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Mucuna pruriens treatment shows anti-obesity and intestinal health effects in obese rats†

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This study evaluated the anti-obesity effect and intestinal health of obese rats treated with *Mucuna pruriens* (MP), focusing on food consumption and somatic, biochemical, and histological parameters. A total of 32 adult male Wistar rats were initially randomized into a healthy group (HG, n = 16) which consumed a control diet and an obese group (OG, n = 16) which consumed a cafeteria diet for eight weeks. They were then subdivided into four groups: healthy (HG, n = 8); healthy treated with MP (HGMP, n = 8); obese (OG, n = 8); obese treated with MP (OGMP, n = 8), with consumption of their respective diets continuing for another eight weeks; the treated groups received 750 mg kg^{-1} of MP extract *via* gavage. Food consumption and body weight were monitored weekly. Glucose and insulin tolerance tests were performed, and feces were collected for bacterial count and quantification of organic acids. The rats were euthanized, their blood was collected for biochemical analysis, organs and adipose tissue for histological analysis and carcasses for body composition. The obese rats showed a preference for processed meat, stuffed biscuits, popcorn, hot dog sausages, Bologna and ham. The OGMP exhibited lower caloric intake (17%), body weight (14%), fat mass (44%), triglycerides (68%), insulin (58%), leptin (40%), C-reactive protein (75%) and alpha1-glycoprotein acid (62%) and increased HDL (45%) compared to the OG. Moreover, MP reversed changes in liver and adipose tissues induced by obesity and increased counts of lactic acid bacteria and organic acids in feces. The MP treatment demonstrated an anti-obesity effect with improvement in body composition, biochemical profile, and intestinal health of obese rats.

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1. Introduction

Obesity is a public health problem of increasing prevalence, becoming a global epidemy.[1] The development of this disease is mainly related to westernized eating habits in the current population. Indeed, increased consumption of processed foods, which in general have high energy density, high content of salt, sugars, fat (total, trans, and saturated), with concomitant reduction in fibre content, characterizes the Westernized food pattern.[2]

In the long term, a Western diet is one of the main factors which predisposes obesity, resulting in chronic inflammation

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in adipose tissue, in turn resulting in pathophysiological complications and thus increasing the risk of developing diabetes,[3] endocrine[4] and psychiatric disorders,[5] cardiovascular diseases, hepatic steatosis,[6] immunological changes,[7] various types of cancers[8] and changes in food reward processes.[9]

Recent studies have shown that changes in intestinal microbiota, such as an increased firmicute/bacteroidete ratio, play an important role in the pathophysiology of obesity. The underlying mechanisms involve increased conversion of energy and lipogenesis, hormonal and immunological changes caused by greater intestinal permeability, provoked endotoxemia and induced inflammatory response.[10–12] A reduced production of short-chain fatty acids (SCFA), which could mitigate inflammation, regulate satiety, glucose and lipid metabolism and bring benefits to the host,[13] is also involved.

Much research has been done on treating obesity in view of its severity and its comorbidities, ranging from behavioural changes to the use of allopathic medicines.[14] However, there is no standard drug for treating obesity, since it is a multifactor disease. There are drugs approved by the United States Food and Drug Administration for chronic weight management such as orlistat, phentermine/topiramate, naltrexone/bupropion and liraglutide. However, they cause adverse effects and even weight re-gain after treatment.[15–18]

In this sense, the use of herbal medicines has become an alternative to the use of allopathic medicine. *Mucuna pruriens* L. (MP) has stood out among herbal medicines as a possible alternative for treating obesity, since it has a rich nutritional composition,[19,20] in addition to hypoglycemic,[21] hypolipidemic,[22] antioxidant,[23] anti-inflammatory[24] and anxiolytic effects.[25] However, such effects of MP have been reported in the literature in non-obese animals and humans, and our research group recently demonstrated weight loss, anxiolytic and antidepressant effects of MP in obese rats[16] for the first time in the literature. Considering the possible influence of MP treatment on the intestine-brain axis of obese rats, this study aimed to assess consumption and food preference, intestinal health, and somatic, biochemical and histological parameters of obese rats treated with MP.

2. Materials and methods

2.1 Mucuna pruriens samples

After botanical identification of the plant and depositing the specimen at the *Herbário Lauro Pires Xavier* at the Federal University of Paraíba (UFPB), the hydroalcoholic extract of MP seeds was obtained according to the methodology described by Muthu and Krishnamoorthy[26] with adaptations.[19,20] The chemical characterization of hydroalcoholic extract of MP was performed in a previous study by our research group.[20] The MP hydroalcoholic extract has bioactive compounds such as 1.42% of soluble fibres, 1.01% of insoluble fibres, citric acid (31.76 mg per 100 g), oligosaccharide 1-kestose (20.70 mg per 100 g) and levodopa (14.08%). The main phenolic compounds quantified in 100 g of this extract were catechins (57.83 mg), chlorogenic acid (49.32 mg), trans-resveratrol (21.41 mg) and kaempferol 3-glycoside (19.34 mg).[20]

2.2 Study design

The biological test was initiated after approval of the experimental protocol by the Ethics Committee on the Use of Laboratory Animals at UFPB (CEUA – UFPB), under protocol number 4657230418, and followed the guidelines of the Brazilian Society of Science in Laboratory Animals (SBCAL) and Institute for Laboratory Animal Research[27] (ESI, Fig. S1†).

A total of 32 male Wistar rats (average initial weight 157.78 ± 10.35 g) at ±40 days of age were used, kept under standard conditions of light (12/12 hours light/dark cycle, light-off at 6 p.m. and light-on at 6 a.m.), humidity (55 ± 10%) and temperature (22 ± 2 °C). The rats were kept in collective cages (two rats per cage) and received filtered water and diets *ad libitum*. The rats were initially randomized into two groups in the first eight weeks of the experiment: healthy group (HG, $n = 16$) and obese group (OG, $n = 16$). Next, they were divided into four groups in the following eight weeks: healthy group (HG, $n = 8$); healthy group treated with MP (HGMP, $n = 8$); obese group (OG, $n = 8$); obese group treated with MP (OGMP, $n = 8$).

The rats consumed their diets and filtered water *ad libitum* for 16 weeks. A previously validated cafeteria diet was offered to the two groups of obese rats (OG and OGMP) to induce obesity composed of control chow diet (Presence, Paulínia, Brazil) and processed foods such as sausages, chips, cookies, cakes, corn chips, among other foods considered hypercaloric, sources of salt, fat and sugars[12] (ESI, Table S1†). The rats in the HG and HGMP groups received a control chow diet (Presence, Paulínia, Brazil). Nutritional composition of the control and cafeteria diets is shown in Table S2 (ESI, Table S2†).

The HGMP and OGMP groups received MP seed hydroalcoholic extract (750 mg kg^{-1} of body weight) diluted in 1 mL of distilled water *via* gavage daily during the final eight weeks of the experiment.[25] The chosen MP extract dose (750 mg kg^{-1}) was established based on a pilot study and on previous studies which reported anxiolytic and slimming effects,[20,25] as well as a lack of adverse effects and toxicity.[28] The extract was obtained as described by Tavares *et al.*[19] The rats in control groups (HG and OG) received gavage with 1 mL of distilled water. The dose of MP seed extract was selected from a pilot study and based on previous studies.[25,28,29]

Consumption and food preference were evaluated daily at the same time, being calculated by the average of the difference, in grams, between the food offered and the residual.[29] Next, the consumption of calories, carbohydrates, proteins, total and saturated fats, dietary fibres, and sodium were calculated for each rat during the experiment based on the nutritional tables of the commercial ration and the foods offered in the cafeteria diet.

The rats' body weight was checked weekly, on the same day and time by directly weighing each rat on an electronic scale (Prix 3/1, Toledo, São Bernardo do Campo, Brazil). Total weight gain was calculated by the difference between the

weight at the beginning and at the end of the experimental protocol.

2.3 Microbial counts in faeces

Faecal samples were collected for three consecutive days in the last week of the experiment to count microorganisms. Faecal samples were diluted (1 : 9) in sterile peptone water and inoculated (20 μL) using the microdrop technique on selective agar for counting lactic acid bacteria (Man's agar, Rogosa and Sharpe – MRS, HiMedia, India), *Enterobacteriaceae* (MacConkey agar; HiMedia, India), *E. coli* (HiColiform chromogenic agar; HiMedia, India) and *Streptococcus*/*Enterococcus faecalis* (Bateroides Bile Esculina agar – Bbe; Acumedia, USA). Agar plates for counting lactic acid bacteria and Bacteroidetes were incubated in anaerobic conditions (Anaerobic System Anaerogen; Oxoid Ltd, Wade Road, UK) and agar plates for counting *Enterobacteriaceae* were incubated under aerobic conditions, all at 37 °C for 48 hours. The characteristic colonies were counted in the selective media at the end of the incubation period, and the results were expressed as a log of colony-forming units per g of faeces (log 10 CFU g^{-1}).[30,31]

2.4 Quantification of organic acids in faeces

Organic acids were assessed in Tavares *et al.*[20] First, organic acetic, butyric, propionic, formic, citric, lactic and malic acids were quantified by liquid chromatography (HPLC) using an LC 1260 Infinity system (Agilent Technologies) coupled to a PDA detector (G1315D; Agilent Technologies). An Agilent Hi-Plax H column (300 × 7.7 mm) with a particle size of 8.0 μm was used for analysis; the column was protected with a PL Hi-Plax H protection column (5 × 3 mm) (Agilent Technologies). Column temperatures were maintained at 50 °C. Each sample was diluted in ultrapure water, filtered through a 0.45 μm pore membrane with an injection volume of 10 μL at a flow rate of 0.5 to 5 mL min^{-1} and at a duration of 20 min. The phase was 4.0 mm H_2SO_4 in ultrapure water. The obtained data was processed using the Open LAB CDS Cessation Edition (Agilent Technologies). The peaks of the HPLC samples were identified by comparing their retention times with those of the organic acid standards.[32] Duplicate injections were performed, and average peak areas were used for quantification. The formic acid standard was obtained from Sigma-Aldrich (St Louis, MO, USA), and the acetic, butyric, propionic, citric, lactic and malic acids standards were obtained from Vetec (Rio de Janeiro, RJ, Brazil), all with a purity of ≥99%. Ultrapure water was obtained from the Scientific Mars System (São Paulo, SP, Brazil) and sulfuric acid was obtained from Merck (Darmstardt, Germany).

2.5 Glucose and insulin tolerance tests

Glucose (GTT) and insulin (ITT) tolerance tests were performed at the end of the experimental protocol, on the antepenultimate and penultimate day of the experiment, respectively.

GTT was performed after a 6 h fast during the light-on cycle, and the initial blood glucose (T0) was measured *via* incision in the rat's tail, followed by gavage administration of a 25% glucose solution at the dose of 2 g of glucose per kg of body weight, measuring blood glucose at 30, 60, 90 and 120 min. Initial blood glucose determined fasting blood glucose and blood glucose at 30 min determined postprandial blood glucose.

ITT was then performed on the next day in fed rats at baseline and after intraperitoneal administration of regular insulin (Novolin® R, Novo Nordisk, Bagsvaerd, Denmark), equivalent to 0.75 IU insulin per kg of body weight, diluted in sterile 0.9% saline, and measuring blood glucose at 0, 30, 60, 90 and 120 min. Blood glucose was measured using an Accu-check Performa glucometer (Jaguaré, Brazil).[12]

The absolute glucose levels measured over each 120 min GTT and ITT were also used to calculate glucose area-under-the-curve (AUC), comparing the AUC of the experimental groups with that of the control group using the trapezoidal rule.[33]

2.6 Euthanasia, collection of biological material and biochemical analyses

The rats were fasted for 8 h at the end of the 16-week experiment and then euthanized by beheading, according to the ethical principles of the National Council for Animal Science and Experimentation (CONCEA). The liver (left lobe), intestine (colon) and adipose tissue were collected for histological analysis, blood for biochemical analysis and carcass was used for body composition analysis.

The blood was collected in sterile tubes and centrifuged (1040*g* per 10 min) to obtain the serum for analyses. Hepatic lipids were extracted based on the method described by Folch *et al.*,[34] and triglycerides and total cholesterol hepatic were then quantified from this lipid extract. Quantification of triglycerides (TG, Labtest ref-87 analysed at 500 nm), total cholesterol (TC, Labtest ref-76 analysed at 500 nm), high density lipoprotein (HDL, Labtest ref-98 analysed at 550 nm) and low density lipoprotein (LDL, Labtest ref-146 analysed at 546 nm), C-reactive protein (CRP, Labtest ref-3002 analysed at 570 nm) and acid alpha-glycoprotein (AAG, Labtest ref-356 analysed at 600 nm) were performed in a Labtest LabMax 240 Premium automatic analyser (Belo Horizonte, Brazil). The obesity-associated hormones (insulin and leptin) were analysed using the electrochemiluminescence immunoassay (Roche Cobas e601 analyzer) and Enzyme-Linked Immunosorbent Assay – ELISA (MilliporeSigma, EZHL-80SK read spectrophotometrically at 450 nm), respectively.[35]

Based on the results of these analyses, the metabolic load index (MLI, eqn (1))[36] and homeostasis model assessment (HOMA-IR, eqn (2))[37] were calculated as equations described below:

$$\text{MLI} = \text{Tryclicerides} + \text{Postprandial blood glucose} \quad (1)$$

$$\text{HOMA-IR} = \frac{\text{Fasting blood glucose} \times 0,0555 \times \text{Fasting insuline}}{22,5} \quad (2)$$

2.7 Somatic parameters

Epididymal, visceral and retroperitoneal fats were collected, weighed, and used to calculate the adiposity index using the formula described in eqn (3):[38]

$$\text{Adiposity index} = \frac{\text{Epididymal} + \text{visceral} + \text{retroperitonial fats}}{\text{Body weight}} \times 100 \quad (3)$$

The rat carcasses were eviscerated, weighed, the skin was removed, ground in a Bermar mill, BM 23 NR (São José do Rio Preto, São Paulo, Brazil) and stored at −20 °C to analyse the rats' body composition. The fat free mass was quantified by adding the moisture determinations by drying in an oven at 105 °C, ash by incineration in a muffle furnace at 550 °C and proteins by the Kjeldahl method.[39] The fat mass was quantified by extracting cold lipids with chloroform and methanol (2 : 1).[40]

2.8 Histological analysis

The liver (left lobe), intestine (colon) and adipose tissue were fixed in 10% buffered formaldehyde and processed according to routine histological technique. The slides obtained were stained using the Harris Hematoxylin and Eosin (H&E) technique, performing the assembly between slide and coverslip with synthetic resin (Entellan®, Merck, Darmstadt, HE, Germany) for analysis in increasing objectives and photographed in increasing 20× and 40× in a standard optical microscope (Motic BA 200, Santa Monica, USA). The slides were reassessed by the same pathologist in order to confirm the observations after being randomized by an independent person and the general agreement between the two analyses was considered as an evaluation criterion.[12,31]

2.9 Statistical analysis

Data were presented as mean and standard deviation of the mean, submitted to the normality test by the Kolmogorov–Smirnov test. One-way ANOVA analysis of variance was used to compare data with a single factor for groups, while two-way ANOVA was used for independent measurements with a factor for group and a factor for time. When there was a difference between the variables, the Tukey post-test was performed with a significance level of 5% ($p \leq 0.05$). The analyses were performed using the Instat 3.0 software program (GraphPad, San Diego, CA, USA).

3. Results

3.1 Calories and nutrients intake, weight gain and body composition

The OG and OGMP consumed a higher content of calories, carbohydrates, proteins, fat (total and saturated) and sodium compared to groups which only consumed the commercial diet (HG and HGMP). MP treatment in obese rats (OGMP) was able to reduce the consumption of proteins, total and saturated fats and sodium compared to untreated rats (OG, $p \leq 0.05$) (Fig. 1). Obesity-induced rats showed food preference for processed meat, sandwich biscuit, popcorn, hot dog sausage, Bologna and ham, in that order, as well as for commercial feed (ESI, Fig. S2†).

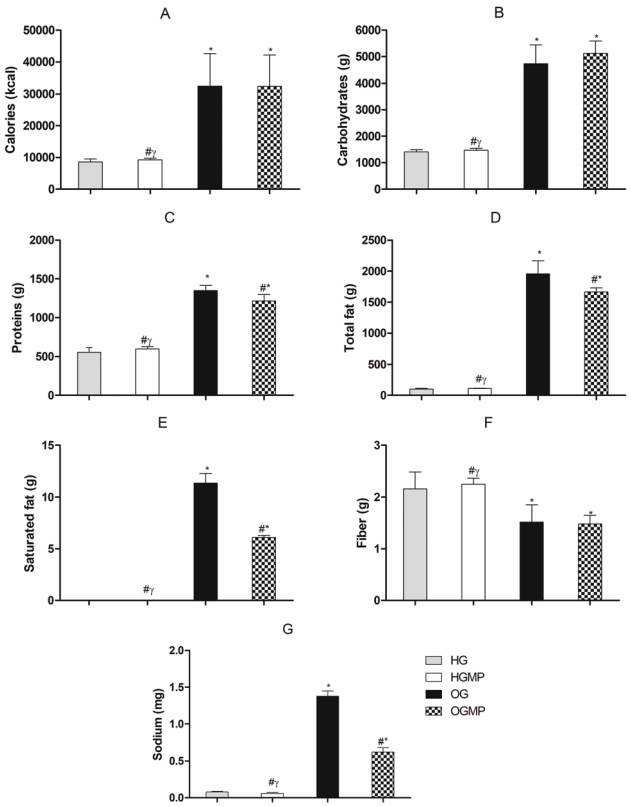

Fig. 1 Calorie intake (panel A), carbohydrates (panel B), proteins (panel C), total fat (panel D), saturated fat (panel E), fibre (panel F) and sodium (panel G) from healthy and obese rats treated or not with MP. One-way ANOVA, $p \leq 0.05$ Tukey's *post hoc* test. * different from HG; # different from OG; γ different from OGMP.

Treatment with MP in obese rats (OGMP) was able to reduce weight gain, the adiposity index and the fat mass percentage compared to OG ($p \leq 0.05$), to the point of normalizing these parameters in comparison with healthy groups, thus presenting differences in food consumption profile (Fig. 2).

3.2 Biochemical analysis

In the GTT, OGMP showed lower blood glucose ($p \leq 0.05$) at 0 min (101.98 ± 3.10 mg dL^{-1}), 30 min (142.28 ± 15.36 mg dL^{-1}), 60 min (161.66 ± 13.40 mg dL^{-1}) 90 min (142.82 ± 6.74 mg dL^{-1}) and 120 min (131.97 ± 10.29 mg dL^{-1}) compared to OG (112.67 ± 6, 09; 156.50 ± 14.49; 184.83 ± 20.76; 176.83 ± 15.66; 168.83 ± 8.75 at 0, 30, 60, 90 and 120 min, respectively). In ITT, blood glucose of the OGMP group decreased ($p \leq 0.05$) (Table 1) at 0 (112.29 ± 5.39 mg dL^{-1}) and 120 min (119.86 ± 8.82 mg dL^{-1}) compared to OG which presented 128.86 ± 8.86 mg dL^{-1} at 0 min and 168.83 ± 8.75 mg dL^{-1} at 120 min ($p \leq 0.05$). Lastly, treatment with MP in healthy rats did not cause any change in blood glucose in the GTT and ITT tests (Fig. 3).

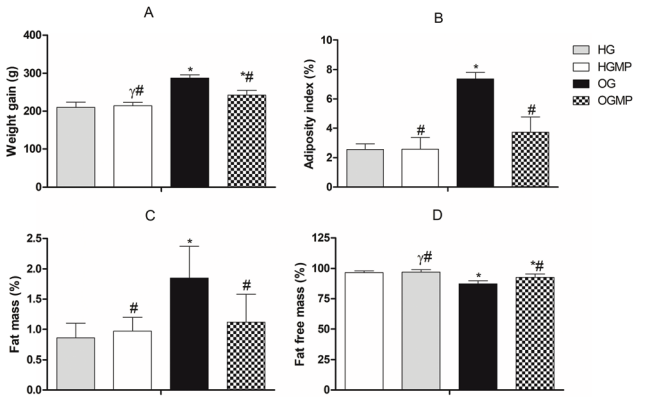

Fig. 2 Weight gain throughout the experiment (A), adiposity index (B), fat mass (C) and fat free mass (D) of healthy and obese rats treated or not with MP. One-way ANOVA, $p \leq 0.05$ Tukey's *post hoc* test. * different from HG; # different from OG; γ different from OGMP.

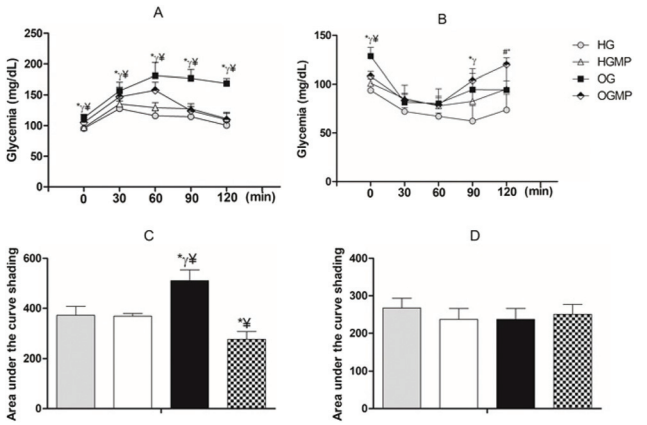

Fig. 3 Glucose tolerance test (A) and insulin tolerance test (B) of healthy and obese rats treated or not with MP. (C) Area under the curve shading of glucose tolerance test and (D) area under the curve shading of insulin tolerance test. Two-way ANOVA, $p \leq 0.05$ Tukey's *post hoc* test. * different from HG; # different from OG; γ different from OGMP; ¥ different from HGMP.

Changes in lipid profile associated with obesity in OG were identified, as these rats had higher of total serum cholesterol, LDL, hepatic cholesterol, hepatic triglycerides, CG and ICM levels compared to HG ($p \leq 0.05$). In turn, MP treatment in OGMP rats reduced LDL, TG and MLI compared to OG, in addition to increasing HDL levels ($p \leq 0.05$) to the point of reversing the changes caused by the cafeteria diet consumed, since these values were statistically similar to those of the HG group ($p \leq 0.05$). The effect of treatment with MP on the lipid profile could also be identified even in healthy rats (HGMP), since MP was able to reduce LDL, serum TG, and hepatic cholesterol in this group, regardless of obesity induction ($p \leq 0.05$). MP treatment reduced insulin, leptin, HOMA-IR, CRP and AAG levels in OGMP rats compared to OG ($p \leq 0.05$), which confirms the anti-obesity effect of MP from a metabolic point of view (Table 1).

3.3 Microbial counts and quantification of organic acids in faeces

Treatment with MP increased the of lactic acid bacteria counts in the faeces of both healthy (HGMP) and obese (OGMP) rats compared to the other groups (Table 2).

Higher succinic acid levels were quantified in HG and HGMP healthy rats and in the OGMP, lower levels of acetic and propionic acids in the OG, and lactic and formic acids in the OG and OGMP obese rats ($p \leq 0.05$) (Table 3).

3.4 Histological analysis

A histological section of liver stained in hematoxylin and eosin showed hepatocytes in cords with preservation of the cell structure compatible with a healthy liver (Fig. 4A and E). The

Table 1 Biochemical parameters of healthy and obese rats treated or not with MP

	Groups			
Parameters	HG	HGMP	OG	OGMP
Total cholesterol (mg dL^{-1})	63.40 ± 7.33	58.14 ± 6.38#	72.50 ± 8.28	69.66 ± 3.83¥
LDL (mg dL^{-1})	12.14 ± 3.33	10.60 ± 2.79*#	23.75± 4.99*	15.33± 2.33#¥
HDL(mg dL^{-1})	32.14 ± 4.88	30.00 ± 5.17	27.75 ± 3.65	40.5 ± 5.82#*¥
Triglycerides(mg dL^{-1})	109.80 ± 11.71	79.85 ± 8.93*#	185.60 ± 32.09*	59.66 ± 6.80#*
Hepatic cholesterol(mg dL^{-1})	21.32 ± 2.07	9.00 ± 2.14*#	39.03 ± 10.80*	36.41 ± 6.18*¥
Hepatic triglycerides(mg g^{-1})	42.44 ± 7.30	45.48 ± 5.07#	251.83 ± 48.71*	211.21 ± 2.86*¥
Metabolic Load Index (mg g^{-1})	233.17 ± 22.46	231.00 ± 18.05#	305.83 ± 30.18*	232.67 ± 18.49#
Fasting Blood Glucose(mg dL^{-1})	94.21 ± 5.15	91.30 ± 3.49	112.67 ± 6.09*	101.98 ± 3.10#¥
Postprandial Glucose (mg dL^{-1})	129.05 ± 12.38	123.53 ± 7.92#	156.50 ± 14.19*	142.28 ± 15.36
HOMA-IR	0.11 ± 0.02	0.12 ± 0.03#	0.28 ± 0.02*	0.13 ± 0.04#
Insulin (ng mL^{-1})	0.50 ± 0.07	0.50 ± 0.14#	1.20 ± 0.04*	0.50 ± 0.10#
Leptin (ng mL^{-1})	0.62 ± 0.04	0.66 ± 0.05#	1.11 ± 0.06*	0.66 ± 0.04#
C-reactive protein(mg dL^{-1})	0.42 ± 0.19	0.40 ± 0.07#	1.60 ± 0.60*	0.40 ± 0.07#
Acid alpha-glycoprotein(mg dL^{-1})	1.67 ± 0.82	1.29 ± 0.49#	4.40 ± 0.70*	1.67 ± 0.50#

Legend: HG = healthy group; HGMP = healthy group treated with MP; OG = obese group; OGMP = obese group treated with MP; LDL = low density lipoprotein; HDL = high density lipoprotein. One-way ANOVA, $p \leq 0.05$ Tukey's *post hoc* test. *different from HG; # different from OG; ¥ different from HGMP.

Table 2 Bacteria count in faeces of healthy and obese rats treated or not with MP

Parameters (log 10 UFC g^{-1})	Groups			
	HG	HGMP	OG	OGMP
Lactic acid bacteria	8.21 ± 0.70	9.42 ± 0.08*#	8.51 ± 0.20	9.42 ± 0.08*#
Enterobacteriaceae	8.80 ± 0.11	8.72 ± 0.58	8.20 ± 0.22	8.80 ± 0.21
Escherichia coli	7.91 ± 0.65	7.79 ± 0.81	8.26 ± 0.24	8.75 ± 0.11
Streptococcus/Enterococcus faecalis	8.64 ± 0.75	8.91 ± 0.59	8.89 ± 0.50	9.30 ± 0.04

Legend: HG = healthy group; HGMP = healthy group treated with MP; OG = obese group; OGMP = obese group treated with MP. One-way ANOVA, $p \leq 0.05$ Tukey's *post hoc* test. * different from HG; # different from OG.

Table 3 Quantification of organic acids in faeces of healthy and obese rats treated or not with MP

Organic acids (μmol g^{-1})	Groups			
	HG	HGMP	OG	OGMP
Citric acid	n.d.	0.05 ± 0.01	n.d.	0.03 ± 0.01
Succinic acid	2.00 ± 0.26	3.12 ± 0.37	0.23 ± 0.09*	0.24 ± 0.09*
Latic acid	3.92 ± 0.06	2.4 ± 0.05*	0.23 ± 0.09*¥	0.24 ± 0.09*¥
Formic acid	0.49 ± 0.06	0.33 ± 0.15	1.88 ± 0.66*¥	1.76 ± 0.26*¥
Acetic actid	0.84 ± 0.04	0.66 ± 0.03	0.35 ± 0.01*¥	0.59 ± 0.08
Propionic acid	4.66 ± 0.71	4.99 ± 0.50	2.58 ± 0.48*¥	7.42 ± 0.24#

Legend: HG = healthy group; HGMP = healthy group treated with MP; OG = obese group; OGMP = obese group treated with MP; n.d. = not detected. One-way ANOVA, $p \leq 0.05$ Tukey's *post hoc* test. * different from HG; # different from OG; ¥ different from HGMP.

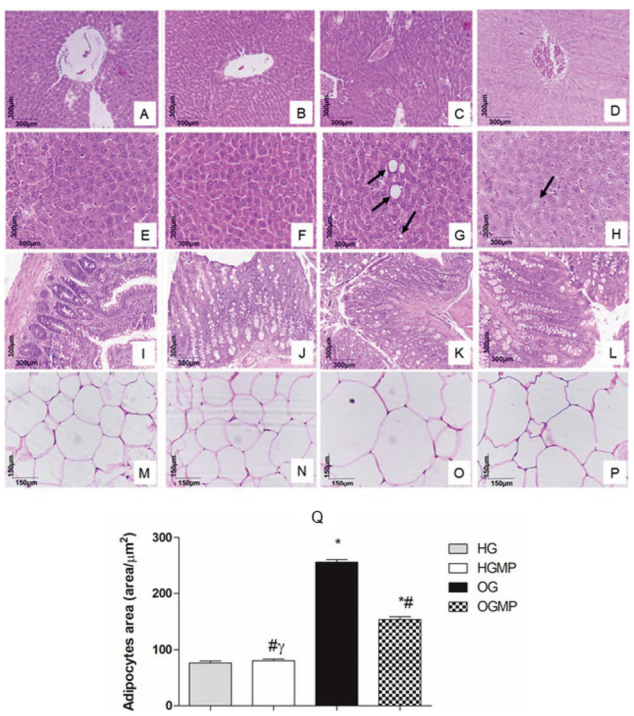

Fig. 4 Histological analysis of liver with magnification 20× (A, B, C, D) and 40× (E, F, G, H), intestine (I, J, K, and L), adipose tissue (M, N, O and P) and morphometry (M, N, O and P) of healthy and obese rats treated or not with MP. One-way ANOVA, $p \leq 0.05$ Tukey's *post hoc* test. * different from HG; # different from OG; γ different from OGMP.

rats in the HGMP group (Fig. 4B and F) exhibited similar liver architecture to that of healthy rats. Rats in the OG group presented focal hepatocytes with lipid vacuoles in their cytoplasm and eccentric nucleus represented by the arrow (Fig. 4C and G). Rats in the OGMP group (Fig. 4D and H) exhibited rare hepatocytes with small lipid cytoplasmic vacuoles (arrowhead) to a lesser extent than those in the OG group. In the histological analysis of the intestine (colon), it was observed that there was preservation of the intestinal epithelium in all groups and all the layers which compose the organ, as presented in panels I, J, K and L of Fig. 4.

The adipocytes of rats in the HG (Fig. 4M) and HGMP (Fig. 4N) groups showed a monotone and homogeneous appearance without major changes in the cell area. However, rats in the OG group (Fig. 4O) and the OGMP group (Fig. 4P) revealed large adipocytes with a broad cytoplasm. The rats in the OG group had a larger area compared to the other groups in the morphometry of these cells, with adipocyte hypertrophy being characterized in obesity.

4. Discussion

Obesity can be caused by hyperphagia (among other factors) and the consequent increase in caloric intake. A cafeteria diet is recognized for activating the hedonic response and stimulating the pleasure induced by sensory food stimuli, favouring

high consumption of food, even when there is sufficient energy in the body.[12]

As in the present study (data not shown), Tavares *et al.*[20] observed a reduction in the cafeteria diet intake by obese rats treated with MP, but this effect was not observed in healthy rats. This result may be related to the fact that the MP extract is rich in levodopa, a precursor to dopamine,[20] which is a neurotransmitter that influences the hedonic system, regulating eating behaviour and energy balance.[41,42]

In this study, the rats' food preference for processed meat, sandwich biscuits, popcorn, hot dog sausages, Bologna and ham resulted in higher consumption of sodium, total and saturated fats in OG. Thus, metabolic changes were identified in relation to the biochemical profile and intestinal health in these rats.

The adiposity index can be used[38] in order to diagnose obesity in animal models. The index considers above 6.3% as a cut-off point for obesity values and an increase in the number and hypertrophy of adipocytes compared to control.[12] MP treatment was able to reduce the accumulation of epididymal, visceral and retroperitoneal fat in rats that consumed the cafeteria diet (OGMP) with a reduction in this index to below 6.3%, which characterizes the normal adiposity index and eutrophic nutritional status.

Another indicator for diagnosing obesity is to evaluate body composition and not only the body weight gain as a diagnosis of nutritional status.[12] In the present study, rats induced to obesity had a higher body fat percentage and a lower fat free mass percentage than healthy rats ($p \leq 0.05$). We previously demonstrated that MP has a slimming effect in obese Wistar rats based on the murinometric parameters of BMI and the Lee index.[20] This slimming effect of MP was ratified in the present study due to the reduction in the fat percentage and accumulation of adipose tissue in comparison with OG, including similar parameters to HG ($p > 0.05$).

Histological analyses of adipose tissue also demonstrated the obesogenic capacity of the cafeteria diet, since the OG and OGMP rats showed an increase in the adipocyte size, which is associated with an increase in the inflammation characteristic of obesity.[43] However, adipose tissue morphometry demonstrated that treatment with MP, rich in phenolic compounds such as catechin, chlorogenic acid, trans-resveratrol and kaempferol 3-glucoside, was able to reduce the size of fat cells in the OGMP group. The literature reports that phenolic compounds can reduce adipocyte differentiation and growth by decreasing liver lipogenesis and fatty acid biosynthesis.[44,45]

Metabolic outcomes were also identified in addition to the weight loss effect, with an emphasis on improvement in the glycemic profile. A previous study demonstrated increasing hypoglycemic action according to the increase in MP extract doses in rats induced to type 1 diabetes.[21] However, no study has yet observed the effect of MP treatment in obese animals, highlighting its ability to normalize blood glucose in both GTT and ITT in relation to the HG and HGMP healthy groups.

Insulin resistance identified in the GTT and ITT tests in the OG rats is related to decreased insulin sensitivity due to increased blood glucose concentration, weight gain and adiposity in these rats. These results agree with the higher hormone insulin concentration in OG rats which is characteristic of the hormonal changes identified in the pathophysiology of obesity.[46]

In addition to these changes in GTT and ITT, liver tissue also demonstrated histological changes characteristic of obesity, such as hepatic steatosis. This condition can be caused by excessive energy consumption from lipids and carbohydrates which can be stored in the body, mainly in the form of triglycerides.[47] Kaempferol, catechins and chlorogenic acid quantified in the MP extract have been shown to have a regulatory effect on hepatic and glucose metabolism,[48,49] since histological changes in the OGMP group were less frequent.

In further assessing glucose metabolism, HOMA-IR was calculated to assess insulin resistance.[37] It is known that obesity has a strong relationship with insulin resistance, increasing the risk of developing type 2 diabetes, so controlling these levels represents a positive effect for treating obesity and comorbidities.[50]

Leptin is an anorectic hormone, responsible for controlling hunger and satiety. However, the excess of adipose tissue in obesity triggers leptin resistance, which in turn is responsible for hyperphagia.[51] Thus, the association between leptin resistance and hyperphagia observed in the OG rats, but not in obese rats treated with MP (OGMP), may help to clarify this effect of MP on weight loss.

Elevated leptin levels may contribute to the *meta*-inflammation present in obesity, as it is also considered a pro-inflammatory cytokine[46] and increases the risk of cardiovascular events[52,53] as the relationship between insulin resistance, hypertriglyceridemia and decreased HDL levels has already been demonstrated, constituting one of the main metabolic abnormalities of insulin resistance.[6] It was also possible to identify lower insulin resistance, reduced TG, and increased HDL in the OGMP rats in the present study.

Dyslipidaemia is an important comorbidity of obesity, as high concentrations of total cholesterol, triglycerides and LDL associated with low HDL concentration promote forming atheroma plaques in the arteries and increase cardiovascular risk.[54] Previous studies have demonstrated hypolipidaemic activity of MP extract in healthy animals,[21,22] which was also observed in the present study regarding the TG and HDL levels; it is important to highlight the effect of treatment with MP in reducing TG in both obese rats (OGMP) and in healthy rats (HGMP).

The accumulation of cholesterol and triglycerides in the liver of obese animals is consistent with the plasma changes of the lipid profile and with the histological findings, which characterizes the injury, inflammation and hepatic steatosis.[54] These changes cause important metabolic consequences, such as increased concentration of LDL and VLDL and risk of developing cardiovascular diseases and type 2 diabetes,[55] with these conditions also being observed in obese rats in this study.

Another parameter which should be considered in the context of cardiovascular health is the metabolic load index (MLI), which associates postprandial elevations in blood glucose and triglyceride levels in order to assess the metabolism of carbohydrates and lipids after a meal. Treatment with MP was able to reduce MLI values to the point of normalizing them compared to healthy rats. This index is an important cardiovascular marker, since the metabolism of carbohydrates and lipids is interrelated and individuals with cardiovascular diseases have greater metabolic challenges involving these markers.[36]

The increased caloric intake and the consumption of a nutritionally inadequate diet by OG rats contributed to developing inflammation characteristic of obesity, as proven in the present study through the highest concentrations of CRP to AAG in this group. Chronic low-grade inflammation is associated with leptin and insulin resistance, atherogenesis and worsens intestinal health, and other metabolic and somatic parameters are directly related to intestinal health,[56,57] which were also identified in the OG. The ingestion of fibres and anti-inflammatory substances present in the MP extract has been shown to reduce inflammation, prevent the development of diabetes and cardiovascular diseases and promote intestinal health.[58]

Faecal organic acids were quantified based on the important relationship between metabolic parameters and intestinal health, as well as the bacterial count in faeces, and then the histological analysis in the colon was performed. Although the obesogenic diet used in the present study did not alter the colonic tissue, less production of acetic and propionic short-chain fatty acids (SCFA) was observed in the OG when compared to the other groups ($p \leq 0.05$). Such acids play an important role in glucose homeostasis and insulin sensitivity and regulating glucagon-like peptide1 (GLP1) and peptide YY (PYY), which are responsible for reducing caloric intake with a consequent reduction in body weight.[59,60]

On the other hand, a reduction in the amount of succinic and lactic acids was found in the faecal samples of the OG and OGMP groups, which have immunological function, modulate intestinal inflammation and can be used by intestinal bacteria as intermediates for the production of other SCFA.[61] An increase in the formic acid concentration was also identified in these groups, corroborating other studies which used a high fat cafeteria diet and demonstrated the possible dietary impact on the production of organic acids.[31,62]

In turn, MP treatment increased the latic acid bacteria (*Bifidobacterium* and *Lactobacillus*) count in the HGMP and OGMP, regardless of the diet consumed. The phenolic compounds, 1-kestose and the fibres present in MP, known as prebiotic components, may have reflected the increase in the count of these bacteria in the treated rats (HGMP and OGMP), thus presenting several metabolic benefits in response to the control of energy homeostasis such as better metabolisation of energy from polysaccharides, control of food intake and body weight, regulated insulin response, glycemic and lipidic profile, and reduced inflammatory effects which culminated in the weight loss effect.[32,59,63,64]

Obesogenic diets increase the firmicute count and reduce bacteroidetes, decreasing the lactic acid bacteria count. Phylum bacteroidetes have fermentative bacteria which beneficially modulate the immune system, while the phylum firmicutes have species related to the induction of inflammation and chronic diseases, such as obesity.[59] This imbalance in the firmicute : bacteroidete ratio facilitates the passage of lipopolysaccharides through the intestinal barrier reaching the blood circulation and causing several metabolic changes related to cardiovascular health, leptin deficiency and consequent increase in caloric intake, and inducing the inflammatory response, among others.[65] On the other hand, the sodium-rich diet does not alter the intestinal microbiota.[66]

Together, our results demonstrate that the effects observed from treatment with MP are associated with its rich bioactive composition. Tavares *et al.*[20] quantified high citric acid, oligosaccharide 1-kestose, levodopa, phenolic compounds catechin, chlorogenic acid, trans-resveratrol and kaempferol 3-glycoside contents, constituting substances which have a direct or indirect effect on weight loss.

Organic acids, including citric acid, can reduce lipogenesis while stimulating lipolysis, resulting in controlling and reducing body weight.[67] Furthermore, 1-kestose, a non-digestible oligosaccharide, can be fermented in the intestine and promote the growth of beneficial bacteria for intestinal health, such as *Bifidobacterium.*[68] Levodopa is recognized as a regulator of food intake.[20] Phenolic compounds are responsible for reducing adipocyte differentiation, inhibiting phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling pathways, presenting metabolic and anti-obesogenic effects.[69] Among phenolics, kaempferol quantified in MP has an anti-diabetic effect by regulating gluconeogenesis and improving insulin response.[48] Catechin and chlorogenic acid, major phenolic compounds quantified in MP, play a role in weight loss, which can occur in response to the modulation of the intestinal microbiota, rebalancing the firmicute : bacteroidete ratio *in vitro*, which leads to an improvement in the lipid profile and glucose metabolism.[49]

It is also noteworthy that only our group has evaluated the effect of MP in obese rats,[20] contributing to developing a new therapeutic approach to treat obesity. It is encouraged that new studies are developed to test different combinations between the implemented dose and treatment time, in addition to better elucidating the action mechanisms of MP on obesity. For future translational studies, it is important to note that the MP dose of 750 mg kg^{-1} of animal weight used in this study should be adjusted based on the body surface area according to the human equivalent dose (HED), being 120 mg kg^{-1} for humans.[70]

5. Conclusions

MP treatment had an anti-obesity effect in obese rats, demonstrated by the loss of body weight, lower fat mass, reduction of the adiposity index and reversion of metabolic alterations

usually present in the pathophysiology of this disease. Such effects may be associated with an increase in the lactic acid bacteria count and the amount of SCFA produced. The composition of MP with a high content of bioactive compounds may have contributed to these outcomes, which confirms its use as a promising strategy in the treatment of obesity.

Conflicts of interest

The authors declare they have no conflicts of interest.

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