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Research paper

Taxifolin and gastro-adhesive microparticles containing taxifolin promotes gastric healing in vivo, inhibits *Helicobacter pylori* in vitro and proton pump reversibly *in silico*

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ABSTRACT

Taxifolin (3,5,7,3,4-pentahydroxy flavanone or dihydroquercetin, Tax) was identified as a gastroprotective compound and a gastroadhesive formulation was recently developed to prolong its residence time and release in the stomach. So, the gastric healing effectiveness of Tax and gastro-mucoadhesive microparticles containing Tax (MPTax) against the acetic acid induced-gastric ulcer in rats was investigated in this study. Moreover, the interactions between Tax and H^+/K^+-ATPase were investigated *in silico*, and its anti- *H. pylori* activity was determined in vitro. The oral treatment with MPTax (81.37 mg/kg, containing 12.29% of Tax) twice a day for seven days reduced the ulcer area by 63%, compared to vehicle-treated group (Veh: 91.9 ± 10.3 mm^2). Tax (10 mg/kg, p.o) reduced the ulcer by 40% but with a $p = 0.07$ versus Veh group. Histological analysis confirmed these effects. Tax and MPTax increased the gastric mucin amount, reduced the myeloperoxidase activity, and increased the glutathione reduced content at ulcer site. However, only MPTax decreased the lipoperoxide accumulation at ulcer site. Besides, Tax and MPTax normalize the catalase and glutathione *S*-transferase activity. Tax showed reversible interaction with H^+/K^+-ATPase *in silico* and its anti-*H. pylori* effects was confirmed (MIC = 625 μg/mL). These results suggest that the antiulcer property of Tax involves the strengthening of the gastric protective factors in parallel to its inhibitory interaction with H^+/K^+-ATPase and *H. pylori*. Considering that ulcer healing action displayed by Tax was favored by gastroadhesive microparticles, this approach seems to be promising for its oral delivery to treat acid-peptic diseases.

1. Introduction

Taxifolin (3,5,7,3,4 -pentahydroxy flavanone or dihydroquercetin, Tax) is a flavanonol abundant in nature, commonly found in onion, milk thistle, French maritime pine bark and Douglas fir bark. Moreover, some commercial preparations such as Legalon® and Pycnogenol® contain Tax [1]. Furthermore, several pharmacological studies have evidenced the health-promoting properties of Tax, including hepatoprotective [2], neuroprotective [3], vasodilating [4] and cardioprotective effects [5]. Tax is an antiplatelet agent [6] and is useful in the treatment of inflammation pain [7], cancer [1] and hypercholesterolemia [8]. Moreover, Schauss et al. [9] showed that the Dahurian Iarch extract, which contains more than 90% of Tax, did not promoted genotoxic or toxicological actions in human lymphocytes or rodents.

The antiulcer potential of Tax was first described in a bio-guiding study of methanolic extracts of *Mimusops balata* (Sapotaceae) edible fruits, that identified this compound as antiulcer agent against acute ulcer model and showed its in vitro inhibitory effect on the H^+/K^+-ATPase activity [10]. Recently, Tax pretreatment was able to rescue the oxidant/antioxidant balance in the gastric mucosa of rats submitted to

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celiac artery ligation [11]. However, Tax has low water solubility, is instable in alkaline medium forming dimers under oxidation medium due to the susceptibility of hydrogen atom of C2' and is degraded by the intestinal bacteria flora [12–16]. To solve these hurdles, a gastroadhesive formulation was proposed to prolong Tax residence time and release at stomach [17].

The exposure of the gastric mucosa surface to acidified environment and hostile stimulus requires protective factors to maintain the homeostasis of the organ [18]. Moreover, alcohol consumption, prolonged treatment with non-steroidal anti-inflammatory drugs, stress, and *Helicobacter pylori* infection favors the imbalance between aggressive and protective mucosal factor. These are the etiological factors of the gastric ulcer, a chronic disease that affects millions of people around the world and has high relapse rates [19,20]. The current pharmacological therapy of gastric ulcer is based in the use of anti-secretory drugs, mainly antagonists of histamine receptor type 2 (*e.g.*, ranitidine) and H^+/K^+-ATPase irreversible inhibitors (*e.g.*, omeprazole), as well as antibiotics against *H. pylori*, when applicable. Despite the effectiveness of this therapy, crescent evidence has suggested that prolonged acid suppression, especially achieved with H^+/K^+-ATPase irreversible inhibitors, triggers several adverse effects [21] and promotes a poor gastric healing process, which in consequence has been linked to ulcer recurrence [22]. Indeed, concern is growing about the wide range of potentially serious adverse events and mortality linked to chronic proton pump inhibitor (PPI) use and its overuse. In this context, off-label prescriptions must be eradicated, whereas its long-term use for clinical indications must continue, until emergence of strong evidence to support serious adverse events and mortality [23]. Besides, as reviewed by Waldum et al. [24], epidemiological studies showed that long-term treatment with PPI increased the risk of gastric cancer due to hypergastrinemia. Therefore, if long-term PPI treatment is necessary, the dose should be adjusted according to the determination of chromogranin A, which reflects 24-h gastrin exposure.

The gastric ulcer healing is a complex process that works in tandem to correct the mucosal imbalance and involves mitigation of the aggressive luminal factors, filling of mucosal defect with proliferating and migrating epithelial cells and connective component to reconstruct the mucosal architecture [25]. Despite the promising results early presented [10,11,17], the effects of Tax on the gastric healing process in already installed ulcers is still unknown. In addition, the gastroprotective effect of a substance does not guarantee its gastric healing activity in a chronic and more complex model of gastric ulcer. Therefore, this study was designed to experimentally evaluate the hypothesis that gastroadhesive microparticles containing Tax accelerate the gastric healing process that occur in the acetic acid-induced gastric ulcer in rats, a suitable model resembling the pathology in humans [26]. Moreover, the putative mechanism of action that Tax inhibits the H^+/K^+-ATPase activity was investigated by docking assay and molecular dynamics, and lastly, the anti-*H. pylori* activity of Tax was also evaluated.

2. Material and methods

2.1. Tax and tax loaded gastroadhesive microparticles (MPTax) production

Tax (>99% purity) was isolated from *M. balata* crushed seeds and characterized as described previously [27]. Briefly, the seeds of the fruit of *M. balata* were peeled and only the seed coat had been used, macerating in methanol for 7 days. The extract was concentrated on rotaevaporator and partitioned with chloroform and ethyl acetate. The ethyl acetate fraction containing Tax was chromatographed on silica gel open column with eluent system of chloroform: methanol. The collected fractions were chromatographed by TLC and the fractions apparently pure were gathered and analyzed by HPLC, as previously described [12].

The mucoadhesive microparticles were prepared as described by Stenger Moura et al. [17]. Briefly, Tax was solubilized in ethanol and Syloid® AL-1 (mesoporous silica), was added in a ratio of 30:70, w/w, keeping on stirring for 2 h at room temperature. Ethanol was successively evaporated under vacuum, and the inclusion product (Tax-Syl) was stored in desiccator. A 0.2% chitosan solution was prepared in 0.25% acetic acid and HPMC was added under magnetic stirring until dissolution (HPMC:chitosan ratio of 1:8 w/w). Tax-Syl complex (30.0% of Tax) was dispersed in water and added to chitosan solution (Tax: chitosan ratio of 1:3.33 w/w) under stirring until dispersion, in an ice bath. The suspensions were then immediately spray-dried (inlet temperature of 120 °C, aspiration rate of 27 m^3/h, pump flow rate of 2 mL/min, and atomizing air flow of 357 L/h) using a mini spray-dryer B290 (Büchi, Cornaredo, Italy). The final dried microparticles are characterized by a Tax content value of 12.29%.

2.2. In vivo studies

2.2.1. Animals

The in vivo experimental procedures were conducted using female *Wistar* rats, (200–250 g, 3–4 months old) provided by the Universidade do Vale do Itajaí (UNIVALI, Itajaí-SC, Brazil). All experimental protocols were approved by the institutional animal ethics committee of the UNIVALI (approval number 50/17p.) and were carried out following the international standards and the ethical guidelines on animal welfare. The animals were housed in propylene cages at 22 ± 2 °C under 12 h light/dark cycle with access to food and water *ad libitum*, except 8 h prior to the experiments, when they were deprived of food.

2.2.2. Acetic acid-induced gastric ulcer

Chronic gastric ulcers were induced in female rats as described by Okabe et al. [28] with slight modifications. First, the rats were randomized into five groups (n = 6), anaesthetized with xylazine and ketamine (10 mg/kg and 100 mg/kg, respectively, i.p.) and the stomach was exposed though a laparotomy. To induce the ulcer, 500 μL acetic acid (80%, v/v) was instilled using plastic tube with 6 mm of diameter applied to the serosal surface of the stomach for 1 min. The acetic acid was then aspirated, and sterile saline was applied to the instilled area. The stomach was replaced in the peritonea, the abdominal wall was sutured, and each rat was observed during its anesthesia recovery. At the second day after ulcer induction, the animals were treated orally with vehicle (water plus 1% tween, 1 mL/kg), omeprazole (20 mg/kg, an inhibitor of gastric H^+, K^+-ATPase and positive control), or Tax (1, 3 and 10 mg/kg), twice daily for 7 days.

In a second set of the experiments, the rats were randomized into three groups (n = 6) and the acetic acid-induced ulcer was performed under anesthesia as described above. From the second day after ulcer induction, these rats received orally the vehicle (water plus 1% tween, 1 mL/kg), gastroadhesive microparticles containing Tax (MPTax, 81.37 mg/kg, which is equivalent to 10 mg/kg of Tax) or gastroadhesive microparticles without Tax (MP, 81.37 mg/kg), twice daily for 7 days.

In both experiments, the suspension of omeprazole and gastroadhesive microparticles were prepared immediately before their administration and the rats were euthanized on the day after the treatment period. Furthermore, one group of animals was not submitted to ulcer induction, named non-ulcerated or naïve group, which was used to allow comparison of the treatment groups to a non-ulcerated group. Their stomachs were removed and opened along the great curvature and the ulcer area (mm^2) was measured using a ruler.

2.2.3. Histological and histochemical analyses

Histological analyses were performed similarly to Da Silva et al. [29]. Briefly, the site of the gastric ulcer was fixed in a solution composed by 85% ethanol, 10% formaldehyde, and 5% acetic acid for 24 h. Following, the ulcer was dehydrated, embedded in paraffin, sectioned into 5 μm thick slices and stained with hematoxylin and eosin. The slices were analyzed and photographed using an optical microscope with a magnification of 100×. The histochemistry quantification of

mucin was performed using the periodic acid of Schiff (PAS)- staining technique, which has been used to detect the presence of glycoproteins found in the mucus. The PAS-stained slices were photographed using an optical microscope (400× of magnification) and the glycoproteins (mucins) stained in pink were quantified using ImageJ® program.

2.2.4. Preparations of stomach tissues for analysis

Ulcer samples were weighed and homogenized with 200 mM potassium phosphate buffer (pH 6.5). The total homogenate was used to measure the reduced lipid hydroperoxides (LOOH) and glutathione reduced (GSH) amount. After, the homogenate was centrifuged at 11,000 rpm (20 min, 4 °C) and the supernatant was used to measure the superoxide dismutase (SOD), catalase (CAT) and glutathione *S*-transferase (GST) activity. The protein concentration was determined by the Bradford method and results were interpolated using a bovine serum albumin (2.5–20 μg/mL) standard curve.

2.2.5. Determination of LOOH content

The levels of LOOH were determined using the method of ferrous oxidation-xylenol orange 2 (FOX2) as described by Jiang et al. [30]. Briefly, 50 μL of methanol was added to 50 μL of homogenate, mixed, and centrifuged at 4,000 rpm (20 min, 4 °C). The supernatant was added to FOX2 reagent (4 mM butylated hydroxytoluene, 250 mM $FeSO_4$, 25 mM H_2SO_4, and xylenol orange 100 mM), and incubated for 30 min at 25 °C. The absorbance at 560 nm was determined and the results expressed as μmol/mg of tissue.

2.2.6. Quantification of GSH levels

Like Da Silva et al. [29], aliquots of homogenate were deproteinized with 12.5% trichloroacetic acid and centrifuged at 4,000 rpm (15 min, 4 °C). After, 10 μL of the supernatant was added to 280 μL of 0.4 M TRIS-HCl buffer (pH 8.9), and 10 μL of 5,5′-dithiobis-2-nitrobenzoic acid at 10 mM. The absorbance was measured after 20 min at 405 nm. The values were interpolated using a standard curve of GSH (1–10 μg/mL) and expressed as μg GSH/g of tissue.

2.2.7. Determination of the SOD activity

The SOD activity was determined as described by Marklund and Marklund [31]. The aliquots of the supernatant were mixed with 1 mM Pyrogallol plus buffer solution composed by 1 mM Tris HCl and 5 mM EDTA (pH 8.5). The reaction was incubated during 20 min and stopped using 1 M HCl addition. At the end of the reaction, the absorbance at 405 nm was registered and the enzymatic activity expressed as U/mg of protein.

2.2.8. Determination of the CAT activity

The CAT activity was measured as described by Aebi [32]. The aliquots of the supernatant were mixed with a solution containing 30% H_2O_2, ultrapure water and 5 mM Tris EDTA (pH 8.0). The absorbance was measured at 240 nm and the results expressed as mmol/min.mg of protein.

2.2.9. Determination of the GST activity

The total GST activity was quantified in accordance with Habig et al. [33]. The supernatant aliquots were added to 1 mM 1-chloro-2,4-dinitrobenzene, and 1 mM GSH plus 100 mM potassium phosphate buffer (pH 6.5) at 25 °C. The reaction of GSH was monitored at 340 nm for 90 s. Specific activity was calculated using an extinction coefficient of 9.6/mM.cm for GSH, and the results were expressed as mmol/min.mg of protein.

2.2.10. Determination of the MPO activity

The neutrophils infiltration into the ulcerated mucosa was assessed indirectly by MPO activity according to Bradley et al. [34] and De Young et al. [35]. The precipitate from the homogenate was mixed with 80 mM of potassium phosphate buffer (pH 5.4), which contains hexadecylmethylammonium bromide, and centrifuged at 20,000 rpm (20 min, 4 °C). The MPO activity in the presence of H_2O_2 and 3,3′, 5, 5′-tetramethylbenzidine was determined at 620 nm in the supernatant and expressed in units of optical density (O.D)/mg of protein.

2.3. In silico assays

2.3.1. Molecular docking

The H^+/K^+-ATPase proton pump in E2P conformation from *Sus scrofa* (PDB code: 4UX1) [36] was downloaded and processed using the Protein *Preparation Wizard* tool as implemented in *Maestro* software package (Schrödinger Release 2019-3: Maestro, Schrödinger, LLC, New York, NY, 2019). The chemical structure of Tax (**1**) was also designed using *Maestro* and refined (tautomer and protonation state) using *MoKa* (Molecular Discovery Ltd). Molecular docking was carried out using *Glide* (Schrödinger Release 2019-3: Glide, Schrödinger, LLC, New York, NY, 2019), with the standard precision (SP) method and applying an enhanced sampling protocol (n. 2 sampling) [37]. Specifically, a grid box was defined with its centre located on the centre of mass of residue Tyr801 into the reference structure 4UX1. Previous mutagenesis experiments reported residue Tyr801 (conserved in human, rat, and rabbit H^+/K^+-ATPase) as important for the interaction of acid pump antagonists (APAs) to H^+/K^+-ATPase [38]. The inner grid box was sized 10 × 10 × 10 Å. Docking studies were carried out using the *g-score* (kcal/mol) scoring function, storing the top ten scored binding poses for Tax (**1**). The docked pose showing the best *glide g-score* (kcal/mol) was selected for molecular dynamic simulations.

2.3.2. Molecular dynamics

Molecular dynamic simulations of Tax (**1**) bound to H^+/K^+-ATPase proton pump in E2P conformation were performed with *Desmond* (Schrödinger Release 2019-3: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2019. Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2019). Specifically, a simulation system was generated building membrane (POPC) around the ligand bound target complex as resulting from docking study, adding water (TIP3P) and 0.15 M of salt (NaCl). Periodic boundary conditions were applied to avoid finite-size effects. Molecular dynamic simulations were performed using the OPLS3e force field [39]. Because the large size of the system (>150K atoms), the simulation protocol included starting relaxation steps and a final production phase of 20 ns, carrying out three independent runs with the generation of three independent simulation trajectories. Trajectories were analyzed using the Simulation Interaction Diagram tool implemented *in Maestro*, calculating the occupancy (%) of ligand/target interactions along each trajectory. Stable interactions were selected and plotted in Fig. 3 as those showing an occupancy value ≥ 20%.

2.4. Anti-H. pylori assay

2.4.1. Bacterial strain and inoculum preparation

Helicobacter pylori (ATCC 43629) was provided by the Oswaldo Cruz Foundation, National Institute for Quality Control in Health, Collection of Reference Microorganisms in Health Surveillance (Rio de Janeiro). It was grown on Mueller-Hinton agar (Kasvi, Spain) supplemented with aged (≥2 weeks old) sheep blood (5% v/v) and incubated at 37 °C for 3–7 days under microaerobic conditions generated by using a microaerobic GasPak EZ (Becton, Dickinson) in an anaerobic jar (Merk). To prepare inoculate, test bacteria was suspended in saline (NaCl 0.89%) by adjusting to 0.5 McFarland standard (1.5×10^8 CFU/mL) and diluting in Mueller Hinton broth (MHB) (Kasvi, Spain) supplemented with 10% Bovine Fetal Serum (BFS) (Cultilab) to obtain the equivalent of 5×10^4 CFU/mL.

2.4.2. Anti-H. pylori activity

A broth micro dilution method carried out in a 96-well sterilized

microtiter plate was used to determine the minimum inhibitory concentration (MIC) of the compound. Tax was dissolved with DMSO and diluted with the culture medium to a concentration of 40 mg/mL. Serial twofold dilutions of the samples of Tax were mixed with MHB supplemented with 10% BFS in 96-well microtiter plates (Corning, USA) in an initial concentration of 2.5 mg/mL. The final concentrations of 2500, 1250, 625, 312.5, 156.25, 78.12, 39.06, 19.53, 9.76, 4.88 μg/mL were tested. Each well was inoculated with *H. pylori* at a final concentration of 5×10^5 CFU/mL. The plates were incubated for 3 days in a microaerobic atmosphere at 37 °C. Following incubation, the plates were examined visually and the lowest concentration showing complete inhibition of growth was recorded as the MIC for that compound. The test was performed in triplicates. The proportion of DMSO did not exceed 1% in the medium. The negative control consisted of culture medium, solvent (DMSO) and inoculum of the bacteria. Ampicillin (Sigma) was used as positive control drug.

2.5. Statistical analysis

Statistical analysis was performed using the software GraphPad version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). The results were expressed as means ± standard error of the means (S.E.M.) of 6 rats in each group. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was applied to verify the differences between means. A $p < 0.05$ was considered significant.

3. Results

3.1. Tax and MPTax in vivo gastric healing study

The treatment with MPTax (81.37 mg/kg) or omeprazole (Ome, 20 mg/kg) twice a day for seven days significantly reduced the ulcer area induced by acetic acid (80%, v/v) by 62.8% and 57.1%, respectively, compared to vehicle-treated group (91.9 ± 10.3 mm^2, Fig. 1A). Interestingly, the administration of Tax at 10 mg/kg reduced the ulcer area by 40.5% with a *p* value equal to 0.07 compared to vehicle-treated group. On the contrary, the administration of Tax at doses of 1 or 3 mg/kg was unable to accelerate the healing process. As expected, the administration of microparticles without Tax (MP) did not reduced the ulcer area when compared to vehicle-treated group.

As for the macroscopic appearance (Fig. 1B), the microscopic observation allowed to verify that acetic acid instillation produced an extensive deep damage in the gastric mucosa of the vehicle-treated group. On the other hand, the regeneration of gastric mucosa promoted by omeprazole (20 mg/kg), Tax (10 mg/kg) or MPTax also is evident in histological appearance of the ulcer site.

As observed in Fig. 2A, the PAS-staining at the ulcer margin from animals treated with Tax (10 mg/kg) or MPTax (81.37 mg/kg) was increased by 264% and 104%, when compared to vehicle-treated group (Veh: $3.7 \pm 1.3 \times 10^4$ pixels/field). The representative images of the PAS staining at the ulcer margin from each experimental group are shown in Fig. 2B.

3.2. Effects of Tax and MPTax on the LOOH and GSH levels in the acetic acid-induced gastric ulcer

As shown in Table 1, acetic acid-induced gastric ulcer reduced by 76.3% the GSH amount and enhanced the LOOH levels by 131% in the vehicle-treated group, compared to non-ulcerated group (Naive: 585.6 ± 17.5 μg/mg of tissue and 11.1 ± 0.4 μmol/mg of tissue, respectively). However, the oral treatment with Tax (10 mg/kg) or omeprazole (20 mg/kg) avoided the GSH depletion, but not the lipoperoxidation, at ulcer site, compared to vehicle-treated group. Interestingly, the groups treated with MPTax (81.37 mg/kg) or microparticles without Tax (10 mg/kg) showed GSH and LOOH at similar levels to those found in the non-ulcerated group (naive group).

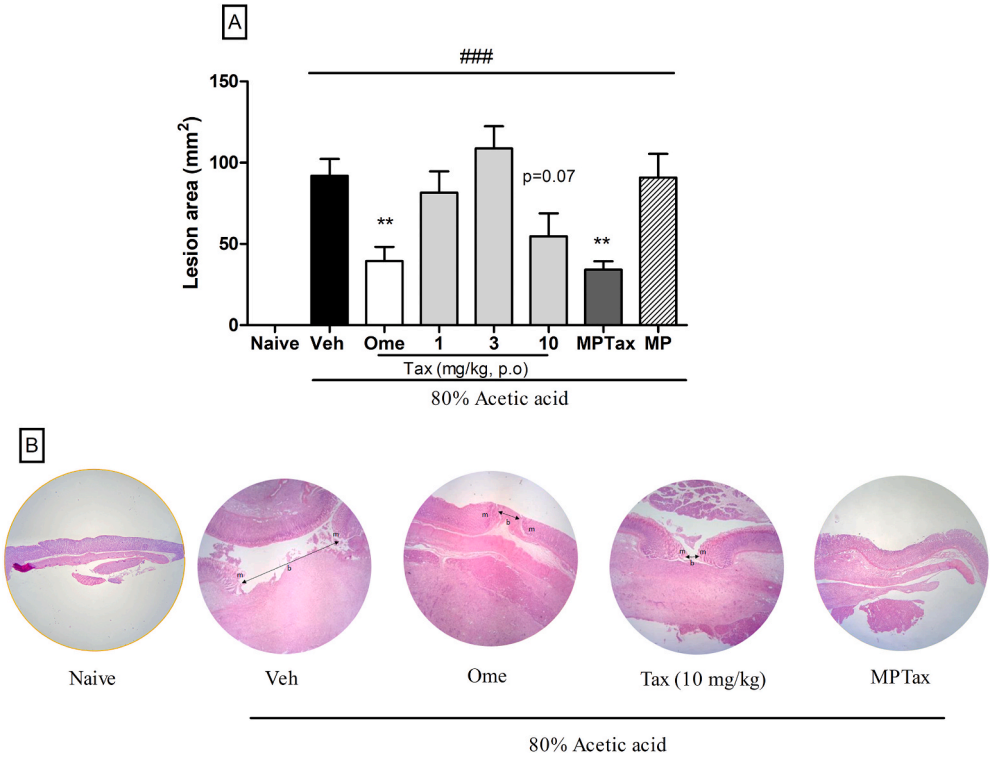

Fig. 1. Effect of oral administration of Tax (1–10 mg/kg) and MPTax (81.37 mg/kg) in acetic acid-induced gastric ulcer in rats. Panel A: Results were expressed as means ± SEM (n = 6). One-way ANOVA followed by Bonferroni's test. $^{**}p < 0.01$, compared to the vehicle-ulcerated group (Veh), $^{###}p < 0.001$, compared to the naive group. Panel B: representative images from histological analysis of experimental groups. Ome: omeprazole. m = ulcer margin and b = ulcer base.

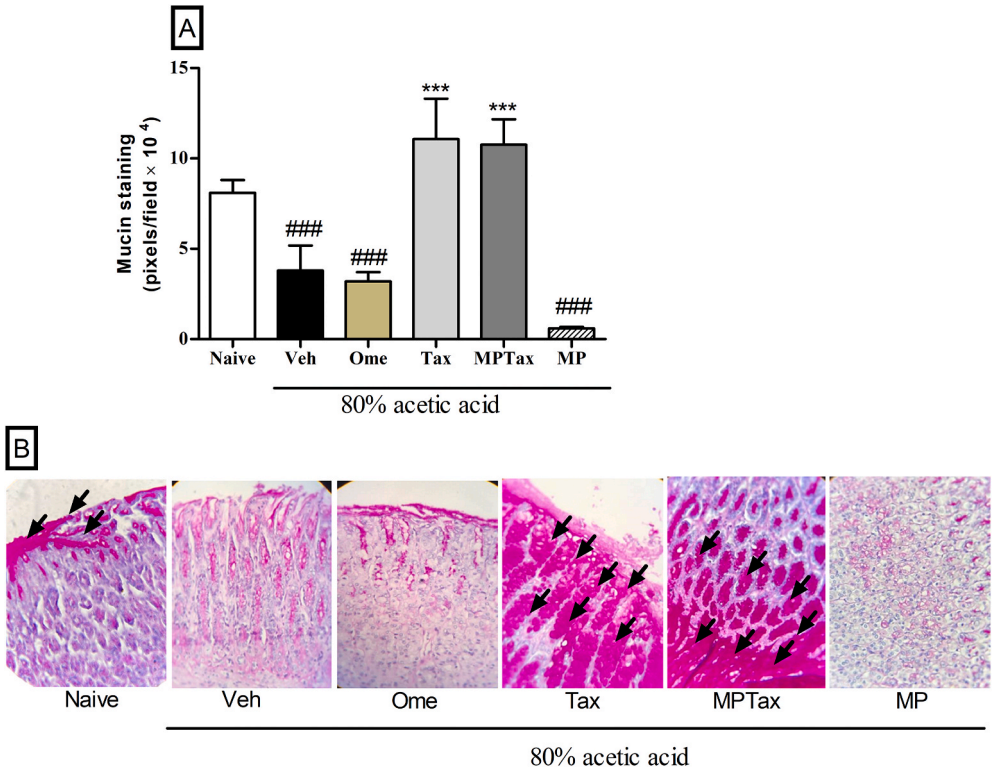

Fig. 2. Effect of oral administration of Tax (10 mg/kg) and MPTax (81.37 mg/kg) in mucin-like glycoproteins amount in acetic acid-induced gastric ulcer in rats. Panel A: quantification of mucin staining (pink areas). Panel B: representative macroscopic images. Ome: omeprazole. The results were expressed as mean ± SEM (n = 6). One-way ANOVA followed by Bonferroni's test. ***p ‹ 0.001, compared to the vehicle-ulcerated group (Veh).

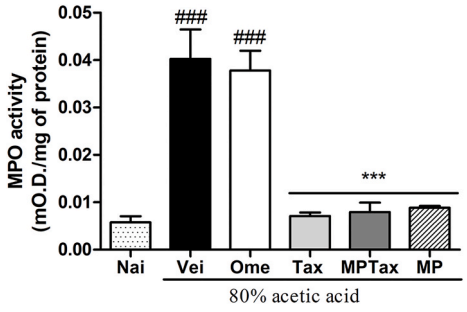

Fig. 3. Effect of oral administration of Tax (10 mg/kg) and MPTax (81.37 mg/kg) on MPO activity. The results were expressed as mean ± SEM (n = 6). One-way ANOVA followed by Bonferroni's test. ###$p < 0.001$ when compared to the naive group; ***$p < 0.001$, compared to the vehicle group (Veh). Nai: naive, non-ulcerated group. Ome: omeprazole.

3.3. Effects of Tax and MPTax on the SOD, CAT and GST activities in the acetic acid-induced gastric ulcer

The rats treated with vehicle experienced a reduction of the SOD ($p < 0.001$), but not of the GST ($p > 0.05$), activity compared to non-ulcerated group (control: 2.86 ± 0.19 U/mg of protein and 0.28 ± 0.04 μmol GSH/mg of protein/min, Table 1). The SOD activity was normalized at basal levels in the ulcerated group treated with Tax, but not in groups that received omeprazole (20 mg/kg), MPTax (81.37 mg/kg) or microparticles without Tax (81.37 mg/kg) twice a day for seven days. In a non-expected manner, rats orally treated with Tax (10 mg/kg), MPTax (81.37 mg/kg) or microparticles without Tax (81.37 mg/kg), but not omeprazole (20 mg/kg) showed an increase of 178, 142 and 257%, respectively, in the GST activity at ulcer site (Table 1).

3.4. Effects of Tax and MPTax in the MPO activity in the acetic acid-induced gastric ulcer

As shown in Fig. 3, the MPO activity was significantly increased in

Table 1
Effects of Tax and MPTax in oxidative parameters at ulcer site.

Oxidative parameters	Non-ulcerated (Naive)	Vehicle (1 mL/kg, p.o)	Omeprazole (20 mg/kg, p.o)	Taxifolin (10 mg/kg, p.o)	MPTax (10 mg/kg, p.o)	MP without taxifoline
GSH (μg/mg of tissue)	585.6 ± 17.5	138.7 ± 0.9[a]	495.2 ± 11.5[b]	445.3 ± 28.3[b]	502.0 ± 11.7[b]	560.9 ± 54.1[b]
LOOH (mmol/mg of tissue)	11.1 ± 0.4	25.7 ± 0.7[a]	28.5 ± 0.6[a,c]	21.4 ± 0.4[a]	14.1 ± 1.0[b]	13.7 ± 1.2[b]
SOD (U/mg of protein)	2.86 ± 0.19	2.04 ± 0.01[a]	2.04 ± 0.01[a]	3.52 ± 0.14[b]	2.05 ± 0.01[a]	2.05 ± 0.01[a]
CAT (mmol H_2O_2/mg of protein/min)	0.44 ± 0.10	2.00 ± 0.48[a]	1.44 ± 0.31[a]	0.19 ± 0.16[b]	0.28 ± 0.08[b]	0.46 ± 0.21[b]
GST (μmol GSH/mg of protein/min)	0.28 ± 0.04	0.14 ± 0.04	0.13 ± 0.06	0.39 ± 0.09[c]	0.34 ± 0.13[c]	0.50 ± 0.16[b]

GSH: glutathione reduced; LOOH: lipoperoxides; SOD: superoxide dismutase; CAT: catalase; GST: glutathione S-transferase. MPTax: gastro-adhesive micro particles incorporating taxiifolin. MP: gastro-adhesive microparticles. Results are expressed as means ± S.E.M (n = 6). One-way ANOVA faloowed by Bonferroni's test. [a] $p <$ 0.001 vs naive, [b] $p < 0.001$ vs veh, [c] $p < 0.05$ vs veh.

acetic acid-ulcerated stomachs when compared to non-ulcerated group ($p < 0.001$). In contrast, oral administration of Tax (10 mg/kg), MPTax (81.37 mg/kg) or microparticles without Tax (81.37 mg/kg) reversed the increased MPO activity at the ulcer site to the basal level. However, omeprazole (20 mg/kg) did not avoid the increase in the MPO activity.

3.5. Molecular docking

Docking study of Tax (**1**, 2*R*,3*R*-2-(3′,4′-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one) into the antagonist binding cleft of the proton pump in E2P conformation suggested a top scored binding pose with a *g-score* of −6.986 kcal/mol (Fig. 4). Specifically, hydrogen bond interactions were observed between the hydroxyl groups of Tax in positions 7 of the 2,3-dihydrochromen-4-one nucleus and 3′ of the dihydroxyphenyl group, and the backbone and side chain of Glu797 and Asp139, respectively. Aromatic and hydrophobic contacts were also found between the 2,3-dihydrochromen-4-one scaffold and residues including Leu798, Tyr801, Tyr804 and Cys815. A further intramolecular hydrogen bond was also observed between the 4-carbonyl group and the 5-hydroxyl group of Tax (**1**).

3.6. Molecular dynamics

Three independent molecular dynamic simulations of Tax (**1**) bound to H^+/K^+-ATPase proton pump in E2P conformation were performed to investigate the stability of ligand/target interactions. As depicted in Fig. 5, the binding pose of Tax (**1**) showed some rearrangements, with hydroxyl groups of the ligand engaging several residues in direct hydrogen bonds and/or water-bridge mediated hydrogen bonds.

In particular, the hydroxyl group in position 7 of the 2,3-dihydrochromen-4-one nucleus is involved in the formation of hydrogen bonds with the backbone carbonyl of Glu797 (1st simulation, occupancy 72%) and Leu812 (2nd simulation, occupancy 100%), whereas the 5-hydroxyl group makes a stable intramolecular hydrogen bond with the 4-carbonyl group in all simulation trajectories. Moreover, the 4-carbonyl group of Tax (**1**) is also found in hydrogen bond contact with Gln129 (3rd simulation, occupancy 27%). The remaining 3-hydroxyl group of the 2,3-dihydrochromen-4-one nucleus forms stable hydrogen bonds with Gln129 and Asp139 (3rd simulation, occupancy 50% and 86%), Thr136 (1st simulation, occupancy 34%), as well as water-bridge mediated hydrogen bonds with Asp139 (2nd simulation, occupancy 20%). Likewise, the 3′,4′-catechol group of Tax drags in the formation of an extensive hydrogen bond network polar residues including Asp139 (1st simulation, occupancy 88%), Asp134 (2nd simulation, occupancy 20%), Glu902, Gln926 and Tyr804 (3rd simulation, occupancy 69%, 42% and 20%). This network is further reinforced by water-bridge mediated hydrogen bonds that are observed between the catechol group and Glu902 in the 2nd simulation (occupancy 20%). In agreement with previous mutagenesis data (ASANO et al., 2003), a conserved and stable π-stacking interaction is observed between the 2,3-dihydrochromen-4-one aromatic nucleus and Tyr801 (1st simulation, occupancy 39%; 2nd simulation, occupancy 35%; 3rd simulation, occupancy 45%). Overall, molecular dynamic simulations of Tax (**1**) bound to H^+/K^+-ATPase suggest a dynamic binding mode of the ligand that is stabilized by a key aromatic interaction with Tyr801 and an extensive dynamic network of hydrogen bonds with polar residues of the binding cleft of acid pump antagonist.

3.7. Antimicrobial activity of Tax against Helicobacter pylori

To determine the MIC of Tax, the range of concentration investigated was between 5.27 μg/mL and 2.5 mg/mL. Minimal *H. pylori* growth was observed in wells containing Tax up to 312.5 μg/mL. In wells treated with 625 μg/mL Tax, no growth was observed so that the MIC was estimated at the value of 625 μg/mL.

4. Discussion

Previously, Stenger Moura [17] demonstrated that Tax permeates the gastric mucosa and formulated a gastric mucoadhesive system using chitosan microparticles to favor Tax absorption in the stomach, achieving rapid therapeutic onset in the treatment of gastric ulcer, and avoiding Tax degradation in the small intestine. In continuity to already established, in the current study we described that Tax and MPTax accelerate the gastric healing process in a suitable model of chronic gastric ulcer in rats. In parallel, in vitro results evidenced the effects of Tax against *H. pylori* and *in silico* trials showed the reversible inhibitory interactions between Tax and H^+/K^+-ATPase.

The chronic gastric ulcer model induced by acetic acid instillation has been developed to examine the healing process occurring in the gastric mucosa. This model is simple and allows to obtain reproducible, round, and deep ulcers in the stomach of rodents that highly resemble human ulcers in terms of pathological features and healing mechanisms [40]. Indeed, in our results omeprazole administration reduced damages of the gastric mucosa of ulcerated rats, while this benefic effect was not observed in the vehicle-treated group.

The Tax dose used in this research was based on previous reports, where Tax prevented the ethanol-induced gastric ulcer in mice at a dose of ~1 mg/kg [10]. Given the complexity of the model employed here, we chose to evaluate a dose of up to 10 times the gastroprotective dose. The administration of Tax at 10 mg/kg reduced the ulcer area with borderline statistical significance ($p = 0.07$), whereas the administration

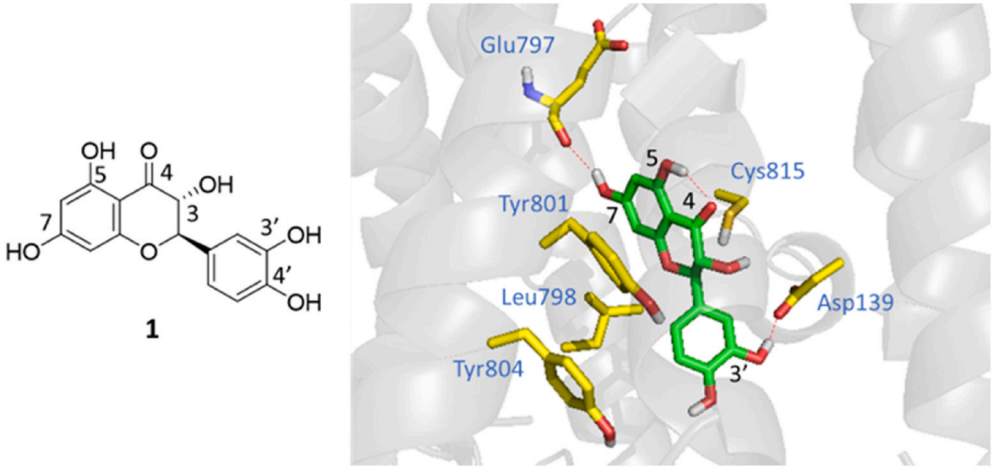

Fig. 4. Interactions of Tax (**1**) into the binding site of acid pump antagonist of H^+/K^+-ATPase proton pump in E2P conformation as resulting from the docking study.

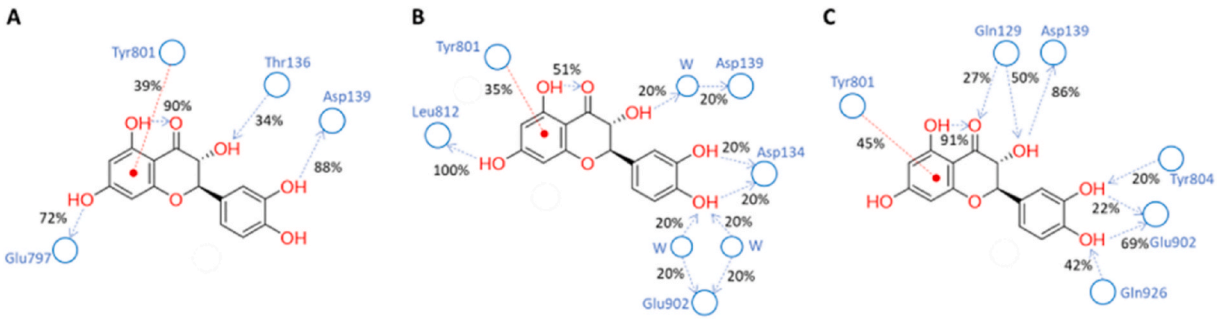

Fig. 5. Plots of ligand/target interactions of Tax into the binding site of acid pump antagonist of H^+/K^+-ATPase proton pump in E2P conformation as resulting from the trajectories of the three independent simulations (A–C).

of MPTax reduced the ulcer area reaching a p value < 0.001. Although there is no statistical difference between the results of the groups treated with Tax or MPTax, it was possible to verify a better homogeneity in the data obtained in the group treated with MPTax. This suggests that Tax loaded in a gastric mucoadhesive system [17], namely composite chitosan-HPMC microparticles, favors Tax effects and by improving gastric absorption, which prevents the flavonoids degradation in the intestinal lumen. However, it is possible that the beneficial effects of chitosan, for example its antioxidant activity, may have influenced the results achieve in MPTax-treated group. Therefore, further pharmacokinetic studies would be necessary to prove this hypothesis.

It is important to emphasize that the microparticles contained 12.29% of Tax and to equalize the Tax dose between the Tax- and MPTax-treated groups, the dose of MPTax was 81.37 mg/kg, corresponding to 10 mg/kg of Tax. Another group received 81.37 mg/kg of microparticles without Tax to evaluate the influence of chitosan and HPMC in the observed effects. As expected, no effect was observed in the ulcer area in the group treated with microparticles without Tax, indicating that the healing process was orchestrated by Tax flavonoid action.

The gastric healing effects of Tax and MPTax were also demonstrated by the regenerative and repairing actions as shown in the histopathological findings, where the recovery of gastric epithelial and glandular architecture was verified. Consequently, a reduction of the base and an increase of the margin of the ulcer were obtained. These results agree with Hou et al. [41] which described the gastroprotective effect of mucoadhesive microparticles used for the gastroretentive delivery of the flavonoid puerarin. However, those previous report assessed the preventive effects in an acute gastric ulcer model and to the best of our knowledge this research is the first study that shows the gastric healing capacity of a flavonoid into gastroadhesive microparticles.

An interesting finding in our study was the enhancement of mucin PAS-staining in ulcer margin only in ulcerated groups that received Tax (10 mg/kg) or MPTax, indicating that the enhancement of this protective factor is pivotal in the acceleration of gastric healing process. The mucin gastric content creates the stolid gastric mucus layer and maintain a stable pH above the gastric mucosa, preventing in parallel the layer enzymatic attack by acid and pepsin [42]. In view of this, it is possible to infer that the increase in the protective barrier of mucus, which creates a favorable microenvironment for cell proliferation and in turn gastric healing, can be a primary effect to achieve the regeneration of the gastric mucosa elicited by Tax. Despite its anti-inflammatory effects [43] and the well-known ulcerogenic effects of NSAIDs due the inhibition of cyclooxygenases and reduction of prostaglandin and mucus barrier [44], the increase in gastric mucin amount promoted by Tax puts this compound in a prominent place in the search of anti-inflammatory resources with good gastrotolerability.

MPO is a heme-containing enzyme abundantly found in neutrophils, which catalyzes the reaction between chloride and hydrogen peroxide to generate hypochlorous acid, a potent oxidant [45]. Despite its role in innate immune defense mechanism, the excessive generation of MPO-derived oxidants has been linked to tissue damage in several inflammatory diseases, including in the gastric ulcer and elevated MPO activity has been evidenced in the acetic acid-ulcerated vehicle-treated group [46]. In contrast to elevated activity found in the vehicle-treated ulcerated group, rats treated with Tax or MPTax showed reduced levels of MPO activity, reflecting the reduction in neutrophil migration at ulcer site. In accordance, Tax has been reported as an antiadhesive agent down-regulating the expression of intercellular adhesion molecule-1 (ICAM-1) on leukocyte [47]. However, rats treated with microparticles without Tax also experienced reduction in MPO levels at ulcer site. The chitosan-neutrophil interaction is encouraged by material properties, including the acetylation degree that favors the interactions [48]. The microparticles was produced using chitosan with a lower degree (7.6%) of N-acetylation [17] and no effects in neutrophil migration were expected [49]. Despite paradoxical, the reduction in MPO activity in intestinal mucosa by chitosan was previously reported by Ref. [50].

The loss of redox homeostasis is involved in the ulcer pathogenesis; thus, the enhancement of antioxidant protective barrier can contribute to gastric epithelium regeneration. A key non-enzyme antioxidant resource in the stomach is the glutathione, which is generally present in its reduced form, GSH. Together with glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione *S*-transferases (GST) form the glutathione system in the gut mucosa, serving as an antioxidative barrier [51]. Several studies have evidenced that the GSH viability is decreased in the ulcer site [45,52,53] and this finding was reproduced in our results obtained with the vehicle-treated group. In contrast, omeprazole, Tax and MPTax restored the GSH levels in parallel to healing effects. The antioxidant properties of Tax are already reported in the literature by Refs. [54,55] that described the synergic effect of Tax and GSH, increasing the free radical scavenging potential of both molecules. In addition, the role of lipid peroxidation in the pathogenesis of gastrointestinal diseases is accepted [56] and, together with the increase in GSH amount, the Tax and MPTax treatment reduced the LOOH accumulation at the ulcer site.

Interestingly, the treatment with microparticles without Tax also increased the GSH availability and decreased the LOOH levels at the ulcer site. These data can be justified by the antioxidant properties of chitosan [57]. However, it is important to note that only the antioxidant activity of a substance does not guarantee its gastric healing effect. Indeed, despite its recognized antioxidant properties, the administration of ascorbic acid (250 mg/kg, p.o, twice a day for seven days) did not promoted the healing of acetic acid-induced gastric ulcer despite it increased GSH content and reduced LOOH amount at the ulcer site [58].

The enzymatic antioxidants SOD and CAT provide major antioxidant defenses against reactive oxygen species (ROS). Reduced SOD activity was early showed in the acetic acid-gastric ulcer, and increased SOD activity has been associated with ulcer healing in rodents [29] and our results are in accordance with these data. However, only rats treated with free Tax experienced an increase in SOD activity. In contrast, the CAT activity was increased in the vehicle-treated group. Together with

SOD, some enzymes were capable of rapidly increasing local H_2O_2 levels, including NADPH oxidases and other oxidases such as xanthine oxidase and 5-lipoxygenase [59], which have been related to the inflammatory response and in the redox signalization at the gastrointestinal tract [60]. Thus, in the vehicle-treated group, despite the inactivation of SOD, the overproduction of H_2O_2 from other oxidases ensure high availability of this substrate in a level that justifies the increase in catalase activity observed in the vehicle-treated group. In contrast, the CAT activity was normalized in ulcerated group treated with Tax, MPTax or MP without Tax, indicating a balance in the redox status of the gastric mucosa of these animals. Interestingly, the GST activity also increased in these three groups, which indicates a greater tissue detoxifying potential.

It is important to emphasize that the antioxidant benefit showed in the gastric mucosa of animals treated with MP without Tax was not accompanied by the acceleration of the gastric healing, in the same way as previously observed for ascorbic acid in this same experimental model [57]. Thus, it is evident that an antioxidant effect is necessary but not sufficient for a gastric healing effect.

H. pylori is an important human pathogen related to several malignant and non-malignant diseases, being the third most common cause of cancer death worldwide and associated with common conditions such as dyspepsia and peptic ulcer [61]. In this context, the source of compounds with anti- *H. pylori* effect that promote gastric ulcer healing are of great interest. To confirm this property, the MIC was evaluated in this study, but unexpectedly, the MIC value of Tax was founded to be 25 times higher to that reported by Ref. [62]. This difference was probably due to differences in the *H. pylori* strain and/or cultivation conditions. However, considering the weight of rats (~233 g) and their gastric volume (~7 mL), the Tax concentration in the gastric juice after oral administration of 10 mg/kg would be ~333 μg/mL, thus lower than the estimated MIC of Tax in our results.

The allometric extrapolation of the Tax dose which was able to promotes gastric healing in rats to a 70 kg human is 168.8 mg. Given that the volume of human gastric contents is on average 50 mL in fasting [63], the Tax concentration at the gastric lumen of a human treated with the allometric dose (168.8 mg) will be 2.4 mg/mL. Therefore, after the administration of the allometric dose to humans, the Tax concentration in gastric lumen matches with the concentration range that MPTax can dissolve in the gastric contents, that is 3.4 mg/mL [17]. In addition, Tax from MPTax is released in stomach for up to 5 h, which corresponds to the time that microparticles remains adhered to pig mucosa [17].

Furthermore, according to the literature, the *in silico* antibacterial mechanism predicted, estimated by Tax molecular docking, suggests the inhibition of cell envelope synthesis of gram-negative bacteria, through the interaction of the hydroxyls of the C-4 'and C-5′ of the B ring with the amino acid residues Arg38 and Phe308 of the enzyme [64].

Finally, a significant prevalence of gastric ulcers in women has been observed since the 1980s [65] and that continues to be reported to the present day, including deaths associated with gastric perforations in these women [66] support the use of female rats in this study.

5. Conclusions

Taken together, the results achieved confirm the antiulcer value of Tax and indicate the involvement of the strengthening of the gastric protective factors, mainly the mucus barrier and antioxidant defenses, in parallel to its reversible inhibitory interaction with H^+/K^+-ATPase and *H. pylori*. Considering that the Tax ulcer healing action was favored using gastroadhesive microparticles, this approach seems to be promising for the oral delivery of Tax to treat acid-peptic diseases.

Credit author statement

Fernanda Cristina Stenger Moura: phytochemical procedures, obtaining the gastro-adhesive system and writing- original draft preparation. Valdir Cechinel Filho: phytochemical procedure and conceptualization. Francesco Antonio Greco, Antonio Macchiarulo, Aurélie Schoubben and Maurizio Ricci: data curation, formal analysis, and in silico investigation. Larissa Venzon, Mariane Caroline Meurer, Tauani Caroline dos Santos França, Bruna Longo, Lincon Bordignon Somensi and Luisa Nathalia Bolda Mariano: in vivo investigation, data curation and visualization. Tania Mari Belle Bresolin and Luísa Mota da Silva: writing- reviewing and editing., conceptualization and supervision.

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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