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Research Article

Inhibitory Effect of *Carallia Brachiata* Extract Through Regulation of Adipogenesis Pathways in 3T3-L1 Cells

Linda Chularojmontri[1], Urarat Nanna[1], Rawiwun Kaewamatawong[2], Sudarat Homhual[2], Wanwisa Suwannaloet[3,*]

ABSTRACT

Background: Pharmacological effects of *Carallia brachiata* Merr. has been reported to show antioxidant effects against the development of diabetes. However, the mechanism underlying antiadipogenic activity have not been investigated. **Objective:** Effect of *Carallia brachiata* ethanolic extract was determined on inhibition of adipogenesis in 3T3-L1 adipocytes. **Materials and Methods:** Adipose tissue development was performed in preadipocyte 3T3-L1 cells culture. *Carallia brachiata* leaf (CL) and stem (CS) part were selected for measuring cytotoxicity, accumulation of lipids, and genes involved in adipogenic differentiation. **Results:** During the adipogenic differentiation, CS down-regulated gene expression of adipogenic transcription factors (PPARγ, C/EBPα, aP2, FAS, LPL and SREBP1c). However, CL only suppressed SREBP1c and aP2 genes. The accumulation of lipids was suppressed by CS, but CL could not show this effect. **Conclusion:** Our findings suggest that ethanol extract of *Carallia brachiata* stem has a better anti-adipogenesis effect than the leaf part by suppressing adipogenesis-related gene expression. Moreover, inhibition of lipid storage could be decreased insulin resistance risk.
Key words: *Carallia brachiata*, Adipogenesis, 3T3-L1 adipocytes.

Linda Chularojmontri[1], Urarat Nanna[1], Rawiwun Kaewamatawong[2], Sudarat Homhual[2], Wanwisa Suwannaloet[3,*]

[1]*Department of Preclinical Science, Faculty of Medicine, Thammasat University, Pathum Thani 12120, THAILAND.*

[2]*Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, 34190, THAILAND.*

[3]*College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani, 34190, THAILAND.*

Correspondence

Wanwisa Suwannaloet

College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani, 34190, THAILAND.

E-mail: wanwisa.su@ubu.ac.th

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INTRODUCTION

The relevance of an increased number of adipocytes and obesity has been documented.[1] Obesity is associated with various diseases, such as type 2 diabetes mellitus, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD).[2] Adipogenesis is a process of proliferation and differentiation of adipocyte precursor cells into mature adipocytes.[3] Numerous transcriptional factors regulate adipogenesis. In the early stage of preadipocyte differentiation, CCAAT/enhancer-binding protein β (C/EBPβ) gene is highly expressed. It induces the expression of peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα for the intermediate stage.[4] C/EBPα and PPARγ are important factors in the regulation of adipocyte-specific genes such as adipocyte acetyl-CoA carboxylase (ACC), adipocyte fatty acid-binding protein 2 (aP2), cluster of differentiation 36 (CD36), fatty acid synthase (FAS) and lipoprotein lipase (LPL) in the terminal stage of differentiation.[5] Moreover, other transcription factors have relevance in inducing adipogenesis, such as sterol regulatory element-binding protein-1 (SREBP-1), Kruppel-like factors (KLFs) and signal transducer and activator of transcription 5 (STAT5).[4] Increasing proliferation and differentiation of adipocytes are significant in obesity and risks of developing diabetes mellitus.

Carallia brachiata is a tree in the family Rhizophoraceae and is found in South and Southeast Asia.[6] A study reports that the *Carallia brachiata* stem bark has an effect on wound healing in a rat model.[7] *Carallia brachiata* bark also has antioxidant activity and inhibits the xanthine oxidase enzyme.[8] A recent study has revealed that hydro-alcoholic extract (3:7 mixture) of *Carallia brachiata* leaf has hypoglycemic activity in diabetic rats.[6] However, there are few studies about the efficacy of the *Carallia brachiata* extract. Therefore, the present study investigated the pharmacological activity of two parts of *Carallia brachiata* on the regulation process of adipogenesis in 3T3-L1 adipocytes.

MATERIALS AND METHODS

Plant extraction

Carallia brachiata leaf (CL) and stem (CS) were identified and collected for analysis by the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Thailand. A voucher herbarium specimen is CB-UBUPH00310. The CL and CS were dried, extracted with 95% ethanol by maceration (48 hours × 3 times) and evaporated for drying extracts. The yields of CL and CS were 10.01% and 14.20%, respectively.

Determination of total phenolic content

Total phenolic content was estimated by Folin-Ciocalteu assay with modification.[9] The aqueous extract (1 mg/mL) was mixed with 10% Folin-Ciocalteu reagent and 7.5% sodium carbonate solution and allowed to stand for 30 minutes. The absorbance was measured at 765 nm. Total phenolic content was calculated as mg gallic acid equivalents (mg GAE)/g dry weight using the equation based on the calibration curve of gallic acid. The analysis was performed in triplicate.

Determination of total flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric assay with modification.[9] The solution of 2% aluminium chloride was mixed with the extract in methanol

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(1 mg/mL) and allowed to stand for 10 minutes. The absorbance was determined at 415 nm. Total flavonoid content was expressed as mg quercetin equivalents (mg QE)/g dry weight by the equation based on the calibration curve of quercetin. The analysis was performed in triplicate.

3T3-L1 adipocyte culture and differentiation

All protocols of this study were approved by the Thammasat University Institutional Biosafety Committee (TU-IBC 031/2562). Mouse 3T3-L1 preadipocytes (American Type Culture Collection, VA, USA) were plated in 24-well plates (2×10^4cells/well) in expansion media, Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum and 0.1% penicillin/streptomycin at 37°C in 5% CO_2, as recommended by the manufacturer. After 2 days, when cells reached 100% confluence, cells (day 0) were removed from the expansion medium. Then 3T3-L1 preadipocytes were differentiated by differentiation medium DMEM supplement with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1.0 µM dexamethasone and 1.0 µg/mL insulin and 10% fetal bovine serum (FBS) for 48 hours. During the induction, various concentrations of CL or CS (3, 10, 30, and 100 µg/mL) were added to observe their effects on 3T3-L1 adipocyte differentiation for 8 days. Culture media were changed every other day. Three independent experiments of treated groups were performed and compared with the control group.

Oil Red O staining

After 8 days of treatment, the cells were washed with phosphate buffer saline (PBS) and fixed with 10% neutral buffered formalin for 1 hour. After washing the cells with PBS (3 times), 0.6% Oil Red O solution was added and incubated at room temperature. After 1 hour, cells were washed five times with double-distilled water until clear and photographed lipid droplets by randomly the field on each well (Primovert, Carl Zeiss, NY, USA, at ×20 magnification). Later, lipid content quantitation was detected by eluting stained cells with isopropanol. The elution was transferred to a new plate and absorbance was measured at 540 nm.

Cell viability assay

The differentiated adipocyte cells (1×10^4 cells/well) were plated into a 96-well plate and treated with various concentration of CL or CS (0-100 µg/mL) in a culture medium for 48 hours. The cell viability was determined with MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) for 2 hours at 37 °C. The absorbance was measured at 570 nm.[10]

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from treated cells using TRIzol reagent (Invitrogen, CA, USA) and complementary DNA (cDNA) was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The RT-qPCR was performed using SYBR Green Master Mix and LightCycler® 480 Instrument II (Roche Molecular Systems, Pleasanton, CA, USA). Relative expressions were normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. The following list of mouse primers was used: C/EBPα (forward 5′-TGCCTATGAGCACTTCACAA-3′ and reverse 5′-AACTCCAGCACCTTCTGTTG-3′), PPARγ (forward 5′-TGGGAACCTGGAAGC TTGTCTC-3′ and reverse 5′TGTGGTA-AAGGG CTTGATGT-3′), SREBP1c (forward 5′-GGGCTCTGCTG-GACCAC-3′ and reverse 5′-TGGCCTTGTCAATGGAACTG-3′), FAS (forward 5′-GCACCTATGGCGAGGACTT-3′ and reverse 5′-ATGGATGATGTTGATGATGGA-3′), LPL (forward 5′-GGC-CAGATTCATCAACTGGAT-3′ and reverse 5′-GCTCCAAGGCTG-TACCCTAAG-3′), aP2 (forward 5′-GCACCTATGGCGAGGACTT-3′ and reverse 5′-ATGGATGATGTTGATGATGGA-3′) and housekeeping gene, GAPDH (forward 5′-CTGGAGAAACCTGCCAAGTA-3′ and reverse 5′-AGTGGGAGTTGC TGTTGAAG-3′).

Statistical analysis

All data are presented as mean ± SEM. One-way analysis of variance followed by Tukey's post-hoc test was used for testing a significant difference between groups using IBM SPSS Statistics for Windows, version 26.0 (IBM Corporation, Somers, NY, USA). The statistical significance was indicated at $P < 0.05$.

RESULTS

Phytochemical contents

Total phenolic and flavonoid contents of the *Carallia brachiata* extracts were shown in Table 1. The flavonoid content of the leaf extract was found higher than the stem extract. The stem extract of *Carallia brachiata* showed slightly increase in the amount of total phenolic content more than the leaf extract.

Effect of CL and CS extracts on adipogenesis

Oil Red O staining on day 8 of treatment showed lipid droplet formation and accumulation in both CL (3-100 µg/mL) treated and non-treated group (Figure 1A). The measurement of the amount of lipid content has shown that CL extracts could not significantly reduce the lipid accumulation in 3T3-L1 adipocytes compared to the non-treated group (Figure 1B). Moreover, there was no cytotoxic effect on any of the concentrations of CL extracts (Figure 1C).

Interestingly, stained oil droplets were suppressed in CS treated group (3-100 µg/mL) (Figure 2A). Consistent with the amount of lipid content, the absorbance value was significantly reduced in CS treated group (3-100 µg/mL) (Figure 2B). Furthermore, CS extract also did not exhibit any cytotoxic effect on 3T3-L1 adipocytes (Figure 2C).

To determine the effect of CL and CS extract on the down-regulation of genes in adipogenesis, results were shown in the Figure 3. In the CL-treated group, the expression of the SREBP1c gene was significantly reduced at 10-100 µg/mL, and the expression of the aP2 gene was also reduced at 100 µg/mL compared with the non-treated group, while other genes were slightly decreased but statistically nonsignificant (Figure 3A). In addition, the expressions of PPARγ, **C**/EBPα, and SREBP1c genes, including the adipocyte-specific genes aP2, FAS, and LPL were significantly reduced in CS extracts (10-100 µg/mL) (Figure 3B) compared to the non-treated group. Therefore, the stem extract could be more effective in reducing lipid droplet size and down-regulating the expression of adipogenesis-related genes.

DISCUSSION

Numerous pharmacological effects such as antioxidant,[11] antibacterial,[12] anti-cancer[13] and anti-obesity[14] activities have the relation to the amounts of flavonoid and phenolic contents that are found in the plants. Few studies report *Carallia brachiata* extracts contain several phytochemical compounds both phenolics and flavonoids such as sterols or triterpenoids, flavonoids, phenols, tannins, carbohydrates,

Table 1: Total phenolic and flavonoid contents of *Carallia brachiata* stem and leaf extracts.

Carallia brachiata parts	Total phenolic content (mg GAE)/g	Total flavonoid content (mg QE)/g
Leaf extract	343.33 ± 16.12	61.95 ± 0.09
Stem extract	350.64 ± 22.77	11.56 ± 0.43*

Data are expressed as mean ± SEM (n=3). * $P<0.05$ vs. the leaf extract. GAE=gallic acid equivalent, QE=quercetin equivalent

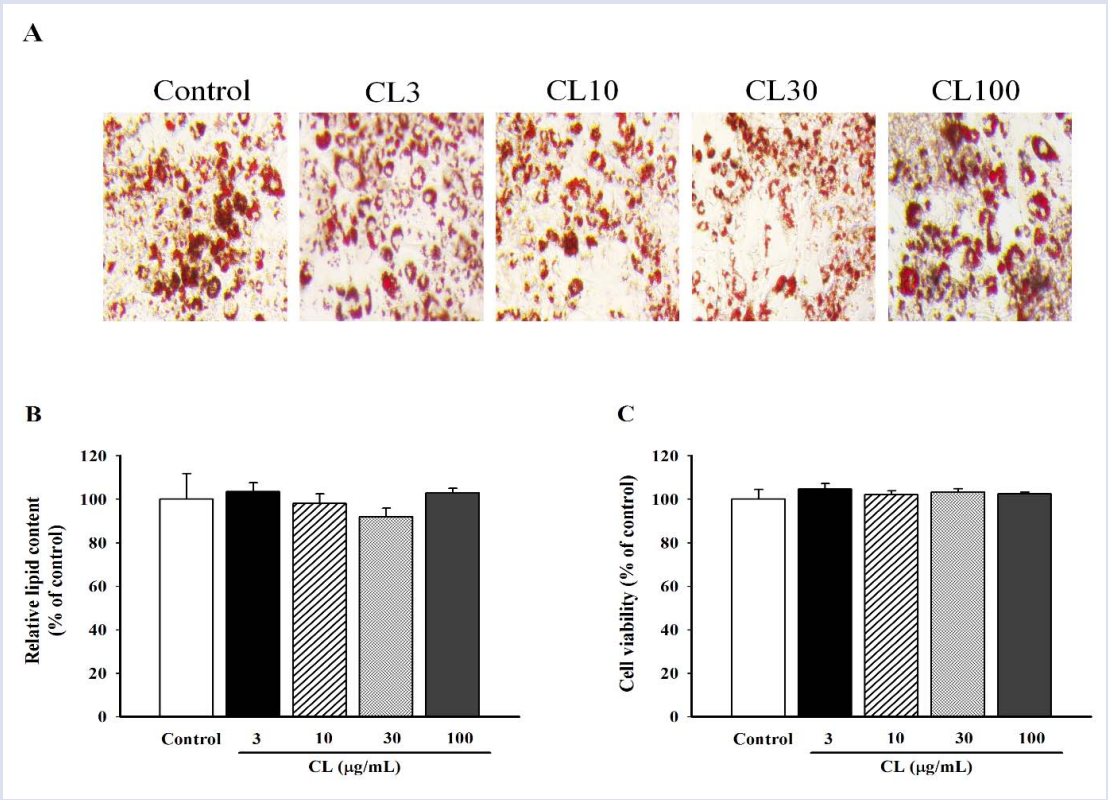

Figure 1: Effect of CL in differentiated 3T3-L1 adipocytes on lipid accumulation based on Oil Red O staining. Lipid droplets were photographed using a microscope (×20 magnification) (A). Lipid content was extracted and quantified by measuring the absorbance at 540 nm (B) and the viability of adipocyte cells using the MTT assay (C). Data are expressed as mean ± SEM (n=3). * P<0.05 vs. the control group (non-treated cells). CL: *Carallia brachiata* leaf ethanolic extract.

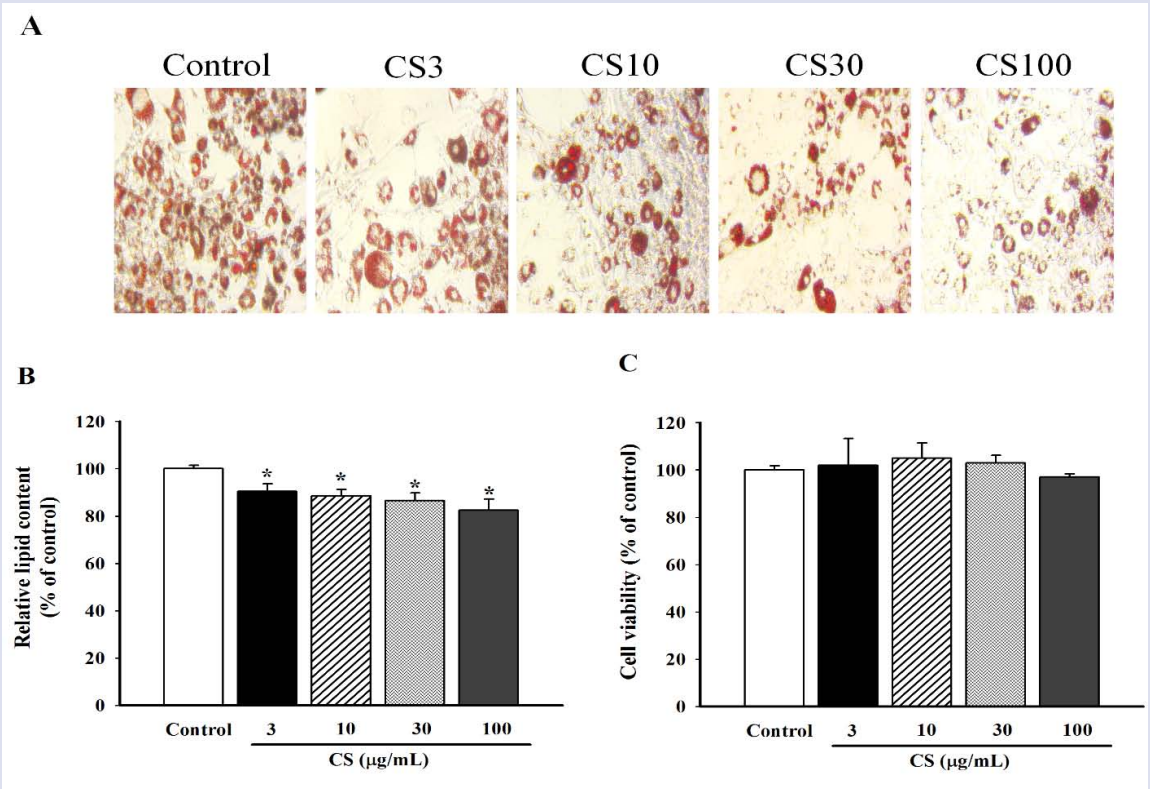

Figure 2: Effect of CS in differentiated 3T3-L1 adipocytes on lipid accumulation based on Oil Red O staining. Lipid droplets were photographed using a microscope (×20 magnification) (A). Lipid content was extracted and quantified by measuring the absorbance at 540 nm (B) and the viability of adipocyte cells using the MTT assay (C). Data are expressed as mean ± SEM (n=3). * P<0.05 vs. the control group (non-treated cells). CS: *Carallia brachiata* stem ethanolic extract.

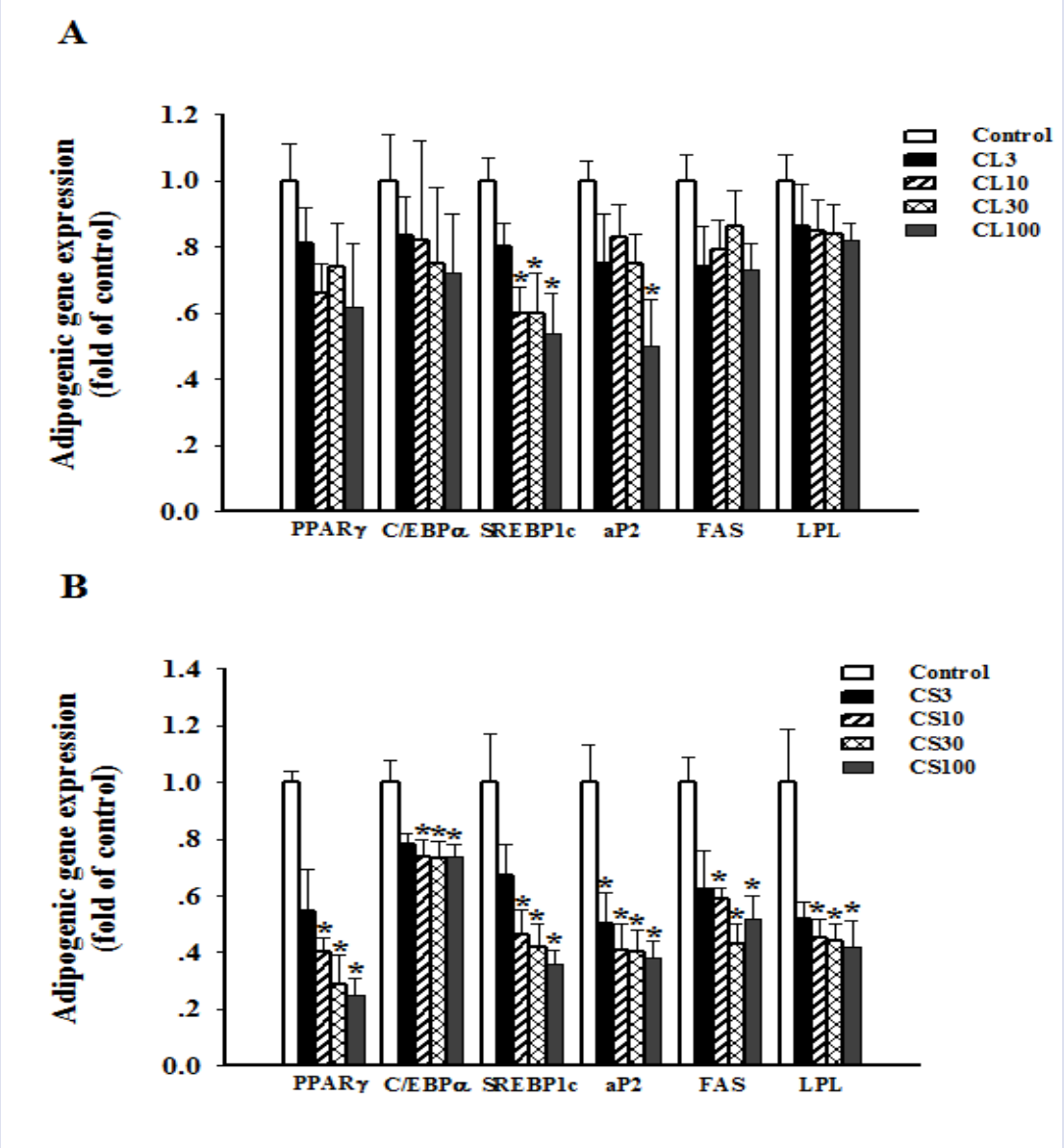

Figure 3: Effect of CL (A) and CS (B) on adipogenic gene expressions in 3T3-L1 adipocytes

GADPH was used as an internal control. Values are expressed as mean ± SEM (n=3). * $P<0.05$ vs. the control group (non-treated cells). CL: *Carallia brachiata* leaf ethanolic extract. CS: *Carallia brachiata* stem ethanolic extract.

fixed oils and fats,[7,8] while flavonoid glycosides, megastigmane glycoside and glycolipids were found in the leaf extract.[15] The present study also found that the *Carallia brachiata* extracts have the amount of total flavonoid and total phenolic contents. These contents may have involved in the inhibitory effect of *Carallia brachiata* extracts on adipogenesis process.

Adipogenesis is the differentiation process of fibroblast-like preadipocytes into mature adipocytes influenced by other factors, including the expression of adipogenic and lipogenic genes.[16] The interaction between PPARγ and several co-regulators such as C/EBPα, and SREBP1c is necessary to regulate the transcriptional cascade controlling adipogenesis.[17,18] Increasing of these adipogenic regulators induce expression of other adipogenic transcription factors and lead to the development of adipocyte and lipid storage. Thus, inhibition adipogenesis by suppressing the activity of key adipogenic regulators can improve to control obesity and to treat metabolic diseases including type-2 diabetes.

The present study investigated the effect of leaf and stem parts of *Carallia brachiata* ethanolic extract in 3T3-L1 adipocytes. The results show that the extract of leaf part inhibited the SREBP1c and aP2 gene expression, which is terminal markers in adipocyte differentiation. Decreased expression of aP2 is partially resistant to obesity-induced insulin resistance and exhibits protection against atherosclerosis.[19] Therefore, inhibition of aP2 may act as a point for managing obesity and diabetes.[20,21] In particular, the stem part of *Carallia brachiata* ethanolic extract can suppress lipid accumulation and also down-regulate the expression of adipogenesis genes such as C/EBPα, PPARγ, and SREBP1c and subsequently down-regulates the expression of aP2, FAS and LPL[5] which all are key transcriptional regulators of adipocyte-specific genes[5]. LPL is a major lipid synthesis enzyme that hydrolyzes triglycerides in plasma lipoproteins and releases fatty acids stored and taken up by the adipose tissues.[22] FAS is also a lipid synthesis enzyme that catalyzes and synthesizes long-chain fatty acids.[23] Up-regulation of FAS and aP2 gene leads to lipid accumulation within

the adipocytes, which is associated with obesity and type II diabetes and other pathologies.[24] Together, our results indicate that decreasing level of LPL and FAS genes in adipose tissue showed a reduction in lipid accumulation. These data demonstrated that the stem part extract have a better effect on the inhibition of transcription factors and their downstream adipogenic genes than the leaf part.

In conclusion, the data suggest that *Carallia brachiata* extracts can inhibit intracellular lipid accumulation and the adipogenic differentiation of 3T3-L1 pre-adipocytes by suppressing the transcription factors PPARγ, C/EBPα, and SREBP1c genes, including the adipogenic-specific genes, aP2, FAS, and LPL. In addition, between the leaf and stem ethanolic extracts, the stem tends to be more effective in inhibiting adipogenesis. Therefore, the anti-adipogenic activity of *Carallia brachiata* extracts may be considered one of the alternative agents for the management of obesity. However, there are some limitations to this study. The cell culture model does not completely show the absorption and metabolism of *Carallia brachiata* extracts. Therefore, it is necessary to perform further studies on animals and humans.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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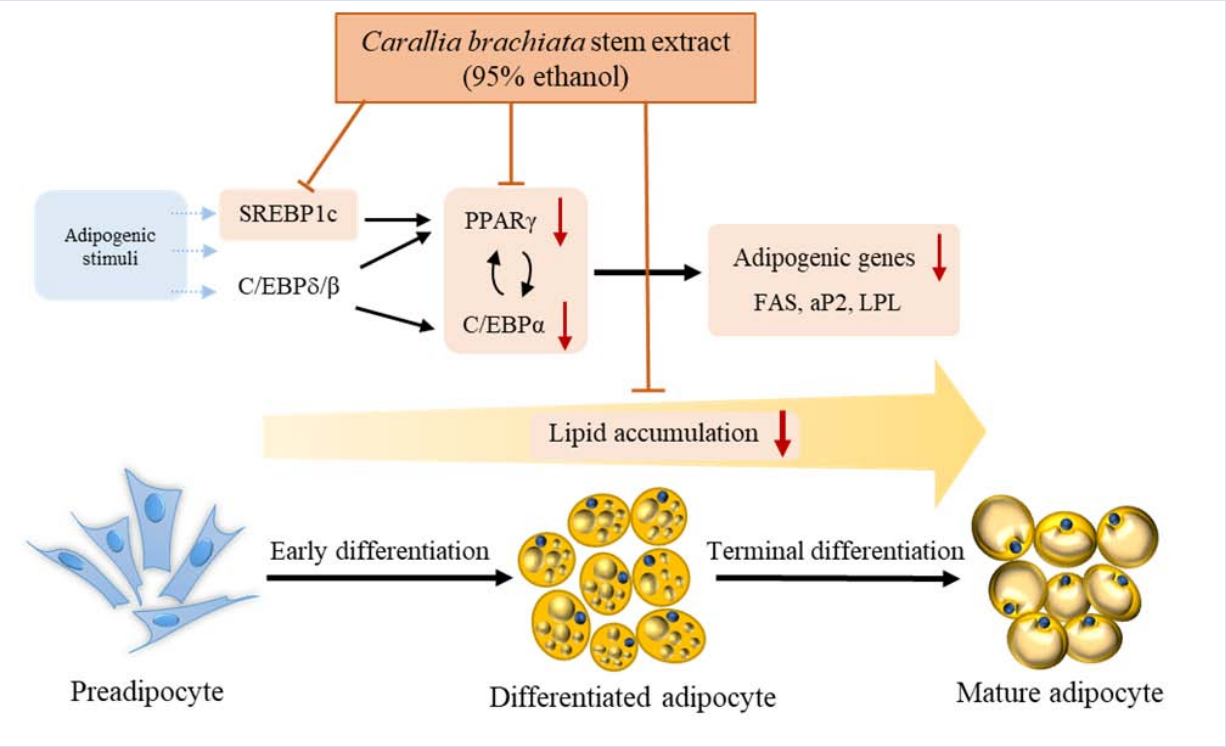
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Linda Chularojmontri is a lecturer at the Department of Preclinical Science, Faculty of Medicine, Thammasat University. Research interest in cardiovascular medicine, skin model, and phytopharmacology of antioxidant-containing herbs.

Urarat Nanna is a lecturer at the Department of Preclinical Science, Faculty of Medicine, Thammasat University. Research interest in pharmacological activity of medicinal plants.

Rawiwun Kaewamatawong is a lecturer at the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University. Research interest in phytochemistry and quality control of phytomaterials and products.

Sudarat Homhual is a lecturer at the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University. Research interest in phytochemical analysis, structure elucidation and biological study of natural products.

Wanwisa Suwannaloet is a lecturer at the College of Medicine and Public Health., Ubon Ratchathani University. Research interest in pharmacological effects of plant extracts and their anti-inflammatory, anti-diabetic, chondrogenic activity.

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