ORIGINAL ARTICLE

Hypolipidaemic and Cardioprotective Effects of Bee Bread Harvested from Stingless Bee (*Heterotrigona itama*) in High-Fat Diet-Induced Obese Animal Model

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ABSTRACT

INTRODUCTION: Bee bread has reached its research interest because of its high antioxidant and nutritional values. Our study aimed to characterize hypolipidaemic and cardioprotective effects of high-fat (F) diet-induced obese rats co-administered with bee bread. **MATERIALS AND METHODS:** Male Sprague-Dawley rats were randomly assigned as N group (rats fed with normal diet), F group (rats fed with F diet), FB group (rats fed with F diet and 0.5 g/kg/day bee bread), and FO group (rats fed with F diet and 10 mg/kg/day orlistat for 6 weeks). **RESULTS:** The FB group significantly had decreased levels of Lee obesity index, total fat pad, total cholesterol and low-density lipoprotein, and significantly had increased cardiac superoxide dismutase, glutathione peroxidase and catalase activities compared to the F group. Furthermore, the FB group demonstrated significant decreases in the levels of cardiac lipid peroxidation marker and fatty acid synthase activity with improvement in histological findings of heart and adipose tissues. **CONCLUSION:** These findings suggested that bee bread exerted hypolipidaemic and cardioprotective effects owing to its modulatory action on *de novo* synthesis of fatty acid.

Keywords
Bee Bread, Cardioprotective, High-fat diet, Anti-Obesity, Fatty Acid Synthase.

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INTRODUCTION

Obesity has gained primary attention among healthcare professionals as it complicates serious health issues. More than 115 million people had obesity-related conditions that were linked to cardiovascular disease (CVD), stroke, diabetes mellitus, and certain types of cancer.[1] Obesity appears to be left untreated in some individuals probably due to lack of awareness and safety issues related to medication.[2] Prolonged high-fat diet consumption promotes detrimental effects on metabolic health due to the risk of development of oxidative stress, redox imbalance, lipid dysregulation, and pro-inflammatory cytokines.[3] Inhibition of fatty acid synthase (FAS) has been shown to promote weight loss and a positive therapeutic approach against hyperlipidaemia.[4] Although excess adipose tissue accumulation is clearly described as the determinant of obesity, there is still a lack of scientific evidence supporting the treatments or interventions to reduce the accumulation of adipose tissue.

Major attention has been spared on the naturally derived products, which have a high potential to directly modulate the antioxidant defense system and a cascade of lipid metabolism. Stingless bee *Heterotrigona itama* (*H. itama*) produces bee bread from a combination of bee pollen, bee's digestive enzyme, and honey. It is stored in the bee comb or pot, allowing for a fermentation process that catalyses the metabolic content into active bio-ingredients.[5] It is rich in antioxidant activity and contains multinutrients, including carbohydrates, fats, proteins, minerals, and vitamins.[6,7,8,9] Multibiological activities of bee bread have been reported such as antibacterial,[10] anticancer,[11] and hepatoprotective properties.[12] However, there is still a lack of scientific-based evidence focusing on bee bread specifically to define its role in obesity and its related cardiovascular complications. To date, no study has been conducted on the cardioprotective effects of bee bread and its potential benefits towards oxidative stress and lipid metabolism. This study intended to determine the possible hypolipidemic and cardioprotective effects of bee bread in high-fat diet (F)-induced obese rats by evaluating the levels of Lee obesity and adiposity indexes, serum lipid profile, cardiac oxidant/antioxidant status, total fat pad (TFP), FAS, as well as the changes on histology of heart and adipose tissues.

MATERIALS AND METHODS

Preparation of bee bread

Bee bread sample harvested from *H. itama* was acquired from a local beekeeper farm, Mentari Technobee PLT, Kelantan, Malaysia. The received fresh sample was dried in a laboratory dehydrator at 35 °C for 2-3 hours until a consistent weight was reached. The dried bee bread was kept frozen at –21 °C, and it was blended into powder form.

Animals and diets

Thirty-two male *Sprague-Dawley* rats aged 8-10 weeks with weights ranging from 180-230 g were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Kelantan. The animals were acclimatised for a week with food and water *ad libitum*. They were housed in a well-controlled room adapted to a ventilation setting of 22-24 °C, humidity setting of 52-54%, and maintained under a 12-hour light/dark cycle. The experimental protocol was approved by Animal Ethics [USM/Animal Ethics Approval/ 2016/ (98) (744)] and conformed to the Guidelines for the Care and Use of Laboratory Animals by the National Institute of Health.

Normal chow consisted of Altromin pellet (Altromin Spezialfutter GmbH & Co. KG, Lage) (P1324) imported from Germany. A high-fat (F) diet with 31% fat was used to induce a rapid increase in adipose tissue accumulation. It was freshly prepared and modified from the previous study.[13] It consisted of 64 g ground normal chow pellet, 32 g animal ghee, 300 mg calcium and 100UI vitamin D, and 12% of cholesterol powder. The regular chow diet consisted of 64% carbohydrate, 12% fat, and 24% protein (318.8 kcal/100 g), whereas the F diet had 46% carbohydrate, 31% fat, and 12% protein (516.5 kcal/ 100 g). Our preliminary study has shown that rats that received F diet for 6 weeks had higher Lee obesity index and fat adiposity index in comparison to rats that received a normal chow diet showing that this F diet could induce obesity in rats (unpublished data).

Experimental design

In the present study, four groups of animals (n=8/group) were randomly assigned as N group (rats fed with normal diet), F group (rats fed with F diet), FB group (rats fed with F diet and 0.5g/kg/day bee bread), and FO group (rats fed with F diet and 10 mg/kg/day orlistat for 6 weeks). The daily dosage of orlistat used in the present study was consistent with the earlier study.[14] The dose of bee bread was based on our preliminary dose study in which rats that received F diet+0.5g/kg/day bee bread had decreased Lee obesity index, total cholesterol (TC), triglyceride (TG) and low-density lipoprotein (LDL) in comparison with rats that received F diet only, F diet+1.0 mg/kg/day bee bread and F diet+1.5mg/kg/day bee bread for 6 weeks (unpublished data). The doses of bee bread and orlistat were determined based on the rat's body weight and separately suspended in distilled water to make the end suspension volume of 1 mL. Bee bread and orlistat were administered via oral gavage for 6 weeks on a daily basis (8.00 to 9.00 am). After 6 weeks, all rats were anesthetised with intraperitoneal injections of 90 mg/kg ketamine and 5mg/kg xylazine. The thoracic and abdominal cavities of the rats were explored. Blood was obtained from the posterior vena cava. The heart was transversely cut at the base of the aortic and pulmonary branches.

Measurement of anthropometrical and nutritional parameters

The body weight and food consumption of rats were measured daily. Obesity was considered when the Lee obesity index rises beyond the level of 315.[15] The Lee obesity index was calculated using the formula below:

$$\text{Lee obesity index} = \frac{\sqrt[3]{\text{Body weight (g)}}}{\text{Nasoanal length(cm)}} \times 1000$$

Measurement of serum lipid profile

Blood was centrifuged (10 min, 4000 rpm) and allowed to coagulate for two hours. The supernatant was collected, aliquoted, and kept at –80 °C for the next analysis. The TC and TG levels were determined by enzyme-linked immunoassay using available commercial kits (ARCHITECT-c Cholesterol and Triglyceride Reagent, Abbott, USA). The high-density lipoprotein (HDL) level was ascertained using the Biosino Direct HDL reagent kit (Biosino Bio-Technology and Science Inc, Beijing, China). The formula used to calculate LDL was described as follows;[16]

$$\mathrm{LDL}\left(\frac{\mathrm{mmol}}{\mathrm{L}}\right) = Total\ cholesterol - HDL - \frac{Triglyceride}{5}$$

Biochemical measurement

Dissected heart was carefully separated from adjacent blood and fat, and washed with ice-cold normal saline. The heart was weighed and homogenized (15 min, 4 000 rpm) in cold phosphate buffer solution (10% w:v). The obtained supernatant was aliquoted for the measurement of cardiac antioxidant enzymes and malondialdehyde (MDA) as well as FAS. Enzymatic activities of cardiac superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were analyzed using commercially available kits from Bioassay (San Francisco, CA, USA). Commercial kits from Northwest (Vancouver, WA, USA) and Cloud-Clone (Houstan, TX, USA) were used to measure cardiac MDA level and FAS activity, respectively. The protein level was determined by the Lowry method, which was used to standardise all the results.[17]

Fat pad and adiposity index measurement

Subcutaneous fat was removed from the abdominal and epididymal regions. The total of these regional fats was used to calculate the total fat pad (TFP). The following formula was used to calculate the adiposity index: AI= (total fat pad/final body weight) × 100.

Histology of heart and adipose tissue

The heart and adipose tissues were preserved in 10% formalin. The tissues were submerged in various ethanol concentrations before being embedded into paraffin blocks. The blocks were sectioned at 5 μm and stained with haematoxylin-eosin. Slides were examined under a light microscope at ×100 magnification. The mean surface area of the adipose tissue was determined from 10 adipocyte cells per section using Image J software (ImageJ, NIH-Bethesda, MD, USA).

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical differences, and post-hoc Tukey test was used to analyse the multiple comparisons. All the results were analysed using Statistical Package for Social Science (SPSS) version 24.0. and were reported as means, and standard deviations. The P values were considered statistically significant if the values were less than 0.05.

RESULTS

Effects of bee bread on anthropometrical and nutritional parameters

The baseline body weight was almost similar in all the experimental groups. After 6 weeks of the experimental period, the F group showed a significant increase in Lee obesity index in comparison to the N group. Whereas the group supplemented with bee bread showed a significant decline in Lee obesity index in comparison to the F group. There was no discernible variation in the amount of food consumed across all the experimental groups. Additionally, the F, FB, and FO rats consumed more calories than the rats in the N group (Table 1).

Effects of bee bread on lipid profile

The TC and LDL levels were significantly increased in the F group in comparison to the N group. These lipid fractions were significantly decreased in the FB group in comparison to the F group. The LDL level in the FO group was significantly lower compared to the F group. In contrast, the TG and HDL levels did not significantly differ across any of the experimental groups (Figure 1).

Table 1: Effect of bee bread on anthropometrical and nutritional parameters.

Parameters	N	F	FB	FO
Baseline body weight (g)	202.03 (24.34)	229.69 (23.10)	229.88 (22.24)	237.71 (22.81)
Final body weight (g)	322.05 (42.96)	347.55 (26.26)	335.19 (26.74)	355.35 (26.74)
Body weight gain (g)	110.23 (38.38)	120.90 (20.52)	99.54 (31.49)	105.13 (19.70)
Lee obesity index	312.59 (5.28)	336.35 (12.75)[a]	317.84 (7.68)[b]	322.50 (12.95)
Food intake (g/day)	20.32 (3.23)	18.09 (1.47)	18.86 (2.54)	20.78 (1.00)
Calorie intake (Kcal/day)	56.61 (6.57)	93.18 (7.57)[a]	97.14 (13.10)[a]	107.02 (5.14)[a,b]

Data are presented in mean (standard deviation), *n*=8 per group. N; normal, F; high-fat diet, FB; high-fat diet and bee bread at 0.5 g/kg/day, FO; high-fat diet and orlistat at 10 mg/kg/day. [a] $P<0.05$ compared with the normal group, [b] $P<0.05$ compared with the F group. Data was analysed using One-way ANOVA followed by Tukey post-hoc test.

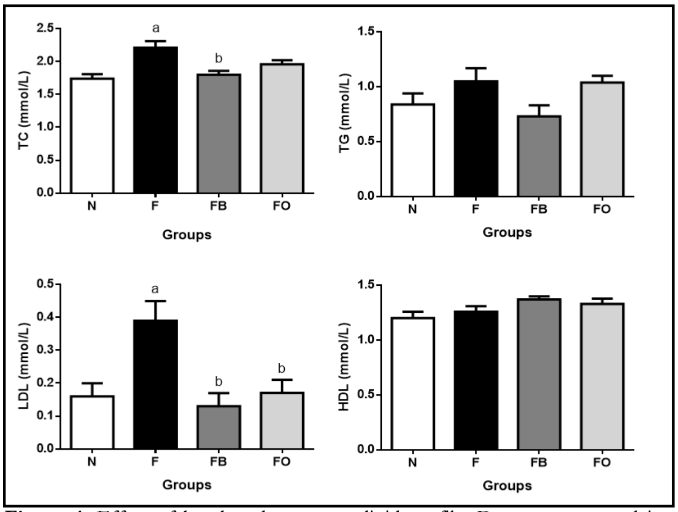

Figure 1: Effect of bee bread on serum lipid profile. Data are presented in mean (standard deviation), n=8 per group. TC; total cholesterol, TG; triglyceride, LDL; low-density lipoprotein, HDL; high-density lipoprotein. N; normal, F; high-fat diet; FB; high-fat diet and bee bread at 0.5g/kg/day, FO; high-fat diet and orlistat at 10 mg/kg/day. [a] $P<0.05$ compared with the normal group, [b] $P<0.05$ compared with the F group. Data was analysed using One-way ANOVA followed by Tukey post-hoc test.

Effects of bee bread on the levels of cardiac oxidant/ antioxidant status and fatty acid synthase

There was a significant increase in cardiac MDA level of the F group compared to the N group. Among the supplemented groups, the FB group showed a significant decrease in cardiac MDA level, whereas, in the FO group, the cardiac MDA level showed no significant change when compared to the F group. Cardiac SOD activity was significantly decreased in the F group compared to the N group. The activities of cardiac CAT and GPx showed no differences in the F group when compared to the N group. The FB group improved these antioxidant enzyme activities, as evidenced by significant increases in cardiac SOD, GPx, and CAT activities when compared to the F group. The FAS activity was significantly increased in the F group compared to the N group, and the level was significantly decreased in the FB group when compared to the F group (Table 2).

Table 2: Effects of bee bread on cardiac oxidant/antioxidants status and fatty acid synthase activity.

Parameters	N	F	FB	FO
Malondialdehyde (nmol/mg protein)	0.09 (0.01)	0.12 (0.03)[a]	0.09 (0.01)[b]	0.11 (0.03)
Superoxide dismutase (U/mg protein)	4.79 (1.40)	2.97 (0.63)[a]	4.19 (0.89)[b]	4.79 (0.92)[b]
Catalase (U/mg protein)	0.97 (0.25)	0.72 (0.30)	1.17 (0.31)[b]	0.83 (0.28)
Glutathione peroxidase (U/mg protein)	440.40 (14.62)	431.11 (3.53)	583.57 (20.39)[a,b]	566.79 (17.48)[a,b]
Fatty acid synthase (pg/ml)	509.80 (137.95)	954.66 (393.78)[a]	524.92 (279.53)[b]	716.09 (370.65)

Data are presented in mean (standard deviation), *n*=8 per group. N; normal, F; high-fat diet; FB; high-fat diet and bee bread at 0.5 g/kg/day, FO; high-fat diet and orlistat at 10 mg/kg/day. [a] $P < 0.05$ compared with the normal group, [b] $P < 0.05$ compared with the F group. Data was analysed using One-way ANOVA followed by Tukey post-hoc test.

Effects of bee bread on total fat pad and adiposity index

The F group exhibited significant increases in abdominal fat, TFP, and adiposity index when compared to the N group. In comparison to the F group, the weight of epididymal fat was significantly lower in the FB group. Conversely, the TFP was significantly lower in the FB and FO groups in comparison to the F group. In addition, all groups that received the F diet had a significantly higher adiposity index in comparison to the N group (Table 3).

Table 3: Effects of bee bread on total fat pad and adiposity index.

Parameters	N	F	FB	FO
Abdominal fat (g)	3.31(1.54)	8.48(3.9)[a]	5.77(1.72)	6.18(1.46)
Epididymal fat (g)	3.32(0.92)	5.15(0.57)	2.97(1.28)[b]	3.89(1.29)
Total fat pad(g)	7.63(1.35)	13.63(3.87)[a]	8.74(2.37)[b]	10.07(1.30)[b]
Adiposity index	1.93(0.78)	3.66(1.47)[a]	3.35(0.88)[a]	3.20(0.60)[a]

Data are presented in mean (standard deviation), n=8 per group. N; normal, F; high-fat diet; FB; high-fat diet and bee bread at 0.5 g/kg/day, FO; high-fat diet and orlistat at 10 mg/kg/day. [a] $P<0.05$ compared with the normal group, [b] $P<0.05$ compared with the F group. Data was analysed using One-way ANOVA followed by Tukey post-hoc test.

Effects of bee bread on the histology of heart and adipose tissue

The N group exhibited a structurally arranged and well-organized pattern of the cardiac fibers. Whereas the F group demonstrated the presence of necrotic patches encircled by inflammatory cells, lipid droplets, and foam cells. The cardiac fibers in the FB and FO groups showed the presence of small cholesterol cleft in between cardiac fibers, with the absence of necrotic patches in these groups (Figure 2). Figure 3 depicts normal size and organization of adipose tissue in the N group, along with the appearance of a signet ring characterised by a thin rim of cytoplasm with the nucleus at the cell edge. The adipocyte size was larger in the F group in comparison to the N group and smaller in the FB and FO groups in comparison to the F group. Significant findings were similarly found for the mean adipocyte surface area among the groups (Table 4).

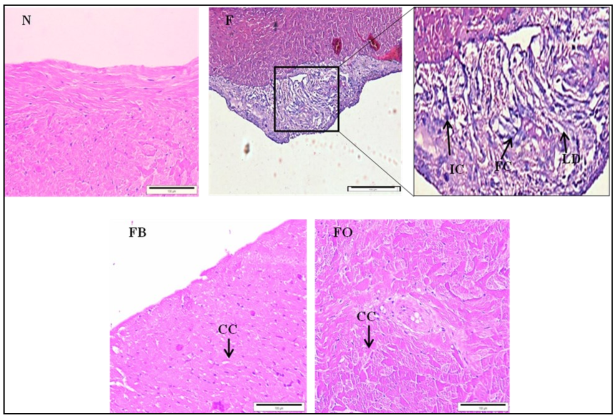

Figure 2: Histology of myocardial tissue in all groups. The N group shows a normal appearance of myocardium fibers with no pathological changes. The F group shows the presence of a necrotic patch, which consists of foam cells (FC), inflammatory cells (IC), and lipid droplets (LD) (black arrow). The dark blue staining indicates the inflammatory cells that infiltrate surrounding the necrotic area. The FB and FO groups show the presence of minimal cholesterol cleft (CC) in between the myocardium fibers. N; normal, F; high-fat diet, FB; high-fat diet and bee bread at 0.5 g/kg/day, FO; high-fat diet and orlistat at 10 mg/kg/day. (Haematoxylin and Eosin staining, magnification ×100, scale bar=100 μm).

Table 4: Effects of bee bread on surface area of adipocyte.

Parameters	N	F	FB	FO
Surface area ($\times 10^{-8}$ mm^2)	0.29 (0.09)	1.92(0.24)[a]	0.57(0.21)[a,b]	0.69(0.12)[a,b]

Data are presented in mean (standard deviation), *n*=8 per group. N; normal, F; high-fat diet; FB; high-fat diet and bee bread at 0.5 g/kg/day, FO; high-fat diet and orlistat at 10 mg/kg/day. [a] P<0.05 compared with the normal group, [b] P < 0.05 compared with the F group. Data was analysed using One-way ANOVA followed by Tukey post-hoc test.

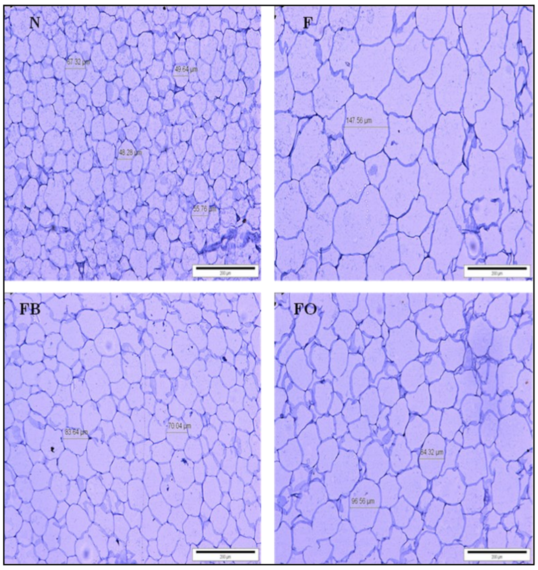

Figure 3: Photomicrograph of adipose tissue in all groups. The N group shows normal signet ring appearance of adipocyte cells. The F group shows larger adipocyte cell size. The FB and FO groups show smaller adipocyte cell size compared to the F group. N; Normal, F; high-fat diet, FB; high-fat diet and bee bread at 0.5 g/kg/day, FO; high-fat diet and orlistat at 10 mg/kg/day. (Haematoxylin and Eosin staining, Magnification = ×200, Scale bar = 200 μm).

DISCUSSION

Obesity-related cardiovascular complications such as hypertension and atherosclerosis have been implicated in the highest death rate. [18] Our study aimed to assess the hypolipidaemic and cardioprotective effects of bee bread harvested from stingless bees (*H.itama*) in high-fat diet-induced obese rats. Obesity was confirmed to be successfully established as the calculated Lee obesity index had exceeded the value of 315, reflecting a high level of body fat.[15] Our finding indicated that the Lee obesity index increased significantly in the F group compared to the N group, which was consistent with the earlier study's findings.[19] Meanwhile, the FB group demonstrated an improvement in the Lee obesity index as evidenced by a significant decline in this index when compared to the F group, and this finding was not observed in the FO group. This might indicate that bee bread had a greater ability to reduce fat buildup in comparison to orlistat. The amount of food consumed by each experimental group did not significantly vary. This might indicate that the supplementation of bee bread and orlistat did not interfere with or suppress the feeding. The present study showed a significantly higher calorie intake found in groups that received F diet (F, FB, and FO groups) in comparison to the N group. This finding might imply that the F diet, containing 31% of total fat energy, had contributed to excessively generated energy in these groups. This, in turn, raised the need to accommodate the surplus of lipids, which in turn caused a rise in the number or size of adipocyte cells.

Hypercholesterolaemia is defined as an elevated level of cholesterol, and it is the commonest risk factor for the progression of CVD.[18] The risk of death implicated by CVD could potentially be decreased by lowering the level of circulating cholesterols. Our finding demonstrated that the F group had significantly higher levels of TC and LDL when compared to the N group. High dietary fat consumption has been found to increase the concentration of circulated free fatty acids and their uptake by hepatocytes, leading to increased TG and very low-density lipoprotein (VLDL) production.[20] The elevated amount of LDL seen in the F group of the present study might be explained by the excess VLDL in plasma, which in turn increased the catabolism of fat into LDL- and intermediate-density lipoprotein.[20] The isolated elevation of TC concentration found in the F group might also be associated with the elevation of LDL.[21] Interestingly, the FB group showed significantly lower TC and LDL levels compared to the F group, indicating that bee bread possesses hypolipidaemic property. The circulating LDL is shown to majorly contribute to the early onset of CVD, due to its strong atherogenic property. Hence, administration of bee bread and orlistat for 6 weeks in F diet-induced obese rats was able to reduce the elevated atherogenic LDL- level as a significant reduction of LDL level was found in FB and FO groups in comparison with the F group.

Obesity-related hypercholesterolaemia is thought to be a key amplifier for developing cardiac failure due to adverse cardiac remodeling, which leads to worse health outcomes.[22] Lipid peroxidation has been evoked by an increase in fat storage and circulating fatty acids. It can react with an oxygen molecule to produce a highly radical, unstable form of fatty acid.[23] Lipid peroxidation signifies an increment in the MDA level, one of the crucial markers for ROS. In the present study, the cardiac MDA level in the F group was significantly increased, with a concomitant decrease in cardiac SOD activity when compared to the N group. A significant decrease in cardiac SOD in the F group could be due to its increased utilization in scavenging free radicals or due to the destruction of the primary antioxidant system by ROS. The mismatch between oxidant and antioxidant status might also account for the development of a necrotic patch area observed in the myocardium of the F group, which was associated with the infiltration of the inflammatory cells. This could be explained by the oxidative stress environment, which could cause cellular function and structure changes, leading to cellular lipid membrane disruption.[24] Obesity-related cardiac necrosis in the present study was related to the accumulation of TC and LDL in non-adipose tissues or cells such as myocardial cells. These accumulated lipids might be transported by the fatty acid transporter proteins such as FAT/CD36, fatty acid transport proteins 1,4, and 6.[25] Our study found that bee bread might have the greater potentiality to combat lipid peroxidation or oxidative stress compared to orlistat as indicated by a significant decrease in the cardiac MDA level in the FB group. This finding was associated with significant increases in cardiac SOD, CAT, and GPx activities demonstrated in the FB group, suggesting a suppression effect of antioxidant enzymes against oxidative stress. These results could account for the improvement in the myocardium histology in the FB group, in which only minor cholesterol clefts were observed. These findings might also indicate that bee bread not only reduced TC and LDL levels by its anti-hypercholesterolaemic effect but also improved cardiac antioxidant status and decreased lipid peroxidation, thus sparing the cardiac tissue from further damage, which was comparable to orlistat.

A high level of adiposity index corresponds to increased fat accumulation.[26] This is further supported by a significant increase in TFP value concomitant to the significant increases in size and surface area of the adipocyte in the F group compared to the N group. The present study indicated that the abdominal fat was the major site for fat deposition in comparison to epididymal fat, which was remarkably increased in the F group. Significant reductions were also seen in the TFP value and adipocyte size in the FB and FO groups, which might indicate that bee bread and orlistat could dampen the development of large adipocytes in rats fed with the F diet. The FAS catalyses the synthesis of fat by mediating the creation of *de novo* saturated long-chain fatty acids, which are the final byproduct of acetyl and malonyl-CoA.[27] In the present study, cardiac FAS was found to be significantly elevated in the F group with respect to the N group, which could be responsible for its high levels of TC, LDL, TFP and adiposity index, with concomitant larger size and surface area of adipocyte cell. The finding was consistent with the previous study, in which cardiac FAS activity was responsible for the increased TC and TG concentrations as well as the deposition of cardiac lipid in Oil-Red O-stained sections following the increased de novo long-chain fatty acids synthesis in the cardiac cells in mice fed with high-fat diet.[28] In contrast, a significant decrease in FAS activity was found in the FB group, which could explain for the lower levels of TC and LDL, and the reduced size and surface area of adipocytes that were comparable to the FO group. Therefore, in the present study, it is plausible to suggest that one of the contributing factors in lipid metabolism changes is due to the presence of the bioactive compounds in bee bread which might have an inhibitory effect against FAS. However, future study is needed to precisely determine the presence of its bioactive compounds as well as its effect on lypolytic enzyme activity.

CONCLUSIONS

Administration of bee bread at 0.5 g/kg/day for 6 weeks significantly improved the Lee obesity index, total fat pad, lipid profile, cardiac oxidant/antioxidant status as well as histology of heart and adipose tissues in F diet-induced obese rats. These findings might be related to the hypolipidaemic and cardioprotective effects of bee bread, probably due to its modulatory activity on FAS, which demands future study to determine its exact molecular mechanism of action.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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