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Upcycled Romaine Lettuce Powder as a Dietary Supplement for Control of Metabolic Syndrome

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ABSTRACT: Romaine lettuce outer leaves are discarded in the field during harvesting, presenting an opportunity for upcycling. We previously developed a romaine lettuce powder (RLP) from discarded outer leaves with high antioxidant capacity, high levels of fiber, and high concentration of total soluble phenolics. In this study, we evaluated the health benefits and preventive effects of RLP in metabolic syndrome (MS) induced by a high-fat diet fed to mice for 12 weeks. Supplementation with 10% RLP in a high-fat diet lowered glucose and insulin levels and prevented the development of insulin resistance in mice. Moreover, adipose tissue weight was lower in mice fed with a high-fat diet supplemented with 10% RLP compared to mice fed with a control high-fat diet. Liver weight was also lower in mice fed with a high-fat diet supplemented with 10% RLP compared to those fed with low-fat and high-fat diet groups, suggesting a positive effect of lettuce powder on liver health. Here, we show that RLP has a preventive effect on MS by regulating body weight, lowering lipid levels, and improving glucose metabolism. Our results indicate that incorporating RLP into mice's diet has potential health benefits.

KEYWORDS: *romaine lettuce, upcycling, metabolic syndrome, dietary supplement, fiber, antioxidants, phenolics*

■ INTRODUCTION

Global food waste is a complex issue that arises during different stages of agricultural production, particularly during processing and postharvesting. Shockingly, about 14% of the world's food production is lost between the harvest and retail stages.[1] Consumer demand for aesthetically perfect fruits and vegetables leads to the disposal of products that do not meet these standards, which could have been used as food.[2,3] Additionally, the outer parts and extremities of fruits and vegetables, which are often discarded during processing and cutting, contain valuable bioactive compounds that can be used in the production of nutraceutical supplements.[4−6] The disposal of such products leads to environmental issues.[3] To address this problem, the United Nations signed Agenda 2030 and its 17 Sustainable Development Goals (SDGs) in 2015. Objectives 2 and 9 of the SDGs focus on sustainability in agriculture and industry and provide solutions to issues concerning waste disposal and food waste. Therefore, there is a growing emphasis on adopting a circular economy approach that promotes sustainability and reduces the environmental impact of various materials, including food byproducts.[7,8]

Romaine lettuce (*Lactuca sativa* L.) is one of the most consumed vegetables around the world due to its crunchy, fresh green appearance, and pleasant aroma, as well as the presence of bioactive phytochemicals, such as phenolic compounds.[9] Despite being approximately 95% water, lettuce is a source of carotenoids such as lutein, zeaxanthin, and β-carotene, vitamins E and C, and a variety of nonvitamin secondary metabolites (phytochemicals), which are thought to reduce the incidence of chronic diseases. Phytonutrients present in lettuce leaves have been recognized for their health benefits, including a lower incidence of cardiovascular and neurodegenerative diseases, cancer, and obesity.[10]

Metabolic syndrome (MS) is described as a series of anomalies associated with an increased level of visceral white adipose tissue. These disturbances include insulin resistance, hypertension, and dyslipidemia.[11] The prevalence of MS is not well-defined, as variations in criteria and diagnosis between organizations or authors are often observed. To induce the development of obesity and MS within an animal model, hypercaloric diets rich in carbohydrates or fats are utilized. These diets cause the development of obesity, hypertension, alterations in glucose homeostasis, dyslipidemia, and non-alcoholic fatty liver disease.[12]

Currently, romaine lettuce outer leaves are discarded in the field during the harvest period, keeping only the product's hearts for commercial trade. This waste implies significant food and economic loss. The discarded outer leaves also represent a disadvantage in terms of nitrogen management in agricultural

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soils. Therefore, our research team found that romaine lettuce outer leaves are an excellent opportunity for upcycling.[13]

In previous work by our group, we developed a romaine lettuce powder (RLP) from discarded outer leaves and evaluated its nutritional components. Interestingly, a high antioxidant capacity, high levels of fiber, and a high concentration of total soluble phenolics (TSP) were observed.[13] Given its nutritional and potential therapeutic benefits, RLP could contribute to a higher intake of natural antioxidants, thus contributing to improve the health of consumers.

Among the nutritional components of RLP, dietary fiber is one of the most predominant, and it is an important ingredient of a healthy diet. Evidence suggests that dietary fiber intake may reduce the risks of cardiovascular disease, type 2 diabetes mellitus, and some types of cancer. Dietary fiber has salutary effects on MS, including regulation of body weight, lowering lipids levels, improving glucose metabolism, and controlling blood pressure.[14] Polyphenols, at certain doses, may delay or prevent the onset of MS by lowering body weight, blood pressure, blood glucose, and improving abnormal lipid metabolism.[15] Specifically, lutein and its zeaxanthin isomer are potent free radical scavengers due to their polarity and conjugated double bonds, which delay the development of metabolic diseases by increasing mRNA expression of antioxidant enzymes and decreasing proinflammatory cytokines.[16] In addition, lutein and zeaxanthin are mainly transported by HDL particles, and research shows that they correlate with the size and concentration of HDL "good cholesterol".[16] Also, vitamin C has been suggested to be beneficial in reversing abnormalities associated with MS. Plasma vitamin C concentration was inversely associated with the body mass index (BMI), percentage of body fat, and waist circumference. Vitamin C supplementation resulted in significant decreases in blood glucose and is a potent antioxidant by acting as a reducing agent preventing oxidation of biomolecules.[17]

This study aimed to evaluate the MS preventive effect of a powdered byproduct of agricultural waste of romaine lettuce outer leaves in a murine model with MS induced by a high-calorie fat diet.

METHODS

Lettuce Powder Preparation. The outer leaves of the organic romaine lettuce vindicate cultivar were collected in the Salinas Valley in California, USA, in the summer of 2022. The whole lettuce outer leaves were arranged in a single layer on nine plastic mesh trays in a model 3900, 600 W cabinet dehydrator (Excalibur Products, Sacramento, CA), dried at 62.8 °C for 22 h, and then packaged in metalized bags flushed with N_2 to reduce oxidation during cold storage at 2 °C. Subsequently, they were ground using a Retsch ZM 200 ultracentrifugal mill (Retsch GmbH, Germany), for approximately 20 s to a particle size of 500 μm.[13]

Mice and Diets. The study protocol for the use of animals was approved by the Institutional Committee and Animal Use (Facultad de Ciencias de la Nutrición y Gastronomía, Universidad Autónoma de Sinaloa, Sinaloa, Mexico). Male C57BL/6N (CIRCULO ADN SA DE CV, Iztapalapa, CDMX) mice (15−21 g), aged 4−5 weeks, were housed in polycarbonate cages in a room with controlled temperature and humidity (20−22 °C, 60% RH) under a 12 h light−dark cycle. After 1 week of acclimation with the standard diet (LabDiet #5001, PMI International, Redwood, CA, USA), mice were assigned by weight range into treatment groups. The characteristics of the four different diets are shown in Table 1. The low-fat diet LFD was LabDiet #5001 (PMI International, Redwood, CA, USA); the high-fat diet HFD was LFD supplemented with lard (39.5% of total kcal); HFD5 was LFD supplemented with lard and 5% RLP; finally, the HFD10 was LF diet with added lard and 10% RLP.

Table 1. Nutritional Components of RLP

nutritional component of RLP	%
protein	16.08 ± 0.09
fat	5.74 ± 0.05
soluble fiber	6.9
insoluble fiber	27.9
total fiber	34.8
ash	6.46 ± 0.02
moisture	6.85 ± 0.15
carbohydrates (by difference)	54.86

Body Weight, Food Consumption, and Calories Intake Estimation. The body weights of the mice were recorded once a week with a precision electronic scale (BAPRE-3, Rhino, CDMX, México). To measure the body weight, the mice were positioned individually inside a metal basket placed on a tared scale, and the results were expressed in grams (g).

Food consumption was measured daily on a precision electronic scale (BAPRE-3). To calculate food consumption, the amount of food placed in the cage was recorded, and the leftover food was measured on the previously tared scale the next day. To determine the amount consumed, the initially given amount of food was subtracted from the amount of leftover food.

The diet calories were measured with the information provided by the manufacturer of each macronutrient, the amount of lard added to HFD diets, and the proximate analysis performed for lettuce powder (HFD5 and HFD10).

Romaine Lettuce Powder Proximate Analysis. Protein content was measured using an FP-628 TrueSpec N analyzer (Leco Corp., St. Joseph, MI, USA). The samples of RLP were analyzed, and the nitrogen content of the diet samples was multiplied by *a* factor of 6.25 to convert it into the protein content.

For fat analysis, stainless steel extraction cells were used, where 2 g of each mixture was mixed with Ottawa sand. Each measurement was performed in an accelerated solvent extractor (Dionex ASE 350, Thermo Scientific, MO, USA). Solvent extraction of each sample was performed in petroleum ether at 125 °C and 1.034 MPa pressure with nitrogen gas. Warming up to start the cycle took 1 min, followed by heating for 6 and 25 min of static time, and 120 s of purging. The wash volume was 60% of the volume of the extraction cell.

To measure the ash content, the sample was placed in a Lindberg/Blue M box furnace (Thermo Fisher Scientific) at 550 °C for 16 h. Before the final weighting, the crucibles containing the ash were carefully relocated in a desiccator to reach room temperature.

Soluble and insoluble dietary fiber content was measured by Medallion Laboratories (Medallion Laboratories, Minneapolis, MN, USA) based on the fiber method AOAC 991.43 with modifications.

Mice Blood and Organs Collection. After 12 weeks of feeding with the four different diets, the mice were prepared for euthanasia. Blood samples were collected by cardiac puncture in tubes. The serum was separated by centrifugation at 2500 rpm for 8 min and then stored at −80 °C in an ultralow temperature freezer (Forma 900 Series, Thermo Fisher, Waltham, MA, USA) until analysis. Abdominal adipose tissue and organs such as the liver, heart, and kidneys were removed from the mice to later be weighed.

Mice Blood and Organs Analysis. Glucose was measured once per week from blood obtained from the mice's tails using a glucose test strip (Accu-Chek Performa test strips, Roche, Basel, Switzerland) and blood glucometer (Accu-Chek Performa II, Roche, Basel, Switzerland). Plasma insulin concentrations were determined using a mouse ultrasensitive insulin ELISA kit (80-INSMS-E01, E10; Alpco, Salem, NH, USA), following the manufacturer's instructions. Abdominal adipose tissue and organs were weighed by a Sartorius TE64 Talent Analytical Balance (Sartorius, Göttingen, Germany) and a watch glass, the scale was tared with the watch glass previously, and the organs were weighed later and obtained results expressed in grams. Cholesterol

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analyses were realized with a cholesterol assay kit (ab65390; Abcam, Cambridge, UK), following the manufacturer's instructions to determine free cholesterol, total cholesterol, HDL cholesterol, and the LDL/VLDL fraction of lipoproteins.

Statistics. All data are presented as mean ± SEM. Statistical significance of differences was analyzed by one-way ANOVA, followed by post hoc Tukey multiple comparison tests, Student's *t* or Kruskal–Wallis *H* test using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA). Data were considered statistically different when $p < 0.05$.

RESULTS AND DISCUSSION

Proximate Analysis of RLP. The nutritional analysis of RLP revealed significant findings regarding its composition. RLP exhibited a protein content of 16.08 ± 0.09% and a fat content of 5.74 ± 0.05%. In terms of dietary fiber, RLP contained 6.9% soluble fiber and 27.9% insoluble fiber, contributing to the total fiber content of 34.8%. Additionally, RLP exhibited an ash content of 6.46 ± 0.02% and a moisture content of 6.85 ± 0.15%. The carbohydrate content in RLP, calculated by the difference, was determined to be 54.86%.

According to the American Heart Association, fiber can help to protect against heart disease, diabetes, diverticulitis, inflammatory bowel syndrome, obesity, and colorectal cancer. Fiber can help to flush toxins from the body, lower cholesterol, and promote weight loss because it helps people feel fuller while consuming fewer calories.[18]

Fiber can be classified as soluble or insoluble. Bacterial fermentation of soluble fiber releases short-chain fatty acids, which give energy to colonocytes and other benefits, including prevention of obesity-induced obesity diet, decreased storage of adipose tissue, and reduction of the glycemic response, leading to decreased hepatic cholesterol synthesis by insulin. In the case of the insoluble fiber, it is generally considered to be nonfermentable and therefore does not promote gut bacterial growth and its associated effects.[19,20]

Proximate Analysis of Mice Diets. LFD had the highest protein concentration (24.1%) compared with the other groups HFD (20.19%), HFD5 (20.02%), and HFD10 (19.85%). As expected, fat was lower in the LFD (5.1%) than in the HFD (20.5%), HFD5 (19.77%), and HFD10 (19.03%). HFD10 presented the highest concentration of total fiber (7.48%), followed by HFD5 (5.96%), LFD (5.3%), and finally HFD5 (4.44%). The ash content in each diet was LFD (7.2%), HFD (6.03%), HFD5 (6.55%), and HFD10 (7.07%). The moisture concentrations were highest in LFD (12%), then HFD5 (9.1%), later HFD (8.6%), and finally HFD10 (6.5%). For carbohydrates, HFD5 was (44.56%), HFD (44.68%), then HFD10 (47.55%), and with a highest concentration the LFD (51.6%) (Table 2).

Table 2. Estimated Nutritional Components of Diets

nutritional component	LFD (%)	HFD (%)	HFD5 (%)	HFD10 (%)
protein	24.1	20.19	20.02	19.85
fat	11.5	25.86	24.86	23.85
total fiber	5.3	4.44	5.96	7.48
ash	7.2	6.03	6.55	7.07
moisture	12	8.6	9.1	6.5
carbohydrates (by difference)	**51.6**	**44.68**	**44.56**	**47.55**
nitrogen-free extract (by difference)	46.3	40.24	38.6	40.07

Energy Estimation of Mice Diets. To determinate the total calorific value of the mice diet, we use the 4–9–4 method. This system applies energy conversion factors to the macronutrients carbohydrate, fat, protein, and fiber. The average values of energy are expressed as the number of calories per 1 g of the macronutrient. The Atwater general factor system includes energy values of 4 kcal per gram (kcal/g) (17 kJ/g) for protein, 4 kcal/g for carbohydrates, and 9 kcal/g (37 kJ/g) for fat.[21] HFD had the highest energy concentration (487.9 kcal/100 g of dry diet) compared to the other groups LFD (408 kcal/100 g of dry diet), HFD5 (480.19 kcal/100 g of dry diet), and HFD10 (472.58 kcal). The percentage energy from protein was 26.68, 18.31, 17.95, and 17.63% in LFD, HFD, HFD5, and HFD10, respectively. The percentage energy from fat was 16.19, 41.19, 42.11, and 40.13% in LFD, HFD, HFD5, and HFD10, respectively, and the percentage energy from carbohydrates was 57.13, 40.51, 34.94, and 42.24% in LFD, HFD, HFD5, and HFD10, respectively (Table 3).

Table 3. Estimated Nutritional Energy of Mice Diets

energy (kcal % derived from)	LFD	HFD	HFD5	HFD10
protein	26.68	18.31	17.95	17.63
fat	16.19	41.19	42.11	40.13
carbohydrates (by difference)	57.13	40.51	39.94	42.24
energy content (kcal/100 g of dry diet)	408	487.9	480.19	472.58

Food Intake and Body Weight. After 12 weeks of feeding of the four different diets, each group's body weight and food intake were measured. The mice fed with the LFD had the highest food consumption, compared with HFD, HFD5, and HFD10 groups ($p < 0.05$). The HFD-fed mice showed lower food consumption than LFD and HFD10 groups, but higher than the HFD5 group. HFD5 was the group with the lowest food consumption compared to the three other groups (Figure 1A,B). In caloric intake, the LFD group had higher caloric intake than the HFD and HFD5 groups ($p < 0.05$), as well as higher caloric intake than the HFD10 group. The HFD group reported the lowest caloric intake compared to the three remaining groups, and the HFD5 had a lower caloric intake than the HFD10 (Figure 1C,D). Interestingly, although the LFD group consumed more food and had a higher caloric intake, they were the group with the lowest weekly body weight gain throughout the study, showing a final weight gain of 25.8%. The HFD, HFD5, and HFD10 showed higher weekly body weight percentage gains, with a body weight gain of 34.5, 34.8, and 33.6%, respectively ($p < 0.05$) (Figure 1E). For the body weight increased in g, the results were similar to the weight gain in percentage. At the end of the study, the LFD group increased 7.3 g, the HFD group 10.5 g, the HFD5 group 10.1 g, and a final increase of 8.9 g was observed for the HFD5 group ($p < 0.05$) (Figure 1F).

In a similar study, Xu et al.[22] investigated the potential advantages of incorporating β-glucans (BG) and mulberry leaf extract (MLE) in the diet of mice fed with a high-fat diet (HFD). The authors did not observe any significant differences in food intake between the groups. However, when comparing weight gain, the HFD groups exhibited the highest increases in weight compared with the control group. Interestingly, the groups supplemented with BG and/or MLE had lower weight gains.[22] In another study that evaluated the metabolic benefits of a polyphenol-rich extract in diet-induced obese mice, researchers

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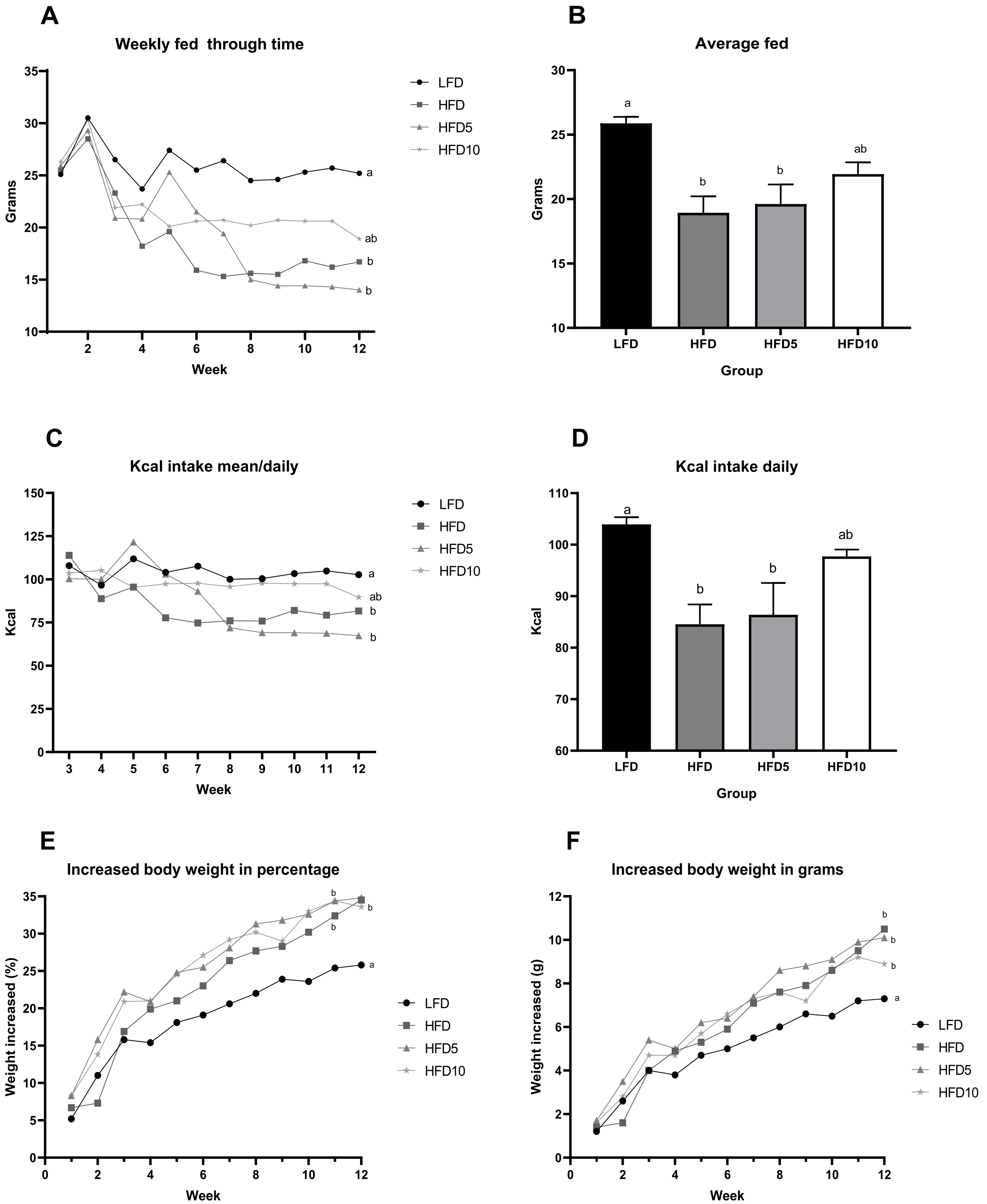

Figure 1. Analysis of the different diets on feed intake and increased body weight. (A,B) Weekly feeding of mice in grams during the duration of feeding. (C,D) Caloric intake daily expressed in kcal. Values are expressed as mean ± SEM. (E) Weekly body weight of mice in weight gain percentage during the duration of feeding. (F) Weekly body weight increased in g during the duration of feeding. LFD: low-fat diet; HFD, high-fat diet; HFD5, high-fat diet with 5% RLP; HFD10, high-fat diet with 10% RLP. Different subscripts denote a significant statistical difference $P < 0.05$.

observed less weight gain in mice fed a high-fat diet supplemented with the extract compared to the high-fat diet group. Similar to our study, the control group in their experiment had a higher average food intake.[23]

Various signals are produced during food consumption including sensory and hormonal signals. Nutrients like fiber and fats create high viscosity in the stomach and intestine leading to a reduction of appetite in the short term.[24,25] Peptide

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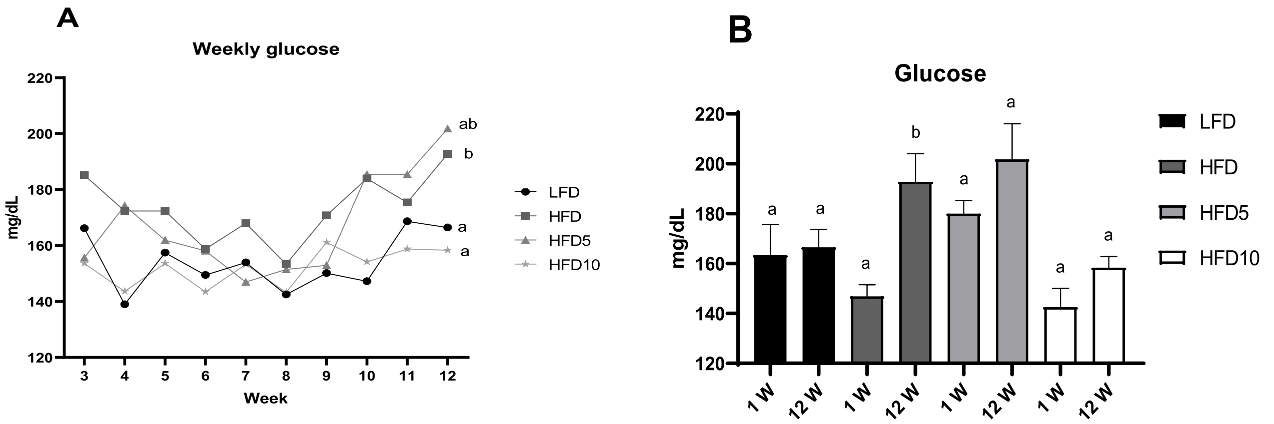

Figure 2. Comparison between diets on glycemic parameters. (A) Weekly capillary glucose of mice in mg/dL. LFD: low-fat diet; HFD, high-fat diet; HFD5, high-fat diet with 5% romaine lettuce outer leaves powder; HFD10, high-fat diet with 10% romaine lettuce outer leaves powder. (B) Initial glucose vs final capillary glucose in mg/dL. LFD: low-fat diet; HFD, high-fat diet; HFD5, high-fat diet with 5% RLP; HFD10, high-fat diet with 10% romaine lettuce outer leaves powder. Values are mean ± SEM, Different subscripts denote a significant statistical difference $P < 0.05$.

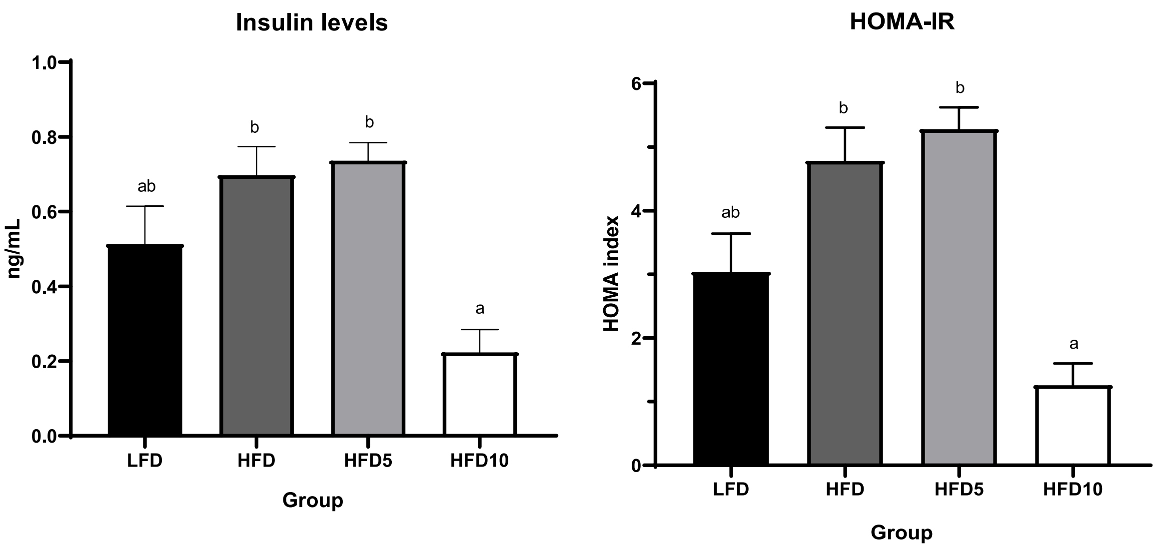

Figure 3. RLP improves insulin sensitivity in mice with MS. (A) Insulin levels in ng/mL. LFD: low-fat diet; HFD, high-fat diet; HFD5, high-fat diet with 5% RLP; HFD10, high-fat diet with 10% RLP. (B) HOMA index to determine insulin resistance in ng/mL. Values are mean ± SEM, different subscripts denote a significant statistical difference $P < 0.05$.

YY (PYY) and cholecystokinin (CCK) are gut anorexigenic hormones that stimulate gall bladder contraction and pancreatic exocrine secretion, thus regulating gastric emptying and intestinal transit and inducing satiety. The most potent stimuli for the secretion of PYY are lipids; a lipid-rich meal results in a significant and more sustained elevation in PYY than glucose and proteins.[26−28] Despite not having evaluated the satiety hormone levels, in our study, food and energy intake were lower in the HFD mice with or without RLP in their diets, and this effect might be due to the satiating effect of fiber and fat in the diet.

Glucose Analysis. At the end of the study, the LFD group's serum glucose level was lower than the HFD groups ($p < 0.05$). The HFD group showed similar serum glucose levels to HFD5 and higher than the LFD and HFD10 groups ($p < 0.05$). Interestingly, the serum glucose level of HFD10 was lower than the rest of the groups ($p < 0.05$). These results indicate a positive effect of RLP in serum glucose levels when it is incorporated into the diet at a 10% concentration (Figure 2A).

In addition, initial and final glucose samples were compared, where the LFD group showed similar glucose concentration at the end than at the beginning. The HFD group had a higher glucose level at the end compared with the beginning of the study ($P < 0.05$). In the case of the HFD5 group, it showed similar glucose levels at the beginning than at the final of the study. HFD10 was the group with less concentration of glucose levels at the final, compared with the other three groups, and showed similar levels in the initial and final glucose levels, which further confirms the protective effect of RLP in the increase of blood glucose levels. It was interesting to note that this protective effect was observed at the beginning and end of the study (Figure 2B).

Cheng et al.[29] showed similar results. They observed that supplementation with 300 mg/kg of a polyphenol rich Rutgers Scarlet Lettuce extract lowered and improved hyperglycemia in high-fat diet mice vs the control group. Other powders and extracts from leaves and plants rich in polyphenols have shown antidiabetic effects, several mechanisms may be involved, however, one of those observed is the α-amylase and α-

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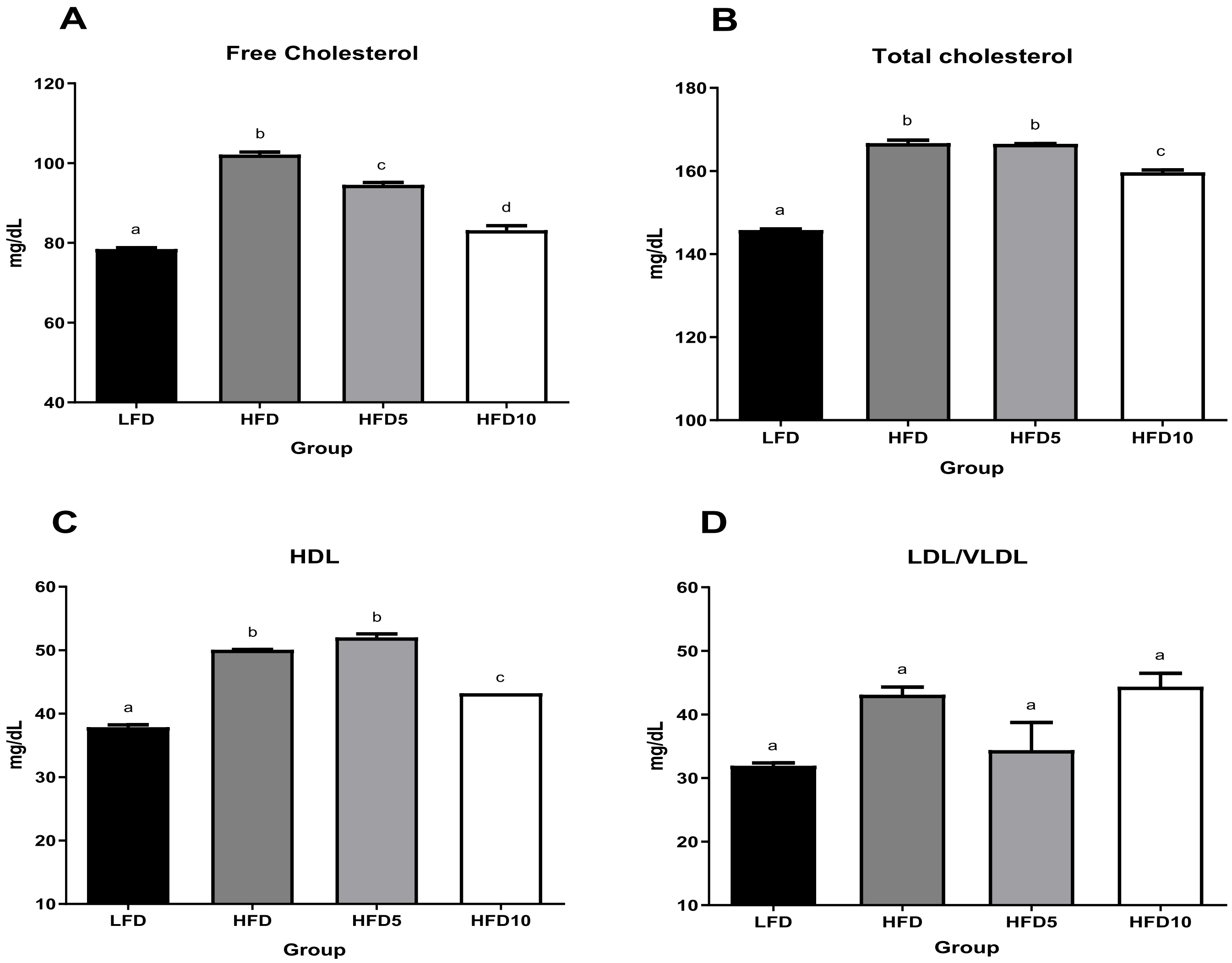

Figure 4. Analysis of the diet's effect on lipidic parameters. (A) Free cholesterol expressed in mg/dL in mice fed with low fat diet (LF), high fat diet (HF), HF diet and RLP 5% (HF5) and HF diet and RLP 10% (HF10). (B) Total cholesterol represented in mg/dL. Low fat diet (LF), high fat diet (HF), HF diet and RLP 5% (HF5), and HF diet and RLP 10% (HF10). (C) HDL cholesterol in mg/dL. Low fat diet (LF), high fat diet (HF), HF diet and RLP 5% (HF5), and HF diet and RLP 10% (HF10). (D) LDL/VLDL cholesterol expressed in mg/dL. Low fat diet (LF), high fat diet (HF), HF diet and RLP 5% (HF5), and HF diet and RLP 10% (HF10). Values are mean ± SEM, different subscripts denote a significant statistical difference $P < 0.05$.

glucosidase inhibitory activity of different fractions of phenolic compounds.[30,31]

Insulin Analysis and HOMA-IR Index. The LFD group showed lower, but not significant, insulin levels than HFD and HDF5 ($p > 0.05$). Interestingly, the LFD group showed higher levels of insulin than HFD10, suggesting a positive effect of the lettuce powder in insulin signaling. The HFD group had similar levels to the HFD5 group and increased levels than the HFD10 ($p < 0.05$). Similarly, the HDF5 group showed higher insulin levels than the HFD10 ($p < 0.05$) (Figure 3A). The results of de HOMA index were the same as those found in the insulin level, which indicates insulin resistance according to the HOMA index in HDF and HFD5 groups, which presented a HOMA index of 4.8 and 5.2, respectively. HFD and HFD5 groups had a significantly higher HOMA index than the HFD10 group ($p < 0.05$) (Figure 3B). Finally, the LFD group and the HFD10 groups had no statistical differences in the HOMA index with a HOMA index of 2.9 and 1.4, respectively, suggesting that the RLP at a concentration of 10% can prevent the effect of obesity on insulin signaling.

Within the dietary causes of MS, a high intake of carbohydrates with a high glycemic index (GI) is associated with insulin resistance and has an impact on the development of diabetes mellitus (DM). Therefore, low GI diets, more abundant in fiber, are recommended since they increase satiety, decrease insulin resistance and the risk of developing DM, as well as a healthy food with limited trans fats, saturated fats, sugar additives, and sodium.[32]

In a similar study, Hwang et al. evaluated the antidiabetic effects of *Nelumbinis semen* (NS) powder (5 and 10% of diet) in HFD-induced obese C57BL/6 mice. Results showed that there were no significant differences among all four groups at week 0, but the blood glucose levels for HFD-NS5% and HFD-NS10% remained lower compared with HFD.[33] In addition, Jeong et al. showed that the intake of noodles containing fermented lettuce extract decreased the glucose level and insulin resistance in mice with diabetes induced by intraperitoneal injection of streptozotocin.[34]

Similar results were observed in our study, where HFD10 had a significantly lower insulin level and HOMA-IR index,

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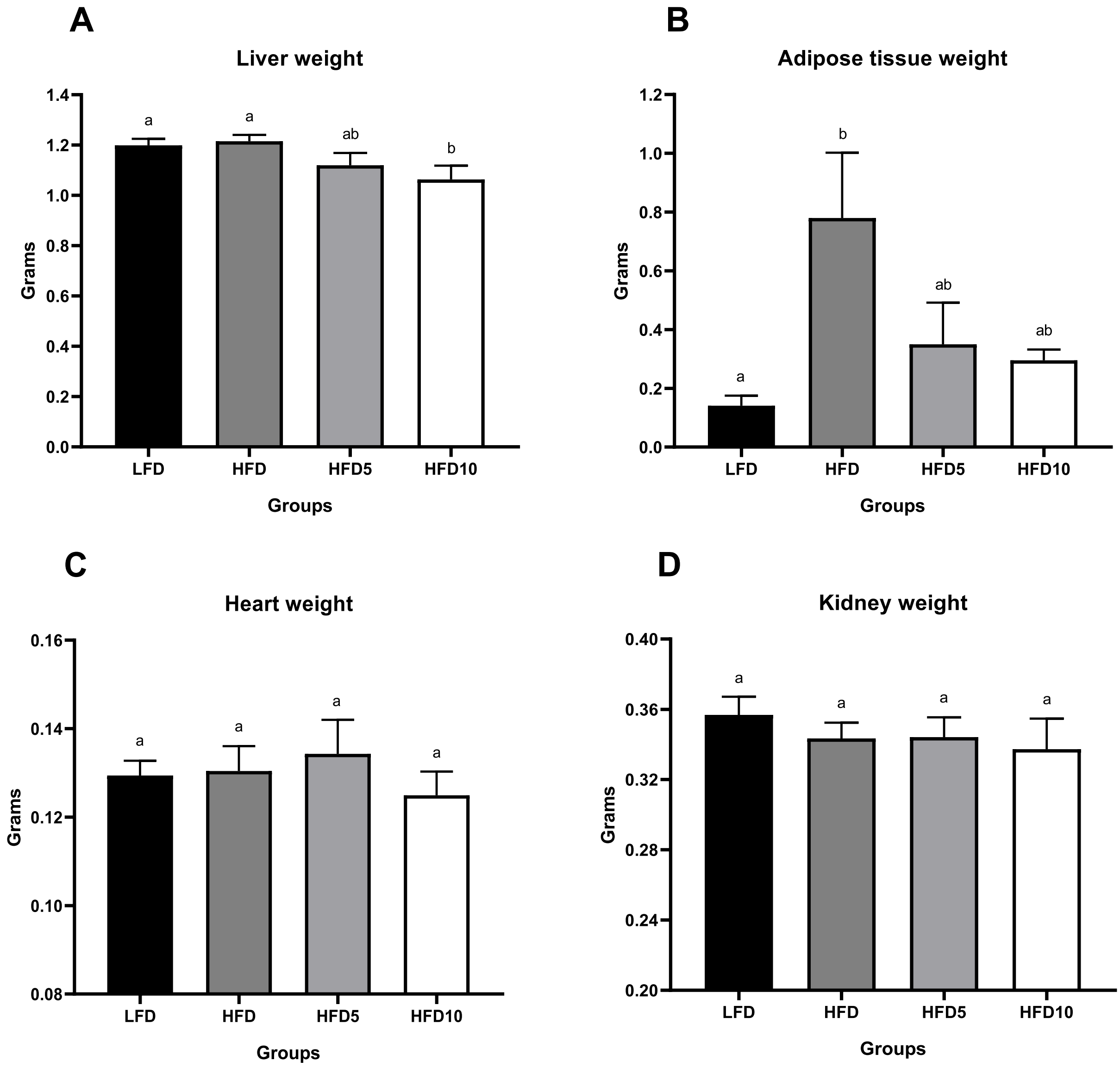

Figure 5. Effects in the weight of the organs with different diets on the MS model: (A) liver weight in grams. (B) Adipose tissue weight in grams. (C) Heart weight in grams. (D) Kidney weight in grams. LFD: low-fat diet; HFD, high-fat diet; HFD5, high-fat diet with 5% RLP; HFD10, high-fat diet with 10% RLP. Values are mean ± SEM, different subscripts denote a significant statistical difference $P < 0.05$.

suggesting that the 10% concentration of RLP had a powerful effect on insulin signaling. It is possible that this effect is caused by the content of fiber and polyphenols contained in the different varieties of lettuce as demonstrated by the study of Cheng et al.[29]

In vitro studies have shown inhibitory effects on the digestive enzymes α amylase and α-glucosidases by polyphenols present in plants, fruits, or leaves as well as inhibition of glucose transporters (SGLT1 and GLUT2) may also contribute to the marked effect on postprandial glucose and insulin response observed.[35,36] In our study, the HFD10 showed similar serum insulin levels that LFD but lower than other HFD groups, Han et al. observed that purple lettuce consumption increased insulin sensitivity in diet-induced obese (DIO) mice,[37] this and our results suggest that the intake green leafy vegetables like lettuces attenuate metabolic disorders in DIO or T2DM mice.

Free and Total Cholesterol and HDL and VLDL/LDL Levels. The LFD group showed lower free cholesterol than the other groups ($p < 0.05$). The HFD group had a higher free cholesterol level than both groups fed with RLP (HF5 and HF10) ($p < 0.05$). The HFD5 group showed increased levels of free cholesterol compared to the HFD10 group ($p < 0.05$) (Figure 4A). These results clearly show an effect of RLP in free cholesterol levels in serum, as the more RLP was added to the diet, the less free cholesterol found in the sera analyzed.

As expected, the group fed with an LFD diet had a lower concentration of total cholesterol than the HFD, HFD5, and HFD10 groups ($p < 0.05$). Interestingly, the HFD group had similar levels of total cholesterol as the HFD5 group and higher level than the HFD10 group ($p < 0.05$). Similarly, the HDF5 group showed higher total cholesterol levels than the HFD10 group ($p < 0.05$) (Figure 4B).

The LFD group showed lower HDL levels than the other three groups ($p < 0.05$). In the HFD group, similar levels of HDL cholesterol as the HFD5 group were observed ($p > 0.05$). Contrary, higher HDL levels were observed in the HFD group than in the LDF and HFD10 groups ($p < 0.05$). The HFD10

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group had a higher level of HDL than the LFD but lower than HFD and HFD5 groups ($p < 0.05$) (Figure 4C).

In the LDL/VLDL fraction of lipoproteins analysis, no statistical differences were found among the groups analyzed ($p > 0.05$). However, a lower concentration in the LFD group than in the HFD and HFD10 groups was found. The HFD had a little lower LDL/VLDL levels than the HFD10 and higher than LFD and HFD5 groups. The HFD5 group was lower than the HFD10 group but with no statistical differences ($p > 0.05$) (Figure 4D).

In serum lipids, the supplementation with 10% of RLP inhibited LDL levels compared with the supplementation with 5% of RLP. With other lipid fractions such as HDL and total cholesterol levels, the RLP showed a tendency to improve blood lipid concentrations. In a similar study, Lin et al. in 2016 evaluated the antihyperlipidemic activity of Allium Chinese bulbs, and they demonstrated a positive effect on the serum lipid level. Same as our study, they observed changes in total cholesterol levels, but unlike us, they showed lower serum concentrations LDL cholesterol; finally, they also showed lower levels of triglycerides.[38] Iftikhar et al. evaluated the effects of rich polyphenols star anis tea in HFD-sugar induced obesity rats, and they observed that the cholesterol levels were lower in the treatment groups.[39] Furthermore, Mbouche Fanmoe et al. in 2021 observed that *Ipomea batatas* Leaf powder reduced serum total cholesterol, LDL-cholesterol, and triglycerides in rats fed a high-fat diet, and this effect was attributed to the high content of polyphenols in the powder.[40]

Liver, Adipose, Heart, and Kidney Weights. The LFD group had a similar liver weight to the HFD and HFD5 groups ($p > 0.05$), but interestingly, it had a higher liver weight than the HFD10 group ($p < 0.05$). The HFD group showed an increase in liver weight compared to HFD10 ($p < 0.05$). The HFD5 and the HFD10 groups had similar levels of liver weight ($p > 0.05$). It was interesting to note that the groups with diets that were supplemented with RLP had a lower liver weight than the LFD group and HFD group, which suggested a positive effect of the RLP on liver fat (Figure 5). As expected, the LFD group had a lower weight of adipose tissue compared to the HFD group ($p < 0.05$), despite being the group with the highest food consumption. The increase in adipose tissue weight was more evident in the HFD group compared with the rest of the groups. The HFD5 had a slightly nonsignificant increase in adipose tissue compared to the LFD group. Finally, the HFD10 inhibited the adipose tissue increment, as it presented lower levels of adipose tissue weight than the HFD group and similar levels to the LFD group. These results suggest that powdered lettuce may, to some extent, inhibit the generation of adipose tissue despite consuming a high-fat diet (Figure 5). The heart weight was similar in the three groups ($p > 0.05$), but the HFD10 group showed a slightly little lower heart weight than HFD and HFD5 groups (Figure 5). Finally, no statistical differences were observed in kidney weight ($p > 0.05$), although the HFD10 group was the group with the lowest kidney weight (Figure 5).

Another important observation in our study is the ability of supplementation with 10% RLP to attenuate weight gain in organs such as the liver, heart, and kidney. Alzahrani et al. observed similar results with the supplementation of Pearl millet grains and their ethanol extract, they showed that rats fed HFD supplemented with grains or extract have lower liver and adipose tissue weights. The significant increase in body weights and visceral fat observed demonstrates the role of the HFD in promoting obesity in rats. Also, the administration of both the powder and ethanoic extract of de Pearl millet grains attenuated the increase in final body and white adipose tissue (WAT) weights in HFD-fed rats, suggesting having a potent antiobesity effect. This result might be occurring by inhibiting adipocyte proliferation, increasing fat cell death, and impairing triglyceride absorption by the inhibition of pancreatic lipase production[41]

This effect could be attributed to the content of polyphenols in the lettuce powder, pure polyphenol compounds, and polyphenol-rich extracts that have been tested in in vitro models of steatosis. Most studies are concordant with the fact that a range of polyphenols reduces hepatocellular lipid accumulation by the inhibition of lipogenesis and promotion of fatty acids catabolism.[42,43]

C57BL/6 becomes obese, hyperglycemic, insulin-resistant, and susceptible to liver steatosis when fed a high-fat diet.[44,45] In this study, the C57BL/6N strain, subjected to high dietary fat for 12 weeks, showed increased efficiency of fat storage and manifested insulin resistance and liver steatosis. Interestingly, supplementation with 10% RLP improved HDF-induced obesity, metabolic disorders, and liver steatosis. These results were consistent with other studies in chronic HFD-fed rodent models.[24,44,46]

Regarding the effect of a high-fat diet on the kidney, Sánchez-Navarro et al. showed that a high-fat diet does not have an effect on the kidney weight; however, the ratio of kidney/body weight (KW/BW) was reduced.[47] However, our study did not show differences in the kidney weight.

Moreover, in a study with Wistar−Kyoto rats fed a high-fat diet, Amirullah et al. showed that the supplementation with ultrasound extract of *Pleurotus pulmonarius* decreased triglyceride and LDL cholesterol levels; however, they did not observe differences in the heart weight.[48]

Several studies have demonstrated that HFD diets favor the development of obesity and, especially, the increase in WAT. WAT is an important initiator of the inflammatory response to obesity and therefore of insulin resistance.[49−51] In our study, the groups supplemented with lettuce powder had a lower caloric intake and greater body weight gain. However, this body weight gain was not at the expense of a body fat increase. According to the five compartments model of Drinkwater and Kerr, the histological or tissue level, consists of various elements, including skeletal muscle, nonskeletal muscle, soft tissue, adipose tissue, and bone.[52] However, in our study, we focused especially on evaluating abdominal adipose tissue. Based on our findings, we can infer that the difference in weight between groups may be attributed to alterations in other tissues, particularly muscle tissue and body water. It was interesting to note that these groups supplemented with RLP had lower adipose tissue gain and better carbohydrate metabolism reflected in lower HOMA IR levels despite the high-fat diet, suggesting that lettuce powder supplementation confers a protective effect against obesity and therefore insulin resistance prior to the development of type two diabetes mellitus.

In conclusion, our work investigated the potential health benefits of RLP made from the discarded outer leaves of lettuce. The results showed that RLP has a preventive effect on MS by regulating body weight, lowering lipid levels, and improving glucose metabolism. RLP is a rich source of soluble and insoluble dietary fiber, antioxidants, and other nutrients such carotenoids (lutein, zeaxanthin), β-carotene, vitamins E and C, proteins, and sugars that contribute to the improvement of consumer health. The study highlights the opportunities for upcycling agricultural waste to produce value-added products that promote a healthy diet and reduce food waste.

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García-Rocha, K. F. collected test data and drafted the manuscript; Capaceta-Osuna, A. collected and analyzed test data; Ochoa Acosta, collected test data and drafted the manuscript; Avena-Bustillos R. J. collected and processed romaine lettuce outer leaves, designed the study, interpreted results, and directed the drafting of the manuscript; Osuna-Martinez, U. designed the study and interpreted results; Cardenas-Torres, interpreted results and analyzed test data; Yokoyama W., advised on mice feeding and testing for this study; McHugh T. H. provided support for lettuce collection and processing; E. Teran-Cabanillas designed the study, interpreted results, and directed the drafting of the manuscript.

Notes

The authors declare no competing financial interest.

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