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Protective effect of *Spirulina* against bone fragility induced by *Garcinia cambogia* in high-fat diet induced obese rats

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Abstract

The present work evaluated the protective effects of *Spirulina* against the bone fragility caused by *Garcinia cambogia* in high-fat diet induced obese rats. High-fat diet and high-fat emulsion (HFD+HFE) were administered via oral gavage to 30 six-week-old female Sprague Dawley rats for six weeks to induce obesity, except for a normal group (n = 6). Following four weeks of treatment, the diet-induced obese groups were orally administered, daily, with (1) *G. cambogia* (GC); (2) *Spirulina* (S); and (3) *G. cambogia* + *Spirulina* (GC+S). The normal and obese control groups were treated with equal volumes of 0.9% saline water. It was found that GC significantly decreased body mass index (BMI) below the obese range (0.68 g/cm^2). Additionally, GC altered bone mineral density (BMD), increased phosphate and calcium levels, and decreased maximum force and mineral apposition rates (MAR) as compared to the obese control group ($p < 0.05$). Bone fragility caused by GC was confirmed by the decrease in bone formation marker osteocalcin (OCN), as well as an increase in bone resorption markers receptor activator of nuclear factor kappa-B ligand (RANKL) and tartrate-resistant acid phosphatase type 5b (TRAP5b) as compared to the obese control group. *Spirulina* also decreased the BMI of the obese rats. *Spirulina* also increased blood bone markers, BMD, maximum force, and Young's modulus. Rats supplemented with GC+S demonstrated higher double-labelled surface (dLS/BS) and MAR as compared to those in the GC group ($p < 0.05$). Meanwhile, the S group demonstrated improvement in all dynamic histomorphometric indices. S and GC+S groups demonstrated bone formation upregulation and bone resorption downregulation, thus indicating a bone protective effect of *Spirulina*. Overall, GC treatment led to bone fragility. GC+S treatment significantly augmented bone formation and mineralisation in obese rats as compared to the GC treatment alone. Rats in the S group demonstrated effective weight reduction while showing no destructive effects on the bone.

Introduction

Obesity is a chronic illness and a public health concern of epidemic proportions based on previous studies in animals, high dietary fat food intake, and weight gain (Smilowitz *et al.*, 2020). Additionally, a high-fat diet produces a poor stimulatory effect on satiation, especially when hunger levels are high, which results in a passive overconsumption of energy as fat. Fat satiates to a lesser degree than carbohydrates or proteins, thus increasing the appetite of an individual on a high-fat diet.

Recent data from epidemiological and animal studies strongly support the concept that fat accumulation (obesity) causes detrimental effects on bone mass, which subsequently leads to bone fragility (Shapses *et al.*, 2017). These detrimental effects are considered to be related to a few possible mechanisms, especially the action of adipocytes in adipose tissue. Adipocytes secrete adiponectin and leptin, which are known to affect bone remodelling. Both of these molecules have the ability to modulate osteoclastogenesis through the RANKL/receptor activator of nuclear factor kappa-B (RANK)/osteoprotegerin (OPG) pathway, thus

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negatively affecting bone metabolism (Lubkowska *et al*., 2017). However, there remains ongoing debate regarding this concept.

Traditionally, obesity was considered to be a protective factor for bone fragility, thereby conflicting with the hypothesis that obesity is detrimental to bone health. It has been suggested that increased loads applied to cortical bone directly stimulate bone formation through leptin, which increases the activity of aromatase and estradiol, thus leading to decreased bone resorption and increased bone formation (Sharma et al. 2014). Bone formation is stimulated by decreased apoptosis and increased proliferation of osteoblasts, which in turn differentiate into osteocytes through the Wnt/β-catenin signaling pathway (Regard *et. al*., 2012).

Several experimental studies on both animals and humans have shown that *Garcinia cambogia* (GC), a fruit native to Southeast Asia and Western Africa, has a beneficial impact on decreasing body weight and increasing fat loss (Sripradha and Magadi, 2015). The presence of hydroxyl citric acid (HCA) in GC extract helps to downregulate insulin and upregulate leptin production. Therefore, GC plays an important role in weight reduction (Chuah *et al.*, 2013). Based on a study by Hayamizu *et al*. (2003), GC treatment prevents fat accumulation in the body through the suppression of leptin. However, changes in serum leptin levels induced by GC may be the cause of the bone fragility seen in high-fat diet induced obese rat models. Preliminary study performed at UCSI University showed that supplementation with GC at 400 mg/kg per day for six weeks caused concomitant weight loss and bone fragility in a rat model (Cho, 2015). Biochemical analysis of the blood samples showed an increase in bone procollagen type 1 amino-terminal propeptide (P1NP), while biomechanical strength testing demonstrated a decrease in Young's modulus values. However, only a few parameters were investigated in this preliminary study.

Approximately 60 - 70% of *Spirulina* is composed of proteins, nucleic acids, and amino acids. *Spirulina* is also a good source of vitamin D and minerals such as magnesium. Magnesium aids in bone health by converting vitamin D into its active form, which is responsible for calcium absorption, and increases calcium deposition in the bone. Vitamin D suppresses bone resorption, and promotes bone remodelling, thus increasing bone mass (Hayamizu *et al*., 2003). Moreover, *Spirulina* has also been studied for its ability to stimulate mineral absorption through the effects on intestinal microflora. It was also found that *Spirulina* has a high content of calcium, and high calcium bioavailability is associated with bone health balance. Additionally, in a study by Moorhead *et al*. (2012), the high calcium and phosphorus contents in *Spirulina* supported bone development as well as remineralisation of teeth.

The present work was therefore undertaken to evaluate the protective effect of either a once daily dose of GC or combined GC with *Spirulina* (GC+S) against bone fragility induced by GC in a high-fat diet obese rat model. Bone health assessments in the present work included bone densitometry measurements using dual-energy X-ray absorptiometry (DXA), bone biochemical testing, bone dynamic histomorphometric indices (calcein), and gene expression level measurements.

Materials and methods

Animals

Thirty 6-week-old female Sprague Dawley rats with average weights of 100 - 150 g were used and housed with six rats each in standard cages. All the rats were placed in an animal room where the temperature was set at 27 ± 2.0°C, humidity at 62%, and lights on a 12-h light/dark cycle. All the rats had free access to a normal diet pellet of 702P (Gold Coin), and filtered tap water for two weeks before the experiment. Each rat in the groups was labelled on the tail to indicate its identity. The ethical committee of Universiti Kebangsaan Malaysia approved this study under the ethical code UCSI/2018/HOR KUAN/25-JULY/939-JULY-2018-APR-2020.

Induction of obesity in experimental rats

A high-fat diet (HFD) in rat pellet form was used to induce obesity. In addition, a high-fat emulsion (HFE) was also fed to the rats to enhance the rate of obesity induction by oral gavage. Both HFD and HFE were used at the same time for six weeks of obesity induction. The rats were divided into two groups; ND group [normal diet pellet 702P + 1mL of 0.9% saline (oral gavage)] and the obese group [HFD (rat pellet) + 1 mL of HFE (oral gavage)] as indicated in Table 1. Weights of rats were monitored weekly using a weighing balance to the nearest 0.01 g, and their nasal-anal lengths measured to the nearest 0.1 cm using a measuring board and ruler. BMI was calculated by dividing the rat's weight

(g) by the square of the nasal-anal length (cm^2). After six weeks of obesity induction, rats with BMIs ranging above 0.68 g/cm^2 were considered obese (Shabbir *et al.*, 2016).

Table 1. Composition of the normal diet (ND) pellets, high fat diet (HFD) pellets, and high fat emulsion (HFE).

ND		HFD	
Nutrient	**%/100 g**	**Nutrient**	**%/100 g**
Carbohydrate	48.8	Carbohydrate	28.2
Protein	21.0	Protein	12.1
Fat	3.0	Fat	42.8
Vitamin and mineral mix	1.2	Vitamin and mineral mix	1.9
Fibre	5.0	Fibre	2.9
Moisture	13.0	Moisture	7.5
Ash	8	Ash	4.6
Total	100	Total	100

HFE	
Nutrient	**Amount (g/mL)**
Fructose (carbohydrate)	30
L-glutamic acid (protein)	1
Animal fat (fat)	10
Cholesterol (fat)	5
Tween 80	10
1,2-propylene glycol	1

Study design, treatments, and measurement of blood glucose level and BMI of experimental rats

After six weeks of obesity induction, all the rats were tested with plasma glucose levels using a quantitative glucometer, Accu-Chek Performa blood glucose monitoring system, and Accu-Chek Performa Glucose strips. Next, rats with high glucose levels were eliminated from the study. The rats were then weighed and divided into five groups ($n = 6$) based on the following experimental design: (1) *G. cambogia* (GC) group (400 mg/kg), (2) *Spirulina* (S) group (300 mg/kg), and (3) *G. cambogia* (400 mg/kg) + *Spirulina* (300 mg/kg) (GC+S) group. The (4) normal and (5) obese control groups were treated with equal volumes of 0.9% saline water. The rats were treated for four weeks.

Body composition and bone densitometry

After the treatment, the rats were anaesthetised using a cocktail of Ketamil (0.1/100 g) and Xylazil (0.01/100 g) (10:1) by intraperitoneal injection. The anaesthetised rat was positioned in ventral recumbency on the scan table. All scans were performed using dual-energy X-ray absorptiometry (DXA) to evaluate BMD of the rats after treatment. DXA scans were performed to confirm and compare the bone degenerative changes. BMD measurements were obtained from scans at the femur bone as a region of interest (ROI). All DXA scans were analysed using the manufacturer's recommended software (Small Animal Analysis Software, Hologic QDR-1000 System).

Blood sample collection for bone biochemical test

After the completion of the bone densitometry measurements, the rats were sacrificed. Briefly, 5 mL of blood sample was collected using a terminal procedure, which was cardiac puncture. Before taking the blood samples from the rats, the rats were fasted for 24 h. During blood sample collection, all the rats were under anaesthesia. Blood was taken slowly from the ventricle to avoid collapsing of the heart. The serum was obtained for bone marker analysis (calcium, phosphate, and 25-OH vitamin D).

Bone processing and histomorphometric analysis

The rats were administered with calcein (Sigma-Aldrich, St. Louis, MO, USA) at 20 mg/kg body weight nine and two days before euthanasia. Right femurs were harvested and then sectioned into

halves sagittally for histomorphometric analysis. The unstained slides were observed using a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan). The calcein-labelled surface of trabecular femur bone was measured manually using a Weibel grid with the aid of an image analyser (MediaCybernetics Image Pro-Plus, Rockville, MD, USA). The dynamic histomorphometric parameters measured included single- (sLS/BS, unit = %) and double-labelled surface (dLS/BS, unit = %), mineralising surface (MS/BS; extent of bone surface actively mineralising, unit = %), mineral apposition rate (MAR; distance between two labels in a double-labelled surface divided by the time between two calcein injections, unit = μm/day), and bone formation rate (BFR; the product of MAR multiplied by the fraction of labelled bone surface, unit = $\mu m^3/\mu m^2$/day).

Bone biomechanical strength test

Left femurs were harvested for bone biomechanical strength test. Three-point bending test was applied to the femur bones to evaluate their biomechanical strength properties. It was done by using a universal mechanical strength test machine AGS-X 500N (Shimadzu AGS-X10N-10kN). Frozen bone samples were thawed at room temperature for 30 min. By using a digital calliper, bone physical parameters including bone diameter, bone length, and bone mid-points were measured and input into the analyser software, Trapezium X software. Extrinsic and intrinsic bone material properties were measured using Trapezium X material testing software (Shimadzu, Japan) on the computer attached to the universal mechanical strength test machine. Parameters such as maximum force and Young's modulus were obtained. The Young's modulus is a measure of the intrinsic stiffness of the bone, which was measured by the ratio of maximum stress over maximum strain.

Gene expressions

Tibia bones for RNA extraction were snap-frozen in liquid nitrogen immediately after they were harvested and cleaned. Next, 20 mg of ground tibia bone tissues were homogenised, and RNA was extracted by using the innuPREP RNA mini kit (Analytik Jena, Germany). Purity and concentration of the extracted RNA were determined using Nanodrop (ND-100) Spectrophotometer (MET, USA). The qPCR reaction mixture was prepared using SensiFAST SYBR HI-ROX kit (Bioline, USA). Six sets of primers, namely alkaline phosphatase (ALP), OCN, OPG, RANKL, TRAP5b, and peroxisome proliferator-activated receptor gamma (PPARγ) were prepared for the qPCR reaction, in addition to three reference genes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin (β-actin), and gamma actin (γ-actin). A qPCR and data analysis were performed using StepOne qPCR system machine (Applied Biosystems). The 3-step cycling condition for each specific gene was set as follows: 95°C for polymerase activation for 2 min, 95°C for denaturation step of 5 s, 60°C annealing temperature for 10 s interval, and lastly 72°C for extension step at 20 s interval. Expression of the six primers as mentioned above were normalised to the three reference genes, and ΔΔCT values were calculated.

Statistical analyses

All data were expressed as mean ± standard error of the mean (SEM) (n = 6). Statistical analysis was performed using SPSS statistical software (IBM SPSS Statistics for Windows, version 21.0; IBM, Armonk, NY). Data of body weight were analysed by paired-independent *t*-test between the groups. All the data for bone assessment tests were analysed by ANOVA, Fisher's LSD *post-hoc* test, followed by Dunnett's multiple comparison tests to evaluate the treatment effects. A significance level of $p < 0.05$ was used for all statistical tests.

Results

Effect of treatments on BMI of rats

BMI of rats in each group at weeks 0 and 4 of treatments are shown in Table 2.

Table 2. Mean BMI of rats in week 0 (initial BMI before respective treatments) and week 4 (final BMI after respective treatments).

Sample	BMI (g/cm^2)	
	Week 0	**Week 4**
Normal	0.64 ± 0.03^{bcde}	0.64 ± 0.04^{b}
Obese control	0.69 ± 0.01^{acde}	0.69 ± 0.01^{acde}
GC	0.72 ± 0.02^{ab}	$0.64 \pm 0.01^{b*}$
S	0.73 ± 0.03^{ab}	$0.66 \pm 0.02^{b*}$
GC+S	0.73 ± 0.02^{ab}	$0.64 \pm 0.01^{b*}$

Values are mean ± SD, n = 6 rats/group. Data were statistically analysed by One-way ANOVA, Fisher's LSD *post-hoc* test, followed by Dunnett's multiple

comparison test. Week 4 in the same row with asterisk (*) are significantly different at $p < 0.05$ as compared to week 0 (paired-sample *t*-test). Means in the same column with different lowercase superscripts are significantly different at $^{a}p < 0.05$ vs normal; $^{b}p < 0.05$ vs obese control; $^{c}p < 0.05$ vs GC; $^{d}p < 0.05$ vs S; and $^{e}p < 0.05$ vs GC+S.

All the groups with treatments were significantly different ($p < 0.05$) between weeks 0 and 4, except normal and obese control groups.

Effect of treatments on bone densitometry test

In the obese control group, rats which were fed with HFD+HFE showed significant lower BMD of femur as compared to the normal group ($p < 0.05$) (Table 3). GC showed a significant decrease in BMD of femur bone as compared to the obese control group ($p < 0.05$) (Table 3). Furthermore, S group did not show any significant difference as compared to the obese control group ($p > 0.05$). GC+S significantly increased BMD of the femur bone as compared to GC ($p < 0.05$), but it maintained the same level as the obese control and S groups ($p > 0.05$) (Table 3).

Effect of treatments on bone biochemical test

Phosphate and calcium levels significantly increased, which were observed in the obese control group as compared to the normal group ($p < 0.05$), while the 25-OH vitamin D level was maintained as compared to the normal group ($p > 0.05$). Besides, GC showed a significantly higher phosphate level as compared to the normal group, and significantly lower than the obese control group ($p < 0.05$). However, GC did not significantly decrease calcium and 25-OH vitamin D levels as compared to the obese control group ($p > 0.05$). Furthermore, GC+S showed a significant decrease in phosphate and calcium levels as compared to GC ($p < 0.05$). *Spirulina* caused significant increase in 25-OH vitamin D level as compared to the obese control group ($p < 0.05$) which was found in GC+S group. All these results are shown in Table 3.

Effect of treatments on the biomechanical strength test

Based on Table 3, the mean maximum force was not significantly decreased in the obese control group as compared to the normal group ($p > 0.05$). However, a significantly lower Young's modulus value was found ($p < 0.05$). GC did not significantly decrease maximum force as compared to the obese control group ($p > 0.05$), but it was significantly lower than normal group ($p < 0.05$). Between groups, there was a significant increase in maximum force in GC+S when compared with GC ($p < 0.05$). When compared with S, GC+S showed significantly higher maximum force ($p < 0.05$). The obese control group showed significantly lower Young's modulus as compared to the normal group ($p < 0.05$). GC+S improved Young's modulus, which increased significantly as compared to both GC and S alone ($p < 0.05$).

Effect of treatments on bone dynamic histomorphometry test

Trabecular bone of GC+S and S showed more calcein double-labelled surface as compared to obese control group by visual observation (Figure 1). Quantification using Weibel grid revealed that obese rats showed a significant decrease in dLS/BS, MS/BS, MAR, and BFR as compared to the normal group ($p < 0.05$) (Table 3). Besides, sLS/BS was significantly higher, and MAR was significantly lower in trabecular bone of GC as compared to the obese control group ($p < 0.05$). S showed significantly higher sLS/BS, dLS/BS, MS/BS, MAR, and BFR as compared to obese control group ($p < 0.05$). With *Spirulina* supplementation, GC+S showed a bone protective effect as compared to GC. Results also showed significantly lower values in sLS/BS, and higher values in dLS/BS and MAR in GC+S as compared to GC ($p < 0.05$).

Effect of treatments on gene expressions

The obese control group showed downregulation of ALP ($p < 0.05$). The group fed with HFD+HFE showed an increase in PPARγ ($p < 0.05$). Furthermore, GC showed downregulation of OCN and OPG, and significantly increased RANKL. Thus, the OPG/RANKL ratio was downregulated in GC ($p < 0.05$). A single daily administration of GC caused upregulation of TRAP5b and PPARγ expressions ($p < 0.05$). In addition, both GC+S and S groups showed significant upregulation of ALP and OCN ($p < 0.05$). *Spirulina* caused downregulation of OPG which was shown in GC+S as compared to GC ($p > 0.05$). GC+S showed downregulation of RANKL but without significant decrease as compared to GC ($p > 0.05$). *Spirulina* caused a decrease in TRAP5b and PPARγ expressions, which was shown in GC+S and S groups. All results are illustrated in Figure 2.

Table 3. Effect of treatments on bone mechanical strength test, biochemical test, and bone dynamic histomorphometry.

Sample	Normal	Obese	GC	S	GC+S
Bone mineral density (g/cm^2)	0.29 ± 0.00^{bcde}	0.28 ± 0.00^{ac}	0.27 ± 0.00^{bde}	0.28 ± 0.00^{ac}	0.28 ± 0.00^{ac}
Maximum force (N)	90.94 ± 1.73^{ce}	90.61 ± 2.19^{e}	84.42 ± 0.68^{ae}	88.77 ± 3.28^{e}	108.94 ± 2.21^{abcd}
Young's modulus (Pa)	95.04 ± 2.35^{acde}	53.11 ± 2.76^{bcde}	83.72 ± 2.04^{bde}	129.15 ± 7.65^{abcd}	168.99 ± 6.39^{abcd}
Single labelled/bone surface (sLS/BS, %)	21.35 ± 2.73^{cd}	20.31 ± 1.64^{cd}	31.72 ± 3.57^{abe}	29.75 ± 4.63^{abe}	22.77 ± 3.07^{cd}
Double labelled/bone surface (dLS/BS, %)	15.05 ± 5.63^{bc}	7.68 ± 0.40^{ad}	4.88 ± 1.14^{ade}	15.75 ± 3.03^{bc}	12.00 ± 1.50^{c}
Mineralising surface/bone surface (MS/BS, %)	1.40 ± 0.34^{bcd}	0.46 ± 0.04^{ad}	0.74 ± 0.14^{ad}	2.37 ± 0.65^{abce}	0.79 ± 0.19^{d}
Mineral apposition rate (MAR, m/day)	2.84 ± 0.14^{bcde}	1.83 ± 0.05^{acd}	1.27 ± 0.08^{abde}	2.62 ± 0.08^{abce}	1.83 ± 0.01^{acd}
Bone formation rate/bone surface (BFR, m^3/m^2/day)	3.98 ± 1.08^{bcde}	0.94 ± 0.07^{ad}	0.84 ± 0.19^{ad}	6.21 ± 1.53^{abce}	1.44 ± 0.35^{ad}
Phosphate concentration (mmol/L)	1.85 ± 0.04^{bc}	2.73 ± 0.04^{acde}	2.14 ± 0.05^{abde}	1.70 ± 0.14^{bc}	1.74 ± 0.03^{bc}
Calcium concentration (mmol/L)	2.71 ± 0.05^{b}	2.87 ± 0.07^{ade}	2.81 ± 0.06^{de}	2.62 ± 0.01^{bc}	2.66 ± 0.02^{bc}
25-OH vitamin D concentration (mmol/L)	47.50 0.67^{de}	51.20 1.12^{de}	50.50 0.67^{de}	79.00 11.63^{abc}	78.50 0.67^{abc}

Values are mean ± SD, n = 6 rats/group. Data were statistically analysed by One-way ANOVA, Fisher's LSD *post-hoc* test, followed by Dunnett's multiple comparison test. Means in the same row with different lowercase superscripts are significantly different at $^{a}p < 0.05$ vs normal; $^{b}p < 0.05$ vs obese control; $^{c}p < 0.05$ vs GC; $^{d}p < 0.05$ vs S; and $^{e}p < 0.05$ vs GC+S.

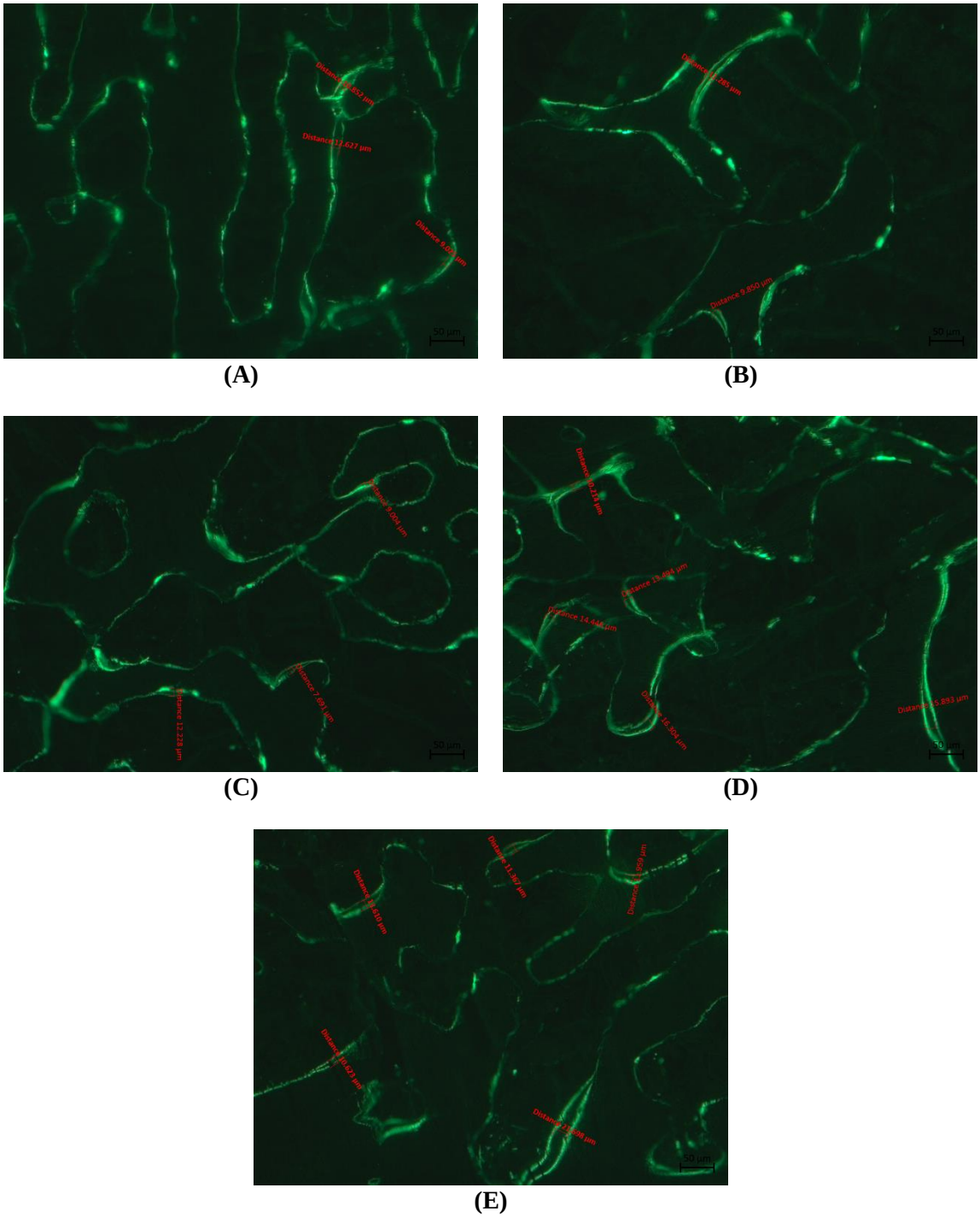

Figure 1. Micrograph of calcein-labelled trabecular bone (20× magnification). Red lines indicate double-labelled surface with distance values between the two labels. (A): normal, (B): obese control, (C): *Garcinia cambogia* (GC), (D): *Spirulina* (S), and (E): *Garcinia cambogia* + *Spirulina* (GC+S).

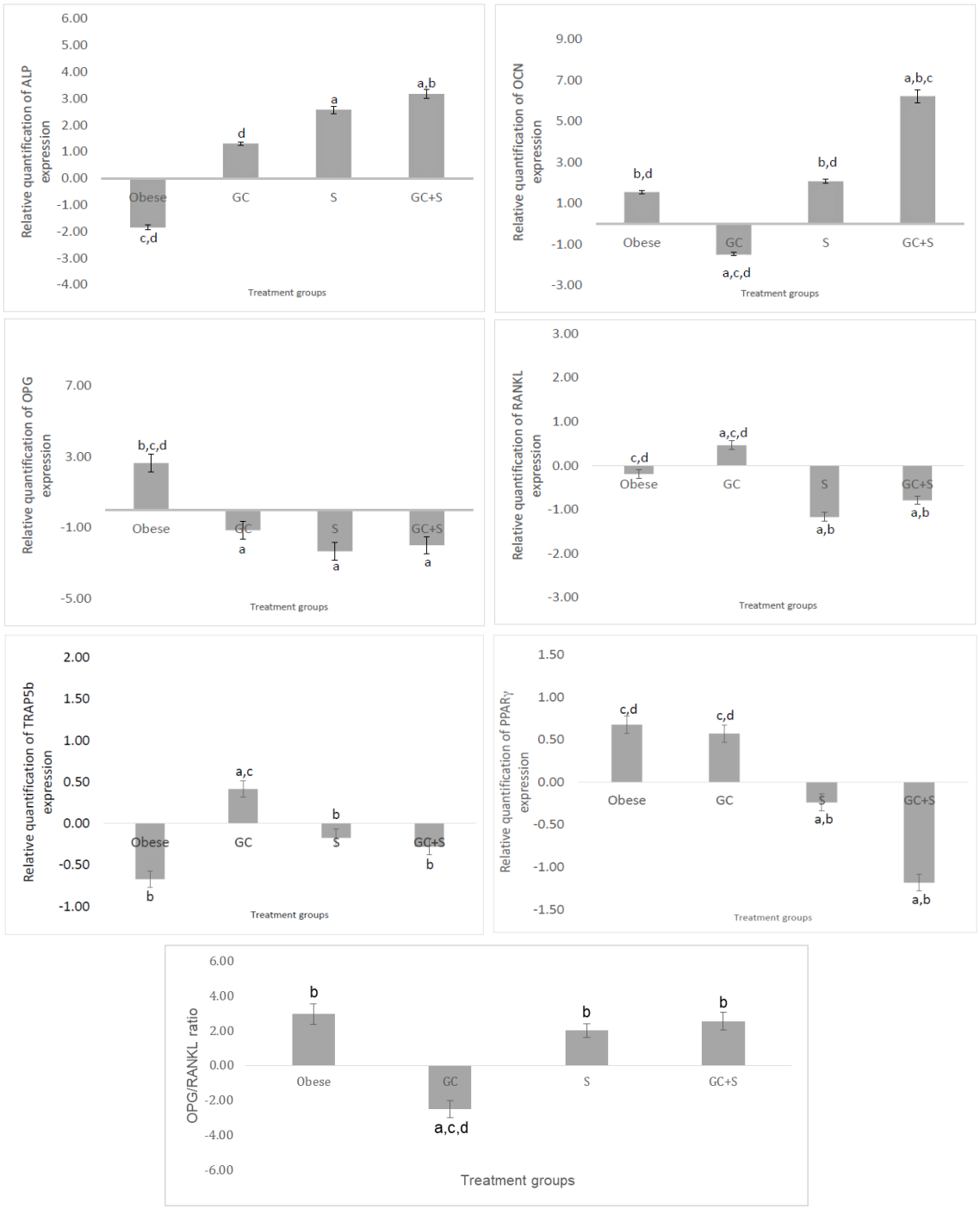

Figure 2. Relative quantification of each gene expression. Data were statistically analysed by One-way ANOVA, Fisher's LSD *post-hoc* test, followed by Dunnett's multiple comparison test. Columns with different lowercase letters are significantly different at [a] $p < 0.05$ vs obese control; [b] $p < 0.05$ vs GC; [c] $p < 0.05$ vs S; and [d] $p < 0.05$ vs GC+S.

Discussion

The rats fed with HFD+HFE had mean BMIs greater than 0.68 g/cm^2 after six weeks, thus confirming that they had become obese (Shabbir *et al.*, 2016), whereas the normal diet used on the control group did not induce obesity. In the present work, female Sprague Dawley rats were used because they are more prone to obesity than males due to the oestrogen hormone (Cao, 2011). Associated with fat metabolism, oestrogen promotes and maintains feminine fat distribution as characterised by adipose tissue accumulation (Lizcano and Guzmán, 2014).

The obese control group which was fed with HFD+HFE showed significantly lower BMD on the femur as compared to the normal group (Table 3). Low BMD is a major risk factor for bone fragility and fractures. A study by Greco *et al.* (2010) showed a similar trend where individuals with higher BMI showed a lower BMD. These were due to the effect of HFD+HFE diet, which was used to induce obesity in the rats. The obese control group showed higher phosphate and calcium levels than the normal group (Table 3). Different dietary lipid and fatty acid compositions in high-fat diet play an important role in bone health. The changes in mineral bioavailability may be influenced by dietary lipid composition in high-fat diet (Xiao *et al.*, 2010). The changes in unsaturated lipid levels and acyl chain lengths may also result in increased intestinal mineral absorption by altering membrane fluidity and function (Xiao *et al.*, 2010). According to Cao (2011), obesity is detrimental to bone despite potential positive effects of mechanical loading on bone conferred by increased body weight with obesity.

Based on Figure 1, the obese control group showed less calcein double-labelled surface as compared to the normal group. This is further highlighted in Table 3, in which the obese control group showed a significant decrease in dLS/BS, MS/BS, MAR, and BFR as compared to the normal group. Therefore, changes in these parameters caused a decrease in bone formation and mineralisation due to the HFD+HFE used to induce obesity in the obese control group. Obesity may increase bone resorption through upregulation of proinflammatory cytokines such as TNF-α that stimulate osteoclastic differentiation (Cao, 2011). According to a study done by Elefteriou *et al.* (2004), leptin secreted by adipocytes in an obese rat model was found to have effects on bone mass. Overproduction of leptin levels resulted in the decrease in osteoblastic activity, and subsequently low bone mass.

Besides Young's modulus value reduction (Table 3), the effects of HFD+HFE on bone fragility could be supported by ALP downregulation and PPARγ upregulation (Figure 2). Obesity could decrease osteoblastogenesis, and increase adipogenesis. This is because osteoblasts and adipocytes are derived from the same multipotential mesenchymal stem cells. Upregulation of PPARγ gene expression decreased osteoblast differentiation, BMD, and trabecular bone mass while increasing adipocyte differentiation and bone marrow adipose tissue volume. Decreased bone mass because of obesity may be due to increased marrow adipogenesis at the expense of osteoblastogenesis, and/or increased osteoclastogenesis because of reduced calcium absorption, upregulation of PPARγ, and/or downregulation of ALP (Cao, 2011). Based on the different parameters shown in Figure 2, HFD could be concluded to exert an adverse effect on the bones. Although obesity is largely known to decrease bone strength, little is known about the mechanism by which obesity could contribute to such damage. The debate is still ongoing with regards to obesity and bone fragility. In the past, obesity was considered as a protective factor for bone health. However, this is not the same given the new concept of obesity which is considered detrimental for bone health (Cao, 2011; Sharma *et al.*, 2014).

In the present work, GC rats had mean BMIs that were lower than the obese range (0.68 g/cm^2). This showed that GC reduced the BMI of the rats significantly after four weeks of treatment (Table 2). This result was supported by Moghaddam *et al.* (2010) who found that GC reduced appetite through neurochemical control of food intake, and inhibited fat synthesis, thus leading to decreased body weight. It is thought that these effects might have been due to the bioactive compound present in GC known as hydroxyl citric acid (HCA). HCA causes a significant spike in serotonin levels that help to reduce appetite, thus playing a role in weight loss in humans. HCA inhibits ATP-citrate lyase, and decreases the synthesis of acetyl-CoA, thus limiting fatty acids in the body. *De novo* fatty acid synthesis inhibition in the adipose tissues is effective with oral intake of GC. GC also helped in the reduction of body fat accumulation in experimental animals (Moghaddam *et al.* (2010). However, recent studies have not supported the anti-obesity properties of GC, and GC

does not appear to help with weight loss in humans despite its popularity. This may be due to the profound difference in the mechanism of GC in rats versus humans. This process, known as *de novo* fatty acid synthesis, is the main weight regulation mechanism in rats, but unfortunately, humans are significantly less reliant on it. This report supported the argument that GC does not aid in reducing fat accumulation due to its appetite suppressant to reduce food intake (Haber *et al.*, 2018).

Next, the present work identified that GC at a dose of 400 mg/kg/day, as recommended by the pharmaceutical industries of health foods, significantly decreased the BMD of femur bone (Table 3). This is further supported by upregulation of TRAP5b which is shown in Figure 2. Upregulation in the relative expression of TRAP5b in GC increased osteoclast differentiation, thus resulting in low BMD and trabecular bone mass (Halling *et al.*, 2017). Furthermore, phosphate levels were higher in GC as compared to the normal group (Table 3). Elevations in phosphorus triggers the release of parathyroid hormone (PTH). This will lead to a high bone turnover, where calcium will be pulled out from the bones. Abnormal calcium and phosphate levels have been associated with bone loss, as both factors are the main constituents of bone minerals that strengthen the mechanical resistance of the bone organic matrix (Lombardi *et al*., 2011). The effects of GC on bone fragility was further reflected in the bone biomechanical analysis. Maximum force was significantly lower as compared to the normal group. GC affected the bone stiffness, and ultimately cannot withstand a higher load at the given displacement.

GC showed significantly lower dLS/BS and MAR, with changes in sLS/BS, dLS/BS, MS/BS, and BFR in bone dynamic histomorphometry indices (Table 3) as compared to the obese control group; this is in agreement with downregulation of OCN expression (Figure 2) following GC treatment. OCN, which is produced abundantly by osteoblasts in bone, primarily plays an important role in bone mineralisation (Zoch *et al*., 2017). Therefore, downregulation of OCN, which acts as a marker for bone formation, led to a decrease in osteoblast differentiation in GC.

GC significantly increased RANKL expression (Figure 2). This showed that GC might have caused a detrimental effect on bone health in the obese rat model. This could have been due to the increase in proinflammatory cytokines, TNF-α in the plasma level by GC. High levels of TNF-α increases the stimulation of osteoclast activity through the regulation of RANKL/RANK/OPG pathway (Cao, 2011). RANKL plays an important role in osteoclastogenesis. OPG is a decoy receptor of RANKL. Osteoclastic differentiation basically can be determined by the biological availability of RANKL and OPG (Hie and Tsukamoto, 2011). Downregulation of OPG and upregulation of RANKL were observed in GC gene expression (Figure 2). This indicated a higher level of osteoclast differentiation.

GC showed a lower PPARγ expression as compared to the obese control group, although there was no observed significant difference between these two groups (Figure 2). GC has been reported to inhibit expression of PPARγ as the proximal effector of adipogenesis (Kim *et al.*, 2007; Kang *et al.*, 2013). In the present work, the effect was not significant probably due to the lower dosage used alongside the shorter duration of GC treatment. GC seemed to play an active role in weight reduction as compared to the obese control group. Meanwhile, several parameters mentioned earlier indicated that GC also promoted bone resorption in the obese rat model.

Spirulina significantly reduced BMI of the rats either alone or in combination (Table 2). BMI reduction was shown in both GC+S and S. *Spirulina* may act as an anti-obesity drug. These results were also supported by several studies on the effects of *Spirulina* in weight management. Individuals with obesity experienced beneficial effects in modulating body weight after receiving *Spirulina* supplementation in a clinical trial (Yousefi *et al*., 2018). Besides that, *Spirulina* decreased triglyceride accumulation; it inhibited adipogenesis through decreased protein expression of the adipogenic regulators (DiNicolantonio *et al.*, 2020). However, as one of the limitations of this study, where only BMI was measured, the actual mechanism and bioactive components present in *Spirulina* responsible for the anti-obesity properties were not elucidated. Further studies are therefore needed to gain more insight into this issue.

Maximum force in the GC+S group was significantly higher than the obese and normal groups. However, for S, it was not significantly lower than the obese and normal groups (Table 3). A previous study by Cho *et al*. (2020) found that *Spirulina* was able to enhance bone strength using rats without high-fat diet on a 7-week treatment. This effect was not seen in the present work, possibly due

to the shorter duration of treatment, and the type of rat model used which was a high-fat diet model. A combination of GC+S may create synergistic effects on the bone that caused bone growth to be greater than normal rats, thus producing higher maximum force and Young's modulus. *Spirulina* helped to improve femur BMD as compared to GC+S and GC (Table 3). According to Napoli *et al.* (2014), *Spirulina* significantly increased the multiple linear regression analysis of BMD. GC+S and S caused a significant increase in Young's modulus values as compared to the obese control group, which means that the intactness and integrity of the bone surface was improved by *Spirulina* (Table 3). This result was due to the vitamin C or collagen properties modulated by *Spirulina*. Bone has a collagen matrix, and contains 90% of type I collagen. Thus, vitamin C, which is a co-factor for collagen synthesis, helped to increase bone stiffness and capacity to absorb the energy of the load. *Spirulina* also contains proteins which may improve the bone collagen structure (Suzer *et al.*, 2020). These results were in line with bone dynamic histomorphometry indices which showed an increase in sLS/BS, dLS/BS, MS/BS, MAR, and BFR in S as compared to the obese control group. GC+S also showed improvement in dLS/BS and BFR as compared to GC ($p < 0.05$) (Table 3).

Phosphate and calcium levels were significantly lower in the S and GC+S groups as compared to obese control and GC groups (Table 3). These observations indicated that both minerals were intact in the bone. There was no leakage of phosphate and calcium from the bones into the blood vessels (Penido and Alon, 2012). A study by Devesh *et al*. (2012) proved that *Spirulina* is rich in minerals such as calcium and phosphate. The presence of these minerals improved the structure and compositions of the bone surface with *Spirulina* supplementation. Vitamin E found in *Spirulina* could protect cells from oxidative damage by scavenging free radicals which could attack osteoblasts and damage cells that are important in bone metabolism (Naina Mohamed *et al.*, 2012). The presence of chlorophyll in *Spirulina* assists in bone formation because magnesium ions are electrically bound to the centre of the heme group in chlorophylls. Magnesium plays a vital role in bone health because it converts vitamin D into the active serum 25-OH vitamin D, which is responsible for calcium re-absorption in the body (Castiglioni *et al.*, 2013). Calcium is critical for bone structure, where it is part of hydroxyapatite in the collagen matrix of the bones (Elbossaty, 2017).

Serum 25-OH vitamin D level was observed to be significantly higher in the *Spirulina* supplementation group, as indicated in GC+S and S groups (Table 3). According to a review article by Radha and Chandra (2018), *Spirulina* is a rich source of vitamin D. Therefore, it might have restored the vitamin D reserve, thus leading to an increase in serum 25-OH vitamin D levels. Vitamin D regulates the expression of several bone proteins, notably osteocalcin (OCN). It promotes the transcription of OCN, and has bidirectional effects on type I collagen as well as alkaline phosphatase (ALP) gene transcription. This aligned with the results of ALP and OCN gene expressions of S as compared to the obese control group (Figure 2). GC+S improved ALP and OCN expressions as compared to GC.

OPG, RANKL, and TRAP5b, which are the markers for bone resorption, were downregulated in GC+S and S (Table 3). The ratio of OPG and RANKL was used to signify osteoclastic differentiation leading to bone resorption. The OPG/RANKL ratio was upregulated with *Spirulina*, which means increased osteoblastic differentiation, thus leading to bone formation (Figure 2). TRAP5b is a marker that reflects the number and activity of osteoclasts, and it was found to be downregulated with *Spirulina*. This means that there was a reduction in osteoclast activity and subsequent bone resorption. This could be because *Spirulina* is a rich source of vitamin B_{12} which improves osteoblast maturation, thus reducing osteoclastogenesis (Devesh *et al.*, 2012).

PPARγ was downregulated in S and GC+S. This showed that *Spirulina* could possibly promote osteoblast-specific transcription factor Runx2 expression through *in vivo* differentiation of mesenchymal cells. The fact that *Spirulina* downregulated PPARγ is further supported in studies by Seo *et al.* (2018) and Yang *et al.* (2020). As a result, an increase in osteoblast differentiation improved the BMD and trabecular bone mass with *Spirulina*. The inhibition of PPARγ was greater when GC was combined with another substance (Oak *et al.*, 2018). This was shown in GC+S which might have produced synergistic effects on PPARγ. Similar effect was also seen in OCN. However, further study is needed to elucidate these suspected synergistic effects of GC+S.

Spirulina, a new natural ingredient was discovered in the present work. In fact, *Spirulina* was

able to function as an anti-obesity drug while protecting from bone fragility. Discovery of *Spirulina* is a promising but comparatively new field; thus, it could be further developed as a potent anti-obesity supplement. *Spirulina* can potentially substitute GC as an ingredient for weight loss especially after considering the adverse effect caused by GC. As of 2009, the FDA has urged consumers to avoid weight loss supplements containing HCA, due to its hepatotoxicity (FDA, 2020). Therefore, further studies on *Spirulina* at different dosages can be performed on obesity and bone fragility to confirm its function as an anti-obesity drug as well as for bone protective effects.

Conclusion

The present work identified the effects of various treatments on BMI and bone health in a high-fat diet obese rat model. Results suggested that oral administration of GC for four weeks lowered BMD, and altered biochemical markers and dynamic histomorphometry indices in the high-fat diet obese rat model. The bone fragility induced by GC might have been due to the imbalance in bone remodelling rates, in turn due to reduction of osteoblastic and elevation of osteoclastic differentiation. Therefore, further research is needed to elucidate the underlying mechanisms behind the induction of bone turnover in an obese rat model by GC. *Spirulina* supplementation exerted a beneficial protection against bone fragility in diet-induced obesity through the upregulation of gene expression for bone formation genes (ALP and OCN), and suppression of bone resorption genes (OPG, RANKL, TRAP5b, and PPARγ). These effects might have been due to the presence of high concentrations of functional bioactive nutrients in *Spirulina* which stimulated osteoblast differentiation, thus improving skeletal integrity. Further research to improve the understanding on the relationship between this newly discovered herbal product as well as its effects on obesity and bone metabolism may help identify a new formula that can increase osteoblastogenesis, while inhibiting adipogenesis and osteoclastogenesis. Ultimately, this knowledge may lead us to develop new therapeutic interventions to prevent both obesity and bone fragility.

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References

Cao, J. J. 2011. Effects of obesity on bone metabolism. Journal of Orthopaedic Surgery and Research 6(30): 1-13.

Castiglioni, S., Cazzaniga, A., Albisetti, W. and Maier, J. 2013. Magnesium and osteoporosis: current state of knowledge and future research directions. Nutrients 5(8): 3022-3033.

Cho, J. A., Baek, S. Y., Cheong, S. H. and Kim, M. R. 2020. *Spirulina* enhances bone modelling in growing male rats by regulating growth-related hormones. Nutrients 12(4): 1187-1205.

Cho, X. 2015. Investigation of effect of *Garcinia cambogia* on bone fragility in high fat diet obese rats. Malaysia: UCSI University, BSc thesis.

Chuah, L. O., Ho, W. Y., Beh, B. K. and Yeap, S. K. 2013. Updates on anti-obesity effect of *Garcinia* origin (-)-HCA. Evidence-based Complementary and Alternative Medicine 2013: article ID 751658.

Devesh, C., Kritika, M., Anroop, N., Kumar, S. P. and Summet, G. 2012. *Spirulina* reverses histomorphological changes in diabetic osteoporosis in Pioglitazone treated rats. Journal of Diabetes and Metabolism 1: article no. 006.

DiNicolantonio, J. J., Bhat, A. G. and OKeefe, J. 2020. Effects of *Spirulina* on weight loss and blood lipids: a review. Open Heart 7(1): article ID e001003

Elbossaty, W. 2017. Mineralization of bones in osteoporosis and osteomalacia. Annals of Clinical and Laboratory Research 5(4): 1-4.

Elefteriou, F., Takeda, S., Ebihara, K., Magre, J., Patano, N., Ae Kim, C., ... and Karsenty, G. 2004. Serum leptin level is a regulator of bone mass. Proceedings of the National Academy of Sciences 101(9): 3258-3263.

Food and Drug Administration (FDA). 2020. FDA recommends against the continued use of Meridia (sibutramine). Retrieved on July 1, 2020 from FDA website: https://www.fda.gov/drugs/drug-safety-and-availability/fda-drug-safety-communication-fda-recommends-against-continued-use-meridia-sibutramine

Greco, E. A., Fornari, R., Rossi, F., Santiemma, V., Prossomariti, G., Annoscia, C. and Migliaccio, S. 2010. Is obesity protective for osteoporosis? Evaluation of bone mineral density in individuals with high body mass index. International Journal of Clinical Practice 64(6): 817-820.

Haber, S. L., Awwad, O., Phillips, A., Park, A. E. and Tam, M. P. 2018. *Garcinia cambogia* for weight loss: alternative therapies. American Journal of Health-System Pharmacy 75(2): 17-21.

Halling, C., Barbro, L., Rylander, E., Krumpel, M., Norgård, M., Narisawa, S., … and Göran, M. 2017. Bone alkaline phosphatase and tartrate-resistant acid phosphatase: potential co-regulators of bone mineralization. Calcified Tissue International 101(1): 92-101.

Hayamizu, K., Hirakawa, H., Oikawa, D., Nakanishi, T., Takagi, T., Tachibana, T. and Furuse, M. 2003. Effect of *Garcinia cambogia* extract on serum leptin and insulin in mice. Fitoterapia 74(3): 267-273.

Hie, M. and Tsukamoto, I. 2011. Vitamin C-deficiency stimulates osteoclastogenesis with an increase in RANK expression. Journal of Nutritional Biochemistry 22(2): 164-171.

Kang, E. S., Ham, S. A., Hwang, J. S., Lee, C. K. and Seo, H. G. 2013. Effects of *Garcinia cambogia* extract on the adipogenic differentiation and lipotoxicity. Korean Journal for Food Science of Animal Resources 33(3): 411-416.

Kim, Y., Kim, K., Kim, M., Lee, J., Lee, K. and Park, T. 2007. A mixture of the aqueous extract of *Garcinia cambogia*, soy peptide and l-carnitine reduce the accumulation of visceral fat mass in rats rendered obese by a high fat diet. Genes and Nutrition 2(4): 353-358.

Lizcano, F. and Guzmán, G. 2014. Estrogen deficiency and the origin of obesity during menopause. BioMed Research International 2014: article ID 757461.

Lombardi, G., Di Somma, C., Rubino M., Faggiano, A., Vuolo, L., Guerra, E., ... and Colao, A. 2011. The roles of parathyroid hormone in bone remodeling: prospects for novel therapeutics. Journal of Endocrinological Investigation 34: 18-22.

Lubkowska, A., Radecka, A. and Mieszkowski, J. 2017. Adiponectin as biomarker of osteoporosis. In Preedy, V. (ed). Biomarkers in Disease - Methods, Discoveries and Applications. Netherlands: Springer.

Moghaddam, G. A., Jonaidi, H., Abbasnejad, M., Yaghoobi, M. M. and Sepehri, H. 2010. Central effect of mammalian oxyntomodulin on food intake in non-fasted and fasted chicks. Iranian Journal of Veterinary Research 11: 51-55.

Moorhead, K., Capelli, B. and Cysewski, G. R. 2012. Nature's superfood: *Spirulina*. 3rd ed. Hawaii: Cyanotech Corporation.

Naina Mohamed, I., Borhanuddin, B., Shuid, A. and Mohd Fozi, N. 2012. Vitamin E and bone structural changes: an evidence-based review. Evidence-Based Complementary and Alternative Medicine 2012: article ID 250584.

Napoli, N., Strollo, R., Sprini, D., Maddaloni, E., Rini, G. B. and Carmina, E. 2014. Serum 25-OH vitamin D in relation to bone mineral density and bone turnover. International Journal of Endocrinology 2014: article ID 487463.

Oak, M., Yi, E., Sharma, K., Kang, S., Gong, D., Oh, S. and Park, E. 2018. Combination of *Garcinia cambogia* extract and pear pomace extract additively suppresses adipogenesis and enhances lipolysis in 3T3-L1 cells. Pharmacognosy Magazine 14(54): article no. 220.

Penido, M. G. and Alon, U. S. 2012. Phosphate homeostasis and its role in bone health. Pediatric Nephrology 27: 2039-2048.

Radha, P. and Chandra, V. 2018. Therapeutic uses of *Spirulina*: a review. International Journal of Current Innovation Research 4: 975-979.

Regard, J. B., Zhong, Z, Williams, B. O. and Yang, Y. 2012. Wnt signaling in bone development and disease: Making stronger bone with Wnts. Cold Spring Harbor Perspectives in Biology 4(12): a007997.

Seo, Y., Kim, K., Choi, J., Koh, E. and Lee, B. 2018. *Spirulina maxima* extract reduces obesity

through suppression of adipogenesis and activation of browning in 3T3-L1 cells and high-fat diet-induced obese mice. Nutrients 10(6): article no. 712.

Shabbir, F., Khan, S., Yousaf, M. J., Khan, M. A. and Rajput, T. A. 2016. Comparison of effect of high fat diet induced obesity and subsequent atorvastatin administration on different anthropometric measures in Sprague Dawley rats. Pakistan Armed Forces Medical Journal 66(5): 699-704.

Shapses, S., Pop, L. and Wang, Y. 2017. Obesity is a concern for bone health with aging. Nutrition Research 39: 1-13.

Sharma, S., Tandon, V., Mahajan, S., Mahajan, V. and Mahajan, A. 2014. Obesity: Friend or foe for osteoporosis. Journal of Mid-Life Health 5(1): 6-9.

Smilowitz, J., German, J. and Zivkovic, A. 2020. Food intake and obesity: the case of fat. Retrieved on April 4, 2020 from NCBI website: https://www.ncbi.nlm.nih.gov/books/NBK53555/

Sripradha, R. and Magadi, S. G. 2015. Efficacy of *Garcinia Cambogia* on body weight, inflammation and glucose tolerance in high fat fed male Wistar rats. Journal of Clinical and Diagnostic Research 9(2): 1-4.

Suzer, B., Seyidoglu, N., Tufekci, K. and Inan, S. 2020. The relationship between *Spirulina platensis* and selected biomechanical indicators of tibiae in rats. Veterinární Medicína 65(1): 18-24.

Xiao Y., Cui J., Shi Y. H., Sun J., Wang Z. P. and Le, G. W. 2010. Effects of duodenal redox status on calcium absorption and related genes expression in high-fat diet-fed mice. Nutrition 26: 1188-1194.

Yang, Y., Du, L., Hosokawa, M. and Miyashita, K. 2020. Effect of *Spirulina* lipids on high-fat and high-sucrose diet induced obesity and hepatic lipid accumulation in C57BL/6J mice. Journal of Functional Foods 65: article ID 103741.

Yousefi, R., Mottaghi, A. and Saidpour, A. 2018. *Spirulina platensis* effectively ameliorates anthropometric measurements and obesity-related metabolic disorders in obese or overweight healthy individuals: a randomized controlled trial. Complementary Therapies in Medicine 40: 106-112

Zoch, M. L., Clemens, T. L. and Riddle, R. C. 2017. New insights into the biology of osteocalcin. Bone 82: 42-49.