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조직배양된 *Gymnema sylvestre* 추출물의 C/EBP-α 및 지방축적 억제에 따른 항비만 효과

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Tissue-cultured *Gymnema sylvestre* Leaf Extract Enhance Anti-adipogenic Effects through Inhibition of C/EBP-α Expression and Lipid Droplet Accumulation

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요 약: 한국에서 '가가이모과잎'이라고 불리는 인도의 전통 식물 약재인 *Gymnema sylvestre*는 당뇨의 원인인 생체 내 당의 대사를 조절하는 약재로 이용되어 왔다. 본 연구에서는 *Gymnema sylvestre* 잎으로부터 인공적으로 조직 배양한 캘러스를 대량 배양하였다. 배양된 캘러스부터 추출물을 얻은 후 지방 전구 세포인 3T3-L1 세포를 이용하여 항비만 효과를 관찰하였다. 결과적으로, *Gymnema sylvestre* 잎으로부터 조직 배양한 캘러스 추출물은 지방 전구 세포의 지방세포로의 분화뿐만 아니라 세포 내 지질 축적을 억제 하였다. 또한 지방세포의 형성을 조절하는 초기 지방세포 내의 전사인자인 C/EBP-α 발현을 억제하면서 인슐린에 의해 유도된 지방세포의 분화를 억제하였다. 따라서 이러한 결과들은 피부를 위한 화장품 성분으로서 응용 가능성을 제공할 수 있을 것으로 사료된다.

Abstract: *Gymnema sylvestre*, a traditional India medicine called a 'Gagaimogouip' in Korea, has been used to regulate sugar metabolism in patients with diabetes mellitus. First, we induced callus from *Gymnema sylvestre* leaf and mode extract from cultured callus. In this study, we investigated the anti-obesity effect of tissue-cultured *Gymnema sylvestre* leaf extract in 3T3-L1 cells. The tissue-cultured *Gymnema sylvestre* extract inhibited the cytoplasmic lipid accumulation as well as adipogenic differentiation of preadipocytes. The tissue-cultured *Gymnema sylvestre* extract inhibited insulin-induced predipocyte differentiation through the inhibition of expression of the early adipogenic transcription factor C/EBP-α that regulate adipogenesis. These results indicate that tissue-cultured *Gymnema sylvestre* extract may be potential natural ingredient for slimming cosmetic products.

Keywords: *Gymnema sylvestre, adipogenesis, C/EBP-α, anti-obesity, callus*

1. Introduction

Gymnema sylvestre known as GURMAR, whose Hindi name literally means 'sugar destroyer' has been used in Ayuveda for centuries to regulate sugar metabolism[1]. *Gymnema sylvestre* is a woody, vine-like plant which climbs on bushes and trees in the Western Ghats in South India. *Gymnema sylvestre*, which is available as a herbal supplement, has been used as natural products for the treatment of obesity and diabetes mellitus[2]. *Gymnema sylvestre* has been reported to affect lipid metabolism including anti-allergic, antiviral, suppressing sweet taste, inhibition of oleic acid absorption and induction of weight loss as well as antioxidant effect[3,4]. Adipocyte differentiation is influenced by a

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large number of growth factors. Insulin, IGF-1, fibroblast growth factor, platelet-derived growth factor and epidermal growth factor promote preadipocyte proliferation but only insulin or IGF-1 can induce adipocyte differentiation. Various cytokines, including TNF-α, interleukins-1, 6 and interferon-γ inhibit adipocyte differentiation. Preadipocyte differentiation is characterized by changes in cell morphology, hormone sensitivity and gene expression in anti-obesity study. Upon induction of proteins that facilitate triglyceride synthesis such as lipoprotein lipase (LPL), adipocyte specific fatty-acid-binding protein aP2, and a lipid-droplet associated protein perilipin, the cells accumulate lipid droplets. During the early stages of differentiation, mRNAs for C/EBP-β and C/EBP-δ appear transiently. Expression of C/EBP-β and to a lesser extent C/EBP-δ results in the induction of two other transcription factors, the peroxisome proliferator activated-receptor (PPAR-γ) and C/EBP-α, PPAR-γ, and C/EBP-α then act through a synergistic mechanism to induce several proteins that are essential in forming the terminally differentiated adipocyte[5-7]. In the present study, we artificially cultured *Gymnema sylvestre* leaf for this study whether tissue-cultured *Gymnema sylvestre* leaf extract have anti-obesity effect like a natural *Gymnema sylvestre* as reported previously. We induced callus from *Gymnema sylvestre* leaf and then, harvested and extracted from cultured callus. We investigated the efficacy of anti-obesity about tissue-cultured *Gymnema sylvestre* leaf extract in order to identify the merit as a cosmetic ingredient.

2. Materials and Methods

2.1. Cell Culture

The 3T3-L1 preadipocyte line is derived from the Swiss 3T3 mouse (Green *et al.*, 1974a; Green *et al.*, 1974b) and was obtained from American Culture Collection. A preadipocyte line 3T3-L1 were maintained in Dulbecco's modified Eagle's medium (DMEM, WelGENE, Korea) supplemented with 10 % heat-inactivated calf serum (Gibco BRL, USA), 100 U/mL penicillin (Gibco BRL, USA) and 100 μg/mL streptomycin (Gibco BRL, USA) at 37 ℃ in a humidified atmosphere containing 5 % CO_2.

2.2. Reagents

Antibodies against CCAAT element binding protein (C/EBP-α) and β-actin were purchased from Santa Cruz Biotechnology (USA). Insulin, dexamethasone, isobutylmethylxanthine (IBMX) and Oil Red O were purchased from Sigma-aldrich (USA).

2.3. Preparation of Tissue-cultured *Gymnema sylvestre* Leaf Extract

Cultivation using young leaves of *Gymnema sylvestre*, we initiated callus formation in culture tubes containing a MS medium supplemented with sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), and gelrite (Duchefa, Netherlands). The media pH was adjusted to 5.8. Cultures were maintained at 25 ± 2 ℃ under a photoperiod and with a light intensity. These cell suspensions were sub-cultured at 2-weeks intervals. The cultures were transferred to conical flasks containing liquid medium, and were grown for 15 days in the dark on a gyratory shaker (95 rpm) at 20 ± 2 ℃ and were subcultured in MS liquid medium with plant growth regulators.

2.4. Extraction

After dried, tissue-cultured *Gymnema sylvestre* leaf were immersed in 70 % ethanol solution and 1,3-butyleneglycol (1,3-BG) for sufficient time and mixed enough for 48 h in 45 ℃ using agitator. Then, the solution was filtered through a filter paper, followed by the removal of ethanol from the filtrate at 50 ~ 60 ℃ to obtain a tissue-cultured *Gymnema sylvestre* leaf extract.

2.5. Pre-adipocyte (3T3-L1) Differentiation

To induce differentiation, pre-adipocytes were grown in 24-wells plates until 2 days post-confluence. Confluent cells were differentiated by incubation with MID hormone mixture (10 μg/mL insulin, 0.5 μM dexamethasone, and 1 mM IBMX) in 10 % fetal bovine serum (FBS)/DMEM for 36 h and maintained in

post-differentiation medium containing 10 μg/mL insulin for more 6 days.

2.6. Oil Red O Staining Assay

The degree of anti-adipogenic effects, with and without the tissue-cultured *Gymnema sylvestre* leaf extract, was evaluated by observation of the lipid accumulation with Oil Red O Staining. At 6 days after the initiation, cells were washed twice with PBS and then fixed for 20 min with 3.7 % formaldehyde. Cells were washed twice in PBS and the lipidic content of the cells is evaluated by the red oil coloration method. Briefly, cells were stained for 10 min using 0.5 % red oil in isopropanol/water at room temperature. Then the stain was solubilized in pure isopropanol, and the absorbance was measured at 540 nm on a spectrophotometer.

2.7. Cell Cytotoxicity by MTT Assay

Cells (1×10^5 cells/well) were seeded in 10 % BCS/DMEM and incubated in 5 % CO_2 incubator at 37 ℃ after treatment with tissue-cultured *Gymnema leaf* extract for indicate times. Measurement of mitochondrial activity to form purple formazan by MTT was used to assess the cytotoxicity of cell following extract treatment: MTT (0.5 mg/mL), one tenth of the original culture volume, was added to each culture and incubated for 3 h at 37 ℃ in 5 % CO_2. The purple formazan formed by viable cells was dissolved by the addition of DMSO and absorbance at the dual ranges of 540 nm and 630 nm was measured by using spectrophotometer.

2.8. Western Blot Analysis

Cells were treated with various dose and lysed in lysis buffer as described previously[8]. After differentiation, cells were lysed in lysis buffer. The lysates were clarified by centrifugation at 12,000 × g for 15 min at 4 ℃ and protein content was measured by 12.5 % SDS-PAGE and blotted to nitrocellulose membrane (0.2 mM, Amersham, USA). The membrane was blocked with 5 % nonfat skim milk in TBS-T and incubated with the primary and secondary antibodies. Immunoblots were visualized by enhanced chemiluminescence (Amersham, UK), according to the manufacturer's protocol.

2.9. Free Radical Scavenging Activity Assay (DPPH Test)

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical using determination of radical scavenging assay. The radical scavenging activity was determined by a previous report of Haraguchi *et al.*[9]. Then, its anti-oxidative activity (%) was calculated as compared with blank control.

2.10. Superoxide Radical Scavenging Activity Assay (NBT Assay)

Superoxide dismutase (SOD) activity was measured using xanthine-xanthine oxidase system for a source of superoxide and nitroblue tetrazolium (NBT) as a scavenger for this radical. The superoxide anion scavenging activity was determined by a previous report of K. Furuno[10].

2.11. Statistical Analysis

Data are presentated as mean ± S.D. Comparisons between groups were used to the paired Student's *t*-test. Asterisk (**, $0.05 < p < 0.01$; *, $p < 0.05$) was considered to be statistically significant.

3. Results

3.1. Cell Cytotoxicity by the Tissue-cultured *Gymnema sylvestre* Extract

To investigate inhibition of the cytoplasmic lipid accumulation as well as adipogenic differentiation of preadipocytes by the tissue-cultured *Gymnema sylvestre* extract, we first studied the effect of cytotoxicity in 3T3-L1 cells treated with the tissue-cultured *Gymnema sylvestre* extract in indicated dose and time. Cell cytotoxicity was measured by MTT assay. As shown in Figure 1, the tissue-cultured *Gymnema sylvestre* extract have not cytotoxicity until 0.5 % concentration for 72 h.

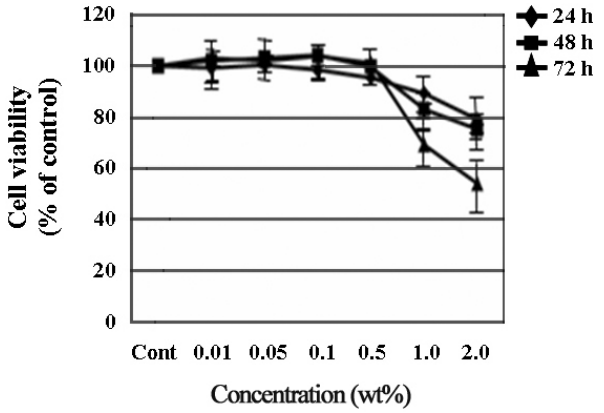

Figure 1. Cell viability of tissue-cultured *Gymnema sylvestre* extract. Cells were treated with various concentrations and time of the extract and cell viability measured by MTT assay as described in Materials and Methods. Each values represent as means ± standard deviation (S.D.) of three independent experiments.

3.2. Free Radical Scavenging Activity of the Tissue-cultured *Gymnema sylvestre* Extract

We next studied the effect on the free radical scavenging activity and superoxide anion scavenging activity to investigate whether the tissue-cultured *Gymnema sylvestre* extract have antioxidant effect like a natural *Gymnema sylvestre* as reported previously. The extract generally has an anti-oxidant effect. The tissue-cultured *Gymnema sylvestre* leaf extract enhanced the free radical scavenging activity in a dose dependent manner in DPPH and NBT assay as shown in Figure 2.

3.3. An Inhibitory Effect of Tissue-cultured *Gymnema sylvestre* Extract on the Lipid Accumulation of 3T3-L1 Cells

To investigate whether tissue-cultured *Gymnema sylvestre* extract have anti-obesity effect, we examine a cytoplasmic lipid droplet accumulation by the differentiation of preadipocytes into matures adipocytes in presence of MID hormone mixture. After incubation for 6 days with both MID and tissue-cultured *Gymnema sylvestre* extract, cells reduced a lipid droplet accumulation. In Figure 3, we investigated whether tissue-cultured *Gymnema sylvestre* extract inhibit a cytoplasmic lipid droplet by Oil red O staining assay as described in

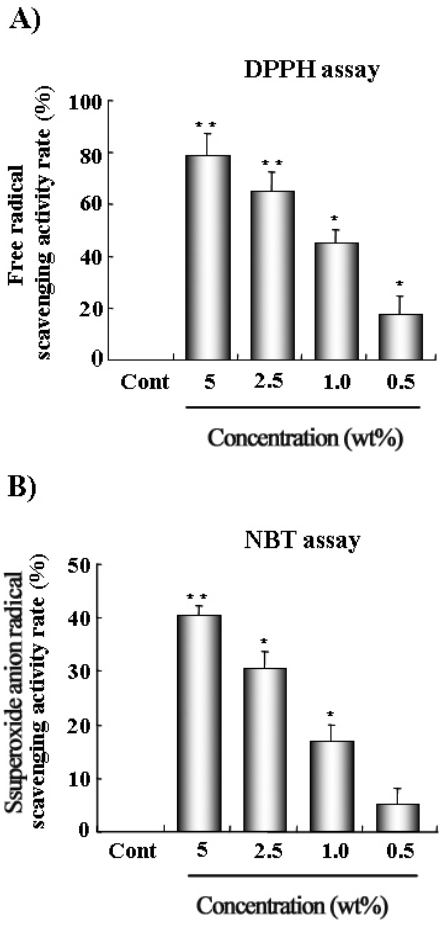

Figure 2. Anti-oxidant effect of tissue-cultured *Gymnema sylvestre* extract. Assays were treated with various concentrations of the extract and anti-oxidative activity measured by DPPH and NBT assay as described in Materials and Methods. Each values represent as means ± standard deviation (S.D.) of three independent experiments. **, $p < 0.01$; *, $0.01 < p < 0.05$ versus control.

Materials and Methods. In result, tissue-cultured *Gymnema sylvestre* extract, tested at 0.05 %, 0.1 % and 0.5 % inhibits by 33 %, 40 % and 44 % the differentiation of pre-adipocytes into adipocytes after incubation with both MID hormone mixture and extract at indicated concentrations as shown in Figure 3.

3.4. Suppression of C/EBP-α (Adipogenic Transcription Factor) Expression by Tissue-cultured *Gymnema sylvestre* Extract

After induction of differentiation, we next examined

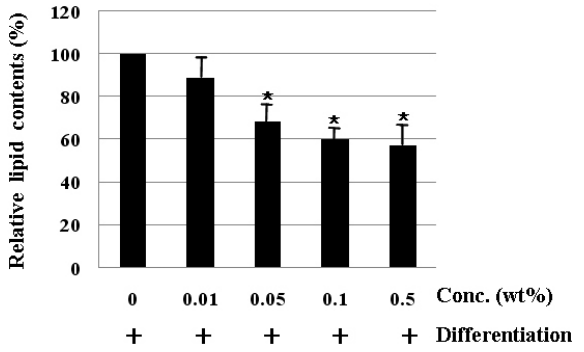

Figure 3. Inhibitory effect of the tissue-cultured *Gymnema sylvestre* extract on the lipid accumulation of 3T3-L1 cells. We compared a lipid contents after cotreatment of the tissue-cultured *Gymnema sylvestre* extract with MID hormone mixture. Lipid accumulation after differentiation for 8 days was measured by Oil Red O staining as shown in Figure 3. Results were represented as a relative percentage of fully differentiated cells. Bars represent as mean of standard deviation (S.D.) from three independent experiments. Each values are represented as means ± standard deviation (S.D.) of three independent experiments. *, $p < 0.05$ versus control.

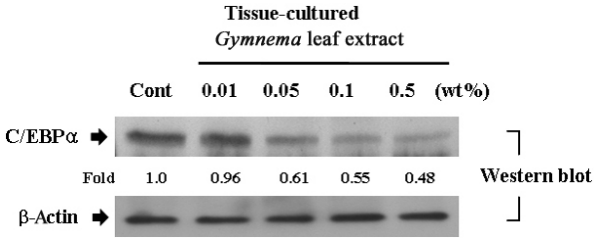

Figure 4. Suppressive effect of C/EBP-α (adipogenic transcription factor) protein expression by tissue-cultured *Gymnema sylvestre* extract. Confluent 3T3-L1 cells were induced to differentiate with or without the tissue-cultured *Gymnema sylvestre* extract for 2 days with indicated concentrations. Expression of C/EBP-α was detected by western blot analysis. Bands were subjected to densitometric scanning using the Sicon Image NIH Image program. Immunoblots are representative of at least twice independently. Detailed experimental procedures are described in Materials and Methods.

that cell lysates were subjected into 10 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and blotted with antibodies against C/EBP-α and actin to further know whether tissue cultured *Gymnema sylvestre* extract inhibit expression of adipogenic transcription factor. As shown in Figure 4, tissue cultured *Gymnema sylvestre* extract dramatically suppressed expression of C/EBP-α in a dose-dependent manner.

4. Discussion

Generally, tissue-cultured plant has a benefit to solve the drawbacks such as a rareness and long time and high cost for growing herb. Although the *Gymnema sylvestre* has well known to herb for remedy a diabetes mellitus and anti-obesity as a Traditional India Medicine, we investigated whether the tissue-cultured *Gymnema sylvestre* leaf have effect of anti-obesity at the molecular level of adipogenic transcription factor and cytoplasmic lipid accumulation of 3T3-L1 cells or not. As a results, suppressive effect of C/EBP-α and lipid droplet accumulation of tissue-cultured *Gymnema sylvestre* as well as a natural *Gymnema sylvestre* provide possible of potential benefits applied to cosmetic ingredient for skin care like a inhibition of lipogenesis and improvement of puffiness about intracellular lipids. In addition, future work will be required to elucidate whether this tissue-cultured *Gymnema sylvestre* occurs in experiment by clinical test, these findings provide possible cosmetic ingredient for skin care.

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