

Methanolic Extract of *Clinacanthus nutans* Leaves can Alter Adipocyte Cellularity, Inflammation and Acetyl Cholinesterase Activity in Male Obese Mice

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Abstract

Background: The adverse effects of obesity on human health necessitate the development of effective interventions for the prevention and treatment of obesity. Mixture of natural products, including crude extracts and isolated pure natural compounds can cause a body weight reduction and prevent diet-induced obesity. Consequently, they have been widely utilized in treating obesity.

Methods: A total of sixty imprinting control region (ICR) male mice (39.01 ± 1.03 g BW) were divided into six groups (10 per group) and randomly assigned to either a Normal diet (ND), High fat diet (HFD)+normal saline, HFD +Orlistat (15.9 mg/kg BW/day), HFD+CN500, 1000 and 1500 mg/kg BW/day, and fed daily for three weeks. Adipose cellularity was determined by histological methods (Haematoxylin and Eosin), whereas plasma lipid profiles were measured by using a Hitachi 902 automatic clinical analyzer, meanwhile cytokines with a Benchmark Plus Microplate Spectrophotometer (RT-2100C) and AChE was detected in muscle and heart by using acetylcholine as a substrate.

Results: Mice supplemented with HFD+CN1000 and 1500 mg/kg had significantly lower adipocyte area, size, and diameter compared to those fed other diets. The mean adipocyte number of mice supplemented with *C. nutans* was significantly lower compared to the control groups. Mice fed HFD+CN1500 mg/kg had a greater adipocyte cell count compared with those supplemented with HFD+CN1000 and 500 mg/kg. Supplementation of *C. nutans* reduced plasma total cholesterol in mice. Diets had no effect on plasma lipid profile. High dose of methanolic extract have more ant obesity properties with better effects on adipocyte cellularity, cytokines, acetylcholinesterase than low dose. In addition, The *C. nutans* fed mice exhibited decrease but non-significant (P>0.05) in leptin, meanwhile the acetyl cholinesterase activity in the heart and muscle was greater in mice fed *C. nutans* compared with control groups.

Conclusion: Methanolic extract of *C. nutans* leaves can preclude diet-induced obesity.

Keywords: Obesity; *Clinacanthus nutans*; Snake grass; Adipose cellularity; IL6; Leptin; Acetyl cholinesterase

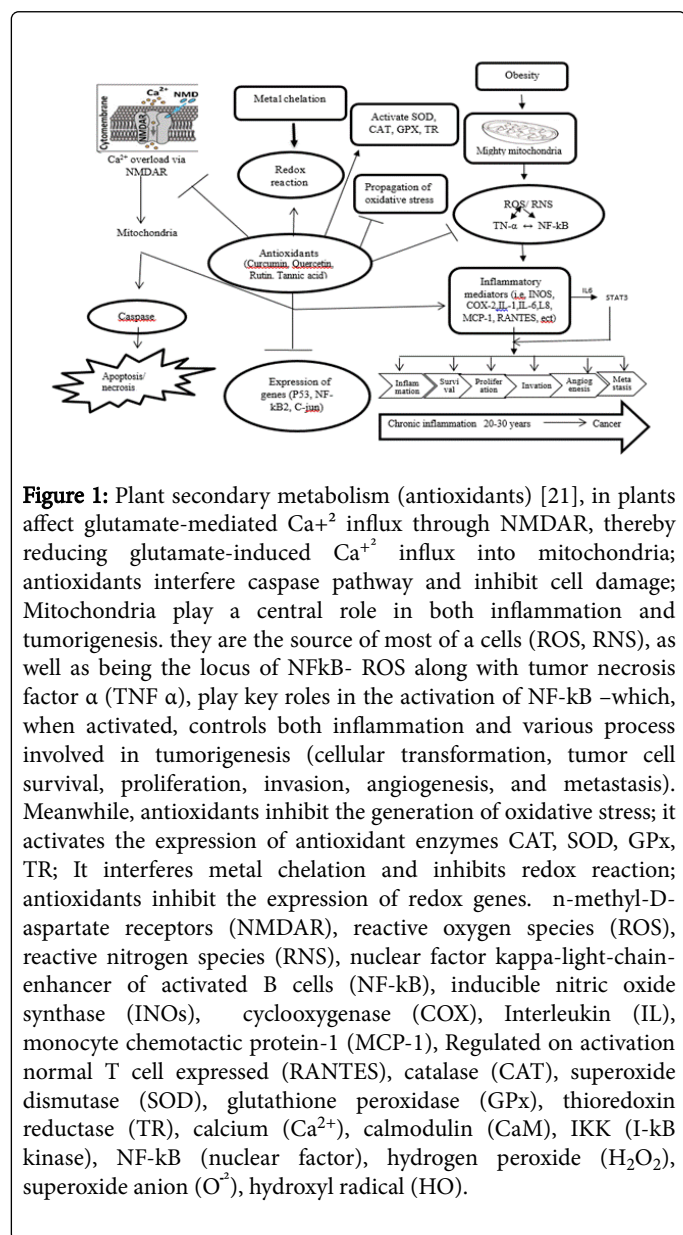
Abbreviations CN: *Clinacanthus nutans*, ICR: Imprinting Control Region, ND: Normal Diet, HFD: High-Fat Diet, BW: Body Weight, TC: Total Cholesterol, TG: Triglycerol, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, IL-6: Interlukin 6, AchE: Acetyl Cholinesterase

Introduction

Obesity is a medical condition characterized by dysregulation of lipid metabolism and excessive increase in adipose tissue mass [1]. Obesity is prevalent in both developed and developing countries [2] and it has deleterious effects on human health, which can cause

reduced life expectancy [3,4]. Though the effects and extents of obesity are still controversial, it is well established that obesity has reached epidemic proportions across the world [5]. In [6] the World Health Organization (WHO) estimated that there were about more than 600 million people classified as obese and 1.9 billion overweight, including 41 million children under the age of five. The prevalence of obesity is on the increase in many parts of the world. There was an increase in the average annual change ranging from 0.2% to 18.5% in developed countries and 0.1% to 35.3% in developing countries [2]. The World Health Organization [7], recognized obesity as a disease in 2007. Nonetheless, recently overweight and obesity is the fifth leading cause of deaths worldwide [8]. To date, the complications related to obesity contribute to the 100,000 to 400,000 deaths per year [9]. Currently, diets high in calories, especially from carbohydrate, saturated fatty acids (SFAs), and long-chain omega-6 polyunsaturated fatty acids

(PUFAs), and sedentary lifestyles lacking physical activity contribute significantly to this obesity [10]. Endogenous and exogenous agents that enhance preadipocyte proliferation or adipocyte hypertrophy have the capability to increase white adipose tissue (WAT) mass and the development of obesity [10]. The expansion of adipose tissue produces numerous bioactive substances, known as adipocytokines or adipokines, which may lead to the growth of various metabolic diseases through altered glucose, lipid homeostasis and inflammatory responses (Dandona et al.). It has been documented that the plasma concentration of inflammatory mediators, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), is increased in obesity and type 2 diabetes [11]. Treatment of obesity is beneficial because the weight loss reduces the hazard of mortality and morbidity [12].



Recently, the Food and Drug Administration (FDA) has approved Orlistat and Sibutramine drugs, which both can induce serious side effects, such as palpitation and hypertension [12]. To ward off the undesirable side effects of these two drugs, dietary concepts look to

play an important role in weight control programs [13]. Alternative medicine and adverse effects of conventional western medicine (WM) [14]. Consequently, a rapidly expanding field in therapeutics is in the use of natural supplements [15,16]. The utilization of plants in different traditional medicine of various cultures has been comprehensively documented [17]. The efficacy of medicinal plants could be attributed to the similarities between plant and mammalian biochemistry and molecular functioning, specifically, the many molecular signaling pathways that are preserved between the taxa and play roles in the synthesis of secondary metabolite within plants [18]. Medicinal plants contain polyphenols, which consist of tannins and flavonoids, which are widely found as secondary metabolites in plants [19]. However, is highly subjective and, therefore these phenolic substances derived antioxidant supplements [20], have been described to quench free radicals and prevent disease like anti-inflammatory, anti-hyperglycemic and anti-hyperlipidemic properties (Figure 1) [21].

The complex pathogenesis of obesity indicates the need of different intervention strategies to confront this problem. Herbal supplements and diet-based therapies for weight loss are among the most common complementary and alternative medicine modalities [22]. The efficacies of medicinal plants in the treatment of obesity are highly variable and inconsistent in the published literature. It was surprising to observe dietary polyphenols are secondary metabolites of the plants have anorectic properties effecting a loss in body weight [23]. To address these concerns, supplementation of chlorogenic acid lowered the body weight gain and adipose weight compared to the high-fat diet HFD control group and caffeic acid group [24]. In chloroplast, thylakoids contain a hundred different membrane proteins, galactolipids and sulpholipids as well as various vitamins (A, E and K), thus a variety of bioactive compounds that could be responsible for the retardation of fat digestion [25], as well as the reduction in the size of the abdominal fat cell could be attributed to the bioactivities of the polyphenolic compounds [19,26]. Different plants contain a large variety of several components with different anti-obesity effects on body metabolism and fat oxidation, and for this reason have been investigated and reported to be useful in the treatment of obesity [27]. This justifies the need for additional studies using novel medicinal plants to give room for tailored decisions and informed choices in the utilization of medicinal plants for the treatment of obesity. One of the medicinal plants of interest is known as *C. nutans*. Lindau (Family: Acantheceae) commonly known as Sabah snake grass [28]. This plant is widely used for treating skin rashes, scorpion and insect bites, gastrointestinal complications, dysuria, menstrual function, anemia, jaundice, setting of fractured bones, diabetes mellitus, fever and diuretics [29]. Numerous bioactive have been demonstrated in *C. nutans* such as phenolics, flavonoids, sitosterol, stigmaterol, and chlorophyll derivatives [29]. In addition, The C-glycosidic flavones such as shaftoside, isoorientin, orientin, isovitexin, and vitexin have been found to be the major flavonoids in the leaves of *C. nutans* [30,31], although protocatechnic acid was one of the most abundant [29]. Nonetheless, despite the established polyphenols in *C. nutans* there is a dearth of information on the anti-obesity properties of the polyphenols. Despite all the known biological activities from previous work, emerging lay commendation and Malaysian newspaper reports indicated that *C. nutans* possesses weight loss (appetite suppression, fat and calorie burning and carb blocking properties) and lowers blood cholesterol levels effect. Nonetheless, these testimonies were not backed up by scientific evidence. We hypothesize that *C. nutans* would affect adipocyte cellularity, lipid profile and acetylcholinesterase activity in muscle and heart of diet-induced obese mice. Thus, the

primary aim of this study was to determine the effects of *C. nutans* adipocyte cellularity, lipid profile and acetylcholinesterase enzyme activity in diet-induced obese mice.

Materials and Methods

Plant materials

Fresh leaves of *C. nutans* (*Burm.f.*) Lindau was harvested at botanical garden in Sermpun II, UPM. Selangor, Malaysia. The botanical identify of *C. nutans* was characterized by the phytomedicinal herbarium, Institute of Bioscience, Universiti Putra Malaysia, Selangor (Voucher no. SK2942/15).

Preparation of extracts

The *C. nutans* leaves were freshly harvested and oven-dried at 40-45°C. The leaves were ground to fine powder and sequentially soaked in methanol and distilled water, incubated for 72 h at room temperature with the gentle shaking, then filtered using filter paper (Whatman No. 1, Fitchburg, WI, USA), and extracted under compact pressure, by using a R-215 rotating evaporator (Buchi, Flawil, Switzerland), and the plants extracts were kept after dehydration and lyophilization by freeze drying machine (Labconco Free Zone 6Pluse Freeze Dry), at -20°C until all tests were performed.

Animals and ethical considerations

Sixty male ICR mice at three weeks of age were purchased and acclimatized for one week prior to the commencement of the trial. They were housed in an air-conditioned animal house with a 12 h light/12 h dark cycle at 22 ± 1°C and 50 ± 10% humidity. Two mice were housed per cage. The use and handling of animals in this study had been approved by the Animal Ethics Committee of Universiti Putra Malaysia (reference no UPM/IACUC/AUP-R083/2016).

Experimental design body weight monitoring and sample collection

To induce obesity, the mice were fed with high-fat diet (HFD) (Rodent Diet D12451 with 45% kcal from fat) (Research Diets, New Brunswick, NJ, USA) [32].

Group number	Group name	Oral feeding daily
1	HFD+distilled water	Positive control
2	ND +distilled water	Negative control
3	HFD+Orlistat	Positive control
4	HFD+CN 500 mg/BW	Low concentration
5	HFD+CN1000 mg/BW	Medium concentration
6	HFD+CN1500 mg/BW	High concentration

CONTROL groups without *C. nutans* (HFD: High Fat Diet; ND: Normal Diet; HFD+Orlistat: High Fat Diet with orlistate). Whereas treatment groups: High Fat Diet(HFD) with the supplementation *C. nutans* (CN); HFD+CN 500: Low concentration *C. nutans*; HFD+CN1000: Medium concentration *C. nutans*; HFD +CN1500: High concentration of *C. nutans*

Table 1: *C. nutans* experimental protocol.

And mice were given with normal chow diet (Rodent Diet D12450B with 10% kcal from fat) (Research Diet, New Brunswick, NJ, USA). In the therapeutic strategy, the male ICR mice were fed the HFD for 16 weeks to induce obesity. Body weights were measured two times a week and housed in 2 mice/cage. The average food intakes were calculated every week. The sample concentrations, duration, and the complete experimental design are shown in (Tables 1 and 2).

Nominal concentration (mg/kg)	Actual concentration (mg/ml)
500	19.5 (0.122)
1000	39 (0.244)
1500	58.5 (0.366)

Table 2: Actual concentration of *C. nutans*.

Methanolic extract of *C. nutans* was dissolved in distilled water while Orlistat was dissolved in ethanol 20 mg/ml. A total of sixty ICR male mice (39.01 ± 1.03) g body weight were divided into six groups (10 per group) and then were randomized to receive the normal diet (ND), and high-fat diet (HFD) control mice were treated with vehicle (normal saline), or Orlistat was used as positive treatments of obesity (15.9 mg/kg per day) [32] and HFD plus *C. nutans* (500, 1000 and 1500 mg/kg per day), daily for another three weeks and administered orally by the oral gavages. Body weight and daily food intake were measured twice per week. After twenty-one days, each mice was made to fast for overnight after the last treatment and then performed under Ketamine and Xylazine (1:10 v/v) anesthesia and sacrificed by exsanguinations of the jugular vein after blood sample collection. Blood samples were collected using syringes with 26-gauge needles and transferred into vacutainer tubes treated with ethylene diamine tetraacetic acid (EDTA, Becton Dickinson, Franklin Lakes, NJ). The blood samples were placed on ice then centrifuged at 3000 rpm for 15 min at 4°C to separate the plasma, Plasma samples were transferred into Eppendorf tubes and stored at -80°C until analysis. The gross morphology examination was performed and the absolute weight of livers and spleens were measured. The peri-epididymal fat, liver, heart and muscle were snap frozen in liquid nitrogen and stored at -80°C. Tissues were either fixed in buffered formalin or processed for histology. The experimental protocols were conducted in accordance with internationally accepted principles for laboratory animal use and care.

Adipocyte measurement

To determine the effects of treatment on adipocyte morphology, visceral and subcutaneous fat was excised immediately after euthanasia with some modified [19]. For analysis of adipocyte size, fat pads of mice from each group were fixed in formalin 10%, and then samples were fixed in Acetone 1:10 for 72 hours, processed by automated tissue processor (Leica, Germany). Molds were prepared by embedding in paraffin Wax and 5 µm thick sections were sliced by Leica tissue microtome. Finally, the slides were kept in an oven at 50°C then stained with Hematoxylin and Eosin. Sections were examined and pictures captured under a light microscope (Motic BA410:Imager ICC50). Adipocytes enumerated and their diameters measured using Image J software (National Institute of Health, USA). While mean an input image of the already prepared slide was taken from the camera attached to the microscope, the original colored input images were

converted into grayscale images from the range starting from (0-255 pixel) and this area represents the white blood cells. The mean adipocyte diameter was calculated as an average of the diameters. To ensure the accuracy of measurement, three images of each animal were analyzed by three different investigators and averaged into a single measurement [33]. Measurements were obtained from three individual animals per group.

Plasma biochemical measures

Measurement of lipid profile: Plasma levels of total cholesterol, triglycerol and low-density lipoprotein (LDL) were determined in duplicate using a Hitachi 902 automatic clinical analyzer (Boehringer, Mannheim, Germany) and commercially available kits (Roche, Mannheim, Germany), high-density lipoprotein values were calculated by subtracting from (Cholesterol-LDL-Triglycerol/2.19).

Measurement of cytokines: Plasma interleukin (IL-6) and leptin concentrations were determined with mouse ELISA kits. Mouse ELISA kits (RayBiotech, USA) were used for IL-6 (sensitivity 2 pg/ml) and leptin (sensitivity 4 pg/ml). The assays were conducted in 96-well microplates according to the manufacturer's instructions, with a Benchmark Plus Microplate Spectrophotometer (RT-2100C).

Preparation of cytosolic fraction of muscle and heart

Preparation of tissue homogenates: muscle and heart tissues were quickly dissected and frozen in dry ice. Frozen samples were weighed and transferred to 50 mL polypropylene tubes (Falcon; Becton Dickinson, Lincoln Park, NJ) containing 0.1 M cold phosphate buffer saline pH 8 (PBS, Sigma USA), (4°C) at a ratio of 4 mL buffer/g wet tissue, and added 1 μ L cold phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich UK). Samples were homogenized for 30 seconds with an electrical homogenizer (Polytron; Brinkmann Instruments, Westminister, NY). Differential centrifugation technique was used to prepare the subcellular organ cytosolic fractions according to the method of [34] with some modifications. Centrifuged at 15000 rpm for 20 minutes at 4°C to separate the cellular. The pellet was discarded and the supernatant was taken and used to determine AChE activity. The protein concentration of content of each cytosolic fraction from muscle and heart was determined by the method of Bradford [35].

Determination of acetylcholine esterase activity

The determination of acetyl cholinesterase (ATC) activity was conducted according to the method described by Ellman et al. [36] adapted for reading on a micro plates reader RT-2100C. The final concentration of the acetylcholine iodide (Sigma, Aldrich) substrate employed was 0.5 mM, while that of the 5,5 Dithio- bis (2-nitrobenzoic acid) DTNB (Sigma- Aldrich) color reagent was 0.067 mM in 0.1 M potassium phosphate buffer pH 8, for both tissues. Absorbance was determined in micro plate reader RT-2100C at 405 nm and the enzyme activity was expressed in μ mol min⁻¹ mg⁻¹.

Statistical analysis

Data obtained from the Adipocyte, plasma biochemical, acetyl cholinesterase, were analyzed using the analysis of variance (ANOVA) procedure of the SAS software package, version 9.1 (SAS Institute Inc., Cary, NC). Significantly different mean values were further elucidated using Duncan's test. The results were expressed as mean and pooled standard error of the means (SEM). Differences were considered to be

statistically significant when <0.05 . Therefore, we conducted univariate linear and nonlinear ANOVA with polynomial contrast. Besides linear trends, this method also examines quadratic (U-shaped) trends [37]. The linear contrast compared the lowest with the highest category and the quadratic compares both middle with the highest and the lowest categories together [38].

Results

Adipocyte cellularity

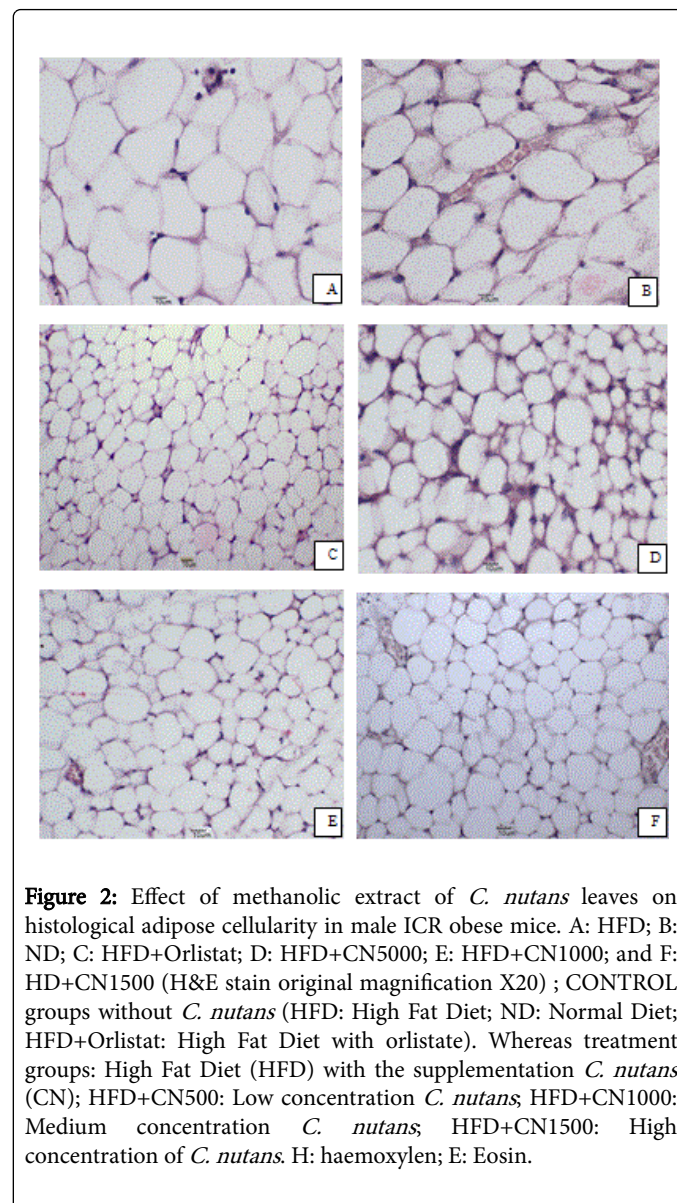


Figure 2: Effect of methanolic extract of *C. nutans* leaves on histological adipose cellularity in male ICR obese mice. A: HFD; B: ND; C: HFD+Orlistat; D: HFD+CN5000; E: HFD+CN1000; and F: HD+CN1500 (H&E stain original magnification X20); CONTROL groups without *C. nutans* (HFD: High Fat Diet; ND: Normal Diet; HFD+Orlistat: High Fat Diet with orlistate). Whereas treatment groups: High Fat Diet (HFD) with the supplementation *C. nutans* (CN); HFD+CN500: Low concentration *C. nutans*; HFD+CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*. H: haemoxylene; E: Eosin.

Based on the results obtained the area size, diameter and number of the adipocytes in male ICR obese mice. The data clearly indicated that the higher concentration of *C. nutans* had a significant effect on fat adipocytes cellularity.

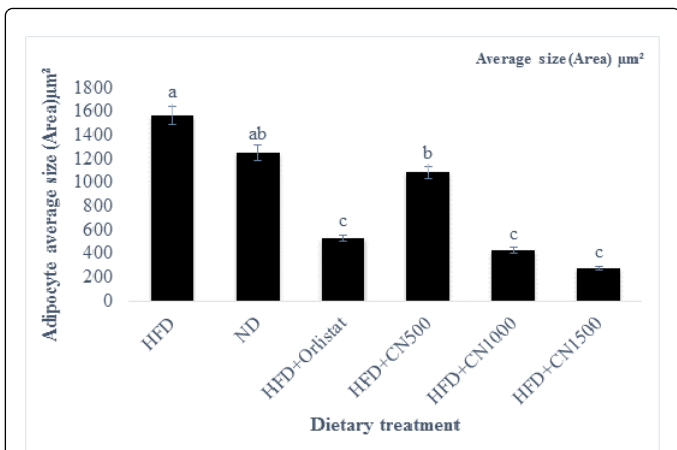


Figure 3: Effects of a methanolic extract of *C. nutans* leaves at three concentrations on adipocytes size (area) in male ICR obese mice compared to the CONTROL groups. The data represent the mean \pm standard error of the means (SEM) of the cell area. $n=10$ per treatment group. The data are presented as Mean \pm SEM; Bars labeled with different letters are statistically different ($P<0.05$). CONTROL groups without *C. nutans* (ND: normal diet; HFD: High Fat Diet; HFD+Orlistat: High Fat Diet with orlistat). Whereas treatment groups: High Fat Diet (HFD) with the supplementation *C. nutans* (CN); HFD+CN 500: Low concentration *C. nutans*; HFD +CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*.

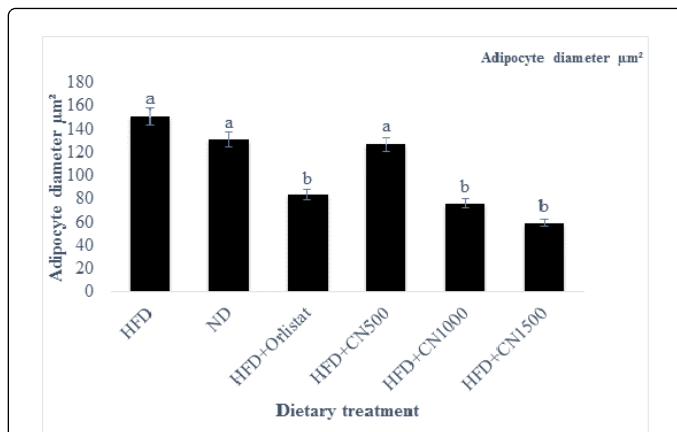


Figure 4: Effects of methanolic leaf extract of *C. nutans* at three concentrations on the adipocytes diameter of male ICR obese mice compared to CONTROL groups. The data represent the mean \pm standard error of the means (SEM) cell diameter, $n=10$ per treatment group. The data are presented as Mean \pm SEM; Bars labeled with different letters are statistically different ($P<0.05$). Control groups without *C. nutans* (ND: normal diet; HFD: High Fat Diet; HFD+Orlistat: High Fat Diet with Orlistat). Whereas treatment groups: high-fat diet (HFD) with the supplementation *C. nutans* (CN); HFD+CN500: Low concentration *C. nutans*; HFD +CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*.

Treated groups HFD+CN1000 and HFD+CN1500 mg/kg have significantly ($P<0.05$) lowered the adipocytes area size and diameter compared to the HFD+500 mg group and HFD and ND CONTROL group are shown in (Figures 2-5) respectively. As the concentration of methanolic extract of *C. nutans* leaves increased, the adipocytes area size and diameter were decreased while cell numbers increased. Treatment with HFD+CN1500 mg/kg has the highest adipocyte cell count compared to the other treatment groups from HFD+CN500 and HFD+CN1000 mg/kg (Figure 5). The mean adipocyte number of *C. nutans* supplemented groups was significantly ($P<0.05$) higher compared to the HFD control groups.

Plasma biochemical measures

Plasma lipids profile: The effects of methanolic extract of *C. nutans* leaves on plasma TC, TG, LDL and HDL for mice were reported in Table 3 The plasma TC, TG and HDL in the treatment *C. nutans* groups were slightly decreased compared to HFD CONTROL. There were no significant ($P>0.05$) differences noted for plasma TC, TG, LDL, and HDL between treatment and HFD and ND CONTROL groups.

Measurement of cytokines

Interleukin IL6 and Leptin: The overall effect of *C. nutans* feeding on IL-6 level of obese mice is depicted in (Figure 6). It is evident that IL-6 was reduced by increasing concentration of dietary *C. nutans* HFD+CN1500 mg/kg significant ($P<0.05$) difference in linear. However, the IL-6 level of mice from the control and *C. nutans* dietary groups were no significantly ($P>0.05$) different.

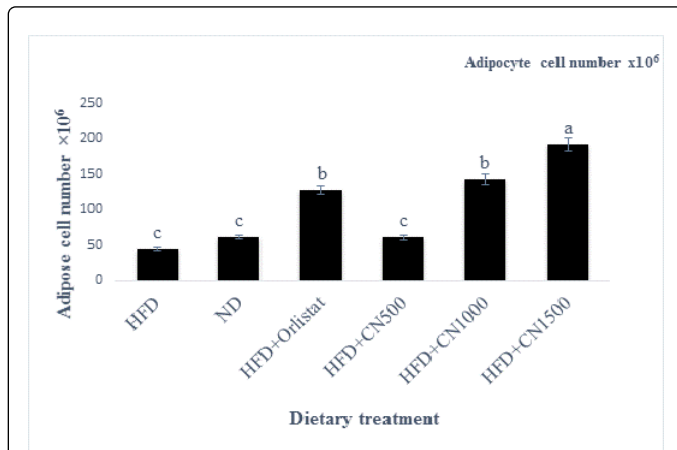


Figure 5: Effects of a methanolic extract of *C. nutans* leaves on adipocyte cell number of male ICR obese mice compared to CONTROL groups. The data represented the mean \pm standard error of the means (SEM) cell number, $n=10$ per treatment group. The data are presented as Mean \pm SEM; Bars labeled with different letters are statistically different ($P<0.05$). CONTROL groups without *C. nutans* (ND: Normal Diet; HFD: High Fat Diet; HFD +Orlistat: High Fat Diet with orlistat). Whereas treatment groups: High Fat Diet (HFD) with the supplementation *C. nutans* (CN); HFD+CN500: Low concentration *C. nutans*; HFD+CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*.

The leptin levels in mice serum were examined (Figure 7). The data showed that the *C. nutans* treated groups have significant lower leptin levels compared to CONTROL ND and HFD groups. As the concentration of *C. nutans* increased, the leptin level was decreasing.

There were no significant ($P>0.05$) differences noted for the leptin plasma in HFD+CN 500, 1000 and 1500 mg/kg with HFD and ND control groups. The leptin level concentrations were the same across all treatment groups ($P>0.05$).

Lipid profile	HFD	ND	HFD+Orlistat	HFD+CN500	HFD+CN1000	HFD+CN1500	SEM	P-Values	
								Linear	Quad
TC (mmol/L) ^{n.s}	5.85	3.48	3.43	4.39	4.33	5	0.77	0.63	0.73
TG. (mmol/L) ^{n.s}	2.44	1.59	1.83	1.33	1.23	1.51	0.52	0.81	0.77
L.D.L. (mmol/L) ^{n.s}	0.75	0.5	0.61	0.61	0.79	0.88	0.25	0.474	0.88
H.D.L (mmol/L) ^{n.s}	3.99	2.25	1.98	3.18	2.98	3.44	0.61	0.77	0.67

Values expressed as mean \pm standard error of the means (SEM). There were no statistically significant differences between the measurements in different groups. ^{n.s}=no significant difference letter. The significant value was set at $P<0.05$; Control groups without *C. nutans* (ND: Normal Diet; HFD: High Fat Diet; HFD+Orlistat: High Fat Diet with Orlistat. Whereas treatment groups: High Fat Diet with the supplementation *C. nutans*. HFD+CN500: Low concentration *C. nutans*, HFD+CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*

Table 3: Effects of oral administration methanolic leave extract of *C. nutans* on lipid profile in male ICR obese mice.

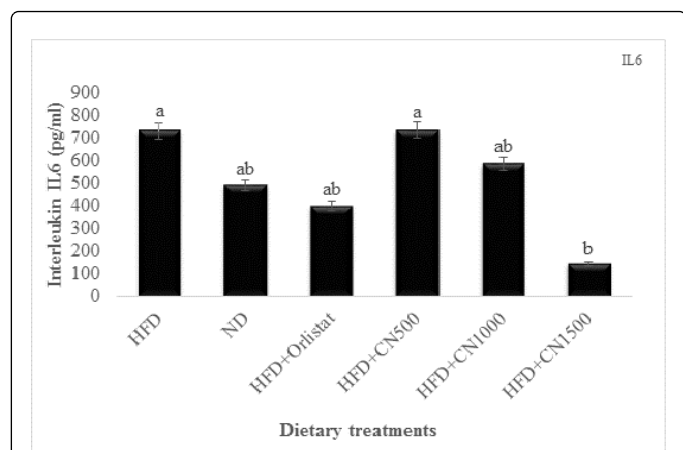


Figure 6: Effects of methanolic extract of *C. nutans* leaves at three concentrations on the interleukin (IL-6) of male ICR obese mice to compare CONTROL groups. n=10 per treatment group. Data represented the mean \pm standard error of the means (SEM) of interleukin (IL-6). The data are presented as means \pm SEM. Bars with different letters are statistically different ($P<0.05$). CONTROL groups without *C. nutans* (HFD: High Fat Diet; ND: Normal Diet; HFD+Orlistat: High Fat Diet with orlistate). Whereas treatment groups: High Fat Diet (HFD) with the supplementation *C. nutans* (CN); HFD+CN500: Low concentration *C. nutans*; HFD+CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*.

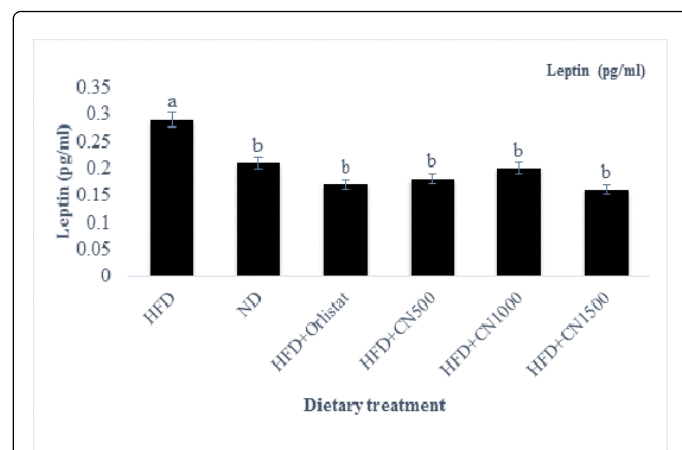


Figure 7: Effects of methanolic extract of *C. nutans* leaves at three concentrations on the leptin of male ICR obese mice to compare CONTROL groups, n=10 per treatment group. Data represented the mean \pm standard error of the means (SEM) of leptin. The data are presented as means \pm SEM. Bars with different letters are statistically different ($P<0.05$). CONTROL groups without *C. nutans* (HFD: High Fat Diet; ND: Normal Diet; HFD+Orlistat: High Fat Diet with Orlistate). Whereas treatment groups: High Fat Diet (HFD) with the supplementation *C. nutans* (CN); HFD+CN 500: Low concentration *C. nutans*; HFD+CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*.

Determination of acetylcholinesterase activity

The activity of acetylcholinesterase (AChE) obtained from the mice heart and muscle at HFD500, 1000, and 1500 mg/kg was higher than the HFD and ND CONTROL group that showed in (Figures 8 and 9). However, there was no significant difference seen in the activity of the heart AChE activity between treatments and HFD and ND Control groups ($P>0.05$).

Discussion

In the current study, our results from histological studies demonstrated that feeding mice with high-fat diet HFD could increase the weight of their abdominal fat and hypertrophy, due to excessive lipid storage within adipocytes [20].

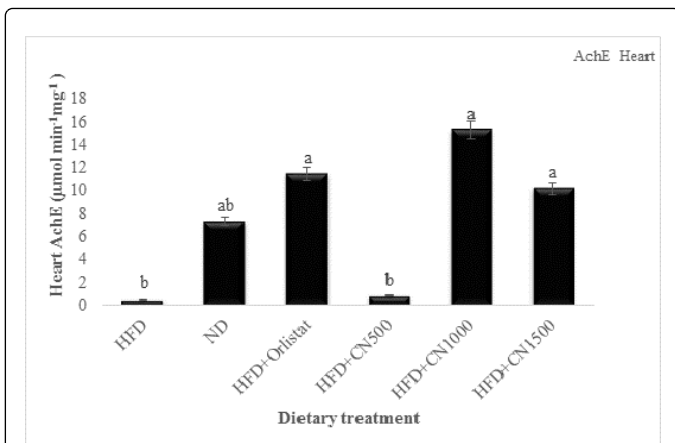


Figure 8: Effects of a methanolic extract of *C. nutans* leaves at three concentrations on the heart acetylcholinesterase of male ICR obese mice to compare control groups, n=10 per treatment group. Data represent the mean \pm standard error of the means (SEM) of heart AchE. The data are presented as Mean \pm SEM. Different superscripts between bars denote significant differences (P<0.05). Control groups without *C. nutans* (ND: Normal Diet; HFD: High Fat Diet; HFD+Orlistat: High Fat Diet with orlistat). Whereas treatment groups: High Fat Diet (HFD) with the supplementation *C. nutans* (CN); HFD+CN 500: Low concentration *C. nutans*; HFD+CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*.

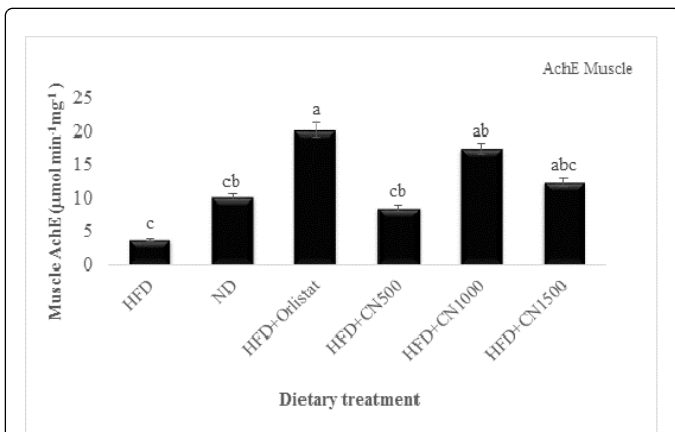


Figure 9: Effects of methanolic extract of *C. nutans* leaves at three concentrations on the muscle AchE of male ICR obese mice to compare control groups, n=10 per treatment group. Data represent the mean \pm standard error of the means (SEM) of muscle AchE activity. Data are presented as means \pm standard error of the means (SEM). Different superscripts between bars denote significant differences (P<0.05). Control groups without *C. nutans* (ND: Normal Diet; HFD: High Fat Diet; HFD+Orlistat: High Fat Diet with Orlistat). Whereas treatment groups: High Fat Diet (HFD) with the supplementation *C. nutans* (CN); HFD+CN 500: Low concentration *C. nutans*; HFD+CN1000: Medium concentration *C. nutans*; HFD+CN1500: High concentration of *C. nutans*.

Meanwhile, our results showed that the concentration of methanolic leaf extract of *C. nutans* had an obvious effect on the fat cell. The supplementation of *C. nutans* reduced the size of the abdominal fat cell in mice. Similar to this study, Wu et al. [39] concluded that dietary mulberry anthocyanins (MACN) had an effect on both number and the size of adipocytes in mice adipose tissue [40]. The reduction in the size of the abdominal fat cell could be attributed to the bioactivities of the polyphenolic compounds in *C. nutans* [19,26]. This adiposity-modulating effect of *C. nutans* extract could be attributed to various reasons. First, polyphenols could positively affect the uptake of lipids from the intestine by manipulating microbiota composition [41]. In addition, polyphenols could enhance oxidation in the liver, increasing the utilization of fatty acids as an energy source. In the adipose tissue, polyphenols inhibited adipocyte proliferation and triglyceride synthesis while stimulating lipolysis, resulting in a reduction in adipocyte size of male mice [41]. Reduction of mean adipocyte size is an effective approach to reduce body fat. Smaller mean abdominal adipocyte volume yields a smaller amount of fat action in mice [42]. This is in line with studies in rats where adipocyte size was increased by high-fat feeding independent of genetic factors, whereas an increase in number was enhanced by high-fat diet only in certain strains [43]. Hence, obesity significantly influences the adipocyte volume.

The effects of methanolic extract of *C. nutans* leaves on plasma TC, TG, LDL and HDL for mice are shown in Table 3. Triglycerides are the main lipids produced by the liver [44]. In other words, lipase is a major enzyme, which catalyzes the hydrolysis of lipids such as triglycerides and cholesterol, to be effectively transported within the body via HDL and LDL [45]. However, in the current study lack of significant differences in plasma TC, TG, HDL and LDL between HFD, ND, and Orlistat CONTROL and treated groups. Our finding is in agreement with findings in oral administration of star fruit (*Averrhoa carambola*) juice which did not effect on serum lipid profiles in Sprague-Dawley (SD) female normal rats [34]. Therefore, measuring these parameters can provide insight into the metabolic state of animals. The results of our experiment showed that the total cholesterol in mice supplemented with *C. nutans* was slightly lower than that of the control mice, and consequently, this observation is in agreement with the findings of Nakazato et al. [19] who reported that the plasma total cholesterol in rats supplemented with tea catechism was significantly lower than that of controls. Interestingly, the relation between plasma lipid profile and plant secondary compound content were further supported by polyphenol combined with pectin lowered rat plasma cholesterol [46], it is possible that tannin, another biologically active polyphenol in *C. nutans* [47], this finding could be related to the inhibition of the absorption of cholesterol from the intestine [48]. It is possible that the polyphenols decreased cholesterol absorption by forming insoluble precipitates of cholesterol [49] and decreasing bile acid-induced micellar solubility. Therefore, the hypocholesterolemic effect of the polyphenols could be due to decreased cholesterol absorption, enhanced cholesterol excretion and inhibition of cholesterol biosynthesis [50]. Although we observed that the highest concentration 1500 mg/kg was less effective than medium concentration 1000 mg/kg of crude methanolic leaf extract of *C. nutans* on plasma lipid profile. The results of this study suggest that consuming high concentration of polyphenol plant secondary metabolism in the form of supplements could lead to an increased health risk [20,51]. To the best of our knowledge, this is the first time that crude methanolic leaf extract *C. nutans* has been evaluated for anti-obesity. Supplementation of crude methanolic leaf extract *C.*

nutans had no effect on the serum lipid profile in mice. This observation could be due to the duration of the trial [19].

White adipose tissue produces a number of adipokines linked to inflammation. The circulating IL-6 is directly correlated with adiposity [52]. The release of IL-6 into the systemic circulation is greater in obese subjects and agrees with findings in mice fed a high fat diet [53]. This supports a possible novel role for IL-6 as a systemic regulator of lipid metabolism and body weight [53]. Accordingly, serum cytokine levels indicate that the inflammatory response was affected by the high-fat diets. This hypothesis is consistent with the current observations in which HFD+CN500 to 1500-fed mice had decreased IL-6 cytokine levels, indicating a lessened inflammatory response. Similar results were observed in mice supplemented with blueberries [54]. In addition, polyphenols, anthocyanin and catechin prevented inflammation by inhibiting nuclear factor kappa-light-chain-enhancer of activated NFB activity [55-57]. Indeed, as recently highlighted by de Melo et al. [58] showed that oleanolic acid a natural triterpenoid when administered daily (50 mg/L in drinking water), to HFD fed adult male Swiss mice for 15 weeks, they confirmed that the triterpene, oleanolic acid (OA) has been described to decrease the IL-6 production. Polyphenols, triterpene, linoleic acid and diterpene extracted and isolated from *C. nutans* leaves [30,31], exhibited anti-inflammatory properties [59]. Thus, the observed decreased in IL-6 production in mice supplemented with *C. nutans* could be attributed to the phenolic contents in *C. nutans*.

Leptin is a cytokine produced mainly by the white adipose tissue, which plays an important role in regulating lipid metabolism and energy homeostasis. In hypothalamus, leptin stimulates anorexigenic pathways and decrease food intake [60]. It is generally known that most obese animals exhibit high serum leptin concentrations [61]. Supplementation of *C. nutans* reduced serum leptin levels in mice. Similarly, supplementation of Blueberry juice or purified blueberry anthocyanins (1.0 mg/ml) in the drinking water were fed male C57BL/6 mice for a period of 72 days [40], and anthocyanin-rich grape-bilberry juice [62], reduced serum leptin levels in mice fed HFD. In addition, supplementation of thylakoid [63] and caffeic acid and chlorogenic acid [24] reduced serum leptin levels in mice. In the current study, Sakdarat et al. [64] have been reported that three chlorophyll derivatives (phaeophytins) were extracted from *C. nutans* leaves, it was expected that thylakoid from plant chloroplasts are useful to elevated the satiety hormone cholecystokinin, which lead to reduce leptin levels [63]. Reduced levels of leptin may suggest enhanced leptin sensitivity [65], which may stimulate fatty acid oxidation. Even thus, the present experiments have confirmed the ability to plant secondary metabolism of chlorophyll, caffeic acid, chlorogenic acid and polyphenol group which are available in *C. nutans* as well to decrease leptin levels and promote satiety signaling in healthy body [63].

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes the neurotransmitter acetylcholine, producing choline and an acetate group [66]. Interestingly, it is clear that the brain both monitors and regulates the energy needs of the body. In turn, both carbohydrate and adipose stores are monitored by the brain using combinations of metabolic and neural signals from the periphery [67]. However, in the highly interconnected conditions of obesity and non-insulin-dependent diabetes mellitus, there appears to be a major resetting of normal homeostatic mechanisms [67]. Results of this study showed that the slightly higher activity of acetyl cholinesterase of muscle and heart in treatment HFD+CN500, 1000 and 1500 mg/kg compare to HFD and ND CONTROL groups. Similarly, de Nigris et al. [68]

showed that regular pomegranate juice and pomegranate fruit extract were fed obese Zucker rats results indicated that acetylcholine (ACh) induced relaxation responses elevated compare to seed oil supplementation. Lau et al. [69] reported that oral administration of a methanol leaves extract of *C. nutans* increased acetyl cholinesterase activity in male normal mice. The observed elevation in acetyl cholinesterase activity might be due to the loss of fat in adipose tissue [70]. Nevertheless, in mice, an increase in acetylcholinesterase activity may be regarded as a general biomarker of plant secondary compound like polyphenol, flavonoids and alkaloids, which work as a natural antioxidant and anti acetylcholinesterase activity [69,71]. However, there was no significant difference seen in the activity of the heart AChE activity between treatments and control group ($P>0.05$). Meanwhile, all the plant extracts contained some level of inhibitory activity against acetylcholinesterase AChE and butylcholinesterase (BChE) [72,73]. Hence, it may be deduced from the current investigations that crude methanolic leaves extract of *C. nutans* leaf can be suitable sources of anti-cholinesterase and antioxidant compounds.

Conclusion

Despite the similar lipid profile in both groups, the changes in adipose cellularity and acetylcholinesterase activity in mice given *C. nutans* suggest that the polyphenols in *C. nutans* enabled the mice to cope with oxidative stress. The lower size of adipocytes in the adipose tissue of mice supplemented with *C. nutans* might due to the decrease in leptin and Interleukin IL-6 secretion and increase acetylcholinesterase activity. Accordingly, the results of this study also suggest that a weight loss agent did not cause any side effects. The best concentration was 1000 mg/kg BW for obese mice. These results could be extrapolated to obese humans, as has been done in the case of many obese animal models. Based on this, *C. nutans* might have beneficial effects against obesity.

Recommendations

The fractions of the plant extract might be repeated several times to isolate sufficient amounts of the plant secondary compound in order to test their bioactivities on the experimental animals.

Future surveys should concentrate on how to swap the herbs effectively from the in vivo animal models to in vitro cell line models, which can establish the adipose tissue efficiency of the plants.

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