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CELLULAR PROLIFERATION IN THE GILLS OF GUPPIES EXPOSED TO PEQUI ETHANOLIC EXTRACTS

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Abstract: The bioactive principles present in the ethanolic extract of "pequi" (*Caryocar brasiliensis*) have proven to be molluscicidal, fungicidal, trypanocidal, insecticide, and leshimanicidal, taking potential use to combat aquatic pests and vectors in public health, and for infectious and invasive diseases in fish. Many plants have proven moluscidida and insecticide effects, and its extracts may contribute to vector control in public health. They also have prophylactic and therapeutic effects in combating diseases in fish, for ornamental and aquaculture systems. Most of them are commonly found in tropical and subtropical countries. However, to be released in environment or used for health and/or combating invasive diseases in fish, the effect of these products on other representatives of aquatic fauna must be better understood. The guppy (*Poecilia vivipara*) is generally used as a bioindicator of environmental toxicity, then, he was elected to the experimental studies carried out to verify the action of these extracts. The gills are the target organ, as they come into direct contact with water and because its morphophysiological activity in demonstrate the effects of substances present in the water. Therefore, the extracts obtained from the stem and leaf of pequi were analyzed chromatographically, and then the extracts rich in flavonoids were selected. The effects in acute tests of these extracts were verified histologically and immunohistochemically using the molecular marker BrdU (5-bromo-2'-deoxyuridine) that binds to cellular DNA during the S phase of their cycle. This work confirms the potential action of "pequi" as inducer of cell proliferation in gills of fish. As the test was conducted in a short period of time and was proven the occurrence of cell proliferation exist the possibility of having future tumor formation, which indicate a need for studies with long-term exposures.

Key words: Phytochemical compounds, ethanolic extract, chromatography, immunohistochemistry, cell proliferation.

Resumo: Os princípios bioativos presentes no extrato etanólico de "pequi" (*Caryocar brasiliensis*) possuem comprovados efeitos moluscicida, fungicida, tripanossomicida, inseticida, e leshimanicida, tendo potencial de uso para combater pragas e vetores aquáticos em saúde pública, e para doenças infecciosas e invasoras em peixes. Muitas plantas têm comprovado efeito moluscidida e inseticida, e seus extratos podem contribuir para o controle de vetores em saúde pública. Elas também têm efeitos profiláticos e terapêuticos contra as doenças em peixes, tanto ornamentais quanto para sistemas de produção em aquicultura. A maioria delas são comumente encontradas em países tropicais e sub-tropicais. No en-

tanto, para lançar esses extratos no ambiente ou usá-los para a saúde pública e/ou combate de doenças invasivas em peixe, o efeito desses produtos em outros representantes da fauna aquática deve ser melhor compreendido. O guppy (*Poecilia vivipara*) geralmente é usado como bioindicador de toxicidade ambiental, e foi escolhido para os estudos experimentais para verificar a ação desses extratos. A brânquia é o órgão-alvo, pois está em contato direto com a água, e por isso, manifesta alterações morfofisiológicas em resposta às substâncias presentes na água. Assim, os extratos obtidos a partir do caule e folha do pequi foram analisados por cromatografia, e em seguida, selecionados os extratos ricos em flavonoides. Os efeitos em ensaios de toxicidade agudos destes extratos foram verificados utilizando-se técnicas histológicas e imuno-histoquímicas, tendo o BrdU (5-bromo-2'-desoxiuridina)como marcador molecular que se liga ao DNA celular durante a fase S do ciclo. Confirmou-se a ação potencial do pequi como indutor da proliferação celular em brânquias de peixes. Como o teste foi em curto espaço de tempo e houve proliferação celular, há possibilidade de haver futura formação de tumores, indicando necessidade de estudos com exposições de longo período.

Palavras-chave: Compostos fitoquímicos, extrato etanólico, cromatografia, imunohistoquimica, proliferação celular.

Introduction

Trademarked products made from plants are frequently used as an alternative for the control of pests and vectors like mosquitoes and trematodes (Kovedan et al., 2012, Singh et al., 2010) and for infectious and invasive diseases in fish (Prieto et al., 2005; Lima et al., 2007; Roesler et al., 2010).

A vegetable biopesticide that is biodegradable and potentially toxic to certain organisms would be a perfect substitute to synthetic pesticides, which toxic substances accumulate in the environment (Kabir et al., 2013; Kovedan et al., 2012; Marimuthu et al., 2012; Singh et al., 2010).

Accordingly, the plants are a source of alternative control agents because they contain a rich variety of bioactive compounds with proven toxic action as flavonoids, triterpenoid glycosides and alkaloids (Kabir et al., 2013; Kovedan et al., 2012; Marimuthu et al., 2012, Singh et al., 2010).

Many plants have proven moluscidida and insecticide effects, and its extracts may contribute to vector control in public health. They also have prophylactic and therapeutic effects in combating diseases in fish, for ornamental and aquacultural systems. Most of them are commonly found in tropical and sub-tropical countries.

Nevertheless, its toxic action in vertebrates like fishes is not yet fully understood.

Today, the use of fish species as indicators of environmental change has wide significance for monitoring toxicity bioassay (Roesler et al., 2010; Mohti et al., 2012; Stacke et al., 2012; Paulo et al., 2012). They serve as a biological model for studying the use of natural compounds extracted from plants (Yonar et al., 2012; Ling et al., 2012).

Euryhaline fish have been used in several studies to verify the potential effects of environmental agents on their bodies. One of these studies looks into cell behavior under environmental change (Araújo et al., 2001; Borges-de-Oliveira et al., 2006; Mohti et al., 2012; Motter et al., 2004; Paulo et al., 2012; Sabóia-Morais et al., 1996; Silva et al., 2002; Stacke et al., 2012).

Several species, such as *Poecilia vivipara* (Block e Schneider, 1801), stand out as bioindicator models in biological studies (Paulo et al., 2012; Stacke et al., 2012). This fish, also called "guppy", is distributed in a neotropical region, is found in geographically widespread fresh water and is generally used in histochemical studies to verify effects caused by environmental agents (Araujo et al., 2009; Cunk, 1993; Chunk & Mendez, 1993; Mohthi et al., 2012; Paulo et al., 2012).

Gills are among the main fish organs used in research due to their various functions. They are the most responsible for gas exchange, metabolic waste excretion, ionic regulation and the blood acid-base balance (Cameron, 1976; Garcia-Santos et al., 2007). This organ presents several cell types that participate in homeostasis and help to maintain the physiological conditions of the body (Pereira & Caetano, 2009).

Responses to toxic changes in the gill epithelium are mediated by different cell types, especially among those chloride cells, which are rich in mitochondria (Garcia Santos, 2007; Perry, 1997). They are located mainly in the interlamellar region, and actively participate in physiological processes such as the acid-base balance (Evans et al., 2005; Podlipaeva & Berger, 2012).

The effects of environmental factors act on fish are crucial to evaluate cell proliferation, and cell cycle dynamics. This can be performed using molecular markers that identify the proliferation rate, proliferating cell types, cytotoxicity (Butterworth et al., 1992; Paulo et al., 2012).

The process of cell proliferation is one of the most important cellular functions and can indicate aquatic environmental changes such as environmental toxicity (Kilemade et al., 2002;

Paulo et al., 2012).

Cytological biomarkers are powerful tools for detecting and characterizing the inductive effect in cell proliferation caused by various environmental factors, among them the exposure to toxic as extracts of plants (Hinton et al., 1987; Santos et al., 2012).

Several methods are commonly used for determining cellular proliferation in animal models, amongst these is the 5-Bromo-2'-deoxyuridine method (BrdU) (Taupin, 2007). This simple and low cost methodology in comparison with other molecular markers expanded the use of BrdU in studies on identification of cell proliferation.

BrdU is a halopyrimidine, similar to thymine, which is incorporated into the cell DNA during the S phase of the cell cycle, and is frequently used for marking and monitoring cellular proliferation by immunohistochemistry using the anti-BrdU antibody (Muskhelishvili et al., 2003; Taupin, 2007).

Thus, this marker is widely used in immunochemical measurements of DNA synthesis *in vivo* and *in vitro*. Another factor that encourages the use of BrdU in proliferation studies is that the 5-bromouracil converted into uracil, either spontaneously or by cell exposure to light, would indicate its lack of interference in the cell division processes (Suzuki et al., 2001).

Exposure to pequi crude extracts correlates with its proven molluscicidal, fungicidal, tripanossomicidal, insecticidal, and leshimanicidal activity (Bezerra, 2002; Herzog-Soares et al., 2002; Ling et al., 2012; Passos et al., 2002; Paula Jr et al., 2006; Pereira et al., 2008; Pereira e Caetano, 2009; Ribeiro et al., 2012), this could justify the use of natural products in the water, providing low–environmental toxicity alternatives for public health, aquaculture and fishkeeping.

Nevertheless, plants extracts (medicinal and fruit) as "pitanga" (*Eugenia uniflora*) and "cagaita" (*Eugenia desynterica*), cause morphological and cytological changes, gills vasodilatation, lamellar fusion and reduction in mitochondrial cell rich density (Balestra et al., 2011; Pires, 2002).

However, before the pequi extract can go into use, possible alterations caused in the fish organism must be verified, particularly in the gills due to their osmoregulatory function.

The purpose of this study is, therefore, to investigate cell proliferation in guppy (*Poecilia vivipara*) gill cells exposed to leaf and stem bark extracts of pequi (Caryocar *brasiliensis* Camb.) using BrdU as a molecular marker.

Materials and methods

Ethanolic Extracts Obtention

Samples of pequi (*Caryocar brasiliensis* - Caryocaraceae - Camb.) leaves and stem bark were collected in Goiânia, Goiás (Latitude 16º40'4"S, Longitude 49º15'14"W). After collected, it was taxonomically identified in the Herbarium in the Biological Sciences Institute in the Federal University of Goiás (voucher 26 871 - HUFG).

The leaves and the stem bark were dried under forced ventilation for 24 hours at 50 ºC, and then ground separately. To obtain the ethanolic extracts, both grounded samples were immersed in 95% ethanol (250g/2000mL), separately, and filtered through cotton after 15 minutes. The filtrated material was evaporated using a rota-evaporator and then frozen at -4oC before undergoing vacuum lyophilization.

The powder of ethanolic extract from the leaves (EEL) and the powder of ethanolic extract from the stem bark (EESB) were used in the treated groups.

Each sample of crude extract from the leaves and bark of *pequi* was fractionated to ensure the chromatographic analysis of its phytochemical compounds. Portions of the samples were diluted in distilled water and ethyl ether (1:1) in a separating funnel. The polarity separation drove the ether fraction to the bottom of the funnel allowing its removal. The aqueous portion, free of grease and chlorophylls, was transferred to a second separating funnel where it received ethyl acetate (1:1), resulting in the ethyl acetate and aqueous fractions. Each of these was evaporated, following the methodology for the extracts and then frozen and lyophilized.

Thin layer chromatography of the crude extracts

To carry out the chromatography, plant samples were dissolved as follows: EEL, EESB and ethyl acetate fractions were dissolved in 95% ethanol; ether fractions in chloroform; and aqueous fractions (1:1) in a water and ethanol solution. All samples (250µL) were placed in double-layer silica plates (stationary phase). An acetone:toluene:formic acid (3:3:1) solution was used for the mobile phase. After contact with the mobile phase, the plates were kept at room temperature for drying before subsequent revelation of the phytochemical compounds.

Fluorescent light at 254nm and 330nm wavelengths was used to verify the presence of coumarins. To detect the presence of other compounds, a vanillin solution (indicative of chlorophyll, carbohydrates, flavonoids and tannins) was sprayed onto one of the plates, and a ferric chloride solution (indicative of phenolic compounds not evidenced by the vanillin) was used on another plate. Gallic acid was used as a reference for ferric chloride revelation results, applied together with the samples. The plates were heated on a hot plate to visualize the bands indicating the presence of phytochemical groups.

Biological model

The biological model used was the teleost guppy (*Poecilia vivipara* - Cyprinodontiformes, Poeciliidae - Block & Schneider, 1801). The animals, males and females, were collected on the Itamaracá Island – Pernambuco - Brazil (Latitude: 07°45'00"S and Longitude: 34º49'30" W. They were previously adapted in laboratory aquaria at least one month, and 24 hours in experimental aquaria, containing water from their original environment and mineral water (1:1), pH 7.2. The fishes were feed once a day with 10% body weight.

The animals were then randomly divided into experimental groups, each totaling five individuals varying in length from 2cm to 3cm. Throughout the experiment, the pH of the tanks was monitored, and the animals were fed once a day with fish commercial feed.

This work is part of a project approved by Ethics Committee of Clinical Hospital, protocol n.046.

Experimental groups

After the acclimation period, four groups of five randomly chosen animals were assembled. Four groups were placed in experimental tanks, and three groups were subjected to contact with the BrdU (30mg.L^{-1}) for marker incorporation in their gills, as recommended by Lopez-Peres (2000).

Bezerra et al. (2002) report that the extract from pequi leaves and stem bark at 200ppm induce 100% mortality of *Bionphalaria glabrata*, and 50ppm, induce only 20% mortality.

On the other hand, in fishes (*P. vivipara*) these same extracts at 20ppm not cause mortality (Carneiro, 2002). These researchers showed that the guppy exposed to 20mg.L^{-1} of pequi crude ethanolic leaf extract, does not presented any mortality. So, this is the dose recommended for release of substances in water sources.

Thus, the groups were distributed as follows: Control group (CG): exposed only to BrdU; Sham group (SG): exposed only to aquaria water; Treated group 1 (TG1): exposed to BrdU and to pequi EEL at 20mg.L^{-1}; Treated group 2 (TG2): exposed to BrdU and to pequi EESB at 20mg.L^{-1}.

The experimental groups (CG, SG, TG1 and TG2) were each exposed to their treated environments for 24 hours (acute exposure).

Subsequently, the animals that survived were decerebrated, and the gills were dissected. The gill leaflets were separated and fixed for 2 hours in 10% neutral formalin.

Anatomo-pathological examination of TG1 animals

The fish in this group were sacrificed after exposure to BrdU associated with EEL and fixed in 10% formalin for histopathological evaluation, and then morphological characteristics of the organs were studied.

Chromatographic evaluation

The silica thin layer chromatography evaluation was performed on the water sample from the aquarium where fish were treated with BrdU and EEL (TG1) to see if there was a reaction between these components, since the treatment killed all the animals in the aquarium. Thus, this water was compared with the EEL (20mg.L^{-1}) and BrdU aqueous solution 30mg.L^{-1} as controls.

After inoculation of 250mcrL samples on the chromatography plate, it was placed in contact with the mobile phase (methanol:chloroform solution, 8:2), dried and revealed with anisaldehyde.

Fixation, inclusion and microtomy

Gill leaflets of *Poecilia vivipara* were fixed for 2 hours in 10% neutral formalin and then submitted to histological procedures for paraffin embedding to visualize the cells that incorporated BrdU.

Histological sections 5µm thickness were made in a microtome (SPENCER - MODEL 820). Five slides covered with Sylane (SIGMA) were prepared from each animal, and only 2 were selected randomly to study tissue and cellular aspects to verify possible cellular alterations.

The amount of material prepared and the number of slides per animal was determined by the statistical analysis for gill research (Rosa et al., 2004).

Immunocytochemical reaction by indirect immunostaining

Monoclonal anti-BrdU (SIGMA, CLONE No. BU-33, B2531, USA) and the secondary antibody B-Gar (SIGMA, USA) were used to detect the BrdU incorporated into the cells in the S phase of the cell cycle.

Immunocytochemical antibody inoculation procedures were divided into two stages. Firstly slide deparaffinization was performed by exposure to kiln dry heat at 60°C/1 hour, followed by two exposures to xylol (10 minutes each), 100% ethanol (10 minutes), 90% ethanol (5 minutes), 70% - 50% - 25% ethanol (3 minutes each) and finally rinsing in distilled water. After this, the slides were incubated with HCl 1N (10 minutes) and HCl 2N (20 minutes at 37°C). Borate buffer 0.1 M was added (20 minutes at room temperature), rinsed with PBS 0.1 M pH 7.4 (5 minutes).

The sections were incubated with hydrogen peroxide 1% in methanol for 10 minutes and washed with PBS for 10 minutes. Then, they were incubated with Triton X-100, 0.58% in PBS for 15 minutes and blocked with bovine serum albumin (BSA) 1% (Sigma, USA) in PBS for 30 minutes. Primary monoclonal antibody anti-BrU was used, diluted in 1% BSA, for the overnight incubation at 37°C. After incubation, the sections were each rinsed three times with PBS for 5 minutes.

The second step was the inoculation of the secondary antibody B-Gar, 120 minutes exposure, followed by washing in PBS (5 minutes), and by exposure to ABC - Avidin Biotin Complex (Vector, USA) for 30 minutes before washing again in PBS (5 minutes). DAB (3,3'-diaminobenzidine, SIGMA, USA) was used as a chromogen for 30 minutes in the dark, then washed with distilled water for 5 minutes and counterstained with toluidine blue 0.5% for 1 minute and 0.5% safranin for 30 seconds. The slides were then assembled with glycerol.

Light microscopy

The two randomly selected slides were analyzed under a LEICA DMLB microscope, and three gill filaments from each slide were also randomly selected. Each selected gill filament had 3 regions (base, middle and peak) analyzed to determine the number of cells marked by BrdU (S-phase of the cell cycle).

Statistical analysis

The analysis of variance (ANOVA) was applied to the number of chlorine cells labeled with BrdU from the gills of fish in the control group (treated only with BrdU) and in the group treated with BrdU and EESB, following recommendations by Rosa et al. (2004). The Tukey test was also applied ($q=2.86$, GL=1; 30 repetitions per region of the filament, $\alpha=0.05$) and 5% significance level.

Results

All the fish in the group treated with BrdU and EEL (ethanolic extract from the leaves) died, and therefore were not subjected to microscopic analysis.

Immunocytochemical and histological procedures were carried out only in the control and BrdU + EESB (ethanolic extract from the stem bark) groups to verify the inductive effects of cell chlorine proliferation due to the effects of EESB, through the BrdU molecular marking.

To compare the variability of phytochemical compounds in the crude ethanolic extracts from pequi leaves and stem bark, crude extract and extract fraction samples underwent thin layer chromatography. This analysis showed that there was a difference between the phytochemical compounds of the pequi crude ethanolic extract from the leaves and the stem bark (Table 1), and their fractions, as evidenced by the bands that indicated significant differences in the types of phytochemicals (Figure 1).

Table 1 lists the phytochemical compounds found in leaf and stem bark of pequi. It's possible to observe that, in both samples, we found sugars, flavonoids glycosides, condensed and hydrolysable tannins, but the compounds chlorophylls and carotenes, and coumarins, respectively, only appear in leaves and steam bark, and that is the only difference between extracts.

Leaf	Steam bark
Sugars	Sugars
Flavonoid glycosides	Flavonoid glycosides
Condensed and hydrolysable tannins	Condensed and hydrolysable tannins
Chlorophylls and carotenes	Coumarin

Table 1 - List of phytochemical components of leaf and stem bark crude ethanol extracts of pequi (Caryocar brasiliensis) evidenced by Silica Thin Layer Chromatography.

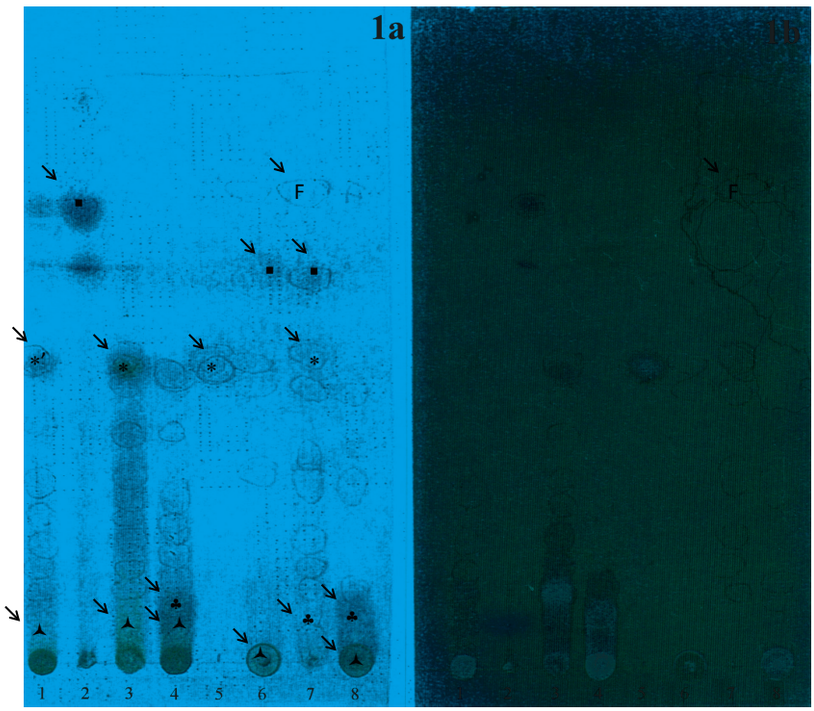

Figure 1: Thin Layer Chromatography of pequi (Caryocar brasiliensis). Application sequence of the fractions: (1) leaf ethanol crude extract; (2) leaf ethyl ether fraction; (3) leaf ethyl acetate fraction; (4) leaf aqueous fraction; (5) gallic acid; (6) stem bark ethanol crude extract; (7) stem bark ethyl acetate fraction ; (8) stem bark aqueous fraction. On the left (1a), revelation by vanillin: (*)yellow bands – flavonoids; (§)green bands - chlorophyll; (©)red bands – tannins; (§)ash bands – sugars; (F) fluorescence bands – coumarins (fluorescent bands revealed by ultraviolet light); On the right (1b), revelation by ferric chloride: (F)fluorescence bands – coumarins; dark bands - phenolic compounds.

Qualitative chromatographic analysis of the phitochemicals

Figure 1 shows the silica thin layer chromatography of ethanolic crude extracts of pequi (*Caryocar brasiliensis*) leaf and stem bark fractions. On the left side of the plate (Fig.1a), revealed with vanillin, the following can be noted: flavonoids, chlorophyll, tannins, sugars and coumarins. The right side of the plate (Fig.1b) revealed by ferric chloride, indicates phenolic compounds (dark bands), and was not stained with vanillin.

Histopathological analysis on fish dead after treatment wth BrdU and ethanolic crude extract from the leaves (EEL)

All animals exposed to the environmental conditions that associate BrdU with EEL died after exposure for 12 to 24 hours. This effect was confirmed by a new exposure (n=5), followed by the anatomopathological analysis revealing characteristics such as redness around the abdominal region; thrombosis points in the gills; edema in anterior digestive tract; excess superficial mucus on the epithelial surface; gallbladder rupture and death of embryos in pregnant females.

Comparative chromatography of the aquarium water

To investigate if the fish mortality was due to possible changes in the aquaria water pattern, as a result of addiction of BrdU and pequi Ethanolic Crude Extracts of the Leaves (EEL) at 20mg.L^{-1}, a comparative thin layer chromatography was performed on aquaria water treated with BrdU and EEL (TG1), EEL aqueous solution (EEL) and aquaria water treated with BrdU (BrdU), which are presented in Figure 2.

It is noted the presence of equal bands among EEL and aquarium water in TG1 (star). Also, another corresponding band appears in TG1 and BrdU (asterisk). This correspondence is confirmed by their presence in the inoculum containing only marker (BrdU). However, is possible to note the absence of a band in the aquarium water TG1 that is present in EEL (arrows).

The water pH showed that the aquaria water, to which BrdU and EEL were added, reached values up to 10.5, differing from the other groups (pH=6.2).

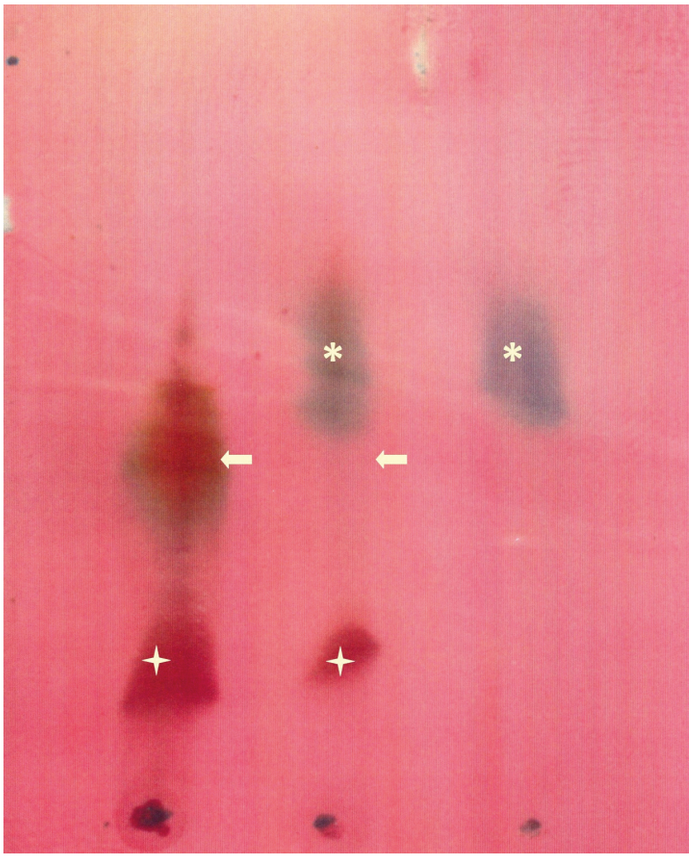

Figure 2 - Comparative Silica Thin Layer Chromatography. Application sequence: (EEL) Ethanolic Extract from pequi Leaves (Caryocar brasiliensis); (TG1) aquaria water of Treatment Group 1 (water + BrdU at 30mg.L-1 + pequi Ethanolic Crude Extracts of the Leaves (EEL) at 20 mg.L-1); and, BrdU (water + BrdU at 30mg.L-1). (*) BrdU; (ï)Unknown substances; ([a]) Flavonoids or Carotenoids.

IDENTIFICATION OF PROLIFERATIVE ACTIVITY IN GILL CELLS

The results of Sham group and Control group show no mortality and no increase or decrease in gill cell number. It also showed no fish mortality and normal morphology. Thus, the experimental conditions not altered the number of gill cells, and confirm that BrDU is not an agent of cell proliferation.

Comparing Treated Group 2 – TG2 (exposed to BrdU and to pequi Ethanolic Extract of Steam Bark – EESB – at 20mg.L^{-1}) to Control Group (exposed only to BrdU at 30mg.L^{-1}) we observed that the gill cells in the S phase of the cell cycle, marked by BrdU (Figure 3; asterisks or dark regions) were identified using an Optical LEICA microscope.

The number of proliferating gill cells in the control group (Figure 3A) and in the fish treated with BrdU and EESB (Figure 3B) underwent statistical analysis to confirm the real effects of the pequi EESB.

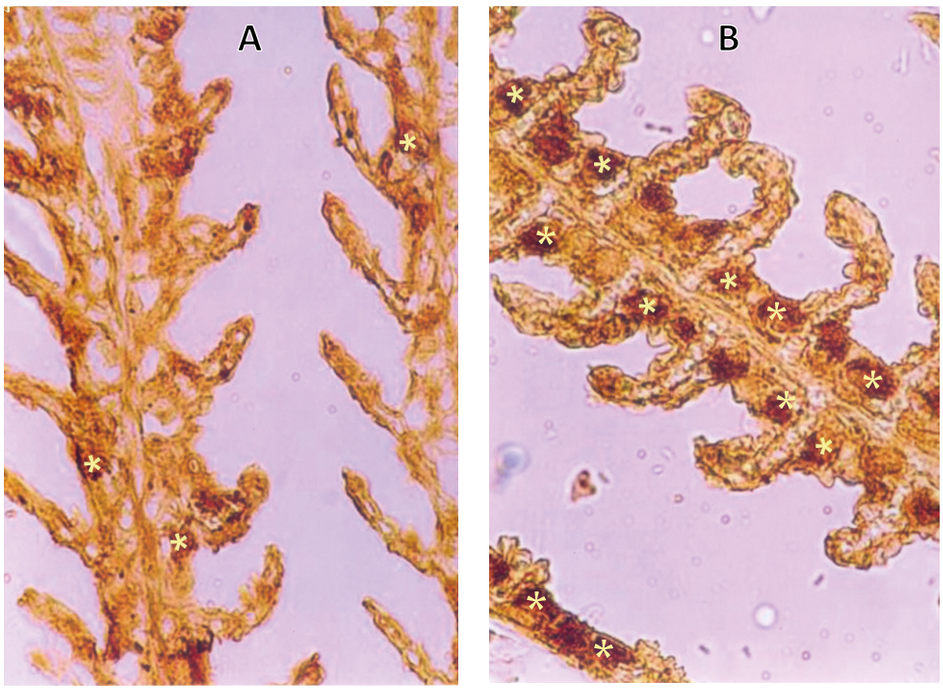

Figure 3 - Histological section (5um) of the middle of the gill filaments from guppy (Poecilia vivipara) exposed for 24h to: A) Control group (water and BrdU at 30mg.L-1); and B) Treated group (water+30mg.L-1 BrdU+20ug.L-1 Ethanolic Extract from the Steam Bark of pequi – Caryocar brasiliensis). Increase: 128,57x. (*) Asterisks indicate S phase cell cycle marked by BrdU. Base and peak did not show histological alteration.

The analysis performed showed that the mean number of cells differs at a significance level of 5% and degree of freedom equal to 1, indicating an increase in number of BrdU marked cells in TG-2 (group exposed to BrdU and to pequi Ethanolic Extract of Steam Bark – EESB – at 20 mg.L^{-1}), confirmed by the Tukey test and the analysis of variance.

Discussion

Studies of crude ethanolic extracts of pequi have demonstrated their molluscicidal, fungicidal, tripanossomicidal, insecticidal, nematicide and leshimanicidal bioactivity (Bezerra, 2002; Herzog-Soares et al., 2002; Ling et al., 2012; Passos et al., 2002; Paula Jr et al., 2006; Pereira et al., 2008; Pereira e Caetaneo, 2009; Ribeiro et al., 2012).

To investigate their effect on fish, researchers have analyzed the activity of extracts and fractions of leaf and stem bark on the gill morphology of *Poecilia vivipara*, verifying no inductive effect on cell division (Silva et al., 2002).

However, Motter et al. (2004) found that the mitotic index of epithelial gill cells of animals treated with the ethyl acetate fraction of the pequi stem bark is different from the control group in two regions of the gill filament epithelium (basal and apical). To elucidate the real effect of pequi EEL and EESB in inducing cell proliferation, in guppy gills, BrdU was used as molecular marker.

The low toxicity of BrdU was confirmed by comparing the results between Sham group (SG - only water) and Control group (CG - water + BrdU), which showed no fish mortality, and normal morphology. Thus, this comparison shows that in our experimental conditions, adding 30mg/liter of BrdU was not able to promote an increase in gill cell number, proving that BrdU is not an agent of cell proliferation.

Histopathological analysis on fish after treatment with BrdU and Ethanolic Crude Extract from the Leaves (EEL)

When comparing the Sham group (SG), Control (CG) and EEL, (water + BrdU + ethanolic leaf extract) we found an extremely toxic effect, that promoted 100% of mortality. This highly toxic effect was also observed by Balestra et al. (2011), studying the effects of medicinal plant (ethanolic leaves extracts, like "pitanga" *Eugenia uniflora*) on the gill epithelium of the guppy (*Poecilia vivipara*). These authors stated that the cytotoxicity of the ethanolic leaves extracts of "pitanga" (*E. uniflora*) is due to the presence of tannins.

Therefore, the thin layer chromatography analyses used to verify the compounds content in leaves of pequi extract, shows a largest range of compounds in the leaf ethanolic extract and fractions.

A study conducted by Carneiro (2002) in guppy gills exposed to 20 $mg.L^{-1}$ of pequi crude ethanolic leaf extract does not present fish mortality. Whereas, we found that, all the guppies died when exposed to Treated group 1 – TG1 (leaf ethanolic extract and BrdU). We assign this effect to a reaction between BrdU and bioactive compounds present in ethanolic leaf extracts, which probably promoted toxic substances arise in water solution.

According to Sabóia-Morais et al. (1996), *Poecilia vivipara* is sensitive species, and despite being euryhaline, requires care, mainly regarding pH and temperature.

The thin layer chromatography carried out to compare the aquaria water treated with BrdU and EEL (TG1), EEL ethanolic solution and BrdU aqueous solution was only performed due to the 100% fish mortality observed in the treated group (TG1) by 12 to 24 hour period.

These results (Figure 2) show a pattern of banding in treated aquaria water (TG1) corresponding to the bands of EEL ethanolic solution and BrdU aqueous solution. Moreover, there is a band present in EEL that is absent in the aquaria water of TG1.

However, it is clear that there is a class of substance present in EEL, that disappears when BrDU and water are added (TG1), because the correspondent band it is not observed in this sample (represented by white arrow in Figure 2). This disappearance may be result of an interaction or reaction of this substance with flavonoids, as the band that represents this last substance has diminished (white star in Figure 2). It is possible that this interaction rise water pH.

This situation could have afforded metabolic reactions that led to death the fishes treated with EEL and BrDU (TG1), a fact that requires specific testing to be proved.

The association of flavonoids with other substances was reported by Ratty and Das (1998) studying the effects of flavonoids on nonenzymatic lipid peroxidation, and by Santos et al. (1999), showing their interaction with natural dyes. It is possible that the reaction between substances of the leaves (EEL), for guppies, have similar chemical behavior, as described by the authors above. However, we note that additional studies must be performed to determine the chemical changes that were detected in this experiment.

Giovannini and Masella (2012) also assert the interactions existence of polyphenols with other substances. These authors report the interaction of these compounds with specific metabolic pathways and/or proteins, which regulate apoptosis processes, according to its concentration and cellular system.

Giovannini and Masella further suggest that due to the ability of polyphenols in modulate cell death, flavonoids have been proposed as chemopreventive and therapeutic agents.

Whereas flavonoids are secondary metabolites, and generally from distinct plants, we must understand that it is plausible that some groups of substances extracted from plants activate different cellular pathways. Thus, for guppies treated with ethanolic extract from the leaves (EEL), there was the promotion of proliferative activity of the gill cells. In this sense, must be understood that there is probably different activation mechanisms, depending on the cell type under consideration.

This different reaction and often opposite, in response to compounds whose well known and described effects for mammals, was also observed in studies on the involvement of beta adrenergic receptors in fish adipose tissue lipolysis. Unlike the known effect in promoting lipolysis in mammal adipose tissue, the beta adrenergic receptors inhibited it in adipose tissue of tilapia (Van Den Thillart et al., 2001; Vianen et al., 2002 e Dias-Jr, 2006).

Thus, there must be some specificity in the use of flavonoids for certain types of cells that are reactive. While other cells may be inert or even respond with opposite behavior.

Despite being proven the fact that, flavonoids interact with other substances, we did not find any paper that reports morphophysiologic effects of flavonoids, neither isolated nor combined with another substance.

Moreover, several studies reported that flavonoids exhibit pharmacological effects such as, antioxidant, anti-inflammatory, antiallergic, antimicrobial, antiviral, and activity against tumor development (Ratty e Das, 1998; Giovannini e Masella, 2012; Chen et al, 2013). In addition, Chen et al. (2013) showed that a synthetic flavonoid is capable of inhibiting cell proliferation.

Also contradicting published reports, our finding shows, as a result of a possible interaction of flavonoids, the death of the fish in the BrdU and EEL treated aquaria. This fact requires more investigation. We found no literature that reports an event like this, because the recent use of fishes for this type of study.

Histopathological analysis on fish after treatment with BrdU and ethanolic crude extract from the stem bark (EESB)

Allen & Smith (2012), Zupank et al. (2012) e Otteson et al. (2002) found that BrdU marked cells progress to the mitosis stage. The gill filament analysis of the guppies, treated with BrdU and pequi Ethanolic Extract of Steam Bark (EESB), showed an increase in the number of cells marked by BrdU in three of the regions examined, base, middle and peak. Hence, it is possible to compare and suggest that the BrdU marked cells of guppies gill filament, had entered the M phase of the cell cycle and, as a result, there was cellular proliferation in comparison with the control group. Thus, these data indicate an increase in

the number of BrdU-reactive cells, in BrdU and EESB treated fish.

By comparing the characteristics of the marked cells with previous identification studies of gill cells by cellular localization and morphologic aspects (Evans et al., 2005) it is possible to infer that the labeled cells were in the S phase of cell cycle and, were rich in mitochondria, which are known as chloride cells. This tag allows cell identification, from which was possible to perform morphometric analysis and confirm the proliferative effect of ethanolic extract of the stem bark of pequi (EESB) in the gills.

These results confirm the proliferative effect of the crude ethanolic extract from the stem bark of *pequi*, which may relate to some proliferation possibilities, such as beneficial cell proliferation, where the extract could be linked to wound healing or cell regeneration, or even neoplastic proliferation, where its action would be harmful and therefore not acceptable for animal treatment and welfare.

This study showed that ethanolic extracts from the pequi stem bark have substances that induce cell proliferation even though the markings made by BrdU refer to the S phase of the cell cycle and do not differentiate neoplastic and normal proliferation.

Although this work does not differentiate neoplastic and normal proliferation, a chronic exposure is necessary for better monitor neoplastic proliferation.

It is important to emphasize that, due to the possible applicability of these data for treatment of the water, fishes and aquaculture, the knowledge of the action of extracted substances from Cerrado biome is relevant. Also, this type of treatment, from bioactive plant extracts, is already used as a treatment of nematodes, trypanosome and leishmaniasis (Ribeiro et al., 2012; Herzog-Soares et al., 2002; Paula-Jr et al., 2006), and it becomes a new possibility for treatments in aquaculture without the use of pollutants and unspecific drugs, as well as contributing to the knowledge of another important vertebrate group.

It is important emphasize that the reactions obtained in the present study indicate that the system model (guppies) is sensitive, and produces cellular and tissue responses that can be biomonitored the use of substances extracted from plants.

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