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Bacupari (*Garcinia brasiliensis*) extract modulates intestinal microbiota and reduces oxidative stress and inflammation in obese rats

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ABSTRACT

The objective of this study was to evaluate the effects of an ethanolic extract of the bark of bacupari (*Garcinia brasiliensis* – EEB) on the abundance of intestinal microbiota, concentration of short-chain fatty acids (SCFAs), oxidative stress, and inflammation in obese rats fed a high-fat diet (HFD). Male Wistar rats were divided into three groups: an HFD-fed obese control group, a group fed HFD plus EEB (BHFD) at a dose of 300 mg per animal per day (42 mg 7-epiclusianone and 10.76 mg morelloflavone), and a lean control group fed an AIN-93 M diet for 8 weeks. EEB decreased ($p < 0.05$) the abundance of organisms belonging to the phyla Firmicutes and Proteobacteria, and increased ($p < 0.05$) the concentration of propionic acid. Liver concentrations of malondialdehyde, nitric oxide, resistin, and p65 nuclear factor-kappa B p65(NF-κB) decreased ($p < 0.05$), while the expression of heat shock protein (HSP)72 and catalase increased ($p < 0.05$) with the consumption of EEB. Moreover, computational molecular modeling studies involving molecular docking between the main constituents of EEB, 7-epiclusianone and morelloflavone, and NF-κB suggested its inhibitory activity, thus corroborating the experimental results. The consumption of EEB may therefore be a promising strategy for the beneficial dietary modulation of the intestinal ecosystem, thereby countering oxidative stress and inflammation in obese rats. This activity is attributable to the presence of bioactive compounds that act individually or synergistically in the scavenging of free radicals or in the inflammatory process.

1. Introduction

Obesity is defined as an abnormal or excessive accumulation of fat that may impair health. Its etiology is multifactorial, involving complex interactions between genetics, hormones, and the environment (Pozza & Isidori, 2018). Obesity increases the risk of developing several diseases, including diabetes mellitus type 2, metabolic syndrome, several types of cancer, cardiovascular disease, and cognitive defects, such as Alzheimer's disease (Proctor, Thiennimitr, Chattipakorn, & Chattipakorn, 2016; Walker, Mcgee, & Druss, 2015). Obesity is aggravated by the consumption of westernized diets, characterized by a high intake of saturated fat, animal protein, and refined sugar (Agus et al., 2016). In addition, changes in intestinal ecology, especially those related to the consumption of saturated fats, can affect the inflammatory and metabolic properties of the intestinal microbiota and, thereby, alter host physiology (Caesar, Tremaroli, Kovatcheva-Datchary, Cani, & Backhed, 2015) to induce obesity, atherosclerosis, diabetes, and nonalcoholic fatty liver disease (NAFLD), among others (Vajro, Paolella, & Fasano, 2013).

Dietary patterns can affect intestinal permeability through the secretion of inflammatory mediators that promote the translocation of lipopolysaccharide (LPS) into the circulation (Kasselman, Vernice, DeLeon, & Reiss, 2018). LPS concentrations in the blood are altered by the intestinal microbiota and primarily by the gram-negative bacteria

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Bacteroidetes, which present LPS on their cell surface that acts as an antigen to stimulate the host immune response, and the gram-positive bacteria Firmicutes, which promote inflammation by increasing the uptake of LPS (Moraes, da Silva, Pititto, & Ferreira, 2014). An increase in the levels of LPS activates toll-like receptor 4 (TLR-4), which binds to LPS and induces low-grade inflammation. Metabolic endotoxemia caused by increased LPS concentration in the bloodstream resulting from intestinal permeability and production of short-chain fatty acids (SCFAs) induces low-grade inflammation, insulin resistance, and adipocyte hyperplasia, thus promoting the development of obesity. The activation of TLR-4 also results in the upregulation of inflammatory pathway mediators, such as nuclear factor-kappa B (NF-κB), which increases the production of inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, IL-8, cyclooxygenase-2, and chemokines, such as monocyte chemotactic protein 1 and inducible nitric oxide synthase (Cartwright, Perkins, & Wilson, 2016; Lappas, Yee, Permezel, & Rice, 2005). Thus, the dysregulation of adipokines and the increase in inflammatory infiltrates in adipose tissue result in chronic inflammation and oxidative stress, which contribute to insulin resistance and increased adiposity (Park, Park, Valacchi, & Lim, 2012; Lin et al., 2000; Kasselman et al., 2018)

Stress leads to rapid transcription and subsequent translation of a series of highly conserved proteins called heat shock proteins (HSP) (Locke, Noble, & Atkinson, 1990; Mayer & Bukau, 2005). HSP72 is the first protein to be induced during stress and performs cytoprotective and anti-inflammatory functions (Drummond, Gomes, Júnior, Gomes, & Natali, 2013). In addition to HSP, the enzymes catalase (CAT) and superoxide dismutase (SOD) act as a first line of defense to combat oxidative stress in order to maintain redox balance in the body (Barbosa et al., 2010). In an attempt to maintain homeostasis, the body may increase the synthesis of stress proteins, thereby increasing the activity of antioxidant enzymes.

Several studies have demonstrated that the ingestion of foods or natural extracts rich in antioxidants may influence metabolic homeostasis as well as the composition of the intestinal microbiota. These are important mechanisms for the prevention and treatment of obesity and for probiotic, antioxidant, and anti-inflammatory effects (Moraes et al., 2014; Nair et al., 2014). These natural extracts include those of *Garcinia* species that have been used in popular Brazilian medicine, as they are rich in phenol derivatives, including benzophenones. Some of the compounds present in these species exert biological activities, such as antifungal, anti-inflammatory, antitumor, and antioxidant effects, and a variety of compounds, including benzophenones, flavonoids, and xanthones, have been isolated from species of the family Guttiferae (García-Conesa, 2015; Martins et al., 2008; Santa-Cecília, 2011). Our research group has observed that an ethanolic extract of the bark of bacupari (*Garcinia brasiliensis* – EEB) was able to reduce obesity, biometry, lipogenesis, and NAFLD in rats fed high-fat diet (HFD), depending on the composition of the extract, including the bioactive flavonoid, morelloflavone, and the benzophenone, 7-epiclusianone (Moreira et al., 2017; Moreira et al., 2018). To further these studies, the objective of this work was to evaluate whether EEB can modulate the intestinal microbiota composition, oxidative stress, and inflammation in HFD-fed obese rats. In addition, computational studies involving molecular docking, which is a computational method that predicts the interaction between two molecules, in this case a protein (macromolecule, specifically NF-κB) and a ligand (small molecule, specifically the main chemical constituents present in EEB, 7-epiclusianone and morelloflavone), were used to generate an interaction model (Prieto-Martínez, Arciniega, & Medina-Franco, 2018) to identify their affinity profiles and possible inhibitory activities.

2. Material and methods

2.1. Plant material

The fruits of *G. brasiliensis* (Mart.) were collected on the campus of the Federal University of Viçosa-MG, Brazil, in February (summer) 2011. Botanical identification was performed at the Horto Botânico of the Federal University of Viçosa by Dr. João Augusto Alves Meira Neto. A voucher specimen (number VIC26240) was deposited at the Herbarium of Federal University of Viçosa.

2.2. Sample preparation, extraction, and characterization

The extract was obtained according to the method described by Castro et al. (2015). The bark of *G. brasiliensis* was used because it contains high levels of bioactive compounds (Chuah, Ho, Beh, & Yeap, 2013). Briefly, the peel of the fruit was first dried in an oven with a circulating air temperature of 40 °C for 8 days, pulverized (1 kg), and subjected to extraction by re-maceration in absolute ethanol at 25 °C for 48 h. EEB was finally concentrated in a rotatory-evaporator under reduced pressure (vacuum) at 40 °C in a water bath. Aliquots for use in the biological assay were stored in an ultra-freezer (−80 °C).

The chromatographic profile and flavonoid content of EEB were published in Moreira et al. (2018).

2.3. Biological assay

2.3.1. Animals/diets

Twenty-four adult male Wistar rats (11 weeks old), supplied by the Animal Laboratory of Biological Science and Health Centre (Federal University of Viçosa, Brazil), were housed under standard conditions in individual cages with 12-h light/dark cycles, humidity of 80 ± 5%, and a temperature of 22 ± 3 °C. Distilled water was provided ad libitum. All experimental procedures were performed in accordance with the Ethics Committee for Animal Research of the Federal University of Viçosa, Brazil (approval number 98/2015). The experimental diets (Table 1) were based on the formulations of RESEARCH-DIETS® and AIN-93 M diet (Reeves, Nielsen, & Fahey Jr., 1993). Eight animals (control group) were fed an AIN93M diet during the entire experiment (15 weeks). The other 16 animals were fed an HFD for 7 weeks to induce obesity. At the end of the induction period, the body weight gain and fasting glycemic levels were recorded and the Lee index was

Table 1
Formulation of the experimental diets (g/100 g).

Ingredients	AIN93M	HFD[b]	BHFD[c]
Casein	14	19.5	19.5
Maltodextrin	15.5	10	10
Corn starch	46.57	5.32	5.32
Sucrose	10	34.1	34.1
Soybean oil (mL)	4	1	1
Swine lard	0	20	20
Cellulose	5	5	5
Mineral mix without iron	3.5	3.5	3.5
Vitamin mix	1	1	1
L-cystine	0.25	0.25	0.25
Cholesterol	0.18	0.18	0.18
Choline bitartrate	0	0.15	0.15
Butylated hydroxytoluene (BHT)	0.0008	0.004	0.004
Calories	308.3	464.7	464.7
Caloric density (kcal/g)	3.8	4.7	4.7
EEB[a] mg/animal/day	–	–	300

[a] EEB: Ethanolic extract of bacupari peel (*Garcinia brasiliensis*). AIN-93M: standard diet; HFD: High-fat diet; BHFD: HFD plus bacupari peel extract (incorporated in eighth week of the experiment - phase treatment of EEB).
[b] Reeves et al. (1993).
[c] Research Diet® (2006).

calculated (Moreira et al., 2017) to identify the presence of metabolic risk. To begin EEB treatment, the obese animal group was divided into two (n = 8 each): an HFD group (fed HFD), and a BHFD group (fed HFD plus EEB), for an additional 8 weeks. The distribution of animals ensured similar metabolic conditions in both groups with no statistical differences between body weight, body weight gain, fasting glycemic level, and Lee index. The extract was incorporated into the HFD at a dose of 300 mg/animal, approximately 674 mg/kg, which corresponds to 42 mg/animal or approximately 140 mg/kg of 7-epiclusianone, and 10.76 mg/animal or approximately 35.86 mg/kg of morelloflavone. This dose was based on previous studies, which demonstrated antioxidant and anti-inflammatory activities of these compounds (Castro et al., 2015; Santa-Cecília et al., 2011). We had used the same dose in our previous studies (Moreira et al., 2017; Moreira et al., 2018), which demonstrated that EEB treatment reduced obesity, biometry, lipogenesis, and NAFLD in HFD-fed rats.

After 15 weeks, the animals were fasted overnight, anesthetized with 100% isoflurane (Isoforine, Cristália®), and euthanized via cardiac puncture. The collected blood was centrifuged in test tubes with or without anticoagulant at 4 °C and 1000 ×g for 10 min (Fanem-204, São Paulo, Brazil) to isolate plasma and serum samples, respectively. The liver was isolated, immediately frozen in liquid nitrogen, and stored at −80 °C.

Feces excreted the day before euthanasia were collected and stored at −80 °C for fecal DNA extraction, microbiological analyses via quantitative polymerase chain reaction (qPCR), and SCFA quantification.

2.4. Analysis of intestinal microbiota

Microbial DNA was extracted from rat fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The composition of intestinal microbiota was evaluated via qPCR (CFX96 Touch™ Real-time PCR Detection System, Bio-Rad, Hercules, CA, USA). All analyses were performed in duplicate using SYBR® Green PCR Master Mix (Promega, Madison, WI, USA) with each well containing 2 µl sample or standard, 300 nM sense and antisense oligonucleotides, and nuclease-free water (total volume = 25 µl). The oligonucleotide primers (AlphaHelix Molecular Diagnostics) used for total bacteria, Bacteroidetes, Firmicutes, and Proteobacteria, are listed in Table 2. The thermal conditions used for PCR were as follows: initial DNA denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing of the primers at 60 °C for 20 s, and final extension at 72 °C for 15 s (Castillo et al., 2006).

Standard curves were constructed for each experiment using five sequential dilutions of bacterial genomic DNA from pure cultures ranging from 20 to 0.032 ng. The abundance of the phyla of bacteria from each fecal sample was calculated in accordance with the relative quantification method described by Stevenson and Weimer (2007). The following strains that were used were obtained from the American Type Culture Collection (ATCC): *Bacteroides ovatus* (ATCC 8483), *Escherichia coli* (ATCC 11775), and the Tropical Culture Collection: *Lactobacillus delbrueckii* (UFV H2b20 CCT 3744).

2.5. Determination of organic acids

Fecal samples (300 mg) were diluted in 2.5 ml water, centrifuged (12,000 ×g, 10 min), and cell-free supernatants were treated according to the method described by Siegfried, Ruckemann, and Stumpf (1984). Concentrations of organic acids (acetic, propionic, and n-butyric acid) were determined via high-performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 double HPLC (Dionex Corporation, Sunnyvale, CA, USA) coupled to a Shodex RI-101 refractive index detector using a Bio-Rad HPX-87H column (300 mm × 4.6 mm; Bio-Rad) maintained at 45 °C. The mobile phase was 5 mmol/l H_2SO_4 and the flow rate was 0.7 ml/min. The following organic acids were used for the standard curve: acetic, succinic, formic, propionic, isovaleric, isobutyric, and butyric acids. All acids were prepared at a final concentration of 10 mmol/l, except isovaleric acid (5 mmol/l) and acetic acid (20 mmol/l).

2.6. Analysis of oxidative stress and antioxidant defenses in liver tissue

2.6.1. Preparation of homogenates

Liver samples (100 mg) were macerated in microtubes before the addition of 1000 µl 1 M phosphate buffer and 1 mM EDTA (pH 7.4). The samples were macerated and centrifuged at 10,000 ×g and 4 °C for 10 min, and then the supernatants were collected and stored in an ultrafreezer until analysis.

2.6.2. Concentration of malondialdehyde (MDA)

MDA concentrations were determined via the thiobarbituric acid reactive substances (TBARS) assay (Kohn & Liversedge, 1944). The final values were calculated using a standard curve and N-oxyl-2,2,2,6,6-tetramethylpiperidine (TMPO) reagent. The results are expressed as nmol MDA per milligram of protein (nmol MDA/mg protein).

2.6.3. Nitric oxide (NO) concentration

NO concentration was determined in liver homogenates using the Griess reaction as a measure of NO production (Green et al., 1982). A standard curve was prepared by the addition of sodium nitrite standard and buffer. After incubation, the absorbance was measured at 570 nm on a Thermo Scientific-Multiskan™ GO reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.6.4. Superoxide dismutase (SOD) activity

The activity of SOD was expressed in relative units (U), with a unit of SOD defined as the amount of enzyme that inhibits the oxidation rate of pyrogallol by 50%. For the sample assay, 30 µl of liver homogenate

Table 2
Sequence of primers used in the RT-qPCR analysis.

Genes	Oligonucleotide	
	Sense (5′-3′)	Antisense (5′-3′)
Total bacteria	GCA GGC CTA ACA CAT GCA AGT C	CTG CTG CCT CCC GTA GGA GT
Bacteroidetes	CAT GTG GTT TAA TTC GAT GAT	AGC TGA CGA CAA CCA TGC AG
Firmicutes	ATG TGG TTT AAT TCG AAG CA	AGC TGA CGA CAA CCA TGC AC
Proteobacteria	CAT GAC GTT ACC CCG CAG AAG AAG	CTC TAC GAG ACT CAA GCT TGC
p65NF-κB	CTG CGC GCT GAC GGC	TCG TCG TCT GCC ATG TTG AA
HSP72	AGG CCA ACA AGA TCA CCA	TAG GAC TCG AGC GCA ATT CTT
SOD	GAG CAG AAG GCA AGC GGT GAA	CCA CAT TGC CCA GGT CTG
GAPDH	AGG TTG TCT CCT GTC ACT TC	CTG TTG CTG TAG CCA TAT TC

p65NF-κB: p65 factor nuclear kappa B; HSP72: heat shock proteins; SOD: superoxide dismutase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

was used. The analysis was carried out on a spectrophotometer (Multiskan Go, Thermo Fisher Scientific) at 570 nm, and the results are expressed as U of SOD/mg protein (Marklund, 1985).

2.6.5. Catalase (CAT) activity

Catalase activity was determined based on its ability to convert hydrogen peroxide (H_2O_2) into water and molecular oxygen, as described by Aebi (1984). The absorbance used for the calculation was the delta obtained from the measured absorbances (absorbance at time 0 − absorbance at 60 s) and the results are expressed as U catalase/mg protein.

2.6.6. Glutathione transferase (GST)

1-Chloro-2,4-dinitrobenzene (CNDB, molecular weight = 202.6 g/mol) was conjugated to glutathione (GSH) by GST, resulting in an increased absorbance at 340 nm (Habig, Pabst, & Jacoby, 1974). The absorbance of the samples was measured in triplicate at 0, 30, 60, and 90 s and the results are expressed as μmol/min/g of protein.

2.7. Gene expression of inflammatory markers and oxidative stress

Total RNA was extracted from the liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The integrity of the RNA was assessed via 1% agarose gel electrophoresis and quantified using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific).

Isolated mRNA was used to synthesize cDNA using a reverse transcription kit (M-MLV Reverse Transcriptase, Invitrogen) according to the manufacturer's protocol. mRNA expression was evaluated via reverse transcription (RT)-qPCR with SYBR® Green fast PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The reaction volume was 10 μl and all primers were used at a final concentration of 100 nmol/l. SYBR® Green fluorescence was quantified using an AB StepOne RT-qPCR System and Primer Express software (Applied Biosystems). The PCR cycle involved denaturation at 95 °C for 20 s, amplification of 40 cycles at 95 °C for 3 s and 60 °C for 30 s, and then generation of the standard dissociation curve. The RT-qPCR data were analyzed via the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

The oligonucleotide (Sigma-Aldrich®, St Louis, MO, USA) sense and antisense primers used to amplify genes related to oxidative stress (p65NF-κB, HSP72, and SOD) are listed in Table 2. The relative mRNA levels were normalized to the endogenous control, rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 2). Normal control-group values were used as standards.

2.8. Western blotting

Liver tissues were homogenized to separate nuclear and cytoplasmic proteins. An NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions. Known amounts (15 μg) of protein from the samples (nuclear and cytoplasmic fractions) and 5 μl of the reference standard (Broad Range Markers; Santa Cruz Biotechnology, Dallas, TX, USA) were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were blocked with 5% blocking solution (blotting-grade blocker, Bio-Rad) and incubated overnight with an anti- p65NFκB- (65 kDa) monoclonal primary antibody (ab13594; Abcam, Cambridge, UK). The membranes were washed with 15 ml 1× Tris-buffered saline (TBS) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (A-9044, Sigma-Aldrich) for 2 h at 4 °C. The bands were detected using 3,3-diaminobenzidine tetrahydrochloride.

2.9. Quantification of resistin

Plasma resistin concentrations were quantified using a commercial kit (Resistin Cat. #SEA847 RA, USCN, USA) according to the manufacturer's instructions. The microtiter plate was coated with an antibody specific for resistin. Standards and samples were added to the enzyme-linked immunosorbent assay (ELISA) plate with a resistin-specific antibody conjugated to biotin. After the incubation period, the absorbance was measured at 450 nm and the results were expressed by comparing the optical density of the samples against that of the standards.

2.10. Molecular modeling studies with molecular docking

All computer applications were run on OpenSUS Tumbleweed. Structures of the ligands morelloflavone and 7-epiclusianone present in EEB were constructed using Maestro 10.2.010 (Schrödinger, 2015a, Schrödinger, New York, NY, USA). The software LigPrep 3.4 (Schrödinger, 2015b; OPLS_3 force field and ionization state for pH 7.0 ± 2.0) was used for the preparation of the ligands involved in these studies. The crystallographic structure of NF-κB (Protein Data Bank (PDB) identifier 1NFK; Ghosh, Van Duyne, Ghosh, & Sigler, 1995; Sharma et al., 2006; Piccagli et al., 2008) was obtained from the PDB and the software Protein Preparation Wizard (Schrödinger, 2015c) was used for the preparation of these receptors. The co-crystallized DNA macromolecule present in 1NFK was removed from the structure and p50 monomers (chains A and B) were selected for docking studies. OPLS3 force field in MacroModel 9.9 was used for optimization. Molecular docking studies between NF-κB and the ligands morelloflavone and 7-epiclusianone were performed using the Induced Fit Docking protocol (Schrödinger, 2015d). The program Prime (Schrödinger) was used for refinement of the compounds and Glide (Schrödinger) provided scores, considering the flexibility of the protein and ligands. The grid box area for NF-κB was defined as 30 × 30 × 30 Å.

2.11. Statistical analyses

The data obtained were subjected to analysis of variance (ANOVA) followed by a Newman–Keuls means test. The level of significance was set at 5%. All statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad, San Diego, CA, USA).

3. Results

At the end of 8 weeks, weight gain in the HFD group was greater ($p < 0.05$) than that in the AIN-93 M and BHFD groups (65.00 ± 13.51, 100.00 ± 15.27, and 68.14 ± 16.17 in the AIN-93 M, HFD, and BHFD groups, respectively). With regard to consumption, rats in the HFD (17.41 ± 0.90) and BHFD (17.03 ± 0.92) groups consumed less food than those in the AIN-93 M group (22.21 ± 1.37; $p < 0.05$), as the energy density of the AIN-93 M diet was lower than that of the HFD. However, calorie intake was similar among groups ($p > 0.05$). Consumption of 7-epiclusianone (41.4 ± 2.4 mg/animal/day) and morelloflavone (10.60 ± 0.55 mg/animal/day), which were present in the extract, may have caused this effect (Moreira et al., 2017).

3.1. Effect of EEB on intestinal microbiota and organic acids

In the BHFD group, the abundance of bacteria from the phylum Bacteroidetes was higher ($p < 0.05$) and that of Firmicutes and Proteobacteria was lower ($p < 0.05$) than that in the obese and normal control groups (Fig. 1A–C). The production of propionic acid was higher ($p < 0.05$) in the group treated with EEB than in the control groups (Fig. 1F). The concentration of acetic acid and butyric acid did not differ among the experimental groups (Fig. 1D and E).

3.2. Effect of EEB on oxidative stress

Liver concentrations of MDA and NO were lower ($p < 0.05$) in the

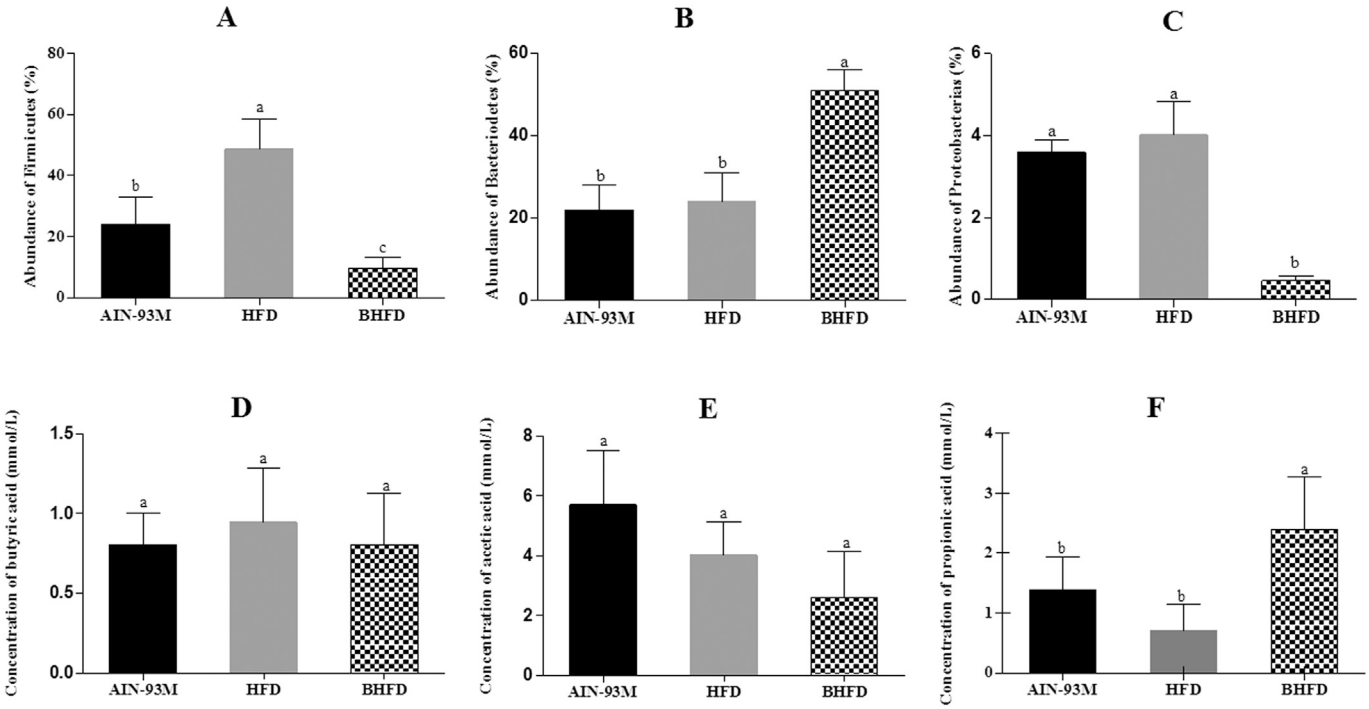

Fig. 1. Abundance of bacteria of the phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* and the concentration of short-chain fatty acids (SCFAs): butyric, acetic, and propionic acids in obese rats fed a high-fat diet supplemented with bacupari extract for eight weeks. Means followed by the same letter do not differ by the Newman–Keuls post hoc test with 5% significance. Normal control group (AIN93M); obese control group fed a high-fat diet (HFD); HFD with bacupari extract (BHFD).

BHFD group than in the obese group (Fig. 2A and B).

SOD expression was higher ($p < 0.05$) in the obese control and BHFD groups (2.04- and 2.554-fold, respectively) than in the normal control group (Fig. 2C). However, SOD levels were lower ($p < 0.05$) in the livers of obese rats treated with EEB than in the livers of rats in the HFD group, matching those of the AIN-93 M group rats (Fig. 2D). The expression of HSP72 (Fig. 2E) and the hepatic activity of CAT (Fig. 2F) were higher in the BHFD group ($p < 0.05$) than in the obese control group, while GST levels showed no difference ($p > 0.05$) between the BHFD and HFD groups. However, GST was lower ($p < 0.05$) in the normal control group (Fig. 2G).

3.3. Effect of EEB on inflammation

The gene expression of p65NF-κB was higher ($p < 0.05$, 1.41-fold) in the obese control group and lower in the BHFD group ($p < 0.05$, 0.82-fold) than in the normal control group (Fig. 3A). Moreover, the band intensity for p65NF-κB was lower ($p < 0.05$) in the BHFD group than in the obese control group (Fig. 3B). The concentration of plasma resistin was lower ($p < 0.05$) in the BHFD group than in the obese control group, although it was similar to that in the normal control group (Fig. 3C).

3.4. Molecular modeling

Morelloflavone presented a docking score value (GScore) of $-7.077\ \text{kcal·mol}^{-1}$ with NF-κB and these interactions occurred mainly through seven hydrogen bonds. In addition to 253 favorable hydrophobic interactions (van der Waals), two salt bridge interactions, one cation-π interaction, and one π-π stacking interaction were observed. 7-Epiclusianone showed a docking score value of $-3.617\ \text{kcal·mol}^{-1}$ with NF-κB, and formed two hydrogen bond interactions, 230 favorable van der Waals interactions, and one salt bridge interaction. The main interactions are shown in Fig. 4 and Fig. 5, demonstrating the relationship between affinity scores and the inhibitor activity of NF-κB.

4. Discussion

The present study investigated the potential benefits of EEB, which contains a high concentration of 7-epiclusianone and morelloflavone and exerts anti-obesity effects (Moreira et al., 2017) and protective effects against NAFLD (Moreira et al., 2018), in modulating the composition of intestinal microbiota and countering oxidative stress and inflammation in HFD-fed obese rats.

In the present study, the decrease in the intestinal abundance of bacteria belonging to the phyla Firmicutes and Proteobacteria and the increase in Bacteroidetes after the consumption of EEB may be attributed to the presence of phenolic compounds in EEB, as was previously observed by our research group (Moreira et al., 2017). Several studies have revealed that intestinal microbiota transform phenolic compounds into bioactive metabolites that contribute to the maintenance of intestinal homeostasis by stimulating the growth of beneficial bacteria (*Lactobacillus* and *Bifidobacterium* spp.) and inhibiting that of pathogenic bacteria, thus exerting a prebiotic effect (Guglielmetti et al., 2013; Parkar, Trower, & Stevenson, 2013; Cheng, Sheen, Wen, & Hung, 2017; Zhu et al., 2018).

The reduction in the intestinal abundance of bacteria from the phylum Firmicutes and increase in the abundance of Bacteroidetes by EEB may also be related to the reduction in body fat measured by the Lee index observed by Moreira et al. (2017) in obese rats consuming the same EEB under equivalent experimental conditions. Our results corroborate those of several animal studies reporting that obesity increases the abundance of the phylum Firmicutes, while abundance of the phylum Bacteroidetes is associated with leanness (Xu, Li, Zhang, & Zhang, 2012; Moraes et al., 2014; Wang et al., 2017; Wang, Hong, Li, Zang, & Wu, 2018, Wang et al., 2018).

The reduction in Proteobacteria abundance in the EEB group can be attributed to the potential of the extract to modulate intestinal microbiota. According to Shin, Whon, and Bae (2015), Proteobacteria abundance is an indicator of an unstable microbial community (dysbiosis) and a potential criterion for the diagnosis of disease. These

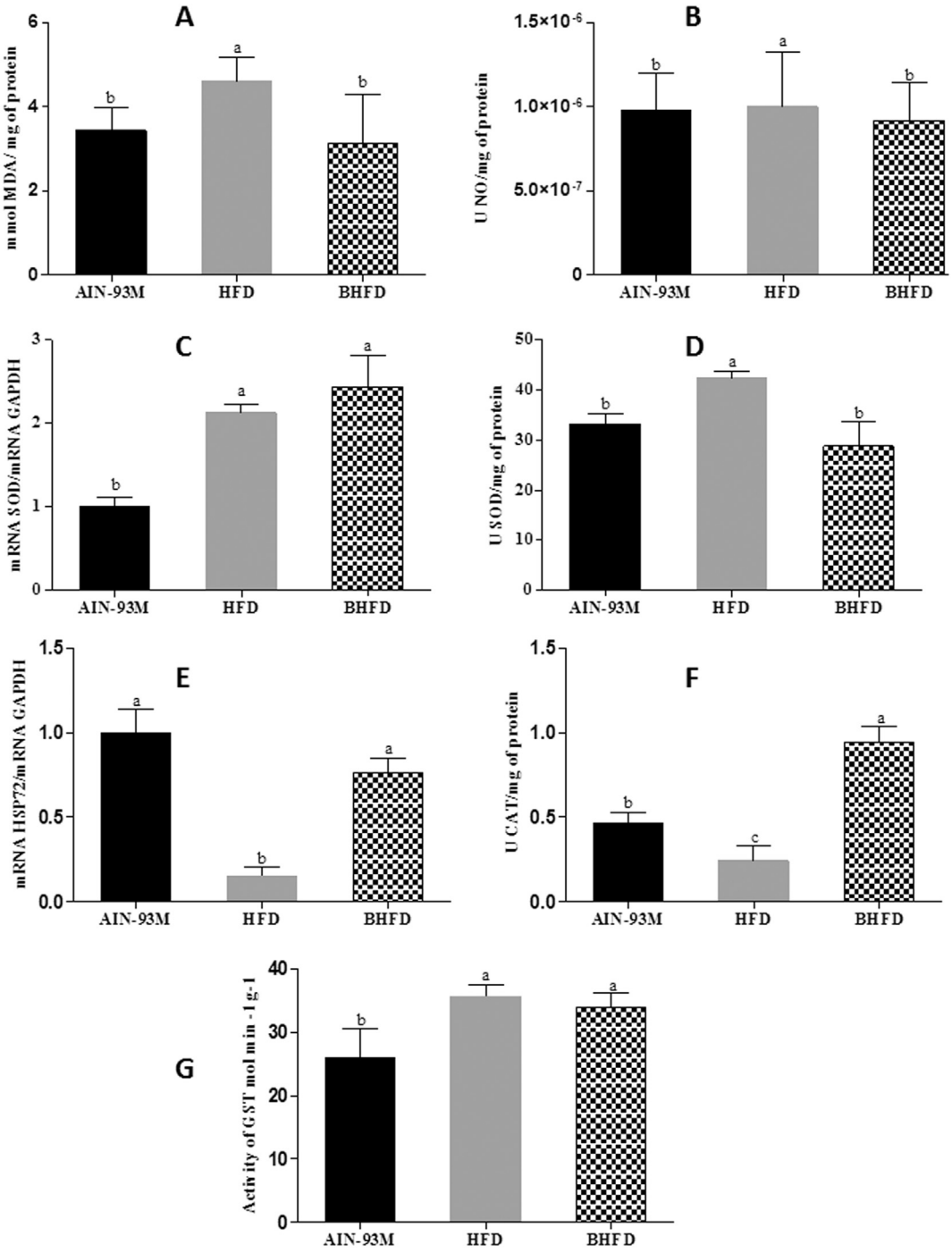

Fig. 2. Concentrations of malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) and relative gene expression of SOD and heat shock protein (HSP72) in obese rats fed high-fat diet along with the extract of bacupari for eight weeks. Means followed by the same letter do not differ by Newman–Keuls post hoc test at 5% significance. Normal control group (AIN-93M); obese control group fed with high-fat diet (HFD); HFD added to extract of bacupari (BHFD).

authors also noted that an increased prevalence of Proteobacteria and dysbiosis is often observed during metabolic disorders.

Modulation of intestinal microbiota by a healthy diet is associated with a reduction in obesity and other diseases as it alleviates inflammation by reducing LPS and improving the health of the host (Grootaert, Marzorati, Van den Abbeele, Van de Wiele, & Possemiers, 2011). However, our results are contradictory to those reported by Duncan et al. (2008), who observed the same proportion of bacteria belonging to the phyla Bacteroidetes and Firmicutes in the feces of lean and obese individuals, and those reported by Schwiertz et al. (2010) and Kasselman et al. (2018), who observed a higher proportion of Bacteroidetes and a lower proportion of Firmicutes in obese individuals. Our study investigated the abundance of phyla, but additional studies should be carried out to assess the abundance of genera and species within each phylum.

The intestinal microbiota are capable of producing a wide range of metabolites including SCFAs in the large intestine through the fermentation of carbohydrates and proteins that escape intestinal absorption during digestion. Acetic acid, propionic acid, and butyric acid are the most abundant and represent 90–95% of the SCFAs present in

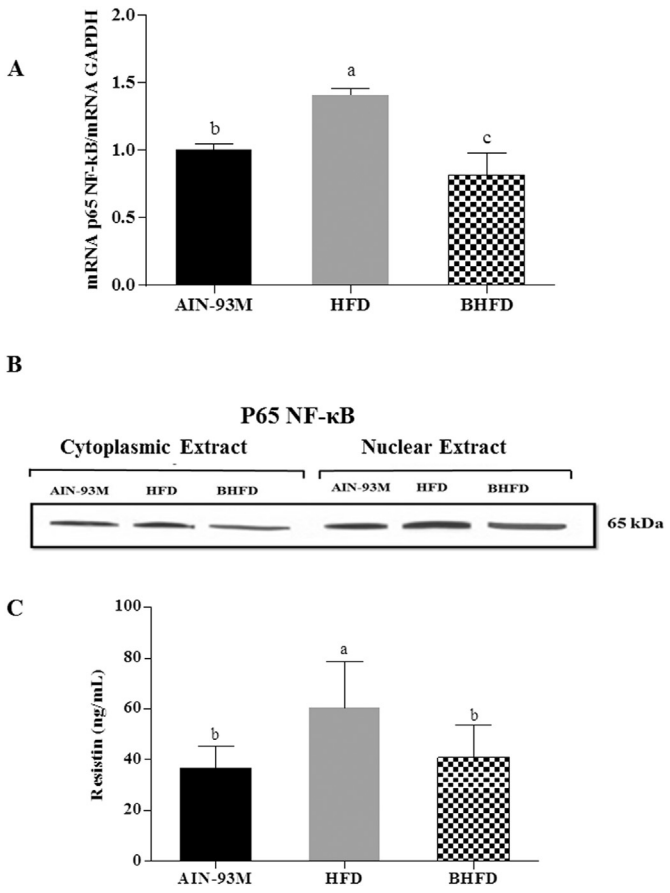

Fig. 3. Relative gene expression of p65 nuclear factor kappa B (p65NF-κB) (A), qualitative analysis of the p65NF-κB protein (B), and quantification of resistin (C) in obese rats fed high-fat diet along with the extract of bacupari for eight weeks. Means followed by the same letter do not differ by Newman–Keuls post hoc test at 5% significance. The lean control group (AIN-93 M); obese control group fed with high-fat diet (HFD); HFD along with extract of bacupari (BHFD).

the colon (Ríos-Covián et al., 2016). These acids can interfere with absorption of the end products of digestion and energy homeostasis of the host (Park, Seo, & Youn, 2013). In our study, the concentration of propionic acid, an important metabolite of the phylum Bacteroidetes, increased in the group treated with EEB. Our previous study (Moreira et al., 2017) showed that EEB reduced weight gain and adiposity indexes. These results are in agreement with those of Shoaie et al. (2013) and Chambers et al. (2014), who reported that propionic acid is related to the release of peptide YY and glucagon-like peptide-1 (GLP-1), which regulate satiety and weight gain. The connection between low levels of bacteria producing propionic acid, the presence of an inflammatory response, and the onset of non-transmissible chronic diseases is also well established (Machiels et al., 2014). In the present study, EEB reduced inflammation in rats by increasing the expression of HSP72, inhibiting p65NF-κB expression, reducing the concentration of resistin, and increasing the concentration of propionic acid. Moreira et al. (2018) also observed that EEB added to the HFD of obese rats increased the concentrations of peroxisome proliferator-activated receptor (PPAR)-α and adiponectin, which suppresses resistin activity (Chen et al., 2014). The flavonoids present in EEB probably promote the growth of bacteria producing propionic acid, thereby reducing inflammation. The presence of morelloflavone in EEB may stimulate this effect as it contains phenolic hydroxyl compounds responsible for scavenging free radicals, in addition to 7-epiclusianone, which displays anti-inflammatory activity in animal models (Santa-Cecília et al., 2011; Gontijo et al., 2012).

The main substrate of the phylum Firmicutes is butyrate, whose level may be influenced by changes in diet, but high levels of indigestible carbohydrates do not increase its plasma levels (Gao et al., 2009; Krupa-Kozak, Markiewicz, Lamparski, & Juśkiewicz, 2017; Shoaie et al., 2013). However, no difference was observed between the experimental groups. This may be a result of the homogeneous nature of the carbohydrates and fiber in the diets.

EEB consumption increased CAT activity and decreased MDA, SOD, and NO levels. These effects were probably exerted by the bioactive compounds in EEB, which may act in conjunction with antioxidant enzymes. Gontijo et al. (2012) observed antioxidant effects of morelloflavone present in the ethyl acetate extract of *G. brasiliensis* peel through its 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, as it contains seven phenolic hydroxyl groups in its chemical structure. Thus, the direct action of non-enzymatic antioxidants present in EEB, which may act together with dietary fiber, probably causes radical scavenging. The synergistic effects of these food components with endogenous antioxidant systems promote cellular redox rebalancing in obese animals. In addition, Moreira et al. (2018) demonstrated that EEB was able to protect against NAFLD, which may be explained by the higher antioxidant activity of diets containing EEB. Our results suggest that EEB decreases reactive oxygen species (ROS) levels by stimulating the activity of CAT and HSP72, without decreasing lipid peroxidation.

Oxidative stress induces rapid induction of HSP72, the first line of

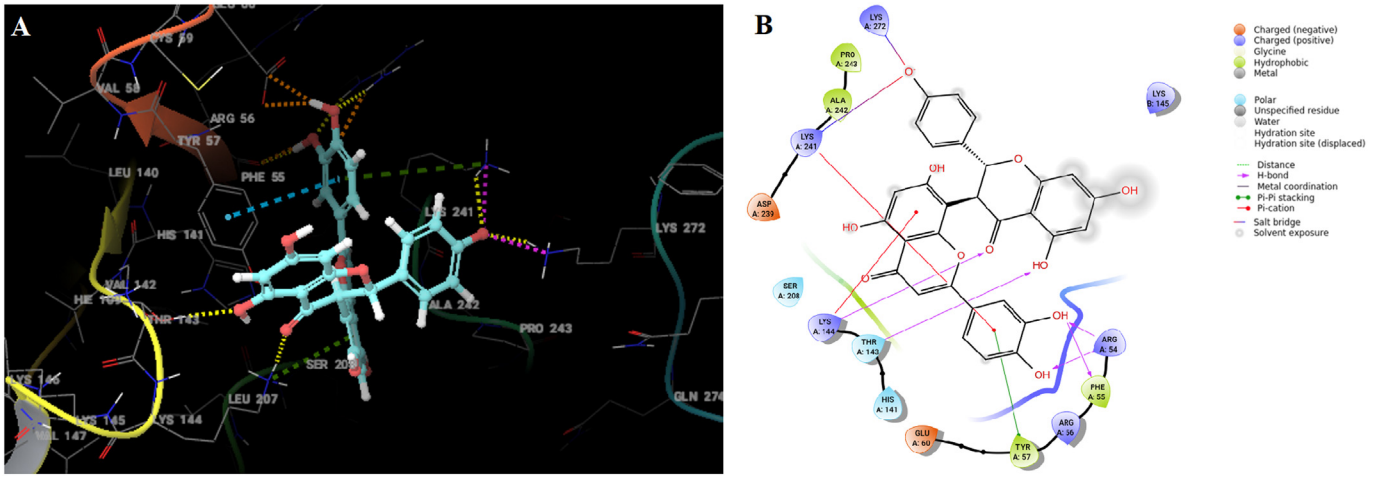

Fig. 4. (A) Representation of the best pose of molecular docking result between morelloflavone and NF-κB transcription factor and (B) interactions between amino acids of NF-κB transcription factor site and morelloflavone.

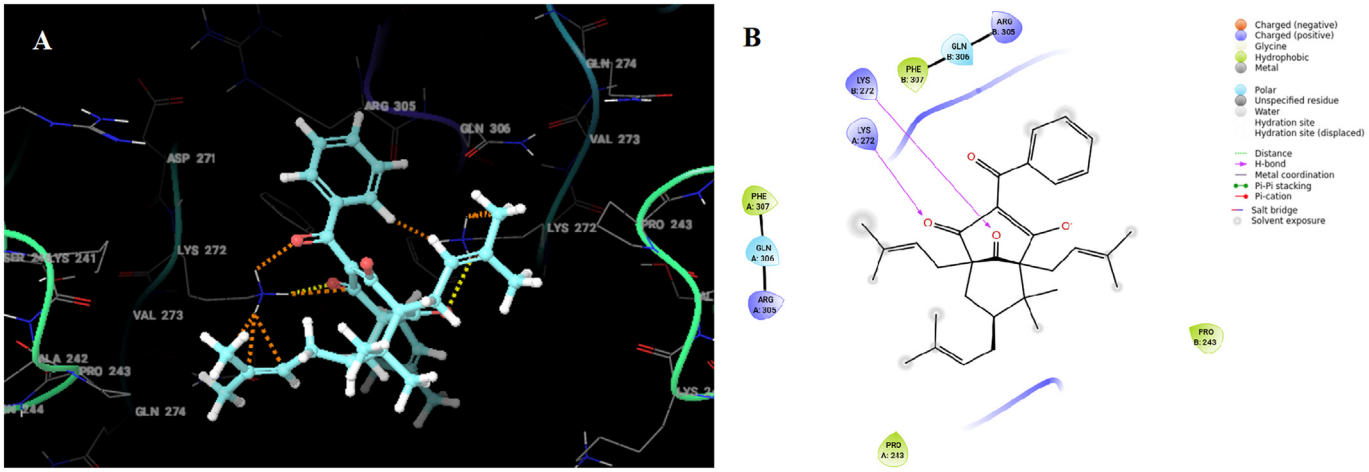

Fig. 5. (A) Representation of the best pose of molecular docking result between 7-epiclusianone and NF-κB transcription factor and (B) interactions between amino acids of NF-κB transcription factor site and 7-epiclusianone.

HSP70 proteins, which exerts cytoprotective and anti-inflammatory activities (Drummond et al., 2013). We observed an increase in the gene expression of HSP72 in the EEB-treated group compared with that in the group fed only HFD. EEB inhibited the expression of NF-κB and decreased the concentration of the proinflammatory cytokine resistin. The increase in HSP72 indicates a possible degradation of p65 through HSP70 chaperones, thus inhibiting the NF-κB pathway. Therefore, the induction of HSP72 reduced inflammation, as confirmed by resistin levels, and the expression of p65NF-κB and TNF-α (Moreira et al., 2017). The reduction in cytokines levels in the animals in our study was probably related to the consumption of EEB, which is rich in bioactive compounds. In a study by Santa-Cecília et al. (2011), EEB administered at doses of 30–300 mg/kg to Wistar rats showed anti-inflammatory and antinociceptive activities. Anti-inflammatory activity was also demonstrated by Moreira et al. (2017), who showed that obese rats fed EEB under equivalent experimental conditions as those used in our study exhibited decreased TNF-α and IL-10 concentrations.

In order to investigate the possible inhibitory activities and binding affinity profiles of the main chemical constituents of EEB (7-epiclusianone and morelloflavone) and NF-κB, computational studies were carried out using a molecular docking approach. Morelloflavone showed a GScore of $-7.077\ \text{kcal·mol}^{-1}$ (Table 3 and Fig. 4), thus displaying high affinity when compared with the docking results of bromopyrogallol red (BPR; Sharma et al., 2006), a standard inhibitor of NF-κB-DNA binding (GScore: $-6.622\ \text{kcal·mol}^{-1}$). The result map of morelloflavone docking with the amino acids of the NF-κB transcription factor site is shown in Fig. 4B. Seven hydrogen bond interactions were formed with the following amino acid residues: Arg54 (chain A; two interactions), Phe55 (A), Thr143 (A), Lys144 (A), Lys272 (A), in addition to 253 favorable hydrophobic van der Waals interactions, two cation-π interactions with Lys144 (A) and Lys241 (A), two salt bridges with Lys241 (A) and Lys272 (A), and one π-π stacking interaction with Tyr57 (A), thus supporting the possibility that morelloflavone inhibits the activation of NF-κB.

Furthermore, results of the molecular docking between 7-epiclusianone and NF-κB revealed a score of $-3.617\ \text{kcal·mol}^{-1}$ (Table 2 and Fig. 5), which, when compared with the docking results of BPR, showed lower affinity. The two-dimensional map (Fig. 5B) of the docking results between 7-epiclusianone and NF-κB revealed two hydrogen bond interactions with Lys272 (chain A) and Lys272 (chain B), 230 favorable van der Waals interactions, and one salt bridge interactions with Lys144 (A). Therefore, the docking studies revealed that morelloflavone, a flavonoid, shows more favorable interactions with the protein than the benzophenone, 7-epiclusianone. This may be because morelloflavone is more electronegative than 7-epiclusianone (electrostatic energy = -1158.201 and $-355.668\ \text{kcal·mol}^{-1}$, respectively), thus resulting in a stronger attraction for the cavity protein.

5. Conclusions

The consumption of EEB countered oxidative stress and inflammation in HFD-fed obese rats, thus revealing a promising strategy for beneficial modulation of the intestinal ecosystem. Therefore, this study demonstrates the potential protective effects of the phenolic compounds, morelloflavone, and 7-epiclusianone, present in EEB.

Author disclosure statement

No competing financial interests exist.

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Table 3
Values of Glide Score (*GScore*), the number of interactions by Hydrogen bonds (*Hbond*), by van der Waals (*good vdW*), by π-π stacking, by salt bridge, and by cation π between the ligands morelloflavone, 7-epiclusianone and Bromopyrogallol Red (BPR - standard drug) with NF-κB transcription factor (Schrödinger Suite, Induced Fit Docking Program).

Ligand	GScore (kcal.mol^{-1})	Hbond	Amino acids that perform HBond	Good vdW	Salt bridge	Cation-π	π-π stacking
Morelloflavone	−7.077	7	Lys144(A)*, Thr143(A), Arg54(A)(2), Phe55(A), Lys272(A)	253	2 Lys241(A), Lys272(A)	2 Lys241(A), Lys144(A)	1 Tyr57(A)
7-epiclusianone	−3.617	2	Lys272(A), Lys272(B)*	230	1 Lys144(A)	–	–
BPR	−6.622	4	TyrR57(B), Lys144(B), Lys145(A)(2)	148	1 Lys144(B)	1 Lys145 (A)	

* (A) and (B) - chain A and chain B of NF-κB transcription factor.

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