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RESEARCH ARTICLE

Effects of methanolic leaf extract of *Clinacanthus nutans* on body weight and fatty acid composition in male obese mice

Samiaa Jamil Abdulwahid-Kurdi[1,3], Yong Meng Goh[2], Mahdi Ebrahimi[2], Zailina Binti Hashim[1]

[1]Department of Environmental and Occupational Health, Faculty of Medicine and Health Sciences, University Putra Malaysia, Serdang, Selangor Darul Ehsan, Malaysia, [2]Department of Preclinical Sciences, Faculty of Veterinary Medicine, University Putra Malaysia, Serdang, Selangor Darul Ehsan, Malaysia, [3]Department of General Science, Faculty of Education, Soran Universitiy, Kurdistan Region of Iraq

Correspondence to: Yong Meng Goh, E-mail: ymgoh@upm.edu.my

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ABSTRACT

Background: In general, obesity refers to abnormal accumulation of fat associated with negative effects on health. It is a severe public health challenge in the 21st century in Southeast Asia and elsewhere in the world. Obesity results in reduced life expectancy and causes enormous economic and social problems. Therefore, the prevention and management of obesity is a key focus of population health. **Aims and Objectives:** This study aims to estimate the potential antiobesity effect of a methanolic leaf extract of *Clinacanthus nutans* (MECN) on high-fat diet-induced male mice by evaluating the body weight, visceral fat, and muscle saturated fatty acid (SFA) compositions (15.57%) (F = 16.24, P = 0.0001). **Materials and Methods:** A total of 60 4-week-old male Institute of Cancer Research mice were randomly assigned into two groups. G**rou**p 1 consists of 10 mice that were fed with normal diet (NC), while G**rou**p 2 consists of 50 mice that were fed with a high-fat diet (HFD) of 60% dietary energy from fat for 16 **week**s. When these mice turned 20 **week**s old, those in G**rou**p 2 were randomly assigned to five groups of 10 mice each, while animals from G**rou**p 1 continued to be fed with the Normal chow diet (NC). The five HFD groups derived from G**rou**p 2 were divided into mice treated with HFD only (HFDC), mice fed with an HFD and orlistat at 15.9 mg/kg (HFD + Orlistat). Mice in three other HFD groups were treated with MECN at 500 mg/kg (HFD + CN500), 1000 mg/kg (HFD + CN1000), and 1500 mg/kg (HFD + CN1500). All animals were then subjected to 21 **day**s of the treatment. **Results:** The results showed that the MECN significantly reduced ($P < 0.05$) the body weight (33.38 ± 1.12 **g**) ($F = 2.46$, $P = 0.04$), visceral fat (1.62 ± 0.27) ($F = 6.39$, $P = 0.0002$), and muscle saturated fatty acid compositions (15.57%) ($F = 16.24$, $P = 0.0001$), especially in mice fed with 1500 mg/kg of MECN compared to the HFDC group. **Conclusion:** Therefore, MECN is a potentially useful natural supplement for alleviating obesity and obesity-mediated metabolic diseases.

KEY WORDS: Obesity; *Clinacanthus nutans*; Body Weight; Fatty Acids

INTRODUCTION

Obesity is a universal health concern that can lead to several severe diseases including type 2 diabetes, dyslipidemia, cardiovascular disease, and hypertension. The current estimations suggest that by 2030, the population of overweight and obese adults worldwide will reach 2.16 billion and 1.12 billion, respectively.[1] Obesity throughout the world affects a large number of people who lead a sedentary life that is dominated by reduced activity and high-calorie intake.[2] According to the World Health Organization, obesity is regarded as a disease.[3] Overweight and obesity have been recently found to be the fifth leading cause of deaths worldwide.[4] Specifically, their complications account for 100,000–400,000 annually.[5] Obese people are sometimes bullied and discriminated against in the workplace and at school, which makes being obese worse than

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it is. Besides this, obese people have been observed to incur about 30% of higher medical bills in comparison with normal weight individuals.[6] The Asia Roundtable on Food Innovation for Improved Nutrition has reported that obesity accounts for 10–19% of the overall health-care costs in Malaysia, totaling RM 4.26–8.53 billion.[7] In the USA, similar reports suggest that every American adult will be either overweight or obese by 2048. This will increase the expenditure attributed to obesity, costing USD 860.7–956.9 billion or 16–18% of the overall US health-care expenditure by 2030.[8] If no serious action is taken soon, obesity may reach the pandemic level in 2040.[4] Obesity refers to excessive fat accumulation in the body. The distribution of accumulating adipose tissue can be classified into lower body fat, abdominal subcutaneous (under the skin), or visceral fat, which is found in the abdominal cavity among organs.[9] The loss of balance between fat intake and energy consumption is thought to be the underlying cause of developing the obesity phenotype.[10,11] Conversely, cutting down the amount of food intake can lower the oxidative stress and increase maximum lifespan. Thus, the quality, quantity, and composition of food intake are crucial for regulating the level of oxidative stress. Attempts at treatment through antiobesity drugs are hampered by their side effects.[12] For example, Ferraz *et al*. studied orlistat, one of the most common antiobesity drugs currently used in clinical settings.[13] It inhibits the gastric and pancreatic lipases and consequently reduces the lipid absorption from the gut.[14] Nature offers a wide range of plants that provide crude extract and isolated compounds which are effective for controlling and reducing weight gain. Dietary phytochemicals have recently created considerable interest as potential therapeutic agents for health promotion and to counteract obesity.[15] A recent work has suggested the effectiveness of *Clinacanthus nutans* Lindau, a plant from the family of Acanthaceae. It is popularly referred to Sabah snake grass in Malaysia, but is known by several other vernacular names: Belalai Gajah (Malay), Dandang gendis (Javanese), Tajam (Sunda) in Indonesia; Phaya Yo, Phaya Plong Thong in Thailand; Twist of Flowers, Alligator Flower, Zuihua in Chinese. It is intensively grown in tropical and subtropical Asian countries including Malaysia, Indonesia, Thailand, China, and Vietnam.[16] *C. nutans* contains important constituents such as phenolics, flavonoids, stigmasterol, β-sitosterol, lupeol, betulin, chlorophyll derivatives, protocatechuic acid, C-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin, 7-O-β-glucopyranoside, orientin, isoorientin, cerebrosides, steroids, triterpenoids, glycerides, monoacylmonogalactosylglycerol, and sulfur-containing glucosides.[16,17] The phytochemical compounds, namely phenols, tannins, alkaloids, steroids, protocatechuic acid, and terpenes, may have the ability to exert hypolipidemic activity.[18] Different plant-based polyphenols have been found to quench free radicals and exhibit anti-inflammatory properties, as well as antihyperglycemic and antihyperlipidemic properties.[19,20] However, there is no evidence demonstrating that *C. nutans* can be utilized as a remedy for obesity. Despite all known biological activities in earlier work, empirical evidence that supports its ability to reduce weight and lower blood cholesterol has not been reported. The present study aims to investigate this plant using doses of methanolic leaf extracts of *C. nutans* (MECN) similar to previous research.[21,22] Recent evidence has shown that MECN improves lipid profiles in rats.[17] However, even though the presence of polyphenols in *C. nutans* has been determined, there is still little evidence about the antiobesity properties and the mechanism by which *C. nutans* could exert antiobesity effects. To date, empirical evidence about the effect of MECN on the high-fat diet (HFD)-induced male mice is still lacking. The present study investigated the effects of MECN supplementation on body weight, organ weight, and lipid profile in HFD-induced obese male mice.

MATERIALS AND METHODS

Plant Materials

Fresh leaves of *C. nutans* (Burm. f.) Lindau were acquired from a botanical garden in Ladang 10, Universiti Putra Malaysia, in Selangor, Malaysia. The botanical identify of *C. nutans* was characterized by the Phytomedicine Herbarium, Institute of Bioscience, Universiti Putra Malaysia, Selangor (Voucher No. SK2942/15).

Preparation of MECN

The extracts were prepared according to the method of Lau *et al*. with some modifications.[22] The *C. nutans* leaves were cleaned under running tap water and then air-dried for 1 week under direct sunlight. These leaves were oven-dried for 24 h at 40°C in an oven, ground to a fine powder by electric grinder (RT-08, Rong Tsong Precision Technology Co., Taiwan) and stored in an air-tight container. The powdered leaves were extracted using 80% methanol (by adding 20% of distilled water) at a ratio of 1:20 (w/v), 1 g of the sample to 20 mL of methanol. The powdered *C. nutans* leaves were left macerated in methanol and shaken for 72 h using a rotary shaker (Liquid Brushless DC motor clock Rotary, Germany). Next, the methanol solution was separated from the powdered leaves using a cloth filter, cotton wool, and Whatman No. 1 filter paper (Whatman No. 1, Fitchburg, WI, USA). The methanol extract was then concentrated under compact pressure with an R-215 rotating evaporator (Buchi, Flawil, Switzerland) at 40°C. The concentrated methanol extract was stored at −80°C and lyophilized with a freeze dryer (Labconco FreeZone 6 plus freeze dryer) to dry powdered form and then kept at −20°C. The yield obtained from the MECN was 15.92% (w/w).

Preparation of Animal Model

All procedures conducted in this work were reviewed and approved by the Universiti Putra Malaysia, Institutional Animal Care and Use Committee, approval No: R083/2016. 3-week-old male Institute of Cancer Research (ICR) mice were bought from Sapphire Enterprise, Malaysia. Before

the trial, the subjects were acclimatized under constant conditions (temperature: 22 ± 1°C; humidity, 40–60%; and light, 12 h of light/dark cycle) for 7 days, before the dietary manipulation. The caloric composition of feed was 20 kcal% protein, 70 kcal% carbohydrate, and 10 kcal% fat. Each gram of the ingredient contained 3.85 kcal calories, including 19 mg cholesterol from lard and yellow dye.

Induction of Obesity

After 7 days of adaptation to the environment, the animals were randomly assigned to two groups. Normal mice were fed normal chow diet (NC) consisting of 10% beef tallow (Research Diets D 12450, New Brunswick, New Jersey, USA) throughout the study. To induce obesity, mice were fed with HFD as provided by purified commercial feeds containing all essential nutrients, vitamins, minerals, and 60 kcal% of fat (Research Diets D12492, New Brunswick, New Jersey, USA).[23] Its caloric composition was 20 kcal% protein, 20 kcal% carbohydrate, and 60 kcal% fat. Each gram of its ingredient contained 5.24 kcal, including 232 mg cholesterol from lard and blue dye. It was kept frozen and could last for 6 months. An amount of 60 kcal% fat was seldom practical in the case of the human diet. On the other hand, for the purpose of inducing obesity in mice more quickly and facilitating the research, 60 kcal% fat was frequently utilized, but when screening the impacts of a drug in genetic manipulation, the requirement may be 45 kcal% fat diet because a high 60 kcal% diet could inhibit a reversal of the effects.[24] In thecurrent study, the mice were fed an HFD for 16 weeks to induce obesity.

Study Design for the Treatment Groups

After 16 weeks of induction, 60 male mice 20 weeks old in six groups were used in an experiment to evaluate the efficacy of MECN against obesity. Five groups were fed continuously with an HFD, one group was fed with the normal chow diet (NC). The assigned groups (10 mice/group) were given treatment for 21 days as stated in Table 1. The five HFD groups were divided into HFD only (HFDC), HFD with orlistat 15.9 mg/kg body weight and dissolved in ethanol 20 mg/mL (HFD + Orlistat),[23] HFD with MECN at 500 mg/kg (HFD + CN500), HFD with MECN at 1000 mg/kg (HFD + CN1000), and HFD with MECN 1500 mg/kg (HFD + CN1500). Distilled water was used as a vehicle to dispense MECN by oral gavage, given once daily for 21 days. The controls in the HFDC and NC groups were given distilled water by oral gavage so that all mice underwent the same gavaging procedure, though without the MECN or orlistat.

Measurement of the Body Weight

For the duration of the experiment, body weight was recorded weekly with a standard weighting balance to monitor the body weight changes. The food was given at 4 g/daily per mice, and mice had access to water at all times. The body weight was recorded throughout the study and changes expressed as percentage (%) at the end of the study. The rate of changes in body weight on supplementation of *C. nutans* was determined. This equation was only used at the end of this study. Weight decreased (%) = initial body weight (g) - final body weight (g)/initial body weight (g) ×100. At the end of the experiment, after a period of 21 days, each mouse was made to fast overnight following the last treatment. The mice were anesthetized with ketamine 80 mg/kg and xylazine 10 mg/kg given intraperitoneal. Visceral fat, liver, kidney, spleen, and intestine were removed immediately, weighed and quickly placed in liquid nitrogen, and stored at −80°C. Visceral fat was fixed in 10% neutral-buffered formalin for further analysis.

Total Lipid Extraction

The extraction of total fatty acids was from muscle tissues employing chloroform to methanol (2:1) (v/v) based on the method of Folch *et al*. and modified by Ebrahimi *et al*.[25,26] The samples of muscle tissues (0.5 mg) were ground by Grinder (IKA Analysentechnik GmBH, Germany) and then homogenized in 40 mL chloroform to methanol (2:1) (v/v) utilizing an Ultra-Turrax T5 FU homogenizer (IKA Analysentechnik GmBH, Germany) in a 50 mL stoppered ground-glass extraction tube. Flushing with nitrogen into the tube was done before stoppering and vigorous shaking for 5 min. The tube was then left to stand for 12 h with intermittent shaking. Following this, about 5 mL was taken from the sample and transferred to a plastic tube. 5 mL of chloroform to methanol mixture in 2:1 concentration was then added to the extracts. Butylated chloroform/methanol was used to prevent oxidation of the samples during sample preparation. The mixture was then vortexed at 5000 rpm for

Table 1: Treatment group assignment of MECN

Group name	Treatment	Name
HFDC	High-fat diet+drinking water	Control
NC	Normal diet+drinking water	Control
HFD+Orlistat	HFD+Orlistat (15.9 mg/kg per body weight)	Control
HFD+CN500	HFD+CN (500 mg/kg per body weight)	Low dose
HFD+CN1000	HFD+CN (1000 mg/kg per body weight)	Intermediate dose
HFD+CN1500	HFD+CN (1500 mg/kg per body weight)	High dose

HFD: High-fat diet, MECN: Methanolic leaf extract of *Clinacanthus nutans*

20 s. Following this, 4 mL of distilled water was added to the mixture. The mixture was then vortexed again at 5000 rpm for 40 s. To allow separation, the mixture was then centrifuged at 3000 rpm for 5 min. The chloroform phase was separated from the aqueous phase using a pipette and transferred to a methylated tube. An amount of 100 µL of an internal standard consisting of heneicosanoic acid (C21:0) (Sigma Chemical, St. Louis, MO, USA) was introduced into every sample before transmethylation to establish the individual fatty acid level in the sample. Following this, the sample was heated in a water bath at 70°C, and the solvent of the sample was dried using nitrogen gas. 2 mL of 0.66 N potassium hydroxide (KOH) (R and M Chemicals, Essex, UK) was then added to the sample, followed by heating in a water bath at 95°C for 10 min. Next, 2 mL of 14% methanolic boron trifluoride (BF3) (Sigma Chemical Co., St. Louis, Missouri, USA) was introduced into the sample and heated in a water bath at 95°

C for 20 min. After cooling, 4 mL of distilled water was added, and 4 mL of petroleum ether (boiling point 40–60°C) was added and the mixture was vortexed for 60 s. Finally, the petroleum ether with the FAME was placed into a 4 mL screw-capped vial (Kimble Glass Inc., USA) followed by flushing with nitrogen. The vial was then closed tightly and kept at 4°C until analysis by gas-liquid chromatography.

Gas-Liquid Chromatography

The methyl esters were quantified by GC (Agilent 7890A) employing a 30m × 0.25 mm ID (0.20 µm film thickness) Supelco SP-2330 capillary column (Supelco Inc., Bellefonte, PA, USA). 1 µl was injected with an autosampler into the chromatograph, equipped with a split/splitless injector and an FID detector. High purity nitrogen (Malaysian Oxygen Bhd., Malaysia) was the carrier gas at 40 mL/min. High purity hydrogen (Dominick Hunter, Parker Hannifin Ltd, UK) and compressed air (Malaysian Oxygen Bhd., Malaysia) were utilized for the flame ionization detector in the gas-liquid chromatography. The injector temperature was programmed at 250°C, and the detector temperature was 300°C. The column temperature program initiated was run at 100°C, for 2 min, warmed to 170°C at 10°C/min, kept constant for 2 min, warmed to 220°C at 7.5°C/min, and then kept constant for 20 min to ensure optimized separation. To identify the fatty acids, the relative comparison was made of FAME peak retention times of samples and standards obtained from Sigma (St. Louis, MO, USA). Both gravimetric calculations and normalized percentage (%) of total FA were employed to establish the variances in FA composition. Peak areas were established and computed with a personal computer integrator (Hewlett-Packard, Avondale, PA). Automatic expression of the peak areas in absolute and percentage amounts of detected fatty acid was obtained with a programmed PC using Microsoft Excel 2010 (Microsoft Corp., Redmond, USA). The quantum of fatty acids was known by their relative proportions (normalized percentages of total fatty acids).[27,28] The normalized percentages explained the interactive and comparable relationships among fatty acids with regard to lipid quality, whereas the gravimetric concentration can indicate the real amount of fatty acids in tissues, in relation to nutritional intake.

Statistical Analysis

Data on body weight, organ weight, serum lipid profile, and fatty acid parameters were analyzed using the one-way analysis of variance (ANOVA) procedure of the SAS software package, version 9.1 (SAS Institute Inc., Cary, NC). This was followed by Turkey's multiple range test for *post hoc* comparison of group means. Differences were accepted as statistically significant when $P < 0.05$. Data were presented as mean ± standard error of the means (SEM).

RESULTS

Body Weight Changes

Models of diet-induced obesity are commonly used in research of non-leptin deficient obesity. This model has been widely used because it mimics human obesity in terms of increased body weight and adiposity. Feeding mice with HFD over a period of 16 weeks increased the body weight of experimental group mice. At the end of the obesity induction period, 20-weeks-old mice demonstrated a significant increase in mean body weight in obese mice (39.33±0.127 g) compared to the normal mice fed with normal chow diet (32.88±1.75 g) ($F = 8$, $P = 0.016$). The HFD mice were then treated with either MECN for an additional 3 weeks and examined for changes in the body weight and biochemical parameters. Figure 1 demonstrates the effect of MECN on the body weight of obese mice, and no notable differences

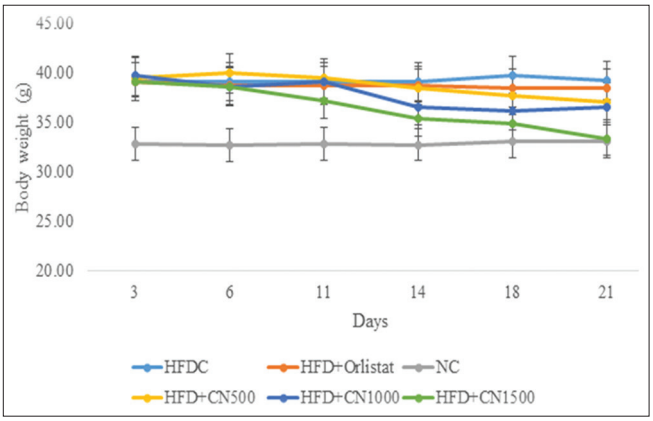

Figure 1: Effect of methanolic leaf extract of *Clinacanthus nutans* (MECN) on body weights of obese mice across treatment groups. HFDC: High-fat diet mice, NC: Normal diet mice, HFD + Orlistat: High-fat diet mice treated with orlistat, HFD + CN500: High-fat diet mice treated with MECN (500 mg/kg), HFD + CN1000: High-fat diet mice treated with MECN (1000 mg/kg), HFD + CN1500: High-fat diet mice treated with MECN (1500 mg/kg) ($n = 6$)

were observed between the mean body weights of HFDC and MECN treatment groups. At the beginning of the treatment period (week 1), the initial body weights were as follows: HFDC (39.12±1.70 g), NC (32.88±1.30 g), HFD + Orlistat (39.12 ± 0.87 g), HFD + CN500 (39.5 ± 1.0 g), HFD + CN1000 (39.75±0.97 g), and HFD + CN1500 (39.12 ± 2.01 g). There were no differences in body weight between groups except for NC ($F = 3.13$, $P = 0.01$). On completion of the supplementation program, after 21 days of treatment, the final body weights were as follows: HFDC (39.25±1.25 g), NC (33.13 ± 1.13 g), HFD + Orlistat (38.5±1.91 g), HFD + CN500 (37.13±0.87 g), HFD + CN1000 (36.63±1.88 g), and HFD + CN1500 (33.38 ± 1.12 g). There was a significant decrease in body weight of mice treated with 1500 mg/kg (HFD + CN1500) compared to the HFDC group ($F = 2.46$, $P = 0.04$). Particularly, HFD + CN1500 group showed the most significant decrease, while HFD + Orlistat and HFD + CN 500 showed a fluctuating trend and were close to the HFDC obese group.

As shown in Figure 2, the percentage of body weight decrease in mice on MECN treatment was computed by subtracting the initial body weight (g) in week 1 from the final body weight (g) in week 3 divided by initial body weight (g) on week 1 multiplied by 100. A comparison was made between the effect of MECN on body weight and decreasing body weight percentage, or changes among different groups, following HFD + CN500 (6.01±2.21 g), HFD + CN1000 (7.86±4.75 g), HFD + CN1500 (14.7±5.81g), and HFD + Orlistat (1.59±4.90 g) treatments. HFD + CN1500 had significantly decreased compared to HFDC group ($F = 2.45$, $P = 0.04$). In addition, both HFDC (-0.33±3.19 g) and NC (-0.7±3.42 g) groups had increased body weight compared to other groups.

Visceral Fat and Weight of Organs

There was also a similar relationship between the fat index and the adverse effect of metabolic syndrome. Table 2 provides information on the relative visceral fat, liver, and kidney. The relative weight of visceral fat of MECN at 1000 mg/kg (HFD + CN 1000) and 1500 mg/kg (HFD + CN 1500) group was significantly lower than that in the HFDC group ($F = 6.39$, $P = 0.0002$), which suggested that MECN treatment had a significant reduction effect on the relative weight of visceral fat. All animals in the HFD + CN500 and HFD + Orlistat did not show significant reduction in visceral fat accumulation. The relative weights of liver and kidney were also reduced by MECN treatment, but this reduction was not statistically significant.

Muscle Fatty Acid Compositions

The fatty acid compositions of the muscle from control groups, HFDC, NC, and mice treated with the MECN are shown in Table 3. The major fatty acids found in the HFDC group consist of the SFA, which are palmitic acids

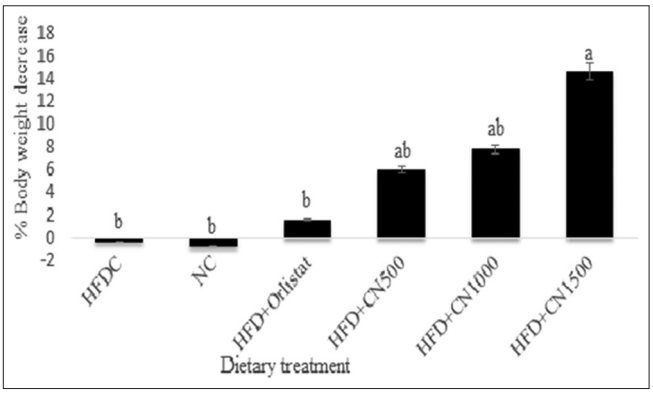

Figure 2: Effects of methanolic leaf extract of *Clinacanthus nutans* (MECN) on percentage body weight decrease in obese mice. Effects of MECN on decreasing body weight percentage in mice across treatments groups. HFDC: High-fat diet control mice, NC: Normal diet control mice, HFD + Orlistat: High-fat diet mice treated with orlistat, HFD + CN500: High-fat diet mice treated with MECN (500 mg/kg), HFD + CN1000: High-fat diet mice treated with MECN (1000 mg/kg), HFD + CN1500: High-fat diet mice treated with MECN (1500 mg/kg). Values are expressed as mean ± standard error of the means ($n = 6$). [a,b]Means with different letters are significantly different ($P < 0.05$, ANOVA) between groups by Tukey's test

Table 2: Effects of MECN on relative organ weights of mice across treatment groups

Dietary treatment group	Visceral fat %	Liver %	Kidney %
HFDC	4.03±0.49[a]	5.82±0.57[a]	1.74±0.09[a]
NC	2.49±0.28[bc]	4.72±0.4[a]	1.42±0.10[a]
HFD+Orlistat	3.26±0.21[ab]	5.34±0.01[a]	1.58±0.03[a]
HFD+CN500	2.70±0.34[abc]	4.88±0.54[a]	1.51±0.09[a]
HFD+CN1000	2.28±0.29[bc]	4.64±0.18[a]	1.47±0.11[a]
HFD+CN1500	1.62±0.27[c]	4.06±0.32[a]	1.38±0.05[a]
P-value	0.0002	0.21	0.1

HFDC: High-fat diet control mice, NC: Normal control mice, HFD+Orlistat: High-fat diet mice treated with orlistat, HFD+CN500: High-fat diet mice treated with *C. nutans* 500 mg/kg, HFD+CN1000: High-fat diet mice treated with *C. nutans* 1000 mg/kg, HFD+CN1500: High-fat diet mice treated with *C. nutans* 1500 mg/kg. Values are expressed as mean±SEM (n=8). Relative organ weight (%) = (weight of organ/weight of mice) ×100. Mean within a column with different superscript letters is significantly different (P<0.05, ANOVA) between groups by Tukey's test. ANOVA: Analysis of variance

(C16:0) at 22.17%. This result was significantly higher than that of the group with high-fat diet treated with MCEN, HFD + CN500 (15.65%), HFD + CN1000 (16.58%), and HFD + CN1500 (15.57%) ($F = 16.24$, $P = 0.0001$). On the other hand, the oleic acid (C18:1n-9), which is one of the unsaturated fatty acids (UFA), was significantly lower in the HFDC group at 41.14%, compared to that of the high-fat diet treated MECN, HFD + CN500 (53.88%), HFD + CN1000 (54.17%), and HFD + CN1500 (54.87%) ($F = 5.99$,

Table 3: Effects of MECN on muscle fatty acid profiles (% of total fatty acids) in mice across treatment groups after 21 days of intervention

Fatty acid profile	Name	HFDC (%)	NC (%)	HFD+Orlistat (%)	HFD+CN500 (%)	HFD+CN1000 (%)	HFD+CN1500 (%)	*P*-value
C14:0	Myristic	1.42±0.15^{a}	1.75±0.02^{a}	1.29±0.19^{a}	1.17±0.19^{a}	1.24±0.21^{a}	1.38±0.19^{a}	0.25
C16:0	Palmitic	22.17±0.68^{a}	15.77±0.21^{b}	17.49±0.96^{b}	15.65±0.44^{b}	16.58±0.30^{b}	15.57±0.74^{b}	0.0001
C16:1	Palmitoleic	6.33±0.64^{a}	5.96±0.82^{a}	4.61±0.92^{a}	5.23±0.84^{a}	5.40±1.40^{a}	4.95±0.47^{a}	0.72
C18:0	Stearic	5.26±0.87^{a}	5.98±0.52^{a}	8.68±1.19^{a}	6.59±1.85^{a}	6.64±1.42^{a}	6.38±0.97^{a}	0.49
C18:1n-9	Oleic	41.14±2.31^{a}	55.82±2.53^{a}	47.45±2.31^{bc}	53.88±2.31^{ab}	54.17±2.53^{ab}	54.87±2.31^{ab}	0.0007
C18:2n-6	Linoleic	15.72±0.71^{a}	9.29±0.77^{b}	9.76±0.71^{b}	10.68±0.71^{b}	9.29±0.77^{b}	10.27±0.71^{a}	0.0001
C18:3n-3	Linolenic	1.54±0.16^{a}	1.25±0.18^{a}	1.11±0.16^{a}	1.12±0.16^{a}	1.30±0.18^{a}	1.31±0.16^{a}	0.49
C20:4n-6	Arachidonic	2.98±0.74^{a}	1.73±0.81^{a}	4.08±0.74^{a}	2.78±0.74^{a}	2.61±0.81^{a}	2.55±0.74^{a}	0.44
C20:5n-3	Eicosapentaenoic	0.21±0.06^{b}	0.19±0.07^{b}	0.49±0.06^{a}	0.23±0.06^{b}	0.22±0.07^{b}	0.26±0.06^{a}	0.04
C22:5n-3	Docosapentaenoic	0.89±0.21^{a}	0.47±0.23^{a}	1.08±0.21^{a}	0.44±0.21^{a}	0.61±0.23^{a}	0.48±0.21^{a}	0.22
C22:6n-3	Docosahexaenoic	2.29±0.64^{a}	1.72±0.70^{a}	3.90±0.64^{a}	2.19±0.64^{a}	1.91±0.70^{a}	1.93±0.64^{a}	0.21
Total SFA		28.86±1.19^{a}	23.52±1.30^{c}	27.47±1.19^{ab}	23.42±1.19^{c}	24.46±1.30^{bc}	23.33±1.19^{c}	0.008
Total MUFA		47.48±2.80^{c}	61.8±3.06^{a}	52.06±2.80^{bc}	59.11±2.80^{ab}	59.58±3.06^{ab}	59.83±2.80^{ab}	0.009
PUFA n-6		18.70±1.12^{a}	11.02±1.23^{b}	13.86±1.12^{b}	13.47±1.12^{b}	11.90±1.23^{b}	12.83±1.12^{b}	0.001
PUFA n-3		4.95±0.95^{a}	3.65±1.04^{a}	6.6±0.95^{a}	3.99±0.95^{a}	4.05±1.04^{a}	3.99±0.95^{a}	0.29
FAR n-6:n3		4.20±0.40^{a}	3.29±0.44^{a}	2.22±0.40^{a}	4.38±0.40^{a}	3.04±0.44^{a}	3.48±0.40^{a}	0.10

HFDC: High-fat diet control mice, NC: Normal control mice, HFD+Orlistat: High-fat diet mice treated with orlistat, HFD+CN500: High-fat diet mice treated with *C. nutans* 500 mg/kg, HFD+CN1000: High-fat diet mice treated with *C. nutans* 1000 mg/kg, HFD+CN1500: High-fat diet mice treated with *C. nutans* 1500 mg/kg. MUFA: Monounsaturated fatty acid, PUFA: Polyunsaturated fatty acid, FAR: Fatty acid ratio. Values were expressed as mean±SEM (n=8). Mean within a row with different letters is significantly different (P<0.05). TSFA=C14:0+C16:0+C18:0), TMUFA=C16:1+C18:1n-9), n-3 PUFA=C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3), n-6 PUFA=C18:2n-6+C20:4n-6). TPUFA=n-3 PUFA+n-6 PUFA. n-6:n-3 FAR=C18:2n-6+C20:4n-6÷C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3

$P = 0.0007$). However, the HFDC group showed a significant increase in total SFA (28.86%) ($F = 3.39$, $P = 0.0008$) and a decrease in monounsaturated fatty (MUFA) acid (47.48%) compared to that of the high-fat diet treated with MECN ($F = 3.83$, $P = 0.0091$).

DISCUSSION

Obesity is characterized by enlarged fat mass and increased lipid concentration in blood.[29] According to Nishikawa *et al*., the obese animal model has different characteristics of obesity, and thus, one should be careful in choosing a model that is suitable for the purpose of the experiment.[30] The characteristics of obesity induced by high-fat diet feeding were influenced by the sex, strain, and age of mice. Sex steroid hormones, hepatic lipid metabolism, and systemic metabolism might be involved in these factors. The data presented in this study may establish a baseline to develop animal models of high-fat diet-induced obesity. The work of finding efficient alternatives has been usually prioritized. In this respect, this study has investigated the MECN and the results have shown, as discussed below, that the extract has been indicated to play an important role in treating obesity.

This study has induced obesity in male ICR mice using HFD for 16 weeks (60% dietary energy from fat). The mice were fed much higher HFD compared to mice fed normal chow diet. The assigned groups (10 mice/group) were given MECN for 21 days. No difference was observed in the food intake between the HFDC group and the high-fat diet group treated with 500, 1000, and 1500 mg/kg of MECN. The male ICR obese mice administered with 500, 1000, and 1500 mg/kg of MECN, respectively, experienced 6.01%, 7.86%, and 14.7% of weight loss after 21 days of treatment. The increase in body weight was also inhibited.

Earlier work has shown that an HFD may elevate energy intake and thus develops obesity.[1,31] HFD usually has 32–60% of calories from fat. From the nutritional perspective, a human diet of 60 kcal% fat would be considered extreme, but these are commonly used to accelerate obesity in rodents and thus save experimental time.[31] Using lard, Zhou *et al*. induced obesity in male Sprague-Dawley rats by feeding them with HFD (46% calories) for 14 weeks.[32] Administering mice with 15.9 mg of orlistat could reduce 1.59% of body weight of obese mice. According to Kumar *et al*., orlistat and sibutramine can promote about 5–10% of weight loss.[33] Administering male mice with 20 mg of orlistat and feeding them with HFD reduced only 0.5% of the body weight after 8 weeks of treatment.[34]

These reports suggest that taking orlistat and consuming HFD may result in weight loss compared to those that continue consuming HFD. The induced obesity in rats using HFD is a common model of investigation in obesity research. This model is valued for its ability to mimic selected physiological aspects of obesity in humans. The HFD is one of the major factors causing obesity, where the long-term consumption of HFD showed significant increases in abdominal fat weight in mammals.[35] In this study, there were no adverse physical changes observed throughout the experiment.

These are important changes that could be useful in managing obesity in humans. Losing a significant amount of initial weight at the onset of a weight management program is crucial as research has shown that around 20% of overweight individuals are successful in the long-term weight loss following significant initial weight losses. These individuals are defined as ones who lose at least 10% of their initial body weight and continue to lose weight for at least 1 year thereafter.[36] The results showed a significantly reduced weight gain in the mice treated with 1000 and 1500 mg/kg MECN compared to the HFDC group.

In the current study, relative weights of visceral fat in mice fed high-fat diet and treated with 1000 and 1500 mg/kg MECN were significantly reduced compared to the HFDC. On the other hand, relative weights of liver and kidney in mice fed high-fat diet and treated with MECN were slightly lower in the groups treated with HFD + CN500 and HFD + CN1000 and HFD + CN1500 compared to the HFDC. Continuous loss of body mass and especially fats is important as visceral fat is known to be more important than the abdominal subcutaneous fat data in predicting future insulin resistance. When the visceral fat depot size is smaller, it is more effective in preventing the future elevation of fasting plasma insulin level.[37] According to Bailey *et al*., the differences in organ weight between groups should always be considered from the perspective of differences in the body weight between groups.[38] *C. nutans* has been noted to have antioxidant action attributed to its polyphenols.[39] In the chloroplast, thylakoids possess a 100 different membrane proteins, galactolipids, and sulfolipids, in addition to various vitamins (A, E, and K), and hence, a range of bioactive compounds that could cause the retardation of fat digestion.[40] Various polyphenols have been known to induce significant weight loss, for example, epicatechin, epigallocatechin, and epicatechin gallate.[18] In addition, other compounds such as chlorogenic acid and anthocyanins which are also present in MECN are also known to contribute to weight loss as well.[41,42] The current findings suggest that the chlorophyll a, b, polyphenol, chlorogenic acid, and caffeic acid in the *C. nutans* may regulate the lipid metabolism by affecting hepatic lipid oxidation and lipogenesis and thus causing an increase in energy expenditure. Therefore, the MECN would be a viable antiobesity option, which also serves to reduce oxidative stress-related disorders. The study suggests that the relative of visceral fat weight in the diet-induced obese

mice could have been affected by a dose of MECN at 1000 and 1500 mg/kg.

The possibility of using MECN to alter the muscle fatty acids in ICR obese mice was investigated. The levels (g/100g total fatty acids in the form of a percentage) of saturated fatty acids (SFAs) and the notable rise in the levels of SFA, namely the palmitic acid (C16:0) in the HFDC mice were also similar to findings reported by Morselli *et al*.[43] The current research also found that those groups with a high-fat diet and treated with MECN 500, 1000, and 1500 mg/kg had a significantly higher amount of oleic acid than the HFDC groups. The results showed significant differences between the palmitic acid and oleic acid levels in the HFDC and MECN groups. Similar to these findings, Graf *et al*. found that the diet had high SFA.[44] It is also well established that the fatty acid composition of the diet influences the fatty acid profile of phospholipids and triacylglycerol's in skeletal muscles, and thus, the MECN-induced changes to muscle fatty acids are very much expected.[45] It is important to note that the photosynthesizing plants are particularly abundantly rich in alpha-linolenic acid, which accounts for up to 55% of the fatty acids present in green vegetables.[46] Interestingly, the *C. nutans* is rich in SFA such as myristic acid, palmitic acid, methyl ester palmitic acid, stearic acid, methyl ester margaric acid, linoleic acid, and ethyl ester linolenic acid.[47] Several positive effects of both n-3 and n-6 PUFA have been previously reported by De Caterina.[46] Their study suggested that incorporating PUFA in cell membranes could affect the membrane fluidity. PUFA has anti-inflammatory effects.[48] The lack of significant effects of MECN supplementation on the rest of the fatty acid composition in the muscle of ICR obese mice could be due to the limited time of the trial and the resilience of phospholipid membranes to sudden change in membrane lipid profiles. Any sudden change in membrane fatty acid profiles would cause catastrophic changes to cell membrane integrity and cellular survival itself. In a study by Köhnke *et al*., the dietary thylakoids (chlorophyll) extracted from spinach leaves were found to reduce serum fatty acids in mice fed with HFD for 100 days.[49] The dose of MECN at 500, 1000, and 1500 mg/kg clearly exhibited significant reduction in SFA and heightened level of monounsaturated fatty (MUSF).

Limitations

This study had some limitations, including a small number of mice and it also used one type of diet, which was HFD to induce obesity due to constraints in research funding. The current study compared only HFD feeding. Other types of hypercaloric diets such as high sucrose were not considered.

CONCLUSION

Based on the results discussed, it can be concluded that the high-fat diet treated MECN (1500 mg/kg) led to significant reduction in body weight and relative visceral fat. The dose of MECN at 500, 1000, and 1500 mg/kg clearly exhibited a significant reduction in SFA and a heightened level of monoun SFA. The results suggested that MECN may be a useful therapeutic candidate for antiobesity. This is a pioneering report on the effect of MECN administration on obese mice.

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