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Lecithin-based nanocapsule loading sucupira (*Pterodon emarginatus*) oil effects in experimental mucositis

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ABSTRACT

Intestinal mucositis (IM) is a frequent adverse effect in anticancer therapy without standard treatment. The oil obtained from sucupira (Pterodon emarginatus) has anti-inflammatory properties, and the soybean lecithin reduces the intestinal toxicity of several xenobiotics. However, their water insolubility impairs the in vivo application. For this reason, we evaluated if the nanoencapsulation of sucupira oil (SO) in lecithin-based nanocapsules (SO-NC) could be a therapeutically effective system for the treatment of IM in murine cisplatin (CDDP)-induced intestinal mucositis model. SO was analyzed by LC-HRMS/MS and HPLC. SO-NC was prepared by nanoprecipitation and characterized using DLS, HPLC, and AFM. Mice body weight and food consumption were assessed daily during experimental mucositis induced by CDDP. The animals were euthanized, and intestinal permeability, inflammatory mediators, and intestinal histology were performed. SO-NC demonstrated adequate characteristics for oral administration as size under 300 nm, IP < 0.3, high EE, and spherical shape. *In vitro* cytotoxicity performed against RAW 264.7 cell lines resulted in cell viability above 80 % confirming the non-cytotoxic profile of SO (IC_{50} 268 μg/mL) and SO-NC (IC_{50} 118.5 μg/mL) up to 117.2 μg/mL. The untreated mice showed intestinal toxicity after i.p. of CDDP, principally weight loss, increased intestinal permeability, and MPO and TNF-α levels. Surprisingly, the administration of SO to CDDP-mucositis animals did not circumvent the CDDP effects and increased intestinal permeability. However, SO-NC proved efficient in mitigating the experimental intestinal mucositis by improving intestinal epithelium architecture, reducing intestinal permeability, and improving the MPO levels. In conclusion, SO-NC can positively impact intestinal mucositis by promoting mucosal recovery. This is a promising strategy for developing a new treatment for intestinal mucositis.

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1. Introduction

Intestinal mucositis is a common complication associated with cancer chemotherapeutics irinotecan, methotrexate, 5-fluorouracil, and cisplatin. Clinically, it can present pain, nausea, vomiting, diarrhea, and constipation [1,2]. These adverse effects significantly impact patients' life since the chemotherapy treatment is modified or suspended, compromising the effectiveness of the therapy [2–4] Mucositis is characterized by damage to the gastrointestinal epithelium and involves intracellular signaling and inflammation. These changes are associated with a disruption of the intestinal epithelium integrity as an increase in intestinal permeability and mucus layer metabolism alteration [5–7]. Besides, inflammatory infiltration and proinflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) are associated with early epithelium damage [2,8]. Despite its clinical importance, intestinal mucositis treatment remains a challenge [2,9]. However, some recent studies have shown the use of immunonutrients [5,10] and vegetal-derived natural products [11] to minimize the side effects of intestinal mucositis in experimental models. In this context, there are reports on the beneficial effects of plants of the genus Pterodon, popularly known in Brazil as "sucupira", on inflammatory responses in animal models [12].

The plant *Pterodon emarginatus* Vogel, Fabaceae (Leguminosae) or sucupira is an aromatic brazilian species, present in the Amazon and Cerrado area. It is widely used by folk medicine to treat rheumatism, sore throat, and respiratory disorders [12–15]. Sucupira oil (SO) has an amber color and can be extracted from the seeds. Its phytochemical composition demonstrates the presence of diverse terpenes, among them β-caryophyllene (CAR) and 6α,7β-Dihydroxyvouacapan-17β-oic acid may be associated with the anti-inflammatory activity [14,16–18]. However, whether SO works and its mechanism in treating intestinal mucositis have not been reported yet. In addition, the lipophilic characteristics of oils and their derived substances in water hinder in vivo application. To circumvent this inconvenience, an efficient approach could employ a carrier system to deliver the drug to the target site.

Nanocarrier-based oral drug delivery is promising for intestinal mucositis drug delivery and targeting due to their accumulation in the inflamed areas prolonging the residence at the site of inflammation [19]. Moreover, it has been reported that nanocarriers can ameliorate oral mucositis [20]. Among these nanocarriers are nanocapsules (NC), a vesicular system consisting of an oily core surrounded by a polymeric wall [21]. Emulsifying substances, such as lecithins, perform the stabilization of these systems. They are natural mixtures of polar and neutral phospholipids, obtained from vegetable or animal sources, with protection properties reported in inflammatory bowel disease [22].

NC have been used for different purposes, among them increasing the dispersibility of oils and lipophilic molecules [23,24], reduce drug toxicity [25], protect the encapsulated substance against inactivation [24], control drug release [23,26], and increase therapeutic efficacy after oral administration [27].

The present study aimed to evaluate nanocapsules as nanocarrier drug delivery systems for intestinal mucositis treatment. SO and lecithin were chosen for the production of NC as potential candidates to obtain an anti-inflammatory product. The evaluation of SO-NC was performed in vivo in murine cisplatin (CDDP)-induced mucositis model.

2. Materials and methods

2.1. Chemicals

Cisplatin (CDDP), β-caryophyllene (CAR) and poly-ε-caprolactone polymer (PCL, 42,500 Da) were obtained from Sigma (St Louis, MO, USA), soybean lecithin (Lec, Lipoid® S75 from Lipoid (Gmbh, Germany), sodium chloride from Synth (São Paulo, SP, Brazil). The $^{99\,m}$Tc (pertechnetateform) was obtained as a saline solution from an alumina-based 99Mo generator (IPEN/Brazil). The SO was previously obtained from fruits by cold pressing and kindly donated by Prof. Edemilson Cardoso da Conceição from Universidade Federal de Goiás (Brazil). All other chemicals used in this study were analytical grade. Water was purified by reverse osmosis (Simplicity 185, Millipore, Bedford, USA).

2.2. Sucupira oil characterization

Sucupira oil (SO) was dissolved in isopropanol to 1 mg mL^{-1}. It was analyzed by liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS) using a Thermo Dionex UHPLC system coupled to a Thermo Quadrupole-Orbitrap QExactive Plus mass spectrometer. The sample was analyzed in negative ion mode using a HESI electrospray ionization source at a voltage of 3.6 kV, with a capillary temperature of 300 °C, sheath, and aux gases of 50 and 10, respectively, and RF voltage level of 50. The LC column was a Waters Xbridge Amide (150 mm × 4.6 mm, 3.5 μm i.d.) maintained at 50 ºC and the sample injection volume was 10 μL. Separation was performed in gradient elution mode at a flow rate of 500 μL min^{-1} using water: acetonitrile: (95:5 %) as mobile phase A and acetonitrile: water (95:5 %) as mobile phase B, both containing 0.1 % of ammonium hydroxide. The gradient was 0–2 min B 95 %, 2–18 min B 95–50 %, 18–21 min B 50 %, 21–28 min B 95 %. The LC-HRMS/MS data were processed in the Thermo Xcalibur software v3, and compound annotation was performed by comparing the MS and MS/MS data to those available in public databases (MassBank of North America, https://mona.fiehnlab.ucdavis.edu/) and based on chemical structures of compounds previously isolated from *Pterodon* species. [28–30] In addition, CAR content presented in SO was quantified by HPLC from previously described assay [31].

2.3. Nanocapsule preparation

Biodegradable nanocapsules containing sucupira oil (SO-NC) and soybean lecithin (Lec) were prepared by interfacial deposition of a preformed polymer followed by solvent evaporation first reported by [21]. Briefly, PCL (0.6 % w/v) was dissolved in 5 mL of acetone solution containing 0.75 % w/v of Lec (Lipoid S75) and 2.5 % v/v of SO. Then, this organic solution was poured into the water (1: 2) with stirring for ten minutes to promote the formation of nanocapsules. Then, the organic solvent and part of the water were evaporated under reduced pressure to 5 mL (Quimis, Brazil).

2.4. Characterization of the nanocapsules

2.4.1. Size and zeta potential

The hydrodynamic diameter and polydispersion of the NC were determined at 25 °C by the dynamic light scattering method (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK) in three different batches. The surface charge or zeta potential (ζ) was performed by microelectrophoresis coupled to laser Doppler anemometry on the same equipment. Samples were analyzed after 1:40 dilution in water. Measurements were performed in triplicate, and results were expressed as the mean diameter ± standard deviation.

2.4.2. Encapsulation efficiency

Untrapped CAR was found in the ultrafiltrate after centrifuging the samples in AMICON device (Microcon, 50 kDa MWCO, Millipore®) (500g for 30 min). HPLC assayed the CAR present in the ultrafiltrate. The total amount of CAR in the NC suspension was assessed by dissolving 100 μL of NC colloidal suspension in acetonitrile (2.5 mL), vortex-mixed for 5 min to disrupt the NC and releases the drug. Then, the volume was completed to 5 mL with acetonitrile, mixed for 5 min, centrifuged, and supernatant filtered (0.45 μm, 25 mm filters) before HPLC injection. The CAR quantification was performed by HPLC method described below. The analyses were performed in triplicate. The process efficiency of CAR encapsulation in the NC was calculated following Eq. (1), as previously reported elsewhere [23]. CAR encapsulation efficiency

(%) represents the weight percentage of feed CLOXB that was encapsulated in the process, considering the not encapsulated fraction was present in the ultrafiltrate.

$$\text{Encapsulation efficiency\%} = \frac{\text{Amount of BCAR in NC}}{\text{Initial BCAR amount}} \text{x100} \quad (1)$$

2.4.3. Determination of CAR by HPLC

The CAR content was assayed on a Waters Alliance 2695 HPLC system, equipped with an autosampler, pump, column oven, and a UV detector (Waters 2996) set at 210 nm. The separation was carried out at 34 °C on a C18-RP LiChroCART® (150 mm × 4.0 mm, 5 μm particle size) and guard (5 mm × 4.0 mm, 4 μm) columns (Merck). The isocratic mobile phase was composed of a mixture of acetonitrile: water (70:30 v/v) at a constant flow rate of 1.2 mL/min as previously reported [31]. The injection volume was 10 μL for all standards and samples. The assay was linear in the 1–100 μg/mL concentration range. The limit of detection (LOD) and limit of quantification (LOQ) were 0.03 and 0.09 μg/mL, respectively. The intra and inter-day coefficients of variation were ± 5 % and within the recommended limits. No interfering peaks were detected in the assay.

2.4.4. NC morphology analysis

A sample of 10 μL of SO-NC (diluted 1/10 in ultrapure water) was deposited onto freshly cleaved mica and analyzed in atomic force microscopy (AFM). The samples were purged with argon and imaged in Tapping® mode on a Dimension Icon multimode AFM (Bruker) using soft Tapping® mode AFM probes (RTESPA-150 from Bruker). Diameter and height analyses were performed using the "particle analyses'' program of the Nanoscope Analysis 1.7 system.

2.4.5. Storage stability

The size distribution and zeta potential of SO-NC was assessed by DLS as a function of time (zero, 7, 14, 21, and 28 days), with the suspensions stored in glass flasks at 4°C and room temperature protected from light. All measurements were performed in triplicate.

2.5. In vitro experiment

2.5.1. Cell cuture

The murine macrophage cell line RAW 264.7 (ATTCC TIB-71) was acquired from ATCC (Manassas, USA), cultured in Roswell Park Memorial Institute (RPMI) supplemented with 10 % heat-inactivated FBS, 1 % Penicillin/Streptomycin, 10 mM HEPES and 3.7 g/L Sodium Bicarbonate at 37 °C in a 5 % CO_2.

2.5.2. Cell viability studies

The cytotoxicity was evaluated in RAW 264.7 cells using the MTT method. Briefly, RAW 264.7 cells were plated at 2×10^4 cells/well. The cells were incubated with SO-NC or SO in equivalent oil concentrations ranging from 58.6 μg/mL to 1875.2 μg/mL for 24 h at 37 °C and 5 % CO_2. Controls with only medium and with medium supplemented DMSO were also included. The MTT assay was performed as previously described [32]. The IC_{50} values for the different formulations were calculated using GraphPad Prism 5 program (USA). All assays were conducted in triplicate.

2.6. In vivo efficacy of SO-NC

An in vivo study was performed in Swiss male mice (25–31 g, 4 weeks; Faculty of Pharmacy, Federal University of Minas Gerais). Animals were housed under standard conditions in an area with a standardized light/dark cycle and received water and food ad libitum. The experimental protocol was approved by the animal experimentation committee of the Federal University of Minas Gerais (Brazil, CETEA 66/2018). Mucositis was induced administering a single dose of CDDP 10 mg/kg intraperitoneally (i.p.) 5 days after the beginning of treatment [33]. The control group received the same intraperitoneal dose of sterile saline. The animals were randomly divided into the five following groups (n = 6/group): control group (without mucositis + water), CDDP group (mucositis + water), SO + CDDP group (mucositis + SO dispersed with 0.75 % of Lec in water) and SO-NC + CDDP group (mucositis + SO-NC). Water, SO, and SO-NC (50 mg/kg/day of SO and 15 mg/kg/day of Lec) were administered daily by oral route on days 0–7. On day 8, the animals were sacrificed, and ileum samples were collected for evaluation of the severity of mucositis. The body weight and food consumption were also monitored during the experiment.

2.6.1. Intestinal permeability (IP)

The intestinal permeability was evaluated using a solution of diethyleneaminepentacetic acid (DTPA) radiolabeled with technetium-99 m ($^{99\,m}$Tc). On the 8th day of the experiment, the animals received 0.1 mL of $^{99\,m}$Tc-DTPA containing 18.5 MBq of activity by gavage. After four hours, mice were anesthetized, and blood collected and placed in appropriate tubes for radioactivity determination. The radioactivity of the standard dose and blood samples was determined in automatic scintillator (Wizard, Perkin Elmer, Finland). A standard dose corresponding to 0.1 mL of the drug solution with $^{99\,m}$Tc-DTPA was also subjected to radiation determination. The results were compared with the standard dose, and data were expressed as % dose, using the following equation: % dose/g = (cpm in g of blood/cpm of standard) × 100) where cpm is counting per minute.

2.6.2. Histological analysis

Ileum segments were processed for histological analysis, as described previously [33]. Briefly, the tissues were rolled up and fixed in a 10 % buffered formalin solution. The histological Section (4-5 μm) were stained with hematoxylin and eosin for a detailed histological study of intestinal inflammation.

2.6.3. Myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) activities

The extent of neutrophil and macrophage infiltration in the ileum tissue was measured by assaying enzyme activities of myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG), respectively, as described previously [34]. The protein content of the samples was determined according to the Lowry method [35]. After protein quantification, the results obtained for MPO and NAG enzyme activities were corrected and expressed per mg protein.

2.6.4. Measurement of cytokine concentrations

The concentration of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) were measured in the ileum by ELISA using commercially available kits and according to the procedures supplied by the manufacturer (R & D Systems, Minneapolis, USA).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). The results were evaluated by Kolmogorov–Smirnov test for normality. Data with normal distribution were tested using Student's t-test or one-way ANOVA followed Newman-Keuls test, whereas non-parametric data were tested using a Kruskal-Wallis ANOVA and Dunn's post-test. Results were expressed as mean ± standard error of the mean (SEM). A P value < 0.05 was considered significant.

3. Results

3.1. Sucupira oil chemical composition

The LC-HRMS/MS base peak chromatogram of SO in negative mode

is shown in Fig. 1. Peaks of high abundance were observed in the first 5 min of the run. The hydrophilic interaction chromatography (HILIC) using an amide stationary phase was chosen for this analysis instead of the typical octadecylsilane (C_{18}) phase to avoid strong interactions and high retention between non-polar compounds commonly found in oil materials with the LC stationary phase. Six vouacapane diterpenoids were tentatively identified based on their high-resolution *m/z* values and MS/MS fragmentation patterns (Table 1, Fig. 1B). They were observed between 1.5 and 2.5 min. Their molecular structures and stereochemical configurations were suggested based on those described for diterpenoids in Pterodon species [16]. In addition to these diterpenes, other compounds were also annotated, as shown in Table 1.

Vouacapane diterpenoids are commonly found with hydroxyl or carboxyl groups in their structures. Thus, verifying fragment losses related to these groups is a helpful tool in identifying the chemical structures of these compounds by MS/MS. According to Table 1, the peak with retention time (Rt) of 2.15 min showed a deprotonated molecule $[M\text{-}H]^-$ at m/z 313.17876, consistent with a molecular formula of $C_{20}H_{25}O_3$ (calc. 313.18092, error = −5.1 ppm). The fragmentation of this ion gave a peak at m/z 269.19229 (loss of a carboxyl group). This compound was annotated as vouacapan-17β,7β–lactone. A hydroxylated derivative of this lactone was detected at the peak with a Rt of 1.93 min. The deprotonated molecule detected at m/z 329.17554 indicated the molecular formula $C_{20}H_{25}O_4$ (calc. 329.175833, error = 0.8 ppm). The fragmentation pattern of this signal showed the ions at 285.18588 ($-CO_2$) and 267.17387 (subsequent loss of H_2O). Thus, the substance was annotated as 6α-hydroxyvouacapan-7β-17-β-lactone. Although the vouacapane diterpene was not found in the LC-HRMS/MS analysis of SO, its hydroxylated derivative 6α-hydroxyivouacapane (Rt 2.36 min) was annotated by the deprotonated molecule at m/z 301.21820 (calc. 301.21730, error = 0.90 ppm), which did not show signs of fragmentation under the conditions of analysis. The fragmentation of the signal at m/z 315.19629 generating the ion at m/z 271.20567 allowed its identification as 17β-vouacapanoic acid. In addition, two other substances with a vouacapan skeleton were detected but could not be noted. Peaks 3 (Rt 20.8, m/z 377.23238) and 7 (Rt 2.20, m/z 331.19202) showed similarities in their fragmentation patterns. The substance of peak 3 generates the substance of peak 7 as a fragment, suggesting that they are analogous compounds. Previously, the composition of *Pterodon pubescens* oil was determined by ESI(+)-MS/MS analysis, resulting in the identification of several diterpenoids, including 14,15-epoxy-geranylgeraniol [36]. In the SO, the percentage amount of CAR was 16 % p/p. Such data demonstrate that our results are in accordance with those described in the literature.

3.2. Physicochemical characterization and stability of lecithin-based nanocapsules loading sucupira oil

The SO-NC had a mean size of 215 ± 12 nm, a PdI of 0.12, and zeta potential of − 39.00 ± 3 mV. The encapsulation efficiency was higher than 88 ± 2 %. To further characterize the formulations, SO-NC was analyzed by AFM. In Fig. 2, it can be shown that NC had a spherical shape. Furthermore, the determined diameter by AFM was 128.8 ± 38.1 nm. The nanocapsules presented a smaller size when using AFM than when measured by DLS. This can be explained by the fact that the NC was not in a hydrated state as they are during the DLS measurements. The diameter/height (D/h) ratios were calculated from the AFM images, and values closer to 1 indicate low flattening. Here the D/h ratio obtained was around 10, in accordance with other polymeric NC [23] indicating the flattening of this nanosystem. Fig. 3 shows the evolution of SO-NC size, PdI, and zeta potential stored at room temperature and 4ºC as a function of time. During the storage period, the sizes were kept under 240 nm and did not change significantly, regardless of the storage condition. The PdI varied for both conditions; however, at 28 days, its value was similar to day 0 ($p > 0.05$) when SO-NC was stored at 4 °C, indicating superior stability in this condition. In addition, all the polydispersity indices remained below 0.3, indicating monodispersed character during the evaluation period. The zeta potential analysis varied for both conditions along 28 days, however the values were above 30 mV (in modulus) providing electrostatic repulsion between nanocapsulesthat generally contribute to particle stability, as observed with our formulation in the storage period.

3.3. In vitro

The MTT test was employed to evaluate the cytotoxicity of SO-NC or SO. Results presented in Fig. 4 show cell viability over 80 % for SO and SO-NC up to 117 μg/mL of oil (corresponding to 31.2 μg/mL of polymer). The IC_{50} of SO was 268 ± 0.03 μg/mL, whereas SO-NC showed an IC_{50} of 118.5 ± 0.03 μg/mL, demonstrating that nanoencapsulation decreased IC_{50} value.

3.4. In vivo therapeutic activity of the lecithin-based nanocapsules loading sucupira oil

3.4.1. Weight changes and food consumption

It can be seen in Fig. 5A and C that oral administration of SO-NC had no significant effect on body weight and food consumption before the mucositis induction ($p < 0.05$) when compared to the control group. However, a difference can be seen in food consumption but not in body weight between SO and SO-NC groups ($p > 0.05$) which may be related to SO palatability. After the mucositis induction, weight loss and a decrease in food consumption were observed in all mucositis-treated groups (Fig. 5B and D) when compared to the control group, demonstrating that mucositis was effectively induced ($p < 0.05$).

3.4.2. Intestinal permeability

IP was higher in the CDDP group compared with the control group (Fig. 6), suggesting intestinal toxicity after intraperitoneal injection of cisplatin. A higher intestinal permeability was observed in OS + CDDP group compared with all groups ($P < 0.05$). However, oral administration of SO-NC in CDDP-induced mucositis mice reduced intestinal permeability compared with the CDDP and SO + CDDP groups.

3.4.3. Histological analysis

The morphological structure of the ileum in the control group presented normal limits, with the preservation of the intestinal villi and epithelium (Fig. 7A). However, the water-treated CDDP group presented modified intestinal epithelium architecture and a reduction in the size of the intestinal villi (Fig. 7B). The worst atrophy data was observed for the SO + CDDP group (Fig. 7C). The treatment with SO-NC presented an intermediate aspect between the mucositis (CDDP) and control group (Fig. 7D).

3.4.4. Inflammatory infiltration and cytokynes in ileum

The MPO activity, an indirect measurement of neutrophil infiltration, had no difference in the ileum between the Control and SO-NC + CDDP groups (Fig. 8A). However, an increase in its levels could be seen for CDDP and SO + CDDP groups. The NAG activity, an indirect measurement of macrophage infiltration, demonstrated no difference between the groups (Fig. 8B). Concerning cytokines, TNF levels were increased for SO + CDDP and SO-NC + CDDP groups with no differences (Fig. 8C). Interestingly, no differences were observed for IL-10 for all groups ($p > 0.05$) (Fig. 8D).

4. Discussion

There is an urgent need for the development of new strategies to reduce the adverse effects on oncological patients. The use of drug delivery systems as the nanocarriers is a promising strategy for intestinal mucositis treatment. In this sense, it has been demonstrated that nanocarriers containing anti-inflammatory molecules are considered

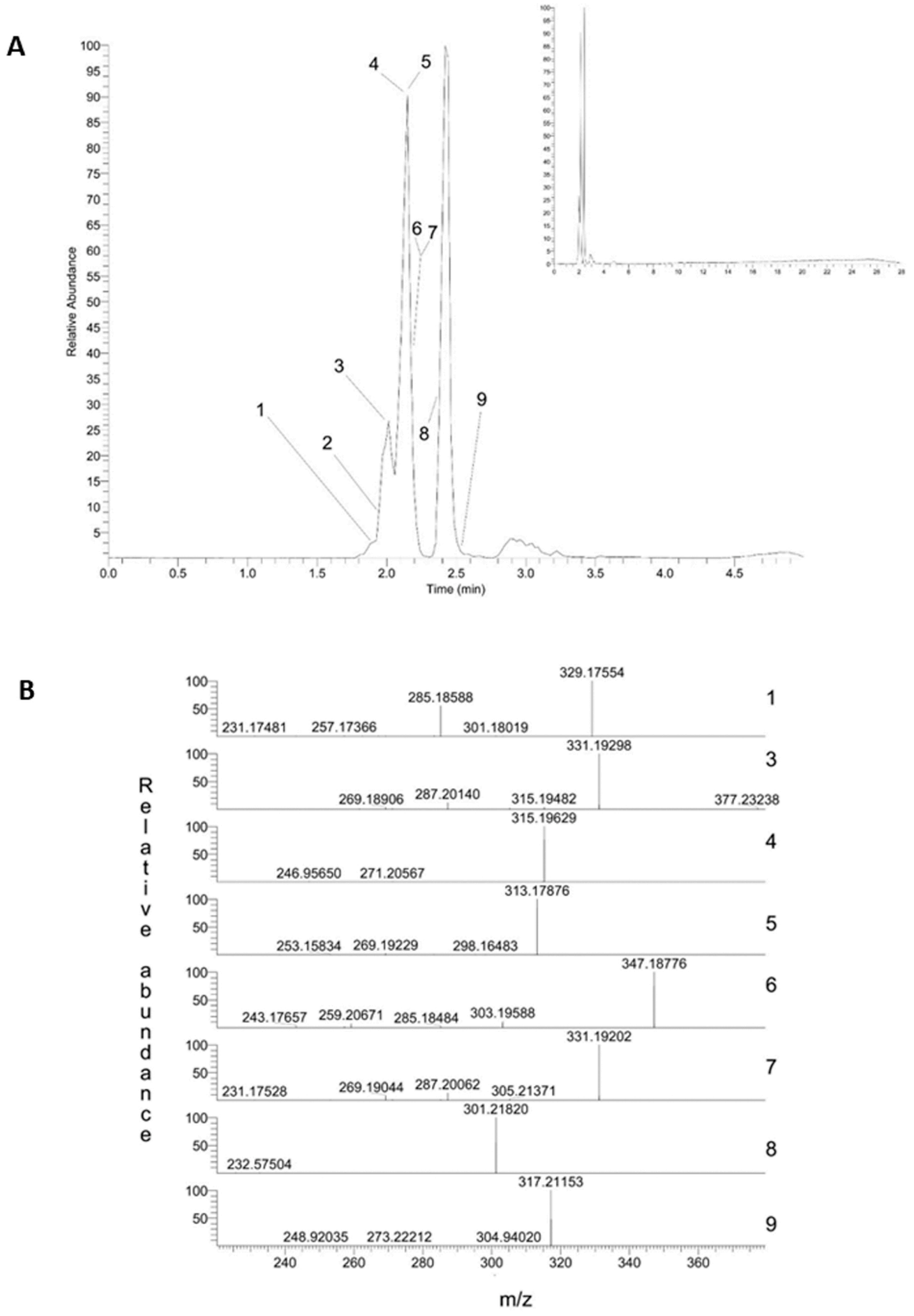

Fig. 1. - LC-HRMS/MS analysis in negative electrospray ionization mode for the *P. emarginatus* oil-resin. A) LC-HRMS base peak chromatogram. B) MS^2 Spectra of peaks 1, 3–9. (Peak 2 was not fragmented due to low concentration).

Table 1
Compounds tentatively identified in *P. emarginatus* oil-resin by LC-HRMS/MS in negative mode.

Peak	t_R (min)	Experimental *m/z*	Molecular formula	Error (ppm)	MS/MS	Compound ID
1	1.93	329.17554	$C_{20}H_{25}O_4^+$	0.8	285.18588 267.17387	6α-hydroxyvouacapane-7β-17 β-lactone
2*	1.97	305.24872	$C_{20}H_{33}O_2$	0.12	–	14,15-epoxygeranylgeraniol
3	2.08	377.23238	$C_{22}H_{33}O_5$	-1.1	331.19298 315.19482 287.20140 269.18906	Unknown
4	2.15	315.19629	$C_{20}H_{27}O_3$	0.9	271.20567	Vouacapan-17-oic acid
5	2.15	313.17876	$C_{20}H_{25}O_3$	-5.1	283.13367 269.19229 253.15834	Vouacapan-17 β, 7β–lactone
6	2.20	347.18776	$C_{20}H_{27}O_5$	-3.9	303.19588 285.18484 259.20671	Vouacapandioic acid
7	2.20	331.19202	$C_{20}H_{27}O_4$	0.83	287.20062 269.19044	Unknown
8	2.36	301.21820	$C_{20}H_{29}O_2$	4.8	–	6α-Hydroxyvouacapane
9	2.51	317.21153	$C_{20}H_{29}O_3$	-2.2	273.22212	Vouacapanoic acid

Rt: retention time. *Peak not fragmented due to low concentration.

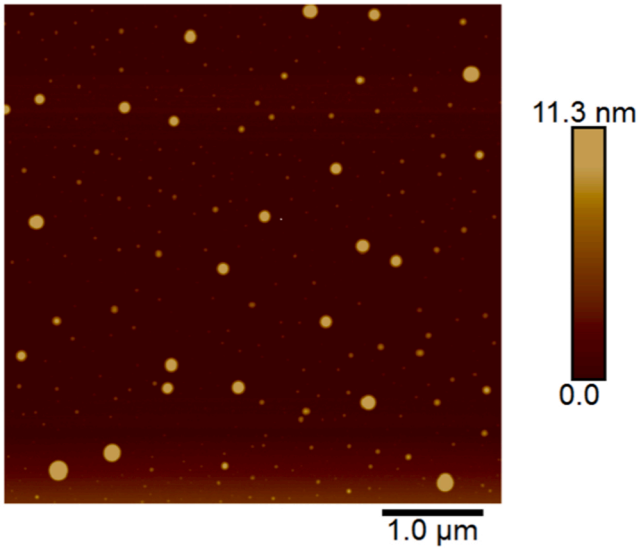

Fig. 2. - Atomic force microscopy images of SO-NC (scale 5 × 5 µm).

potential treatments approach since they are able to accumulate in the inflammed sites [19]. In addition, the presence of lipid excipients or bioactive oils in those systems is reported to have anti-inflammatory effect in vivo [17,19,37]. Regarding its chemical composition, all the annotated compounds by LC-HRMS/MS and HPLC in SO, especially vouacapane diterpenoids and β-caryophyllene, match those already reported in the literature and are putative candidates as the main anti-inflammatory compound in sucupira oil [16]. As SO is rich in anti-inflammatory constituents so it is believed to target different aspects of mucositis pathobiology [12,18,38]. Many of molecules or plant derived products are lipophilic impairing their absorption and consequently action in the organism. To overcome this inconvenient, we have associated the potential bioactive lipids, SO and Lec, with a biocompatible and biodegradable polymeric nanoparticle, seeking to obtain a protective effect on intestinal experimental mucositis.

SO-NC formulations were prepared by a simple and fast method [21]. Data concerning to the physicochemical properties of the SO-NC showed high encapsulation efficiency, size distribution between 200 and 300 nm, and negative zeta potential value, which are in accordance with the literature for polymeric nanocapsules [23,25]. In addition, SO-NC demonstrated maintenance of size and PdI below 0.3 for 28 days when stored at 4 °C. These formulations features indicated that they could be used orally for reaching inflammed intestinal epithelium.

Macrophages are essential for maintaining mucosal homeostasis and are involved in several diseases such as intestinal bowel inflammation (IBD) and mucositis [5,7,19]. The literature well documented that nanocarriers are taken up by several immune-related cells [23,32]. Thus, the following part of the work was carried out to investigate the SO-NC and SO impact on the cellular viability of a murine phagocytic cell (RAW 267.4). Although few works have investigated the cytotoxic effects of SO, the compound(s) responsible for such effect was not explained yet. Yamaguchi and Levy [39] demonstrated that the incubation of CAR (from 50 µg/mL to 200 µg/mL) with RAW267.4 cells suppressed 50 % of their proliferation. Here, SO suppressed RAW267.4 proliferation in concentrations higher than 200 µg/mL. Our result is similar Dutra et al. [40] that demonstrated no toxicity of sucupira essential oil in peripheral mononuclear blood cells (PBMC) until 100 µg/mL. The cytotoxicity of five vouacapanes from *Pterodon pubescens* against cancer cell lines and mouse normal embryonic fibroblasts (3T3) was demonstrated by [41]. The compounds 6α-acetoxy-7β-hydroxyvouacapan, 6α,7β-dihydroxyvouacapan-17β-methylene-ol and methyl 6α,7β-dihydroxyvouacapan-17β-oate were most selective for prostate cancer and presented a IC_{50} between 22 and 34 µg/mL for 3T3 cell line. The IC_{50} results also showed that the encapsulation of SO into NC increased its cytotoxicity when compared to SO, as reported elsewhere [42]. This result can suggest polymeric NC are actively phagocytized as demonstrated in the literature [23] increasing the SO bioactive compounds levels inside the cell.

The delivery of anti-inflammatory compounds in intestinal mucositis foci is an area of great interested. The mucositis damages on intestinal epithelium can increase intestinal permeability, inflammatory infiltrates and mediators and, induce weight loss [5,7,10]. Intestinal permeability is a good parameter to evaluate epithelium damage. We observed that SO-NC administration showed beneficial effect once it reduced the intestinal permeability promoted by CDDP when compared to CDDP group ($p < 0.05$). The intestinal mucosal membranes produce mucus with high concentrations of phosphatidylcholine (PC) forming a hydrophobic surface on the top of mucus layer preventing the invasion of bacteria. PC, presented around 70 % in the Lec (Lipoid S75), is known to have positive therapeutic properties in intestinal inflammation. The use of 12.5 mg/kg of water-soluble PC orally in a rat model of intestinal inflammation were able to reduce intestinal permeability [43], similarly to our results. Thus, maintenance of the PC levels at the hydrophobic surface by supplementation can be important for health and prevention of intestinal inflammation. However, SO administration induced an increase of the intestinal permeability ($p < 0.05$), regardless the presence

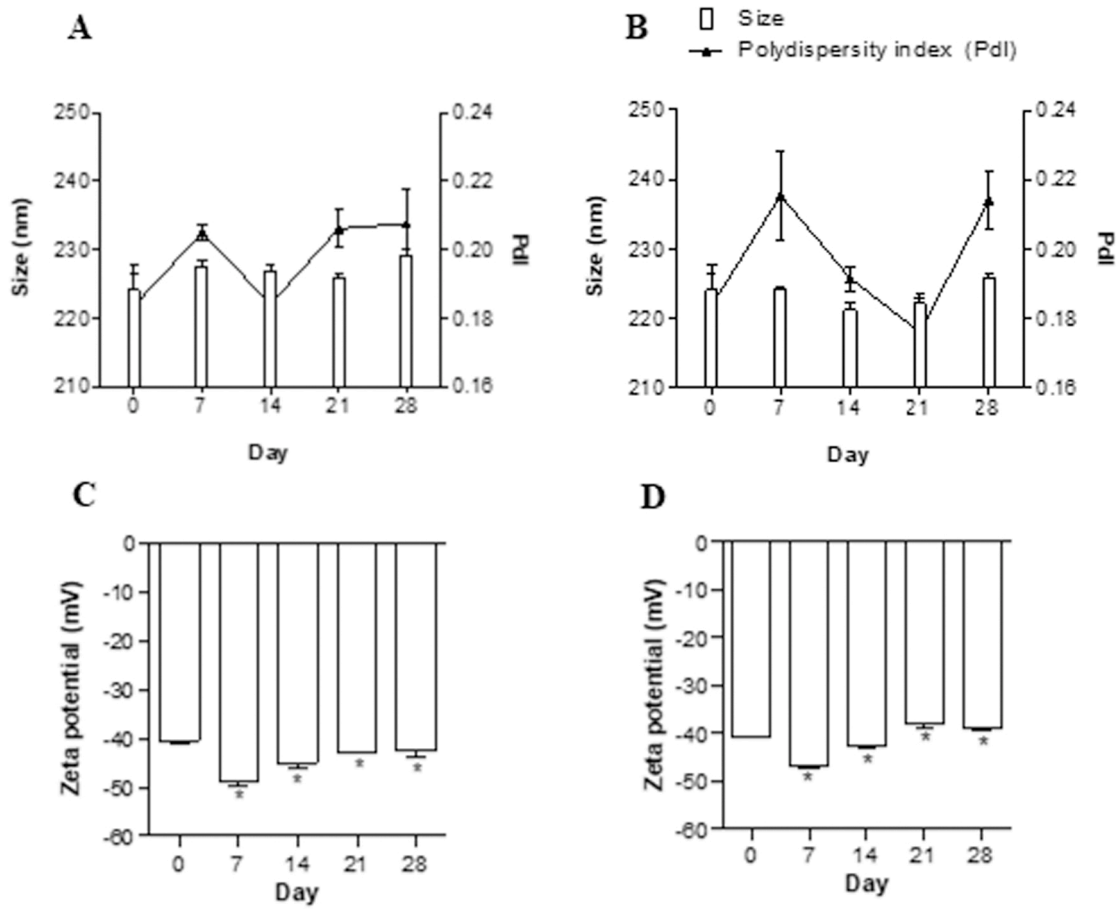

Fig. 3. - Physical stability of SO-NC during storage at 25 °C and 4 °C for four weeks.*versus control; $P < 0.05$.

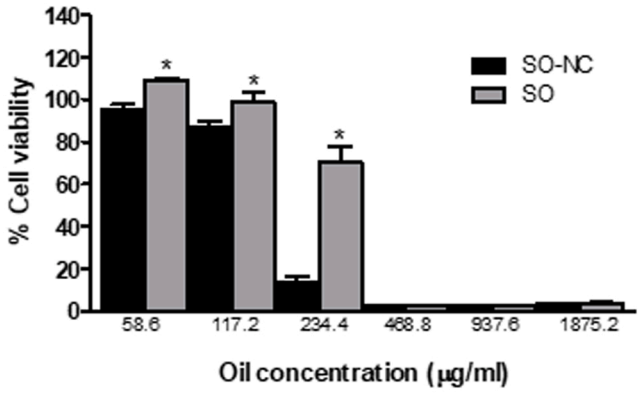

Fig. 4. - Cytotoxicity assay performed in RAW 264.7 macrophage cell line. * $P < 0.05$. Abbreviations: SO, sucupira oil; SO-NC, lecithin based-nanocapsules containing sucupira oil.

of Lec. High IP levels can allow bacterial translocation, increase absorption of molecules which may lead to systemic toxicity and poor action at local level. The mechanism responsible for this effect can be linked to tight junction proteins, as paracellular intestinal permeability is controlled mainly by them [10]. According to the literature CDDP was able to increase intestinal permeability and decrease the expression of tight junction proteins zonulin-1 and occludin 72 h after i.p. administration [7].

Reinforcing these data, the IP results from the in vivo experiments were in line with those from the histological assessment of the intestinal inflammation (Fig. 7). CDDP-induced mucositis and the oral administration of SO clearly modified the architecture of the intestinal tissue, with a reduction of the protective epithelial layer as an increased intestinal permeability. For SO-NC treated CDDP group less intestinal damage was observed when compared to the CDDP group.

The inflammatory response is present in chemotherapeutic-induced mucositis and its modulation has been reported in the literature in the sense to decrease or prevent damage [5,10]. Therefore, the results of the present work showed that the inflammatory process measured by neutrophil infiltration through MPO activity was attenuated with the administration of SO-NC. However, the administration of SO did not decrease the inflammation seen by increased MPO level, corroborating the increased intestinal permeability found for this group. Despite there is no report concerning SO and MPO, Carvalho et al. [15] demonstrated inhibition of neutrophils migration induced by carrageenin to the peritoneal cavity of rats treated with 500 mg/kg orally of sucupira hexanic extract. For SO-NC + CDDP group the MPO values were similar to control, indicating that nanoencapsulation of SO had a protective mechanism.

Regarding NAG activity (representing macrophage infiltration) there was no difference between all groups. Despite there is no data of intestinal NAG and CDDP, a similar result was found by Ferreira et al. [6] after oral administration of the lipophilic compound butyrate on another chemotherapic, 5-FU induced intestinal mucositis. This could be explained due to macrophages are generally seen in later inflammatory stages, depending on the animal sacrifice time and model of mucositis employed [44].

Regarding the pro-inflammatory cytokyne TNF-α, its levels were increased for all groups, except for control group. It is likely that the high levels of TNF-α would be linked to the role of this cytokine to

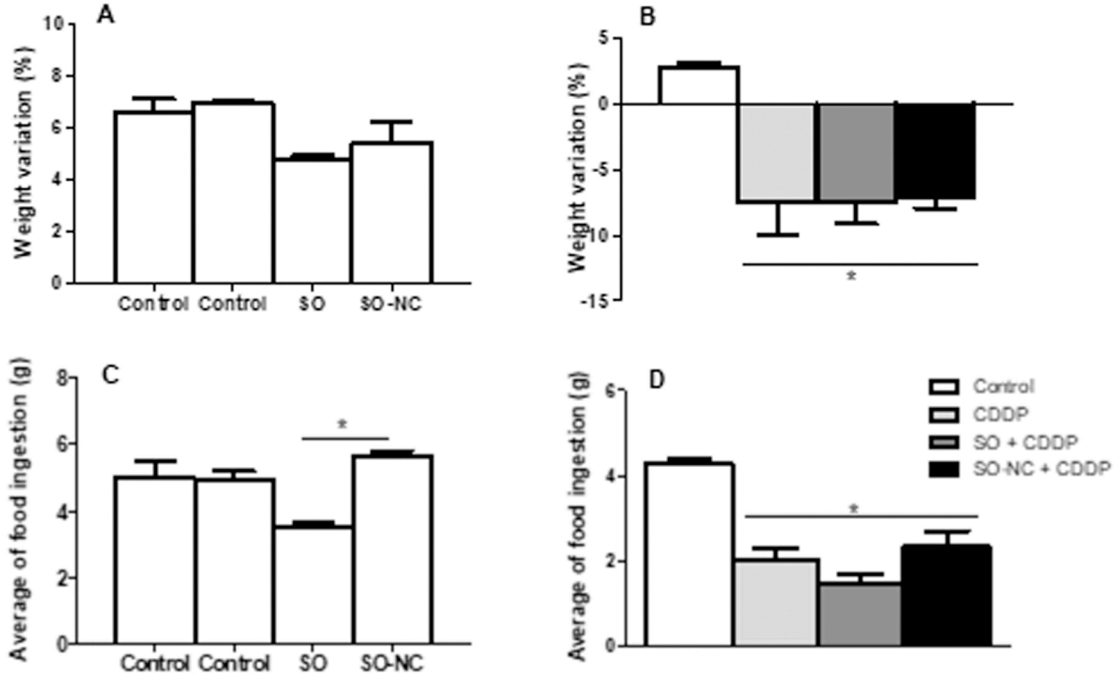

Fig. 5. - Body weight changes and food consumption across groups in (%) healthy (A, C) and CDDP-induced intestinal mucositis (B, D) in Swiss mice. Data are expressed as mean ± SEM of 6 animals per group. *versus day 0; $P < 0.05$. Abbreviations: CDDP: mucositis group; SO, sucupira oil; SO-NC, lecithin based-nanocapsules containing sucupira oil.

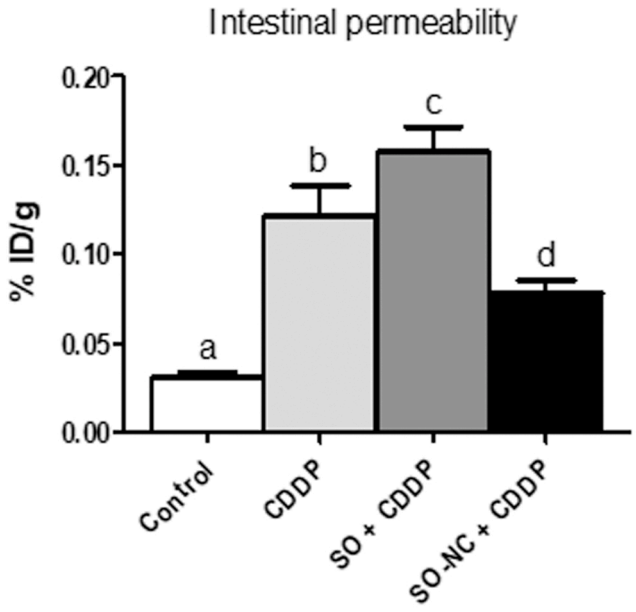

Fig. 6. - Evaluation of intestinal permeability by ^{99m}Tc-DTPA. Data are presented as mean ± SEM of 6 animals per group. Different letters indicate statistically significant differences ($P < 0.05$). Abbreviations: CDDP: mucositis group; SO, sucupira oil; SO-NC, lecithin based-nanocapsules containing sucupira oil; ID/g, injected dose per gram.

recruit and/or activate other inflammatory cells not measured here that could deal with the inflammatory scenario. Dial et al. [45] haven't seen a decrease in TNF-α levels after oral use of 100 mg/kg of water-soluble PC in a rat colitis model, similarly to this work that employed a dose of 15 mg/kg of Lec (around 10 mg/kg of PC). On the other hand, no alteration on IL-10 levels could be detected between the groups. This lack of detection could be also explained by the time peak production of this cytokine once Araújo et al. [7] detected a decrease in its levels after 72 h of CDDP i.p. administration. Recent works have demonstrated that cytokines play an essential role in intestinal mucositis. Increased levels of pro-inflammatory TNF-α and IL-6 are closely correlated to injury after chemotherapy, revealing as good inflammatory response markers [3, 5–8]. In addition, TNF-α activates nuclear factor-kB, resulting in over-expression of pro-inflammatory cytokines by immune cells such as macrophages leading to an amplification of the response and epithelial ulceration [7,46]. The beneficial effect of decreased intestinal TNF-α levels has been demonstrated in the literature after using immunonutrients [5,7,10]. The anti-inflammatory cytokines are not explored as the pro-inflammatory are despite their importance. IL-10 configures the most researched anti-inflammatory cytokine in mucositis [7,8].

The mechanisms of the absence of anti-inflammatory effects of SO on intestinal induced mucositis are still unclear. In the literature the absence of toxicity and mutagenic activity of SO is reported in the literature [47]. In addition, Souza et al., [48] demonstrated no toxicological effects after oral daily treatment with dichloromethane crude extract of *Pterodon pubescens* fruits and vouacapan diterpenes for 110 days in male and female Wistar rats. A possible explanation for the lack of efficacy of the treatment with SO may be the dosage, duration of treatment or inability of this oil-resin to interfere in the pathogenesis caused by the drug. Despite there is no data concerning SO and intestinal mucositis or other IBD, Barbosa et al. [49] showed that copaiba oil did not attenuate the inflammatory damage in acute and subchronic colitis in rats at 1.15 g/kg. The intestinal damage probably occurred by the contact of poorly SO soluble constituents emulsified by lecithin on a pre-damaged epithelium by CDDP causing local irritation. However, it appears that sucupira action depends on the model used to induce inflammation. Carvalho et al. [15] reported action of sucupira hexane extract on carrageenin edema model but not for dextran and histamine edema models indicating that sucupira hexanic extract is able to block the response to prontaglandin E2. This result was confirmed by Santos et al. [50] and linked to terpenic compounds as 6α,7β-dihydroxyvouacapan-17β-oic, also detected in SO of this work. The involvement of prostaglandins in mucositis remains unclear [51,52]. The literature

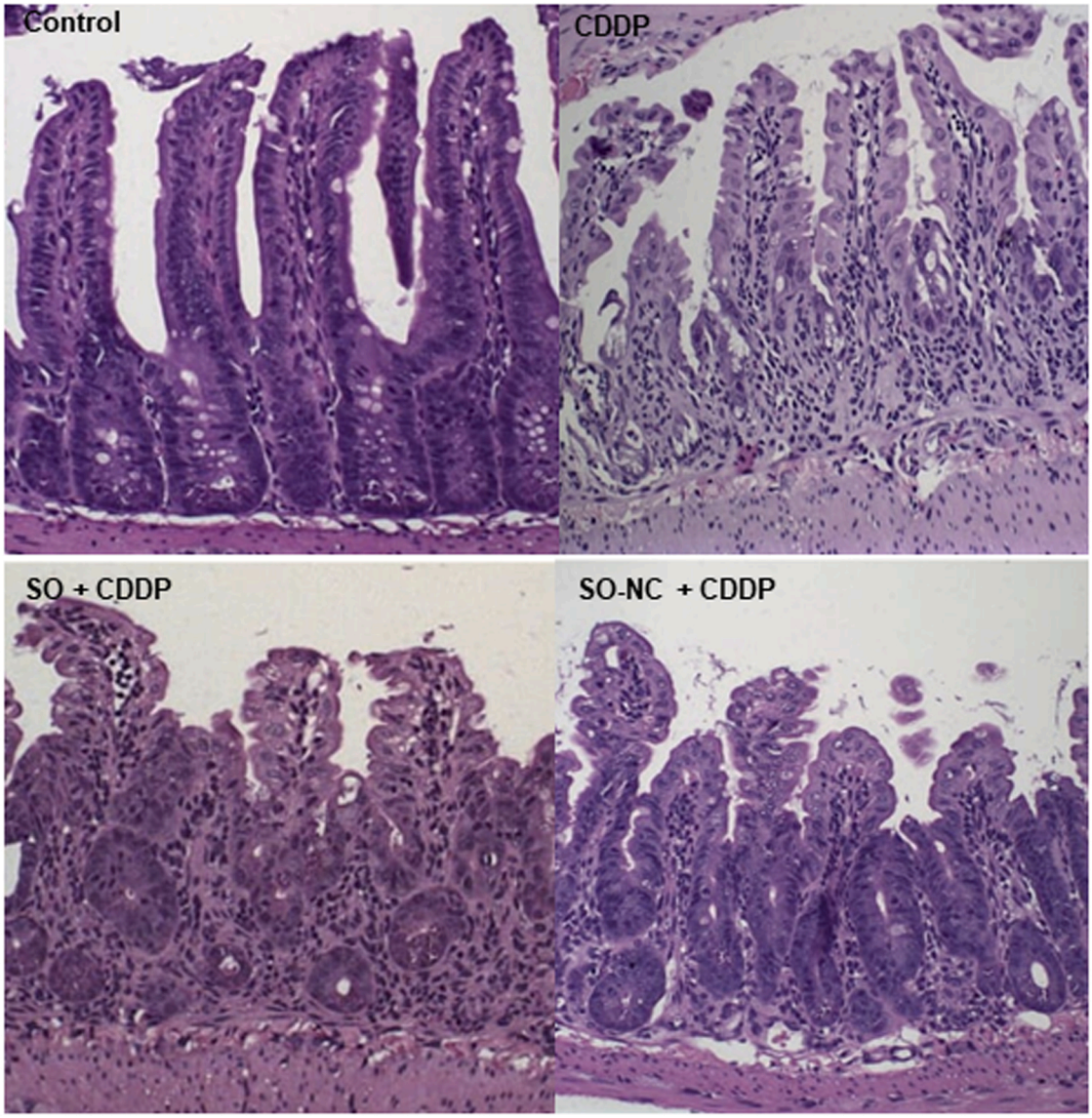

Fig. 7. - Histopathological ileum analysis. Photos are representative images, 20x magnification. Data are expressed as media ± SEM of 6 animals per group.

indicated that administration of prostaglandin E2 had beneficial effects on the outcome of oral mucositis [53], whereas, in others severely aggravated [52,54]. It is possible that prostaglandin E2 has different effects in oral and intestinal epithelial cells. Here we can suggest that oral administration of SO allowed intestinal contact with poor soluble chemical compounds blocking prostagladine E2 response and worsened the intestinal mucositis regarding IP and MPO. This altogether could help to explain the intestinal toxicity after SO administration on CDDP-induced mucositis. In addition, it should be noted that SO obtained here was by cold-pressing, a more ecologic process that can result in an oil with different chemical composition when compared to oil obtained by other techniques as solvent extraction. It could be seen here some substances that could not be identified by LC-MS and that could have induced the adverse effects observed.

Our results demonstrated that polymeric nanocapsules protected the intestinal epithelium from CDDP effects and SO toxicity demonstrated in this work. This could be due to polymeric nanocapsules prevented the direct contact of SO with intestinal epithelium, reducing IP and inflammatory damage observed with SO-NC treatment. This is in accordance with Villalba et al. [25] that demonstrated the reduction of gastrotoxicity associated with the anti-inflammatory meloxicam by encapsulation in NC made of PCL, a non-toxic and biocompatible polymer. To our knowledge, this is the first report that assessed the effects of SO on multiple parameters of the intestinal mucositis. Moreover, a toxic effect of SO on intestinal mucositis has been revealed and suggests careful with the use of natural products in the presence of intestinal damage. Altogether, our results demonstrate that SO-NC alleviated the inflammatory process and the intestinal permeability resulting in improvement of intestinal mucositis induced by CDDP.

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CRediT authorship contribution statement

Jeruza F. F. Di Miceli: Investigation, Methodology, Data curation, Formal analysis, Writing – original draft. **Paula L. A. Carvalho:** Investigation, Methodology. **Anna Eliza M. F. M. Oliveira:** Investigation, Methodology. **Rodrigo A. S. Cruz:** Investigation, Methodology. **Elandia**

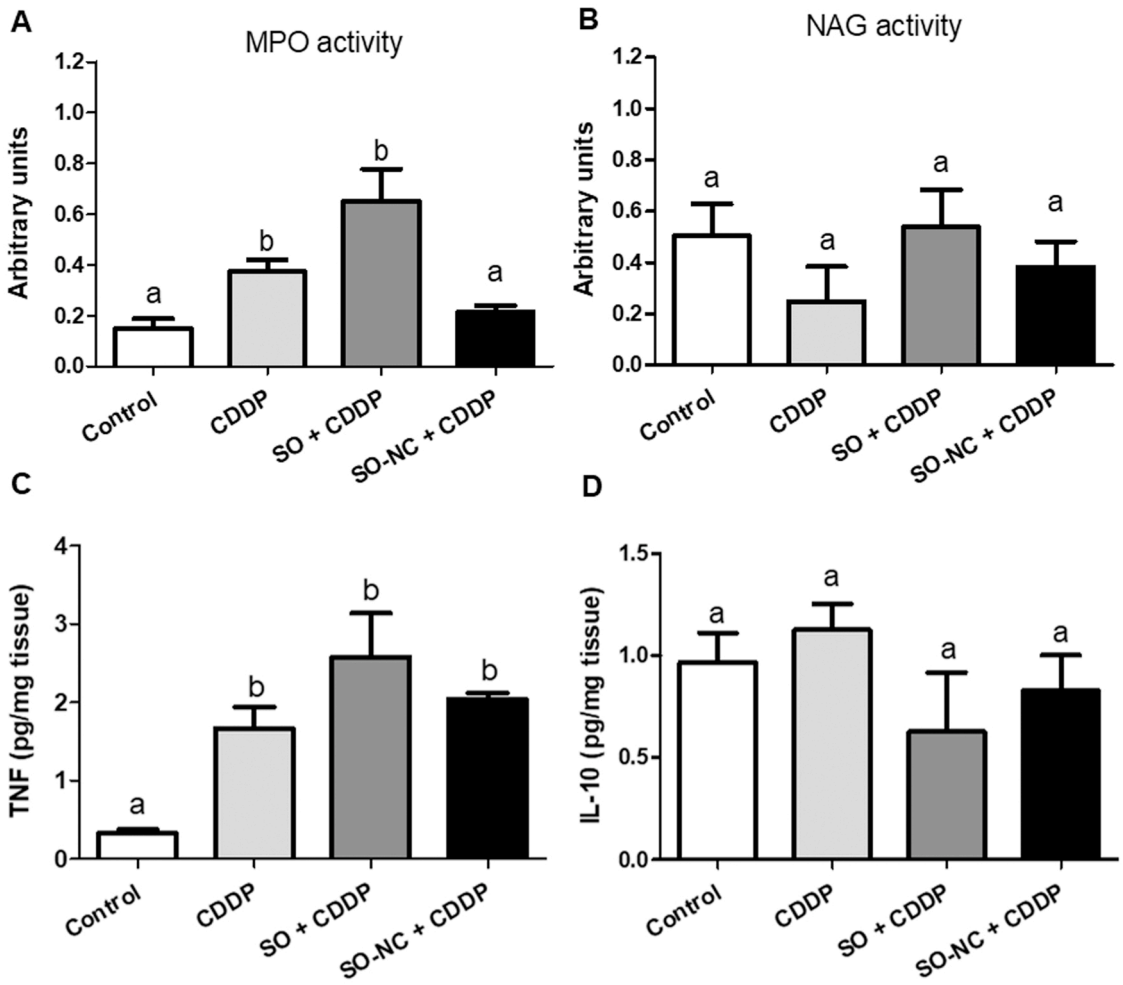

Fig. 8. - Activity of myeloperoxidase (MPO) (A), N-acetylglucosaminidase (NAG) (B), Levels of TNF-α (C), and IL-10 (D) cytokines of the ileum. Data are expressed as media ± SEM of 6 animals per group. Different letters indicate statistically significant differences ($P < 0.05$). Abbreviations: CDDP: mucositis group; SO, sucupira oil; SO-NC, lecithin based-nanocapsules containing sucupira oil.

A. Santos: Investigation, Methodology. **Maria Emília R. Andrade:** Data curation, Writing – review & editing. **Rafael Garrett**: Data curation, Writing – review & editing. **Caio P. Fernandes:** Resources, Visualization. **Vanessa C. F. Mosqueira:** Resources, Visualization. **Geovanni Dantas Cassali:** Resources, Conceptualization. **Jacqueline Isaura Alvarez Leite:** Resources, Conceptualization. **Valbert Nascimento Cardoso:** Resources, Conceptualization. **Raquel S. Araújo**: Conceptualization, Formal analysis, Project administration, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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