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Research Article

Effects of *Citrus aurantifolia* Root Ethanolic Extract on Lipogenesis in Palmitate-Induced Lipid Accumulation in HepG2 Cells

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ABSTRACT

Introduction: *Citrus aurantifolia* (lime) is mostly found in tropical and subtropical region. The lime peel and lime juice extracts have antioxidant, antidiabetic and anti-inflammatory effects. However, the pharmacological effects of the lime root remain widely unknown. Thus, the current study investigated the effects of *Citrus aurantifolia* root ethanolic extract (CA) on lipogenesis induced by palmitic acid (PA) in HepG2 cells. **Methods:** The PA-induced lipogenesis in HepG2 cells was used for measuring lipogenic gene expression and lipid accumulation of CA. Phytochemical content was also determined in CA. **Results:** In PA-treated group showed the state of hepatic lipid accumulation with increased lipogenic gene, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and sterol regulatory element binding protein1c (SREBP1c) as compared to the control group. Interestingly, administration of CA (5-10 µg/mL) effectively reduced lipid storage and significantly decreased the expression of these lipogenic gene in PA-treated cells. Notably, CA treatment increased the gene expression of fatty acid oxidation, carnitine palmitoyl transferase 1A (CPT1A) and peroxisome proliferator-activated receptor α (PPARα). Furthermore, this study found that the major bioactive component from CA was nordentatin (coumarin group). **Conclusions:** The results indicated that the CA treatment might be a useful agent for improving abnormal lipid metabolism in obesity-related nonalcoholic fatty liver disease.

Key words: *Citrus aurantifolia*, Lipogenesis, Nonalcoholic fatty liver disease, Lipid metabolism, Obesity.

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INTRODUCTION

Abnormal lipid metabolism and energy imbalance are associated with the development of obesity.[1] Obesity is linked to establishment of nonalcoholic fatty liver disease (NAFLD), which has the characteristic of hepatic lipid accumulation.[2] NAFLD pathogenesis can increase circulating free fatty acids (FFAs).[3] These FFAs is converted into triglyceride in the liver and stored as lipid droplets.[4] The *de novo* fatty acid synthesis pathway is regulated by two key enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Activity of these enzymes are upregulated by lipogenic transcription factor, sterol regulatory element binding protein 1c (SREBP1c), which is affected by peroxisome proliferator-activated receptor α (PPARα) agonists that bind to SREBP1c in hepatocytes.[5,6] In the initial step, the carboxylation of acetyl-CoA is catalyzed by ACC to produce malonyl-CoA, which is an essential intermediate and also acts as an allosteric inhibitor of carnitine palmitoyltransferase 1A (CPT1A). Inhibition of CPT1A reduces lipid accumulation, thereby slowing the progression of NAFLD by preventing the conversion of acyl-CoA into acyl-carnitine for mitochondrial FA metabolism.[7] Some transcription factors such as PPARα can enhance the expression of CPT1A and other genes related to fatty acid oxidation.[8]

HepG2 cells, derived from human liver carcinoma cell line, serve as a well-established *in vitro* model for NAFLD study.[9] FFAs are commonly implicated as possible drivers of hepatic lipotoxicity. Several experiments suggested that the induction of NAFLD with saturated fatty acids resulted in more severe NAFLD pathogenesis compared to unsaturated FAs.[10-12] Palmitic acid (PA), a dietary saturated fatty acid with a 16-carbon chain, is commonly found in palm oil and meat.[13] The use of PA-supplemented culture media can induce lipid accumulation in HepG2 cells[14] and has become an established *in vitro* NAFLD model.[10-12]

Citrus aurantifolia (lime) is mostly found in tropical and subtropical region.[15] The sour taste of lime juice is famously used as seasoning in Thai food. Several studies such as antidiabetic,[16] antilipidemic,[17] antiobesity,[18] antibacterial,[19] anticancer,[15] antioxidant[20] and anti-inflammatory[21] activities of lime peel and lime juice extracts have been reported. The plant parts of *C. aurantifolia* contain a variety of bioactive compounds. Essential oil can be isolated from the leaves and fruit peels, but its detection is very low in the root part.[22] Sub-chronic toxicity studies have reported mild toxicity of essential oils, associated with low hemoglobin levels and increased liver enzymes.[23] However, the pharmacological effects of the lime root remain widely unknown. In this study, we aimed to investigate the effects of lime root extract on PA-induced lipid accumulation in HepG2 cells and to explore the underlying mechanism.

MATERIALS AND METHODS

Chemicals

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's

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modified Eagle's medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Total RNA Extraction Kit was obtained from Vivantis (Kuala Lumpur, Malaysia). iScript cDNA synthesis kit was purchased from Bio-Rad (Hercules, CA, USA). LightCycler® 480 SYBR Green I Master was purchased from Roche Diagnostics (Indianapolis, IN, USA). Sodium palmitate (PA), Fatty acid-free bovine serum albumin (FA-free BSA), Oil red O and HPLC standards, which included nordentatin, gallic acid, chlorogenic acid, ellagic acid, coumaric acid, vanillin, caffeic acid and ferulic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant extraction

Citrus aurantifolia roots were collected from Udonthani Province and the species was identified by Assoc. Prof. Sudarat Homhual. The voucher specimens are deposited in Faculty of Pharmaceutical Sciences, Ubon Ratchathani University. The plant roots were dried in hot air oven at 50 °C. The dried root powder 30 g was macerated with 100% ethanol (3 × 300mL). The ethanol was then evaporated to yield the dry extract, resulting in a 10% yield of the dry powder of *Citrus aurantifolia* roots ethanolic extract (CA).

Qualitative phytochemical screening

To identify the main classes of compounds (alkaloids, amines, coumarin, flavonoids, and phenolics) in the extracts, phytochemical screening of CA was performed according to standard protocols.[24] The results are expressed as (+) for the presence and (-) for the absence of these specific groups of compounds.

Total phenolic content determination

Folin-Ciocalteu method is used for determination of total phenolic content of CA extract. CA solution (1 mg/mL) was mixed with a 10% Folin-Ciocalteu reagent and 7.5% (w/v) Na_2CO_3. The mixture was incubated in the dark for 30 min, and then its absorbance was measured at 765 nm. Total phenolic content was quantified in terms of mg gallic acid equivalents (mg GAE)/g dry weight.[24]

Total flavonoids content determination

Flavonoid content was assessed using aluminum chloride assay. CA solution (1 mg/mL) was mixed with 5% $NaNO_2$, and after 5 min, 2% $AlCl_3$ was added. After 1 min, 1 M NaOH was added, and the mixture was incubated for 10 min before measuring the absorbance at 415 nm. Total flavonoid content was expressed as mg quercetin equivalents (mg QE)/g dry weight.[24]

High-performance liquid chromatography (HPLC) analysis

A stock standard solution of 1 mg/mL was prepared in methanol. The working solution was made by diluting the stock to concentrations between 0.56 and 36 µg/mL. Accurately weighed 10 mg of dried extracts were dissolved in 1 mL of methanol. The HPLC assay was performed in triplicate using Dionex Ultimate 3000 system (Thermo Fischer Scientific, USA). Mobile phase composition using gradient elution of solvent A (0.1% formic acid) and solvent B (100% acetonitrile). The gradient program was started with 90:10 (A: B) for 5 min, 72:28 for 15 min, 50:50 for 10 min, 35:65 for 10 min, 25:75 for 5 min, and 0:100 for 5 min respectively. The stationary phase was contained in ACE C18 column (250 × 4.6 mm, and 5 µm) at 25°C. The injection volume was 10 µL at a flow rate of 0.8 mL/min, and the absorbance of detection was 254 nm. The compounds were identified by comparison of their retention times and matching their spectral (UV-Vis) characteristics against standards.

Preparation of fatty acid-BSA conjugation

PA was prepared following the method previously described by Qin et al.[25] PA was prepared as a 50 mM stock solution by dissolving in 0.1 M NaOH and heating at 70°C until the solution was clear. The PA stock solution was then diluted into preheated (37°C) DMEM containing 1% FA-free BSA and incubate for 1 h to allow for conjugated complex that can be absorbed and utilized by cells. The PA-BSA conjugated solutions was diluted with DMEM to final concentration 0.25 mM PA and filtered through a 0.22 µm filter for sterilization.

Cell culture

All experiments were approved by the Thammasat University Institutional Biosafety Committee (TU-IBC 035/2566). HepG2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C. After reaching 80% cell confluence, the medium was replaced with DMEM containing 0.25 mM PA (PA conjugated BSA) for 24 h.[26] Then the experiment treatments were as follows: (1) the control group non-treated cell; (2) the PA-treated group; (3) PA-treated with CA 1 µg/mL; (4) PA-treated with CA 5 µg/mL; and (5) PA-treated with CA 10 µg/mL. All groups were incubated for 24 h. Six independent experiments were performed, each in duplicate.

Cytotoxicity assay

HepG2 cells were plated at an initial density 1×10^4 cells/mL in 96-well plates. The cells were then treated with PA with or without various concentrations of CA (0, 1, 5, 10, 50, 100 and 200 µg/mL) for 24 h. After incubation, the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to a final concentration of 0.5 mg/mL. The absorbance was measured at 545 nm using a plate reader (Biochrom EZ Read 2000). Six independent experiments were conducted, each with duplicate samples.

Oil Red O staining

HepG2-treated PA were incubated with CA (0, 1, 5 and 10 µg/mL) for 24 h. Cells were rinsed with phosphate buffered saline (PBS) and fixed in 10% formalin for 1 h. The cells were washed with isopropanol and stained with 0.6% Oil Red O solution for 1 h. The stained cells were photographed by Primovert (Carl Zeiss, NY, USA) at ×400 magnification. To quantify lipid content, the stained lipid droplets were extracted with 100% isopropanol, and the absorbance of the elution was measured at 500 nm (ClarioStar, BMG Labtech).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RNA extraction was performed as recommended (GF-1 Total RNA Extraction Kit) and cDNA was generated using the iScript cDNA synthesis kit. RT-qPCR was performed using SYBR Green I Master Mix in a LightCycler® 480 system with the following PCR conditions: pre-incubation 95°C for 5 min, then 45 cycles for 10 sec at 95°C, 60°C for 15 sec, and 72°C for 10 sec. The sequences of the primers used in this study were shown in Table 1. β-actin was used for normalization, and relative quantitation were calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were analyzed using SPSS 26.0 software. Results were expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to assess statistical differences among experimental groups, followed by Tukey's post-hoc test and considered significant at $p < 0.05$.

Table 1: Primers and their sequences.

Primers	Sequences (5'- 3')
SREBP1c Forward	CCACTTCATCAAGGCAGACTCG
SREBP1c Reverse	CAAGATGGTTCCGCCACTCAC
ACC Forward	CTTGGCCTTGCACATAAGGTCC
ACC Reverse	CCACCTACGGATAGACCGCA
FAS Forward	ATAGTGTGGAAGACGCTGGC
FAS Reverse	CTGGTACACCTTCCCACTCAC
PPARα Forward	CAATGCACTGGAACTGGATGA
PPARα Reverse	GTTGCTCTGCAGGTGGAGTCT
CPT1A Forward	TGTCCAGCCAGACGAAGAAC
CPT1A Reverse	ATCTTGCCGTGCTCAGTGAA
β-actin Forward	GATTCCTATGTGGGCGACGA
β-actin Reverse	AGGTCTCAAACATGATCTGGGT

Table 2: Phytochemical contents of *C. aurantifolia* root ethanolic extract.

Phytochemicals	*C. aurantifolia* root ethanolic extract
1. Alkaloids	-
2. Amines	+ (purple)
3. Coumarins	+ (green fluorescence)
4. Flavonoids	+ (orange)
5. Phenolics	+ (green)
6. Total phenolic content	144.70 ± 1.34 mg GAE/g
7. Total flavonoid content	138.04 ± 3.62 mg QE/g
8. Nordentatin	302.79 ± 0.01 mg/g dry extract
9. Gallic acid	ND
10. Chlorogenic acid	ND
11. Ellagic acid	ND
12. Coumaric acid	ND
13. Vanillin	ND
14. Caffeic acid	ND
15. Ferulic acid	ND

(+) = presence and (-) = absence of groups of compounds.

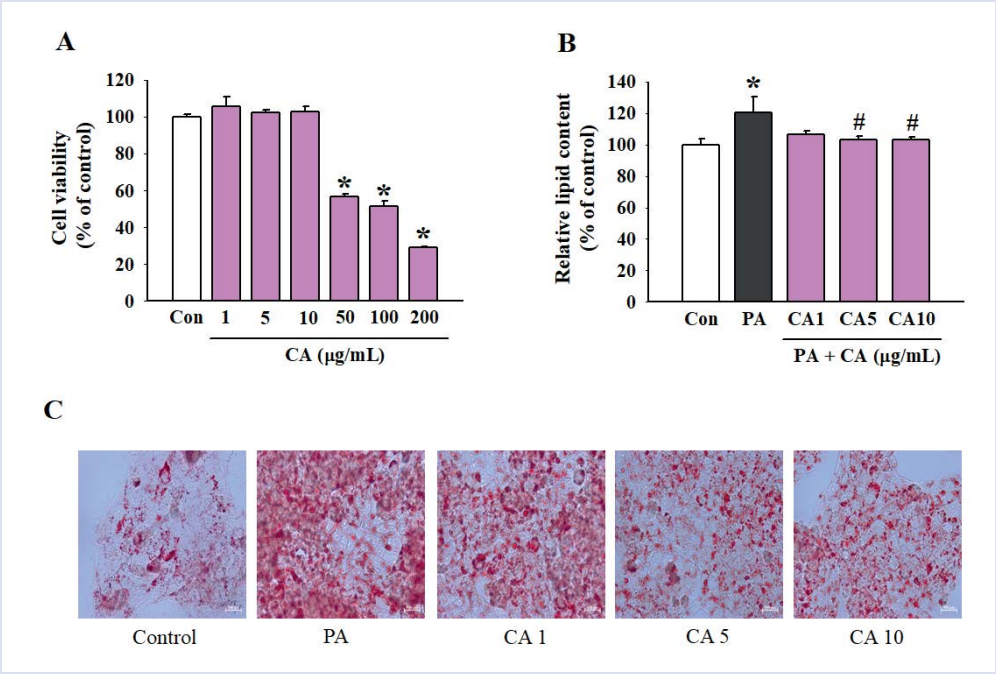

Figure 1: Effects of CA on cell viability and lipid accumulation in PA-induced HepG2 cells. (A) Viability of HepG2 cells was performed by MTT assay. (B) Lipid accumulation was extracted by isopropanol and quantitative content was measured at 500 nm. (C) Oil Red O-stained image of HepG2 cells observed under a microscope (×400). Data are expressed as mean ± SEM (n=6). *$p < 0.05$ vs. the control group (non-treated cells). #$p < 0.05$ vs. the PA-treated group.

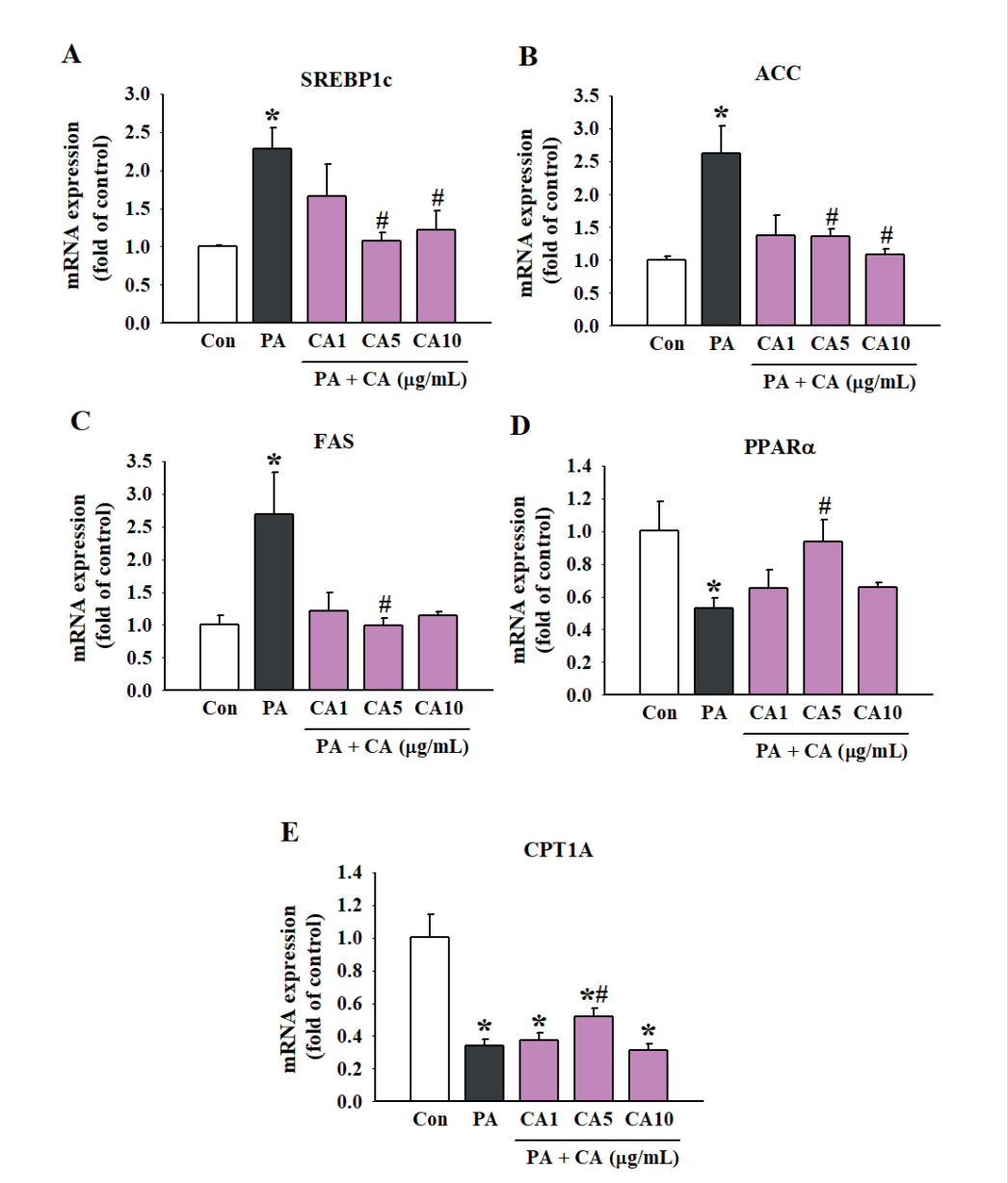

Figure 2: Effects of CA on lipogenic gene expression in PA-induced HepG2 cells. Relative expression levels of lipogenic gene, (A) SREBP1c. (B) ACC and (C) FAS. Relative expression levels of fatty acid oxidation genes, (D) PPARα and (E) CPT1A. β-actin was used as an internal control. Data are expressed as mean ± SEM (n=6). * $p < 0.05$ vs. the control group (non-treated cells). # $p < 0.05$ vs. the PA-treated group.

RESULTS

Phytochemical contents of CA

To determine the presence of alkaloid, amine, coumarin, flavonoid and phenolic in the extract. As shown in Table 2 the amine, coumarin, flavonoid and phenolic compounds were found in CA. Moreover, the measurement of total phenolic and flavonoid contents in CA were found 144.70 mg GAE/g and 138.04 mg QE, respectively. The HPLC analysis showed that the nordentatin was dominantly found in CA (302.79 mg/g dry extract).

Cell viability

The effects of various concentrations of CA on the cell viability of PA-induced lipid accumulation were determined using MTT assay. After 24-h incubation, CA concentrations of 1-10 μg/mL did not affect cell viability (Figure 1A). However, CA concentrations from 50-200 μg/mL significantly showed cytotoxicity. Therefore, this study has chosen the concentrations of CA at 1-10 μg/mL for further investigation of CA on lipid accumulation.

Hepatic lipid accumulation of CA

The quantitative analysis of lipid droplets was significantly increased in PA-treated group, while CA-treated groups significantly decreased the lipid accumulation (Figure 1B). The image of PA-treated group showed widespread lipid droplets by Oil Red O staining as compared to the control group, however, the lipid accumulation was reduced in CA-treated groups in comparison to the PA-treated group (Figure 1C).

Lipogenesis gene expression of CA

The PA-treated group significantly ($p<0.05$) increased the lipogenic gene expressions of SREBP1c, ACC and FAS as compared to the control group (Figure 2A-2C, respectively). Interestingly, the PA-treated cells with CA at 5 or 10 µg/mL significantly suppressed the SREBP1c and ACC genes as compared to the PA-treated group. Moreover, only 10 µg/mL CA could inhibit the FAS gene expression.

The fatty acid oxidation genes (PPARα and CPT1A) were inhibited in PA-treated group as compared to the control group, the concentrations of CA at 5 µg/mL could increase these genes as compared to the PA-treated group (Figure 2D and 2E, respectively).

In the PA-treated group, the fatty acid oxidation genes (PPARα and CPT1A) were inhibited compared to the control group. However, treatment with 5 µg/mL of CA increased the expression of these genes relative to the PA-treated group.

DISCUSSION

Obesity-related NAFLD can alter lipid metabolism in hepatocytes.[2] The hepatic lipid accumulation is the main characteristic of abnormal lipid metabolism in this condition.[1,2] The present study showed that the PA could induce the lipid accumulation in HepG2 cells. The results showed a more widespread deposition of Oil Red O-stained lipid droplets in hepatocytes compared to the control group. Elevation of lipogenic genes (SREBP1c, ACC and FAS) and reduction of fatty acid oxidation genes (CPT1A and PPARα) were significantly expressed in this model as compared to the control group. These results successfully confirm that the PA-induced lipid accumulation in HepG2 cells could use as an obesity-related NAFLD model to explore the activity of CA in regulating hepatic lipid metabolism.

Disorders of lipid synthesis, lipid oxidation and lipid transportation are the main manifestations in NAFLD.[27] The increased hepatic lipogenesis observed in NAFLD model is driven by SREBP1c activation. SREBP1c is a key transcription factor that regulates the expression of lipogenic genes such as FAS and ACC.[11] Elevated SREBP1c activity leads to increased expression of these genes.[28] Our study showed that the treatment of CA (1-10 µg/mL) could reduce lipid droplets in PA-treated HepG2 cells. The gene expressions of lipogenesis, SREBP1c, ACC and FAS were inhibited by CA. Furthermore, the CA treatment could increase the fatty acid oxidation genes, CPT1A and PPARα.[29] PPARα is highly expressed in the liver with the function of preventing hepatic lipid storage, while CPT1A functions as the rate-limiting enzyme in fatty acid oxidation.[30] These findings suggest that CA treatment can alleviate abnormal lipid metabolism by downregulating the expression of lipogenesis together with upregulating fatty acid oxidation genes.

Our study used absolute ethanol, which is one of the most suitable solvents for extracting phytochemicals from various plants.[31] In this study, absolute ethanol was used to extract bioactive substances from lime root. The lime root ethanolic extract revealed the presence of the amines, coumarins, flavonoids and phenolic compounds. The presence of flavonoids and phenolics in the *C. aurantifolia* extract has been reported to exhibit multiple pharmacological properties, such as antioxidant effect and increased renal function,[32] and anti-inflammation by inhibition of NF-κB.[33] Additionally, the total phenolic and flavonoid contents in the extract were 144.70 mg GAE/g and 138.04 mg QE, respectively. However, the main component detected by HPLC analysis was nordentatin (302.79 mg/g dry extract), which belongs to the coumarin group. Plant-derived coumarin are compounds that contain benzopyrone structure and easily soluble in ethanol. The natural coumarins and their derivatives exhibit distinct pharmacological properties that depend on the substituents present in coumarin molecules.[34] Nordentatin, a coumarin derivative isolated from many plant has been reported several pharmacological properties such as antihyperglycemic effect which involves the inhibition of α-glucosidase,[35,36] neuritogenic and antioxidant,[37] antimicrobial[38] and anticancer[38-40] activities. However, there is limited study on the anti-lipogenic properties of *C. aurantifolia* root and its main compound, nordentatin. Therefore, further investigation of *C. aurantifolia* root is necessary to explore these actions.

In conclusions, the present study showed that CA treatment could improve abnormal lipid metabolism in PA-induced HepG2 cells via decreased hepatic lipid droplets, downregulated lipogenic genes; SREBP1c, ACC and FAS, and upregulated PPARα and CPT1A. These findings suggest that the CA may be a useful plant for regulating obesity-related NAFLD.

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AUTHOR DISCLOSURE

The authors declare that there is no conflict of interest.

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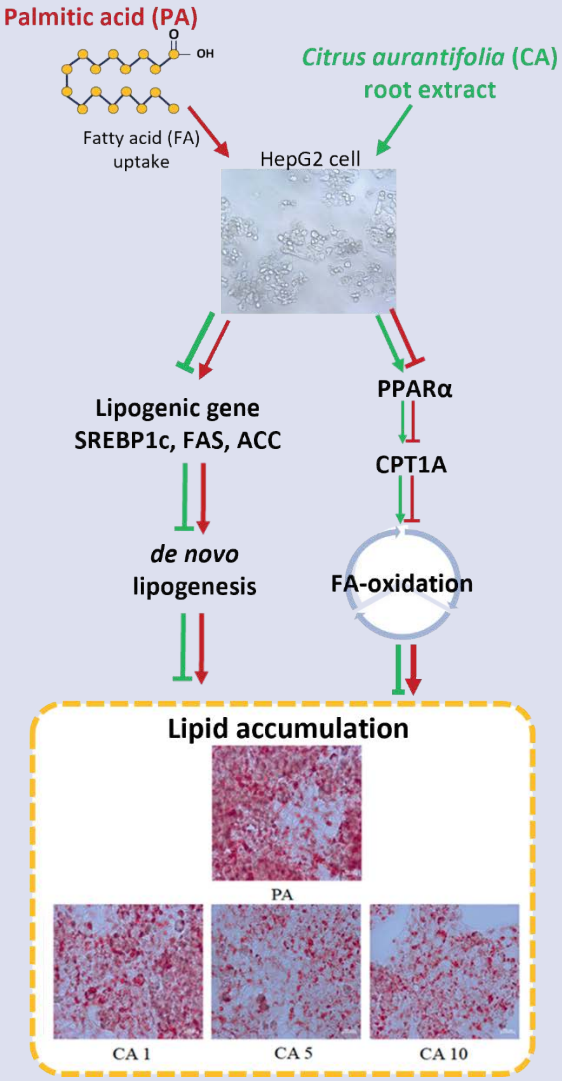
GRAPHICAL ABSTRACT



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