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Chemistry

Evaluating cytotoxicity and genotoxicity of oil extracted from visceral fat of *Caiman yacare* (Daudin, 1802) in Chinese hamster lung fibroblast *in vitro*

Avaliação da Citotoxicidade e genotoxicidade do óleo extraído da gordura visceral do *Caiman yacare* (Daudin, 1802) em fibroblastos pulmonares de hamster chinês *in vitro*

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ABSTRACT

In previous studies, the oil extracted from the visceral fat of *Caiman yacare* (Daudin, 1802) demonstrated a wound-healing effect on the skin of Wistar rats. To enhance our knowledge about the mechanism underlying this effect, we analyzed the oil's toxicological potential *in vitro*. Cytotoxicity, genotoxicity, pro-oxidant, and antioxidant activities were evaluated in a V79-4 cell line. The oil was obtained using the Soxhlet method, and the proportions of the fatty acid profile were previously identified as 43.74 % saturated and 34.65 % unsaturated fatty acids. Protocol 487 of the Organization for Economic Co-operation and Development (OECD) was employed for cell line selection and concentrations. Cytotoxicity was determined using the MTT assay and clonogenic survival. Pro-oxidant and antioxidant activities were analyzed using flow cytometry. Genotoxicity was evaluated using comet and micronucleus assays. The oil did not demonstrate cytotoxicity up to a concentration of 500 µg/mL. At concentrations of 250 and 500 µg/mL, the oil exerted a protective effect against oxidative stress and showed genotoxic effects only at the highest concentration (2000 µg/mL). Like other oils of interest for human health, the oil extracted from the visceral fat of *C. yacare* demonstrated low toxicological potential *in vitro*.

Keywords: Antioxidant; DNA damage; Fatty acids; Oxidative stress

RESUMO

Em estudos anteriores, o óleo extraído da gordura visceral do *Caiman yacare* (Daudin, 1802) demonstrou efeito cicatrizante na pele de ratos Wistar. Para aprimorar nosso conhecimento sobre o mecanismo

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subjacente a esse efeito, analisamos o potencial toxicológico do óleo *in vitro*. A citotoxicidade, genotoxicidade, atividades pró-oxidantes e antioxidantes foram avaliadas em uma linhagem celular V79-4. O óleo foi obtido pelo método Soxhlet, e as proporções do perfil de ácidos graxos foram previamente identificadas como 43,74% de ácidos graxos saturados e 34,65% de ácidos graxos insaturados. O Protocolo 487 da Organização para a Cooperação e Desenvolvimento Econômico (OCDE) foi empregado para a seleção da linhagem celular e das concentrações. A citotoxicidade foi determinada pelo ensaio MTT e sobrevivência clonogênica. As atividades pró-oxidantes e antioxidantes foram analisadas por citometria de fluxo. A genotoxicidade foi avaliada por meio dos ensaios de cometa e micronúcleo. O óleo não demonstrou citotoxicidade até uma concentração de 500 µg/mL. Nas concentrações de 250 e 500 µg/mL, o óleo exerceu um efeito protetor contra estresse oxidativo e mostrou efeitos genotóxicos apenas na concentração mais alta (2000 µg/mL). Assim como outros óleos de interesse para a saúde humana, o óleo extraído da gordura visceral do *C. yacare* demonstrou baixo potencial toxicológico *in vitro.*

Palavras-chave: Antioxidante; Dano no DNA; Ácidos graxos; Estresse oxidativo

1 INTRODUCTION

Caiman yacare is a reptilian species found in the Brazilian Pantanal, with high economic value owing to its meat and leather (Vicente-Neto et al., 2010). However, the growing awareness of sustainability issues has led consumers to seek products with the lowest environmental impact (Kumar et al., 2021). Captive management of *C. yacare* using the ranching method meets this requirement by monitoring the area and conservation of the local biome (Campos et al., 2020).

However, approximately 2.65 kg of viscera are discarded per animal during their slaughter (Romanelli & Schmidt, 2003). This wasted viscera include tissues and organs rich in fats, which are used in the production of cosmeceuticals and nutraceuticals Shima-Prydon & Camacho-Barret, 2007). In Brazil, the northeastern and traditional communities in the Amazon, especially indigenous people, frequently use crocodilian oils and fats as a food source and for the treatment of diseases (Abrão et al., 2021; Mishra et al., 2020). Crocodilian fats and oils are used in the treatment of wounds and infectious diseases, and as an anti-inflammatory agent (Alves et al., 2017).

The wound healing property of oil extracted from visceral fat of *C. yacare* (hereafter referred to as 'CVO') and *Crocodylus siamensis* was demonstrated in a rat

excisional wound model (Azevedo et al., 2020) and in rat burns, respectively (Li et al., 2021). Evidence has indicated the functional and chemical similarity between oils from different crocodilian species, but safety data is only available for *C. siamensis* oil. The oil from this species showed no toxicity in orally supplemented rats (Praduptong et al., 2018), did not decrease the cell viability of macrophages, and demonstrated a protective effect against damage caused by oxidative stress on macrophage DNA (Ngernjan et al., 2022).

In vitro cytotoxic and genotoxic assays are required by regulatory agencies to determine an effective and safe therapeutic range, before animal testing can be conducted (Kramer et al., 2007). Therefore, due to the central role played by DNA in the homeostasis of living organisms, an in vitro cytotoxic and genotoxic evaluation becomes indispensable in the development of wound healing products.

In this study, the in vitro cytotoxicity and genotoxicity of oil extracted from visceral fat of *Caiman yacare* (CVO) were investigated, using the V79-4 cell line of Chinese hamster lung fibroblasts.

2 MATERIALS AND METHODS

2.1 Drugs and reagents

All the following reagents were purchased from Sigma-Aldrich: DCFH-DA (Catalog: D6883, 4091-99-0), Dimethyl sulfoxide (Catalog: D2650, CAS: 67-68-5), Hydrochloride doxorubicin D1515, CAS: 25316-9), N-acetylcysteine (Catalog: A9165-5G, CAS: 616-91-1), Dulbecco's Modified Eagle's Medium (DMEM) - Low glucose without phenol red, (Catalog: D2902), Penicillin G sodium salt (Catalog: P3032-10MU, CAS: 69-57-8), Streptomycin sulfate salt (CAS: 3810-74-0) and MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) (Catalog: M5655-500MG, CAS: 298-93-1). Ciprofloxacin hydrochloride was obtained from Corning (61-277-RG). Hydrogen peroxide was purchased from Supelco (Catalog: HX0636, CAS: 7722-84-1), and formamidopyrimidine

[fapy]-DNA glycosylase (Fpg) enzyme (M0240S) from New England BioLabs. DAPI (4',6-Diamidino-2-henylindole, dihydrochloride) was obtained from Invitrogen (Catalog: D1306, CAS:28718-90-3).

2.2 Ethical statement

This study was conducted in accordance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA), resolution nº. 56, of October 5, 2022, which recognizes alternative methods for the use of animals in research activities in Brazil. We also followed the safety criteria recommended by the National Health Surveillance Agency (ANVISA) and the Organization for Economic Co-operation and Development (OECD).

2.3 Sample preparation

Visceral fats were collected from the *C. yacare* waste produced by zootechnics; the oil was then extracted and the fatty acid profile was characterized as previously described (Azevedo et al., 2020). The mass of crude oil extracted from the visceral fats was determined using an analytical balance (Ohaus Adventure AR2140). The concentrations required for this study were obtained by diluting the crude oil solution (986.6 mg/mL) in dimethyl sulfoxide (DMSO).

2.4 Experimental Design

The concentrations selected for the *in vitro* cytotoxicity, intracellular ROS detection, and genotoxicity assays followed the criteria established by the OECD (protocol 487 for the micronucleus assay) (OECD, 2014). However, the solubility of the oil was a limiting factor for obtaining the required concentrations. The lowest amount of solvent required for diluting the stock solution of the oil extracted from visceral fat of *C. yacare* (CVO) was determined to be 1/5 oil and 4/5 Dimethyl sulfoxide - DMSO (v/v), corresponding to a range of 0.8–0.006% DMSO in the wells with CVO for the cell viability assay and 0.8–0.1% for the other tests.

For the cell viability assay, eight serial concentrations of CVO (15.62–2000 µg/mL) were selected. After screening using the cell viability assay, four concentrations of CVO (250, 500, 1000, and 2000 µg/mL) were selected for the remaining tests. In all assays, a negative control (NC, culture medium + cells) and solvent control (SC, culture medium + 0.8% DMSO) were used. At this concentration, DMSO was shown to be non-toxic and did not interfere with the results obtained.

For the positive control (PC), two concentrations of doxorubicin were used, 50 mM for the cell viability assay and 0.025 µg/mL for the other tests (clonogenic survival, standard alkaline comet assay, and micronucleus assay) (Serpeloni et al., 2011) with modifications. For the assay measuring intracellular levels of reactive oxygen species, hydrogen peroxide at 1 mM (PC, H_2O_2) was used as positive control. N-acetylcysteine (PC, NAC) at 2 mM were used as reference substance in intracellular levels of reactive oxygen species, due to their antioxidant capacity. Hydrogen peroxide (H_2O_2) at 20 µM was used as a positive control (PC) for oxidative DNA damage in the modified alkaline comet assay with the repair enzyme formamidopyrimidine (fapy)-DNA glycosylase (Fpg).

2.5 Cell Culture

The Chinese hamster lung fibroblast cells of the V79-4 lineage were obtained from the Rio de Janeiro Cell Bank (BCRJ) (code: 0244). The cells underwent karyotyping and were subjected to monthly tests to detect mycoplasma contamination using DAPI staining (Young et al., 2010). The V79-4 lineage were cultured in DMEM culture medium supplemented with 1.5 g/L glucose, 2 mM L-glutamine, and 10% fetal bovine serum (FBS), 0.006% penicillin, and 0.01% streptomycin sulphate. They were maintained in a biochemical oxygen demand (BOD) incubator at 37°C. In all assays, cells were seeded 24 hours prior to the initiation of the treatments.

2.6 Cell viability assay

Cell viability analysis was performed using the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay (Kumar et al., 2018) with modifications. Briefly, a total of 1×10^5 cells were seeded in 96-well plates and treated with different concentrations of CVO and control solutions. After 24 h, the supernatant was removed and the MTT formazan blue precipitate was dissolved in 100 µL of DMSO. The absorbance was measured at 540 nm using spectrophotometry (Thermo Scientific Multiskan EX). Three independent assays and treatments (in triplicate) were conducted.

The determination of the percentage of viable cells (%, cell viability) was calculated using the formula (1):

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of treated cells wells}}{\text{Mean absorbance of wells in the SC group}}\right) \times 100 \quad (1)$$

2.7 Clonogenic survival assay

The fraction of surviving cells after treatment with CVO was determined using the clonogenic survival assay (Franken et al., 2006) with modifications. A total of 0.14×10^6 cells were seeded in 12-well plates and treated with different concentrations of CVO and control solutions. After 24 h of treatment, the cells were removed with trypsin and 300 viable cells were seeded in a 25 cm^2 flask per treatment. They were then incubated for 5 d until non-confluent colonies were observed. For colony observation, the cells were fixed with methanol/acetic acid (3:1) and stained with Giemsa. Colonies with at least 50 cells were counted under a binocular stereoscopic microscope (Olympus SZ40). Experiments were performed in triplicate.

The number of colonies formed after cell treatment was expressed as a survival fraction (SF), which was calculated from the plating efficiency (PE) value using the following formulas (2) and (3):

$$PE\ (\%) = \left(\frac{\text{Number of colonies formed after treatment}}{\text{Number of cells seeded}}\right) \times 100 \quad (2)$$

Then, the survival fraction was calculated using the following formula:

$$SF\ (\%) = \left(\frac{\text{Number of colonies formed after treatment}}{\text{Number of cells seeded} \times PE}\right) \times 100 \quad (3)$$

2.8 Measurement of intracellular reactive oxygen species (ROS) levels

The intracellular production of reactive oxygen species (ROS) by CVO (pro-oxidant) was analyzed by flow cytometry using the fluorescent probe DCFH-DA (LeBel et al., 1992) with modifications. Briefly, cells were seeded at a concentration of 0.14 × 10^6 cells per well in 12-well plates and treated with different concentrations of CVO and control solutions for 4 h. Then, 500 µL of 5 µM DCFH-DA solution diluted in Hank's solution was added to each well for 20 min at 37° C in a BOD incubator. Subsequently, excess DCFH-DA was discarded, and cells were washed with Hank's solution and removed with trypsin. Then, centrifugation was performed for 5 min at 1500 rpm. Next, the supernatant was discarded, and the pellet was suspended in culture medium and Hank's solution with 2% FBS. Three independent assays and treatments (in duplicate) were conducted. Measurements were recorded using an Accuri C6 plus flow cytometer (BD Biosciences), and event acquisition was performed using the FACSDiva program (BD Biosciences). The analysis of acquired data was performed using Flowjo software (version 10.8.1). ROS production was depicted in histogram graphs, and the mean fluorescence intensity was related to DCFH detection.

To evaluate the protective effect against oxidative stress (antioxidant), cells were pre-treated with the determined concentrations of CVO and control solutions for 3 h.

Then, the supernatant was discarded, and cells were washed with Hank's solution and treated with 1 mM hydrogen peroxide (H_2O_2) for 1 h. The DCFH-DA labelling protocol was followed, and the measurements were recorded as described above.

2.9 Comet assay

Single-strand DNA breakage damage was analysed using the standard alkaline comet assay (Singh et al., 1988) with modifications. Briefly, cells were seeded at a concentration of 0.14×10^6 cells per well in 12-well plates and treated with different concentrations of CVO and control solutions for 4 h. Approximately 50 µL of the cell solution was mixed with low-melting-point agarose (0.5%, LM agarose) and placed on slides that were previously prepared with normal-melting-point agarose (1.5%, NM agarose), then incubated at 4 °C for 5 min to polymerize the LM agarose. Lysis, electrophoresis, neutralization, and fixation were then performed as described in the procedure proposed by (de Lucca et al., 2015) with the following modifications: incubation in lysis solution for a minimum of 24 h and incubation in electrophoresis solution for 30 min with a current intensity of 0.86 V/cm (25 V/300 mA) for 30 min at 18° C.

The comet assay and its modified version with the formamidopyrimidine(fapy)-DNA glycosylase (Fpg) enzyme to detect oxidative lesions (8-oxoguanine) caused by ROS were performed as described by (Speit et al., 2004) with modifications. Briefly, cells were analysed under an epifluorescence microscope (Nikon Eclipse Ci ProRes MF) using a 516–560 nm filter, a 590 nm filter barrier (total magnification of 400x), and images were processed using the Lucia Comet Assay software. The percentage of DNA in the tail of cells was used as the standard to measure DNA damage. One hundred nucleoids (50 per slide) were analyzed per treatment. Oxidative DNA damage was obtained by calculating the difference between the median values calculated from the percentage of DNA in the tail by treatment in the presence of 1 µg/mL of Fpg enzyme

per slide for 30 min and in the absence of the enzyme (Fpg–). Three independent assays and treatments (in duplicate) were conducted.

2.10 Cytokinesis-block micronucleus assay (CBMN)

The chromosomal anomalies were analyzed in the cytoplasm of interphase cells using the cytokinesis-block micronucleus assay (Fenech, 2007) with modifications. Briefly, approximately 5×10^5 cells were seeded in individual culture flasks (25 cm^2) for each treatment and treated with cytochalasin B (3 µg/mL) and at the determined concentrations of CVO and control solutions for 24 h (OECD, 2014). Then, the cells were subjected to hypotonic treatment with 2.0 mL of cold sodium citrate solution (1%) and fixed in ice-cold methanol and acetic acid solution (3:1; v/v). Next, the cells were placed onto slides (two slides per treatment) and stained in 3% Giemsa solution diluted in phosphate buffer (pH 6.8) for 5 min. For each treatment, the number of micronuclei (MN), nucleoplasmic bridges (NPB), and nuclear buds (Nbuds) were counted using optical microscopy (at 40x) in 2,000 binucleated cells (1,000 cells per slide). Three independent assays and treatments (in duplicate) were conducted.

The percentage of cytostasis, a cytotoxicity marker, was calculated as follows: 500 cells were counted to determine the proportion of mono- (MN), bi- (BN), and multi-nucleated (MuN) cells, and the cytokinesis-block proliferation index (CBPI) values were determined for each treatment following the formulas (4) and (5):

Then, the percentage of cytostasis was calculated using the following formula:

$$CBPI = \frac{((n° \text{ MN cells}) + (2 \times n° \text{ BN cells}) + (3 \times n° \text{ MuN cells}))}{(n° \text{ total cells})} \quad (4)$$

$$\text{Cytostasis } (\%) = 100 - 100 \times \left\{ \frac{(CBPI_{treatment} - 1)}{(CBPI_{control} - 1)} \right\} \quad (5)$$

2.11 Statistical analyses

The Shapiro-Wilk test was used to verify data distribution. Data presented as the mean ± standard error of mean values (SEM). Means were compared using one-way ANOVA and Dunnett's post-hoc test when necessary. $p \leq 0.05$ was considered significant for all statistical analyses. Statistical analyses and graphs were created using GraphPad Prism software (version 9.5). No statistical differences were observed between the negative control (NC) and solvent control (SC) in all assays. Thus, considering the presence and non-interference of DMSO in CVO treatments, comparisons were made with respect to SC (MTT, clonogenic survival, comet, and micronucleus assays). For the assay detecting intracellular ROS levels, comparisons were made with respect to positive control (PC, H_2O_2) (pro-oxidant activity) and N-acetylcysteine (PC, NAC) (antioxidant activity).

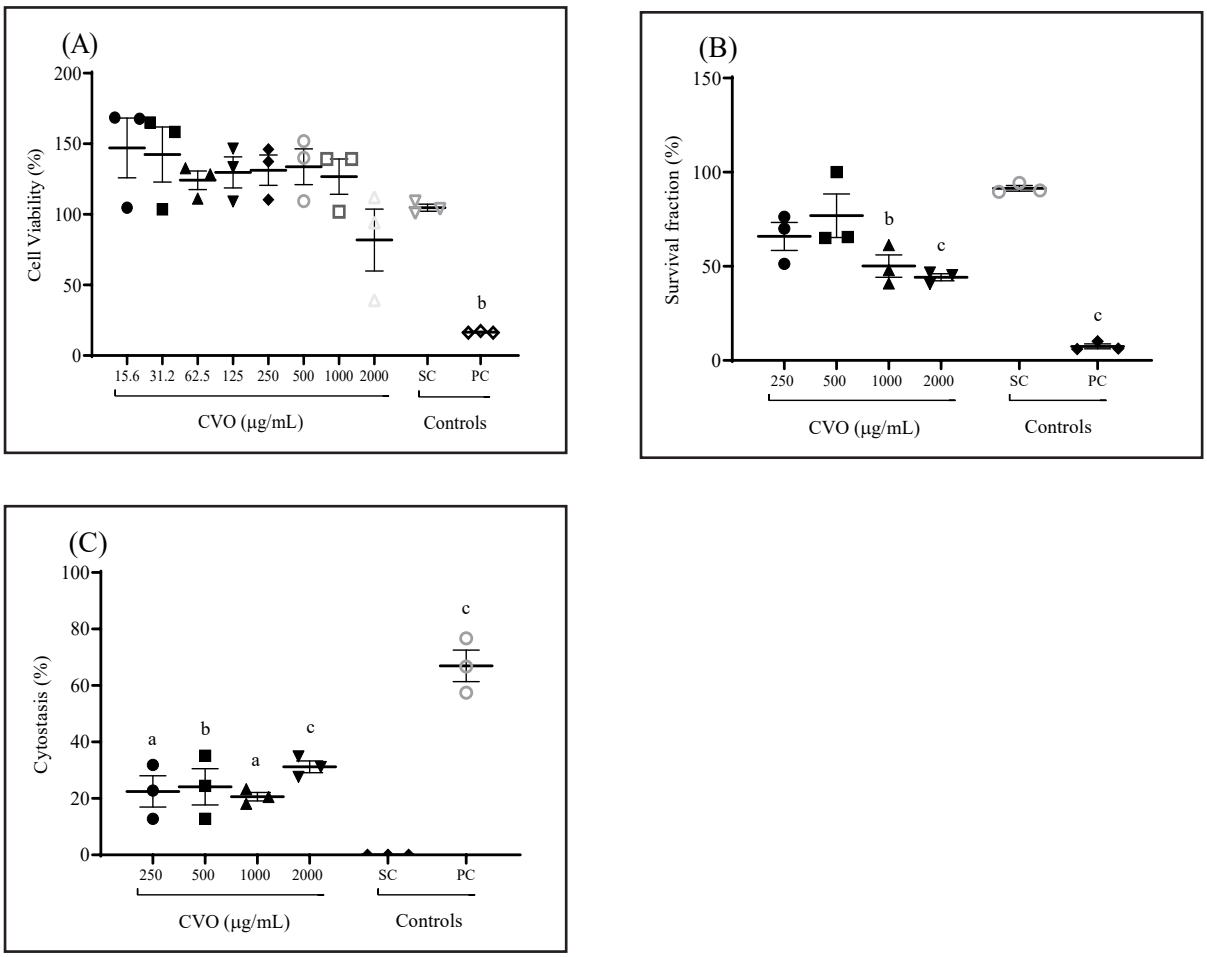
3 RESULTS

3.1 Cytotoxicity: cell viability and clonogenic survival assay

The CVO did not reduce cell viability at any of the evaluated concentrations [$f(9, 20) = 7.97$, $p \geq 0.05$]. In contrast, it increased the conversion of MTT salt into formazan blue at concentrations up to 1000 µg/mL (Fig. 1A), which initially could suggest a proliferative stimulus. However, this hypothesis was discarded by subsequent clonogenic survival assays and the cytostatic effect observed. Cells treated with CVO may be metabolically active but with compromised proliferative capacity due to DNA damage; however, the MTT assay cannot measure this effect. Therefore, the clonogenic survival assay was performed to assess the cytotoxic effects of CVO on both proliferation and cell death. The results demonstrate that CVO treatment reduced the survival fraction (SF) only at the two highest concentrations (1000 and 2000 µg/mL) [$f(5, 12) = 22.26$, $p \leq 0.0001$] (Fig. 1B). The cytostatic effect, another parameter of the cytotoxicity of CVO treatments,

was below 30% [f (5, 12) = 26.38, p ≤ 0.05] (Fig. 1C), which indicates a reduction of cell proliferation potential compared to control.

Figure 1 - Cytotoxicity of Chinese hamster lung fibroblast, V79-4 cell line, after treatment with different concentrations of oil extracted from visceral fat of *Caiman yacare* (CVO) for 24 h



Source: Authors (2023)

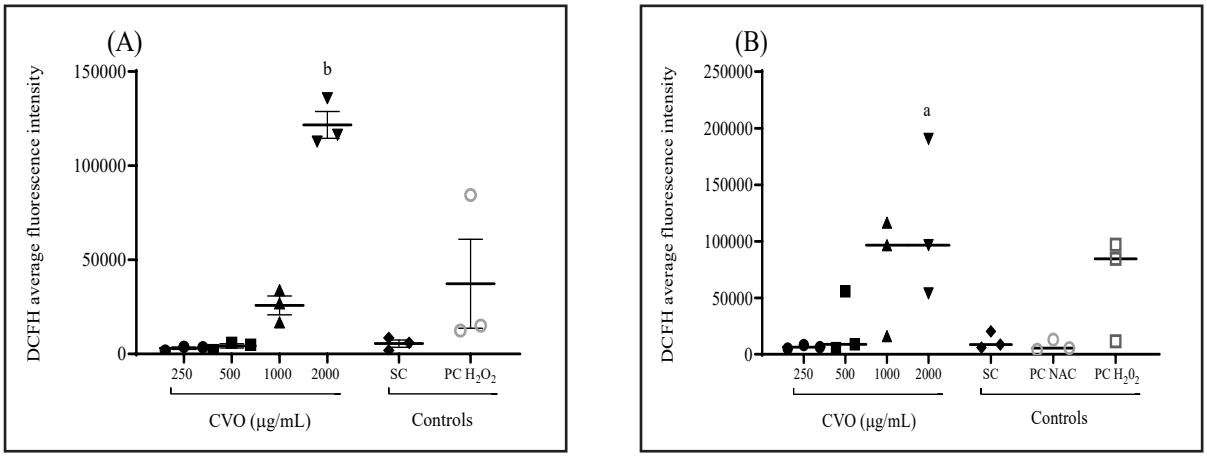
Notes: (A) Cell viability (MTT assay); (B) survival fraction (SF, clonogenic survival assay); (C) amount of cytostasis (%). Data are presented as mean ± SEM of three independent assays (in triplicate to MTT assay and without replication for the other assays). One-way ANOVA test, followed by Dunnett's post-hoc test. Letters above the symbol indicate significance: a, p ≤ 0.05; b, p ≤ 0.01; and c, p ≤ 0.001 versus the solvent control (SC, 0.8% DMSO). Positive control (PC, doxorubicin 0.025 µg/mL,).

3.2 Determining intracellular ROS levels

The treatment of cells with CVO for 4 h resulted in a significant increase in ROS levels (pro-oxidant) only at the highest concentration (2000 µg/mL) [*f* (5, 12) = 19.6, *p*

≤ 0.0001] compared to positive control (PC, H_2O_2) (Fig. 2A). CVO also demonstrated an antioxidant effect, reducing ROS levels in cells treated with concentrations of 250 and 500 µg/mL [f (6, 14) = 3.43, p = 0.026] when compared to positive control (PC, NAC) (Fig. 2B).

Figure 2 - Intracellular ROS production in Chinese hamster lung fibroblasts, V79-4 cell line, treated with different concentrations of oil extracted from visceral fat of *Caiman yacare* (CVO)



Source: Authors (2023)

Notes: (A) For pro-oxidant activity (ROS production), the cells were exposed to different concentrations for 4 h. (B) For antioxidant activity (protective effect), cells were treated for 3 h. Then, treatment solutions were removed, and oxidative stress was induced with hydrogen peroxide (H_2O_2) at 1 mM for 1 h in all cultures. Data are presented as mean ± SEM of three independent assays in duplicate. One-way ANOVA test, followed by Dunnett's post-hoc test. Letters above the bars indicate significance; a, p ≤ 0.05; b 0.001 versus hydrogen peroxide positive control (PC, H_2O_2; 1 mM, pro-oxidant) and positive control with N-acetyl cysteine (PC, NAC; 2 mM, antioxidant).

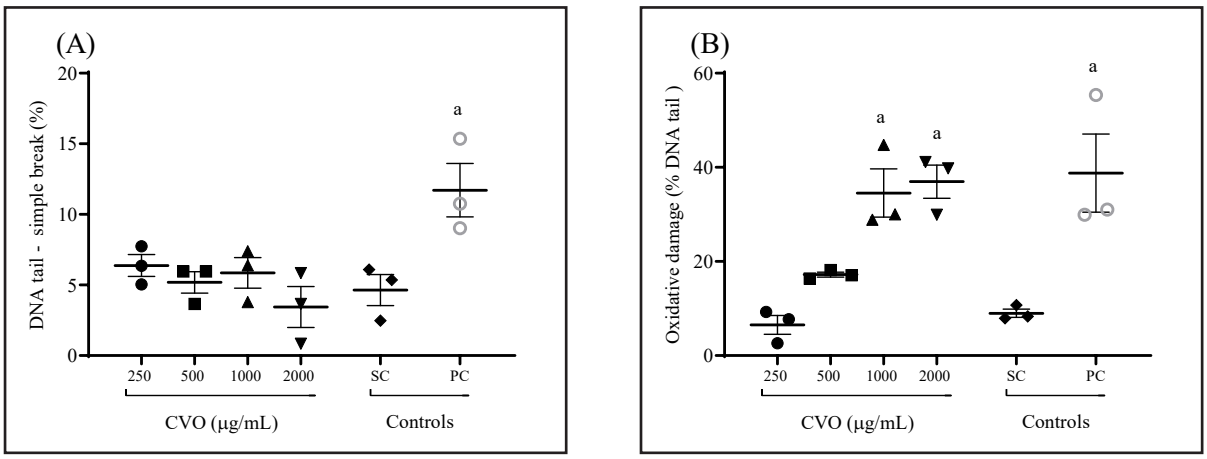
3.3 Evaluation of genotoxicity

3.3.1 Comet assay

CVO did not increase the percentage of single-strand DNA breaks [f (5, 12) = 5.40, p ≥ 0.05] (Fig. 3A). However, it increased the percentage of oxidative DNA damage, but only at the highest concentrations tested (1000–2000 µg/mL) [f (5, 12) = 11.44, p =

0.0003] (Fig. 3B). Interestingly, treatment with the lowest concentration (250 µg/mL) reduced the percentage of oxidative damage compared to the solvent control.

Figure 3 - Percentage of DNA damage in Chinese hamster lung fibroblasts, V79-4 cell line, after treatment with different concentrations of oil extracted from visceral fat of *Caiman yacare* (CVO) for 3 h



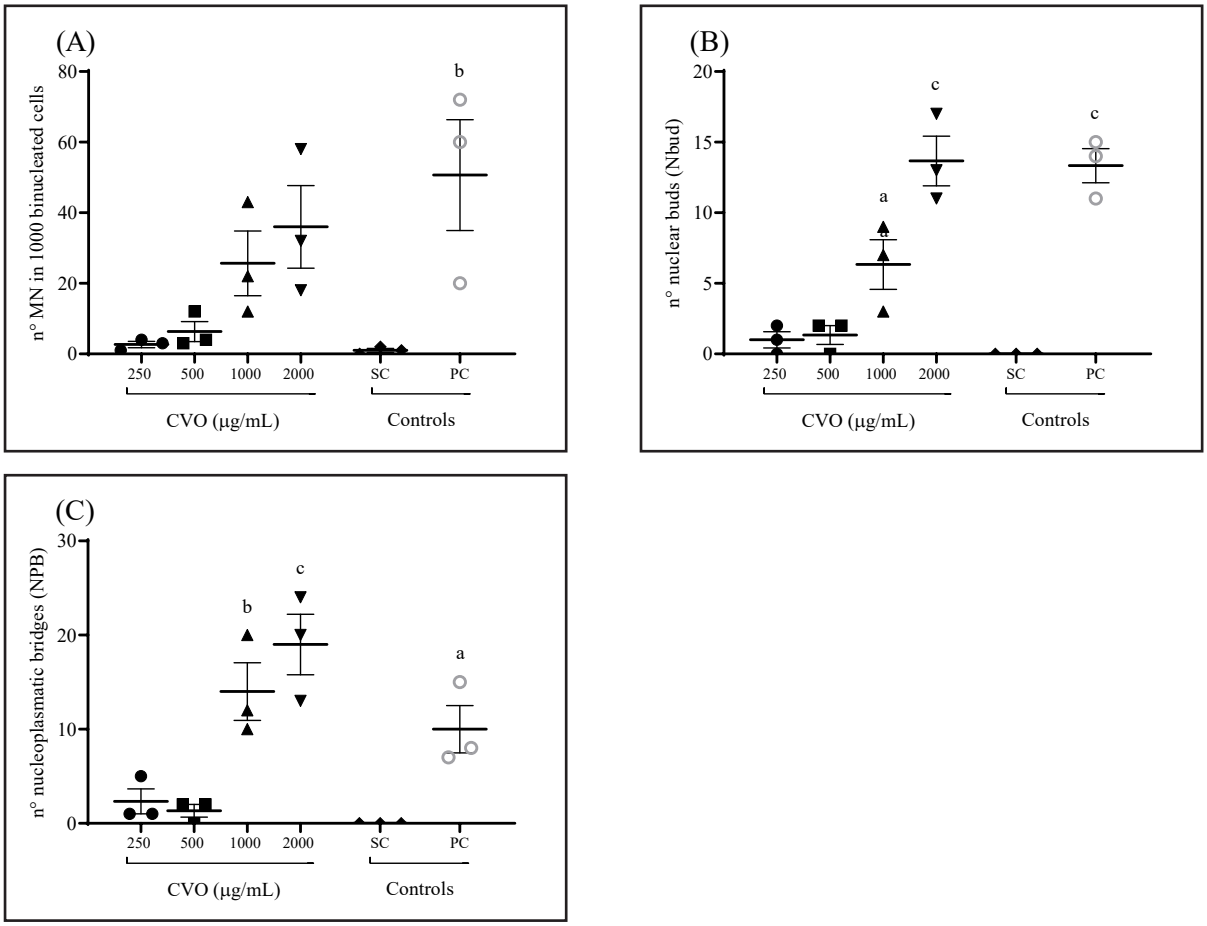
Source: Authors (2023)

Notes: (A) Single-strand breaks in DNA (standard alkaline comet assay); (B) oxidative DNA damage (modified alkaline comet assay with the repair enzyme formamidopyrimidine(fapy)-DNA glycosylase (Fpg). Data are presented as mean ± SEM of three independent assays in duplicate. One-way ANOVA test, followed by Dunnett's post-hoc test. Letters above the bars indicate significance; a, p ≤ 0.01 versus solvent control (SC, 0.8% DMSO). Positive control (PC, doxorubicin 0.025 µg/mL, standard alcaline comet assay) and (PC, H_2O_2 20 µM, modified alkaline comet assay with fpg).

3.3.2 Cytokinesis-block micronucleus assay (CBMN)

Cells treated with CVO had increased numbers of micronuclei (MN) [f (5, 12) = 5.22, p = 0.008], nuclear buds (Nbuds) [f (5, 12) = 27.77 $p \leq 0.0001$] and nucleoplasmic bridges (NPB) [f (5, 12) = 12.81, $p \leq 0.0002$] only at higher concentrations (1000–2000 µg/mL) (Fig. 4A, B, and C).

Figure 4 - Chromosomal instability in Chinese hamster lung fibroblasts, V79-4 cell line, after treatment with different concentrations of oil extracted from visceral fat of *Caiman yacare* (CVO) for 24 h



Source: Authors (2023)

Notes: A) Number of micronuclei, (B) nuclear buds, and (C) nucleoplasmic bridges in binucleated V79-4 cells (cytokinesis-block micronucleus assay). Data are presented as mean ± SEM of three independent assays without replication. One-way ANOVA test, followed by Dunnett's post-hoc test. Letters above the symbol indicate significance: a, $p \leq 0.05$; b, $p \leq 0.01$; and c, $p \leq 0.001$ versus the solvent control (SC, 0.08% DMSO). Positive control (PC, doxorubicin 0.025 µg/mL).

4 DISCUSSION

The cytotoxic and genotoxic effect of CVO was only observed at the highest concentrations tested in this study. It is worth noting that at lower concentrations, CVO demonstrated antioxidant effects and decreased oxidative DNA damage. These results are similar to those previously described in human retinal pigment epithelial

cells (RPE-1) treated with CVO (Azevedo et al., 2020) and murine macrophages treated with *C. siamensis* oil (Ngernjan et al., 2022).

The cytotoxic and cytostatic effects of CVO were accompanied by an increase in ROS production and/or oxidative DNA damage. ROS cause various lesions in DNA, with 8-oxo-guanine, recognized by Fpg glycosylase, being the most common lesion (Cooke et al., 2003). Oxidative DNA damage can activate cell cycle checkpoints, blocking the division progression for damage repair or activation of apoptosis when damages exceed the cell's processing capacity (Barzilai & Yamamoto, 2004). The cell's processing capacity is time-dependent, which explains why CVO's cytotoxic/cytostatic effects are observed as colony formation and cytostasis decreases.

As we described previously, CVO is composed of a nearly equal proportion of saturated (42.95%) and unsaturated (43.74%) fatty acids. Among the saturated fatty acids, palmitic (21.61%) and stearic acids (18.50%) are the most abundant, while oleic acid (32.31%) is the most abundant unsaturated fatty acid. Both saturated fatty acids have been recognized for their *in vitro* cytotoxic effect in T and B cells and in primary cultures of hepatocytes (Lima et al., 2002; Moravcová et al., 2015; Takahashi et al., 2012). In HepG2 cells, palmitic acid increased cytotoxicity and ROS production concomitantly with mitochondrial dysfunction and release of inflammatory markers (Alnahdi et al., 2019), indicating the mechanisms by which this fatty acid can contribute to ROS production. The increased presence of fatty acids in CVO may have triggered oxidative stress due to increased mitochondrial metabolism. MTT salt is converted to formazan blue in the presence of NADPH, and increased mitochondrial metabolism in cells overloaded with fatty acids can result in higher NADPH (Kastaniotis et al., 2017; Stockert et al., 2018). The increase in formazan blue density in the MTT assay suggests an increase in mitochondrial metabolism due to fatty acid overload from CVO, rather than cell proliferation, as indicated by the clonogenic survival assay, which showed no proliferative effect of CVO on cells.

The CVO also presented mono- (MUFA) and poly-unsaturated fatty acids (PUFA) in its composition, mainly oleic (ômega-9), linoleic, and arachidonic acids (ômega-6). Linoleic and arachidonic acids, when oxidized, generate trans-4-hydroxy-2-nonenal (HNE), which are genotoxic even at low concentrations (Chung et al., 2003; Speit et al., 2004). However, it has been shown that unsaturated fatty acids, such as oleic and linoleic acids, can have beneficial effects at certain concentrations, counteracting the deleterious effects of palmitic acid, decreasing ROS production, DNA damage, and apoptosis (Alnahdi et al., 2019; Beeharry et al., 2003; Moravcová et al., 2015). It is possible that a positive balance between PUFA and saturated fatty acids was achieved at lower, but not at higher concentrations tested in this study, explaining the absence of cytotoxic and genotoxic effects.

As evidenced by the comet assay, treatment with CVO increased DNA breaks. Considering that this effect was also observed at concentrations where there was no increase in oxidative DNA damage, this result suggests that the oil may cause other types of DNA lesions besides oxidative damage. The presence of DNA breaks correlated with higher concentrations of PUFA and saturated fatty acids, but not with an increase in oxidative damage, has been reported previously in lymphocytes *ex vivo* (Thorlaksdottir et al., 2007). However, the mechanism involved in generating this type of lesion remains unclear. Nevertheless, the damage caused at lower concentrations was low and the cells could recover. No increase in the frequency of micronuclei was observed at 250 µg/mL.

The oxidative damage caused at higher concentrations exceeded the cell's recovery capacity, resulting in increased micronuclei, nuclear buds, and cytoplasmic bridges. These three nuclear anomalies can originate from DNA breaks, which can be caused by the simultaneous repair of proximal oxidative lesions (M. Fenech et al., 2011; Kisurina-Evgenieva et al., 2016). Oxidative damage can also compromise telomeres, generating chromosomal fusion processes that produce dicentric chromosomes, evidenced as nuclearplasmic bridges (Wang et al., 2010).

Although the higher concentrations tested in this study caused damage that exceeded the cell's recovery capacity, the concentration of 250 and 500 µg/mL was able to protect the cell from oxidative stress caused by hydrogen peroxide. Interestingly, at this same concentration, CVO showed a lower level of oxidative DNA damage than the solvent control, indicating that it may have some positive influence on lesion removal mechanisms. These results suggest that CVO may have antioxidant or pro-oxidant effects depending on the concentration used.

5 CONCLUSION

In this study, CVO demonstrated its safety regarding cytotoxic and genotoxic effects in vitro, which is an important step in the development of wound healers or even nutraceutical products. We have also demonstrated that CVO reduced the percentage of intracellular ROS and oxidative DNA damage, potentially increasing the mitochondrial metabolism. The present study is a first step in the development of new medicinal and biotechnological products, such as wound healers or even nutraceutical products. In fact, this study is an obligatory preliminary phase before proceeding to pre-clinical or clinical studies in humans. Thus, further studies are necessary to discover new properties of the oil and confirm the popular use of Pantanal alligator fat.

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