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Dietary Fiber and Dietary Protein of Lemongrass (*Cymbopogon Citratus* Stapf.) as Potential Functional Food in Risk Prevention for Diabetes Mellitus and Cardiovascular Disease: an Animal Model

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Abstract

This study determined the glucose-lowering and cholesterol-lowering effects of large molecules, dietary fiber (DF) and protein, of lemongrass in an animal model using completely randomized design. Total DF and protein were extracted; dietary protein (DP) was further digested to obtain protein hydrolyzates (PH). Sprague-Dawley rats were initially fed with high sugar, high fat and high cholesterol diet for two weeks, then administered with total DF, DP, PH, and a combination of DF and DP for another two weeks while maintained with the diet. Blood samples were obtained for determination of fasting blood sugar (FBS), total cholesterol, HDL and LDL+VLDL levels, and the differences before and after treatments were compared. There was a total of six treatment groups, including Untreated and Acarbose+Pravastatin treatment, which served as controls. Administration of DF, DP, PH, and DF+DP resulted to lower increase of FBS in comparison with control groups. However, PH treatment led to the greatest decrease in total cholesterol levels among the treatments. HDL cholesterol levels were not affected by the treatments. The rise in LDL+VLDL cholesterol levels was least in rats treated with DP, but the group treated with PH did not increase. Thus among the treatments, protein hydrolyzates exerted the most effective glucose- and cholesterol-lowering effects in rats fed with high sugar, high

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fat, and high cholesterol diet. Furthermore, the treatments appeared to have reduced the extent of injury on liver and kidney cells caused by the diet. This study supports the potential of lemongrass as a functional food in mitigating the risk of diabetes mellitus and cardiovascular disease (CVD) through prevention of hyperglycemia and hypercholesterolemia.

Abbreviations

AP – acarbose + pravastatin
BW – body weight
DF – dietary fiber
DP – dietary protein
DPP IV – dipeptidyl peptidase IV
CVD – cardiovascular disease
IACUC - Institutional Animal Care and Use Committee
FBS – fasting blood sugar
FNRI-DOST – Food and Nutrition Research Institute-Department of Science and Technology
GI – gastrointestinal
HDL – high-density lipoprotein
LDL – low-density lipoprotein
TC – total cholesterol
PH – protein hydrolysates
VLDL – very low-density lipoprotein

Introduction

Diabetes mellitus (DM) and CVD are among the chronic diseases that have affected millions of people worldwide with their prevalence being much greater in low- and middle-income countries, due to limited access to quality healthcare. In the Philippines, ischaemic heart diseases, cerebrovascular diseases, and diabetes mellitus were the first, third, and fourth leading cause of deaths, respectively, recorded for 2022 and 2023.[1]

Diabetes mellitus is characterized by hyperglycemic blood levels because of impaired production of insulin (Type 1), or impaired use of insulin (Type 2). Type 2 DM is more prevalent, comprising 95% of people with diabetes; it develops undetected through the years in overweight adults with less physical activity, and is increasing in children as well.[2] Type 2 DM is an important risk factor for CVD, and both chronic diseases are closely linked with each other. Insulin resistance in type 2 diabetics leads to dyslipidemia, with elevated levels of fatty acids, triglycerides, LDL and VLDL cholesterol circulating in the bloodstream. Furthermore, elevated levels of glucose in the blood promote the formation of advanced glycated end products, which lead to plaque formation in the blood vessels. Conventional treatment for patients with type 2 DM and CVD include drug therapy to lower cholesterol levels and improve glycemic control, treatment of plant phytochemicals with antioxidant activity, and lifestyle management.[3]

Dietary fiber is a group of carbohydrates which are non-digestible by the human gut but are associated with health benefits. These include cellulose, fructans, galacto-oligoasaccharides, pectin, and resistant starch. Intake of dietary fiber has been demonstrated to reduce the risk of diabetes and CVD, and is strongly correlated with weigh loss. DF's ability to protect against diabetes, obesity, and CVD is attributed to its capacity in modulating the expression of key enzymes involved in carbohydrate and lipid metabolism. Expression of synthesis enzymes HMG-CoA reductase, fatty acid synthase, and acetyl-CoA carboxylase are inhibited in animal models fed with DF.[4]

Dietary proteins provide health benefits aside from their nutritional role. Proteins from plant sources have gained increasing interest in the past years as they are cheaper and readily available compared to those from animal sources.[5] Plant proteins are considered more beneficial than animal proteins in risk prevention for CVD lipid markers.[6] Proteins encrypt bioactive peptides within their native structure, and such peptides are released during gastrointestinal digestion, food processing, or by the action of other proteolytic enzymes. These peptides were documented having over 44 different biological potentials, with inhibition of angiotensin-I converting enzyme being the most studied. Antioxidant, anti-inflammatory, antimicrobial, and anti-diabetic through DPP IV inhibition, are among the other widely studied bioactivities of the potential peptides.[7]

Lemongrass (*Cymbopogon citratus* Stapf.) from the Poaceae family is a traditional aromatic herb

used in many countries worldwide. It has often been consumed as an herbal tea prepared by decoction or infusion for treatment of kidney and digestive problems, as well as diabetes and hypertension.[8] In the past years, studies on the health-promoting benefits of lemongrass have focused on bioactivity-guided isolation of phytochemicals,[9-10] as well as characterization and bioactivity of the essential oil.[11] More recently, interest has shifted to investigation of the larger molecules of lemongrass. Hot water-soluble polysaccharides have been extracted from lemongrass and shown to possess free-radical scavenging activity and anti-cancer activity.[12] Polysaccharides were also shown to exert anti-tumor and immunomodulatory effects in tumor-bearing mice.[13] Dietary fiber from lemongrass was demonstrated to have antihyperglycemic and cholesterol-lowering potential,[14] while protein hydrolyzates were shown to exert cholesterol-lowering activity.[15]

In the Philippines, lemongrass is abundant and grows in various places, marginalized areas or in backyards. However, there are very few functional food products and nutraceuticals developed out of lemongrass despite the accumulation of scientific evidence on the various bioactivities and phytochemicals of lemongrass. Lemongrass, as a potential functional food ingredient, has been underutilized for the many health benefits it can offer, especially in a developing country where access to quality healthcare is limited. Since the bulk of available literature on lemongrass is focused on the small molecules, this study was conducted to demonstrate the potential of the biomolecules of lemongrass to help mitigate the development of risk of type 2 DM and CVD in an animal model. These biomolecules, dietary fiber and proteins, are present in greater amounts than the small phytochemicals, and is expected to promote the development of functional foods and nutraceuticals from lemongrass in the Philippines in risk prevention for DM and CVD.

Materials and Methods

Collection of Plant Material

Lemongrass plants were collected from Brgy. Baong, in Alimodian Iloilo, Philippines, washed, and air-dried. Samples were authenticated and submitted to the UST herbarium (Certificate Acc. No. USTH 014150). Dietary fiber analysis and extraction utilized freeze-dried plant samples, while dietary protein extraction made use of oven-dried plant materials at 50 – 60 °C, until moisture content is 5 – 6 %. The dried samples were ground to a fine powder.

Analysis and Extraction of Total DF

Total dietary fiber was analyzed in duplicates using AOAC Official Method 985.29 and 991.43.[16] The same method was also used to extract total DF. Briefly, the sample was added with MES-TRIS buffer, and sequentially digested with heat stable amylase, then protease, and finally amyloglucosidase, and TDF was precipitated from the enzyme digest using ethanol.

Extraction and Digestion of Total DP

Proteins were extracted and digested based on our previous work.[15] Briefly, dried lemongrass powder was defatted and proteins were extracted through alkali solubilization and acid precipitation method. Tannins were removed by binding the protein extract with polyamide. The resulting tannin-free protein extract was digested *in vitro*, simulating gastrointestinal conditions.

Animal Manipulation Protocols and Treatments

The protocol was carried out as approved by the UST IACUC with Animal Research Permit Ref. No. AR2018-061 issued by the Bureau of Animal Industry, and conducted at the animal facility of the Thomas Aquinas Research Center, UST. The experiment was carried out using completely randomized design. Thirty Sprague-Dawley rats of mixed sexes, about six to eight weeks old, and weighing 150 – 180 g, were obtained from an accredited breeder in good health condition, free of pests, parasites, and diseases. The day after arrival, the animals were bathed with mild liquid soap and water, and immediately dried with clean towels to further ensure that they are pest-free. They were allowed to acclimatize for one week and were housed in stainless steel cages lined with wood shavings as beddings. The rats were exposed in a well-ventilated room with a daily cycle of 12 h light and 12 h dark, room temperature of 22-25°C and humidity of 55 - 60%. The males were kept in separate cages from the females. Standard rat pellets were fed to the animals *ad libitum*, and free access to purified drinking water was allowed. Cleaning of cages was done twice in a week, by scrubbing with mild detergent solution containing 0.1% hypochlorite, and flushing thoroughly with

water. Feeding cups and water bottles were replaced every other day; water bottles were cleaned by washing with mild detergent solution containing 0.1% hypochlorite solution and rinsing thoroughly with water. When the rats have acclimatized, the diet was modified *ad libitum* to a high cholesterol, high sugar diet consisting of 60% standard pellets, 15% lard, 10% egg yolk powder, and 15% sucrose in the next four weeks. After the first two weeks of the modified diet, the rats were fasted overnight, and blood samples were drawn from the tail vein for analyses of total, HDL and LDL+VLDL cholesterol.

The rats were grouped into six groups of five animals each, consisting of a mix of male and female animals, and age-matched per group (Figure 1). The treatments and dose were as follows:

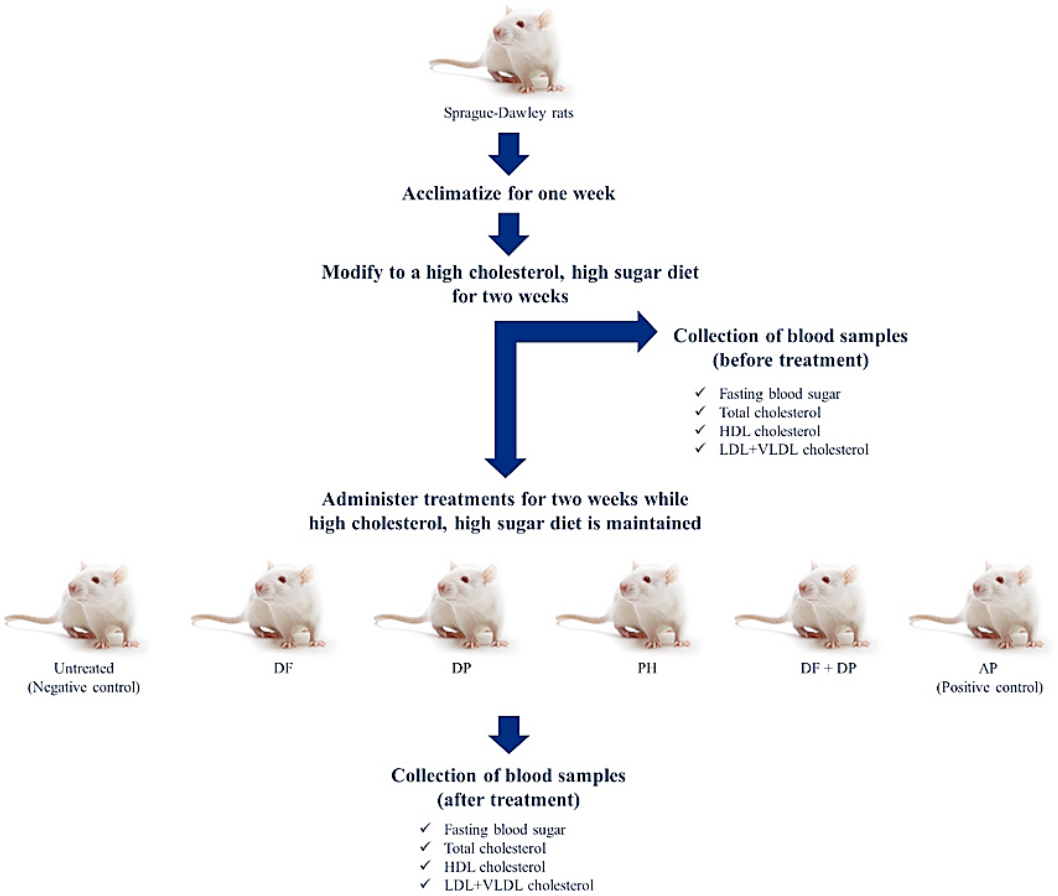

Fig. 1. Flowchart for animal treatments

- Group 1 – untreated (negative control) group, which received saline only.
- Group 2 – total dietary fiber (DF group), at 400 mg/kg BW, following the recommended daily intake of the FNRI-DOST Philippines.[17]
- Group 3 – dietary protein (DP group), at a dose of 0.07 mg/kg BW, which is safe for the renal parameters and blood pressure of human subjects.[18-19]
- Group 4 – protein hydrolysate (PH group), at 0.05 mg/kg BW based on digestion yield of 74%.[15]
- Group 5 – combination of dietary fiber and dietary protein (DF+DP group), administered separately at the same doses as individual groups.
- Group 6 – combination of acarbose and pravastatin (AP group; positive control), recommended at 40 mg/kg BW and 10 mg/kg BW, respectively.[20-21]

The treatments were administered via oral gavage in 0.5% saline vehicle for two weeks on a dily basis, while the animals were maintained on the high cholesterol, high sugar diet. The body weight of the animals were monitored every 4 days for dosing of treatments. Only saline was given to the untreated group. At the end of the treatment, the rats were fasted overnight and anesthesized with 0.1 mg/kg Zoletil. The collection of blood samples was carried out using intracardiac puncture, and the animals were sacrificed by Zoletil overdose. All blood samples were submitted for analysis of total, HDL, and LDL+VLDL cholesterol, using the standard CHOD-PAP method for TC and direct measure-PEG for HDL while LDL+VLDL was computed as the difference between TC and HDL.[22] The results obtained were within the range values for Sprague-Dawley rats.[23] Representative liver and kidney organs per group were immersed in 10% buffered formalin, submitted to routine histopathological examination, and interpreted by a histopathologist.

Statistical Analysis

One-way ANOVA and Duncan's multiple range test was employed to determine differences between treatment groups using an SPSS Software (IBM SPSS Statistics).

Table 1. Yield of dietary fiber, protein and protein hydrolyzates from lemongrass

	g per 100 g dry weight ± SEM
total dietary fiber	65.68 ± 0.26
total dietary protein	0.033±0.007
protein hydrolyzates	0.025±0.003

Results

Table 1 shows the yield of total dietary fiber, protein, and protein hydrolyzates. The yield of TDF is high, which is more than 50% of the plant dry weight. However, total dietary protein yield is very low at 0.033 g per 100 g dry weight, while protein hydrolyzates yield is even lower at 0.025 g.

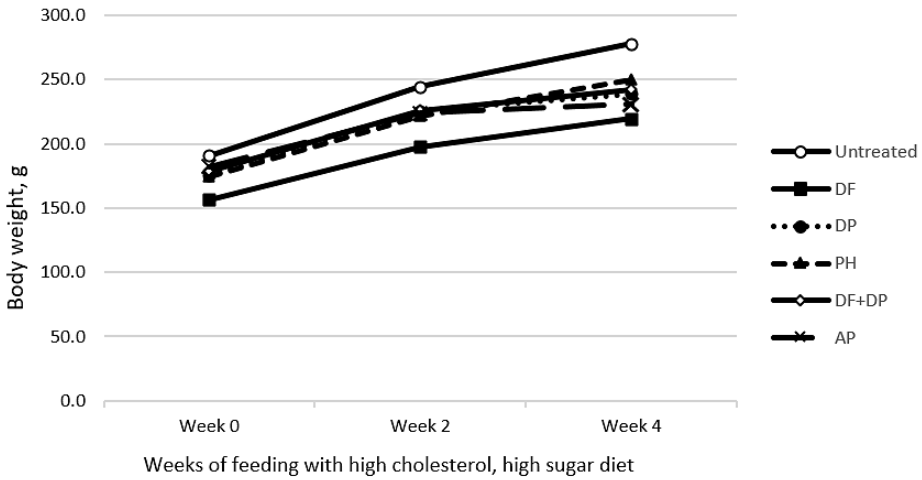

Fig. 2. Body weight of rats for the duration of the high cholesterol, high sugar diet

Figure 2 shows the body weight of the rats according to the corresponding treatment. The body weights of the rats increased gradually throughout the course of the experiment.

Figure 3 shows elevated levels of fasting blood glucose as a result of the high cholesterol, high sugar diet fed to rats. The untreated group had the highest increase, and also the AP group. The group treated

with DF, protein hydrolyzates, and a combination of DF+DP were significantly lower than the untreated group. Rats treated with dietary protein had the lowest increase in FBS.

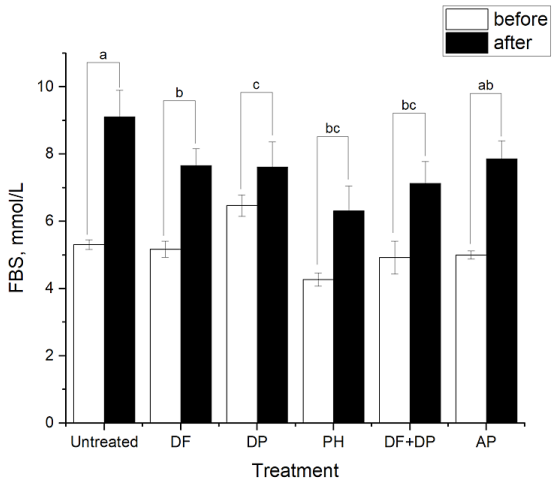

Fig. 3. Influence of dietary fiber (DF), dietary protein (DP), protein hydrolyzates (PH), and combination of dietary fiber and protein (DF+DP) on FBS of rats. Connecting bars refer to an increase in FBS levels before and after treatment. a–cMeans with different letters were significantly different (p < 0.5).

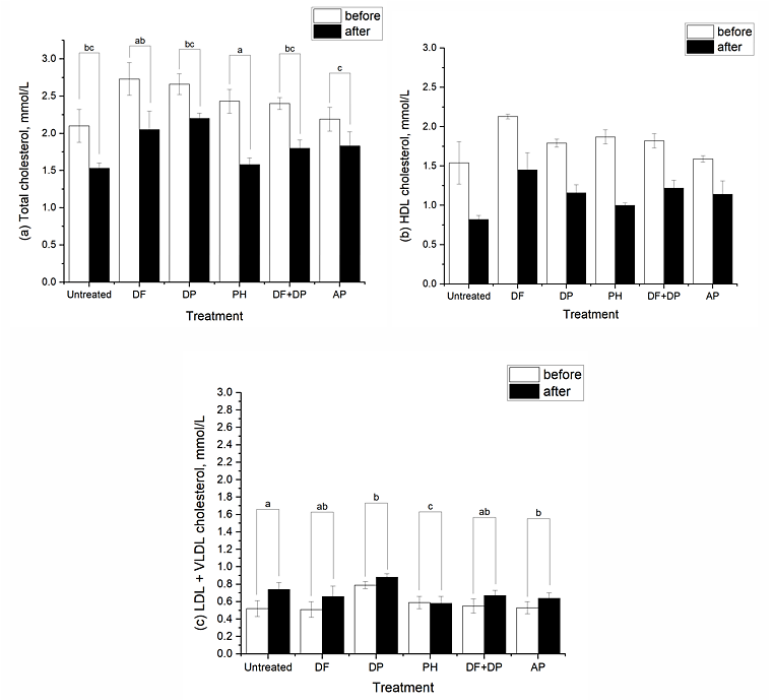

Fig. 4. Influence of dietary fiber (DF), dietary protein (DP), protein hydrolyzates (PH), and combination of dietary fiber and protein (DF+DP) on the serum (a) total cholesterol levels; (b) HDL cholesterol; and (c) LDL+VLDL cholesterol of rats. Connecting bars refer to a decrease in total and HDL levels, and an increase in LDL+VLDL levels, before and after treatement. a–cMeans with different letters were significantly different (p < 0.5)

On the other hand, the high cholesterol, high sugar diet fed to rats resulted to decreased levels of serum cholesterol levels (Figure 4). The total cholesterol of the PH group had the highest decrease, as well as DF group. However, the decrease in TC was the same for the groups DF, DF+DP, DP, and untreated. The group treated with acarbose and pravastatin had the lowest decrease in cholesterol. This decrease may be due to the lowering of HDL cholesterol. Levels of LDL+VLDL cholesterol increased, with the untreated group having the highest increase, which was the same in DF and DF+DP groups. AP and DP groups had lower increase compared to the untreated group. The group treated with PH did not experience any increase in LDL+VLDL cholesterol. Thus the protein hydrolyzates from lemongrass exerts cholesterol-lowering effects by lowering HDL-cholesterol but preventing the increase of LDL+VLDL cholesterol.

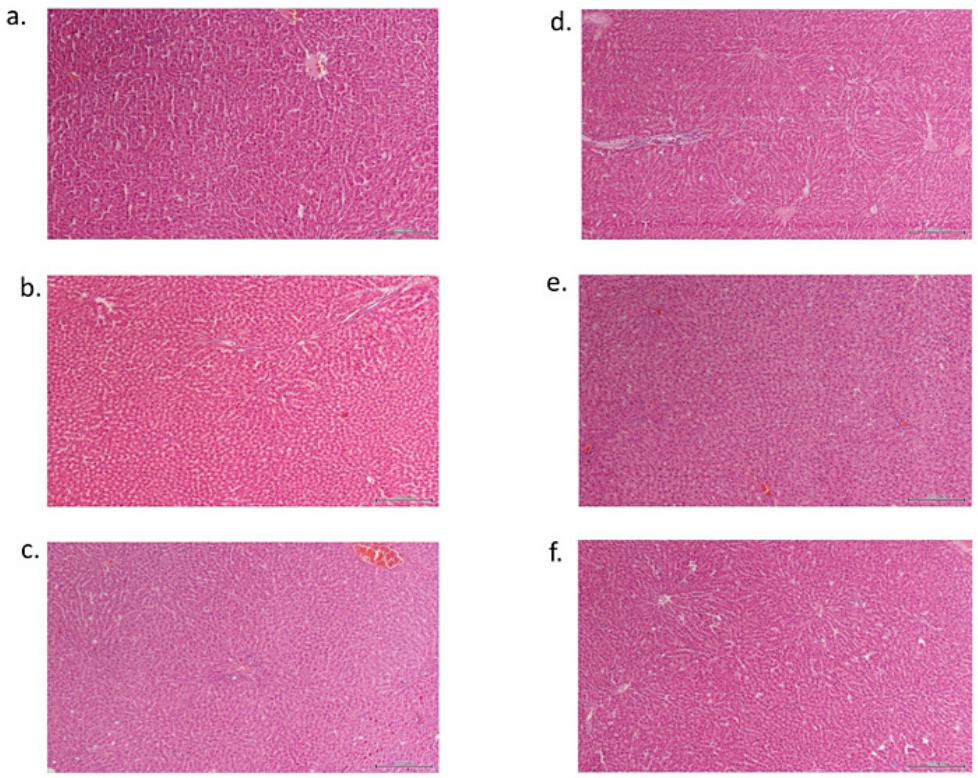

Fig. 5. Cross-section of liver tissues stained with H&E. a, untreated; b, DF; c, DP; d, PH; e. DF+DP; f, AP. Scale at the lower right is 200 µm

Figure 5 shows representative cross sections of liver samples stained with hematoxylin and eosin (H&E). Liver injury was observed in all rat groups due to the high fat, high cholesterol diet for four weeks. Generally, the sections of liver tissues showed widespread lytic necrosis of hepatocytes, disintegrated cells and loss of hepatocytic individualization, vacuolar degeneration, and the presence of inflammatory cells in the portal tracts and blood vessels. The untreated group and AP group appeared to have a similar extent of liver injury, but DF-, DP-, PH, and DF+DP-treated groups have reduced extent of hepatocyte damage.

Representative cross sections of kidney samples are shown in Figure 6. Necrosis of tubules and swelling of glomeruli are observed, accompanied with atrophy of few glomeruli and inflammation of interstitium surrounding blood vessels and tubules. The DF-, PH-, DF+DP, and AP-treated rats have reduced extent of renal damage than the untreated and DP-treated rats.

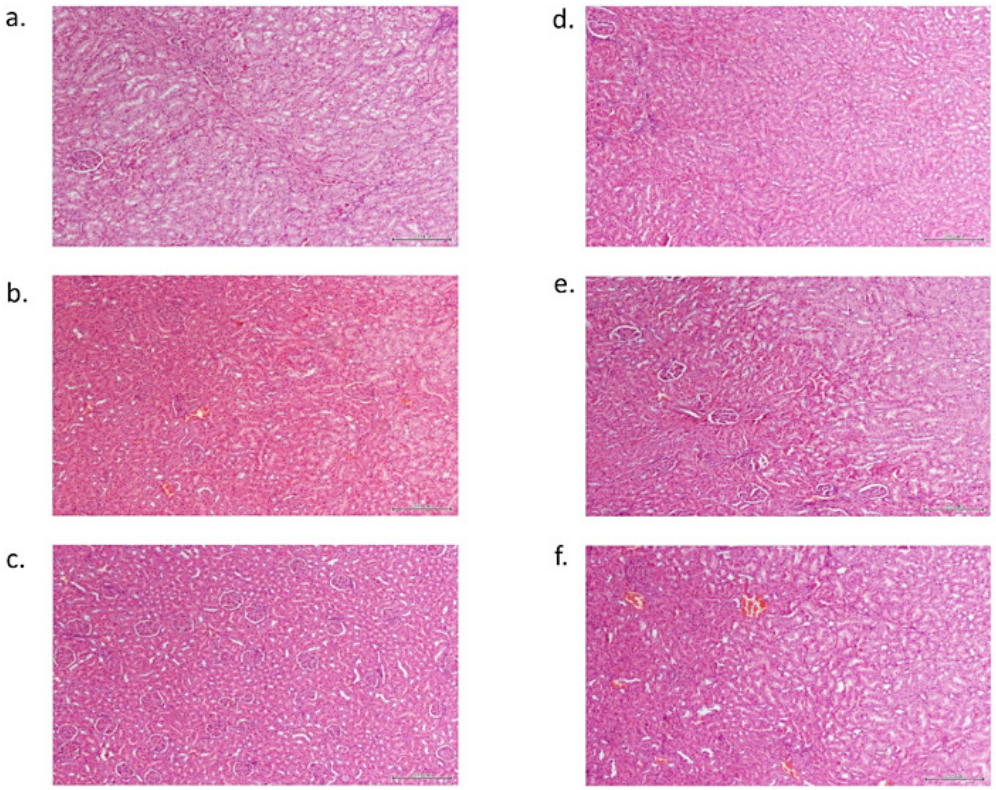

Fig. 6. Cross-section of kidney tissues stained with H&E. a, untreated; b, DF; c, DP; d, PH; e. DF+DP; f, AP. Scale at the lower right is 200 µm.

Discussion

Lemongrass is reported to have high fiber content, consisting mostly of cellulose, lignin and hemicellulose 24. On the other hand, reports on crude protein of lemongrass ranged from 4.56%[25] to 19.79% [26]; both were determined using the standard Kjeldahl method for nitrogen and a conversion factor of 6.25 to determine the protein content. Yet protein content of leaves from various plant species are low, and the 6.25 factor does not always apply.[27] Antinutrients in plants, e.g. tannins, bind to proteins during extraction and reduce protein extraction yield.[28] In this study, proteins were obtained using alkali solubilization followed by acid precipitation. The proteins obtained had residual tannins detected using the $FeCl_3$ test (data not shown) which may interfere with the treatments, and removal of the tannins using polyamide further resulted to lower yield of tannin-free proteins. Simulated gastrointestinal digestion of the tannin-free proteins gave a good yield of protein hydrolyzates at 74% (Table 1), but digestion of proteins without tannin removal gave a much lower yield (data not shown). Significant sugar- and cholesterol-lowering effect of TDF, tannin-free protein, and protein hydrolyzates was observed in rats fed with high sugar, high cholesterol. Dietary fiber is known to lower sugar and cholesterol through several mechanisms. Soluble and insoluble DF binds or adsorbs glucose, cholesterol and bile acids in the gastrointestinal tract, thereby preventing their absorption into the bloodstream and subsequent rise in serum levels after a high sugar, high cholesterol meal.[29-30] Consumption of carbohydrate foods with high DF content is associated with a lower glycemic index in humans, thus resulting to reduced postprandial serum glucose levels and insulin responses.[31] The amount of fiber administered to the corresponding animal group was dosed at 400 mg per kg body weight, which is based on the recommended daily intake of 20-25 g for Filipino adults. This DF amount is sufficient enough to bind significant levels of cholesterol and sugar for excretion, thus preventing absorption into the bloodstream, which may account

for the sugar- and cholesterol-lowering effect in the animal groups treated with TDF.

DF is fermented in the colon to produce the short chain fatty acid products, which has been shown to regulate key enzymes involved in carbohydrate and lipid metabolism. Propionate slows down gluconeogenesis while increasing glycolysis rates in the liver,[32] and inhibits fatty acid synthesis and cholesterol synthesis.[33] We have reported in an earlier study that propionate is produced from *in vitro* fermentation of lemongrass DF, and exerted mild inhibition on key enzymes involved in postprandial hyperglycemia and cholesterol synthesis, α-amylase and HMG-CoA reductase, respectively.[14] This inhibitory effect on the enzymes further contribute to the sugar- and cholesterol-lowering effect in the serum of the animal groups.

On the other hand, many plant-derived dietary proteins exert potent physiological effects in the intact form, or encrypt bioactive peptides in their structure, which are released during digestion, fermentation, or hydrolysis. Antidiabetic effects of bioactive proteins or peptides are determined *in vitro*, through inhibition of carbohydrate-digesting enzymes α-amylase and α-glucosidase,[34] or DPP-IV enzyme, thereby improving insulin secretion.[35] For example, protein hydrolyzates from beans had *in vitro* α-amylase and α-glucosidase inhibitory activities, antihyperglycemic effect in rats, and hypoglycemic activity in mice,[36] while amaranth grain protein hydrolyzates exerted *in vitro* DPP-IV inhibitory activity, and improved glucose tolerance in diabetic mice.[37] We have tested for the inhibitory activity of lemongrass tannin-free proteins and protein hydrolyzate fractions on α-amylase and α-glucosidase, but there is no substantial inhibitory activity on both enzymes (data not shown). Yet the group treated with dietary protein and protein hydrolyzates gave the least elevation in FBS. This might be due to DPP-IV inhibition of intact protein and hydrolyzates.

Protein hydrolyzates and bioactive peptides lower cholesterol through inhibition of HMG-CoA reductase, the key enzyme in endogenous cholesterol synthesis. Statins are well-known cholesterol-lowering drugs through inhibition of HMG-CoA reductase,[38] and statins cause lowering of LDL cholesterol levels.[39] We have extracted proteins from lemongrass, subjected the proteins to simulated *in vitro* gastrointestinal digestion, and obtained protein hydrolyzate fractions. Preliminary results showed that the fractions have *in vitro* HMG-CoA reductase inhibitory activity in comparison with pravastatin control.[15] These *in vitro* results support the observation that the protein hydrolyzates administered to rats resulted to the greatest reduction in total cholesterol levels. Furthermore, the groups fed with dietary protein and pravastatin had low increase in LDL+VLDL cholesterol levels, but the group fed with protein hydrolyzates completely prevented the rise in LDL cholesterol levels. This demonstrates the potency of the protein hydrolyzates in lowering cholesterol levels through statin-like action, especially inhibition of endogenous cholesterol synthesis.

Animal models fed with an atherogenic diet, e.g. high fat (15-40%), high cholesterol (1.25-5%), or a combination of both, lead to chronic fatty liver disease and non-alcoholic steatohepatitis; liver tissues are usually characterized to have hepatocyte necrosis, inflammation, cellular ballooning, and fibrosis, depending on the length of time of diet administration.[40] Co-supplementation of functional foods with the high fat, high cholesterol, high sugar diet, helped ameliorate the hepatic injury due to the diet. Rats fed with a high fat diet and co-treated with streptozotocin resulted to necrosis of hepatocytes, loss of regular cell architecture, and infiltration of inflammatory cells. However, co-supplementation of cucurmin obtained from turmeric, helped alleviate hepatocyte damage and reduced inflammatory in rats fed with a high fat diet and co-treated with streptozotocin; serum cholesterol levels were also lower in these rats with cucurmin co-supplementation than those without.[41] Galacto-oligosaccharides treatment also reduced degree of liver damage in mouse hepatocytes fed with high fat, high sugar diet, which was accompanied with lower cholesterol levels in serum and liver.[42]

Similarly, a high fat diet also causes renal injury in animal models. Histopathological changes in nephrons of rats and mice fed on a high fat diet include necrosis and atrophy of glomeruli and other tubules, inflammation, and glomerulosclerosis; nephrons are generally deformed and degenerated.[43-44] Administration of functional foods to animal models of diabetes or obesity fed on high fat, high cholesterol diet were also shown to exert protective effects on

renal cells by minimizing the structural damage of nephrons. Diabetic rats fed with high cholesterol diet showed pronounced glomerulosclerosis and tubular lesions, but co-treatment with cucurmin decreased the degree of nephron damage.[45]

In our results, the treatments do not show toxicities to liver or kidney cells, but help minimize the hepatocyte and nephron damage due to the high cholesterol, high sugar diet. This suggests that dietary fiber, dietary protein, and protein hydrolyzates exert protective effects on liver and kidney injury or damage caused by high cholesterol, high sugar diet. This study was limited to the use of a mixed sex animal model, with each group having the minimum number of animals required to give statistical significance. Thus, the animals were distributed in a way that all the groups have similar average weight, and all animals were exposed to the same environment conditions during the duration of the study. However, the results obtained provided substantial evidence to demonstrate the serum glucose- and cholesterol-lowering effect of lemongrass dietary fiber, dietary protein, and protein hydrolyzates, which was further supported by *in vitro* results in earlier studies.[14,15]

Conclusion

Lemongrass dietary fiber, dietary protein, and protein hydrolyzates resulted to significant prevention of blood glucose elevation, and lowering of cholesterol levels when administered to rats maintained on a high sugar, high fat, high cholesterol diet. The extent of rat hepatocyte and nephon damage caused by the diet also appears to be reduced by the treatments. Thus dietary fiber, protein and protein hydrolyzates obtained from lemongrass serve as a promising functional food in helping prevent the risk for diabetes mellitus and CVD through prevention of hyperglycemia and hypercholesterolemia.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Availability Statement

The manuscript incorporates all data produced throughout this research study.

Ethics Statement

The animal study protocol was evaluated and approved by the UST IACUC under Code No. RC2017-950925, with Animal Research Permit Ref. No. AR2018-061 issued by the Bureau of Animal Industry.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author Contributions

- **Mizpah Cervera Villalobos:** conceptualization, funding acquisition, methodology, data acquisition and analyses, visualization, writing-original draft and final manuscript.
- **Marilou Gagalac Nicolas:** conceptualization, supervision, evaluation, writing-original draft and final manuscript.
- **Trinidad Palad Trinidad:** conceptualization, funding acquisition, project administration, evaluation.
- **Niel-Ju Angelle Caigoy Cadiao:** methodology, data acquisition.
- **Mary Ann Julyn Ferrer Catalan:** methodology, data acquisition.
- **Rosario delos Santos Sagum:** funding acquisition, project administration, writing and approval of final manuscript.

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