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Phenolic Profile, Antioxidant, Gastroprotective and Ulcer Healing Activities of Pot-pollen Produced by the Stingless Bee [*Melipona compressipes fasciculata* (Smith, 1854)]

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Abstract The gastric ulcer is definited as erosion of the stomach's mucosal wall, which affects over 4.6 million people per year. The objective was to investigate the phenolic profile and the antioxidant activity of ***Melipona compressipes*** fasciculata pot-pollen collected at seasons rainy (PPMF1) and dry (PPMF2), as well as the gastroprotective and ulcer healing activities of PPMF2, as **PPMF1** did not have a gastroprotective effect in screening protocols. The phenolic profile revealed compounds such as caffeic acid, quercetin, myricetin and naringenin. In models of gastric lesions by ethanol, ischemia/reperfusion (I/R) and acetic acid, PPMF2 reduced the lesion area (at doses 3 to 80 mg/kg, p.o.) and volume at doses (3 to 24 mg/kg, p.o.) with protection at percent 52 to 92%. PPMF2 too, significantly ($p<0.05$) elevated mucus and non-protein sulfhydryl groups (SH-NP) with an increase of 122% and 92% respectivelly compared to vehicle group, and decreased malonaldehyde (MDA) in rats (36% compared to vehicle group). As for cicatrizant activity in acetic acid-induced ulcer, PPMF2 promoted healing rates until 97%, caused significant microscopic gastric recovery, elevation of SH-NP (103%), attenuation of MDA (84%) levels and improvement of biochemical profile ($p<0.05$). This is a relevant and pioneering research associating stingless bee (*M. compressipes fasciculata*) in gastroprotective activity studies.

Keywords: *stingless bees, oxidative stress, phenolics, gastroprotection, pot-pollen*

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1. Introduction

Gastric ulcer is definied as erosion of the stomach's mucosal wall and has a fatality rate of one death per 10,000 cases, affects over 4.6 million people per year. This high morbidity rate leads to increased medical costs and decreases in the quality of life [1]. This gastrointestinal disorder has a multifactorial and complex etiology that involves an imbalance between protective factors of the gastric mucosa (mucus-bicarbonate barrier, blood flow, cell renewal and migration, antioxidant system) and aggressors (hydrochloric acid, alcohol, stress, reactive oxygen species - ROS). Other factors that contribute to gastric ulcers include excessive consumption of nonsteroidal antiinflammatory drugs (NSAIDs) and nutritional deficiencies [2].

The drugs used in the treatment of gastric ulcer are proton-pump blockers (omeprazole). H2-receptor antagonists, anticholinergic drugs, and antacids. This drugs are related to undesirable side effects and poor

gastric healing quality, promoting ulcer recurrence [3]. In this context, the searches for effective therapies, with minor and accessible side effects or for products capable of conferring an auxiliary protection against gastric ulcer continue and studies are turning to natural products [4].

Bee pollen is a natural material with high nutritional and medicinal value, commonly used in folk medicine in the treatment of a number of diseases, such as ulcers [5], with gastroprotective effect already reported in the literature [6]. It results from the flower pollen agglutination with nectar and salivary substances [7]. In hives, bees mix this collected pollen with their digestive enzymes, store and preserve it with honey and wax. Such a mixture is chemically altered by microorganisms, enzymes, temperature and humidity, and after two weeks is called "bee bread". This raises the nutrient availability and bioactive components [8]. The term "bee bread" is more commonly used for pollen stored in *Apis* nests and "pot-pollen" for pollen in stingless bees' nests [9].

Scientific data on botanical origin, chemical composition, bioactive compounds or bee pollen pharmaceutical applications are abundant. However, for bee bread, and especially for pot-pollen, such information is still rare [10]. The pot-pollen is widely appreciated in popular culture as food and natural medicine [11]. In comparison to bee pollen, stored pollen (bee bread or pot-pollen) has higher stability, nutritional value and therapeutic potential, with more bioactive polyphenols and richer chemical composition. It is better absorbed by the human organism, since its constituents undergo partial fermentation, being assimilated more easily than is pollen [12].

The bee bread stimulates factors of human body protection, normalizes the metabolism and increases tissues regeneration. It also presents biological activities such as antioxidant (*in vivo* and *in vitro*), immunomodulatory, antimicrobial, hepatoprotective and adaptogenic [12-16]. Because it has enzymatic antioxidants (such as glucose oxidase and catalase) and non-enzymatic (such as phenolic acids and flavonoids), it may be especially useful in the prevention of diseases in which free radicals are involved [17]. In this context, the aim of the present study was to investigate the phenolic profile and the antioxidant activity of (PPMF1) and (PPMF2), as well as the gastroprotective and ulcer healing activities of PPMF2.

2. Material and Methods

2.1. Reagents

For the chromatographic analysis, the following solutions were used: catechin, epicatechin, epigallocatechin-gallate, rutin, isoquercitrin, naringenin, quercitrin, protocatechic, vanillic, homovanilic, caffeic, p-coumaric, m-coumaric, benzoic and rosmarinic acids (Sigma-Aldrich, Steinhein, Germany); gallic acid, siringeic acid and quercetin (Fluka Chemie AG, Buchs, Switzerland); procyanidin B1 (Extrasynthese, Genay, France); p-hydroxybenzoic acid (Alfa Aesar, Karlsruhe, Germany). Reagents and drugs: absolute ethanol (Vetec PA, Brazil), acetic acid, hydrochloric acid, carbenoxolone and N-acetylcysteine (NAC) (Sigma Chemical Co., St. Louis, USA), cimetidine (Medley, Campinas, Brazil).

2.2. Pot-pollen Samples

Samples of pot-pollen produced by stingless bees *Melipona compressipes fasciculata* (Smith, 1854), known as *tiúba*, were collected directly from colonies of a meliponary located in São Luís, Maranhão, Brazil. These were collected at two stations and coded as: PPMF1 - collected in the rainy season, in March; and PPMF2 - dry season, November, both in 2017. Afterwards, they were lyophilized (30.10^{-3} mmHg, -50°C, 72 h), crushed and stored at - 20°C until the moment of the analysis. In the tests of gastroprotective activity, the pot-pollen of *M. compressipes fasciculata* was codified as PPMF2 (because of this phenolic profile, a greater presence of composites with antioxidant and gastroprotective activities has been mentioned in the literature). Processed and lyophilized pot-pollen was administered in doses ranging from 3 to 80 mg/kg, suspended in 0.5% carboxymethylcellulose (CMC) and administered to the animals at a dosage of 1 mL/100 g body weight.

2.3. Phytochemical Analysis: Antioxidants and Phenolics

The samples (PPMF1 and PPMF2) were submitted to extraction according to the methodology described [18] and their phytochemical profile was analyzed as follows.

2.3.1. Antioxidant Activity *in vitro* - DPPH Method (2,2-diphenyl-1-picrylhydrazyl)

A method adaptation of the ones reported [19] was used to evaluate the antioxidant activity. The absorbance (515 nm) was measured in t (0 and 30 min). The coefficient of determination was 0.996. The results were also expressed in % elimination of the DPPH radical.

2.3.2. Profile of Phenolic Compounds

2.3.2.1. Method of High-performance Liquid Chromatography (HPLC)

Chromatographic analysis was performed using an Agilent 1200 HPLC system (Waldbronn, Germany), equipped with a quaternary pump, automatic sampler, thermostatic column housing, degassing device, *Diode Array Detector* (DAD) and an *Agilent ChemStation* Software for liquid chromatographic systems (LC). Analytes were separated on a Kinetex® EVO C18 100Å (150 × 3 mm i.d, 5 µm) analytical column (Phenomenex, Torrance, CA, USA) maintained at 30°C. The injection volume was 20 µL and the flow rate was 0.6 mL/min. The mobile phase consisted of: water-acetic acid (99.9:0.1 v/v) - solvent A; methanol-acetic acid (99.9:0.1 v/v) solvent B. The separation was performed in 46 min under the following conditions: 0 min, 5% B; 3 min, 10% B; 10 min, 20% B; 18 min, 30% B; 25 min, 70% B; 33 min, 100% B; 40 min, 100% B; 41 min, 5% of B.

Chromatographic peaks were identified by comparing their retention times and ultraviolet spectral data with those of the pure and confirmed patterns by overloading

the samples with the standard solutions. Chromatograms were acquired at wavelengths of 278, 300 and 360 nm, depending on the maximum absorption of the compound identified. The results were expressed in µg of the compound per g of sample. The detection limit (DL) of phenolic compounds ranged from 0.05 to 0.48 µg/g; quantification limit (QL) ranged between 0.15 and 1.54 µg/g. The concentration range used for the phenolic standards was between 0.025 and 40.00 µg/g and the coefficient of determination in this interval was always higher than 0.99.

2.3.2.2. High-performance Liquid Chromatography Coupled to Mass Spectrometry (HPLC-MS/MS)

In order to confirm the presence of the phenolic compounds in the pot-pollen samples, a HPLC-MS/MS system composed of Accela automatic sampler, Accela 1250 pump with degasser coupled to a TSQ Quantum Access max quadruple mass spectrometer controlled by Xcalibur software (Thermo Fisher Scientific, San Jose, CA, USA) was used. The mass spectrometer operated in electrovaporization mode (ESI) positive for the detection of quercetin and negative for the detection of all other phenolic compounds. Chromatographic conditions were the same as those used in HPLC analysis. The compounds were characterized according to their retention time in relation to an external standard, precursor ions and the main product ions obtained. The gas used was nitrogen, with a pressure of 25 psi, a spraying voltage of 2500 V, a vaporization temperature of 340°C and a capillary temperature of 350°C. Detection was performed in a Single Reaction Monitoring (SRM).

2.4. Gastroprotective and Ulcer Healing Activities

2.4.1. Experimental Animals

Wistar rats (180–220 g) were kept under controlled conditions (24 ± 1 °C, 12-h dark/ light cycle), with food and water ad libitum. They fasted for 18 h and then were acclimatized to the test environment for 2 h prior to each experiment. The animals were randomly assigned to different groups (n = 6 animals/group). After experimental procedures, animals were euthanized by means of sodium thiopental (100 mg/kg, i.p.). All experimental protocols were carried out according to the standards established by the National Council for Animal Experimentation (CONCEA) and the protocols were approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Piauí, Teresina, Brazil (number 380/2017).

2.4.2. Gastric Lesions Induced by Absolute Ethanol

Acute gastric lesions were induced in rats by oral route (1 mL/animal) of absolute etanol [20]. Vehicle (CMC, 0.5%), PPMF2 (3, 8, 24 and 80 mg/kg) or carbenoxolone (100 mg/kg) were administered orally 1 h before applicationof the ulcerogenic agent. Animals were euthanized 30 min after ethanol administration and stomachs were removed and opened along the greater curvature. The area of gastric lesions was measured using Image J software-NIH® and was calculated as follows: lesion area (%) = lesion area (in square millimeters) × 100/total area (in square millimeters), [21]. After measuring the lesion area, the stomachs were stored at -80°C for the study of the in vivo antioxidant activity.

2.4.3. Gastric Lesions Induced by Ischemia/Reperfusion

Rats were orally treated with vehicle (CMC, 0.5%), PPMF2 (8, 24 and 80 mg/kg) or N-Acetylcysteine (200 mg/kg). After 30 min, animals were anesthetized with ketamine and xylazine (50 and 50 mg/kg, i.m) and subjected for 30 min to ischemia induced by celiac artery occlusion (microvascular clamp) followed by reperfusion for 1 h [22]. Thereby, animals were euthanized and stomachs were excised and analyzed for gastric damage. The area of gastric lesions was measured using ImageJ-NIH®, as previously described.

2.4.4. Determination of Mucus Content in the Gastric Wall

Rats were anesthetized with ketamine and xylazine (50 and 50 mg/kg, i.m), submitted to the protocol of pylorus ligation [23] and treated with intraduodenal vehicle (CMC, 0.5%), PPMF2 (24 mg/kg) or carbenoxolone (100 mg/kg). The mucus content was determined according to the methodology proposed by reference [24].

2.4.5. Gastric Ulcers Induced by Acetic Acid and Histopathological Evaluation

Rats fasted for 18 h and then were anesthetized with ketamine and xylazine (50 and 50 mg/kg, i.m). The abdominal cavity was opened (an approximate 2-cm incision), and the stomach was exposed. To induce gastric ulcer, a glass tube 8 mm in diameter and 2 cm long was used, in contact with the serosa of the stomach to limit the area that would be injured. Seventy microliters of 80 % acetic acid was added to the tube, which remained in contact with the serosa of the stomach for 1 min. After the acetic acid was removed with the help of an automatic pipette, the site was washed with saline solution [25]. The stomach was accommodated in the abdominal cavity, and the abdominal region was sutured. One day after ulcer induction, daily oral treatment was begun with vehicle (CMC, 0.5%), cimetidine (100 mg/kg) or PPMF2 (3, 8 and 24 mg/kg) for 7 days. After the chronic treatment, the animals were euthanized, and the stomach was removed (opened at the greater curvature). The ulcer volume (in cubic millimeters) was calculated by measuring the ulcerated area x depth of the ulcer [26]. After this measurement, part of the stomach in the ulcer area of each rat was carefully removed for histopathological studies and the other part was stored at -80 °C for the study of *in vivo* antioxidant activity as described below.

2.4.6. Histological Evaluation of Gastric Tissue

The stomach tissues were fixed in 10% neutral buffered formalin followed by embedding in paraffin. Sections with 5 µm thickness were cut and stained with hematoxylin and eosin (H & E). Gastric microscopic damage was evaluated using a scoring system with a 0 to 3 (0 - negative, 1 - weak, 2 - moderate, 3 - intense) scoring

system [27]. The damages evaluated were: epithelial erosion, submucosal edema, presence of inflammatory cells, hemorrhagic damage and total damage (score: 0-12).

2.4.7. Antioxidant Activity *in vivo*

Part of the stomach of the animals treated (previous induction of injury with absolute ethanol or later with acetic acid) orally with the vehicle (CMC, 0.5%) and PPMF2 (in dose of 24 mg/kg (which was chosen because it was the lowest effective dose in the ethanol injury model and the dose that promoted greater cicatrization in the acetic acid ulcer model) was used, as well as part untreated and without induction of injury or ulcer (group - Sham). The following parameters were quantified: levels of non-protein sulfhydryl groups (SH-NP) [28] and malondialdehyde analysis (MDA) [29].

2.5. Statistical Analysis

Statistical analyzes were performed using Student's t-test for chemical tests and analysis of variance ANOVA and Tukey's post-hoc test, for the tests of gastroprotective activity. Regarding the microscopic gastric damages by acetic acid, non-parametric Kruskal-Wallis test was applied followed by Dun's post-test for multiple comparisons. All tests were done using the GraphPad Prism™ 5.0 software (San Diego, CA, USA). Values were considered statistically significant when the value of $p < 0.05$.

3. Results

3.1. Phytochemical Analysis: Antioxidant and Phenolics

The results of the antioxidant activity (DPPH) found for pot-pollen from the stingless bees *M. compressipes fasciculata* collected in two seasons was: PPMF 1 - 62.93 ± 0.51% elimination of the DPPH radical; PPMF2 - 58.86 ± 1.10% elimination of the DPPH radical. In the present work, the average value of *M. compressipes fasciculata* pot-pollen collected in the rainy season (PPMF1) and dry (PPMF2) did not differ statistically from each other ($p < 0.05$).

M. compressipes fasciculata bee pot-pollen extracts were also submitted to HPLC-DAD analysis, whose complete chromatographic profiles of the phenolic compounds showed multiplicity of peaks. Of these, 21 compounds were investigated qualitatively and quantitatively, of which 13 were found in the samples. Of the 21 compounds investigated initially, only 3 of them (protocatechic, p-hydroxybenzoic acid and syringeic acid) could be detected in the samples using HPLC-DAD; the other 10 needed to be analyzed through HPLC-ESI-MS-MS (Table 1). Quantification using HPLC-ESI-MS-MS was performed by comparison with known concentration standards. Table 2 presents the phenolic compounds in pot-pollen samples of *M. compressipes fasciculata* detected by means of HPLC-DAD and HPLC-ESI-MS-MS.

Table 1. Phenolic compounds detected through HPLC-DAD and HPLC-ESI-MS-MS

	Compounds	Retention time (min)	λ nm – maximum absorbance	Molecular formula	[M–H]⁻ *m/z*	Products MS/MS *(m/z)*	Structural class	Collision Energy (V)
1	Gallic acid	2.27	270	$C_7H_6O_5$	168.9	125.9, 79.12	Phenolic acids	-17; -27
2	Protocatechuic acid	4.00	260, 294	$C_7H_6O_4$	153	109.0, 108.0	Phenolic acids	-16; -26
3	P-hydroxybenzoic acid	6.33	256	$C_7H_6O_3$	136.7	93.00; 65.20	Phenolic acids	-18; -31
4	Caffeic acid	9.56	322	$C_9H_8O_4$	179.3	135.0, 133.8	Phenolic acids	-65; -29
5	Syringic acid	10.87	274	$C_9H_{10}O_5$	196.9	166.90,181.90	Phenolic acids	-21; -16
6	P-coumaric acid	13.21	310	$C_9H_8O_3$	163.0	119.0, 93.1	Phenolic acids	-19; -37
7	Rutin	22.52	256, 356	$C_{27}H_{30}O_{16}$	609.1	299.8, 270.7	Flavonoids	-39; -58
8	Isoquercitrin	22.46	256, 356	$C_{21}H_{20}O_{12}$	463.0	299.9, 270.6	Flavonoids	-27; -53
9	Myricetin	23.27	372	$C_{15}H_{10}O_8$	317.2	178.8, 150.9	Flavonoids	-22; -26
10	Rosmarinic acid	24.12	330	$C_{18}H_{16}O_8$	358.8	160.9, 196.9	Phenolic acids	-20; -20
11	Naringenin	24.76	290	$C_{15}H_{12}O_5$	271.2	150.9, 119.0	Flavonoids	-21; -31
12	Quercetin*	24.85	256, 372	$C_{15}H_{10}O_7$	274.2	88.1, 70.2	Flavonoids	+23; +26
13	Quercitrin	24.85	256, 350	$C_{21}H_{20}O_{11}$	447.0	330.8, 299.8	Flavonoids	-25; -28

Table 2. Phenolic compounds in *M. compressipes fasciculata* pot-pollen (PPMF) samples detected through HPLC-DAD and HPLC-ESI-MS-MS

Phenolic compounds	PPMF1 (rainy season) (µg/g)	PPMF2 (dry season) (µg/g)
Gallic acid	1.98±0.01	ND
Protocatechuic acid	442.49±13.31	252.72±25.78
P-hydroxybenzoic acid	780.73±20.54	1757.05±18.32
Caffeic acid	0.17±0.00	0.42±0.02
Syringic acid	188.22±7.03	116.83±5.96
P-coumaric acid	0.28±0.00	0.42±0.01
Rutin	3.39±0.09	2.71±0.07
Isoquercitrin	1.38±0.02	1.11±0.02
Myricetin	9.77±0.23	16.49±0.84
Rosmarinic acid	0.02±0.00	ND
Naringenin	0.27±0.01	2.64±0.08
Quercetin	2.34±0.03	2.75±0.01
Quercitrin	0.73±0.03	1.96±0.08
Sum	*1431.77*	*2155.10*

Results expressed as mean ± SEM; ND: Not Detected.

3.2. Gastroprotective and Ulcer Healing Activities

3.2.1. Effect of PPMF2 on Gastric Lesions Induced By Absolute Ethanol

Oral administration of ethanol induced lesions in the form of hemorrhagic erosions in the glandular portion of the gastric mucosa of rats. In the vehicle group (CMC, 0.5%), severe gastric lesions were observed in the mucosa, such as thick linear hemorrhages and hyperemia, with a lesion area (LA) of 21.17 ± 1.73%. The pretreatment with PPMF2 prevented the development of gastric lesion induced by ethanol, significantly decreasing, compared to the vehicle group, at doses of 24 (10.24 ± 1.52%) and 80 mg/kg (9.68 ± 1.52%), whose percentages of were 52% and 54%, there was no significant difference in the reduction of the lesions among such doses. Animals receiving carbenoxolone (100 mg/kg) also had reduced LA (0.83 ± 0.33%) and protection of 96% (Figure 1).

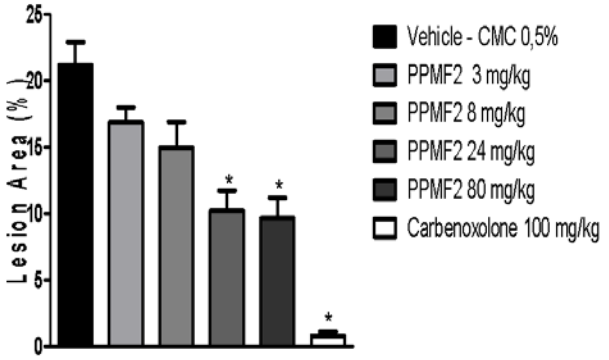

Figure 1. Effect of *M. compressipes fasciculata* pot-pollen (PPMF2) on ethanol-induced gastric ulcers in rats and illustrative images of the stomachs. Animals were treated orally with vehicle (0.5% CMC), PPMF2 (3, 8, 24 mg/kg) or carbenoxolone (100 mg/kg) for seven days after induction (n = 6). Data are expressed as mean ± SEM. *p<0.05 when compared to vehicle control. (One-way ANOVA followed by Tukey's test)

3.2.2. Effect of PPMF2 on Gastric Lesions Induced by Ischemia/Reperfusion

Compared with the vehicle (LA = 13.49 ± 1.57%), the pretreatment with PPMF2 prevented the development of gastric lesions, induced by the ischemia and reperfusion period, promoting a significant reduction of the LA at all doses studied: 8 (3.74 ± 1.27%), 24 (3.15 ± 1.02%) and 80 mg/kg (1.09 ± 0.51%), showing protection of 72, 77 and 92%, respectively, as well as the positive control N-acetylcysteine (LA of 0.83 ± 0.15% and protection of 96%). No significant differences were observed between all doses of PPMF2, as well as between such doses and N-acetylcysteine ($p < 0.05$) in Figure 2.

3.2.3. Effect of PPMF2 on the Production of Gastric Mucus

The rats submitted to pylorus ligation and treated with an intraduodenal vehicle, showed a significant reduction of the gastric mucus content (50.20 ± 2.07 µg of Alcian blue/g of tissue), in relation to the group not submitted to pylorus ligation - Sham group (103.5 ± 8.00 µg Alcian blue / g tissue). In contrast, rats treated at intraduodenal with PPMF2 at 24 mg/kg, contents of gastric mucus (111.3 ± 7.80 µg of Alcian blue/g) were significantly higher ($p < 0.05$), with an increase of 122% in relation to the animals treated with the vehicle. Carbenoxolone significantly increased gastric mucus content by 80% (90.17 ± 8.98 µg of Alcian blue/g, $p < 0.05$). Significant statistical differences between treatment with **PPMF2** (24 mg / kg), carbenoxolone (100 mg / kg) and sham group were not found ($p < 0.05$) (Figure 3).

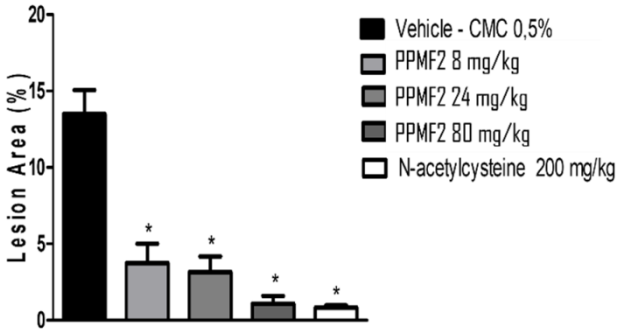

Figure 2. Effect of *M. compressipes fasciculata* pot-pollen (PPMF2) on ischemia and reperfusion -induced gastric ulcers in rats and illustrative. The animals were orally treated with vehicle (0.5% CMC), PPMF2 (3, 8, 24 mg/kg) or N-acetylcysteine (200 mg/kg) for seven days after induction (n = 6). Data are expressed as mean ± SEM. *p<0.05 when compared to vehicle control. (One-way ANOVA followed by Tukey´s test)

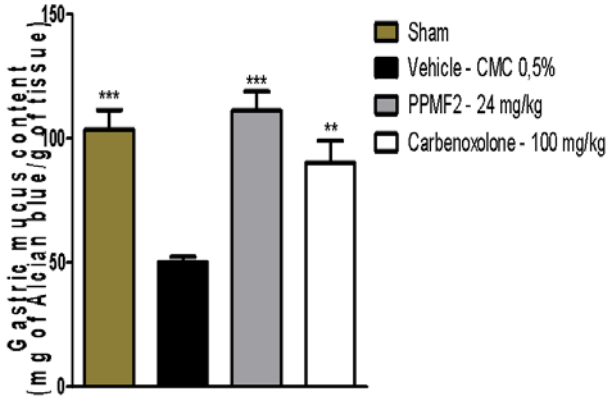

Figure 3. Effects of PPMF2 on gastric muco in absolute ethanol-induced gastric damage in mice. Results are expressed in mean ± SEM. ANOVA followed by Tukey's test. P<0.05 compared with vehicle.

3.2.4. Effects of PPMF2 on Gastric Markers of Oxidative stress in Absolute Ethanol-induced Gastric Damage in Mice

The level of groups SH-NP in the glandular region of the gastric mucosa of untreated and uninjured animals (Sham group) was 455.8 ± 56.31 µg/g tissue. Ethanol administration reduced gastric contents of groups SH-NP in vehicle-pretreated animals (250.3 ± 19.73 µg/g tissue) when compared to the uninjured group. However, pretreatment with PPMF2 (24 mg/kg) was able to maintain SH-NP groups (480.80 ± 22.70 µg/g tissue) at baseline, with a significant increase of 92% when compared with the vehicle. In the study, no statistically significant difference was observed between the group

treated with PPMF2 at a dose of 24 mg/kg and the Sham group ($p < 0.05$) (Figure 4A). The level of MDA of non-injured animals (Sham group) was 1.81 ± 0.42 µM/g tissue. In animals pretreated with vehicle, it was observed that administration of ethanol caused elevation of this level (11.26 ± 0.48 µM/g tissue). However, pretreatment with PPMF2 at a dose of 24 mg/kg significantly ($p < 0.05$) reduced MDA levels (7.19 ± 0.58 µM/g tissue) in 36% comparing with the vehicle group (Figure 4B).

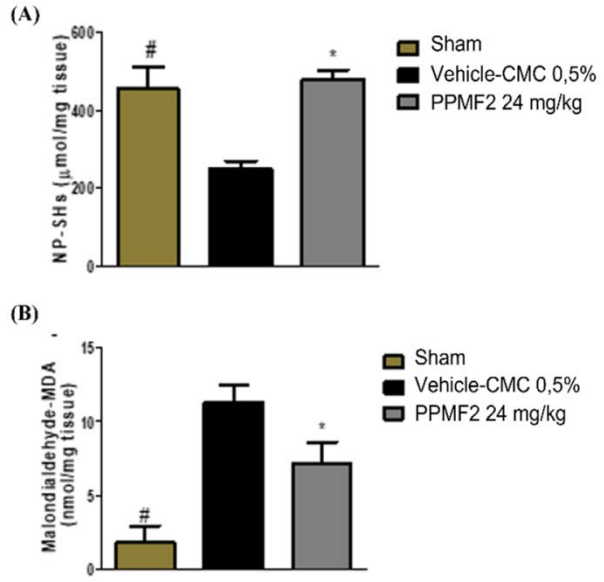

Figure 4. Effects of PPMF2 on gastric markers of oxidative stress in absolute ethanol-induced gastric damage in mice: (A) NP-SHs, Non-protein sulfhydryls and (B) MDA, malondialdehyde. Results are expressed in mean ± SEM. ANOVA followed by Tukey's test. * and # P<0.05 compared with control (vehicle)

3.2.5. Effect of PPMF2 on Gastric Ulcers Induced by Acetic Acid

Oral treatment for seven consecutive days with PPMF2 promoted a reduction in the volume of previously induced ulcer and, thus, a greater healing of these ulcers in rats. PPMF2 significantly reduced ($p < 0.05$) the volume of gastric ulceration, induced by acetic acid, at doses 3 (152.67 ± 20.53 mm^3), 8 (34.89 ± 10.36 mm^3) and 24 mg/kg (7.09 ± 1.86 mm^3), compared to the vehicle - CMC group (237.68 ± 15.47 mm^3). Administration of PPMF2 at a dose of 24 mg/kg was found to provide a superior improvement in the 97% reduction/healing percentage of the ulcer resolution process followed by 8 (85%) and 3 mg/kg (36%), as well as cimetidine (78%). In the investigation, statistical similarity was verified between the groups treated with PPMF2 at doses 8 mg/kg and 24 mg/kg and cimetidine 100 mg/kg (Figure 5).

3.2.6. Effects of PPMF2 on Histological Changes In Gastric Tissue Induced by Acetic Acid

The results of the analysis of the microscopic gastric damage of the animals submitted to the chronic ulcer protocol by acetic acid is shown in Table 3. The examination of gastric sections showed that the application of acetic acid caused deep erosion of surface epithelial cells with intense production of edema, a high presence of inflammatory cells and severe hemorrhage in vehicle-treated animals. The healing effect of **PPMF2** treatment was corroborated by the histological analysis. In treated animals, for seven days, PPMF2 (24 mg/kg) showed a reduction of all gastric damage significantly, when compared with vehicle group ($p < 0.05$). This reduction was also observed in animals treated with cimetidine (Figure 6).

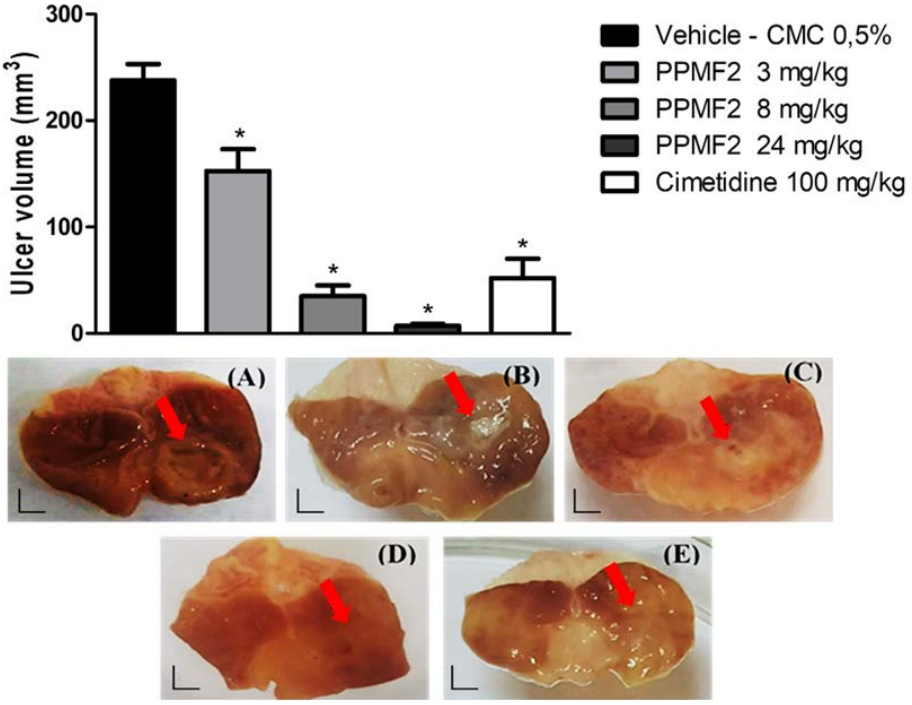

Figure 5. Effect of *M. compressipes fasciculata* pot-pollen (PPMF2) on acetic acid-induced gastric ulcers in rats and representative macroscopic photographs showing healing effects of vehicle-CMC 0,5% (A), **PPMF2** 3 mg/kg (B), **PPMF**2 8 mg/kg (C), **PPMF**2 24 mg/kg (D) or cimetidine (E) on acetic acid induced gastric ulcer . Data are expressed as mean ± SEM. *p<0.05 when compared to vehicle control (One-way ANOVA followed by Tukey´s test)

Table 3. Effect of *M. compressipes fasciculata* pot-pollen (PPMF2) on microscopic gastric damage induced by acetic acid

Experimental Group	Sham	Vehicle – CMC 0.5%	PPMF2 - 24 mg/kg	Cimetidine – 100 mg/kg
Epithelial erosion (score 0-3)	0[a]	3 (3-3)[b]	0,5 (0-2)[a]	0,5 (0-2)[a]
Submucosal edema (score 0-3)	0 (0-0)[a]	3 (3-3)[b]	0 (0-1)[a]	0 (0-1)[a]
Inflammatory cells (score 0-3)	0 (0-0)[a]	3 (3-3)[b]	0 (0-0)[a]	0 (0-1)[a]
Hemorrhage (score 0-3)	0 (0-0)[a]	3 (3-3)[b]	0 (0-2)[a]	0 (0-2)[a]
Total (score 0-12)	0 (0-2)[a]	12 (12-12)[b]	1 (0-3)[a]	1 (0-4)[a]

Data are expressed as median with the minimum and maximum scores in parentheses (0 – negative, 1 – weak, 2 – moderate, 3 – strong). Non - parametric Kruskal - Wallis test followed by Dun 's post - test for multiple comparisons. [a]$p < 0.05$ when compared to the vehicle group (negative control). [b]$p < 0.05$ when compared to the sham group. [c]$p < 0.05$ when compared to the cimetidine group (positive control - standard drug).

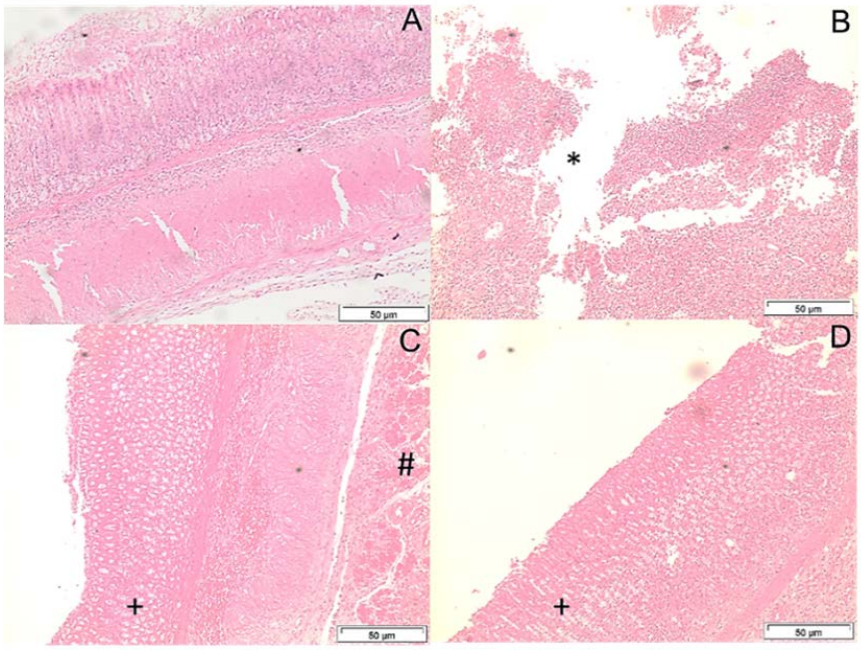

Figure 6. Effect of *M. compressipes fasciculata* pot-pollen (PPMF2) treatment on stomach sections of rats submitted to acetic acid ulceration stained with hematoxylin-eosin. A - sham group (no ulcer induction), showing normal gastric architecture. B - vehicle group (CMC - 0.5%), displaying the ulcer area and complete degassing of the mucosa and submucosa (*). C - PPMF2 group (24 mg / kg), demonstrating a normal glandular pattern with restored mucosa (+) and presence of granulation tissue (#). D - cimetidine group (100 mg / kg), also demonstrating normal pattern. Magnification of 100x

3.2.7. Effects of PPMF2 on Gastric Markers of Oxidative Stress in the Acetic Acid-induced Gastric Ulcer Model

Acetic acid caused a decrease in the gastric content of of non-protein sulfhydryl groups (SH-NP) in the animals treated for seven days with vehicle (286.4 ± 11.22 µg/g tissue) compared to the non-ulcerated group (Sham) 434.8 ± 45.48 µg/g tissue. In contrast, treatment with PPMF2, at the dose of 24 mg/kg, increased the levels of the SH-NP groups (581.5 ± 18.71 µg/g tissue) in 103%, relative to the vehicle; as well as it promoted higher results than the group without ulcer induction (Sham). This treatment with PPMF2 presented a significant difference when compared to the vehicle and also to the Sham group ($p < 0.05$) (Figure 7A). In the stomachs of rats submitted to treatment for seven days with vehicle after the induction of chronic ulcer by acetic acid, high lipid peroxidation was observed, denoted by the high levels of MDA (7.90 ± 1.01 µM / g of tissue) when compared to animals without ulcer induction (Sham) (2.13 ± 0.24 µM / g tissue). Treatment with PPMF2, at a dose of 24 mg / kg, promoted a significant reduction in the level of MDA (1.30 ± 0.15 µM/g tissue) in 84%, when compared to the vehicle group; resulting in a decrease in MDA levels to values similar to those in the sham group ($p < 0.05$) (Figure 7B).

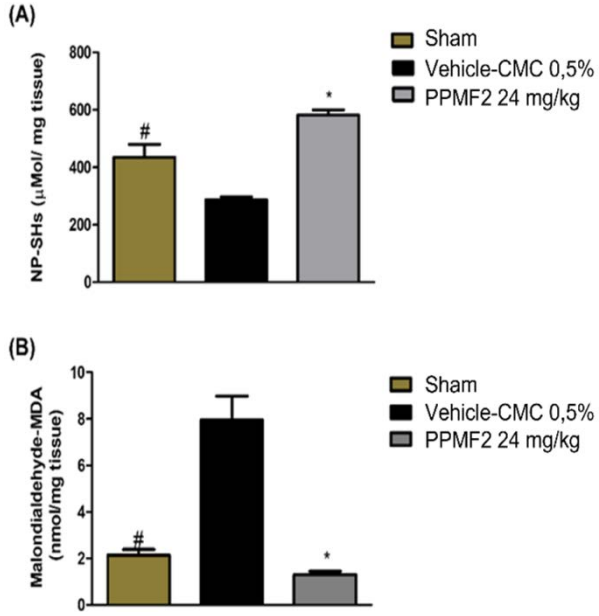

Figure 7. Effects of PPMF2 on gastric markers of oxidative stress in acetic acid-induced gastric ulcer model. (A) NP-SHs, Non-protein sulfhydryls. And (B) MDA, malondialdehyde. Results are expressed in mean ± SEM. ANOVA followed by Tukey's test. * and # $P<0.05$ compared with control (vehicle)

4. Discussion

There are few data in the literature on the antioxidant activity of pollen synthesized and stored by stingless bees (pot-pollen), especially from species of the genus *Melipona*. The values of antioxidant activity found in this study were higher than those obtained by reference [30], whereby pollen from three species of stingless bees from Malaysia (Trigona toracica, Trigona itama and Trigona apicalis) showed percentages of DPPH inhibition of, respectively, 6.7%, 14.3% and 39%. The reference [31] shows pot-pollen from *M. rufiventris*, found antioxidant activity of 50% inhibition of DPPH. The bee pollen collected in different seasons and regions may exhibit distinct antioxidant capacity; the same holds true for bee bread and pot-pollen [32]. The determination of the antioxidant capacity is strongly influenced by the experimental conditions and heterogeneity of the matrix [33], which may hinder the comparison between results.

In the present study, the quantitative and qualitative phenolic profile analyses were carried out using high-efficiency liquid chromatography (HPLC) and high-efficiency liquid chromatography coupled to mass spectrometry (MS/MS), pot-pollen *M. compressipes fasciculata* collected in the rainy and dry season. As for the phenolic compounds that were investigated and quantified through these analyses, it was verified that the sample collected in the dry season (PPMF2) showed a larger sum of these (Table 4). This variation can be correlated with the fact that phenolics are secondary plant metabolites associated to the protection and elevation of plant survival, with increased biosynthesis under different environmental factors and in response to stress conditions (such as water) and the addition of UV radiation [34].

For the class of phenolic compounds investigated in the research, the phenolic acids were the major ones. In relation to these, the p-hydroxybenzoic and protocatechuic acids followed by the syringe were the most prevalent. Among the constituents of bee pollen, the following phenolic acids and their esters have already been described: benzoic acid derivatives (gallic acid, p-hydroxybenzoic acid, vanillic acid, succinic and protocatechuic acid) and cinnamic acid derivatives (ferulic acid, p-coumaric acid, caffeic acid and its glycerol esters). Other derivatives, such as rosmarinic acid dihexoside, were also found [35].

In the present work, the following flavonoids were detected: myricetin, rutin, quercetin, isoquircetin, quercitrin and naringenin. In other studies, several phenolics such as quercetin, rutin, kaempferol have been described as the most abundant in bee pollen samples from different countries [36]. In the study of investigation of the chemical composition of beebread, the authors identified by HPLC method, the chemical substances: quercetin, naringenin, kaempferol, apigenin and isorhamnetin [37].

The phenolic profile of the bread of bee is equal to the pollen of the corresponding flora (collected manually) and to bee pollen [38]. Despite the current biochemical transformations during the fermentation process inside the hive, it appears that these phenolic compounds are unaffected and remain unchanged and preserved. Of the compounds present in pollen and bee bread, phenolics form an important group of bioactive structures and are useful in several applications [39]. These compounds are described as the main bioactive agents in bee pollen. It is known that phenolic compounds trigger antioxidant activities, acting as free radical scavengers, and may reduce the risk of various pathologies [40]. The phenolic compounds have antiulcerogenic effects related to cytoprotective activity, such as phenolic acids, caffeic acid, p-coumaric acid, cinnamic acids, among others [41]. The protocatecous acid also has cyproprotective and gastroprotective activity [42]. Certain flavonoids have also demonstrated antiulcerogenic and gastric lesion prevention actions in *in vivo* experimental models such as rutin, naringenin, quercetin, kaempferol, among others [43,44].

Thus, due to the rich phenolic composition and other bioactive compounds reported in the pollen literature collected and stored by bees, and the lack of research on the effects of pot-pollen produced by stingless bee *M. compressipes fasciculata* on the gastric mucosa, the gastroprotective activity of this product in rats was investigated in this study.

Initially, the ability of *M. compressipes fasciculata* pot-pollen (PPMF2) to protect the gastric mucosa against lesions induced by absolute ethanol was investigated. In this study, the experimental results demonstrated that the administration of a single dose by gavage caused marked damage to the gastric mucosa of rats, especially characterized by macroscopic elongated lesions with intense hyperemia and bleeding, similar to the effect verified by other authors [45,46]. Acute gastric lesions by ethanol in experimental animals are the most used model in the preclinical evaluation of substances with potential gastroprotective effects, since ethanol has been considered as one of the main ulcerogenic agents in humans. Its use in animals enables the analysis of the cytoprotective activity of potentially active products [46].

Because it is a direct acting necrotizing agent, ethanol changes protective factors and causes lesions in the gastric mucosa due to the rupture of the muco-bicarbonate barrier, disorders in the microcirculation and ischemia, with consequent high production of reactive oxygen species (ROS). Thus, there is an increase in oxidative stress and lipid peroxidation, reduction of glutathione (GSH) and activation of inflammatory signaling pathways [47-48]. In this work, pretreatment with PPMF2 inhibited the development of gastric lesions induced by absolute ethanol, suggesting a possible cytoprotective action.

The antioxidant action is one of the most important mechanisms of gastric protection, which motivated the investigation also to the protocol of acute lesions induced by ischemia-reperfusion (I/R). The I/R gastric lesion model is used to analyze the response of products in ulcerogenic processes without the use of chemical agents, pathogens or somatic stress, isolating the ulcerative factors pertinent to free radicals developed by vascular and inflammatory events [49,50]. Ischemia alone is capable of injuring the gastric mucosa; however, it is after reoxygenation (reperfusion) that the most damaging events occur, with the lesion content increasing by approximately three-fold, compared to those caused by ischemia [51].

The experimental data evidenced the presence of damage in the glandular portion of the stomachs of the rats

after the ischemia and reperfusion processes, which were significantly decreased with the PPMF2 pretreatment. It is known that both the ischemia/ reperfusion-induced gastric injury model and ethanol are directly linked to the release of ROS, which causes oxidative damage in cellular biomacromolecules and that can lead to apoptosis and tissue damage [52,53]. Thus, the action of **PPMF2** to prevent the development of gastric lesions in this model by I/R, thus exhibiting gastroprotective activity, denotes antioxidant or sequestering action of EROs.

Interestingly, the treatment with PPMF2 prior to the injury induction during the I/R period promoted effects with no significant difference in relation to N-acetylcysteine, a potent antioxidant with proven action. An effect similar to that promoted by N-acetylcysteine (200 mg/kg) was observed even with pretreatment with PPMF2 at the lowest dose studied (8 mg/kg), a dose that is considerably lower than that of N-acetylcysteine.

The mucus wall that lines the gastric mucosa acts as a physical barrier against the aggressive action of gastric acid secretion. Mucus also has antioxidant action, so weakening of the mucus barrier is directly responsible for gastric lesions [54]. In the present study, treatment with PPMF2 at a dose of 24 mg/kg, elevated the mucus content in the gastric mucosa in the protocol of ligature of the pylorus (in relation to the vehicle group), which suggests that the PPMF2 protects the gastric mucosa by increasing this important antioxidant and cytoprotective agent. Interestingly, it has also been found that this treatment with PPMF2 promoted results similar to those obtained with treatment with carbenoxolone (standard drug that stimulates mucus secretion) at a dose of 100 mg/kg, although the **PPMF2** dose is about four times lower, corroborating the gastroprotective effect of PPMF2 and evidencing its pharmacological potentiality.

In this context, antioxidants and free radical scavengers have been identified as inhibitors of oxidative stress and, consequently, of the progression of lipid peroxidation. Studies have showed that substances with antioxidant properties may act as defenders of the gastric mucosa in various models of lesion or ulcer induction [55,56,57]. In the present research, it was first analyzed whether PPMF2 would be able to sequester free radicals in an *in vitro* model through the use of DPPH radicals, as well as investigations on total phenolic content and analysis of detection and identification of these phenomena. The pot-pollen has been shown to have an antioxidant capacity in vivo and in vitro, as well as the presence of phenolic acids and antioxidant flavonoids that have already had reported gastroprotective action, such as caffeic acid, quercetin, myricetin, rutin and naringenin [43,44]. Thus, because of these characteristics, administration of PPMF2 in this study has possibly promoted improvements in cellular antioxidant defenses, which may increase the gastroprotective effect of this product against induced lesions in rats.

Since PPMF2 showed antioxidant effect *in vitro* and subsequent to the proving of gastroprotective activity in models of lesions induced by absolute ethanol, it was interesting to find out whether the mechanism of action was related to cellular antioxidant defenses. The participation of non-protein sulfhydryl groups (SH-NP) and the involvement of lipid peroxidation (MDA) were evaluated. In these approaches, PPMF2 was used at a dose of 24 mg/kg, which was the lowest effective dose in the model of gastric injury induced by absolute ethanol.

The gastric mucosa has high levels of reduced glutathione representing the main component of the non-protein sulfhydryl groups (SH-NP). Oral administration of ethanol decreases SH-NP levels in the mucosa [58]. This reduction may be related to its binding with acetaldehyde (produced by the enzyme alcohol dehydrogenase), oxidation by toxic metabolites of ethanol and also by the reduction of GSH production [59].

These SH-NP groups participate in mucosal protection by the effect of binding to free radicals and by forming disulfide bonds between the mucus subunits, in order to avoid their dissociation. Hence, the depletion of these groups seems to be involved in the formation of the lesion [60,61]. They are important in the reduction of oxidative stress and peroxides, protecting proteic cellular structures [62,63]. Thus, they constitute an important component of intracellular defense mechanisms and maintenance of the antioxidant balance against noxious stimuli.

In the research, it was verified that the administration of absolute ethanol caused a decrease in the levels of non-protein sulfhydryl groups (SH-NP); on the other hand, pretreatment with PPMF2 was able to maintain these normal levels, promoting a significant increase when compared to the vehicle. This increase in SH-NP levels may be responsible for the gastroprotective action of PPMF2, causing potentiation of the endogenous antioxidant defense and reduction of oxidative stress damage generated by the administration of absolute ethanol.

One of the main implications of the release of ROS is the production of lipid peroxides that cause damage to biological membranes [53]. Malondialdehyde (MDA) is the metastable end-product of lipid peroxidation, and its plasma levels have been added after this process [64]. MDA is used to evaluate levels of lipid peroxidation; the existence of a relationship between gastric ulcer and the increase in MDA levels is suggested and an increase in its production after ethanol-induced tissue injury has been reported [65]. Antioxidants are capable of both decreasing lipid peroxidation and capturing free oxygen radicals, and thus are likely to serve as antiulcer agents [66]. In the present study, PPMF2 was able to significantly reduce lipid peroxidation as measured by MDA (marker of oxidative stress). This suggests that PPMF2 acts as a sequester of ROS, since it was able to protect the gastric mucosa against lipid peroxidation induced by absolute ethanol, thus exhibiting gastroprotective activity possibly related to elevation of the antioxidant effect.

After the observation of the preventive effect of PPMF2 on acute gastric ulcer models and with the purpose of better understanding this gastroprotective effect, the cicatrizant activity was evaluated against the chronic ulcer model induced by acetic acid. Gastric ulcers in this model exhibit many similarities to those observed in humans, in terms of pathological aspects, as well as in the healing processes, the difficulty of treatment and an extended period of cure [67]. In general, healing of gastric ulcers is a multifactorial process involving stages such as cell proliferation, migration, differentiation and regeneration, angiogenesis and deposition of the extracellular matrix,

leading to the development of a scar. These processes are orchestrated by multiple hormones, cytokines, transcription and growth factors [68]. The re-epithelialization stage (migration of ulcer margin epithelial cells to restore epithelial continuity) begins three days after the ulcer induction and is essential for the healing of gastric ulcers, as this continuous epithelial barrier protects tissue from granulation against injuries and infections [69]. The quality of ulcer healing is a key point in the pathogenesis of gastric ulcers, since abnormalities in mucosal regeneration within cicatrized ulcer scars have been reported, as well as the continuation of chronic inflammation evidenced by the presence of increased infiltration of macrophages and neutrophils, these processes being the basis for ulcer recurrence. Thus, the search for new therapeutic agents should also focus on improving this quality of healing [70].

The results of the present study showed that the treatment with PPMF2 for seven days significantly reduced the volume of gastric ulcer induced by acetic acid, promoting improvement in the **healing** response. This healing action of PPMF2 was provided even at a low dose (3 mg/kg). Interestingly, the healing effect of cimetidine (reference drug used and widely used clinically for the treatment of gastric ulcers) at a dose of 100 mg/kg showed no significant difference in the effect promoted by PPMF2 (at doses of 8 and 24 mg/kg), although these doses are considerably smaller. PPMF2 (at a dose of 24 mg/kg) also resulted in a regression of the microscopic gastric damage ascertained and induced by the direct application of acetic acid, causing gastric mucosal patterns similar to normal, corroborating its healing effect. Thus, these results indicate that PPMF2 modulates protective factors of the gastric mucosa, with healing effects on the pre-existing ulcers, being a product that can be used in the treatment of gastric ulcers.

In order to identify the possible signs of toxicity or side effects of PPMF2 on the general physiological function, behavioral follow-up of the animals during the course of the protocol of chronic gastric ulcer induced by acetic acid was verified, being verified absence of mortality, of any abnormalities or signs of toxicity. The evaluations of body weight gain and absolute body weight were also performed. All animals gained weight, with no statistical difference between the groups, and had organs with weights within the normal range for rats, indicating that the treatment with pot-pollen did not cause changes.

The use of acetic acid in the gastric mucosa induces the appearance of the gastric ulcer resulting from the oxidative stress, indicated by the reduction of the level of SH-NP groups and elevation of the lipid peroxidation evidenced by the increase of MDA [71]. As discussed, antioxidant compounds are useful in the defense of the gastric mucosa against oxidative stress [61]. Thus, it was evaluated whether the healing effect of PPMF2 administered seven days after the induction of chronic ulcer by acetic acid was also related to the endogenous antioxidant system. For this purpose, the gastric tissue of rats treated with PPMF2 at a dose of 24 mg/kg was used, since this dose was the highest healing index (97%).

The continuous treatment (for seven days) with PPMF2 increased considerably, relative to the vehicle, the content of the non-protein sulfhydryl groups, depleted by the elevation of ROS generated by the direct application of acetic acid to the gastric mucosa. Interestingly, this treatment with PPMF2 also promoted levels of SH-NP groups higher than those of the non-ulcerated group. These findings suggest that PPMF2 acts on the potentiation of the antioxidant system, promoting conditions that are more conductive to the healing and healing processes, since SH-NP groups are able to bind to reactive free radicals blocking their deleterious effects [60].

It was also found that this administration of PPMF2 significantly reduced the lipid peroxidation caused by the acetic acid ulcer and evidenced by the levels of the oxidative stress marker (MDA), suggesting action against ROS. Positively also, this treatment with **PPMF2** promoted levels of MDA similar to those found in non-ulcerated animals, corroborating that PPMF2 acts as an antioxidant and strengthens the body's defenses. Similarly, bee pollen from Cairo and Egypt led to a significant decrease in malonaldehyde (MDA), as well as a notable increase in the levels of superoxide dismutase (SOD) and glutathione (GSH) in the blood and brain of rats, an antioxidant system against the toxicity of sodium fluoride [72].

Comparatively, these results with treatment for seven days post gastric ulcer induction were even more expressive than those found for these same single-dose pretreatment analyses in the absolute ethanol induction model, denoting that the administration of pot-pollen from *M. compressipes fasciculata* (PPMF2) in a continuous way is able to potentiate its gastroprotective effects.

5. Conclusion

The pot-pollen of *M. compressipes fasciculata* analyzed presents antioxidant activity *in vitro* (**PPMF1** - 62.93 ± 0.51% elimination of the DPPH radical; PPMF2 - 58.86 ± 1.10% elimination of the DPPH radical), as well as the presence of phenolics with proven in **PPMF2**, possibly be involved in the antioxidant (increasing gastric contents of groups SH-NP and promoted a significant reduction in the level of MDA) and gastroprotective and ulcer healing activities (in the lesions gastric protocol induced by etanol, ischemia/reperfusion and acetic acid) possibly by mechanisms of potentiation of the antioxidant system, negative modulation of inflammation and restoration of the gastric mucosa with increased mucus production. Thus, pot-pollen, in addition to the already known nutritional constituents, presents several bioactive substances with potential antiulcerogenic effect, being a source of potential new antiulcerogenic products.

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