Research Article

The Antihyperglycemic Effect of Crude Liang (*Gnetum gnemon* var. *tenerum*) Leaves Powder on Wistar Rats

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1.Introduction

Diabetes mellitus (DM) is a serious global health disorder. In 2022, more than 400 million diabetic patients were recorded, and numbers are projected to increase to 783 million by 2045 [1]. Diabetes results in health expenditures of at least USD 966 billion, with increased cost of care management [2]. Metabolic disorders of DM are either inadequate production or impaired utilization of insulin [3]. When not enough insulin is present or when cells cease responding to insulin, the metabolism of carbohydrates, fats, and proteins is disturbed [4]. Most plants contain chlorophyll, a green pigment for photosynthesis [5]. Chlorophyll is the most abundant natural pigment and has been considered as hypoglycemic agent via mechanism of free radicals’ inhibition [6]. Chlorophyllin is a semisynthetic substance that is created by replacing the chlorophyll magnesium ion with another metal and removing the phytol tail [7]. Recent studies have shown that both chlorophyllin and metformin (a medication for diabetes) inhibit gluconeogenesis and increase glucose uptake by peripheral muscles [8, 9].

Liang (Thai name) is a native vegetable of southern Thailand [10]. Liang may have high potential to lower blood glucose due to its high protein, fiber, and chlorophyll...
character [11] as a natural antidiabetic nutrient [12]. However, a comparison of the antidiabetic properties of crude plants containing chlorophyll compared to those with induced chlorophyllin is lacking. Therefore, this study compared the antidiabetic activity of crude Liang leaves, Liang leaves treated with CuSO₄ (Liang chlorophyllin), and commercial chlorophyllin in a Wistar male rat model.

2. Materials and Methods

2.1. Liang Leaves Preparation for Chlorophyllin Induction. The species of Liang used in this study was Gnetum tenerum var. tenerum, with voucher specimen number QBG No. 43621 [13]. Liang leaves in the edible stage were divided into two preparations. Preparation 1 comprised crude Liang leaves powder (CLL) and preparation 2 was Cu-chlorophyllin Liang leaves powder (CCL) treated with CuSO₄. CLL was prepared by removing the leaves from the petiole and washing with 100 ppm chlorinated solution for 15 minutes as decontamination before drying. CCL was prepared by treatment with CuSO₄ to obtain Cu-chlorophyllin Liang leaves (CCL). The leaves were tied together with a small brush and the stem was slant cut to allow more contact area to absorb 20% CuSO₄ solution for 120 minutes at 60°C before washing twice in tap water to remove excess CuSO₄.

2.2. Liang Leaves Powder Preparation. The drying process was performed using a vacuum microwave dryer at 3600 watts for 14 minutes per 0.5 kg leaves to achieve \( a_w < 0.6 \). The dried leaves were ground and sieved through 280 mesh size. Liang CCL and CLL powders were analyzed for proximate composition following AOAC 2019 assay [14].

Experimental Animals. Twelve male Wistar rats (250–300 g), 5 weeks old, were obtained from Nomura Siam International and checked and housed for 7 days for acclimatization to laboratory conditions. After acclimation, the rats were administered with 30% fructose solution every day for 3 weeks to get diabetic symptoms. Throughout this experiment, the rats were reared at the Animal Laboratory Center, Prince of Songkla University, Thailand, according to the animal welfare care system with temperature 22 ± 3°C, relative humidity 55% ± 10%, light intensity 130–325 lux, lighting schedule 12/12 hours (bright/dark), and given filtered water using a reverse osmosis system. All rats received water ad libitum and a standard laboratory diet.

2.3. Ethical Approval. The study was approved by the Institutional Animal Care and Use Committee, Ref. No. 55/2021, Prince of Songkla University.

2.4. Induction of Diabetes. A literature review and preliminary tests suggested that a high dose (>50 mg/kg bw) of streptozotocin (STZ) was typically used to induce type 1 diabetic (T1D) rats but not used for insulin resistance induction [15] while using long-term fructose feeding at 3-4 weeks which could develop insulin resistance [16]. Therefore, using fructose with low STZ was an effective way to induce diabetic symptoms. The experimental rats were administered with 30% fructose solution instead of drinking water for 4 weeks and then fasted overnight (10–12 hours) before STZ injection [17]. A single intraperitoneal injection of streptozotocin (STZ) dose 35 mg/kg body weight was prepared in 0.1M citrate buffer (pH 4.5) [18]. After 48–96 hours of STZ injection, blood was drawn by tail incision after the antiseptic process. Blood glucose level was determined using a glucometer (Bluetooth SIG, Inc., UK). Rats with nonfasting blood glucose levels greater than 250 milligrams per deciliter (NFBG >250 mg/dl) were diagnosed as diabetic [19].

Experimental Groups. Normal and diabetic rats (NFBG level >250 mg/dl) were divided into four groups (3 rats/group) as follows.

- Group A: Normal rats receiving distilled water.
- Group B: DM + 100 mg/kg of CHL
- Group C: DM + 0.97 g/kg of CCL
- Group D: DM + 0.97 g/kg of CLL

The rats were fasted overnight and received the designed agents via oral gavage every day for 7 days, with NFBG levels assessed on the 1st, 3rd, and 7th day to determine antihyperglycemic activity.

2.5. Toxicity Study. At the end of the study (7 days), the rats were fasted overnight (16–18 hours) before injection with 120 mg/kg of sodium thiopental. After euthanasia, the heart, kidney, spleen, and liver were collected, rinsed with normal saline, and dried with tissue paper. Organ weight was measured using a precision balance for a 2-digit calculation of organ index as shown in the following equation:

\[
\text{Organ index} = \frac{(\text{organ weight} \times 100)}{\text{body weight}}. \tag{1}
\]

2.6. Biochemical Analysis. At the end of the study (7 days), blood was collected by cardiac puncture. Serum was prepared to determine the function of the liver (serum glutamate-pyruvate transaminase, SGPT, and serum glutamic oxaloacetic transaminase, SGOT), kidney (blood urea nitrogen, BUN, and creatinine), and lipid profile using a Mindray BS-200 clinical analyzer.

2.7. Histopathological Study. Internal organ tissues including the liver and kidneys were separately cut and fixed in 10% formalin for 3 days before being dehydrated. Thereafter, the samples underwent automated tissue processing, starting with paraffin wax embedding, thin sectioning at 5 mm thickness, and hematoxylin and eosin staining. The slides were examined under a light microscope to identify any indications of unusual symptoms.
2.8. Hematological Analysis. Blood samples from the rats were collected in tubes containing EDTA for hematological analysis (complete blood count (CBC) including hemoglobin (Hb), hematocrit (Hct), total RBC, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, and white blood cells count (WBC)) by using a Nihon Kohden Celltac Es MEK-7300 automated hematology analyzer.

2.9. Statistical Analysis. The Statistical Package for the Social Sciences (SPSS) version 28.0 was used to analyze all data (Chicago, IL, USA) with one-way ANOVA followed by Tukey’s post hoc test performed to compare differences between the experimental groups. Results were reported as mean ± SEM and a significant value was set at $P < 0.05$.

3. Results

Table 1 shows the proximate compositions of CCL and CLL. Both Liang leaves samples contained mainly protein and dietary fiber. As expected, CCL had a much higher copper level than CLL (almost 110 times), confirming the success of dietary fiber. As expected, CCL had a much higher copper content than CLL, almost 110 times, confirming the success of dietary fiber. Besides Cu content, CuSO₄ treatment process did not change the proximate composition of CuSO₄ treatment process. Besides Cu content, CuSO₄ treatment did not change the proximate composition of CuSO₄ treatment process.

Table 2. CHL rats showed lower WBC, with higher RBC mass presented by higher Hct, more RCB, and higher Hb levels but not MCV, MCHC, and MCH. Interestingly, the CCL group had lower platelet count than the normal, CHL, and CLL groups.

4. Discussion

Results confirmed the antihyperglycemic effect of CHL, concurring with Abani et al. [8] who reported that 50 mg/kg BW of CHL effectively reduced blood glucose levels in STZ-induced diabetic mice. Their proposed CHL mechanism for lowering BG was a metformin-like effect wherein it enhances glucose uptake by promoting the expression of GLUT4 (data is in publication process). However, our findings showed that the effect of CHL was rapid but not durable. CHL lowered BG rapidly within 3 days but could not sustain this through 7 days. Based on the NFBG result, CHL treatment was superior to treated CCL and CLL rats. The suspected chlorophyllin contained in CCL was equal to CHL but its action was different, while CLL without chlorophyllin induction demonstrated a marginal antihyperglycemic efficacy. Chlorophyllin (CHL) is used to control blood glucose levels by decreasing gluconeogenesis in hepatocytes [8]. However, it was hypothesized that Cu-chlorophyllin (CCL) in this experiment was too high and harmed hepatocytes and impaired liver function. As a result, CCL was not safe to use to reduce blood glucose levels compared to crude chlorophyll (CLL). Interestingly, results suggested that although the antihyperglycemic activity of CCL was not as rapid as CHL, BG levels of rats receiving Liang products, particularly CCL, reduced while those receiving CHL increased. However, the duration of 7 days may not be long enough to give credence to this efficacy. Furthermore, the
The antihyperglycemic effects of Liang products may not result from chlorophyllin. Liang leaves contain high fiber and protein, with low carbohydrates as beneficial factors in glucose control [11].

Apart from their marginal effect on blood glucose homeostasis, both CCL and CLL, particularly CLL, could express as protective agents in maintaining body weight. Their high protein content is very important for muscle synthesis,

<table>
<thead>
<tr>
<th>Test list</th>
<th>Unit</th>
<th>Cu-chlorophyllin Liang leaves powder (CCL)</th>
<th>Crude Liang leaves powder (CCL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>g/100 g</td>
<td>24.38</td>
<td>24.73</td>
</tr>
<tr>
<td>Fat</td>
<td>g/100 g</td>
<td>5.39</td>
<td>4.72</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>g/100 g</td>
<td>58.82</td>
<td>56.86</td>
</tr>
<tr>
<td>Dietary fiber</td>
<td>g/100 g</td>
<td>40.83</td>
<td>39.27</td>
</tr>
<tr>
<td>Soluble dietary fiber</td>
<td>g/100 g</td>
<td>3.01</td>
<td>2.79</td>
</tr>
<tr>
<td>Insoluble dietary fiber</td>
<td>g/100 g</td>
<td>37.82</td>
<td>36.48</td>
</tr>
<tr>
<td>Ash</td>
<td>g/100 g</td>
<td>6.58</td>
<td>6.53</td>
</tr>
<tr>
<td>Copper</td>
<td>mg/kg</td>
<td>1309.44</td>
<td>11.81</td>
</tr>
</tbody>
</table>

Figure 1: Antihyperglycemic study showing change of NFBG level in screening test study. Different uppercase letters indicate significant differences within a group (P < 0.05). Different lowercase letters indicate significant differences within a day (P < 0.05). Non-DM is the normal rat group; DM/CHL is the diabetic rat group treated with 100 mg/kg of commercial chlorophyllin powder; DM/CCL is the diabetic rat group treated with 0.97 g/kg of Cu-chlorophyllin Liang leaves powder; DM/CLL is the diabetic rat group treated with 0.97 g/kg of crude Liang leaves powder.

Figure 2: Effects of different treated groups on the body weight of rats. Different uppercase letters indicate mean significant differences within a group. Different lowercase letters indicate significant differences within a day (P < 0.05). Non-DM is the normal rats administered with only distilled water; CHL commercial is the diabetic rats treated with 100 mg/kg of commercial chlorophyllin powder; Liang with CuSO₄ is the diabetic rats treated with 0.97 g/kg of Cu-chlorophyllin Liang leaves powder; crude Liang is the diabetic rats treated with 0.97 g/kg of crude Liang leaves powder.
leucine contained in Liang leaves may help to influence factors involved in protein synthesis [11]; therefore, high glucose-intolerant elderly. Leucine can activate initiation proved lean body mass, strength, and physical functions in the groups. Borsheim et al. [21] reported that supplementing specific body composition, especially muscle mass between collected at the end of this study to demonstrate changes in [20]. However, no data on body composition of rats were CLL. This weight-sparing effect of Liang leaves may be due to the histological study did not show excess triglycerides in the hepatocyte cytoplasm, and the cause of higher organ index values of the liver remains unknown. By contrast, the kidney in early diabetes is well known to be hypertrophy from hyperfiltration due to the osmotic effect of hyperglycemia [25], leading to a heavier kidney. Changes in lipid profiles in diabetes-induced rats included higher TG, LDL, and HDL. In a hyperglycemic state, the elevation of triglyceride levels triggers an enhancement in the activity of the lipoprotein lipase enzyme [26]. This leads to the increase of insulin resistance and decreased in the effectiveness of insulin [27]. With the limitation of lipoprotein lipase enzyme activity, TG lasts longer, leading to high blood levels. The rise in HDL was large, whereas the increment of LDL was smaller. The proportion of elevated LDL was much larger with different baseline levels, leading to reduced HDL/LDL ratios in all diabetic rats, and lowest in CCL; however, the mechanism of this lipid change remains unexplained.

The toxicity of the studied agents in CCL rats suggested significant liver damage by elevation of liver enzymes (SGOT and SGPT). However, the histological study showed significant liver cell hypertrophy in CHL and CCL. Eventual cell death in CCL may be due to damaged cells by high fructose feeding and STZ as the DM induction process. The European Food Safety Authority (EFSA) [28] regulated that specifications for Cu-chlorophyllin must not exceed 8.0% of the total copper chlorophyllin. In this study, CCL was only 1.20% but was suspected to be harmful to the liver and kidney of DM rats. DM induces increased complication or changes that cause other burdens and diseases.
The histological study showed significant kidney injury in diabetic rats receiving chlorophyllin and both commercial and Cu-chlorophyllin. Kidney injury was found in the histological study, but only mildly elevated BUN and Cr were found in the blood study of the CHL group, with marginal increases in BUN in the CHL group. Intracellular copper was previously reported to lead to kidney fibrosis by stimulating LOX-mediated collagen and elastin crosslinking [29]. The kidney enzyme increased in the early stages and progressed to kidney fibrosis, which is the primary mechanism and the final common route underlying the advancement of all chronic kidney diseases (CKDs) [29]. Copper is

![Figure 3: Hematoxylin and eosin staining of kidney (a–d) showing interstitial edema (E), tubular degeneration (D), and pyknosis (P) and liver (e–h) showing hepatocyte hypertrophy (H), karyolysis (K), pyknosis (P), and central vein (CV). Non-DM is the normal rats administered only distilled water; DM/CHL is the diabetic rats treated with 100 mg/kg of commercial chlorophyllin powder; DM/CCL is the diabetic rats treated with 0.97 g/kg of Cu-chlorophyllin Liang leaves powder; DM/CLL is the diabetic rats treated with 0.97 g/kg of crude Liang leaves powder.](image)

**Table 4: Effect of different treatment groups on rat hematology in the screening test.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Non-DM</th>
<th>DM/CHL</th>
<th>DM/CCL</th>
<th>DM/CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>×10^9/L</td>
<td>4.00 ± 0.84a</td>
<td>2.75 ± 0.49b</td>
<td>4.00 ± 1.56a</td>
<td>4.55 ± 0.07a</td>
</tr>
<tr>
<td>Total RBC</td>
<td>×10^12/L</td>
<td>10.03 ± 0.67ab</td>
<td>11.75 ± 0.07a</td>
<td>9.74 ± 1.22ab</td>
<td>8.88 ± 0.09b</td>
</tr>
<tr>
<td>Hct</td>
<td>%</td>
<td>55.00 ± 4.24a</td>
<td>63.50 ± 2.12a</td>
<td>53.50 ± 7.78ab</td>
<td>47.00 ± 1.41a</td>
</tr>
<tr>
<td>Hb</td>
<td>g/dl</td>
<td>18.50 ± 0.99ab</td>
<td>21.50 ± 0.42a</td>
<td>18.20 ± 2.26ab</td>
<td>16.25 ± 0.35b</td>
</tr>
<tr>
<td>MCV</td>
<td>Fl</td>
<td>55.00 ± 0.00a</td>
<td>54.00 ± 1.41a</td>
<td>54.50 ± 0.71ab</td>
<td>53.00 ± 1.41a</td>
</tr>
<tr>
<td>MCH</td>
<td>Pg</td>
<td>18.45 ± 0.21a</td>
<td>18.30 ± 0.28a</td>
<td>18.70 ± 0.00a</td>
<td>18.35 ± 0.21a</td>
</tr>
<tr>
<td>MCHC</td>
<td>%</td>
<td>33.55 ± 0.07a</td>
<td>33.35 ± 0.21a</td>
<td>33.65 ± 0.07a</td>
<td>32.55 ± 0.21a</td>
</tr>
<tr>
<td>Platelet count</td>
<td>×10^12/L</td>
<td>875.00 ± 140.71a</td>
<td>700.50 ± 77.07a</td>
<td>434.50 ± 465.9b</td>
<td>744.50 ± 115.25a</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (N=3). Different lowercase letters indicate significant differences in rows (P < 0.05). Non-DM is the normal rat group administered only distilled water; DM/CHL is the diabetic rat group treated with 100 mg/kg of commercial chlorophyllin powder; DM/CCL is the diabetic rat group treated with 0.97 g/kg of Cu-chlorophyllin Liang leaves powder; DM/CLL is the diabetic rat group treated with 0.97 g/kg of crude Liang leaves powder.
a hepatotoxic substance that induces acute or chronic hepatitis by damaging DNA, thereby causing oxidative stress through activation of Kupffer cells [30], [31]. Abnormalities found in the liver also include hepatocyte hypertrophy, karyolysis, and pyknosis which are signs of cell injury and lead to cell death [32, 33]. This finding strongly suggested that toxicity studies should include not only blood but also histological studies. Toxic effects on the liver and kidney were not observed in diabetic rats receiving crude Liang leaves treatment. The hematologic safety study also showed lower WBC in the CHL group, lower platelet counts in CCL, and mild anemia in CLL rats. These hematologic changes suggested significant toxicity of chlorophyllin, both in the commercial and in-house products. Crude Liang leaves caused mild anemia, and this should be monitored further since low WBC and platelet count can lead to severe infection and bleeding.

5. Conclusions

Our study showed the efficacy of CLL in lowering blood glucose in diabetic rats with weight maintenance and no toxicity category. The antihyperglycemic effect of CLL was shown to be less and slower than commercial chlorophyllin which can lower blood glucose fast but provide liver, renal, and hematologic toxicity. Rats treated with CLL showed more firmness with healthy fur. Further studies on the longer-term effects of Liang leaves are required using a larger number of Wistar rats to draw more accurate conclusions.

Data Availability

The data supporting the results are included in the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

AS, SS, and CTY conceived and designed the study. AS undertook data collection, conducted statistical analysis, and drafted the first manuscript, while SS, CTY, and RL provided critical feedback and revisions. All authors have approved the manuscript for submission.

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