivity of *Artemia salina* Leach to the submitted extract. This can be explained thanks to the high sugar content found in the pulp of *Ananas comosus* L. Merril and, consequently, in its extract, directly affecting the microcrustacean, which is adapted to saline environments.

In a test carried out by Evangelista *et al*. (2012) that also aimed to determine the LC_{50} against the microcrustacean *Artemia salina* Leach, both pineapple pulp and skin extracts of *A. comosus* presented 100% mortality in the highest concentration of 1000 µg/mL. We believe that this divergence of results occurred mainly due to the different methodologies used. Evangelista *et al*. (2012) produced ethanolic extracts and applied a procedure based on the writings of Cantoria (1994), Peteros & Uy (2010), with small modifications, while the present study produced hydroalcoholic extracts and applied a technique according to the methodology of Meyer *et al*. (1982).

This same research exposed 10 metanauplius of the microcrustacean to each concentration, whereas in this one 12 metanauplius were exposed; finally, seawater was used as the natural habitat of *Artemia salina*, while the cited study used a mixture of 2.5 litres of water and 9.5 grams of sea salt obtained from a pet store (Evangelista *et al*. 2012). Among the differences found, it is believed that the production of the ethanolic extract and the use of water and sea salt to simulate the environment for *Artemia salina* were the main points that favoured the death of the species.

In agreement with the results found in this study, there is a research by Adoum (2009) that, despite the difference in the production of the sample to be tested, it concluded that the species presents low toxicity against *Artemia salina* Leach. The study developed fractions of different residues from the pineapple skin: an ethanolic fraction and other with water fraction. These were obtained by percolation with different solvents aiming the solubilization and separation of the compounds. The fractions presented values of toxicity above 1000 µg/mL, corroborating with the results found in the current study, which affirms the low toxicity of the *A. comosus* species.

Erythrocyte osmotic fragility

According to De Carvalho *et al*. (2017), red blood cells are susceptible to blood pH in several pathologies, leading to changes in acid-base balance and making them more prone to cell lysis, in other words, increasing their osmotic fragility.

Considering this, the method consists in determining the fragility of the red cells in a buffered solution when submitted to vegetal extracts. From the value of the duplicate of the tested concentrations, the mean and then the average absorbances were calculated.

The control group presented a percentage of haemolysis of 0.22%, while the pineapple crown leaves extract presented 7.19% at the highest concentration of 1000 µL/mL (*Table 1*) and may be seen as moderately toxic. On the other hand, the skin extract showed a percentage of haemolysis of 1.66%, with a lower lysis of red blood cells when compared to the crown leaves extract (*Table 2*). Therefore, the extract is considered of low toxicity in relation to the tested parameters and conditions, corroborating with the results of the bioassay against *Artemia salina* Leach.

Regarding the pineapple pulp extract, the erythrocyte osmotic fragility study presented a percentage of haemolysis of 0.83% at a concentration of 1000 µg/mL, being classified as practically non-toxic (*Table 3*).

Table 1. Haemolysis percentage of the DCE of *A. comosus* crown leaves.

Concentration (µg/mL)	Absorbance 1	Absorbance 2	Mean Absorbance	Percentage of haemolysis (%)
1000	0.094	0.095	0.095	7.19
750	0.070	0.075	0.073	5.53
500	0.045	0.048	0.047	3.56
250	0.029	0.025	0.027	2.04
100	0.014	0.015	0.015	1.13
50	0.002	0.005	0.0035	0.27

Table 2. Erythrocyte osmotic fragility of the skin DCE of *A. comosus.*

Concentration (µg/mL)	Absorbance 1	Absorbance 2	Mean Absorbance	Percentage of haemolysis (%)
1000	0.020	0.024	0.022	1.66
750	0.018	0.019	0.018	1.36
500	0.018	0.015	0.016	1.21
250	0.011	0.011	0.011	0.83
100	0.007	0.007	0.007	0.53
50	0.006	0.005	0.005	0.37

Table 3. Percentage of haemolysis of the pulp DCE of *A. comosus.*

Concentration (µg/mL)	Absorbance 1	Absorbance 2	Mean Absorbance	Percentage of haemolysis (%)
1000	0.010	0.011	0.011	0.83
750	0.004	0.003	0.004	0.30
500	0.003	0.003	0.003	0.22
250	0.000	0.001	0.0005	0.037
100	0.002	0.001	0.001	0.075
50	-	0.000	0.000	0

In a study carried out by Lopes Neto *et al*. (2015), the haemolytic activities of extracts of the crown leaves and the pulp of the *A. comosus* were tested. The technique consisted in incorporating extracts in different concentrations (50 and 25 µg/mL) in sterilized paper disks, through the diffusion method of the samples in Blood Agar. In such manner, it is possible to detect the presence of haemolysis halos in the Petri dish. The results obtained in this study displayed that both extracts showed no toxicity at the tested concentrations, since the haemolytic activity is related to the toxicity of the plant.

According to Kaiser *et al*. (2010), the haemolytic activity is directly linked to the presence of saponins in the plant; although the *A. comosus* species is rich in saponins, these were not able to cause haemolysis in the performed test under the tested concentrations. This confirms the results of this study, in which, even at the maximum concentration of 1000 µg/mL, the pulp extract exhibited a percentage of haemolysis lower than 1%, whilst the crown leaves extract presented a percentage of 0.27% in the concentration of 50 µg/mL, the same concentration as the study of Lopes Neto *et al*. (2015), as can be seen in *Table 1*.

The low toxicity of *A. comosus* is confirmed by its presence in the National Relation of Medicinal Plants of Interest to the Brazilian Unified Health System (Renisus). Despite this, the performed research sought to certify the coherence of the species in the list, finally agreeing with it. In view of the found results and since its low toxicity is confirmed by studies carried out with different types of samples from the species, such evidences lead one to believe that the pineapple can be used to manufacture pharmaceutical

products according to their already proven therapeutic activities. However, further toxicological studies regarding the species are still required.

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References

Adoum O.A. Determination of toxicity levels of some savannah plants using brine shrimp test (BST). BAJOPAS.2009;2(1):135-8.

Cantoria M.C. Selected Topics in Pharmacognosy. Taguig: NAST. National Academy of Science and Technology, 1994.pp 417-30.

Chakraborty S., Rao P.S., Mishra H.N. Effect of combined high pressure–temperature treatments on color and nutritional quality attributes of pineapple (*Ananas comosus* L.) puree. Innov Food Sci Emerg Technol. 2015a;28:10-21.

Chakraborty S., Rao P.S., Mishra H.N. Kinetic modeling of polyphenoloxidase and peroxidase inactivation in pineapple (*Ananas comosus* L.) puree during high-pressure and thermal treatments. Innov Food Sci Emerg Technol. 2015b;27:57-68.

Crestani M., Barbieri R.L., Hawerroth F.J., Carvalho F.I.F., Oliveira A.C. Das Américas para o Mundo - origem, domesticação e dispersão do abacaxizeiro. Cienc Rural. 2010;40(6):1473-83.

Da Paixão J.A. Caracterização química e testes de atividade biológica *in vitro* em abacaxizeiros silvestres. Dissertação (Pós-Graduação em Recursos Genéticos Vegetais). Bahia: Feira de Santana. 2016.

Darcie J.V., Lewis S.M. Practical Hematology. Churchill Livingstone. 5th ed. London. 1975.

De Carvalho L.A.L., Cruz N.R.N., Bueno P.J., Santana A.E. Avaliação eritrocitária através da biotecnologia de fluxo. Investigação. 2017;16(8):44-9.

Evangelista J.H., De Vera M.J., Garcia R.S., Joven M.G., Nerosa M.J.A., Solidum J.N. Preliminary Assessment of *in vitro* Anticoagulant Activity vs. Heparin 1,000I.U. and Cytotoxicity of Selected Philippine Medicinal Plants. IJCEE. 2012;3(6):371-6.

Franco L. Crescimento, produção e qualidade do abacaxizeiro 'Pérola' sob diferentes lâminas irrigação por gotejamento. Dissertação (Pós-graduação em produção vegetal no semiárido), Minas Gerais: Montes Claros. 2010.

Kaiser S., Pavel C., Gonzales Ortega, G. Estudo da relação estrutura-atividade de saponinas hemolíticas e/ou imunoadjuvantes mediante uso de análise muito variada. Rev. Bras. Farmacogn. 2010;20 (3):300-9.

Kerbauy G.B. Fisiologia vegetal. 1st ed. Rio de Janeiro: Guanabara Koogan. 2004.

Lopes Neto J.J., Veras K.S., Rosa C.S. Estudo botânico, fitoquímico e avaliação de atividades biológicas do fruto de *Ananas comosus* var. comosus (L.) Merril (Bromeliaceae). Gaia Scientia. 2015;9(1):164-71.

Manetti L.M., Delaporte R.H., Laverde Jr A.R. Metabólitos secundários da família Bromeliaceae. Quim. Nova. 2009;32(7):1885-97.

Meyer B.N., Ferrigni N.R., Putnam J.E., Jacobsen L.B., Nichols D.E., McLaughlin J.L. Brine shrimp: a convenient general bioassay for active plant constituents. Plant. Med. 1982;45(5):31-4.

Offia-Oluan B.I., Ekwunife O.A. Production and evaluation of the physico-chemical and sensory qualities of mixed fruit leather and cakes produced from apple (*Musa pumila*), banana (*Musa sapientum*), pineapple (*Ananas comosus*). Niger Food J. 2015;33(1):22-8.

Parle M., Goel P. Is pineapple a fine apple?. Annals of Pharmacy and Pharmaceutical Sciences. 2010;1(2):134-41.

Peteros N.P., Uy M.M. Antioxidant and Cytotoxic Activities and Phytochemical Screening of Four Philippine Medicinal Plants. J Med Plants Res. 2010;4(5):407-14.

Pinto M.R. Utilização de materiais de origem vegetal em produtos farmacêuticos e cosmé-

ticos de aplicação cutânea. Dissertação (Mestrado em Ciências Farmacêuticas) Portugal: Lisboa. 2013.

Rodrigues H.G., Batista M.T.A., Fonseca L.C., Aversi-Ferreira T.A. Efeitos de pesticidas sobre a fragilidade osmótica de eritrócitos – Uma breve revisão. Biotemas. 2009;22(1):7-16.

Sant'ana V.A.C., Birgel E.H., Mourao G.B., Mirandola R.M.S. Fragilidade osmótica dos eritrócitos de bovinos das raças holandesa, girolando e gir, criados no estado de São Paulo Cienc Rural. 2001;31(4):609-14.

Seyfried M. Triagem da atividade antitumoral de extratos vegetais utilizando ensaios de toxicidade *in vivo* sobre *Artemia salina* e *in vitro* sobre células da linhagem HeLa. Monografia (Pós-Graduação em Biologia Celular). Paraná: Curitiba. 2010.

Silva S., Tassara H. Abacaxi. In: Silva S, Tassara H, editors. Frutas no Brasil. São Paulo: Nobel, 2001. p.25-27.

Vieira D.A.P., Portes T.A., Stacciarini-Seraphin E., Teixeira J.B. Fluorescência e teores de clorofilas em abacaxizeiro cv. pérola submetido a diferentes concentrações de sulfato de amônio. Rev Bras Frutic. 2010;32(2):360-8.