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The Synergistic Effect of Lotus Leaf, Chinese Hawthorn, Cinnamon, Ginger, and Red Pepper on Anti-obesity

Hui-Chun Chang[1], Kai-Wen Kan[1], Jia-Haur Chen[1], Yung-Hao Lin[2], Yung-Hsiang Lin[1], Chen-Meng Kuan[1,*]

[1]Research & Design Center, TCI CO., Ltd., Taipei, Taiwan
[2]Global Business Center, TCI CO., Ltd., Taipei, Taiwan
*Corresponding author: Chenmeng.Kuan@tci-bio.com

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Abstract Lotus leaf, Chinese hawthorn, cinnamon, ginger, and red pepper possess noticeable anti-inflammatory, hypolipidemic, and anti-obesity effects in traditional Chinese medicine. Their availability for anti-obesity has been well investigated in the past studies, but the synergistic effect of these components on inhibition of adipogesis of adipocytes and hepatocytes and improvement in lipid metabolism are elusive. The objective of this *in-vitro* study is to investigate the efficacies of the combination of these ferments (called lotus leaf ferment) regarding anti-obesity and reduction of lipid accumulation in adipocytes and hepatocytes. O Red O staining assay and gene analysis were introduced into the study. In comparison with the control group, the oil content levels in OP9 cells and HepG2 cells after treatment with lotus leaf ferment solutions could be improved by 38% and 56%, respectively. Also, lotus leaf ferment could significantly down-regulate the expression of *CEBPa* and *GLUT4* genes as well as enhance the expression of *PLIN1* gene in OP9 cells; the improvement effects on *CEBPa*, *GLUT4*, PLIN1 genes were 0.46, 0.44, and 0.23 fold, respectively. On the other hand, as compared with the control group, the expression levels of *SCD*, *PPAR-γ*, and *PPAR-α* were able to be ameliorated by 0.33, 0.4, and 0.4 fold, respectively. In summary, the lotus leaf ferment can improve the lipid reduction and the expression of the lipolysis-, adipogenesis-related genes in adipocytes and hepatocytes. Although the results unveil the early evidences for the efficacy of the lotus leaf ferment, we believe that the combination of herbs (lotus, Chinese hawthorn, cinnamon, ginger, and red pepper ferments) has potential to improve metabolic disorders and manage weight in humans.

Keywords: *lotus leaf, Chinese hawthorn, cinnamon, ginger, red pepper, anti-obesity*

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1. Introduction

Overweight and obesity (BMI ≥ 30 kg/m^2) are global epidemic and cause heavy healthcare burden on healthcare systems (e.g., additional 42% of healthcare expenditure on obese individuals in the U.S.A.) [1]. According to the report of World Health Organization (WHO), 1.9 billion adults were overweight and 650 million of overweight people were obese in 2016 [2]. UK government has estimated that 60% of adult men and 50% of adult women will suffer from obesity by 2050 in the UK [3]. Obesity is the leading cause to disability and several non-communicable diseases (NCDs), such as cardiovascular diseases, diabetes mellitus, and cancers [4]. The dilemma can be managed with minimal effort by weight control and diet modification together with moderate exercise. However, the self-disciplinary actions are difficult for general people. In clinical practice, physicians may treat patients with severe obesity with anti-obesity drugs, but these drugs are often accompanied by some adverse effects (e.g., headache, back pain, fatigue) [5]. Considering the notorious side effects, some researchers have recently focused on the development of harmless herbal remedies for anti-obesity, such as ingredients extracted from turmeric, green tea, or chili pepper [6]. Moreover, the approach has also been used in the development of nutraceutical supplements with respect to weight loss or improvement of fat metabolism [7].

This work demonstrates an herbal-based formula mainly composed of the ferments of lotus leaf, Chinese hawthorn, cinnamon, ginger, and red pepper for improvement in lipid metabolism. Lotus (*Nelumbo nucifera*) is a commonly used plant in Asian regions; lotus leaf and seed can provide the benefits of anti-inflammatory, hypolipidemic, anti-diabetic, and anti-obesity effects given that the bioactive compounds of louts enable the suppression of carbohydrate and fat adsorption, along with enhancement of lipid degradation and energy depletion [8-12]. Chinese hawthorn (*Crataegus pinnatifida*), commonly known as Shan Zha, accounts for 50% of the antihyperlipidemic remedies in traditional Chinese medicine, and it has also been proved to be beneficial for improvement of the activity of hepatic fatty acid oxidation enzyme and the inhibition of adipogenesis

[13,14]. Moreover, cinnamon, ginger, and red pepper, the well-known folk food ingredients, has potential to ameliorate lipid metabolism. As demonstrated by *in-vitro* and animal models, their extracts can reduce the occurrence of adipogenesis, enhance insulin resistance and lipolysis, and improve thermogenic efficiency [15-18]. Accordingly, these herbs are potent materials for the development of efficacious anti-obesity supplements. We take advantage of the combined ferments to investigate their synergistic effect on lipid metabolism in this study.

2. Material and Methods

2.1. Materials

Lotus Leaf Enzyme Drink [IBESTHIN, Mageline, China; ingredients: water, fermented vegetable extracts (cinnamon, ginger, pepper, 20 wt%), fermented lotus leaf and Chinese hawthorn extracts (10 wt%), apple juice, young guava fruit extract, lactitol, isomaltooligosaccharide, glucose, polydextrose, pear jucie, resistant dextrin, balsam pear powder, corn silk powder, chitosan-oligosaccharide, grapefruit juice, pectin, citric acid, and food flavor], OP9 cells (ATCC® CRL-2749™), HepG2 (ATCC® HB-8065™), culture medium of OP9 cells [90% minimum essential medium alpha medium (Gibco), 20% fetal bovine serum (FBS, Gibco), and 1% penicillin-streptomycin (Gibco)], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco), Oil-red O staining reagent (Sigma), culture medium of HepG2 cells [Dulbecco's Modified Eagle Medium (Gibco), 10 % FBS (Gibco) and 1 % penicillin/streptomycin (Gibco)], oleic acid (OA, Sigma), bovine serum albumin) (BSA, Bio Basic Inc), formaldehyde (ECHO), isopropanol (ECHO), phosphate buffered saline (PBS, Gibco), Microscopy (ZEISS), ELISA reader (BioTek), RNA extraction kit (Genaid Biotech), nCounter® platform (NanoString Technologies).

2.2. Cell Viability Assay

8×10^4 OP9 cells and 1×10^5 HepG2 cells in 0.2 mL of culture media were put into each well in 96-well plates and were incubated at 37°C for 7 days and 18 hours, respectively. Afterwards, the OP9 cells and HepG2 cells were treated with different concentrations of lotus leaf ferment followed by 7-10 days and 24 hours incubation, respectively. 15 μL of MTT (4 mg/mL) was added to each well followed by 3 hours interaction. Finally, 50 μL of DMSO in each well was used to dissolve formazan crystals and measure the absorbance at 570 nm via an ELISA reader.

2.3. Adipocytic Lipid Accumulation Assay

8×10^4 OP9 cells in 0.5 mL of culture medium were incubated at 37°C in each well of 24 well plates for 7 days for cell differentiation and lipid formation. Afterwards, the cells were treated with lotus leaf ferment solutions, and incubated for another 7-10 days. Following the cell fixation procedure (PBS, 10% formaldehyde, and 60% Isopropanol), the cells were stained with Oil red O staining reagent. Finally, the staining results were recorded with a microscopy and analyzed by an ELISA reader.

2.4. Hepatic Lipid Accumulation Assay

1×10^5 HepG2 cells in 2 mL of culture medium were incubated at 37°C in each well of 6 well plates for 18 hours. Subsequently, the cells were treated with lotus leaf ferment solutions in 2% FBS medium for 24 hours. After sample treatment, OA solutions were added into the cells to induce the lipid formation. Afterwards, the cells were stained with Oil red O staining reagent followed by the cell fixation procedure. In the end, the staining results were recorded with a microscopy and analyzed by an ELISA reader.

2.5. Analysis of mRNA Expression

1.5×10^5 OP9/HepG2 cells in 2 mL of culture medium with 0.125% or 0.25% lotus leaf ferme were added into each well of 6 well plates for 24 hours incubation. Next, we collected the OP9/HepG2 cells and extracted their total RNA by the RNA extraction kit. The mRNA expression analysis was completed by the nCounter platform.

2.6. Statistical Analysis

The statistical significance of each experimental result was analyzed by Student's t-test in Microsoft Excel software; p value < 0.05 represents a significant difference.

3. Results

3.1. Lipid Metabolism in Adipocytes

In this study, we employed OP9 and HepG2 cells as research models to investigate the synergistic effect of the lotus leaf ferment on lipid metabolism. OP9 cells, a type of embryonic stem cells, are indentified as a reliable and efficient cell line to study adipocyte differentiation [19]. Figure 1 shows the cell viability results of OP9 cell after treatment with different concentrations of lotus leaf ferment solutions. In comparison with the control group, the proliferation efficiencies of OP9 cells were improved by 34%, 16%, and 4% by the corresponding treatments of 0.06%, 0.13%, and 0.3% lotus leaf ferment solutions. The cell viability trend was inversely correlated with the increasing level of lotus leaf ferment. Figure 2 shows the triacylglycerol droplet accumulation results of OP9 cells after treatment with 0.06%, 0.13%, 0.3%, and 0.5% of lotus leaf ferment solutions. All levels of lotus leaf ferment solutions could significantly inhibit lipid synthesis and accumulation as compared with the control group; 0.06%, 0.13%, 0.3%, and 0.5% of lotus leaf ferment solutions could improve the oil content levels of OP9 cells by 29%, 38%, 19%, and 18%, respectively. Especially, low concentration of lotus leaf ferment got the better improvement effect than high concentration of lotus leaf ferment. The improvement effect also reflected the gene expression result (Figure 3). The expression of

CEBPA (CCAAT enhancer binding protein alpha, CEBPα), *GLUT4* (glucose transporter type 4), and *PLN1* (perilipin-1) genes in OP9 cells was significantly improved by treatment with lotus leaf ferment. The expression levels of *CEBPa* in 0.06%, 0.13%, 0.25%, and 0.5% groups were down-regulated by 0.46, 0.44, 0.23, 0.16 fold, respectively, as compared with the control group. The improvement of *CEBPa* expression showed a dose-dependent effect. The expression levels of *GLUT4* in 0.06%, 0.13%, 0.3%, and 0.5% groups were improved by 0.28, 0.17, 0.29, and 0.3 fold, respectively, as compared with the control group. The expression levels of *PLN1* in 0.06%, 0.13%, 0.3%, and 0.5% groups were improved by 0.65, 0.56, 0.59, and 0.59 fold, respectively, as compared with the control group. However, different concentrations of lotus leaf ferment solutions did not demonstrate distinctive influences on *GLUT4* and *PLN1* expression.

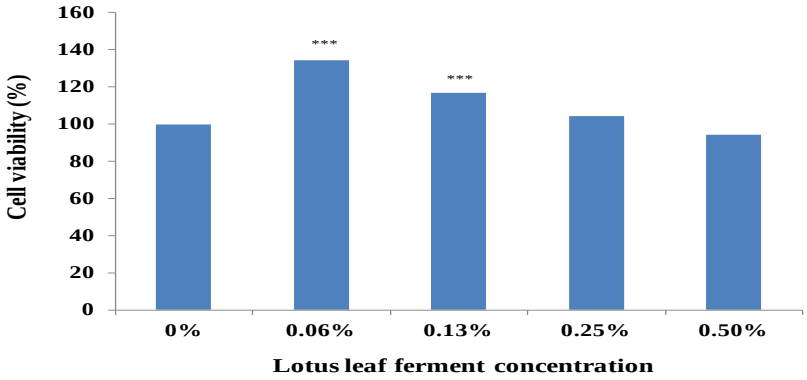

Figure 1. Result of cell viability in OP9 cells after treatment with lotus leaf ferment. (n = 3; mean value ± S.D.) (***, $p < 0.001$)

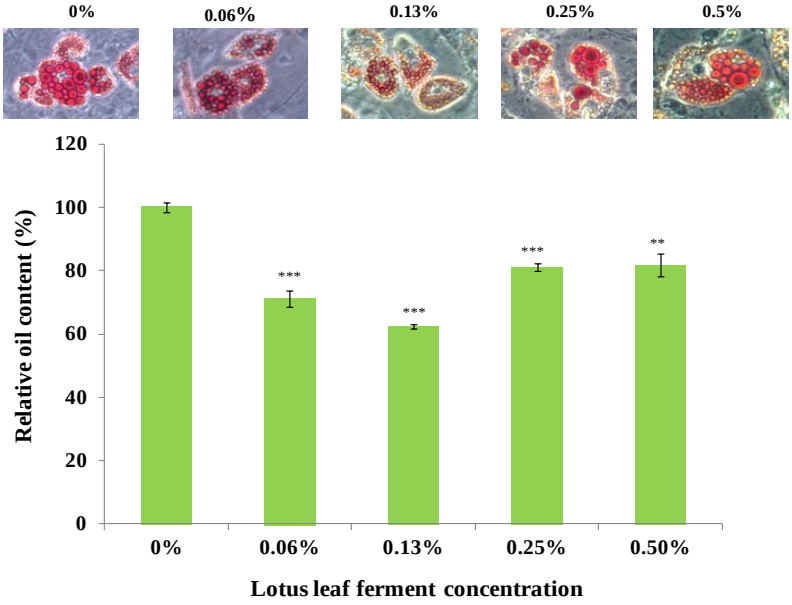

Figure 2. Adipocytic lipid accumulation result. (n = 3; mean value ± S.D.) (**, $p < 0.01$; ***, $p < 0.001$)

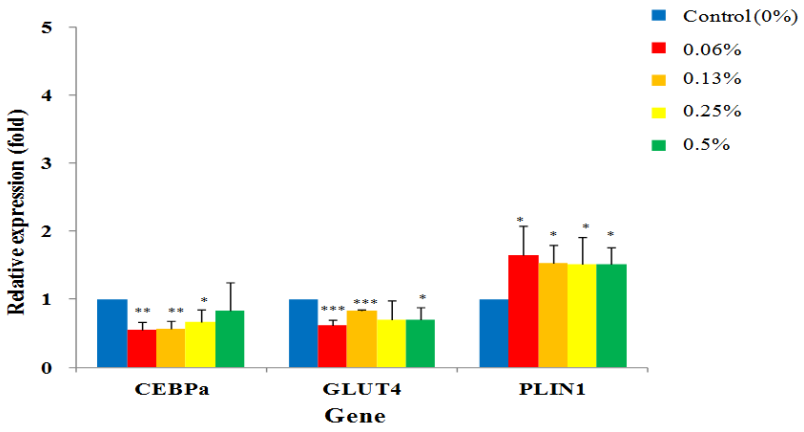

Figure 3. Gene expression analysis of OP9 cells. (n = 3, mean value ± S.D.; **, $p < 0.01$; ***, $p < 0.001$)

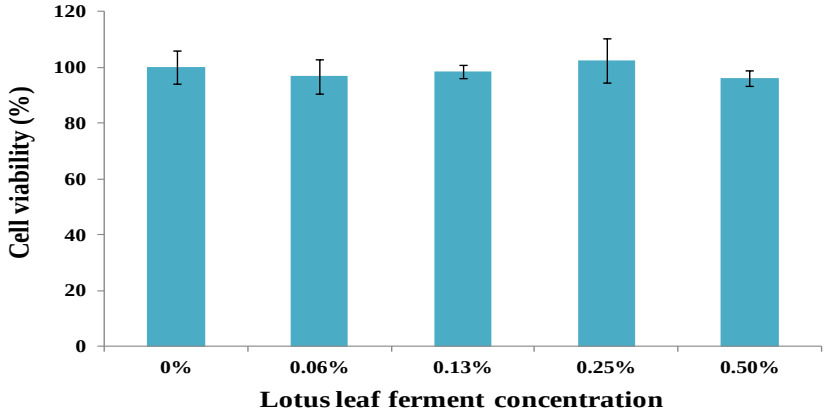

Figure 4. Result of cell viability in HepG2 cells after treatment with lotus leaf ferment. (n = 3; mean value ± S.D.)

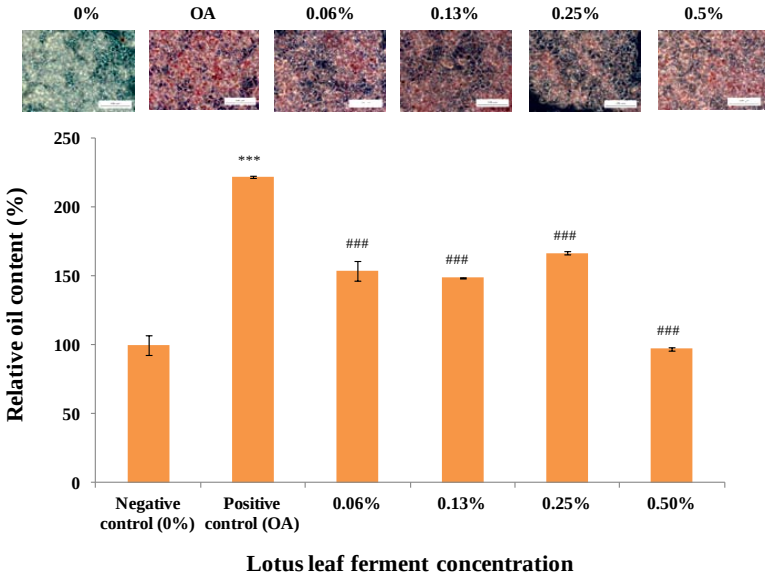

Figure 5. Hepatic lipid accumulation result. (n = 3; mean value ± S.D.) (Corresponding to the negative control; ***, $p < 0.001$) (Corresponding to the positive control; $^{###}$, $p < 0.001$)

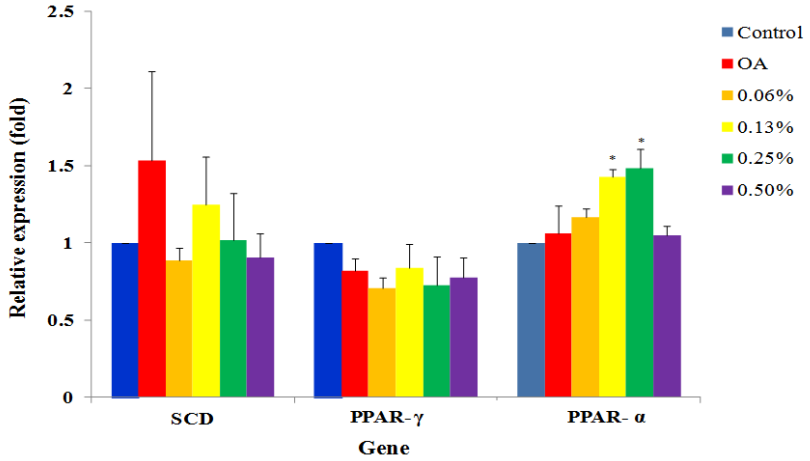

Figure 6. Gene expression analysis of HepG2 cells. (n = 3; mean value ± S.D.) (Corresponding to the positive control; *, $p < 0.05$)

3.2. Lipid Metabolism in Hepatocytes

We used oleic acid (OA) to induce the formation of lipid droplets in HepG2 cells and treated the cells with lotus leaf ferment solutions to observe the lipid metabolism phenomenon. Figure 4 shows the cell viability results of HepG2 cells after treatment with different concentrations of lotus leaf ferment solutions. We discovered that 0.06%, 0.13%, 0.3%, and 0.5% of lotus leaf ferment solutions did not show remarkable improvement effects in comparison with the control group, and each concentration demonstrated the similar improvement progress. In addition, as compared with the OA group, the oil contents in HepG2 cells were improved by 31%, 33%, 25%, and 56% after treatment

with corresponding 0.06%, 0.13%, 0.3%, and 0.5% of lotus leaf ferment solutions (Figure 5). We also measured adipogenesis-associated gene expression levels to evaluate the influence of lotus leaf ferment at molecular level (Figure 6). Lotus leaf ferment could suppress the expression levels of *SCD* (stearyl-CoA-desaturase) and *PPAR-γ* (peroxisome proliferator-activated receptor gamma) genes and might significantly up-regulate the expression level of *PPAR-α* (peroxisome proliferator-activated receptor) gene. In comparison with OA group, the expression levels of *SCD* gene in 0.06%, 0.13%, 0.3%, and 0.5% groups were down-regulated by 0.42, 0.18, 0.33, and 0.41 fold, respectively; on the other hand, the expression levels of *PPAR-γ* gene in 0.06%, 0.13%, and 0.3% groups were down-regulated by 0.1, 0.34, and 0.4 fold, respectively. Moreover, the expression levels of *PPAR-α* gene in 0.06%, 0.13%, and 0.3% groups were up-regulated by 0.1, 0.34, and 0.4 fold, respectively.

4. Discussion

Lotus leaf ferment could remarkably reduce the lipid droplet levels in OP9 and HepG2 cells. The underlying mechanisms for inhibition of lipid formation could partly be explained by the molecular results. Lotus leaf ferment significantly up-regulated the lipolysis (*PLIN1*) gene and down-regulated adipogenesis-related gene (i.e., *CEBPa* genes) and *GLUT4* in adipocytes. The expression of C/EBPα determines the fate of adipocyte differentiation, thus its suppression should improve adipogenesis in OP9 cells [20]. C/EBPα not only regulates PPAR-γ but also affects the modulation of GLUT4 [20]. It has been proved that the down-regulation of GLUT4 expression can ameliorate the insulin sensitivity and triglyceridemia in rodents [21]. Our results of *CEBPα* and *GLUT4* genes did correspond to the regulation theory. PLIN1 is the key protein to lipid metabolism in adipocytes by acting as a physical barrier as well as a recruitment site for lipases to the lipid droplet [22]. Enhancement of PLIN1 expression is beneficial for lipolysis. Notably, low concentrations of lotus leaf ferment solutions in this case led to better improvement progresses on lipid metabolism in OP9 cells. This is due, in part, to high concentration (0.5%) may hinder the normal cell activities and functions as indicated by the cell viability result. In light of these experimental results, lotus leaf ferment might be able to improve the efficiency of lipolysis and intervene in the adipogenesis, along with increasing insulin sensitivity in adipocytes. In addition, lotus leaf ferment also improved the lipid metabolism in hepatocytes. Nonalcoholic fatty liver disease (NAFLD) affect 25% of the global adult population, and it is usually occurred in individuals with metabolic disorders (e.g., obesity, diabetes mellitus, and hypercholesteremia) [23]. NAFLD is associated with the progression of steatosis and steatohepatitis, so the management of the deposition of hepatic fat is imperative to impair the development of NAFLD [24]. SCD plays the vital role in lipogensis in human by catalyzing the formation of monounsaturated fatty acids [25]. On the other hand, PPAR-γ is associated with the modulations of adipogenesis, lipid metabolism, and glucose homeostasis [26]. PPAR-α is involved in fatty acid oxidation and enables to drive the thermogenesis in brown fat tissue [27]. According to the results of gene analysis, lotus leaf ferment positively influenced the lipolysis and lipogeneiss in HepG2 cells. Although some experimental conditions did not reach much improvement progresses in gene regulation, lotus leaf ferment still significantly improved the lipid metabolism as evidenced by the hepatic lipid accumulation result. In brief, lotus leaf ferment may confer the preventive effect on the development of fatty liver and lipid accumulation.

5. Conclusion

In summary, the lotus leaf ferment can improve the lipid reduction and the expression of the lipolysis-, adipogenesis-related genes in adipocytes and hepatocytes. Although the results unveil the early evidences for the efficacy of the lotus leaf ferment, we believe that the combination of herbs (lotus, Chinese hawthorn, cinnamon, ginger, and red pepper ferments) has potential to improve metabolic disorders and manage weight in humans.

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