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Chemistry

Chemical profile analysis and therapeutic potential of *Caiman yacare* paracloacal gland oil ethanolic extract (Daudin, 1802)

Análise do perfil químico e potencial terapêutico do extrato etanólico do óleo das glândulas paracloacais de *Caiman yacare* (Daudin, 1802)

Natasha Rayane de Oliveira Lima[I], Lucas Polizzeli Azevedo[II], José Vitor de Menezes Costa[II], Leandro Nogueira Pressinotti[I], Rhavena Graziela Liotti[III], Larissa Maria Scalon Lemos[I], Carmen Lucia Bassi-Branco[II], Rosa Helena dos Santos Ferraz[II], Leonardo Gomes de Vasconcelos[II], Samuel Vandresen Filho[II], Fabrício Rios-Santos[II]

[I]Universidade do Estado de Mato Grosso, Cáceres, MT, Brazil
[II]Universidade Federal de Mato Grosso, Cuiabá, MT, Brazil
[III]Instituto Federal de Educação, Ciência e Tecnologia de Mato Grosso, Cuiabá, MT, Brazil

ABSTRACT

With the aim of expanding our knowledge of the *Caiman yacare* paracloacal gland oil ethanolic extract (Daudin, 1802), we investigated the chemical composition and antimicrobial activity of the ethanolic extract as well as its influence on cell viability. Fourier transform infrared spectroscopy and gas chromatography coupled with mass spectrometry were used to determine the chemical profile. Subsequently, using the broth microdilution method, we evaluated the ethanolic extract for its antimicrobial effect against 15 bacterial strains and two yeast strains as well as its effect on cell viability in different glial cell and skin tissue lineages using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The ethanolic extract was found to have a heterogeneous composition, containing compounds such as nitrosourea, hydrocarbons, ketones, and esters. Additionally, the ethanolic extract exhibited a bacteriostatic effect at concentrations above 200 µg/mL against *Pseudomonas aeruginosa* and above 400 µg/mL against *Streptococcus pyogenes* and *Enterococcus faecalis*. The ethanolic extract reduced cell viability only at the two highest concentrations (500 and 1000 µg/mL) in fibroblasts (GM07492) and at the highest concentration in astrocytes (ACBRI-371) and glioblastoma multiforme (T98G). These findings contribute to a better understanding of the pharmacological potential of *C. yacare* paracloacal gland oil, providing support for future investigations and the development of therapeutic products.

Keywords: Cytotoxicity; Antimicrobial; Chemical composition

RESUMO

Com o objetivo de ampliar o conhecimento acerca do extrato etanólico do óleo das glândulas paracloacais de *Caiman yacare* (Daudin, 1802), investigamos a composição química, atividade antimicrobiana e influência na viabilidade celular desse extrato. Para isso, as técnicas de Espectroscopia de Infravermelho por Transformada de Fourier e Cromatografia Gasosa acoplada à Espectrometria de Massas foram utilizadas para determinar o perfil químico. Posteriormente, foi avaliado capacidade antimicrobiana contra quinze cepas bacterianas e duas cepas de leveduras, pelo método de microdiluição em caldo, e na viabilidade celular em três linhagens de células gliais e duas de tecido cutâneo pelo ensaio de MTT. Os resultados revelaram composição heterogênea, contendo compostos como nitrosurea, hidrocarbonetos, cetonas e ésteres. O extrato apresentou efeito bacteriostático em concentrações acima de 200 µg/mL contra *Pseudomonas aeruginosa* e acima de 400 µg/mL contra *Streptococcus pyogenes* e *Enterococcus faecalis*. Além disso, reduziu a viabilidade celular apenas nas duas maiores concentrações (500 e 1000 µg/mL) nos fibroblastos (GM07492) e na maior concentração em astrócitos (ACBRI-371) e glioblastoma multiforme (T98G). Essas descobertas contribuem para um melhor entendimento do potencial farmacológico do óleo das glândulas paracloacais de *C. yacare*, fornecendo subsídios para futuras investigações e desenvolvimento de produtos terapêuticos.

Palavras-chave: Citotoxicidade; Antimicrobial; Composição química

1 INTRODUCTION

The use of animal organs and tissues by traditional communities for treating illnesses has attracted the interest of the scientific community owing to their potential therapeutic benefits (Medeiros & Alves, 2020). Among the animal species that serve as medicinal resources, crocodilians have garnered attention because of their medicinal properties and the abundance of bioactive compounds in their oils and fats (Alves & Alves, 2011; Leiva et al., 2022). Outside Brazil, the trade and therapeutic use of oils and fats obtained through the slaughter of captive crocodilians has been reported (Shim-Prydon & Camacho-Barreto, 2007). Preliminary studies have revealed the antimicrobial and anti-inflammatory activities of *Crocodylus niloticus* oil (Buthelezi et al., 2012), and the wound healing and immunomodulatory properties of *Crocodylus siamensis* oil (Li et al., 2021), in addition to the presence of unsaturated fatty acids with known biological activity. However, knowledge gaps regarding the chemical composition and therapeutic use of crocodilian oils and fats still exist, especially in the Brazilian context.

The paracloacal gland, located in the distal region of the digestive system of crocodilians, constitutes a relatively unexplored oleaginous source that deserves greater scientific attention (Weldon & Sampson, 1987). More than 234 compounds have been identified in different crocodilian species; however, only the oil derived from the glands of *Caiman yacare* has been documented to exhibit antifungal activity (Azevedo et al., 2018). Therefore, knowledge gaps regarding the chemical composition and pharmacological activity of *C. yacare* paracloacal gland oil (PGO) exist and require further exploration.

In addition to the scientific implications, considering the current profitable economic scenario in the Brazilian Pantanal region related to products derived from the captive breeding of *C. yacare* is relevant (Carreira & Sabbag, 2015). Currently, in Brazil, the zootechnics of these animals exclusively market the meat and leather (Campos & Coutinho, 2006). As a result, captive breeding of *C. yacare* can benefit by adding scientific value to a raw material, PGO, that has remained unused by producers.

A crucial step in the investigation of natural products is the application of analytical chemistry techniques to identify molecules of pharmacological interest and direct research to understand their biological effect (Nováková et al., 2019). Therefore, owing to the relevance of knowing the chemical profiles of these products as an initial step in their development for human consumption, chemical evaluation combining analytical chemistry techniques is indispensable. In this study, we investigated the chemical profile of an ethanolic extract of *C. yacare* PGO using Fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS). After analyzing the profile of PGO, we investigated its antimicrobial potential and influence on the viability of human cells of glial origin and skin tissue.

2 MATERIALS AND METHODS

2.1 Collection and extraction of *C. yacare* PGO

Paracloacal glands were collected from discarded specimens of *C. yacare* from the municipality of Cáceres, Mato Grosso, Brazil. The experiments were conducted in accordance with the current legal norms of the Ethics Committee for Animal Use (CEUA) under protocol No. 003/2018 and the Biodiversity Authorization and Information System (SISBIO) under Permit No. 58681-1.

The glands were washed under running water, followed by immersion in 70% ethanol for 5 min. Subsequently, the samples were washed again with sterile distilled water and manually compressed to extract the oil (Weldon et al., 1989). Subsequently, absolute ethanol was added at a volume equivalent to that of PGO (50%, v/v). The sample-containing vials were stored in sterile containers protected from light and agitated daily for 14 days.

Following the maceration step, the ethanolic extract was subjected to a vacuum concentration process using a rotary evaporator under a pressure of 600 mmHg at a rotation speed of 45 rpm and temperature of 37°C. The obtained extracts were stored in glass ampoule vials, protected from light, and kept refrigerated at -20°C.

2.2 Chemical analysis

2.2.1 FTIR

FTIR was used to determine the chemical composition of PGO. FTIR analysis was performed at the Analytical Center of the Federal University of Mato Grosso (Analytical Center, IQ/UFMT) using a Shimadzu Iraffinity-1 spectrophotometer (Model IRAffinity-1, Shimadzu Corporation).

Sample preparation for FTIR analysis involved the use of potassium bromide (KBr) pellets. KBr pellets were prepared in the laboratory by drying in an oven for 24 h

and subsequently pressed into pellets. A background spectrum was acquired using a KBr pellet weighing 0.07 g. For the preparation of the KBr/sample pellet, a ratio of 0.07 g of KBr to 0.0007 g of PGO ethanolic extract was employed. Pelletization was performed using a hydraulic press system (Model SSP-10A, Shimadzu Corporation). The resulting KBr/sample pellet was then subjected to FTIR analysis. Qualitative acquisition of the FTIR spectra was performed using IRSolution software (Version:1.50) with the following parameters: measurement mode, % transmittance; apodization, Happ_Genzel; number of scans, 200; resolution, 16; range, 400–4000 cm^{-1}; and gain, 1.

2.2.2 GC-MS

GC-MS was conducted at the Analytical Instrumentation Center of the University of São Paulo Analytical Center (Analytical Center-IQ/USP). The analysis was performed using a gas chromatograph coupled with a mass spectrometer (Shimadzu, model QP2020). The instrument was operated under the following conditions: injector temperature of 220°C; column temperature program starting from 60°C and ramping up to 280°C; initial column pressure of 111.5 KPa; split ratio of 5.00; and an injection volume of 1 µL (1% solution in dichloromethane). Compound identification was performed by comparing the mass spectra (NIST 14) and Kovats Index data with literature references to assess the similarity of chemical compounds.

2.3 Experimental design – biological assays

The chemical profile of the PGO ethanolic extract obtained from captive-bred animals was analyzed using two complementary analytical techniques: FTIR and GC-MS. Using these techniques, molecules with potential antimicrobial, antitumor, and wound-healing properties were identified. Based on these findings, we conducted two preliminary *in vitro* biological assays. The first assay was an antimicrobial test in which we evaluated the effectiveness of the extract against a panel of gram-positive bacteria, gram-negative bacteria, and yeast strains. The second assay focused on cell viability, using three glial cell

lineages and two skin tissue cell lineages. These assays allowed us to assess the *in vitro* cytotoxic potential of PGO by examining molecules identified in the extract.

The concentrations for the broth microdilution experiments and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were selected according to the criteria established by the OECD (Protocol 487 for the micronucleus assay) (OECD, 2014). Solubility limitations and a maximum solvent content of 1% in the treatments posed challenges in obtaining the study concentrations. To address this issue, the smallest amount of solvent needed for the dilution of the PGO stock solution was established to be 1/6 PGO and 5/6 absolute ethanol (v/v).

For the antimicrobial assay (broth microdilution), five serial concentrations (100 to 1600 µg/mL) of PGO were selected. A serial dilution of absolute ethanol was performed for use as a solvent control (SC) starting from a concentration of 1% ethanol in the culture medium (v/v). These concentrations of absolute ethanol (1–0.06%) corresponded to the percentage of ethanol present in the PGO treatments. In the MTT assay, seven serial concentrations (15.62 to 1000 µg/mL) of PGO were chosen. A SC of 1% absolute ethanol diluted in culture medium (v/v) was used. At this concentration, absolute ethanol did not affect the cell viability. Positive controls (PCs) included ciprofloxacin at 100 µg/mL for bacteria, amphotericin B at 100 µg/mL for yeasts (microdilution assay), and doxorubicin at 50 mM for human cell lineages (MTT assay).

2.4 Microorganisms

The bacterial strains were sourced from reputable institutions, such as the American Type Culture Collection (ATCC), and veterinary isolates were provided by the Federal University of Mato Grosso/UFMT and the University of São Paulo/USP. The microorganisms used consisted of a panel of 15 bacteria, including five Gram-positive strains: *Enterococcus faecalis*, *Streptococcus pyogenes*, *Staphylococcus aureus* (ATCC 6538), *Staphylococcus aureus* (ATCC 25923), and *Staphylococcus epidermidis*. Additionally, ten Gram-negative bacteria were included in this study: *Pseudomonas*

aeruginosa (ATCC 9027), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae*, *Pasteurella multocida*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Salmonella enterica*, *Enterobacter cloacae*, *Providencia stuartii*, and *Proteus mirabilis*. Two yeast strains, *Candida glabrata* and *Cryptococcus neoformans*, were used in this study. The bacteria were cultivated in Mueller-Hinton agar (MHA) medium, and the yeast strains were cultured in Sabouraud broth. The strains were stored at 4°C and sub-cultivated every two weeks during the study period.

2.5 Cell culture

Normal human keratinocytes (HaCaT lineage) used in this study were obtained from Rio de Janeiro (BCRJ). The cell lines normal human astrocytes (ACBRI-371 lineage), glioblastoma multiforme (T98-G lineage), malignant glioma (M059K lineage), and fibroblasts (GM07491 lineage) were provided by Professor Dr. Elza Tiemi Sakamoto-Hojo of the Department of Genetics of the Faculty of Medicine of Ribeirão Preto/USP. All cell lines were cultured in Dulbecco's modified Eagle's medium, containing 4.5 g/L glucose, 2 mM L-glutamine, 10% fetal bovine serum, 0.006% penicillin, and 0.01% streptomycin sulfate. The cells were maintained in a B.O.D incubator at 37°C. Cells were seeded 24 h before the start of treatment in all assays.

2.6 Antimicrobial activity

2.6.1 Inhibitory concentration

The vulnerability of the microorganisms was assessed using the broth microdilution method (CLSI, 2008; Eloff, 1998). Bacterial inocula were cultured in MHA medium, whereas yeast inocula were cultured in Sabouraud broth. In 96-well microplates containing culture medium, different concentrations of PGO were added, ranging from 100 to 1600 µg/mL. Subsequently, 100 µL of bacterial inocula at 0.5 McFarland (1.5×10^8 CFU/mL) and yeast inocula at 1 McFarland (3×10^8 CFU/mL) were added to the plate. After inoculation, the plates were incubated in a bacteriological incubator at 35°C for

24h for bacteria and at 30°C for 48 h for yeasts. Following incubation, growth inhibition was determined based on the absorbance of the wells, which was measured at 450 nm using a spectrophotometer (Thermo Scientific Multiskan EX).

PC and SC activities were determined as described in Section 2.3. The sterility control was verified in wells containing only MHA or Sabouraud culture medium. The inhibitory concentration (IC) was defined as the concentration at which PGO exhibited significantly greater inhibition than the SC. Three independent assays were performed in triplicate.

2.6.2 Bactericidal and fungicidal concentration

The method used was adapted from assays that qualitatively determined the bactericidal and fungicidal concentrations of natural products (Mbah et al., 2012). In wells showing the IC, 6 µL of the inoculum was withdrawn and cultured under the same conditions mentioned previously in section 2.4. To evaluate the efficacy of PCs, at the end of the IC assay, 6 µL from wells containing inocula treated with PCs were transferred onto the surface of MHA agar for bacteria and Sabouraud agar for yeasts. The treatment plates were then incubated at 35°C for bacteria and 30°C for yeasts and observed for 24 h. A PC was considered effective when it resulted in the death of 99% of the inoculum.

The bactericidal and fungicidal concentrations were defined as the concentrations of PGO that caused the death of 99% of the inoculum. When the inoculum grew, the PGO was considered to be bacteriostatic or fungistatic. Three independent assays were conducted in triplicate.

2.7 Cell viability assay

Cell viability was analyzed using the MTT assay (Kumar et al, 2018) with modifications. A total of $1x10^5$ cells were seeded in 96-well plates and treated with different concentrations of PGO or the respective controls, as described in section 2.5. After 24 h, the supernatant was removed, and the MTT formazan precipitate was dissolved in 100 µL of absolute ethanol. The absorbance was measured at 540 nm using a spectrophotometer (Thermo

Scientific Multiskan EX). Three independent assays were performed in triplicate. The cell viability (% cell viability) was calculated using the following formula (1):

Cell Viability (%) = Absorbance of treated cells/mean absorbance of wells in the solvent control group × 100 (1)

2.8 Statistical analysis

The Shapiro-Wilk test was used to verify the data distribution. For the antimicrobial assay (broth microdilution-inhibitory concentration), the means were compared using repeated-measures one-way ANOVA and Dunnett's post-hoc test. Statistical comparisons were made relative to the percentage of absolute ethanol (SC) in PGO-treated wells because interference from the SC was observed in the IC assay. The ICs of the PGO and solvents were calculated considering that the PC exhibited an IC of 99%, as verified by sub-culturing the samples on a solid medium. Proportional calculations of the PGO and solvent ICs were performed using the rule of three. For the cell viability assay, the means were compared using one-way ANOVA and Dunnett's post-hoc tests. Considering the presence or absence of absolute ethanol in the PGO-treated samples for cell viability analysis (MTT assay), comparisons were made relative to the SC with 1% absolute ethanol. A significance level of $p \leq 0.05$ was used as the criterion for statistical significance for all statistical analyses. Analyses and graphs were generated using GraphPad Prism software, version 9.5.

3 RESULTS

3.1 Chemical determination of the *C. yacare* PGO ethanolic extract

The FTIR data of the PGO revealed twelve absorption bands within the wavelength range of 2924.1 to 725.2 cm^{-1} (Table 1 in supplementary material 1). Based on these preliminary findings, PGO was subjected to GC-MS analysis to confirm the chemical composition of the extract, which revealed the presence of 19 compounds including a nitrosourea, ketones, hydrocarbons, and esters (Table 1).

Table 1 – GC-MS analysis of the *C. yacare* PGO ethanolic extract

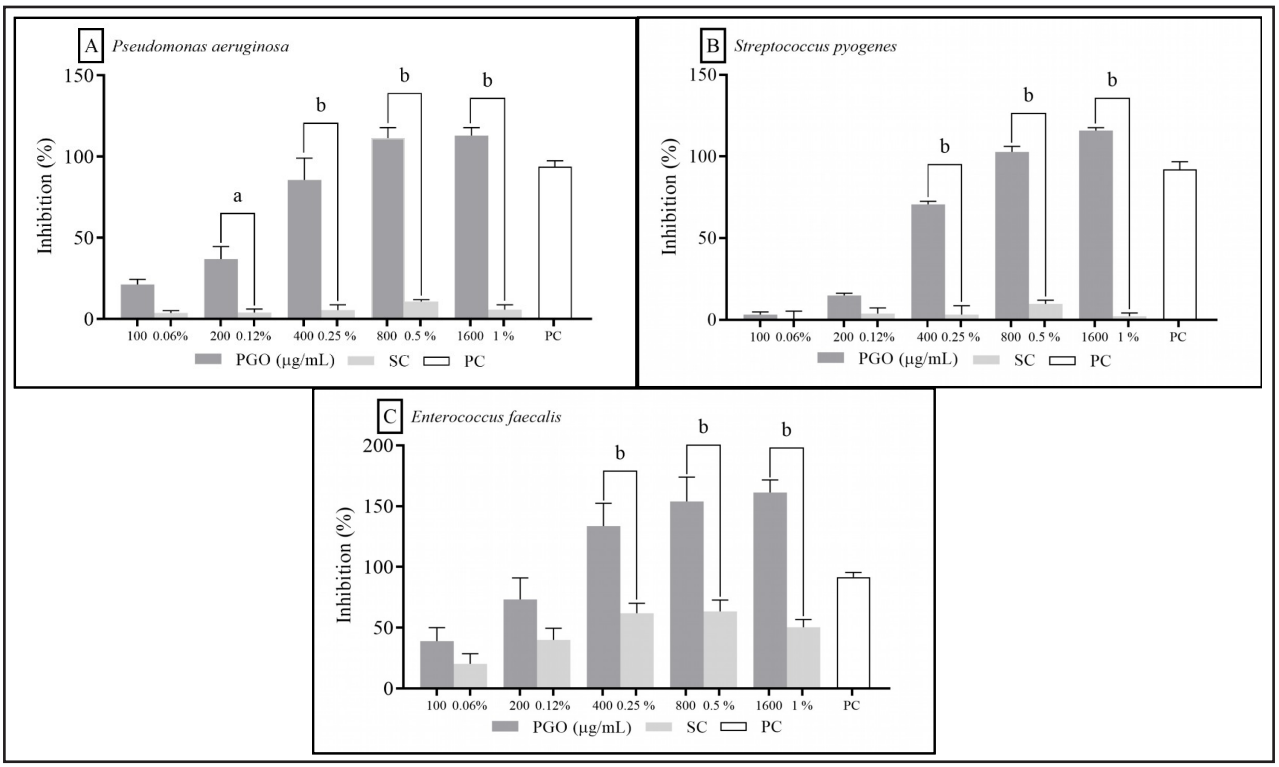
Chemical class	Name (IUPAC)	Common name	RT (min)	Area (%)	Molecular formula
Nitrosourea	1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea	semustine	10.99	1.32	$C_{10}H_{18}ClN_3O_2$
Ketone	heptadecan-3-one	3-heptadecanone	15.08	2.34	$C_{17}H_{34}O$
Ester	ethyl myristate	tetradecanoic acid, ethyl ester	15.37	7.88	$C_{16}H_{32}O_2$
Hydrocarbon	1-dodecyne	dodec-1-yne	15.48	0.55	$C_{12}H_{22}$
Ester	(2E,6E,10E)-3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol	2,6,10,14-hexadecatetraen-1-ol, 3,7,11,15-tetramethyl	15.55	15.04	$C_{20}H_{34}O$
Hydrocarbon	(5E)-2,3,5,8-tetramethyl-1,5,9-decatriene	1,5,9-decatriene, 2,3,5,8-tetramethyl-	16.47	4.28	$C_{14}H_{24}$
Ester	ethyl 9-hexadecenoate	ethyl 9-hexadecenoate	17.20	12.01	$C_{18}H_{34}O_2$
Alcohol	(4E,8E)-5,9,13-triméthyl-4,8,12-tétradécatrién-1-ol	4,8,12-tetradecatrien-1-ol, 5,9,13-trimethyl-	17.31	6.68	$C_{17}H_{30}O$
Ester	ethyl palmitate	hexadecanoic acid, ethyl ester	17.39	15.88	$C_{18}H_{36}O_2$
Alcohol	3,7-dimethyloct-6-en-1-ol	citronellol	18.22	0.50	$C_{10}H_{20}O$
Hydrocarbon	(9Z,12Z)-9,12-octadecadienoic acid	linoleic acid	18.97	1.48	$C_{18}H_{32}O_2$
Ester	ethyl (9E)-9-octadecenoate	(E)-9-octadecenoic acid ethyl ester	19.02	15.54	$C_{20}H_{38}O_2$
Ester	3,7-dimethyloct-6-en-1-yl butyrate	citronellyl butyrate	19.09	2.70	$C_{14}H_{26}O_2$
Ester	ethyl icosanoate	eicosanoic acid, ethyl ester	19.25	1.56	$C_{22}H_{44}O_2$
Ester	[(8E)-3,7,11-trimethyldodeca-8,10-dienyl] acetate	3,7,11,trimethyl-8,10-dodecedienylacetate	19.90	0.62	$C_{17}H_{30}O_2$
Hydrocarbon	(5Z,8Z,11Z,14Z)-5,8,11,14-icosatetraenoic acid	arachidonic acid	20.41	0.57	$C_{20}H_{32}O_2$
Ester	(3β)-cholest-5-en-3-yl carbonochloridate	cholest-5-en-3-ol (3.beta.)-, carbonochloridate	24.12	0.48	$C_{28}H_{45}ClO_2$
Ester	[10,13-dimethyl-17-(6-methylheptan-2-yl)-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl] 2,2,2-trifluoroacetate	5.beta.-cholestan-3.alpha.-ol, trifluoroacetate	24.90	2.62	$C_{29}H_{47}F_3O_2$
Ester	(3β)-cholest-5-en-3-yl carbonochloridate	cholest-5-en-3-ol (3.beta.)-, carbonochloridate	25.07	1.62	$C_{28}H_{45}ClO_2$

Notes: IUPAC: International Union of Pure and Applied Chemistry; RT (min): Retention time in minutes; Area (%): percentage area of the peak curve relative to the total area of all peaks in the chromatogram obtained by GC-MS analysis of PGO
Source: Authors (2023)

Some of the compounds identified by GC-MS exhibited antimicrobial, antitumor, and wound-healing activities, as described in the literature. Therefore, PCO was assessed for its antimicrobial capacity and influence on cell viability in cell lineages of glial and cutaneous tissue origin, which are known to be involved in wound healing.

3.2 Antimicrobial activity

Figure 1 – Percentage of inhibition (broth microdilution assay) after treatment with different concentrations of the *C. yacare* PGO ethanolic extract for 24 h



Notes: (A) *P. aeruginosa*; (B) *S. pyogenes*; (C) *E. faecalis*. One-way ANOVA for repeated measures was followed by Dunnett's post hoc test. Values above the bars represent the mean ± standard error of the mean (SEM) of three independent assays with PGO and the respective SC (percentage of absolute ethanol). The letters (a) and (b) above the lines correspond to the values of (a) $p \leq 0.01$ and (b) $p \leq 0.0001$ versus SCs (0.06% to 1% absolute ethanol). The PC was ciprofloxacin (100 µg/mL)
Source: Authors (2023)

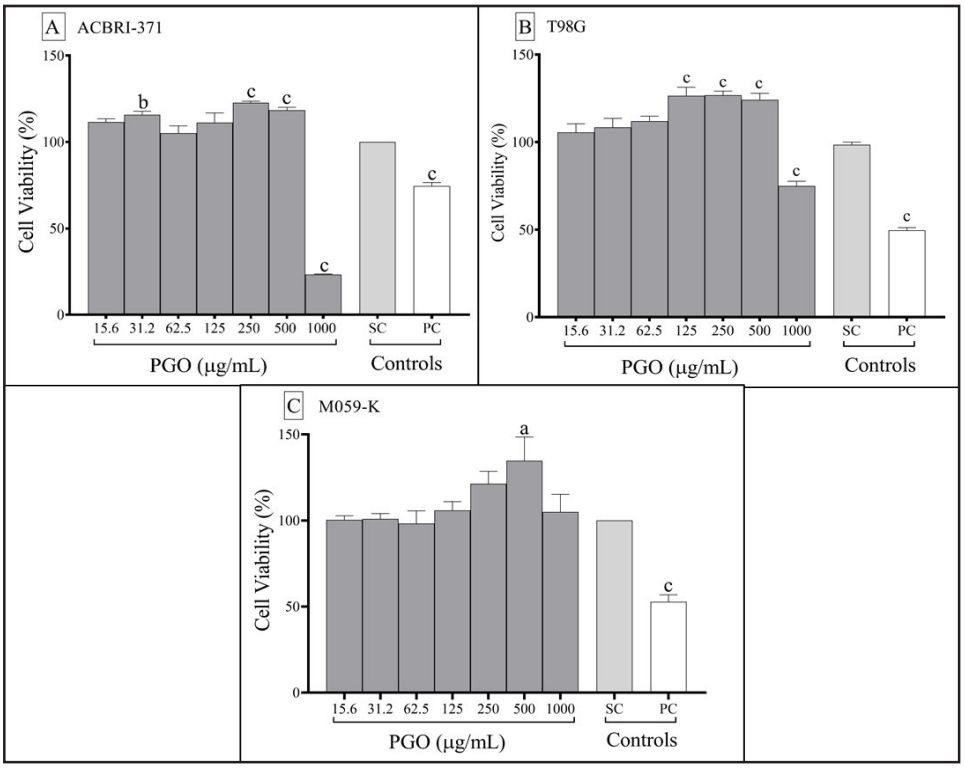
PGO demonstrated inhibitory activity against the proliferation of *P. aeruginosa* [$F(10, 20) = 73.52$, $p \leq 0.0001$] at concentrations equal to or greater than 200 µg/mL, while strains *S. pyogenes* [$F(10, 20) = 348.7$, $p \leq 0.0001$] and *E. faecalis* [$F(10, 20) = 49.57$, $p \leq 0.0001$] were inhibited in a dose-dependent manner at concentrations equal to or greater

than 400 µg/mL (Figure 1A, B, and C). PGO did not exhibit inhibitory activity against *S. aureus* (ATCC 6538), *S. aureus* (ATCC 25923), *S. epidermidis*, *E. coli* (ATCC 25922), *E. coli* (ATCC 25922), *K. pneumoniae*, *P. multocida*, *C. freundii*, *K. oxytoca*, *S. enterica*, *E. cloacae*, *P. stuartii*, or *P. mirabilis*. No inhibitory activity was observed against *C. glabrata* or *C. neoformans*.

Bacteria that exhibited significant growth inhibition in the presence of PGO compared with the SC were cultured again after treatment and resumed growth in the absence of oil. There was no growth in the subculture of microorganisms treated with 100 µg/mL of the PC. Therefore, PGO is considered to be bacteriostatic against *P. aeruginosa, E. faecalis*, and *S. pyogenes*.

3.3 Cell viability

Figure 2 – Percentage of viable cells (MTT assay) after treatment with different concentrations of *C. yacare* PGO ethanolic extract for 24 h



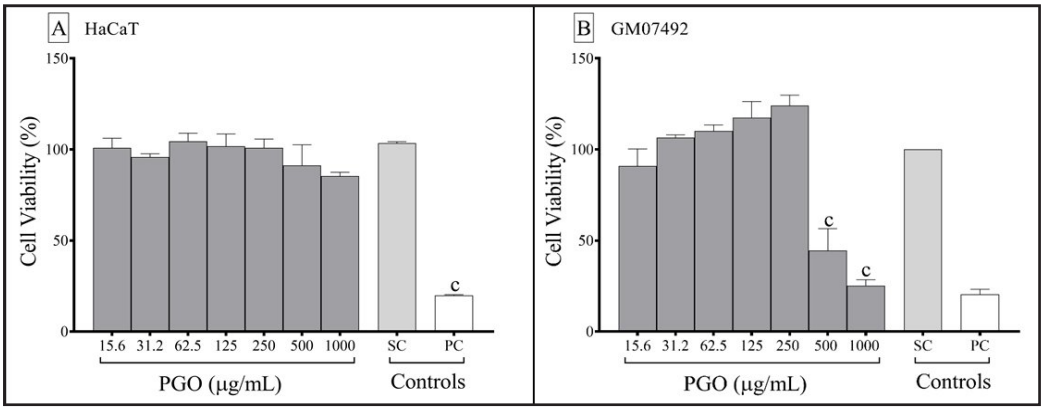
Notes: (A) Human normal astrocytes, ACBRI-371 lineage; (B) human glioblastoma multiforme, T98-G lineage; (C) human malignant glioma, M059K lineage. Values above the bars represent the mean ± SEM from three independent assays. One-way ANOVA followed by Dunnett's post-hoc test. Letters (a), (b), and (c) above the bars correspond to the values of (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$ versus the SC (1% absolute ethanol). The PC was 50 mM doxorubicin

Source: Authors (2023)

PGO demonstrated reduced cell viability of astrocytes ACBRI-371 [F (9,20) = 182.9, p ≤ 0.0001] and glioblastoma T98G cells [F (9,20) = 112.2, p ≤ 0.0001] at the highest concentration (1000 µg/mL). However, there was no reduction in the viability of malignant glioblastoma M059K cells [F (9,20) = 9.40, p ≥ 0.05] (Figure 1A and B). Interestingly, PGO promoted an increase in the conversion of the MTT salt to formazan blue at different concentrations in both tumor and non-tumor glial cell lines.

Regarding keratinocytes (HaCaT), PGO did not affect cell viability at any of the concentrations evaluated [F (9,20) = 35.51, p ≥ 0.05]. However, PGO reduced the viability of fibroblasts (GM07492) at the two highest concentrations (500 and 1000 µg/mL) [F (9,20) = 47.72, p ≤ 0.0001] (Figure 2D and E).

Figure 3 – Percentage of viable cells (MTT assay) after treatment with different concentrations of *C. yacare* PGO ethanolic extract for 24 h



Notes: (A) Human normal keratinocytes, HaCaT lineage; (B) human normal fibroblasts, GM07492 lineage. One-way ANOVA followed by Dunnett's post-hoc test. Values above the bars represent the mean ± SEM from three independent assays. Letters (a), (b), and (c) above the bars correspond to the values of (a) $p < 0.05$, (b) $p < 0.01$, and (c) $p < 0.001$ versus the SC (1% absolute ethanol). The PC was 50 mM doxorubicin
Source: Authors (2023)

4 DISCUSSION

This study is the first to investigate the chemical composition and antimicrobial activity of PGO as well as its, effect on the viability of human glial and cutaneous

cell lineages. Analytical chemical techniques revealed the heterogeneous chemical composition of PGO, including hydrocarbons, esters, ketones, and nitrosourea. *In vitro* biological activity assessment demonstrated the bacteriostatic capability and reduced viability of treated human cell lineages at high PGO concentrations.

The integration of FTIR and GC-MS techniques provided a comprehensive understanding of the structure and presence of compounds in PGO with potential bioactivity. The FTIR data revealed a peak at 2924.1 cm^{-1} corresponding to symmetric stretching vibrations of aliphatic CH and CH_3 groups found in vegetable oils such as canola, corn, olive, soybean, and sunflower oils (Javidnia et al., 2013). Additionally, a peak at 1712.8 cm^{-1} was observed, which was related to the vibration of the carbonyl group C=O present in triacylglycerol esters found in pork fat (Rohman et al., 2011).

FTIR analysis indicating the presence of these chemical groups, was subsequently validated by GC-MS. This strategic approach provides valuable insights into the identification of bioactive compounds. The composition of PGO of crocodilians, although consistent regarding the presence of certain lipids, may vary in relation to these compounds and other molecules, according to the diet, sex, and age of the animal (Vera-Candioti et al., 2021; Weldon et al, 1989). Based on the presence of semustine (a DNA alkylating agent) (Brandes et al., 2016), oleic and linoleic acids (wound healing) (Cardoso et al., 2004), and citronellol (antimicrobial activity) (Singh et al., 2012), assays were conducted to evaluate the antimicrobial activity and effect on the viability of human cell lineages of PGO.

PGO demonstrated inhibitory activity against certain bacterial strains, similar to the activity found in the oil extracted from the body fat of the *Melanosuchus niger* species, which exhibited inhibitory activity against *S. aureus*, *E. coli*, and *Salmonella enteritidis* at concentrations above 1000 µg/mL (Schmeda- Hirschmann et al., 2014). These results corroborate the traditional use of oils derived from crocodilian tissues to treat infections in indigenous communities (Alves et al., 2008).

However, PGO did not exhibit inhibitory activity against other bacterial or fungal strains. Previous studies have reported the antifungal activity of PGO against *Candida*

albicans (Azevedo et al., 2018), but the results of this study demonstrate a lack of activity against yeast strains. However, the susceptibility of microorganisms to natural product constituents is difficult to predict because of the possibility of multiple targets and heterogeneity in oil composition (Hyldgaard et al., 2012). The inhibitory activity of PGO may have been influenced by its solubility in the culture medium. Solubility is a limiting factor for effective activity against microorganisms reported in other studies on antimicrobials (Hood et al., 2003). Notably, a systematic review of 56 randomized clinical trials did not find a significant difference in the efficacy of bactericidal and bacteriostatic antibiotics (Wald-Dickler et al., 2018).

PGO did not selectively decrease the viability of tumor and non-tumor cells. Previous studies have reported wound healing effects in rats by the oil extracted from the visceral fat of *C. yacare* as well as no reduction in the viability of retinal epithelial cells (Azevedo et al., 2020) and in macrophages treated with oil from *C. siamensis* (Ngernjan et al., 2022). Although PGO shares the constituents oleic, linoleic, and arachidonic acids with oils from these species (Dunn et al., 1993; Weldon et al, 1989), the relative quantities of these compounds and the presence of other components in PGO may explain the observed differences in its *in vitro* action.

The responses obtained in the biological assays in this study can be partially explained by the genetic backgrounds of bacterial, fungal, and human cells. All cells have distinct sets of genes that regulate different metabolic pathways, defense systems, and cellular repair mechanisms (Junker & Oudenaarden, 2014; Morinet, 2013). These genetic variations can lead to varying levels of resistance and sensitivity to the compounds found in PGO. For example, DNA damage can activate repair mechanisms, resulting in the blockade of cell division progression to repair the damage or activation of cell death mechanisms when lesions exceed the processing capacity of the cell (Barzilai & Yamamoto, 2004). The different genetic profiles of bacterial, fungal, and human cells observed in this study may partially explain the bacteriostatic effects and decreased viability of cells treated with PGO.

Another result observed was an increase in the conversion of the MTT salt to formazan blue by the GM07492, ACBRI-71, T98-G, and M059K lineages. This may be related to increased mitochondrial metabolism due to the presence of oleic, linoleic, and arachidonic acids in PGO (Kastaniotis et al., 2017). Unsaturated fatty acids play different structural roles, influencing cellular membrane plasticity as well as cellular signal transduction and serving as substrates for ATP production during cellular respiration in the mitochondria (Santos & Preta, 2018; Wymann & Schneiter, 2008). An increase in mitochondrial metabolism can lead to an increase in the conversion of MTT salt, as this salt is converted in the mitochondria of healthy cells (Aslantürk, 2018). Therefore, PGO may increase mitochondrial metabolism in human cell lineages. This effect is evident in the M059K lineage, which possesses a gene (ND6) in the mitochondrial complex I subunit and that results in decreased mitochondrial metabolism (Dehaan et al., 2004; Leipnitz et al., 2018).

Although the highest concentrations tested in this study resulted in bacteriostatic effects and a non-selective reduction in cell viability in both tumor and non-tumor cell lineages, investigating the influence of sex, age, and diet on the composition of PGO is necessary. Understanding the interaction of these factors with the genetic profiles and metabolic pathways of bacterial, fungal, and human cells is essential to assess the toxicity and therapeutic potential of this compound. Studies examining these variables can provide valuable insights for the development of targeted and safe therapeutic strategies based on PGO-containing natural products. Furthermore, the fractionation of PGO may pave the way for the discovery of specific components with more effective biological activities.

5 CONCLUSIONS

Under the experimental conditions used in this study, PGO exhibited a heterogeneous composition of molecules with pharmacological activity, as described in the literature. Additionally, PGO displayed a specific bacteriostatic effect on a

group of bacteria as well as a non-selective decrease in the viability of human glial and cutaneous cell lineages. The development of natural products containing PGO for human consumption could add scientific value to the captive breeding of *C. yacare*. This advancement is an important step towards the future utilization of this raw material, which, until the present study, remained without economic value.

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SUPLEMENTARY MATERIAL

Table 1 – Fourier transform infrared spectroscopy (FTIR) analysis of *the C. yacare* (PGO) ethanolic extract (Continue)

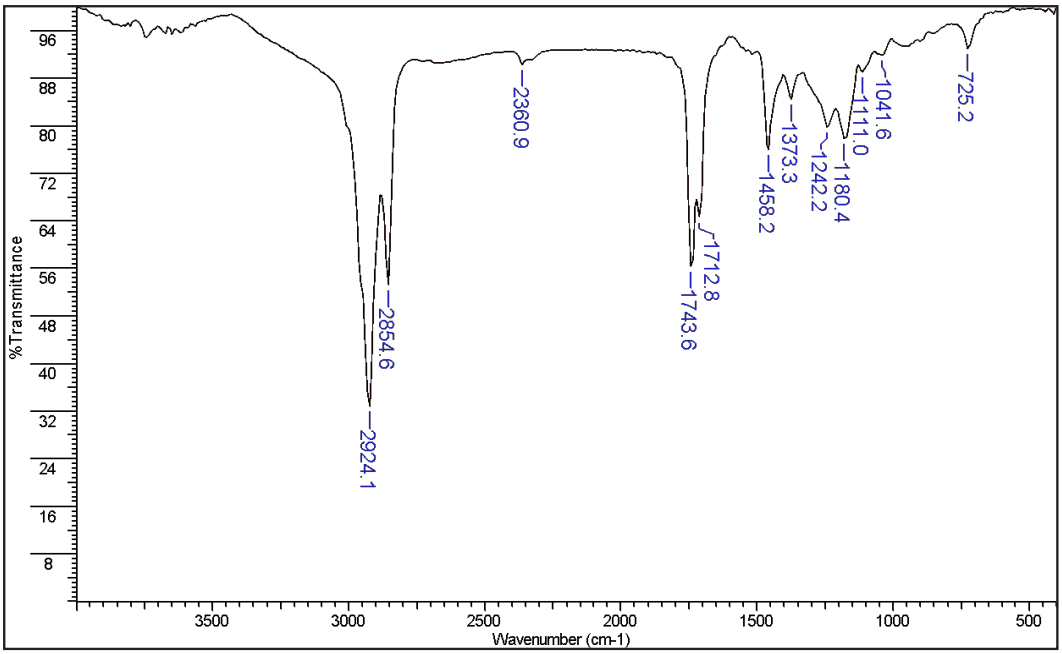
Present in	Wavenumber (cm^{-1})	Assignments	Functional Group Name	Reference
Olive oil, Sunflower oil, Corn oil,Vegetable oil, Margarine, Butter	2924,1	-C-H (CH2), asymmetrical stretching vibration	Double bond hydrocarbons	(Guillén; Cabo, 1997)
Soybean oil	2854,6	-C-H, symmetrical stretching	Double bond hydrocarbons	(Christy; Egeberg, 2006)
Unidentified component	2360,9	-C≡N, sretching	Nitrile group	(Socrates, 1994)
Olive oil, Sunflower oil, Corn oil,Vegetable oil, Margarine, Butter, Peanut, Safflower, Canola	1743,6	C=O of triglycerides, stretching	Free fatty acids	(Guillén; Cabo, 1997) (Muik Et Al., 2007)
Olive oil, Sunflower oil, Corn oil,Vegetable oil, Margarine, Butter	1712,8	C=O of triglycerides, stretching	Free fatty acids	(Guillén; Cabo, 1997) (Rohman, 2017)
Olive oil, Sunflower oil, Peanut, Safflower, Canola	1458,2	-C-H (CH2) *cis*, bending	Simple hydrocarbons	(Muik Et Al., 2007)
Olive oil, Sunflower oil, Corn Oil, Vegetable oil, Margarine, Butter, Peanut, Safflower, Canola	1373,3	C-H-C, symmetrical bending vibration	Methyl group	(Guillén; Cabo, 1997) (Muik Et Al., 2007)
Propolis, Pomegranate, Sage, Dragon's blood	1242,2	C-O-C, asymmetrical stretch	Ether	(Oliveira, R.n Et Al., 2016)

Table 1 – Fourier transform infrared spectroscopy (FTIR) analysis of *the C. yacare* (PGO) ethanolic extract (Conclusion)

Present in	Wavenumber (cm^{-1})	Assignments	Functional Group Name	Reference
Unidentified component	1180,4	-C-H, stretching Quaternary dimethil carbon compounds have a characteristic absorption near 1180 cm^{-1} (8,48 ~µm).	Hydrocarbons	(Socrates, 1994)
Olive oil, Sunflower oil, Corn oil, Vegetable oil, Margarine, Butter	1111	C-C(=O)-O, asymmetric coupled vibrations	Carbonyl group	(Guillén; Cabo, 1997)
Olive oil, Sunflower oil, Corn oil, Vegetable oil, Margarine, Butter	1041,6	O-C-C, asymmetric coupled vibrations	Carbonyl group	(Guillén; Cabo, 1997)
Vegetable oil	725,2	-C-H (CH_2), bending (rocking)	Hydrocarbons	(Mirghani; Che Man, 2003)

Notes: Wavenumber (cm^{-1}): Wavelength of absorbed spectra. Unidentified component: Author didn't identified the sample's origin
Source: Authors (2023)

Figure 1 – Fourier transform infrared spectroscopy (FTIR) spectrum of the *C. yacare* (PGO) ethanolic extract



Source: Authors (2023)

Autorship contributions

1 – Natasha Rayane de Oliveira Lima

Doctorate degree student in Environmental Sciences at Universidade do Estado de Mato Grosso
https://orcid.org/0000-0002-7238-8476 • natasha.rayane@unemat.br
Contribution: Conceptualization; Investigation; Methodology; Project administration; Data curation; Writing – original draft

2 – Lucas Polizzeli Azevedo

Doctorate degree student in Health Sciences at Universidade Federal de Mato Grosso
https://orcid.org/0000-0002-5704-5950 • lpa.ufmt.enf@gmail.com
Contribution: Conceptualization; Investigation; Methodology; Project administration; Data curation; Writing – review & editing; Funding acquisition

3 – José Vitor de Menezes Costa

Master's degree student in Health Sciences at Universidade Federal de Mato Grosso
https://orcid.org/0000-0002-9038-8859 • jose.vitor@unemat.br
Contribution: Data curation; Investigation; Visualization; Validation; Writing – review & editing.

4 – Leandro Nogueira Pressinotti

PhD in Cellular and Tissue Biology at Universidade de São Paulo
https://orcid.org/0000-0003-2682-1598 • microtomo@unemat.br
Contribution: Conceptualization; Methodology; Data curation; Formal Analysis; Funding acquisition; Supervision

5 – Rhavena Graziela Liotti

PhD in Biotechnology and Biodiversity from Universidade Federal de Mato Grosso
https://orcid.org/0000-0001-8492-8823 • rhavena.liotti@ifmt.edu.br
Contribution: Conceptualization; Methodology; Data curation; Formal Analysis

6 – Larissa Maria Scalon Lemos

Doctorate in Health Sciences, with an emphasis on Pharmacology of Natural Products, from Universidade Federal de Mato Grosso
https://orcid.org/0000-0003-2706-0305 • larissa@unemat.br
Contribution: Conceptualization; Methodology; Data curation; Formal Analysis

7 – Carmen Lucia Bassi-Branco

PhD in Biological Sciences (Genetics) from Universidade de São Paulo
https://orcid.org/0000-0003-4981-6556 • carmen.bbranco@gmail.com
Contribution: Conceptualization; Data curation; Formal Analysis; Validation; Writing – review & editing

8 – Rosa Helena dos Santos Ferraz

Doctorate in Anatomy of Domestic and Wild Animals from Universidade de São Paulo
https://orcid.org/0000-0002-9980-4994 • rhsferraz@gmail.com
Contribution: Conceptualization; Data curation; Writing – original draft

9 – Leonardo Gomes de Vasconcelos

PhD in Biotechnology from Rede de Biodiversidade e Biotecnologia da Amazônia Legal
https://orcid.org/0000-0002-2886-1887 • vasconceloslg@gmail.com
Contribution: Conceptualization; Data curation; Formal Analysis; Methodology

10 – Samuel Vandresen Filho

PhD in Neurosciences from Universidade Federal de Santa Catarina
https://orcid.org/0000-0002-4718-2900 • samuelvandresen@yahoo.com
Contribution: Conceptualization; Data curation; Formal Analysis; Writing – review & editing

11 – Fabrício Rios-Santos

Master's and Doctorate in Pharmacology from Universidade de São Paulo, post-doctorate in Pharmacology of Natural Products
https://orcid.org/0000-0001-7151-1631 • fabriciorios.juris@gmail.com
Contribution: Data curation; Formal Analysis; Funding acquisition; Validation; Writing – review & editing

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