To investigate the effect of Rhinacanthus nasutus leaf extracts on impaired glucose and lipid metabolism in obese ICR mice.

**Objective:** To investigate the effect of Rhinacanthus nasutus (R. nasutus) leaf extract on impaired glucose and lipid metabolism in obese ICR mice.

**Methods:** Obesity was induced in male ICR mice by feeding them a high-fat diet (60 kcal% fat) for 12 weeks. After the first six weeks of the diet, the obese mice were administered with the water extract of *R. nasutus* leaves at 250 and 500 mg/kg per day for the next six weeks. Subsequently, the blood glucose, lipid profiles, insulin, leptin, and adiponectin levels were measured. The liver and adipose tissues were excised for histopathological examination and protein expression study.

**Results:** After six weeks of the treatment, *R. nasutus* extract (at 250 and 500 mg/kg per day) was found to reduce the elevated blood glucose level, improve the insulin sensitivity, decrease the serum leptin, and increase the serum adiponectin levels. The obese mice treated with *R. nasutus* were found to have a reduction in the increased lipid concentrations in serum and liver tissues. Moreover, treatment with *R. nasutus* reduced the fat accumulation in the liver and the large adipocyte size in the fat tissues. Interestingly, the administration with *R. nasutus* extract was marked by an increase in the hepatic peroxisome proliferators-activated receptor alpha, fat cell adiponectin, and glucose transporter 4 proteins.

**Conclusions:** To the best of our knowledge, the present study is the first report on the impact of *R. nasutus* extract in improving the impaired glucose and lipid metabolism in high-fat diet-induced obesity in mice via stimulating the insulin sensitivity in the liver and adipose tissues.

1. **Introduction**

Abnormal lipid metabolism in obesity can impair insulin signaling by inhibiting the release of glucose from the liver and its uptake by the fat and muscle cells [1]. An overloaded long-term high-fat diet (HFD) leads to obesity, which can induce insulin resistance in many tissues such as the liver, skeletal muscle, and adipose tissues. In the insulin-resistant state, insulin is unable to inhibit lipolysis, which results in increased circulating free fatty acid (FFA) [2]. The elevated FFA levels increase the chronic hyperglycemia and hypertriglyceridemia. Hepatic insulin resistance can increase the hepatic gluconeogenesis and lipogenesis [3]. FFA accumulation in the liver results in hepatic steatosis and contributes towards dysfunctional insulin signaling [4]. Moreover, the adipose tissues in the obese state are largely expanded and function abnormally in the regulation of cytokine release as well as fatty acid metabolism and its storage [4].

Insulin is the most important hormone for the regulation of blood glucose level. Glucose transport, the rate-limiting step in carbohydrate metabolism, is facilitated by glucose transporters (GLUT) across the cell membranes. GLUT4, a major GLUT, stimulates glucose uptake into fat and muscle cells. Its function is crucial for the condition of insulin sensitivity [5]. It has been reported that the overexpression of GLUT4 gene reduces the hyperglycemic and hyperinsulinemic conditions in HFD-fed transgenic mice [6]. Adiponectin is an adipokine secreted by the adipose tissues that...
regulates glucose and lipid metabolism [7]. Peroxisome proliferators-activated receptor alpha (PPARα) plays an important role in the regulation of lipid metabolism in the liver. PPARα enhances the uptake, utilization, and catabolism of the fatty acids by up-regulating the genes, which are involved in the transport, binding, and β-oxidation of fatty acids [8]. It has been shown that PPARα agonist improves hepatic and muscle insulin resistance, decreases hepatic and intramuscular fat content, decreases plasma FFA, and enhances adiponectin expression in rodents [9–13].

*Rhinacanthus nasutus* (*R. nasutus*) is found naturally in Indonesia, South China, and Southeast Asia including Thailand. *R. nasutus* has been reported to have several bioactivities such as anti-allergic [14], neuroprotective [15], antidiabetic [16], antitumor [17], and neuroprotective activities [18]. However, the effects of the water extract of *R. nasutus* leaves on obesity have not been clearly demonstrated yet. Therefore, given the increasing incidence of obesity, the aim of this study was to investigate the effect of the extract of *R. nasutus* on impaired glucose and lipid metabolism in HFD-fed mice.

2. Materials and methods

2.1. Plant extraction

Leaves of *R. nasutus* were collected from Buriram, Thailand, between July and September 2014. A voucher herbarium specimen (SKP 001 18 14 01) was given by the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The dried leaves were extracted with water at 100°C for 30 min. The extract was then concentrated and freeze-dried. After this procedure, the yield was 25.11% of the initial dry weight of the leaves. The obtained *R. nasutus* extract was kept at −20°C until it was further used. The amount of the total polyphenol and flavonoid were calculated to be (91.10 ± 0.97) mg gallic acid equivalents per gram of extract and (85.65 ± 0.40) mg catechin/g extract, respectively. The prior was determined by Folin-Ciocalteu method[19], while the later was from the method derived by Sumczynski et al. [20].

2.2. Animals, diets, and obesity induction

All animal experiment protocols were approved by the Animal Care and Use Committee of Thammasat University, Pathum Thani, Thailand (Record No. AE 006/2014). Thirty-two male ICR mice weighing 2025 g were obtained from the National Laboratory Animal Center of Mahidol University, Nakhon Pathom, Thailand (Record No. AE 006/2014). Thirty-two male ICR mice were divided into three groups with eight mice per group and were orally treated as follows: obese control mice (OB) treated with 5% gum arabic, obese mice treated with *R. nasutus* 250 mg/kg per day, and obese mice treated with *R. nasutus* 500 mg/kg per day. In the normal control group (NC), the mice were also treated with 5% gum arabic. All mice were administered for six weeks. Gum arabic at 5% concentration was used for dissolving *R. nasutus* extract. The doses of the extract were selected based on the preliminary test. We also studied an acute toxicity test and found that the *R. nasutus* water extract did not show any toxicity in mice at the doses of 250, 500, 1000, and 2000 mg/kg for a week (data have not been shown). Changes in the body weight, food consumption, and caloric intake were monitored weekly. After six weeks of treatment, mice were fasted for 6 h and anesthetized with isoflurane. Blood samples were collected from the hearts of all mice to determine their blood glucose, lipid profile, insulin, leptin, and adiponectin levels. After the blood collection, the liver and epididymal fat tissues were removed from the mice and subjected to histological examinations. The rest of these tissues were stored at −80°C until analysis.

2.4. Intraperitoneal glucose tolerance test (IPGTT)

After five weeks of treatment, an IPGTT was performed on the mice to evaluate the effect of each treatment on their glucose tolerance. After 6 h of fasting, the mice were injected with glucose (2 g/kg), followed by the collection of blood samples from the tail vein at 0, 20, 60, and 120 min.

2.5. Measurement of serum insulin, leptin and adiponectin levels

After six weeks of treatment, the serum insulin, leptin, and adiponectin concentrations in mice at fasting condition were measured by using ELISA kit (EMD Millipore, Billerica, MA, USA). The index of the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as an indicator of insulin sensitivity according to the following formula: [insulin (μIU) × glucose (mmol/L)]/22.5.

2.6. Measurement of serum and liver lipid profiles

After six weeks of treatment, serum total cholesterol (TC), triglyceride (TG), and non-esterified fatty acid (NEFA) concentrations were determined by using commercial kits (Wako, Osaka, Japan). For determination of liver TG and NEFA accumulations, 100 mg of the liver was used to extract lipids according to the previous study [21]. TG and NEFA concentrations were measured by using commercial kits (Wako, Osaka, Japan).

2.7. Western immunoblotting

Liver and epididymal fat tissues were homogenized and extracted with TPER® and Halt® protease inhibitor (Thermo Scientific, Rockford, IL, USA). For Western blot analysis, 40 μg of liver PPARα and 20 μg of fat cell adiponectin and GLUT4 proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skimmed milk in Tris-buffered saline containing 0.01% Tween 20 for 1 h. The blocked membrane was incubated...
overnight with anti-PPARα, anti-adiponectin, anti-GLUT4, and anti-actin primary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature, the immunoreactive proteins were developed and the intensities of each band were quantified by densitometry with an ImageQuant™ 400 imager (GE Healthcare Life Sciences, Piscataway, NJ, USA). Actin was used as a loading control to normalize the target proteins.

2.8. Histopathology

Liver and epididymal fat tissues were fixed in 10% formalin and embedded in paraffin. Three-micron sections were cut and stained with hematoxylin and eosin for examination of the liver and adipose tissue histology (CX31; Olympus, Tokyo, Japan). The fat cell size was calculated by using an Image J Software program (National Institute of Health, Bethesda, MD, USA).

2.9. Statistical analyses

Data were expressed as the mean ± SEM. The groups of data were compared by One-way ANOVA followed by the Tukey’s post hoc test (SigmaStat Software, CA, USA). Statistical analyses with the P value less than 0.05 were considered significant.

3. Results

3.1. Effect of R. nasutus on metabolic abnormalities in HFD-induced obese mice

After six weeks of induced obesity, the body weight was found to be significantly higher in the OB group compared to the NC group (Figure 1A). However, the body weight of OB mice treated with either 250 or 500 mg/kg R. nasutus did not show any significant decrease in comparison to the OB group. No significant difference was observed in food intake among the groups (Figure 1B). However, all of the OB groups showed a significant increase in the energy intake compared to the NC group (Figure 1C).

The OB group significantly increased the epididymal fat weight compared to the NC group (Figure 2A). Interestingly, the obese mice treated with both 250 and 500 mg/kg R. nasutus demonstrated the reduction in the body fat. Moreover, their enlarged fat cell size significantly decreased compared to the OB group (Figure 2B,C).

After six weeks of R. nasutus (250 and 500 mg/kg) treatment, the fasting blood glucose was significantly reduced compared to the OB group (Figure 3A). The administration of R. nasutus (250 and 500 mg/kg) showed a significant reduction in the elevated insulin level too (Figure 3B). The reduced fasting blood glucose and insulin levels in the obese mice treated with 250 and 500 mg/kg R. nasutus were found to be effective in decreasing the index of HOMA-IR (Figure 3C). The serum leptin level was
significantly increased in OB group compared to the NC group, but it reduced significantly after being treated with 250 and 500 mg/kg R. nasutus (Figure 3D). After six weeks of treatment with R. nasutus, the serum adiponectin levels were significantly higher than that of the obese control mouse group (Figure 3E).

After glucose injection, the blood glucose level of the obese control group was significantly increased compared to the NC group (Figure 3F). However, the obese mice treated with R. nasutus (250 and 500 mg/kg) showed a significant reduction in the high blood glucose levels at 20, 60, and 120 min compared to the OB group.

After six weeks of treatment, the R. nasutus (250 and 500 mg/kg) slightly decreased the serum TC, which was not significant compared to the OB group (Figure 4A). In comparison to the OB group, the groups treated with R. nasutus showed a significant reduction in the serum TG and NEFA levels (Figure 4B,C). The weight of the liver of the OB group increased compared to the NC group, but the obese mice treated with R. nasutus recorded a decrease in the weight of their livers (Figure 4D). Moreover, the storage of hepatic TG and NEFA were significantly reduced by R. nasutus treatment (Figure 4E,F). The accumulation of lipid droplets in the liver tissue was related to the results of hepatic TG and NEFA content. The OB group clearly showed increased hepatic fat accumulation, but its treatment with R. nasutus markedly reduced the fat accumulation (Figure 4G).

Figure 3. Effect of R. nasutus on fasting blood glucose (A), serum insulin (B), HOMA-IR index (C), serum leptin (D), serum adiponectin (E), and blood glucose levels in IPGTT (F) in HFD-induced obese mice. Values are represented as mean ± SEM; (n = 8). #: P < 0.05 when compared with the NC group; *: P < 0.05 when compared with the OB group.

Figure 4. Effect of R. nasutus on serum total cholesterol (A), serum triglyceride (B), serum non-esterified fatty acid (C), liver weight (D), liver triglyceride (E), liver non-esterified fatty acid (F), and histology of liver (hematoxylin and eosin-staining, 40×) (G) in HFD-induced obese mice. Liver histological examination confirmed that the RN administration for six weeks decreased lipid accumulation in the liver. Hepatocytes of obese mice were filled with macrovesicular fat deposits while microvesicular fat deposits to a lesser extent were found in obese mice treated with RN; Values are represented as mean ± SEM; (n = 8). #: P < 0.05 when compared with the NC group; *: P < 0.05 when compared with the OB group.
3.2. Effect of *R. nasutus* on PPARα, adiponectin, and GLUT4 protein expressions

The OB group showed a significant reduction in hepatic PPARα, fat cell adiponectin, and fat cell GLUT4 proteins expression as compared with the NC group (Figure 5A,B and C, respectively). Interestingly, *R. nasutus* strongly reversed the decreased protein expressions of PPARα, adiponectin, and GLUT4.

![Figure 5](image)

Figure 5. Effect of *R. nasutus* treatment on liver PPARα (A), fat adiponectin (B), and fat GLUT4 (C) protein expressions in HFD-induced obese mice. The RN administration for six weeks significantly increased the expressions of PPARα, adiponectin, and GLUT4 proteins.

Values are represented as mean ± SEM; (n = 8). *: P < 0.05 when compared with the NC group; #: P < 0.05 when compared with the OB group.

4. Discussion

To the best of our knowledge, the present study is the first to demonstrate the effect of *R. nasutus* on impaired glucose and lipid metabolism in the HFD-induced obese mice. Our findings stated that HFD can produce a hyperglycemic condition with decreased glucose tolerance and insulin sensitivity, and increased insulin and leptin levels in obese mice. This HFD-induced obesity model also showed an increase in the serum TC, TG, and NEFA levels, which were similar to those in the case of human obesity. Furthermore, the HFD-fed mice showed a large amount of lipid accumulation and increased TG and NEFA levels in the liver tissue.

The weight of the body and visceral adipose tissue of the HFD-fed mice (60% kcal fat) was more than that of the mice fed with a low-fat diet [22,23]. From our study, we found that the HFD-fed mice also showed a significant increase in the epididymal fat weight. However, the *R. nasutus* treatment slightly reduced the body weight, epididymal fat weight, and the amount of food intake of the obese mice. The energy intake was, yet, significantly higher in HFD mice groups.

The feeding of HFD increased the cholesterol levels via an increase in the cholesterol absorption by the small intestine [24]. The hypertriglyceridemia observed in the HFD model may be due to the increased absorption and formation of TG, and decreased TG uptake by the fat tissues [25]. Many studies have reported that the elevated fat storage in the liver tissue and the abnormal circulating FFA are also associated with the increased insulin resistance [26-28]. Noticeably, all doses of *R. nasutus* treatment strongly decreased the hepatic serum TG and NEFA levels by decreasing the hepatic fat accumulation in the histological examination.

PPARα is the master regulator of lipid metabolism [29]. PPARα agonists stimulate lipid oxidation, decrease the levels of circulating TG, increase high-density lipoprotein cholesterol, and exhibit anti-atherosclerotic activity [30,31]. In our studies, we found that *R. nasutus* can reduce the high levels of serum lipid profiles and hepatic fat accumulation. Therefore, the effect of *R. nasutus* on the expression of PPARα protein is an interesting aspect to investigate. After six weeks of *R. nasutus* treatment, the protein expression of PPARα significantly improved compared to the obese control mice. This may lead to the possibility of an improvement in the impaired lipid metabolism by inhibiting the serum lipid profiles, liver fat accumulation, and stimulating the PPARα expression through *R. nasutus* treatment.

Either deficiency of insulin or insulin-resistant condition can induce the dysfunction of GLUT4 resulting in its retention inside the cell. This dysfunction causes a reduction in the glucose uptake into the muscle and adipose tissues leading to the increase in the blood glucose levels. These reduction and retention may be the reason for causing the obese state [6]. In our study, the HFD-fed mice showed elevated blood glucose, increased serum insulin levels, and increased HOMA-IR values. Interestingly, the OB mice treated with *R. nasutus* can improve the insulin sensitivity by reducing the levels of high blood glucose, insulin, and HOMA-IR. Restoration of GLUT4 levels would be helpful in controlling the hyperglycemic and hyperinsulinemic conditions via an enhancing glucose uptake by the fat tissues. The present study also showed how the small adipocyte in the *R. nasutus*-treated groups could help in increasing the GLUT4 protein expression. These results indicated that *R. nasutus* may be stimulating insulin sensitivity in adipose tissues.

Leptin is an adipokine related to the impaired insulin sensitivity condition. It has been reported that leptin is more likely to be increased in the obesity state [32,33]. In our study, the OB mice showed an increase in the serum leptin levels, but the levels were reduced after six weeks of *R. nasutus* treatment. Adiponectin has an effect on glucose homeostasis. The circulating level of adiponectin correlates with insulin sensitivity both in the humans and rodents [34-36], and it is found to be reduced in humans with obesity and type 2 diabetes [37]. The induction of adipose tissue adiponectin expression and consequently, the increase in circulating adiponectin levels could represent a novel potential enhancement of whole-body insulin sensitivity [38]. After the obese mice had been treated with *R. nasutus* for six weeks, we found that the levels of serum adiponectin and protein...
expression levels increased significantly. Thus, the treatment with *R. nasutus* may cause an increase in the peripheral insulin sensitivity in the obesity-induced insulin resistant condition.

In conclusion, *R. nasutus* administration has an impressive effect on the glucose and lipid metabolism in HFD-induced obese mice. The effects are as follows: (a) reduction of serum TG and NEFA levels and lipid accumulation in the liver, (b) reduction of blood glucose and improvement of insulin sensitivity, and (c) increase of small adipocyte numbers in fat tissue. Furthermore, *R. nasutus* may mediate these effects via its positive effect in stimulating hepatic PPAR, fat adiponectin, and fat GLUT4 protein expressions. Thus, to the best of our knowledge, our findings are the first to support the usefulness of *R. nasutus* in regulating the abnormalities of glucose and fat metabolism in HFD-induced obesity condition.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**Acknowledgments**

This research was supported by the research grant from the Faculty of Medicine, Thammasat University (Contract number: GEN2-05/2015).

**References**


