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CRediT author statement

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Signature

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Bergamot leaf extract treats cardiorenal metabolic syndrome and associated pathophysiological factors in rats fed with a high sugar fat diet

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Abstract

Bergamot citrus (Citrus bergamia Risso et Poiteau), have been used as a strategy to prevent or treat comorbidities associated with metabolic syndrome parameters, such as cardiorenal metabolic syndrome (CRMS). The aim was to test the effect of bergamot leaf extract on CRMS and associated pathophysiological factors in rats fed with a high sugar-fat diet. Animals were divided into two experimental groups with control diet (Control, *n*=30) and high sugar-fat diet (HSF, *n*=30) for 20 weeks. Once CRMS was detected, animals were redivided to begin the treatment with Bergamot Leaf Extract (BLE) by gavage (50mg/Kg) for 10 weeks: control diet + placebo (Control, n=09), control diet + BLE (Control+BLE, n=09), HSF diet + placebo (HSF, n=09), HSF + BLE (n=09). Evaluation included nutritional, metabolic and hormonal analysis; and renal and cardiac parameters. HSF groups presented obesity, dyslipidemia, hypertension, hyperglycemia, hyperinsulinemia, insulin resistance. BLE showed protection against effects on hypertriglyceridemia, insulin resistance, renal damage, and structural and functional alterations of the heart. Conclusion: Bergamot leaf extract shows potential as a therapeutic to treat CRMS in animals fed with a high sugar-fat diet.

Key words: Bergamot, CRMS, Western diet, metabolic syndrome

1. Introduction

In recent decades, the Western lifestyle demands more practical and quick meals, usually composed by industrialized products with high energy density and rich in sugars and fats, which associated to physical inactivity, resulting in positive energy balance resulting in adipose tissue expansion and, consequently, obesity (Popkin et al., 2012). The adipose tissue expansion due to adipocyte hypertrophy causes an imbalance in the secretion of adipokines which are associated with several complications and diseases, such as insulin resistance, hypertension, dyslipidemia and chronic inflammation, all risk factors for cardiorenal metabolic syndrome (CRMS)(Rangaswami et al., 2019)(Whaley-Connell and Sowers, 2014)

In addition to hypertrophy, consumption of high sugar-fat diets can cause mitochondrial stress leading to an increase in reactive oxygen species (ROS) and a decrease in antioxidant capacity, by inhibiting the nuclear factor erythroid 2 related factor 2 (Nrf2), a transcription factor that plays a crucial role in the maintenance of redox and metabolic homeostasis in response to reactive species and inflammation by regulating genes involved in the response against oxidative stress, promoting redox homeostasis(Cuadrado et al., 2018; Echeverría et al., 2018; Wu et al., 2021). Thus, the redox imbalance scenario favors CRMS and contributes to the development of complications that trigger this syndrome.

The CRMS, defined as combined disorders of heart and kidney, is characterized by impaired coronary blood flow, impaired diastolic relaxation, impaired ischemic reconditioning, renal hyper-filtration, proteinuria, glomerular sclerosis, tubule-interstitial fibrosis, and decreased GFR (glomerular filtration rate), with significant impact on life expectancy and mortality(Francisqueti et al., 2017; Rangaswami et al., 2019; Silva Junior et al., 2017; van der Velde et al., 2011; Whaley-Connell and Sowers, 2014). In order to prevent or treat cardiorenal metabolic syndrome, it is important to consider treatment strategies for modulating the pathophysiological processes involved in this disease. Within this context, researches about the effect of bioactive compounds present in natural products, as fruits, have been emerged mainly because these compounds have anti-inflammatory capacity, modulating the macrophage profile, and antioxidants acting as scavengers or stimulators of Nrf2(Gugliandolo et al., 2020).

Bergamot citrus (*Citrus bergamia Risso et Poiteau*) has shown antioxidant and anti-inflammatory potential(Formisano et al., 2019). In addition, studies also report to be effective in treating metabolic syndrome parameters, especially due the hypolipemic and hypoglycemic effect(Mollace et al., 2011; Risitano et al., 2014). These benefits are attributed to the polyphenolic fraction of bergamot fruit that consists predominantly of flavonoids (naringin, neo-peridin, neoeriocitrine, brutieridin and melitidine)(Di Donna et al., 2009). However, investigations show that for some plants, the phenolic composition of the leaf can be similar or even higher than the fruit, indicating that they may be used as an alternative source for the development of food supplements, nutraceuticals or functional foods(Baron et al., 2021; Ferlemi and Lamari, 2016).

Based on this, Baron et al (2021), conducted a study characterizing the bioactive compounds present in the leaf and fruit extract of bergamot. The results showed that the leaves have a higher amount of polyphenols and a greater antioxidant and anti-inflammatory capacity *in vitro*(Baron et al., 2021). Thus, since there are no studies that evaluated the effect of bergamot leaf extract on metabolic parameters neither in cardiorenal metabolic syndrome *in vivo* model, this investigation becomes original and extremely relevant. In addition, because the bergamot fruit is a valuable raw material that is in high demand, has many applications, and is cultivated in a limited region of Southern Italy, the opportunity to identify the leaves as a source of bioactive polyphenols with

a chemical and bioactive profile overlapping that of fruit polyphenols, allows for the availability of a renewable and low cost source for this important raw material. These results may lead to a sustainable product with pharmacological action to attenuate Cardiorenal Metabolic Syndrome. Therefore, the aim of this study was to test the effect of bergamot leaf extract on Cardiorenal Metabolic Syndrome and associated pathophysiological factors in rats fed with a high sugar-fat diet.

2. Materials and Methods

2.1. Experimental Protocol and Groups Characterization

The experiments and procedures were approved by the Animal Ethics Committee (271/2021) and were performed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Male Wistar rats (±187 g) were kept in an environmental controlled room (22 ◦C ± 3 ◦C; 12 h light-dark cycle and relative humidity of 60 ± 5%) and randomly distributed into 2 experimental groups by 20 weeks. During this period, the animals received, ad libitum, a control diet + water (Control, n = 30) and high sugar-fat diet + water containing 25% sucrose (HSF, n = 30). The diets were designed in our laboratory and previously published(Francisqueti et al., 2017). After this period, when CRMS was detected- determined by cardiac dysfunction and stage 0 of renal disease(Romão Junior, 2004) the presence of renal disease risks detected, a 95% confidence interval (CI) was built for the cardiac dysfunction and risk factors for renal diseases from the HSF and control groups. Was adopted as the separation point (SP) between the groups, the midpoint between the upper limit of the control group and the lower limit of the HSF group. From the data, the CRMS was characterized(Rangaswami et al., 2019; Whaley-Connell and Sowers, 2014). This criterion was adopted because animals submitted to different diet models do not always present the expected response. This fact can lead to erroneous animal classification and, consequently, false conclusions. Accomplished, these animals were randomly redistributed into 4 experimental groups: control diet + placebo (Control, n=09), control diet + bergamot leaf extract (Control+BLE, n=09), high sugar-fat diet + placebo (HSF, n=09), high sugar-fat diet + bergamot leaf extract (HSF+BLE, n=09). This procedure was performed over a period of 10 weeks to verify the effect of the treatment of bergamot leaf extract on cardiorenal metabolic syndrome. The animals that didn't developed CRMS were excluded from the experiment.

2.2. Bergamot leaf extract (BLE)

The bergamot leaves were harvested in a farm located in the Reggio Calabria region, Italy, and the dry extract was obtained at H&AD (Herbal & Antioxidant Derivatives S.r.l.) plant located in Località Chiusi, 89032 Bianco (RC), Italy (www.head-sa.com). The leaves were immediately chopped and added to a solution of water / ethanol (30/70 % v / v) to extract the polyphenols. The extraction procedure was repeated twice for one hour and the resulting solution was distilled to recover the ethanol. The clarified water solution was passed through a layer of absorbent polystyrene resins with pores with a diameter between 100 and 150 Ångstrom to retain polyphenols, which were subsequently eluted from the resin bed flowing pure ethanol. Then, the obtained ethanol solution was distilled in vacuum at temperatures of up to 40°C, obtaining a concentrate containing residual water and polyphenols, which were dried in a spray drying system, obtaining a powder with moisture content less than 4.0%.

The administration was carried out daily by gavage, at a concentration of 50mg/Kg of weight and the extract was diluted in filtered water. The dose was established according to literature data that use this concentration from fruit juice, as there are no studies using leaf extract(Musolino et al., 2020).

2.3. Nutritional, Metabolic and Hormonal Analysis

The nutritional profile was evaluated according to caloric intake, body weight and adiposity index. Calorie intake was determined by multiplying the energy value of each diet (g × Kcal) by the daily food intake. For the HSF group, it also included calories from water (0.25 × 4 × mL consumed). The animals' body weight was evaluated weekly. The adiposity index was used as an indicator of obesity at 30th week, since it accurately assesses the amount of body fat in the animals. After euthanasia, the epididymal, visceral and retroperitoneal fat deposits were dissected from the animals. The sum of deposits normalized by body weight [(epididymal+retroperitoneal+visceral)/body weight x 100] is considered the adiposity index(Luvizotto et al., 2013).

After 8-h fasting, blood was collected and the plasma was used to measure insulin and biochemical parameters. Glucose concentration was determined by using a glucometer (Accu-Chek Performa; Roche Diagnostics, Indianapolis, IN, USA); triglycerides levels were measured with an automatic enzymatic analyzer system (Chemistry Analyzer BS-200, Mindray Medical International Limited, Shenzhen, China). The insulin level was measured using the enzyme-linked immunosorbent assay (ELISA) method using commercial kits (Linco Research Inc., R&D Systems, Millipore e B-Brigde International Inc.) and the reading was performed in a Spectramax 190 microplate spectrophotometer (Molecular Devices®, Sunnyvale, CA, USA). The homeostatic model of insulin resistance (HOMA-IR) was used as an insulin resistance index, calculated according to the formula: HOMA-IR = (fasting glucose (mmol/L) × fasting insulin (μU/mL))/22.5(Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF et al., 1985).

2.4. Systolic Blood Pressure

Systolic blood pressure (SBP) evaluation was assessed in conscious rats by the non-invasive tail-cuff method with a NarcoBioSystems® Electro-Sphygmomanometer (International Biomedical, Austin, TX, USA). The animals were kept in a wooden box (50 × 40 cm) between 38–40 °C for 4–5 min to stimulate arterial vasodilation(Gonc et al., 2014). After this procedure, a cuff with a pneumatic pulse sensor was attached to the tail of each animal. The cuff was inflated to 200 mmHg pressure and subsequently deflated. The blood pressure values were recorded on a Gould RS 3200 polygraph (Gould Instrumental, Valley View, OH, USA). The average of three pressure readings was recorded for each animal.

2.5. Renal Function

Plasma and urine were used to evaluate the renal function. During 12 hours, urine was collected through metabolic cages for further analysis of total protein and creatinine using commercial colorimetric kits (CELM® kits, Barueri, São Paulo, Brazil). To assess kidney damage, proteinuria through protein/creatinine ratio was determined; glomerular filtration rate (GFR) = ((urine creatinine × flux)/plasma creatinine) was calculated as marker of kidney function(Anna et al., 2010).

2.6. Structural and Functional Cardiac Function

Doppler echocardiographic evaluation was performed by a single examiner at the 20th and 30th weeks. Animals were anesthetized with ketamine (50 mg/kg, i.p.) and xylazine hydrochloride (1 mg/kg, i.p.). After trichotomy of the anterior chest region, the animals were placed in slight left lateral decubitus for the exam. The equipment used was model Vivid S6 (General Electric Medical Systems, Tirat Carmel, Israel) with a multifrequency ultrasonic transducer 5.0 to 11.5 MHz. To implement structural measurements of the heart, the images were obtained in one-dimensional mode (M-mode) guided by the images in two-dimensional mode with the transducer in the parasternal position, minor axis.

Left ventricular (LV) evaluation was performed by positioning the cursor M-mode just below the mitral valve plane at the level of the papillary muscles. The images of the aorta and left atrium were obtained by positioning the M-mode course to plan the level of the aortic valve. The following cardiac structures were used to analyze cardiac morphology: left ventricular diastolic diameter (LVDD); left ventricular systolic diameter (LVSD); left atrium (LA); LA/AO; relative wall thickness (RWT). The LV systolic function was assessed by the following parameters; endocardial fractional shortening (FS%) ((LVDD − LVSD)/LVDD) × 100; posterior wall shortening velocity (PWSV). The LV diastolic function was evaluated using the following indices: peak velocity of early diastolic filling (E wave). The study was supplemented by evaluation by tissue Doppler systolic displacement (S′), early diastolic (E′) and late (A′) of the mitral annulus (arithmetic average travel speeds of lateral and septal walls), and E/A and E/E′ wave ratios.

The LV systolic function was assessed by the following parameters: Left ventricle posterior wall shortening velocity (LVPWSV) and endocardial fractional shortening (EFS%) ((LVDD-LVSD/LVDD) x100). The LV diastolic function was evaluated using the following indices: E/E' ratio and E wave deceleration time (EWDT). The study was supplemented by evaluation by tissue Doppler systolic displacement (S′), early diastolic (E′) and late (A′) of the mitral annulus (arithmetic average travel speeds of lateral and septal walls), and E/E′ wave ratios(Sahn et al., 1978).

2.7. Nrf2 activation

The bergamot leaf ability to modulate the antioxidant response pathway induced by Nrf2 activation was tested using NRF2/ARE Responsive Luciferase Reporter HEK293 stable cell line (Signosis, Santa Clara, CA, USA). Cells were grown in Dulbecco modified Eagle medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), 1 % Penicillin/Streptomycin (Lonza) and 50 μg/mL of G418 sulfate solution (Promega Corporation, Madison, WI, USA). HEK293 cells were treated with different concentrations (100, 150, 200 and 250 μg/ml) of BLE for 18 h after seeding in white 96-well plate (BRANDplates®, cell grade) at 10000 cells/well. Subsequently, to avoid any interference on the reading of luciferase activity, media was removed and 100 μL/well of PBS was added. ONE-Glo™ Luciferase Assay Substrate (purchased from Promega Corporation, Madison, WI, USA) (100 μL/well) was directly added to the wells, followed by a luciferase measurement performed using a luminometer (Wallac Victor2 1420, Perkin-Elmer™ Life Science, Monza, Italy). Experiments were performed with biological and technical replicates; values are shown as mean ± SD compared to untreated control cells. Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple comparisons test ($p < 0.05$ was considered significant).

The cell viability was assessed with MTT assay on HEK293 cells treated with all the concentrations of the BLE as reported previously(Baron et al., 2021).

2.8. Statistical Analysis

Data are presented as means ± standard deviation (SD) or medians (interquartile range). Differences among the groups were determined by two-way analysis of variance. Statistically significant variables were subjected to the two-way ANOVA test with Tukey post-hoc. Statistical analyses were performed using Sigma Stat for Windows Version 3.5. (Systat Software, Inc., San Jose, CA, USA). A p value of 0.05 was considered as statistically significant.

3. Results

Nutritional and metabolic parameters: triglycerides, insulin and insulin resistance were attenuated with 10 weeks of treatment with BLE.

The HSF diet promoted increased caloric intake, adiposity index, final body weight, systolic blood pressure and blood glucose, characterizing an experimental model of obesity accompanied by comorbidities that are risk factors for cardiorenal metabolic syndrome. These parameters were not attenuated with the treatment of bergamot leaf extract (Figure 1 A, B, C, D and F). On the other hand, triglycerides, insulin and insulin resistance were also found to be elevated in the HSF group, but were attenuated by the extract treatment (Figure 1 E, G and H).

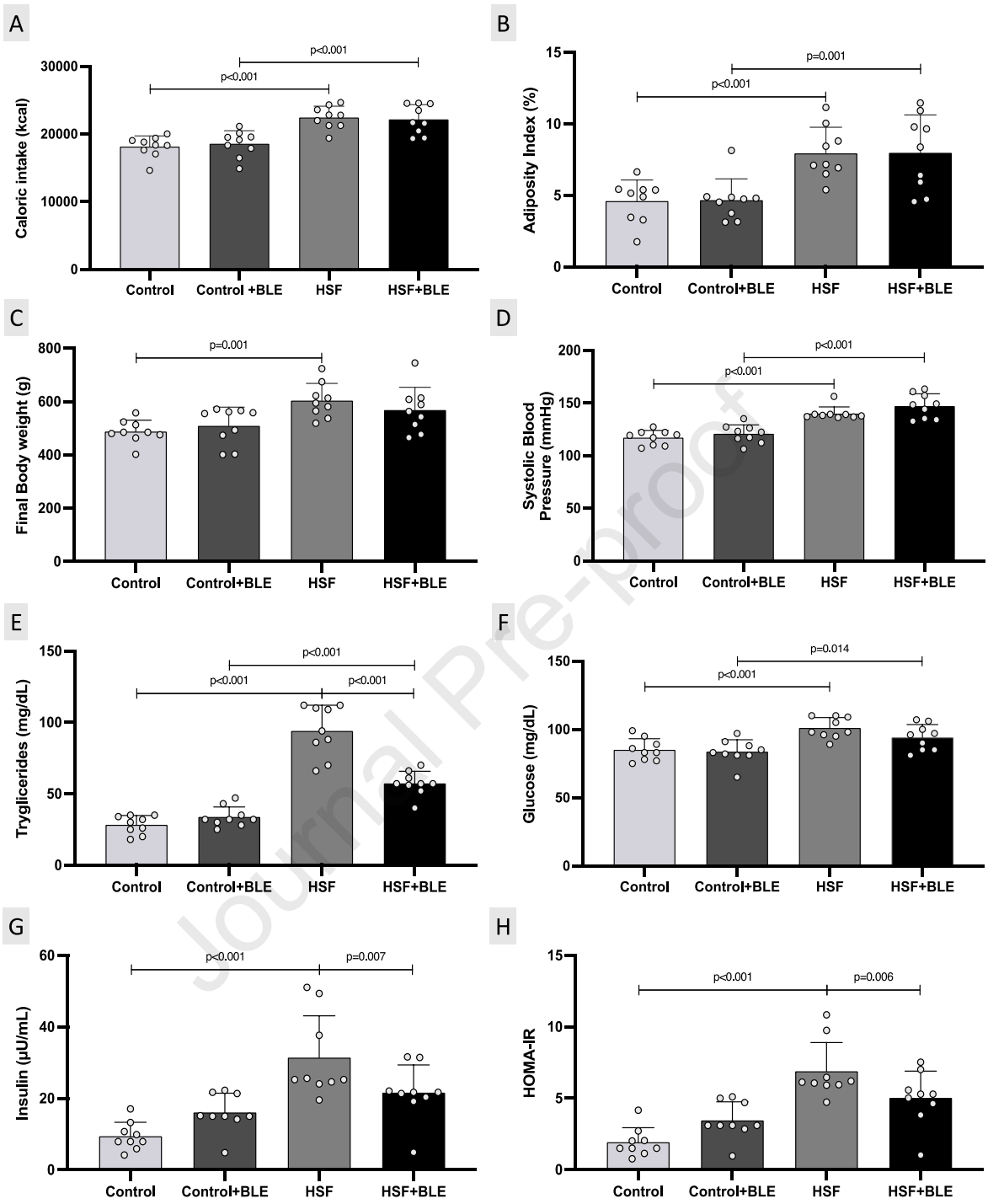

Figure 1. Nutritional, metabolic and hormonal analysis in the 30th week. (A) Caloric intake (kcal); (B)Adiposity index (%); (C) Final body weight (g); (D) Systolic blood pressure (mmHg); (E) Triglycerides (mg/dL); (F) Glucose (mg/dL); (G) Insulin (µU/ml); and (H) HOMA-IR. Data are expressed as mean ± standard deviation. Comparison by two-way ANOVA with Tukey post-hoc. BLE– Bergamot Leaf Extract. HSF—high sugar fat diet.

Renal function parameters: treatment with BLE attenuated proteinuria and improved renal function

It is possible to note that the HSF group developed renal impairment when compared to the control group, characterizing by increased protein/creatinine ratio and reduced glomerular filtration rate. The treatment

with the extract provided full recovery of proteinuria and improved renal function, shown by increased glomerular filtration rate (Figure 2 A and B).

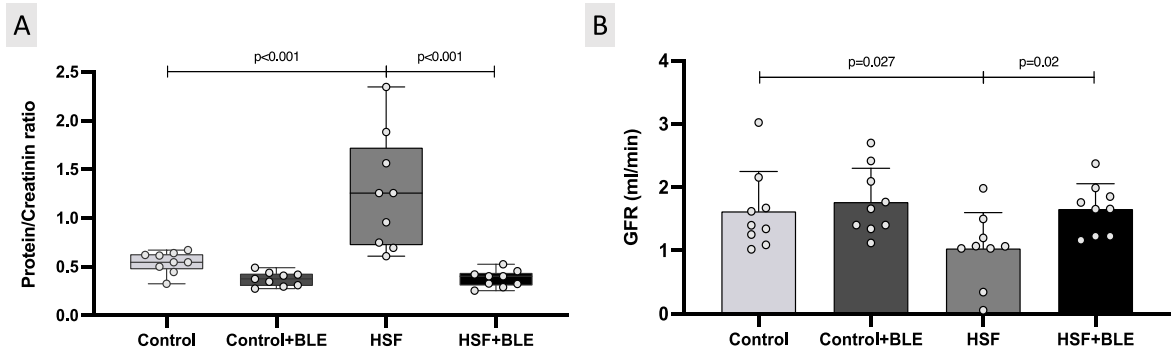

Figure 2. Renal function in 30 weeks. (A) Protein/creatinine ratio; (B) Glomerular filtration rate– GFR (mL/min). Data are expressed as median (min-max) and mean ± standard deviation. Comparison by two-way ANOVA with Tukey post-hoc. BLE– Bergamot Leaf Extract. HSF—high sugar fat diet.

Cardiac function: BLE attenuated cardiac remodeling

The echocardiographic parameters are presented in the Table 1. The HSF group presented cardiac remodeling characterized by changes in morphological variables (PWT, IST, LA, LVM and LVRT), diastolic deterioration (lower EWDT and E/E') and systolic dysfunction (higher LVPWSV) in comparison to the control group. Bergamot leaf extract treated the cardiac remodeling and the deterioration of diastolic and systolic functions compared to the HSF group.

Table 1. Cardiac function in 30 weeks.

	Groups				Effects		
	Control	Control + BLE	HSF	HSF + BLE	Diet	BLE	Interaction
PWT (mm)[1]	1,53(1.53-1.44)	1.50(1.51-1.45)	1.79(2.04-1.58)*	1.53(1.61-1.52) ▲	0.016	0.096	0.055
IST (mm)[1]	1.53(1.53-1.50)	1.53(1.56-1.50)	1.79(2.14-1.53)*	1.60(1.62-1.55) ▲	0.036	0.220	0.128
LA(mm)	4.71 ± 0.29	4.69 ± 0.25	5.51 ± 0.65*	5.05 ± 0.57 ▲	<0.001	0.143	0.174
LVM (g)	0.69 ± 0.09	0.73 ± 0.17	1.03 ± 0.20*	0.77 ± 0.10 ▲	<0.001	0.036	0.004
LVRT	0,43 ± 0.02	0.43 ± 0.04	0.55 ± 0.07*	0.42 ± 0.02 ▲	0.002	<0.001	<0.001
LVPWSV (mm/s)	82.44 ± 6.77	80.56 ± 6.33	61.24 ± 10.54*	74.25 ± 2.80 ▲	<0.001	0.026	0.004
EWDT (ms)[1]	44.00(46.00-43.00)	46.00(46.75-44.50)	58.50(62.00-51.00)*	48.00 (51.50-44.00) ▲	<0.001	0.024	0.004
E/E'	13.92 ± 1.42	12.85 ± 1.56	22.94 ± 3.71*	15.75 ± 1.69 ▲	<0.001	<0.001	<0.001
EFS (%)	63.81 ± 3.87	59.99 ± 6.04	54.17 ± 4.61*	57.01 ± 2.91	<0.001	0.747	0.034

PWT– Diastolic thickness of the left ventricle posterior wall; IST– diastolic thickness of the interventricular septum; LA—left atrium; LVM– Left ventricle mass; LVRT– Left ventricle Relative Thickness; LVPWSV– Left ventricle posterior wall shortening velocity; EWDT– E wave deceleration time; EFS– Endocardial fractional shortening. Data expressed in mean ± standard deviation. [1]median (interquartile range). Comparison by Comparison by two-way ANOVA with Tukey post-hoc. $p<0.001$. HSF—high sugar fat diet. BLE–Bergamot Leaf Extract. *$p<0.001$ vs. C; ▲ $p<0.001$ vs. HSF

Correlation between nutritional, metabolic, renal and cardiac parameters

Figure 3 presents Pearson correlation among the variables. Regarding metabolic parameters, triglycerides, glucose, insulin and HOMA-IR were correlated with proteinuria, while only triglycerides were correlated with glomerular filtration rate. About the cardiac parameters, systolic blood pressure, triglycerides, glucose, insulin and HOMA-IR showed correlation.

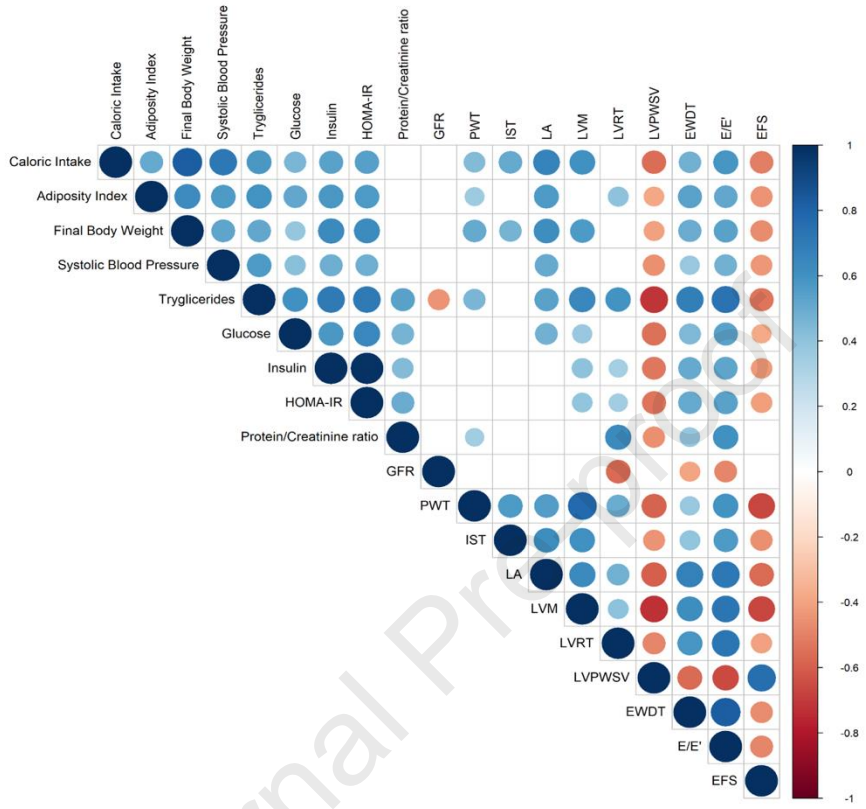

Figure 3. Correlation matrix among the variables. Positive correlations are presented in blue and negative correlations are presented in red color. Color intensity and the size of the circle are proportional to the correlation coefficients. Correlations with p>0,05 were excluded from the matrix. Glomerular filtration rate– GFR; PWT– Diastolic thickness of the left ventricle posterior wall; IST– diastolic thickness of the interventricular septum; LA—left atrium; LVM– Left ventricle mass; LVRT– Left ventricle Relative Thickness; LVPWSV– Left ventricle posterior wall shortening velocity; EWDT– E wave deceleration time; EFS– Endocardial fractional shortening.

Since triglycerides were the only metabolic parameter associated with both renal and cardiac variables, figure 4 highlights Pearson Correlation among these variables.

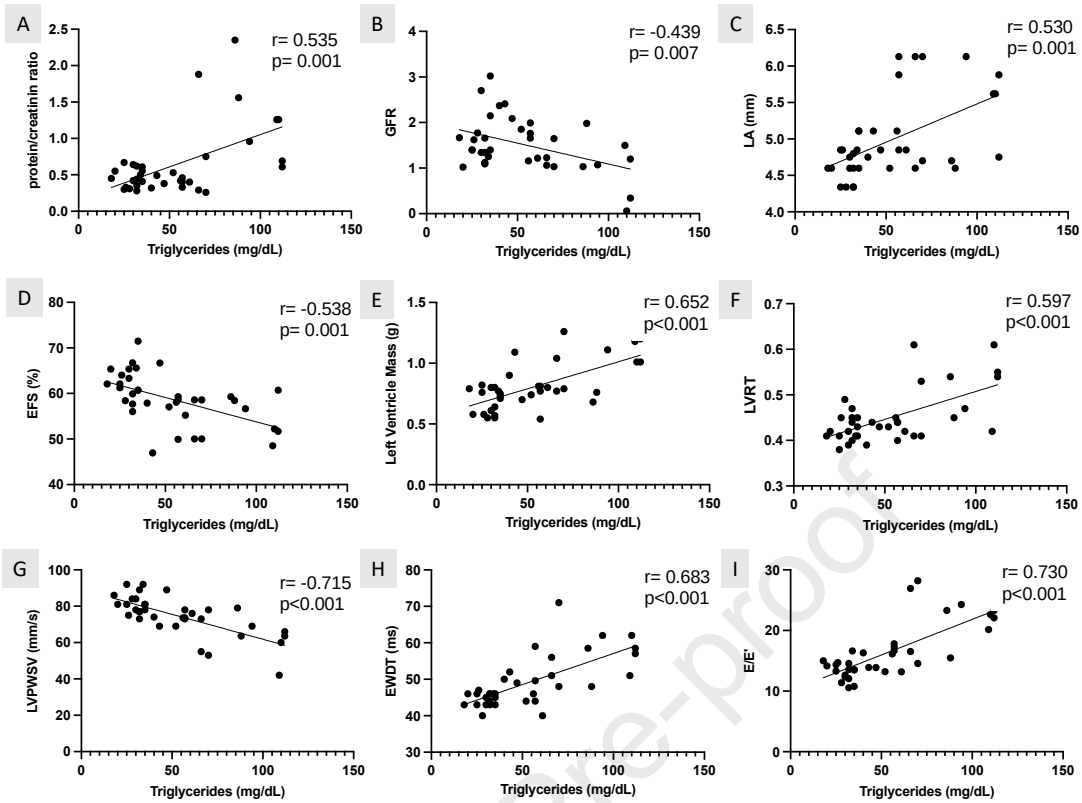

Figure 4. Pearson correlations among triglycerides and Cardiorenal metabolic syndrome parameters. (A) Triglycerides (mg/dL)/protein/creatinine ratio; (B) Triglycerides (mg/dL)/Glomerular filtration rate–GFR (mL/min); (C) Triglycerides (mg/dL)/Left atrium–LA(mm); (D) Triglycerides (mg/dL)/Endocardial fractional shortening–EFS(%); (E) Triglycerides (mg/dL)/Left Ventricle Mass (g); (F) Triglycerides (mg/dL)/Left ventricle Relative Thickness–LVRT; (G) Triglycerides (mg/dL)/Left ventricle posterior wall shortening velocity–LVPWSV (mm/s); (H) Triglycerides (mg/dL)/E Wave deceleration time–EWDT (ms); (I) Triglycerides (mg/dL)/E/E'; Pearson regression was used to examine the association between variables.

Nrf2 activation

The dose-dependent effect of BLF to activate the Nrf2 pathway was tested using HEK293 stable cell line with a luciferase gene reporter. The results are reported in the graph bar of Figure 5 and show the fold increase of the luciferase expression and hence of Nrf2 nuclear translocation in respect to control cells. BLF was effective in a dose-dependent manner, from 100 μg/ml and 250 μg/ml being the highest concentration tested.

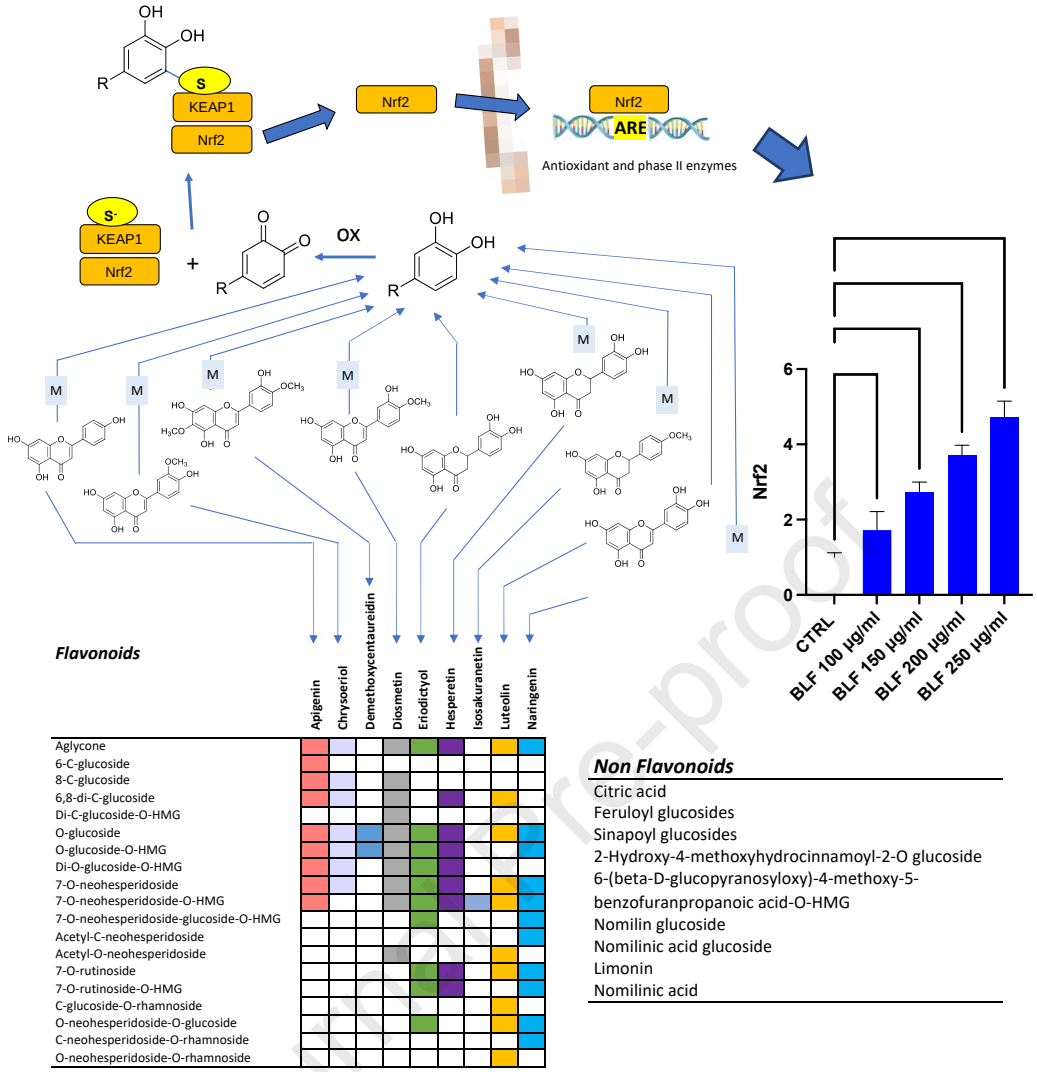

Figure 5. BLF activates the Nrf2 pathway. The graph bar shows the dose-dependent effect of BLF as Nrf2 activator as determined in NRF2/ARE Responsive Luciferase Reporter HEK293 stable cell line. The main flavonoid and non flavonoid constituents of bergamot leaves as reported in Baron et al. are summarized in the two tables(Baron et al., 2021). The structure of each aglycone is depicted and linked by an arrow to the ortho-diphenol moiety which is the required structure for Nrf2 activation through a mechanism shown in the upper part of the figure and described in the text. The link arrow is labelled by an M when a phase I metabolic activation (O-demethylation and/or aromatic hydroxylation) is required for the conversion of the compound to the ortho-diphenol moiety.

4. Discussion

The aim of this study was to test the potential of BLE on CRMS in animals fed with a high sugar-fat diet. At the end of the experimental protocol, both groups that consumed the HSF diet presented obesity, systolic arterial hypertension, dyslipidemia, higher glucose and insulin levels and insulin resistance. These results demonstrate that the diet promoted the risk factors for CRMS in the HSF groups(Francisqueti et al., 2017). Corroborating these findings, the literature shows that a diet rich in simple carbohydrates, such as those administered to the HSF animals, due to their lower molecular weight, are absorbed more quickly, resulting in higher fat deposition. Also, this energy imbalance caused by a hypercaloric diet can impair some molecular alterations, such as insulin

signaling, resulting in impaired glucose transport, dysregulated lipolysis and dyslipidemia(Rodríguez-Correa et al., 2020).

The confirmation of CRMS in the HSF group was through the increase in protein/creatinine ratio, decrease in the GFR and cardiac remodeling characterized by changes in morphological variables, diastolic deterioration and systolic dysfunction in relation to the control group. These results can be explained by the presence of obesity-related disorders in the HSF group triggered by the chronic inflammation state common in obesity, consequence of adipocyte hypertrophy and altered adipokine and adipocytokines pro-inflammatory secretion, among them, insulin resistance(Bischoff et al., 2017).

The presence of insulin resistance appears to play an important role in both obesity-related heart and kidney failures, since impaired insulin metabolic signaling reduces bioavailable nitric oxide (NO), increases oxidant stress and inflammation, and activates renin-angiotensin-aldosterone system (RAAS), resulting in maladaptive myocardial tissue remodeling and interstitial fibrosis that contribute to impairments in diastolic function in heart and induces glomerular dysfunction and tubulointerstitial fibrosis in kidneys(Whaley-Connell and Sowers, 2012). Moreover, insulin resistance is also associated with increased lipolysis in adipose tissue that releases a large amount of free fatty acids (FFAs) into the bloodstream. The imbalance between the excessive influx of FFAs and the saturation of oxidative capacity (mitochondrial β-oxidation) results in bioenergetic incompatibility with accumulation of TG in the hepatocyte and greater mobilization to the circulation of very low-density lipoproteins (VLDLs) rich in TG, leading to hypertriglyceridemia(Mashek, 2021). This condition of hypertriglyceridemia also promotes ectopic accumulation of fat in non-fat-storing organs such as kidneys and heart, which induces oxidative stress and promotes a mitochondrial dysfunction, intensifying the reduction-oxidation imbalance contributing to the activation of pro-inflammatory, pro-fibrogenic, and pro-apoptotic pathways, causing cellular injury. Therefore long-term HSF diet feeding results in obesity, metabolic syndrome, which are risk factors for CRMS(Sun et al., 2020; Wali et al., 2020).

Furthermore, the FFAs overload also results in an increase ROS generation as a metabolism product through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation locally in adipose cells or in other organs cells, aggravating the oxidative stress(Matsuda and Shimomura, 2013). The accumulation of ROS contributes to vasoconstriction, inflammation and impaired vascular which contributes to the pathogenesis of CRMS(Ammar et al., 2021; Rubattu et al., 2013). The increase of oxidative stress markers can lead to structural changes in the myocardium, which can result in cardiac remodeling, decrease in contractility and heart failure(Spinale et al., 1998). Also, increased ROS are related to reduced renal function, leading to kidney hyperfiltration through compensatory mechanisms that contributes to glomerular hypertension, proteinuria or even loss of function (Cachofeiro et al., 2008).

In opposition, animals that consumed BLE in addition to the HSF diet presented lower triglycerides, insulin levels and insulin resistance compared to the group that consumed just the HSF diet. The amelioration of these parameters, which are risk factors for CRMS, are in accordance with studies that evaluated the same parameters with bergamot fruit, highlighting the power of leaves as a possible adjuvant in the treatment of these comorbidities(Mollace et al., 2011). However, the mechanisms responsible by the positive effects of BLE are not fully elucidate. Bergamot fruit and leaf extract share the same class of polyphenolic compounds, and some of them, like flavones and flavanones, in higher concentrations in the leaf, showing promising results treating complications associated with the HSF diet consumption(Baron et al., 2021). Some of these flavonoids, such as

naringin, neoeriocitrin and rutin, have been shown to exert an antiatherogenic effect on animal models and low density lipoproteins (LDLs) oxidation inhibition, contributing to the potential hypolipidemic effect in the BLE(Miceli et al., 2007; Mollace et al., 2011). The buteridine, naringin and melitidine of BPF are structurally similar to HMG-CoA reductase substrate statins and thus can competitively inhibit HMG-CoA reductase(Leopoldini et al., 2010). The flavonoids may also lower cholesterol levels by binding bile acids and increasing the turnover rate of blood and liver cholesterol, reducing TG accumulation in the liver, and enhancing the excretion of fecal neutral sterols and total bile acids(Miceli et al., 2007; Parafati et al., 2015). Naringin and neohesperidin may reduce hepatic TG accumulation by inhibiting the activity of phosphatidate phosphohydrolase, a TG synthetic enzymes and by reducing the availability of lipids for assembly of lipoproteins via reduced activities of acyl CoA:cholesterol acyltransferases.

As above described, oxidative stress is a condition associated with cardiorenal metabolic syndrome. In this sense, in order to alleviate oxidative stress, different mechanisms are activated, such as Nrf-2, increasing the production of endogenous antioxidants, preventing and controlling the formation of free radicals and consequently the damage caused by them(Ishii et al., 2002). By using a gene reporter cell model of NRF2 activation, we here report the ability of the extract to induce, in the concentration range 100-250μg/ml, the Nrf2-ARE pathway which regulates the expression of a large battery of genes involved in the cellular antioxidant and anti-inflammatory defense as well as mitochondrial protection. Figure 5 reports the main flavonoid and non flavonoid constituents of bergamot leaves(Baron et al., 2021). The structure of each aglycone is depicted and the derivatives reported in the table. In the figure, each aglycone structure is linked by an arrow to the ortho-diphenol moiety which is the required structure for Nrf2 activation for this class of compounds, through a mechanism shown in the upper part of the figure. In particular, the di-phenol moiety is activated by an oxidative stress environment to the corresponding quinone which being an electrophilic derivative it binds the thiol of KEAP-1, inducing the release of NRF2 which, upon translocation at the nuclear level, binds to the antioxidant response elements (AREs) in the regulatory region of target genes that encode proteins involved in redox homeostasis, xenobiotic metabolism and survival responses. When a phase I metabolic activation (O-demethylation and/or aromatic hydroxylation) is required for the conversion of the compound to the ortho-diphenol moiety, the link arrow is labelled by an M. Eriodictyol and luteolin do not require a metabolic conversion because they already contain the ortho-diphenol moiety and hence they act as direct Nrf2 activators. We believe that the overall Nrf2 activity of the extract is more likely underestimated because many polyphenol compounds require a metabolic conversion which should occur in vivo, mainly in liver and not in cell lines, as already demonstrated for resveratrol and sylibin which are activated to the ortho-diphenol derivatives by the cytochrome P450 enzyme CYP1B1 and by CYP mediate O-demethylation, respectively(Potter et al., 2002; Xie et al., 2019).

The polyphenol content also exerts antioxidant and inflammatory effects, as demonstrated by Baron et al(Baron et al., 2021), in a mechanism that seems to be associated with inhibition of NF-κB activation. The literature reports that NF-κB translocation to the nucleus encodes inflammatory mediators such as tumoral necrosis factor alpha (TNF-α) and interleukin 1-beta (IL-1β) and interleukin- 6 (IL-6). This inflammatory condition causes serine kinase phosphorylation of insulin receptor substrate -1 or 2 (IRS-1 or IRS-2), which may block insulin signaling and finally lead to the occurrence of insulin resistance(Chen et al., 2015). Therefore, the improvement in insulin sensitivity can be result of the antioxidant and antinflammatory effect of BLE, especially addressed to flavanone and flavone glycosides, as well as to their aglycones, including naringin and

hesperidin, diosmetin, apigenin, and luteolin glycosides(Baron et al., 2021). This antioxidant and antinflammatory effect can also explain the protection against heart and kidney dysfunction even in the presence of hypertension, an independent factor for CRMS. Some studies of our research group showed a local antioxidant and antinflammatory effect of lycopene in the heart of obese animals and the same positive effect of gamma-oryzanol in the kidney of obese animals, preserving renal function(Ferron et al., 2020; Francisqueti et al., 2018). This same effect may be exerted BLE in the heart and kidney of animals from this study. Figure 6 presents a summary of the observed effects.

Despite there are no clinical trials evaluating the effect of bergamot leaf extract in humans, the extract may be a promising candidate in preventing and improving factors associated with the development and progression of Cardiorenal Metabolic Syndrome.

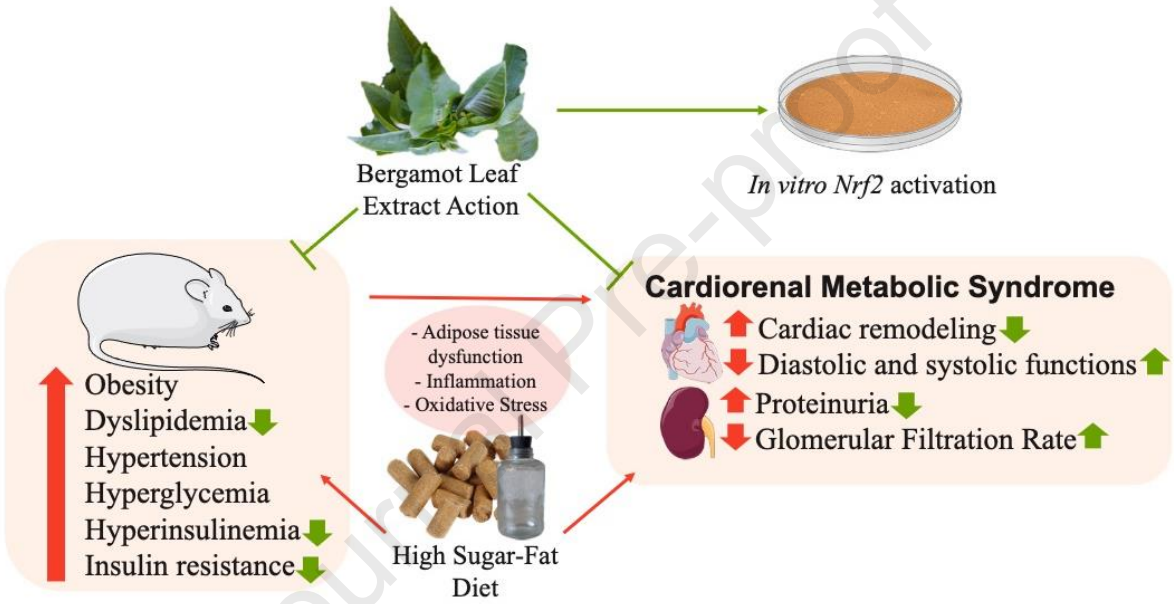

Figure 6. Role of Bergamot Leaf Extract in the pathophysiological process of Cardiorenal Metabolic Syndrome (CRMS). The results demonstrate that the high sugar-fat diet promoted the risk factors for CRMS in the HSF groups, such as obesity, systolic arterial hypertension, dyslipidemia, higher glucose, insulin levels and insulin resistance. The high consumption of high sugar-fat diets contributes to adipose tissue dysfunction, inflammatory pathways activation and reactive oxygen species production, promoting oxidative stress. These conditions contribute directly with CRMS development by the presence of increased protein/creatinine ratio, decreased glomerular filtration rate and cardiac remodeling characterized by changes in morphological variables, diastolic deterioration and systolic dysfunction. Bergamot (Citrus bergamia) Leaf Extract was efficient in controlling and treating CRMS and pathophysiological process involved with the disease. In addition, the extract was able to induce the Nrf2-ARE pathway, which regulates the expression of a large battery of genes involved in the cellular antioxidant and anti-inflammatory defense as well as mitochondrial protection.

5. Conclusion

In summary, this study showed that the HSF diet induced obesity, insulin resistance, dyslipidemia, systolic blood pressure, and cardiorenal metabolic syndrome. On the other hand, the HSF group treated with bergamot leaves extract presented amelioration on insulin resistance, dyslipidemia and cardiorenal metabolic syndrome. Therefore, it is possible to conclude that bergamot leaf extract was able to treat cardiorenal metabolic syndrome in animals fed with a high sugar-fat diet.

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Bergamot leaf extract promoted lower dyslipidemia and Insulin Resistance levels.

The extract treated cardiorenal metabolic syndrome, improving organ's function.

Bergamot leaf extract stimulates Nrf2 activation.