

Thermogenic Blend Alone or in Combination with Whey Protein Supplement Stimulates Fat Metabolism and Improves Body Composition in Mice

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ABSTRACT

Background: Certain food ingredients promote thermogenesis and fat loss. Similarly, whey protein improves body composition. Due to this potential synergistic effect, a blend of thermogenic food ingredients containing African mango, citrus fruit extract, *Coleus forskohlii*, dihydrocapsiate, and red pepper was tested alone and in combination with a whey protein supplement for its effects on body composition in sedentary mice during high-fat diet. **Objective:** The objective of this study was to evaluate the interaction of thermogenic foods on improving body composition during consumption of an unhealthy diet. **Materials and Methods:** C57BL/6J young adult male mice ($n = 12$) were placed on a 60% high-fat diet for 4 weeks and subsequently randomly assigned to receive daily dosing by oral gavage of vehicle, the novel blend alone or with whey protein supplement for another 4 weeks. Body composition, thermal imaging of brown adipose tissue (BAT), mitochondrial BAT uncoupling protein 1 (UCP1), and plasma levels of leptin were assessed. **Results:** Novel blend alone and in combination with protein supplement attenuated body weight gain, fat, and increased surface BAT temperature in comparison to vehicle control and to baseline ($P < 0.5$). The combination of novel blend and whey protein supplement also significantly increased UCP1 protein expression in BAT mitochondria in comparison to vehicle control and novel blend alone ($P < 0.5$). **Conclusions:** These data indicate that this novel blend stimulates thermogenesis and attenuates the gain in body weight and fat in response to high-fat diet in mice and these effects were improved when administered in combination with whey protein supplement.

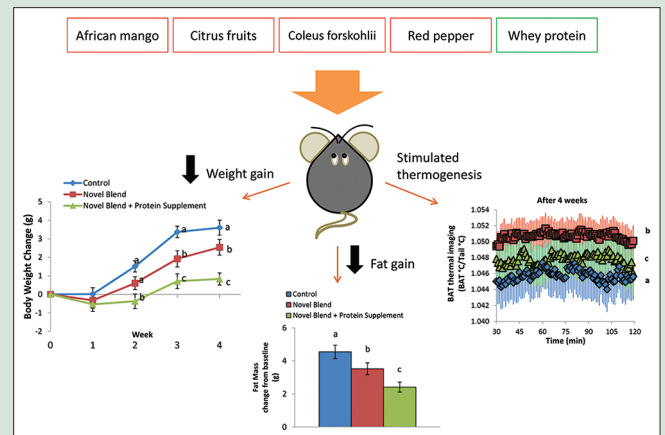
Key words: African mango, capsiate, forskolin, synephrine, thermogenesis, whey protein

SUMMARY

- 30 days oral administration to mice of a novel blend containing African mango seed extract, citrus fruits extract, *Coleus forskohlii* root extract, dihydrocapsiate and red pepper fruit extract reduced body weight and fat gain in response to high-fat diet without impairing muscle mass.
- The novel blend stimulated thermogenesis as shown by the increased

thermal imaging and UCP1 protein expression in brown adipose tissue, indicating that improvement in body composition potentially occurred due to a fat-burning effect.

- The positive effects on body weight, fat, and thermogenesis were improved when the novel blend was administered in combination with a whey protein supplement suggesting that protein provides a synergistic fat-burning effect.



Abbreviations Used: BAT: Brown adipose tissue, UCP1: Uncoupling protein 1, DEXA: Dual-energy X-ray absorptiometry

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INTRODUCTION

The sedentary lifestyle of the current modern society coupled with more frequent consumption of high-fat and/or high-sugar foods has led to an elevated proportion of people with high body fat. According to the National Health and Nutrition Examination Survey, a significant increase was observed for obesity among men and women from 1999 to 2010, with more than 2/3rd of adults in the US being considered overweight.^[1] This unhealthy body composition is associated with low quality of life and several other conditions.^[2] Thus, due to the high prevalence of overweight people and negative impact on health, ways of improving body composition are needed.

Thermogenesis is the physiological process of generating heat. An important site of thermogenesis is the brown adipose tissue (BAT).^[3] BAT generates heat in response to cold temperatures for protection against hypothermia, as well as in response to feeding for dissipation of excess energy from food.^[3,4] Since BAT can convert energy into heat, this body region can be

considered a site for active metabolism. Particularly, BAT has been considered an organ that burns off excess fat, with an area of recent intense research, and an interesting target for improving body composition.^[5,6]

Diet-induced thermogenesis, and a potential stimulation of fat metabolism, can be achieved by consumption of certain foods. Capsinoids, which consist of capsaicin, capsiate, and dihydrocapsiate, are the naturally occurring spicy components in *Capsicum annum* peppers,

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and known to activate thermogenesis via β 3-adrenergic receptors^[7] and upregulation of uncoupling protein 1 (UCP1),^[8] a downstream signal from β 3-adrenergic receptors in BAT.^[6] In clinical studies, capsinoid administration reduced body weight and fat.^[9] The seed extract of *Irvingia gabonensis*, also known as African mango, was shown to reduce body weight and fat in clinical trials^[10-12] by potentially modulating PPAR γ and leptin genes.^[13] Similarly, the root extract of the plant *Coleus forskohlii*, extensively known for its stimulation on intracellular cyclic adenosine monophosphate production, increases UCP1 mRNA and protein *in vitro*^[14] and reduces weight gain and body fat in rodents and humans.^[15-17] Finally, *P*-synephrine, the natural stimulant present in *Citrus aurantium* and other citrus fruits, increases energy expenditure in humans.^[18,19]

Extensive research has also demonstrated that whey protein supplementation promotes satiety, improves body composition, and enhances weight loss.^[20-22] Particularly, previous published preclinical research on the currently tested whey protein supplement revealed significant effects on biomarkers of fat burning, metabolism, and satiety in rats.^[23] Due to this potential synergistic effect of whey protein and thermogenic foods, a novel supplement containing thermogenic food ingredients was tested alone and in combination with a whey protein supplement for effects on body composition and thermogenesis in this current pilot study in mice.

The primary aim of the current study was to assess whether a combination of the above-mentioned food ingredients (novel blend) reduces the gain in body weight and fat that is caused by high-fat diet. Furthermore, it was hypothesized that the addition of whey protein supplementation would improve the benefits of the novel blend in reducing body weight and fat without altering muscle mass. To test these hypotheses, we assessed body weight, fat mass, and muscle mass before and after treatment with novel blend alone or in combination with whey protein in mice fed a high-fat diet. We further carried out experiments to explore the potential mechanism by which novel blend attenuates body weight and fat gain. These experiments suggest that novel blend attenuates body weight and fat gain in response to high-fat diet mainly via stimulation of thermogenesis in BAT rather than reduction in appetite in mice.

MATERIALS AND METHODS

Subjects

C57BL/6J male mice (The Jackson Laboratory, Bar Harbor, ME, USA) initially aged 4 weeks old were ear notched for identification and housed in individually and positively ventilated polysulfonate cages with high-efficiency particulate absolute-filtered air. The animal room was lighted entirely with artificial fluorescent lighting, with controlled 12 h light/dark cycle (6 am to 6 pm light). The normal temperature and relative humidity were 22°C \pm 4°C and 50% \pm 15%, respectively. Food and water were available *ad libitum*. Food consumption was measured twice a week. All experiments were performed by The Jackson Laboratory, approved by The Jackson Laboratory Institutional Animal Care and Use Committee, and conducted in accordance with the NIH guide for care and use of animals.

Study protocol

Animals ($n = 12$ per group) were placed on a 60% high-fat diet at wean age (4 weeks of age) and remained on high-fat diet throughout the study. High-fat diet was supplied by Research Diets, Inc., and irradiated prior to shipment to The Jackson Laboratory. At 8 weeks of age, baseline measures were collected as described below, and subsequently animals were randomly assigned to receive daily dosing by oral gavage of vehicle, the novel blend alone or in combination with whey protein supplement.

At 12 weeks of age, posttreatment measures were collected as described below.

Supplements

The test article is a blend that contains African mango (*I. gabonensis*) seed extract (daily human equivalent dose of 300 mg; Nutrigenesis, Vermont, USA), citrus fruit extract from *Citrus aurantium*, *Citrus sinensis*, and *Citrus paradisi* standardized to 5% synephrine and 80% bioflavonoids (daily human equivalent dose of 1000 mg; Novel Ingredients, NJ, USA), *Coleus forskohlii* root extract 10% forskolin (daily human equivalent dose of 500 mg; Suan Farma, NJ, USA), dihydrocapsiate (2.4 mg daily human equivalent dose; Glanbia, Ireland), and red pepper (*Capsicum annum*) fruit extract 2% capsinoids (1.56 mg daily human equivalent dose). The novel blend was dissolved in 20-mL 0.5% carboxymethyl cellulose at 250 mg/kg. Control group received 20-mL 0.5% carboxymethyl cellulose. The human equivalent dose was calculated per ingredient as described by Reagan-Shaw *et al.*, 2008.^[24]

Safety of the novel blend was first evaluated in an acute maximum tolerated dose study (data not presented) in which 12 ICR mice were placed on 60% fat diet for 8 days and subsequently treated with 125, 250, or 500 mg/kg of the novel supplement by oral gavage daily for 5 days. No observable adverse event was noted at any given dose. The middle dose of 250 mg/kg was selected for the current study. This experiment was independently performed and analyzed by Wasatch Scientific (Murray, Utah, USA).

Protein supplement consisted of 4LifeTransform® PRO-TF (4Life Research, LLC, UT, USA), a blend containing whey protein concentrate, extensively hydrolyzed whey and egg white protein, bovine colostrum, and egg yolk extract. Protein supplement was administered once daily via oral gavage 4 h apart from novel blend treatment at the human equivalent dose of 10 g (2000 mg/kg mouse dose) dissolved in 10-mL deionized water. Novel blend was administered in the morning and protein supplement was administered in the afternoon. Due to a limited amount of gavage material a mouse can take, the supplements were not administered at the same time. The choice of administration of whey protein in the afternoon was to match previous rodent study that demonstrated that this particular whey protein supplement administered in the early afternoon to rats produces significant effects on markers of body composition.^[23] Future studies are necessary to evaluate the impact of the timing of administration of novel blend on body composition.

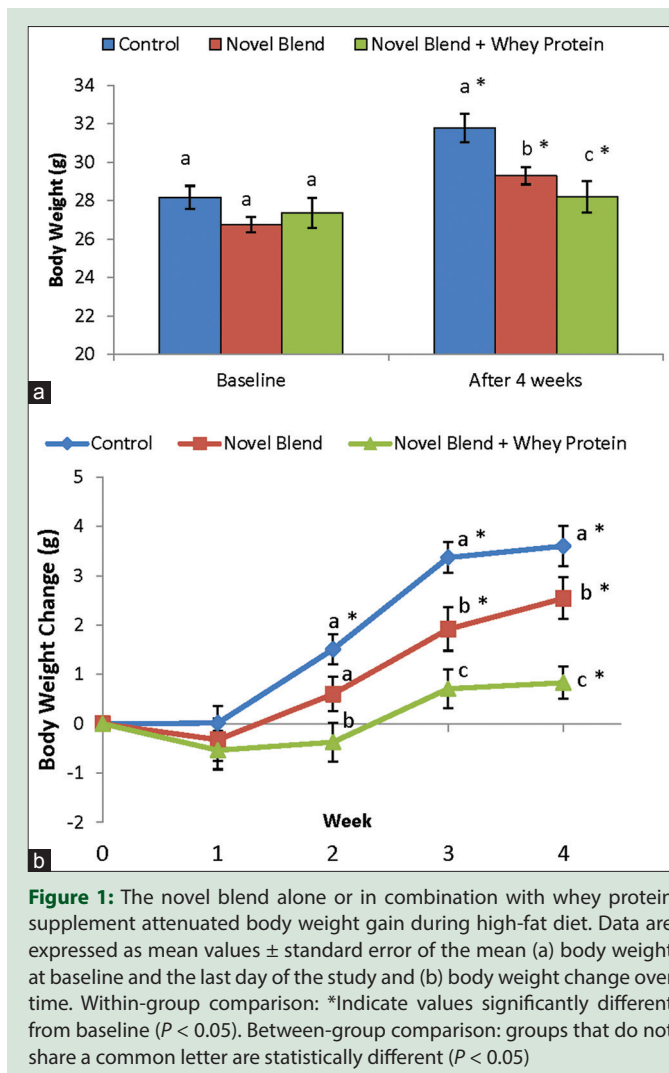
Body weight and composition

Body weight was measured three times a week for the entire course of the study. Just prior to treatment (baseline) and after 4 weeks of treatment, body weights, fat mass, and lean muscle mass were measured using dual-energy X-ray absorptiometry (DEXA). Mice were anesthetized via gaseous anesthetic (isoflurane) for approximately 10 min during recording. Body weights shown in Figure 1a and body weights at week 4 in Figure 1b were assessed via DEXA.

Thermogenesis

Surface thermal imaging of BAT and tail regions were obtained using the FLIR A6703sc thermal camera and ResearchIR software (FLIR Systems, OR). Mice were anesthetized via gaseous anesthetic (isoflurane) and fur removed from the subscapular region and at the base of tail using a small electric shaver. During this procedure, mice were set on a heated platform (37°C) to maintain body temperature. The following day, mice were again anesthetized, placed on heated platform, and temperature reading was recorded over the course of 0–120 min. Outcome measures were obtained before and after 4 weeks of treatment.

Mitochondrial UCP1 protein expression was assessed via Western blot from isolated intrascapular BAT harvested after the final thermal



imaging scan ($\sim 2\frac{1}{2}$ h postfinal dose) and snap-frozen on dry ice. Mitochondria were isolated from BAT using Abcam Mitochondrial Isolation Kit (ab110168, Abcam, MA, USA). Protein quantification was determined using DC™ Protein Assay (BioRad Life Science Research, CA, USA). UCP1 protein quantification was determined using ProteinSimple Wes™ system (ProteinSimple, CA, USA) using 0.5- μ g protein loading, Ucp1 antibody at 1:100 dilution, and Cox 4 antibody (mitochondrial housekeeping) at 1:100 dilution.

Leptin levels

Plasma level of leptin was assessed using Mouse Metabolic Kit (K15124C-3) from Meso Scale Diagnostics, MD. Whole blood was collected from mice via retro-orbital eye bleed (~ 200 μ l) into BD P800 vacutainers and processed in a refrigerated centrifuge set to 4°C and spun at 14,000 rpm for 10 min to obtain plasma. Leptin level was obtained before and after 4 weeks of treatment.

Statistical analysis

Statistical analyses were conducted using Statistica software (Dell, Oklahoma, USA). The baseline and posttreatment outcome measures were analyzed using one-way ANOVA followed by Newman-Keuls *post hoc* test for determination of significance among groups. Within-group comparisons were obtained using paired *t*-tests. Difference among

groups was considered significant if the probability of type I error was $< 5\%$ ($P < 0.05$).

RESULTS

Body weight

All animals received high-fat diet throughout the study, and as expected and shown in Figure 1, all groups gained weight over time ($P < 0.05$). However, the amount of weight gain differed among groups. Results presented in Figure 1a reveal that animals on high-fat diet treated with the novel blend weighed significantly less than animals on high-fat diet treated with vehicle control at the end of 4 weeks of daily oral supplementation ($P = 0.0168$ vs. control). The addition of whey protein supplementation led to a further reduction in body weight gain [Figure 1a; $P = 0.0028$ vs. control]. As shown in Figure 1b, within-group comparison analysis of body weight change revealed that animals treated with vehicle control gained significant amounts of weight at weeks 2, 3, and 4 in comparison to baseline; whereas, a significant amount of weight gain was observed only at weeks 3 and 4 in animals treated with the novel blend alone, and at week 4 in animals treated with the combination of novel blend and whey protein ($*P < 0.05$ vs. baseline). Similarly, between-group comparison revealed that body weight gain was greater in animals treated with vehicle control at weeks 3 and 4 in comparison to animals treated with the novel blend alone, and at weeks 2, 3, and 4 in comparison to animals treated with the combination of novel blend and whey protein ($^{a,b,c}P < 0.05$).

Body composition

Body composition was assessed to investigate whether the reduction in body weight gain was due to reduction in fat mass, muscle mass, or both. Results presented in Figure 2a demonstrate that, despite all animals gaining fat over time ($*P < 0.05$ vs. baseline), animals that received the novel blend alone and in combination with the whey protein supplement had significantly less fat mass than animals that received vehicle control ($^{a,b}P < 0.05$). Analysis of fat mass change from baseline demonstrated that novel blend alone reduces fat mass gain in comparison to vehicle control [Figure 2b; $^{a,b}P < 0.05$]. The addition of whey protein supplement further reduced fat mass gain [Figure 2b; $^{a,b,c}P < 0.05$ vs. control and novel blend alone]. Lean mass was not significantly different among groups at baseline and at the end of the study [Figure 2c]. However, within-group comparison showed that the novel blend + whey protein supplement group had a reduction in lean mass ($*P < 0.05$ vs. baseline). Between-group comparison of lean mass change from baseline similarly revealed that the novel blend + whey protein supplement group lost lean mass at the end of 4 weeks [Figure 2d; $^{a,b}P < 0.05$ vs. control and novel blend alone].

Fat metabolism and thermogenesis

As an approach to understand the potential mechanism by which novel blend alone and in combination with whey protein supplementation reduces body weight and fat gain, the end points of fat metabolism and thermogenesis were assessed. The novel blend alone and in combination with whey protein supplementation led to significantly greater BAT temperature than animals that received vehicle control [Figure 3a and d; $^{a,b,c}P < 0.05$]. Additionally, data revealed that the novel blend alone increased BAT temperature to a greater extent than the novel blend + whey protein supplement [Figure 3a and d; $^{a,b,c}P < 0.05$ vs. novel blend alone]. Representative image of thermal imaging data is shown in Figure 3b. Thermal imaging data at baseline are shown in Figure 3c. In contrast to the thermal imaging data, as shown in Figure 4, protein expression in BAT of the thermogenic marker UCP1 was

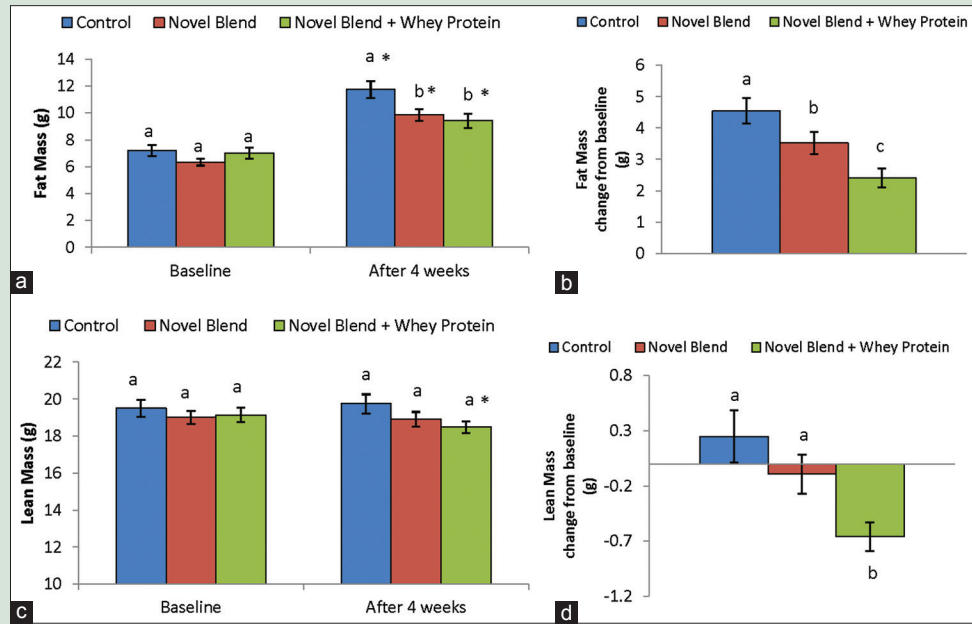


Figure 2: The novel blend alone or in combination with whey protein supplement attenuated body fat gain in high-fat diet with small change in lean mass. Data are expressed as mean values \pm standard error of the mean (a) total fat mass, (b) fat mass change from baseline, (c) total lean mass, and (d) lean mass change from baseline. Within-group comparison: *Indicate values significantly different from baseline ($P < 0.05$). Between-group comparison: groups that do not share a common letter are statistically different ($P < 0.05$)

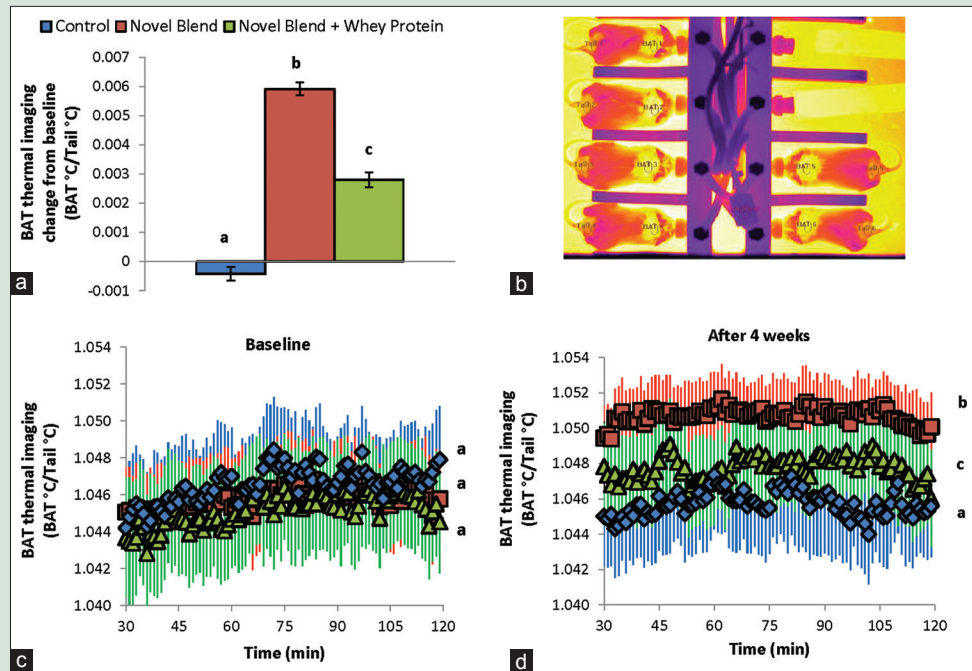


Figure 3: The novel blend alone or in combination with whey protein supplement increased thermal imaging of brown adipose tissue. (a) Data are expressed as mean values \pm standard error of the mean of 0–120 min brown adipose tissue thermal imaging change from baseline. (b) Representative image of thermal imaging data depicting the region of interest of brown adipose tissue positive and negative (tail). Means \pm standard deviation at each time point from 30–120 min are shown for brown adipose tissue thermal imaging collected at (c) baseline, and (d) at the end of the study. Groups that do not share a common letter are statistically different ($P < 0.05$)

significantly greater in animals that received novel blend in combination with whey protein supplement ($^{ab}P < 0.05$), but no significant effect in UCP1 was found in the novel blend alone group.

Food consumption

As shown in Table 1, no treatment effect was found among groups and in comparison to baseline on the food consumed. The amount

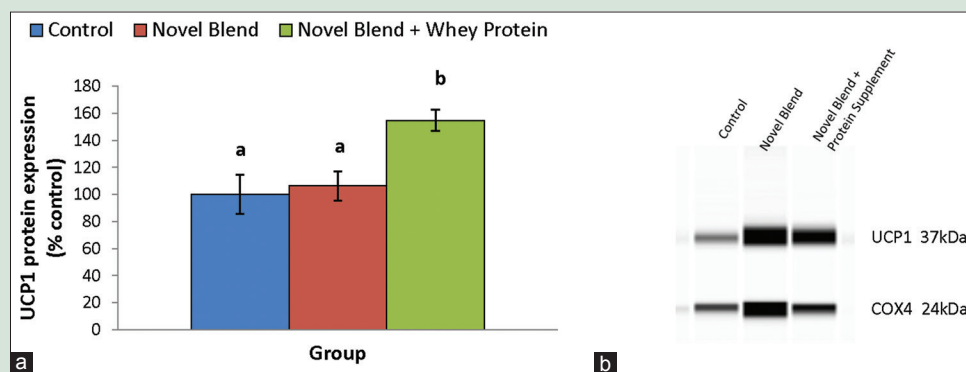


Figure 4: The novel blend in combination with whey protein supplement increased UCP1 protein expression in brown adipose tissue. (a) Data are expressed as percent values \pm standard error of the mean of mitochondrial UCP1 protein expression normalized to COX4 collected at the end of the study. Groups that do not share a common letter are statistically different ($P < 0.05$). (b) Representative Western blot image from the data shown in (a)

Table 1: Food consumption (mg of food/g body weight)

	Control	Novel blend	Novel blend + whey protein
Week 1	84.8 \pm 5.5	84.4 \pm 7.2	73.4 \pm 5.9
Week 2	88.8 \pm 5.5	91.1 \pm 9.9	85.0 \pm 8.6
Week 3	102.8 \pm 7.6	110.5 \pm 13.5	85.9 \pm 7.8

Data expressed as mean \pm SEM. SEM: Standard error of mean

Table 2: Blood leptin (pg/ml)

	Baseline	After 4 weeks	Difference
Control			
Mean	13,614	16,764	3150 ^a
SEM	1726	2243	2023
Novel blend			
Mean	11,805	14,070	2266 ^a
SEM	1393	2297	1833
Novel blend + whey protein			
Mean	14,314	10,275	-4038 ^b
SEM	2645	1691	2053

Between-group comparison: groups that do not share a common letter are statistically different ($P < 0.05$). SEM: Standard error of mean

of food consumed by the novel blend + whey protein supplement group had a tendency to be less in comparison to control and novel blend alone groups, but these effects were not statistically different ($P > 0.05$).

Leptin levels

Pre-/post-comparisons of leptin levels in plasma revealed significant treatment effect of the novel blend + whey protein supplement in comparison to vehicle control and novel blend alone [Table 2; ^{a,b} $P < 0.05$].

DISCUSSION

Current data reveal that 4-week oral administration to mice of a novel blend stimulates thermogenesis and attenuates the gain in body weight and fat in response to a high-fat diet. The positive effects on body weight, fat, and thermogenesis were improved when the novel blend was administered in combination with a whey protein supplement. No significant effects of novel blend alone were observed on lean muscle mass and plasma leptin levels. However, a reduction in lean muscle and leptin levels was observed in mice treated with novel blend + whey protein supplement.

Previous research has revealed that oral administration of *I. gabonensis*^[10] and *C. forskohlii*^[15,25] reduces body weight gain. In a clinical trial with overweight adults, *I. gabonensis* seed extract administered before lunch and dinner for 10 weeks reduced body weight in comparison to placebo control.^[10] In a 6-week study with young rats fed a high-fat/high-sugar diet, oral administration of *C. forskohlii* root extract attenuated body weight gain in comparison to high-fat/high-sugar diet alone in as early as 1 week.^[15] Similarly, in the current investigation, oral administration to mice of a novel blend that contains *I. gabonensis* and *C. forskohlii* attenuated body weight gain after 3 weeks in comparison to high-fat diet alone.

Similar to novel blend alone, novel blend + whey protein supplement reduced body weight gain in response to high-fat diet. Particularly, the reduction in body weight gain was greater in novel blend + whey protein supplement group than that in novel blend alone group. Furthermore, no difference in food consumption was found among groups and thus the significant effect of novel blend and whey protein supplement on body weight gain was likely not due to reduction in food consumption.

The current study also revealed that the novel blend alone or in combination with whey protein supplement reduces fat mass gain in response to high-fat diet. These data resemble previous research conducted on the individual ingredients in the novel blend and in whey protein supplements. In clinical studies, *I. gabonensis* seed extract,^[10] capsinoids,^[9] forskolin,^[17] and whey protein^[21,26] administration showed reduction in total body fat in comparison to placebo. Furthermore, this finding is in agreement with previous clinical studies demonstrating that whey protein,^[21] particularly whey hydrolysate,^[26] reduces body fat.

Protein supplementation, without an exercise component, has also been shown to stimulate muscle protein synthesis in rats^[23] and increase muscle mass in humans.^[20,22,26] In contrast, muscle mass was reduced in the novel blend + whey protein supplement group in comparison to novel blend alone and control groups. These effects potentially occurred due to the significant greater reduction in body weight gain observed in this group.

The mechanism through which the novel blend reduced fat mass can be in part explained by its thermogenic effect. Previous research has demonstrated that capsinoids and forskolin increase BAT temperature and the protein and mRNA expression of the thermogenic biomarker UCP1.^[8,14,27] Furthermore, previous study demonstrated that oral administration of the whey protein supplement used in the current investigation increases other fat metabolism markers, namely, phospho-HSL and PGC-1 α in rats.^[23] In accordance with these prior studies, in the current investigation, the novel blend increased thermal

imaging of BAT in comparison to vehicle control and this effect remained significant when the novel blend was administered with a whey protein supplement. In addition, the thermogenic biomarker UCP1 was increased in the novel blend + whey protein supplement group, but unexpectedly not in the novel blend alone. Unpublished data suggest that a higher dose of novel blend might be necessary for upregulation of UCP1 protein.

The satiety hormone leptin was investigated as another possible mechanism for the reduction in body weight gain. Previous clinical and preclinical studies have demonstrated that bitter orange reduces hunger cravings,^[18] *I. gabonensis* and capsinoids reduce blood leptin levels,^[10,28] and *Coleus forskohlii* reduces food intake.^[16,25] Furthermore, whey protein has also shown to reduce leptin levels in rodents,^[29] although not in humans.^[30] In the current study, blood leptin levels at posttreatment were different in the novel blend + whey protein group in comparison to control and novel blend alone groups. However, since no significant effect was found in the novel blend-alone group, any effect in leptin levels may be due to the whey protein supplement as opposed to the novel blend. Another possibility for the lack of effect in blood leptin in the novel blend-alone group is that blood leptin levels were not significantly increased with 4 weeks of high-fat diet as seen in the within-group comparisons of posttreatment versus baseline in all the three groups. Further studies on the effects of novel blend on blood leptin during high-fat diet are needed to elucidate this issue.

CONCLUSIONS

In conclusion, oral administration to mice of a novel blend containing *I. gabonensis*, citrus fruit extract, *C. forskohlii*, dihydrocapsiate, and red pepper stimulated thermogenesis and attenuated the gain in body weight and fat in response to high-fat diet without affecting muscle mass. The novel blend increased thermal imaging of BAT, indicating that improvement in body composition potentially occurred due to a fat-burning effect. Furthermore, reduction in body weight and fat gain improved when novel blend was administered in combination with whey protein supplement. Particularly, novel blend + whey protein supplement increased UCP1 protein expression in BAT in addition to augmentation of BAT thermal imaging, suggesting that protein supplement provides a synergistic fat-burning effect.

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4Life Holdings, LLC.

Conflicts of interest

All of the authors are currently employees of the funding organization.

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