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EVALUATION OF CYTOTOXICITY OF EXTRACT OF LEAVES AND FRUITS OF THE *Xanthium cananillesii*

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ABSTRACT

Backgrund: The poisoning by toxic plants is one of the factors that most undertake animal health and productivity. *Xanthium cavanillesii*, better known as Sheep'sburrow" or "Sheep'sthorn"is a plant that fits this profile, understudied and very dangerous.

Materials and Methods: *X. cavanillesii* samples were collected and extracts were prepared from the leaves (EL) and the stem (ES) (2% at pH 5 and 10). Assessment of cytotoxicity was measured by the ability to lyse erythrocytes, ALT dosage, HET-CAM test and histopathological evaluation.

Results: The extracts of the *X. cavanillesii* showed hemolytic activity from the concentration 1/64 (LE) and 1/32 for the FE at pH 5.0, while for the extracts at pH 10.0 cell lysis occurred from the concentration of 1/128 (LE) and 1/64for the FE. Cells treated with different concentrations of extracts of *X. cavanillesii* resulted in increased release of ALT levels. The irritation Score (IS) for leaves extract at 1/1 concentration at pH 5 was 6.68, and at pH 10 it was 8.78, whereas at 1/16 concentration at pH 5 it was 2.9 and at pH 4.05. For fruits extracts at 1/1 concentration, the irritation index was 5.89 (pH 5) and 7.04 (pH 10), and at the concentration of 1/16, at pH 5 it was 2.57 and at pH 10 it was 3.04.The result of the histopathological evaluation of ear skin incubated with these extractsshowed no microscopic lesions.

Conclusion: The pH changes in the extraction media altered the toxicological potential of the extracts, since the extracts used for the cytotoxic studies demonstrated toxic activity (pH 5.0 and pH 10.0). This result was also observed in the HET-CAM test (pH

5.0 and pH 10.0). But the toxicological potential is related to the concentration of the extracts, where from the concentration of 1/16 (both extracts) do not present irritating effect to the chorioallantoic membrane of chicken embryo.The prospects of this work refer to identification and physicochemical characterization of this compound(s) present(s) in the leaves and fruits of *X. cavanillesii* plant.

Keywords: Cytotoxicity, cattle, HET-CAM, *Xanthium cavanillesii*.

INTRODUCTION

Many factors compromise animal health, and when one speaks of productivity, poisoning by toxic plants is one of the factors that causes greater losses, both in economic factors and in relation to animal welfare, and this situation only worsens when the effects, and the actual impact of the toxin are not clarified and understood. *Xanthium cavanillesii*, better known as “Sheep'sburrow” or "Sheep'sthorn”, is a plant that fits in this profile, which is not very studied and very dangerous, is easily found in the region of Alto Uruguai Catarinense, which can lead to death of a bovine in a short time[1].

The main economic activities of the West of Santa Catarina are structured in productive chains, in which agroindustries are the determining factors of their trajectories, obeying determinations that come from the market and/or legislation of the sector. Currently, Santa Catarina is the fifth largest milk producer in Brazil and the state still has an annual growth of 10% in production. By the end of 2017 producers from Santa Catarina must reach the three billion liters. Therefore, the reduction of dairy production due to the lack of adequate management by the few could jeopardize the sustainability of rural property[2]. In this way, the objective of this project is to investigate the toxicological properties of *Xanthium cavanillesii*, fomenting discussions about the clinical implications in the dairy herd.

MATERIALS AND METHODS

Specimens of *X. cavanillesii* were collected from the forest of the region, and botanical identification was performed by exsiccate. The extracts were prepared from leaves and stems. The crude extract of the leaves (LE) was prepared from 200 g of leaves with 1000 mL of acidified water (pH 5.0) and was subjected to the extraction process by maceration in the period of 3 days. Already, the raw fruit extract (FE) was prepared with 200 g of small pieces of fruits and 1000 mL of acidified water

(pH 5.0) and proceeded in the same way as the leaf extract. This procedure wasrepeated for bothextractsat pH 10.

Evaluation of cytotoxicity

The evaluation of cytotoxicity was measured by the ability to lyse bovine erythrocytes. The free hemoglobin dosage was performed by subjecting a suspension of 2% red blood cells to different concentrations of *X. cavanillesii* extracts (1/1, ½, ¼, 1/8, 1/16, 1/32, 1/64, 1 / 128, 1/256, 1/512) at pH 5 and 10 for a period of 5 minutes incubation at room temperature. After this period, the formation of a button at the bottom of each tube was verified and, immediately afterwards, the fraction of free hemoglobin (Hemoglobin - Labtest, Minas Gerais, Brazil) was measured. As a negative control, physiological solution (NaCl 0.9%) was used and as a positive control 0.01% KCN solution. This test was performed in triplicate, similarly to the hemoglobin dosing test, the supernatant was used for alanine transaminase dosing. To the supernatant was added 1 mL of the labtest kit reagent (Minas Gerais, Brazil) and the absorbance (A) of each tube was read at 340 nm during the 1 minute interval. The concentration of alanine transaminase (ALT) was calculated according to the equation (ALT (U / L) = [Ateste x 1746]. As a negative control, a physiological solution (NaCl 0.9%) was used and as a positive control hemolyzed bovine blood at 1% and the test was performed in triplicate.

Hen´s Egg Test on the Chorioallantoic Membrane (HET-CAM)

White fertile eggs fresh Lohmann (Lohmann selected Leghorn, LSL) were used in the HET-CAM test. The eggs were kept optimized incubation conditions (temperature between 38 to 39°C and humidity between 55 and 60% at 10 days). On the 10th, the egg shell, around the airspace, was carefully removed with a rotary tool (Dremel, WI). Afterwards, it was added 0.3 ml of each substance (LE at 1/1 concentration at pH 5; LE at 1/16 concentration at pH 5;LEat 1/1 concentration at pH 10; LEat 1/16 concentration at pH 10; FE at 1/1 concentration at pH 5; FE at 1/16 concentration at pH 5;FE at 1/1 concentration at pH 10; FE at 1/16 concentration at pH 10) in each egg, respectively (negative control - 0.9% saline solution; positive control - 0.1 M NaOH solution; EL and ES). The observation of the irritant effect was observed in times of 30 seconds, 2 minutes and 5 minutes after the application of each substance. The result of the irritation score (IS) was given

according to the equation below, on a scale from 0 to 4.9 denoted nonirritant (or practically no irritation) and 5.0 to 21 denoted irritant (moderate/severe or extreme irritation)[3]:

$$IS = \left(\left(\frac{(301 - Hemorrhage\ Time)}{300}\right)x5\right) + \left(\left(\frac{(301 - Lysis\ Time)}{300}\right)x7\right) + \left(\left(\frac{(301 - Coagulation\ Time)}{300}\right)x9\right) \quad (1)$$

Determination of permeation and histopathological evaluation

Tissue samples from adult male pigs, freshly slaughtered at the Institute Federal of Santa Catarina – campus Concordia, were used to evaluate the formation of tissue damage due to the action of the *X. cavanillesii*. Pigs were slaughtered according to the rules of the Brazilian Ministry of Agriculture, respecting animal welfare (Brazil, 2013). The ear skin of pigs was used in this study. The tissues were removed within a period of 5 minutes after the slaughter; the hairs were carefully removed by an electric trimmer and transported to the laboratory in ice-cold Krebs-Hepes buffer. The skin samples were mounted in Franz diffusion cells (Logan Instrument Corp., NJ) with the diffusion area of approximately 1.75 cm^2. The epidermal side of the skin was exposed to a PBS pH 7.0 (negative control) and to 0.1 M NaOH solution (positive control) for a period of 6 hours. The *X. cavanillesii* extracts (LE and FE) in the concentration 1/16at pH 5 and 10was dissolved in a PBS pH 7.0 were used to do this experiment. The epidermal side of the skin was subjected to extract contact with this solution for a period of 6 hours. Fragments of these tissues were harvested, fixed in 10% neutral-buffered formalin, processed routinely and stained with hematoxylin and eosin (HE), and examined under light microscopy. The experiments were carried out in triplicate.

Data analysis

The significance of differences between means of the data was statistically calculated by one-way analysis of variance (ANOVA). The difference between means was considered significant $p<0.05$.

RESULTS

The hemolytic activity of *X. cavanillesii* extracts was determined by the percentage of red cell lysis. According to this study, cell lysis was performed from the 1/64 concentration of the LE and 1/32 for the FE for extracts at pH 5.0, whereas for the extracts at pH 10.0 the cell lysis occurred from the concentration 1/128 LE and 1/64 for SE. Cells treated with

different concentrations of *X. cavanillesii* extracts resulted in increased levels of ALT release (Figure 1).

The figure 2 shows the relationship between IS and the logarithms of the concentrations of the LE and FE extracts. This ratio is expressed by equation 1 as a function of the log concentration for each of the extracts tested. The irritation Score (IS) for leaves extracts at 1/1 concentration at pH 5 was 6.68, and at pH 10 it was 8.78, whereas at 1/16 concentration at pH 5 it was 2.9 and at pH 4.05. For fruits extracts at 1/1 concentration, the irritation index was 5.89 (pH 5) and 7.04 (pH 10), and at the concentration of 1/16, at pH 5 it was 2.57 and at pH 10 it was 3.04. The pH changes in the extraction media altered the toxicological potential of the extracts, since the extracts used for the cytotoxic studies demonstrated toxic activity (pH 5.0 and pH 10.0). This result was also observed in the HET-CAM test (pH 5.0 and pH 10.0).

The result of the histopathological evaluation of ear skin incubated with LE and FE extractsshowed no microscopic lesions (Figure 3).

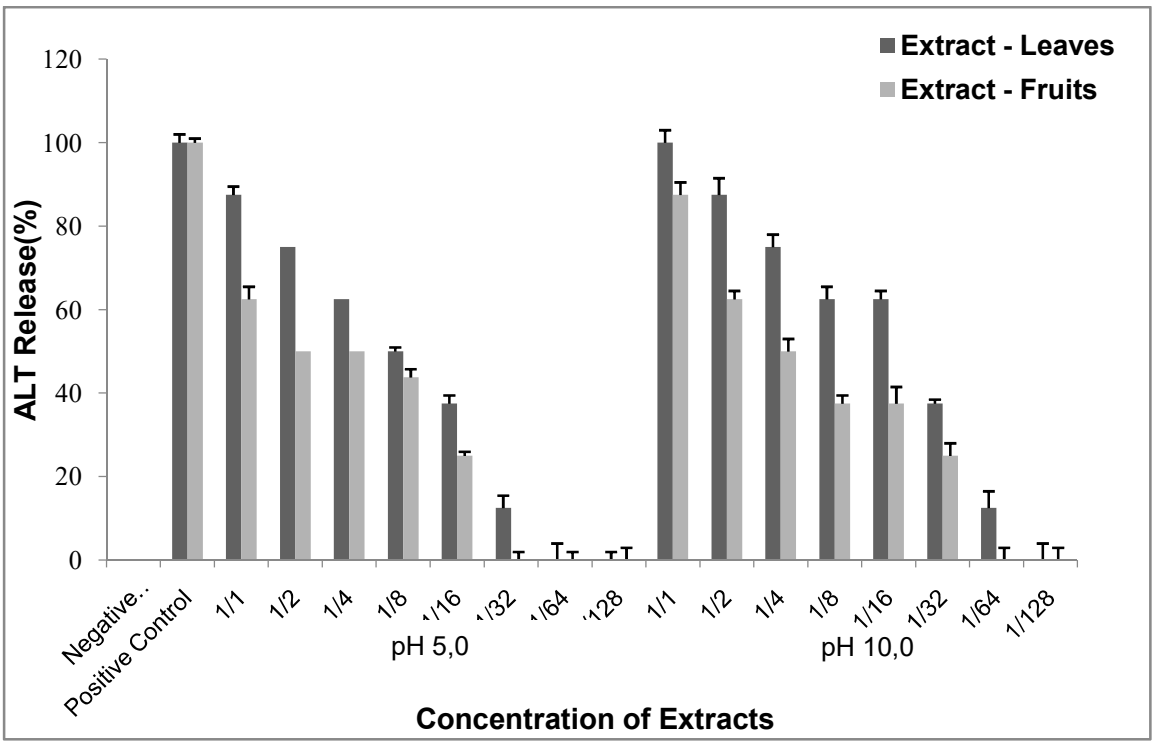

Figure 1: Evaluation of the cytotoxicity of leaves extracts and fruits extracts of *Xanthium cavanillesii* in relation to the percentage of lysed erythrocytes according to the quantification of free alanine aminotranferase (ALT). Extract of leaves of *Xanthium cavanillesii*. Extract of fruits of *Xanthium cavanillesii*.

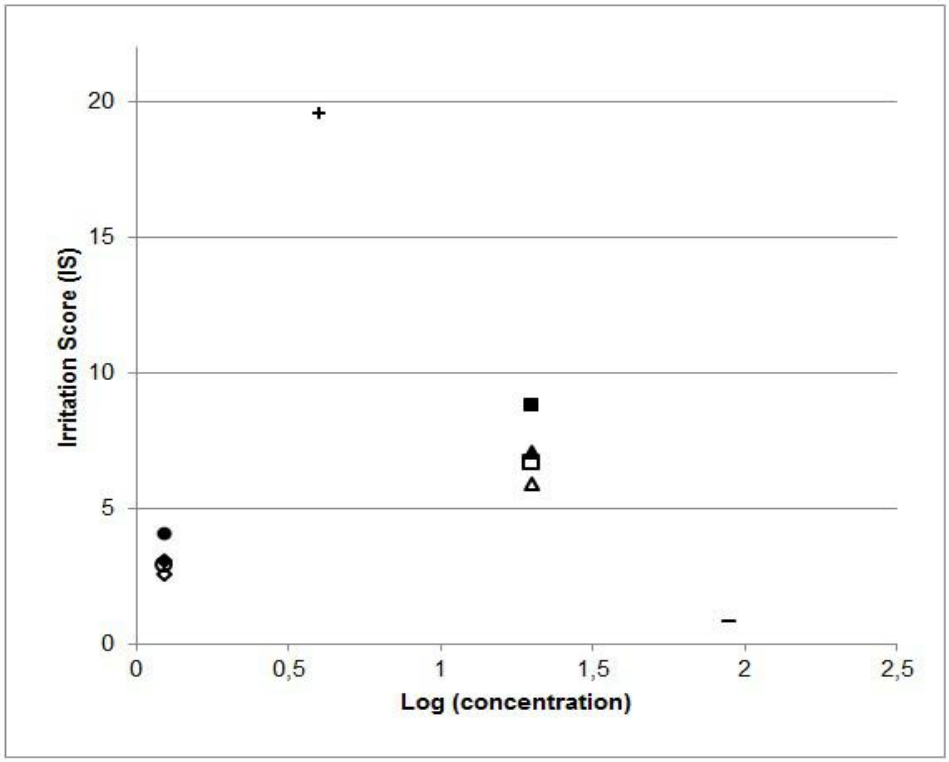

Figure 2: Dose-response relationship for *Xanthium cavanillesii* extracts: (□)leaves extract at 1/1 concentration at pH 5; (■) leaves extract at 1/1 concentration at pH 10; (Δ) fruits extract at 1/1 concentration at pH 5; (▲) fruits extract at 1/1 concentration at pH 10;(○) leaves extract at 1/16 concentration at pH 5; (●) leaves extract at 1/16 concentration at pH 10; (♦) fruits extract at 1/16 concentration at pH 5; (◊) fruits extract at 1/16 concentration at pH 10;(¯) negative control (NaCl 0,9%) and (+)positive control (NaOH 0,1 M). Each point represents an experiment (n = three eggs). The concentrations were transformed logarithmically: 0.0 to 2.0, according to the methodology used

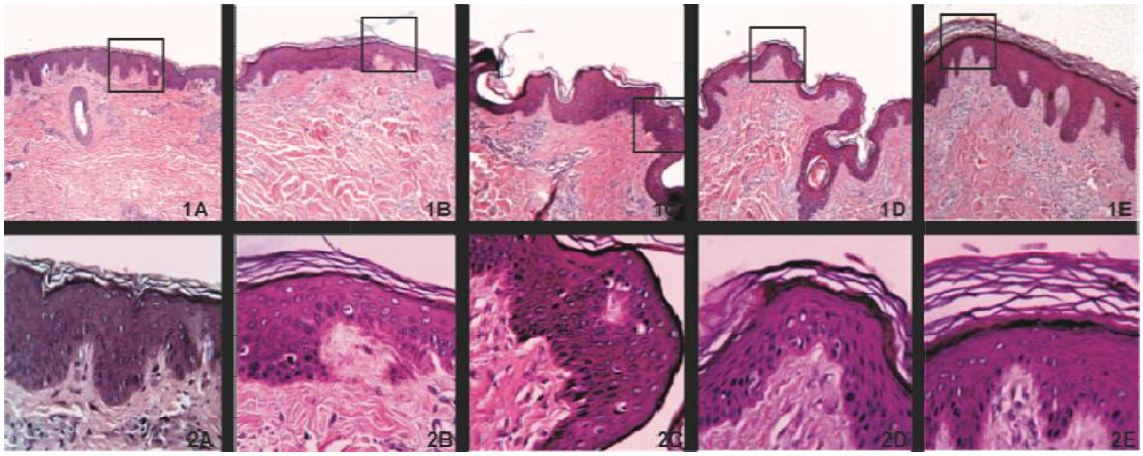

Figure 3: Histopathological evaluation of porcine cells treated with *Xanthium cavanillesii* extractand negative control at 100 and 400 times magnification. 1A. Swine epidermal cells treated with PBS pH 7.0 at 100 times magnification. 1B. Swine epidermal cells treated with leaves extract (concentration 1/16 at pH 5,0) at 100 times magnification. 1C. Swine epidermal cells treated with leaves extract (concentration 1/16 at pH 10,0) at 100 times magnification. 1D. Swine epidermal cells treated withfruits extract (concentration 1/16 at pH 5,0) at 100 times magnification. 1E. Swine epidermal cells treatedwith fruits extract (concentration 1/16 at pH 10,0) at 100 times magnification. 2A. Swine epidermal cells treated with PBS pH 7.0 at 400 times magnification. 2B. Swine epidermal cells treated with leaves extract (concentration 1/16 at pH 5,0) at 400 times magnification. 2C. Swine epidermal cells treated with leaves extract (concentration 1/16 at pH 10,0) at 400 times magnification. 2D. Swine epidermal cells treated with fruits extract (concentration 1/16 at pH 5,0) at 400 times magnification. 2E. Swine epidermal cells treated with fruits extract (concentration 1/16 at pH 10,0) at 400 times magnification.

DISCUSSION

The loss of cell membrane integrity is closely related to the concentration of extracts (LE and FE). Preliminary cytotoxicity analysis of *X. cavanillesii* crude extracts was investigated in bovine erythrocytes. Cells treated with LE and FE showed similar results in this study, in which the extent of cell damage was proportional to the concentration of the respective extracts. Thus, cell viability decreases progressively with increasing extracts concentration. Different levels of cytotoxicity may be related to the biochemical parameters of the cells involved, such as plasma membrane composition and metabolic activity, time of exposure to the toxicant, as well as the toxicity test used[4]. One of the indicators of cell death is the release of intracellular contents into the extracellular medium, and the quantification of ALT in the cell culture medium supernatant indicates cell membrane damage and consequent cell death[5]. The ALT assay therefore indicates the loss of plasma membrane integrity of the treated cells.

The toxicological potential is related to the concentration of the extracts, where from the concentration of 1/16 (both extracts) do not present irritating effect to the chorioallantoic membrane of chicken embryo. This difference of toxicological potential is due to the fact that the active compounds of plants, have different chemical characteristics in different pH ranges[6].

The absence of lesions allowed to continue the permeation studies of these extracts, in order to determine which type of tissue could be the accumulation of the same.

CONCLUSION

Cytotoxicity studies are based on the relation between dose and the chemical structure of these compounds. Thus toxicological studies using models such as HET-CAM, which allow determining the permeation profile of these compounds, and to discriminate the various levels of toxicity by means of the IS calculation. The HET-CAM is a very sensitive test to determine the toxicological parameters, in this way, the use of such methodology is shown to be acceptable, becoming an alternative to other in vivo tests[3,7,8].

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