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Safety Profile of *Caesalpinia spinosa* Aqueous Extract Tested in *Oreochromis niloticus* Toward Its Application in Dermocosmetics

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Herbal extracts have been widely used in dermocosmetics as a source of biomolecules and also as a natural claim. Fruits from *Caesalpinia spinosa* show great potential for their polyphenolic content, preservative, and film-forming features, as previously reported in specialized literature; however, the toxicity requires investigation. We explored *Oreochromis niloticus* (tilapia) in larval, alevins, and juvenile stages to evaluate the *ex vivo* and *in vivo* genotoxicity and *in vivo* acute and chronic toxicity of *C. spinosa* aqueous extract in different concentrations. Cytotoxicity, animal behavior, morphological deformities, and DNA damage were evaluated. Our results showed genotoxic effect in *ex vivo* tests, but no DNA damage in *in vivo* erythrocytes. We suggest a mechanism of cell permeability involved in the toxicity of *C. spinosa* aqueous extract. Internal validation showed the feasibility of *O. niloticus* applied for toxicity evaluation. Further studies could contribute for better understanding the uses and safety of *C. spinosa* in cosmetics and topical pharmaceutical products.

Keywords: *Caesalpinia spinosa*, *Oreochromis niloticus*, herbal extract, natural cosmetic, safety

INTRODUCTION

The largest sources of bioactive compounds in the pharmaceutical and cosmetic industries are derived from plants because of their intrinsic characteristics and presumed safety. Cultivable plants that ensure sustainability in quality and quantity and a reduced negative environmental impact are generally preferred (Lubbe and Verpoorte, 2011). *Caesalpinia spinosa* "tara" is a native species of Peru, with both wild types and those cultivated in various types of soils, from neutral to saline. These plants are resistant to drought and are able to adapt to temperature variations (Lubbe and Verpoorte, 2011). The leaves and fruits contain a high concentration of tannins, ranging from 26.4% to 60.0% (Goycochea, 2010) (water-soluble type equivalent to 55.1 g of gallotannins in 100 g of tara pods) (Chambi et al., 2013). Variations in the content of polyphenols and flavonoids with antioxidant activity have been reported according to the type of extract (aqueous or ethanolic) (Skowyra et al., 2013). Previous studies have demonstrated that the aqueous extract of tara has an IC_{50} value of antioxidants greater than that of ascorbic acid (Callohuari et al., 2017) and is able to control the degradation of hyaluronan (Valachová et al., 2014). In addition to the antioxidant

profile, *C. spinosa* extract contains active compounds for the control of gram-positive and gram-negative bacteria, established by *in vitro* (Aguilar-Galvez et al., 2014) and *in vivo* treatment of tilapia infected with *Flavobacterium columnare* (Prieto et al., 2018). From the isolation of the bioactive fraction of the extract, two compounds, methyl gallate and gallic acid, were identified as responsible for the bacterial inhibition of *Salmonella typhi*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Olmedo-Juárez et al., 2019). This evidence allowed us to consider the use of *C. spinosa* extracts in cosmetic formulations not only as a preservative, but also for inducing the formation of a protective film on the surface of human skin (film-forming effect), reducing moisture loss and skin peeling. A mixture of extracts from *Kappaphycus alvarezii* and *C. spinosa* improved the sensory characteristics and smoothness of the skin when vehiculated in a cosmetic form (de Melo and Maia Campos, 2019).

C. spinosa has also been shown to have antitumor activity when evaluated *in vitro* in tumor cells. The ethanolic extract induced changes in the morphology of the cell membrane, presence of necrotic cells, loss of mitochondrial membrane potential, nuclear fragmentation, and induction of apoptosis (Castaneda et al., 2012). The cytotoxic effect of *C. spinosa* extract was reported in cell lines with multidrug-resistant profiles, and in mice with breast cancer, a synergistic effect was observed in the reduction of neoplastic cells with the combined application of doxorubicin and *C. spinosa* extract (Sandoval et al., 2016). It favors the activation of the immune response that allows for a better prognosis in the elimination of tumor cells from mice with breast cancer (Urueña et al., 2020). Additionally, modulation of the expression of the ligand protein involved in important immune checkpoints in personalized cancer therapy was evaluated, which suggested that the *C. spinosa* extract effects are promising for the treatment of many types of cancer (Lasso et al., 2020).

In a recent study, the absence of toxicity, genotoxicity, and mutagenicity of the *C. spinosa* ethanolic extract (P2Et) was demonstrated in healthy mice through *in vivo* tests. Micronucleus values were observed even below the negative control in groups that received oral P2Et. Similarly, the Ames test showed no mutagenicity in *Salmonella typhimurium* cultures (Ballesteros-Ramírez et al., 2021). Considering this scenario with the purpose of determining the safety of the use of natural extracts as additives in cosmetic formulations, and taking into account that "tilapia" (*Oreochromis niloticus*) has a highly sensitive biological system in genotoxicity tests (Skowyra et al., 2013), we investigated the cytotoxic and genotoxic effect of the *C. spinosa* aqueous extract *ex vivo* and the absence of toxicity and genotoxicity in *in vivo* tests in healthy individuals of *O. niloticus*.

MATERIALS AND METHODS

Biological Material

The dried fruits (pods) of *C. spinosa* were collected from a crop in Chao, La Libertad, Peru (8°36′49.47″ S, 78°43′51.61″ W) in December 2020 (**Figure 1**). Botanical identification was carried out in the Herbarium Truxillense (HUT) of the National University of Trujillo (registration code N° 60715, *Caesalpinia spinosa* "tara").

Larvae, 5 to 10 days old, 15 day-old fingerlings, and 3 month-old juveniles of *O. niloticus* from the Experimental Genetics Center of the National University of Trujillo (8°6′44.66" S, 79°2′19.52" W) were used. This study was approved by the Ethics Committee under consubstantiate report number R.D.N° 051-2016-Fac.CC.BB.Oficio N° 010-2021-CE-Fac.CC.BB/UNT.

Aqueous Extraction of *C. spinosa*

The extract was prepared *via* infusion, with distilled water as the solvent. A total of 200 g of *C. spinosa* fruits (pods without removal of seeds) was introduced into 1 L of distilled water and placed in an oven at 55°C ± 1°C for 7 h. After which time, it was filtered with Whatman paper N° 1 and followed by a second filtration with a 1.0-μm pore size filter, using a vacuum pump. The extract was concentrated in a rotary evaporator at 60°C and dried in an oven at 50°C (Bouabid et al., 2018). The percent yield (% w/w) was calculated using Eq. (1) (Mohammad-Azmin and Mat-Nor, 2020).

$$\%\text{yield} = \text{weight of dry extract(g)} \times 100/\text{weight of dry fruit (g)} \quad (1)$$

Ex vivo Cytotoxicity Assay of *C. spinosa* Extract in *O. niloticus* Blood Cells

Blood was obtained from the caudal vein of the fish (0.1 ml) with a heparinized syringe and immediately mixed with fetal bovine serum (FBS) at a ratio of 1:100 μl (v/v). The previously prepared cell suspension was distributed into seven groups at a volume of 40 μl per tube. Immediately, 10 μl of *C. spinosa* aqueous extract was added to each treatment. Final concentrations were 50.0, 20.0, 10.0, 2.0, 1.0, 0.5, and 0.0 μg/μl. After 1 h of treatment, 20 μl of the sample was used to determine the percentage of cell mortality using propidium iodide stain. A fluorescence microscope (Olympus BX51 microscope, Japan, 400×) was used. The number of cells evaluated was >1,000. The percentage viability and mean lethal dose (LD_{50}) were determined. Before the test, it was necessary to standardize and achieve internal validation of the three basic processes. The viability of blood cells is required to be maintained for periods of at least 2 to 3 h, which is the time that an *ex vivo* experimental trial could last. In this regard, achieving a viability of over 90% was only possible using FBS by mixing the blood immediately after collection from the fish. In our previous tests, PBS and Hank's solutions were used; however, a mortality rate of more than 50% of blood cells was registered. Another internal validation was the sufficiency of the amount of the blood sample due to the total presence of nucleated cells. We only recommended 1 μl per 60 to 100 μl of FBS, as higher amounts of blood prevent individual cells from being evaluated. Finally, for the lysis process, lithium hydroxide was used instead of sodium hydroxide.

Ex vivo Genotoxicity Assessment of *C. spinosa* in *O. niloticus* Blood Cells

Cellular samples of peripheral blood from the caudal vein of *O. niloticus* were used. They were suspended in FBS and exposed

FIGURE 1 | Crops where the dried fruits of *Caesalpinia spinosa* were collected, on December 5, 2020.

to *C. spinosa* aqueous extract concentrations of 10.0, 2.0, and 1.0 μg/μl, as well as negative (FBS) and positive (cyclophosphamide 1.25 μg/ml) controls. For this test, only concentrations with a viability >90% were considered.

Comet Test

Forty microliters of each diluted sample (1.0 μl in 100 μl of FBS) was suspended in 95 μl of low melting agarose (LMPA) at a final concentration of 0.7% and immediately placed on a previously impregnated thin layer on a slide and incubated at 4°C for 15 min. Cell lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% Triton X-100, 10% DMSO, and pH 10, adjusted with lithium hydroxide) was continued for 1 h, followed by alkaline electrophoresis (pH > 13.5 at 20 V and 300 mA for 30 min at 4°C in a buffer of 1 mM Na_2EDTA and 0.3 M NaOH). After neutralization by rinsing three times with 0.4 M Tris buffer at pH 7.5, the slides were stained with Sybr Gold and observed under a fluorescence microscope (Olympus BX51 microscope, Japan, 400×) (Hussain et al., 2018; Bivehed et al., 2020).

In vivo Acute Toxicity Test and Chronic Toxicity Test of *C. spinosa* in *O. niloticus*

Tilapia larvae's acute toxicity assay (Capela et al., 2019). Tilapia larvae were obtained from the oral cavity of females and randomly distributed into fish incubators (∼300 individuals per treatment). Each incubator was maintained with a continuous aerated water flow at 25 ± 1°C. After 48 h of adaptation, the experiment began with four experimental groups: positive control (cyclophosphamide, 1.25 μg/ml) and

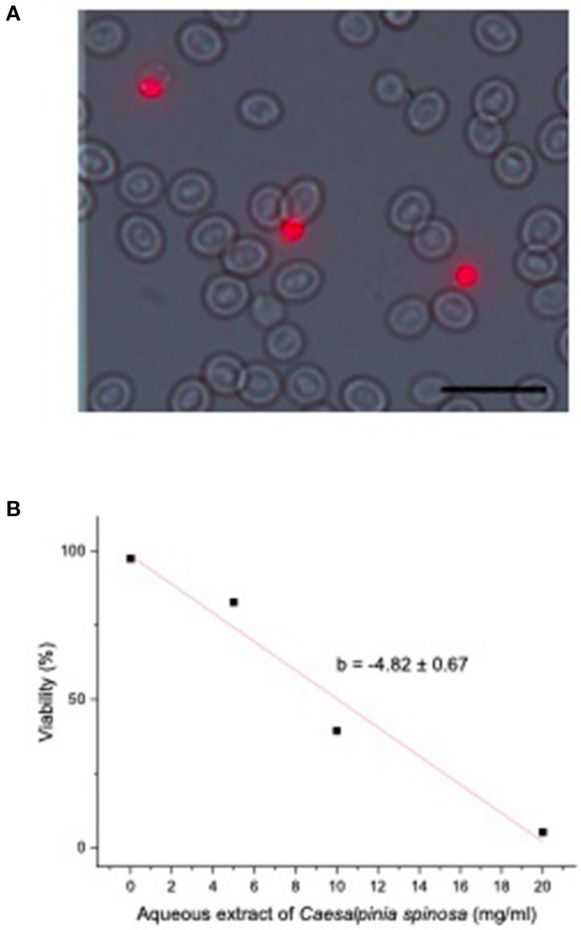

FIGURE 2 | (A) Peripheral blood erythrocytes from *Oreochromis niloticus* stained with propidium iodide. Dead cells can be recognized as red nuclei observed through fluorescence microscope (Olympus B51), 40×. Bar: 25 μm. **(B)** Viability *ex vivo* of erythrocytes from *O. niloticus* exposed to *Caesalpinia spinosa* aqueous extract, DL_{50} = 10.50 mg/ml.

three concentrations of *C. spinosa* extract (0.00, 0.02, and 0.04 mg/ml). Water exchange was performed while maintaining the treatment conditions. After 92 h, the final count of the survivors was determined and expressed as a percentage. Morphological deformities were also identified.

Tilapia Alevin's Acute and Chronic Toxicity Assay

Fifteen-day-old tilapia alevins, with an average weight of 0.09 ± 0.01 g and an average body length of 1.68 ± 0.15 cm, were used to evaluate *C. spinosa* toxicity. The alevins were distributed in groups of 100 individuals in each aquarium containing 260 L of fish culture water. The aqueous *C. spinosa* extract was added in quantities to obtain final concentrations of 0.0, 0.04, and 0.08 mg/ml. Acute toxicity evaluation was performed after 96 h of continuous treatment, the exposure time based on those established to assess the toxicity of a substance in tilapia and chronic toxicity after 25 days (OECD Guideline for the testing of chemical, fish, juvenile growth test = 200) (OECD Guideline for the testing of chemical, 2018). After 92 h and 25 days, the survivors were counted and expressed as percentages.

Tilapia Adult'S Acute Toxicity Assay

The 2.5-month-old juvenile fish, with an average weight of 48.01 ± 10.03 g and an average length of 14.17 ± 1.68 cm, were distributed in aquariums with a capacity of 260 L of water. Ten individuals per group were included in each treatment. After 48 h of adaptation, the treatments were started with the *C. spinosa* extract at concentrations of 0.0, 0.04, and 0.08 mg/ml and a positive control (cyclophosphamide 5.0 mg/ml). After 96 h of treatment, the survival of the individuals was evaluated.

In vivo Genotoxicity Evaluation of the *C. spinosa* Aqueous Extract in *O. niloticus*

After 96 h, the fish treated with the *C. spinosa* extract were anesthetized with clove oil and 0.1 ml of blood was extracted from the caudal vein.

Micronucleus Test

Two microliters of blood was collected from each fish and cell spreading was performed on slides, allowed to dry at room temperature, fixed with 96% alcohol for 10 min, and stained with 2% Giemsa in phosphate buffer for 30 min (Obiakor et al., 2014). Two thousand erythrocytes with or without micronuclei were counted on an Olympus Microscope BX41 at 1000×. The criterion to consider the presence of micronuclei was the observation of a rounded body without connection to the main nucleus and with the same coloration characteristics (Fenech et al., 2003).

Comet Test

One microliter of blood was diluted in 100 μl of FBS, and 40 μl was used for the comet assay as described above. The tail moment and percentage DNA tail were considered genotoxicity parameters using CometScore Software.

Data Processing

A data fit was performed using the normality test and variance homogeneity test between treatments. The data were expressed as the mean values of three independent experiments. Comparison of means between treatments was performed using one-way ANOVA with an additional Tukey test. Linear regression analysis was used to estimate the median lethal dose of the viability percentage of erythrocytes (*ex vivo*), and the results were considered statistically significant at $p < 0.05$, using Origin Pro 2020 Software.

RESULTS

Extraction Yield

The yield percentage of *C. spinosa* extract was 14.93 ± 0.31. One thousand milliliters of water was used as the solvent. After 7 h of extraction at 55°C, 800 ml of liquid extract and 3.15 g/ml of dry extract were obtained (**Table 1**).

TABLE 1 | Percentage yield of *Caesalpinia spinosa* aqueous extract of dried fruits.

n	Fruit weight (w) (g)	Dry weight (w) (g)	% Yield (g) (w/w)	% Yield (mean, g/g) ± SD
1	200	29.33	14.67	14.93 ± 0.31
2	200	30.40	15.20	
3	200	30.40	15.20	
4	200	29.33	14.67	

*Number of replicas (n = 4). % yield = weight of dry extract (g) * 100/weight of dry fruit (g).*

Ex vivo Cytotoxicity and Genotoxicity Tests of *C. spinosa* in *O. niloticus* Blood Cells

After standardization of procedures for *in vivo* and *ex vivo* toxicity and genotoxicity tests with samples of *O. niloticus* erythrocytes, it was possible to clearly differentiate dead and living cells up to 3 h outside the body (**Figure 2A**). The LD_{50} was estimated at 10.50 mg/ml and the slope (b) was -4.82 ± 0.67 mg/ml ($p < 0.05$), with an adjusted correlation of 0.94 (**Figure 2B**).

Toxicity was demonstrated in *O. niloticus* erythrocytes. The lethality caused by the extract induced the formation of cell clusters rather than a homogeneous dispersion, as observed for the negative and positive controls.

Genetic damage in the blood cells of *O. niloticus* outside the individual provoked by the *C. spinosa* extract was evident, as demonstrated by the comet test. In **Figure 3**, the differences were noticed after observing the effects of the *C. spinosa* aqueous extract after 1 h of treatment. The comet tail lengths visibly increased at a concentration of 5.0 mg/ml in comparison with the lowest concentration, and even with those of the positive control, which indicated a clastogenic effect. The mean and median values of percentage DNA tail and tail moment were significantly higher than those of the negative control ($p < 0.05$) (**Table 2**).

In vivo Acute Toxicity Test and Chronic Toxicity Test of *C. spinosa* in *O. Niloticus*

The results of the *in vivo* tests were distinct. Toxicity tests were carried out at the level of larvae (∼5–10 days old), alevins (15 days old), and juveniles (3 months old). In all cases, there were no deaths, behavioral changes that would indicate stress conditions, or morphological alterations of the body structure suggesting malformations were observed.

The larvae exposed to concentrations of 0.02 and 0.04 mg/ml of the *C. spinosa* aqueous extract showed similar reactions to the negative control. After 96 h of treatment, there was no mortality among the specimens, and no morphological alterations were observed; however, cyclophosphamide application caused the presence of individuals with two heads, deformations in the yolk sac, and spine deformity, at frequencies of 0.67%, 2.34%, and 1.34%, respectively (**Figure 4**).

The percentage of surviving fingerlings exposed to the *C. spinosa* aqueous extract for both 96 h and 15 days was 100%. The alevins showed physical appearance of healthy fish with normal swimming group movements (shoal behavior), evident response to noise stimulation, and normal food consumption, as well as complete fins, body color, bright eyes, and transparent cornea.

The unusual presence of the approach of a video camera near the aquarium stimulated changes in the normal movements of the fish. In the negative control group, alterations were observed in the swimming of the fish in different directions, mainly toward one end of the aquarium, creating a partial dispersive confinement (**Figure 5A**). However, in the fish exposed to the *C. spinosa* extract, a confinement was generated mainly in one end of the aquarium (**Figure 5C**), followed by a recovery of the swim toward a single direction (**Figures 5B,D**).

The micronuclei frequency in *O. niloticus* erythrocytes, considering the effect of the *C. spinosa* aqueous extract, was similar to that of the negative control. In contrast, micronuclei frequencies in erythrocytes of individuals exposed to cyclophosphamide were clearly increased with nuclear abnormalities, nuclear buds, and segmentations, among others (**Figure 6**).

Similarly, in the comet assay, there were no differences among treatments with the *C. spinosa* aqueous extract evaluated through DNA parameters, tail percentage, and tail moment ($p > 0.05$) (**Table 2**).

DISCUSSION

Tannins from *C. spinosa* can be extracted with water alone or with water mixed with other solvents, varying the temperature from 4°C to 100°C, as well as extraction times (Fenech et al., 2003). Among these processes, extraction with water prevails due to the greater amount of flavonoids and gallic acid present compared to the ethanolic extraction at 4°C (Skowyra et al., 2013). The presence of a high content of flavonoids and gallic acid in the aqueous extract obtained with water at 55°C for 7 h can be assumed.

The *safety of C. spinosa* extract is essential for cosmetic and/or pharmaceutical applications and, to evaluate its toxic and genotoxic activities, *O. niloticus* constitutes an ideal species as a biological model for *ex vivo* and *in vivo* studies because of its convenience of handling, low cost, and, in particular, its high sensitivity to xenobiotic agents.

Ex vivo Cytotoxicity and Genotoxicity Assay of *C. spinosa* in *O. niloticus* Blood Cells

For *ex vivo* testing of the cytotoxic and genotoxic effects of *C. spinosa* aqueous extract on tilapia peripheral blood erythrocytes (caudate vein), it is essential to preserve the blood cells. We concurred with Ramsdorf et al. (2009) in the use of FBS as an adequate preservative. Erythrocyte viability was maintained for 3 h at room temperature (24°C ± 2°C) in percentages >90%. With the PBS and Hank's solutions, we obtained >50% dead cells, normally unfeasible for the comet assay, which is generally not recommended for use in the preservation of tilapia blood samples.

Toxicity and DNA damage due to the effect of *C. spinosa* extract on isolated erythrocytes from peripheral blood of *O.*

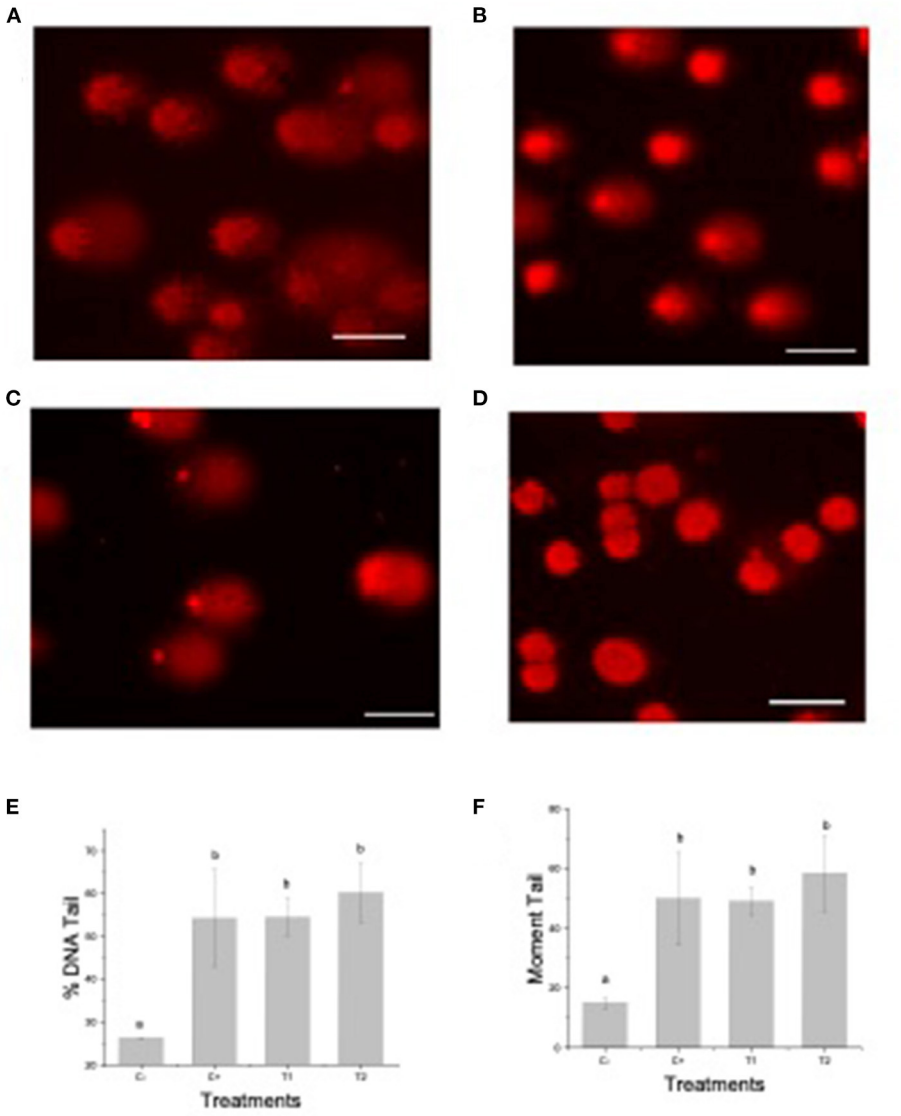

FIGURE 3 | *Ex vivo* testing of *Oreochromis niloticus* erythrocytes by the comet assay. **(A)** *Caesalpinia spinosa* 5.0 mg/ml. **(B)** *C. spinosa* 2.5 mg/ml. **(C)** Positive control (cyclophosphamide 1.25 μg/ml). **(D)** Negative control. Bar 50 μm. **(E)** %DNA Tail and **(F)** Moment Tail (C- Negative control; C+ Positive control; T1 and T2, *C. spinosa* 2.5 and 5.0 mg/ml, respectively). Same letters mean no significant differences.

niloticus were evident in all the experimental tests performed on samples from the same and different individuals. No *ex vivo* or similar tests have been reported in other cell types; however, the effects of *C. spinosa* extract on tumor cell lines in *in vitro* tests have been documented (Castaneda et al., 2012; Sandoval et al., 2016; Urueña et al., 2020).

An important investigation is the immunomodulatory activity of *C. spinosa* in a culture of human dendritic cells, which revealed increased levels of proteins and mRNA of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12p70, and TNF-α (Santander et al., 2011). The functional change of the cells would make them permeable to the compounds from the *C. spinosa* extract, causing toxicity and DNA damage. *Ex vivo* and *in vitro* cytotoxicity and genotoxicity tests require conditions similar to those *in vivo*, which cannot be fully imitated. Therefore, changes in cells to artificial environments modify their functional activity (Tice et al., 2000). This situation was demonstrated in bacterial cells when cultivation under anoxic conditions increased the membrane potential and the propidium iodide dye entered the intracellular space, which would not occur in *in vivo* conditions (Kirchhoff and Cypionka, 2017).

In vivo Acute Toxicity Test and Chronic Toxicity Test of *C. spinosa* in *O. niloticus*

The contrasting results from cytotoxicity and genotoxicity in the *ex vivo* tests *vs.* the absence of toxicity in the *in vivo* tests could be explained by the complexity and sensitivity of the cell structure,

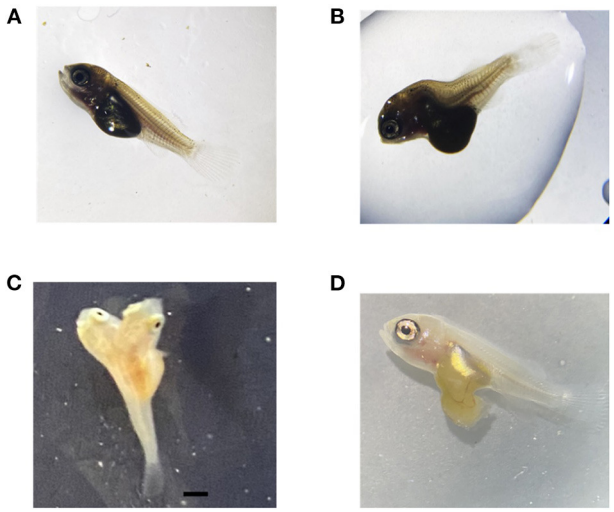

FIGURE 4 | *Oreochromis niloticus* larvae. **(A)** Normal, exposed to *Caesalpinia spinosa* aqueous extract. **(B–D)** Positive control (cyclophosphamide 1.25 μg/ml), spinal malformation, larvae with two heads, and malformed yolk sac, respectively (Stereoscope MOTIC, SMZ-168, China,1.5×, View a and b with backlight, view c and d with light outside). Bar: 1 mm.

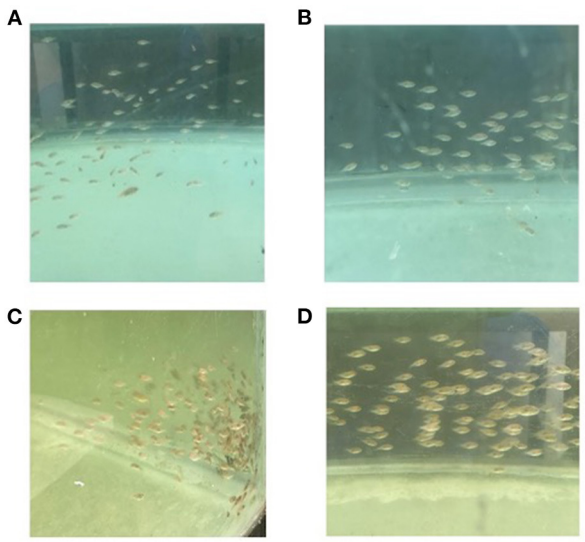

FIGURE 5 | Group behavior of tilapia *Oreochromis niloticus* alevins in aquarium. **(A, B)**. Negative control (culture water). **(C, D)** Treatment with *Caesalpinia spinosa* aqueous extract.

TABLE 2 | %DNA Tail and Moment Tail in *Oreochromis niloticus* erythrocytes treated with *C. spinosa* extract, cyclophosphamide (positive control), and fish culture water (negative control).

Treatments	%DNA Tail	Moment Tail
Negative Control	23.58 ± 10.49^{a}	8.29 ± 6.01^{a}
C. spinosa aqueous extract (0.04 mg/ml)	20.38 ± 1.53^{a}	5.85 ± 1.84^{a}
C. spinosa aqueous extract (0.08 mg/ml)	26.14 ± 5.10^{a}	8.17 ± 4.40^{a}
Positive Control	31.20 ± 3.45^{b}	18.57 ± 2.99^{b}

Different letters in the same column show statistical differences ($p < 0.05$).

which refers to the semi-permeability of the cell membrane. The mechanisms for entry and exit regulating the existing structurally designed biomolecules from the plasma membrane have not been fully explained (Bozelli and Epand, 2020). Cellular differences vs. chemical signaling are other characteristics that confer differences according to the tissue in which they are found and the function they perform. Any change in genetic regulation or changes in its functionality *in situ* or *ex situ* will generate alterations in membrane permeability, as in the case of quantitative transmembrane glycoprotein changes in cancer cell membranes (Fu et al., 2019).

Although the most susceptible stages of the fish life cycle are the embryonic and larval, due to the replication and transcription cycles that regulate the organogenesis processes (Sfakianakis et al., 2015), the larvae exposed to *C. spinosa* aqueous extract (0.02 and 0.04 mg/ml) showed healthy development, supported by the determined parameters. The larval development speed was not affected by any of the treatments with the *C. spinosa* aqueous extract. The positive control (cyclophosphamide) had no alteration in viability due to the very low concentration, although visible malformations were evident in the larval body structure. The individuals with abnormalities, as well as a minority group with alterations in swimming behavior remained in the lower part of the incubator, denoted by difficulty in swimming or delay in development progress (Sarikaya and Selvi, 2005). The indicators of changes in tilapia larval development processes in the positive control were consistent with the reports of exposure to genotoxic contaminants (Mao et al., 2020). Behavioral changes are the most sensitive indication of potential toxic effects (Sarikaya and Selvi, 2005), and according to our results, we have confirmed the absence of damage.

Fingerlings (10–15 days post-larvae) exposed to the *C. spinosa* extract had visibly normal health. To consider the state of visible health of the fish or situations that cause stress in the rearing environment, the parameters to be considered are morphology or physical appearance, and behavior in swimming activity (Martins et al., 2012). Conducting an environmental disturbance either by distributing food or confinement (Sadoul et al., 2014), as in this case, induced a reaction by the fish to the sudden presence of a video recording. Fish swimming activity in the water treated with the *C. spinosa* extract indicated that the fish were not toxically affected. Faced with the sudden presence, by recording or feeding, the fish had a normal reaction. In both cases, fish escaped toward one end of the aquarium. In the control, the escape was partially dispersive, and in the treatment with *C. spinosa* extract, the escape was in a group followed by a rapid recovery and swimming of the group in one direction. This treatment did not have a stressful effect or caused any death of the alevins after 4 and 25 days of continuous treatment. Another peculiarity of this treatment was the improvement of water quality by precipitating algae or residues of uneaten food.

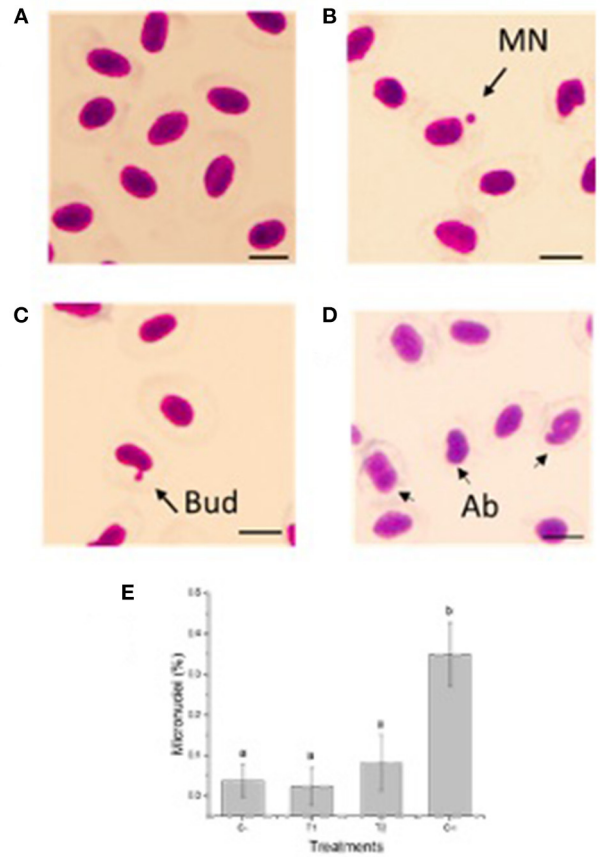

FIGURE 6 | *Oreochromis niloticus* erythrocytes stained with Giemsa observed under an Olympus Microscope, BX41, Japan, 1000×. **(A)** Normal erythrocytes. **(B)** Micronucleus (MN). **(C)** Bud: Nuclear Bud. **(D)**. Ab: nuclear abnormalities. Bar: 20 μm. **(E)** Micronuclei (%), same letters mean no significant differences (Tukey test: $p < 0.05$).

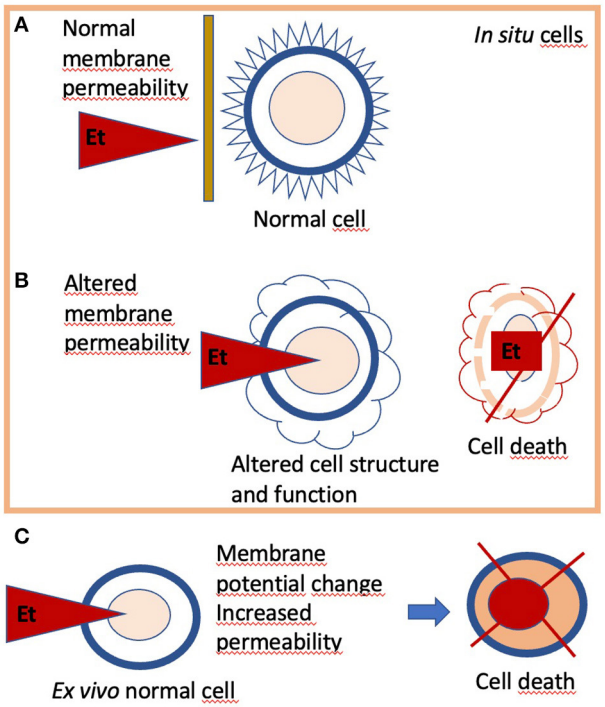

FIGURE 7 | Proposal for the action of the aqueous extract of *Caesalpinia spinosa* (Et). An Et compound has cytotoxic and genotoxic action. The limiting action of this effect is that a normal cell is not permeable for the entry of Et and is therefore not affected **(A)**. However, if the structure of the membrane is modified, as occurs in a neoplastic cell, Et will enter and cause cell death **(B)**. In the case of *ex vivo* or *in vitro* tests, the cells being outside the individual will change their cellular permeability; the Et will enter the intercellular space and produce cell death, according to the dependent dose, as demonstrated in this research and previous reports of *in vitro* tests **(C)**.

Juvenile tilapia are often used as biological models in genotoxic assays with reliable results because of their nucleated erythrocytes, the ability to work with a small amount of sample, and their compatibility with international ethical standards (Brendler-Schwaab et al., 2005). Among the parameters that determine genotoxicity, the working group of the 6th International Workshop on Genotoxicity Testing (IWGT) held in Foz de Iguazú, Brazil, 2013 (Speit et al., 2015) concluded that the combination/integration of the *in vivo* MN assay and the *in vivo* comet assay through estimates of tail intensity (percentage DNA tail) and tail timing (MT) are adequate for detecting DNA-damaging agents (Tice et al., 2000).

As in the previous stages of the tilapia life cycle, the results in the juveniles were similar, with no effect on the viability or well-being of the fish. In the positive control, the presence of micronuclei, nuclear buds, and nuclear abnormalities were evident at significant frequencies with respect to the treatments with the *C. spinosa* extract. According to the specialized literature, the *C. spinosa* ethanolic extract obtained from the pods administered orally (nasogastric tube) to mice, at concentrations of 500 to 2,000 mg/kg of body weight, did not differ from the negative control. Similarly, the Ames test demonstrated the absence of mutations in the *C. spinosa* ethanolic extract (Ballesteros-Ramírez et al., 2021).

From these tests, we deduced that there was a bioactive compound or a mixture of compounds in the *C. spinosa* aqueous extract that had toxic and genotoxic activities. If such compounds cross the membrane, due to changes in the membrane potential or other mechanisms that alter its permeability, as is the case with tumor cells, the *C. spinosa* extract, upon entering the intracellular environment, may induce apoptosis and cell death (**Figure 7**). This proposal could be explained by the results of Gomez-Cadena et al. (2016), Prieto et al. (2018), who reported a reduction in tumors in mice treated with the *C. spinosa* ethanolic extract.

The complexity of the active compounds present in the aqueous and ethanolic extracts of *C. spinosa* pods remains unresolved. In general, polyphenols, flavonoids, tannic acids, and gallic acids (Aguilar-Galvez et al., 2014; Mu et al., 2016), along with 10 types of sesquiterpenoids, have been identified in the extract of mature pods, including Caesalpinin A(1) (Mu et al., 2016) in extracts of immature pods, galactomannan (polysaccharides) (Santander et al., 2011; Skowyra et al., 2013) in

seed endosperm, and diterpenes in extracts of leaves (He et al., 2015). From our results, it can be inferred that as long as the structure and function of normal cells were maintained, there was no risk of DNA damage due to the active compounds from *C. spinosa* aqueous extract. Therefore, according to the properties cited in previous studies, the extract of *C. spinosa* pods could potentially be considered safe for use in dermocosmetic products.

This study was restricted to the cytotoxic and genotoxic evaluation of cells from *O. niloticus* in *ex vivo* and *in vivo* tests of *C. spinosa* aqueous extract. It is important to conduct further studies on the characterization of the extract constituents and to perform independent tests of the active ingredients.

CONCLUSIONS

In conclusion, the *C. spinosa* aqueous extract exhibited toxicity and genotoxic activity that acted on *ex vivo* exposed cells, as demonstrated by the blood cells obtained from the peripheral blood of *O. niloticus*. In contrast, in the *in vivo* evaluation, the aqueous extract did not cause mortality, or phenotypic changes in morphology or behavior evaluated at different stages of the biological cycle (larvae, fingerlings, and juveniles). Similarly, the absence of genotoxicity was demonstrated by the micronucleus tests and the comet assay of blood samples from individuals exposed to the aqueous extract continuously for 96 h. Further research is required to consider the characteristics of the compounds present in the *C. spinosa* extract and the mechanisms of cytotoxic and genotoxic action in cells *ex vivo* and *in vitro*. The reported properties in specialized literature and the safety profile of *C. spinosa* aqueous extract potentiate it as a resource to be considered for inclusion in dermocosmetic and topical pharmaceutical products.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by 010-2021-CE-Fac.CC.BB/UNT, (Universidad Nacional de Trujillo).

AUTHOR CONTRIBUTIONS

ZP and AB: conceptualization. DS, LS-T, NR-P, and MA: methodology. AB, DS, CA, and AC: formal analysis. ZP, AB, and DS: investigation. ZP and AB: writing—original draft preparation. AB and RM: writing—review and editing. ZP and CA: supervision and project administration. ZP and AB: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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